



รายงานวิจัยฉบับสมบูรณ์

โครงการ

บทบาททางสรีรวิทยาของฮอร์โมนโพรแลคตินเพื่อการนำแคลเซียมจากอาหารสู่กระดูกในระหว่าง
ตั้งครรภ์และให้นม: ศักยภาพในการนำไปใช้เพื่อป้องกันภาวะกระดูกบางจากการเลี้ยงลูกด้วยน้ำนม
Physiological roles of prolactin during pregnancy and lactation in the translocation of calcium
from diet to bone: Potential application for the prevention of maternal osteopenia

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Physiological roles of prolactin during pregnancy and lactation in the translocation of calcium from diet to bone: Potential application for the prevention of maternal osteopenia

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บทคัดย่อ

ชื่อโครงการ

บทบาททางสรีรวิทยาของฮอร์โมนโพรแลคตินเพื่อการนำแคลเซียมจากอาหารสู่กระดูกในระหว่างตั้งครรภ์และให้นม : ศักยภาพในการนำไปใช้เพื่อป้องกันภาวะกระดูกบางจากการเลี้ยงลูกด้วยน้ำนม

เป็นที่ทราบกันดีว่าการตั้งครรภ์และให้นมจะทำให้ร่างกายสูญเสียแคลเซียมเป็นจำนวนมากให้แก่ทารกในครรภ์ และการสร้างน้ำนม โดยทั่วไปหญิงตั้งครรภ์จะเสียแคลเซียมราว 200 มิลลิกรัมต่อวัน ส่วนหญิงที่ให้นมบุตรจะเสียแคลเซียมในปริมาณมากกว่า คือประมาณ 250–300 มิลลิกรัมต่อวัน ในสัตว์บางประเภท เช่น วัว ปริมาณแคลเซียมที่สูญเสียไปต่อวันสูงมาก จนอาจทำให้สัตว์เหล่านี้เกิดความผิดปกติ และอาจตายจากปริมาณแคลเซียมในเลือดที่ลดต่ำจนวิกฤต งานวิจัยก่อนหน้านี้แสดงให้เห็นว่าฮอร์โมนโพรแลคตินที่สูงขึ้นในระหว่างตั้งครรภ์และให้นมอาจช่วยกระตุ้นการดูดซึมแคลเซียมจากทางเดินอาหาร และอาจใช้ความรู้นี้พัฒนาวิธีการป้องกันภาวะกระดูกบางในผู้หญิงที่กำลังตั้งครรภ์และให้นมบุตร

การวิจัยที่ผ่านมาในช่วงระยะเวลา 3 ปีแสดงให้เห็นว่า แคลเซียมที่สะสมในกระดูกของหนูที่ตั้งท้องและให้นมมีการเปลี่ยนแปลงอย่างมาก กล่าวคือ มวลกระดูกจะเพิ่มขึ้นในระหว่างตั้งท้อง โดยเฉพาะในกระดูกส่วน cortex ของ long bone และ vertebra ซึ่งผู้วิจัยคาดว่าเกิดจากความพยายามของร่างกายที่จะสะสมแคลเซียมไว้ใช้ในภายหลัง (คือในระหว่างให้นม) เมื่อเข้าสู่ระยะให้นม มวลกระดูกที่เคยเพิ่มขึ้นกลับลดลงอย่างรวดเร็ว ซึ่งเป็นผลจากการที่โพรแลคตินเร่งการนำแคลเซียมจากกระดูกมาใช้สร้างน้ำนม (โดยเฉพาะที่กระดูกส่วน trabecular) จนอาจทำให้เกิดภาวะกระดูกบางในแม่ได้ ซึ่งเป็นปรากฏการณ์ที่สอดคล้องกับที่เกิดในมนุษย์

อย่างไรก็ตาม โพรแลคตินที่เพิ่มขึ้นในระหว่างให้นมสามารถช่วยเพิ่มอัตราการดูดซึมแคลเซียมจากลำไส้ได้ประมาณ 2 เท่า ซึ่งอาจเป็นวิธีหนึ่งที่ร่างกายแม่ใช้ลดการนำแคลเซียมจากกระดูกมาสร้างน้ำนม (กล่าวคือ ฮอร์โมนโพรแลคตินอาจมีบทบาทสำคัญในฐานะฮอร์โมนที่ช่วยนำแคลเซียมจากแม่ไปสร้างน้ำนมเพื่อการเจริญเติบโตของลูก หากแม่รับประทานแคลเซียมอย่างเพียงพอ โพรแลคตินก็จะนำแคลเซียมจากอาหารมาใช้ แต่ถ้ารับประทานไม่เพียงพอ โพรแลคตินก็จะนำแคลเซียมจากกระดูกแม่มาใช้แทน) การกระตุ้นการดูดซึมแคลเซียมของโพรแลคตินอาศัย 2 กลไกหลัก ได้แก่

1. กลไกแบบ genomic ซึ่งเกิดจากการที่ลำไส้สัมผัสกับฮอร์โมนโพรแลคตินที่สูงในเลือดเป็นเวลานาน (> 7 วัน) จากการศึกษาด้วยเทคนิค microarray พบว่า โพรแลคตินกระตุ้นการแสดงออกของยีนจำนวนมากเพื่อช่วยให้การดูดซึมแคลเซียมเพิ่มสูงขึ้น
2. กลไกแบบ non-genomic ซึ่งเกิดจากการที่เซลล์เยื่อบุลำไส้สัมผัสกับโพรแลคตินในระดับที่สูงมาก ๆ (> 400 ng/mL) ในช่วงเวลาสั้น ๆ (15 นาที ถึง 1 ชั่วโมง) เมื่อให้ลูกหนูดื่มนมเพื่อเพิ่มระดับโพร

แลคตินในเลือดของแม่หนูให้สูงประมาณ 400 ng/mL นาน 30 นาที พบว่า อัตราการดูดซึมแคลเซียมเพิ่มขึ้นอย่างมาก ซึ่งสอดคล้องกับสมมติฐานที่เสนอไว้ จึงเป็นการยืนยันในระดับหนึ่งว่า องค์ความรู้ด้านบทบาททางสรีรวิทยาของฮอร์โมนโปรแลคตินมี ศักยภาพในการนำไปใช้เพื่อป้องกันภาวะกระดูกบางจากการเลี้ยงลูกด้วยน้ำนม

การวิจัยเชิงลึกในระดับเซลล์และโมเลกุลพบว่า กลไกการออกฤทธิ์ของโปรแลคตินแบบ non-genomic จำเป็นต้องใช้โปรตีนของเซลล์หลายชนิด ได้แก่ ตัวรับโปรแลคตินชนิดยาว (long-form prolactin receptor), phosphoinositide 3-kinase (PI3K), protein kinase C (PKC) ชนิด "เซต้า" (zeta) และ Rho kinase (ROCK) ซึ่งโปรตีน 3 ชนิดหลังทำหน้าที่เป็น signaling protein ภายในเซลล์ โปรแลคตินใช้กลไกนี้สำหรับกระตุ้นการดูดซึมแคลเซียมไม่ว่าจะเป็นที่ลำไส้เล็กส่วนต้น (duodenum) ลำไส้ใหญ่ส่วนซีคัม (cecum) และเซลล์ลำไส้เพาะเลี้ยง Caco-2 อนึ่งแคลเซียมที่เข้าสู่เซลล์ลำไส้ภายใต้การกระตุ้นของโปรแลคตินยังผ่านโปรตีนขนส่งคนละชนิดกันกับที่ได้เคยมีรายงานไว้ กล่าวคือ โปรแลคตินจะกระตุ้นการนำแคลเซียมเข้าสู่เซลล์ผ่าน L-type calcium channel ชนิด $Ca_v1.3$ ซึ่งแตกต่างจาก vitamin D ที่กระตุ้นการนำแคลเซียมเข้าสู่เซลล์ลำไส้ผ่าน TRPV6 channel งานวิจัยโดยอาศัยเทคนิค siRNA ยังช่วยยืนยันว่า โปรแลคตินทำงานโดยอาศัยเพียง $Ca_v1.3$ เท่านั้น ส่วน TRPV5 และ TRPV6 ซึ่งเป็นโปรตีนขนส่งแคลเซียมเช่นกัน ไม่มีความสำคัญต่อการออกฤทธิ์แบบเฉียบพลันของโปรแลคติน (acute non-genomic)

นอกจากนี้คณะผู้วิจัยยังพบว่า มียีนจำนวนมากที่สามารถเพิ่มประสิทธิภาพการดูดซึมแคลเซียมจากทางเดินอาหารได้ และภาวะใด ๆ ที่สามารถเพิ่มอัตราการดูดซึมแคลเซียม เช่น ภาวะเลือดเป็นกรด ก็มักกระตุ้นยีนชุดนี้ ผู้วิจัยยังได้แสดงให้เห็นว่า ปัจจัยภายนอกร่างกาย เช่น การออกกำลังกาย ก็มีส่วนช่วยเพิ่มอัตราการดูดซึมแคลเซียมเข้าสู่ร่างกาย องค์ความรู้เรื่องปัจจัยภายนอกนี้ ได้จากการประยุกต์ใช้เทคนิคการวัดอัตราการขนส่งแคลเซียมที่พัฒนาขึ้นเพื่อวัดผลของฮอร์โมนโปรแลคติน และยังอาจใช้การออกกำลังกายไปช่วยเสริมฤทธิ์ของโปรแลคตินเพื่อต้านภาวะกระดูกบางในระหว่างให้นมได้อีกด้วย

จากการวิจัยในหนูท้องและหนูที่กำลังให้นมพบว่า ระดับของโปรแลคตินที่สูงขึ้นในช่วงเวลาดังกล่าวกระตุ้นการดูดซึมแคลเซียมในลำไส้เล็กส่วนต้นกว่า 2 เท่า และยังพบว่า การให้แคลเซียมคลอไรด์เสริมทางปาก 30 นาทีก่อนให้นมลูก นาน 2 สัปดาห์ สามารถชะลอการสูญเสียมวลกระดูกในแม่หนูที่กำลังให้นมได้ และปริมาณแคลเซียมที่เหมาะสม คือ 6 mg per kilogram body weight per dose โดยแบ่งให้วันละ 3-4 ครั้ง กล่าวคือ เมื่อแม่หนูได้รับแคลเซียมเสริมในเวลาและปริมาณที่เหมาะสมจะทำให้ได้รับแคลเซียมเข้าสู่ร่างกายในปริมาณสูงเพื่อเสริมความแข็งแรงของกระดูกโดยไม่ต้องพึ่งยาหรือฮอร์โมนอื่น ๆ เสริม โดยแคลเซียมที่เข้าสู่ร่างกายเพิ่มขึ้นนี้จะช่วยเสริมให้กระดูกของแม่แข็งแรง และมีปริมาณแคลเซียมในน้ำนมเพียงพอต่อความต้องการของร่างกายลูก จึงสรุปได้ว่า ฮอร์โมนโปรแลคตินที่เพิ่มขึ้นตามธรรมชาติมีศักยภาพในการนำไปใช้เพื่อป้องกันภาวะกระดูกบางจากการเลี้ยงลูกด้วยน้ำนม

Abstract

Physiological roles of prolactin during pregnancy and lactation in the translocation of calcium from diet to bone: Potential application for the prevention of maternal osteopenia

It has been known that pregnancy and lactation induce tremendous maternal calcium loss for fetal skeletal development and milk production. Generally, pregnant women transfer ~200 mg calcium per day across the placenta, while breastfeeding mothers lose greater amount of calcium of ~250–300 mg/day. In some mammals such as dairy cows, such massive calcium loss may lead to severe hypocalcemia with lethal neurological complications. Our previous investigation strongly suggests that physiological hyperprolactinemia (i.e., the elevated plasma prolactin levels) which normally occurs during pregnancy and lactation may help enhance intestinal calcium absorption, and thus help prevent osteopenia (low bone density) in pregnant and lactating mothers.

Our research during years 2008–2011 has shown that bone calcium metabolism was markedly changed in pregnant and lactating rats. Specifically, bone mass was increased during pregnancy, especially in the cortical parts of long bones (such as tibiae and femora) and in vertebrae. This adaptation was to store calcium in bone for later use during lactation. In the lactating period, prolactin stimulated bone resorption, especially in the trabecular sites (e.g., tibial metaphysis), to release calcium for lactogenesis.

Although prolactin could induce bone loss during lactation, it also enhanced intestinal calcium absorption by ~2-fold (i.e., the main goal of prolactin was to provide adequate calcium for milk production, either from diet or bone). Therefore, a sufficient oral calcium supplementation should shift the calcium source from bone to diet, thereby preventing osteopenia in mothers. We found that prolactin enhanced intestinal calcium absorption by 2 major mechanisms as followed.

1. Genomic mechanism (Step 1): chronic exposure to high plasma prolactin (~100–200 ng/mL) for > 7 days led to the upregulation of genes responsible for intestinal calcium transport, as demonstrated by microarray and real-time PCR in non-pregnant hyperprolactinemic rats. Thus, this genomic mechanism elevated the baseline of calcium flux across intestinal epithelium of pregnant and lactating rats.

2. Non-genomic mechanism (Step 2): acute exposure to extremely high plasma prolactin of > 400 ng/mL (as during the suckling-induced prolactin surge) for 15–60 minutes

further increased the intestinal calcium transport above the newly elevated baseline (i.e., Step-1 calcium transport).

Further studies by using cellular and molecular techniques demonstrated that non-genomic effect of prolactin required the long isoform of prolactin receptors expressed in the intestinal absorptive cells, as well as 3 signaling proteins, namely phosphoinositide 3-kinase (PI3K), protein kinase C (PKC)-zeta, and Rho kinase (ROCK). Prolactin was found to use these signaling proteins in the duodenum, cecum and intestinal epithelium-like Caco-2 monolayer (i.e., cultured epithelial sheet). Moreover, the prolactin-enhanced apical calcium uptake in intestinal epithelial cells was mediated by the voltage-dependent L-type calcium channel $Ca_v1.3$, but not TRPV5 or TRPV6 calcium channels. This information suggested that, in some special conditions such as in lactation, the intestinal cells use atypical calcium channel (i.e., $Ca_v1.3$) for calcium absorption.

In addition to $Ca_v1.3$, we also found that there were a number of genes which participated in the intestinal calcium absorption during some other special conditions, such as chronic metabolic acidosis and endurance exercise. Indeed, the endurance swimming exercise was of interest since it was capable of stimulating intestinal calcium absorption. Thus, it was anticipated that the combined swimming and oral calcium supplementation might have a synergistic effect for the prevention of osteopenia in lactation.

Finally, we have shown that oral calcium supplementation ~30 minutes prior to breastfeeding (i.e., pre-suckling calcium supplement) for 2 weeks could prevent severe bone loss in maternal rats. The optimal calcium chloride dose was 6 mg per kilogram body weight per dose, 3–4 times per day. Therefore, it could be concluded that **oral calcium supplementation before each suckling-induced prolactin surge has a potential for the prevention of maternal osteopenia.**

หน้าสรุปโครงการ

(Executive Summary)

ทุนพัฒนานักวิจัย

1. ชื่อโครงการ

(ภาษาไทย) บทบาททางสรีรวิทยาของฮอร์โมนโพรแลคตินเพื่อการนำแคลเซียมจากอาหารสู่กระดูก
ในระหว่างตั้งครรภ์และให้นม : ศักยภาพในการนำไปใช้เพื่อป้องกันภาวะกระดูกบางจากการเลี้ยงลูก
ด้วยน้ำนม

(ภาษาอังกฤษ) Physiological roles of prolactin during pregnancy and lactation in the
translocation of calcium from diet to bone: Potential application for the prevention of maternal
osteopenia

2. ผู้ดำเนินการวิจัย

2.1 หัวหน้าโครงการ

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2.1.3 ตำแหน่ง	รองศาสตราจารย์
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2.2 ผู้ร่วมโครงการวิจัยลำดับที่ 1

2.2.1 ชื่อ-นามสกุล	นายกู้เกียรติ ทุตปอ Kukiat Tudpor
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2.3 ผู้ร่วมโครงการวิจัยลำดับที่ 2

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2.3.2 คุณวุฒิ B.Sc. (Physiotherapy), M.Sc. (Physiology)
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2.4 ผู้ร่วมโครงการวิจัยลำดับที่ 3

2.4.1 ชื่อ-นามสกุล นางสาวนา แสงอำนาจ
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2.4.2 คุณวุฒิ B.Sc. (Biology)
2.4.3 ตำแหน่ง นักวิทยาศาสตร์ 6
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2.5 ผู้ร่วมโครงการวิจัยลำดับที่ 4

2.5.1 ชื่อ-นามสกุล นางสาวปาหนัน สุนทรสารทูล
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2.5.5 โทรศัพท์ 0-2201-5629
2.5.6 โทรสาร 0-2354-7154

2.6 ผู้ร่วมโครงการวิจัยลำดับที่ 5

2.6.1 ชื่อ-นามสกุล นางสาวกมลชนก ไกรดิษฐ์
Kamonshanok Kraidith

2.6.2 คุณสมบัติ	B.Sc. (Physical Therapy)
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3. สาขาวิชาที่ทำการวิจัย

วิทยาศาสตร์การแพทย์พื้นฐาน – สรีรวิทยาการดูดซึมแคลเซียมในลำไส้ระหว่างตั้งครรภ์และให้นม

4. คำสำคัญ

การดูดซึมแคลเซียม, ลำไส้ใหญ่ส่วนซีคัม, ลำไส้ใหญ่ส่วนต้น, ระยะให้นม, ระยะตั้งครรภ์
calcium absorption, cecum, duodenum, lactation, pregnancy

7. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

7.1 ข้อมูลทั่วไป

7.1.1 ฮอร์โมนโพรแลคตินในขณะตั้งครรภ์และให้นม

โพรแลคตินเป็นเปปไทด์ฮอร์โมน (peptide hormone) ที่หลังจากต่อมใต้สมองส่วนหน้าของมนุษย์และสัตว์เลี้ยงลูกด้วยนม เช่น หนู และเป็นฮอร์โมนที่มีบทบาททางสรีรวิทยากว่า 300 บทบาทครอบคลุมเกือบทุกอวัยวะของร่างกาย เช่น การทำงานของระบบประสาทและพฤติกรรม สมดุลน้ำและเกลือแร่ การทำงานของระบบภูมิคุ้มกัน และการทำงานของระบบสืบพันธุ์ [4]

ในระหว่างตั้งครรภ์และให้นม โพรแลคตินจะมีบทบาทมากเป็นพิเศษ เพราะเป็นฮอร์โมนหลักในการกระตุ้นการสร้างและการหลั่งน้ำนม โดยทั่วไปคนและสัตว์เลี้ยงลูกด้วยนมที่ไม่ได้ตั้งครรภ์จะมีระดับของโพรแลคตินในเลือดประมาณ 7–10 ng/mL แต่เมื่อตั้งครรภ์ จะมีการหลั่งโพรแลคตินเพิ่มขึ้น 10–20 เท่า ทำให้ในเลือดมีโพรแลคตินสูงถึง 100–200 ng/mL ในระยะให้นมร่างกายจะมีการหลั่งโพรแลคตินเพิ่มขึ้นอีก โดยอาจสูงราว 300–400 ng/mL นอกจากนี้ ในระหว่างดูดนม (suckling) จะมีกระตุ้นให้มีการหลั่งโพรแลคตินให้สูงขึ้นเฉลี่ย 650 ng/mL (400–800 ng/mL) ทันทีหลังดูดนม และจะคงที่อยู่ในระดับนี้นานถึง 1 ชั่วโมง ก่อนระดับจะลดลงจนเหลือ 300–400 ng/mL [1, 3, 7]

7.1.2 สรีรวิทยาการขนส่งแคลเซียมของลำไส้

การขนส่งแคลเซียมในลำไส้มีสองทิศทางคือ การดูดซึมเข้าสู่เลือด (absorption) และการขับออกสู่โพรงลำไส้ (secretion) แต่ในลำไส้เล็กส่วนต้นการขับแคลเซียมเกิดขึ้นน้อยมากจนถือได้ว่าไม่มีการขับแคลเซียมเลย คณะผู้วิจัยได้รายงานก่อนหน้านี้ว่า ลำไส้เล็กส่วนต้นของหนูขาวขนส่งแคลเซียมได้ด้วย 2 กลไกหลัก [6–8] คือ

1. Paracellular passive transport กลไกนี้อาศัยความแตกต่างระหว่างความเข้มข้นแคลเซียมในโพรงลำไส้และในเลือด แคลเซียมจะเคลื่อนที่ผ่านชั้นเยื่อทางช่องระหว่างเซลล์ (paracellular space) การดูดซึมลักษณะนี้เกิดขึ้นได้ตลอดเวลาตราบเท่าที่ความเข้มข้นแคลเซียมในโพรงลำไส้สูงกว่าในเลือด แต่เซลล์ลำไส้สามารถควบคุมอัตราการขนส่งได้โดยควบคุมความกว้างของช่องระหว่างเซลล์ (size selectivity) และ/หรือการคัดแยกชนิดของประจุ ขณะที่สารผ่านช่องระหว่างเซลล์ (charge selectivity) [12, 31] ซึ่งต้องอาศัย tight junction protein ประเภท claudin (เช่น claudin-2, claudin-12 และ claudin-15)

2. Active transport กลไกนี้ต้องใช้พลังงานจากเซลล์ในการขนส่งแคลเซียม ซึ่งแบ่งเป็น 3 กลไกย่อย คือ

2.1 Transcellular active transport เป็นกลไกที่เซลล์นำแคลเซียมจากโพรงลำไส้ผ่าน Ca^{2+} channel (เช่น TRPV5 และ TRPV6) เข้าสู่เซลล์ และขับออกอีกด้านหนึ่งโดยใช้โปรตีนขนส่ง Ca^{2+} -ATPase

2.2 Solvent drag-induced transport เป็นกลไกที่เซลล์ขนส่งโซเดียม (ซึ่งเข้าเซลล์พร้อมน้ำตาลกลูโคส) แบบใช้พลังงานไปสะสมอยู่ภายในช่องระหว่างเซลล์ เมื่อช่องระหว่างเซลล์มีความเข้มข้นของโซเดียมสูงจะทำให้น้ำไหลผ่านช่องนี้โดยมีทิศทางจากโพรงลำไส้เข้าสู่พลาสมา ดังนั้น แคลเซียมที่ละลายอยู่ในน้ำก็จะไหลตามน้ำ (solvent drag) เข้าสู่พลาสมาด้วย อย่างไรก็ตาม คณะผู้วิจัยพบว่า การขนส่งด้วยกลไกนี้ขึ้นอยู่กับ size selectivity และ charge selectivity ของช่องระหว่างเซลล์ด้วยเช่นกัน

2.3 Voltage-dependent transport เป็นกลไกต่อเนื่องจากการขนส่งโซเดียมแบบใช้พลังงาน ทั้งนี้เพราะเมื่อเซลล์ขนส่งโซเดียมเข้าสู่กระแสเลือด จะเกิดศักย์ไฟฟ้าที่เป็นลบในโพรงลำไส้ ดึงให้แคลเซียมเคลื่อนจากด้านพลาสมาผ่านช่องระหว่างเซลล์เข้าสู่โพรงลำไส้ ฉะนั้นกลไกนี้จึงเป็น การขับแคลเซียม ไม่ใช่การดูดซึมแคลเซียม การขนส่งด้วยกลไกนี้เกิดขึ้นน้อยมากในภาวะปกติ

7.2 ปัญหาที่ทำการวิจัย

เป็นที่ทราบกันดีว่าการตั้งครรภ์และให้นมจะทำให้ร่างกายสูญเสียแคลเซียมเป็นจำนวนมาก ให้แก่ทารกในครรภ์ และการสร้างน้ำนม โดยทั่วไปหญิงตั้งครรภ์จะเสียแคลเซียมราว 200 มิลลิกรัมต่อวัน ส่วนหญิงที่ให้นมบุตรจะเสียแคลเซียมในปริมาณมากกว่า คือประมาณ 250–300 มิลลิกรัมต่อวัน [27] ในสัตว์บางประเภท เช่น วัว ปริมาณแคลเซียมที่เสียไปต่อวันสูงมาก จนอาจทำให้สัตว์เหล่านี้เกิด ความผิดปกติ และอาจตายจากปริมาณแคลเซียมในเลือดที่ลดต่ำจนวิกฤต [13]

เมื่อต้องสูญเสียแคลเซียมเป็นจำนวนมาก ร่างกายจึงพยายามนำแคลเซียมจากแหล่งต่างๆ มา รักษาระดับของแคลเซียมในเลือด เช่น จากการสลายกระดูก ทำให้หญิงที่ตั้งครรภ์และให้นมมีภาวะ กระดูกบาง (osteopenia) เกิดขึ้น ร่วมกับความหนาแน่นกระดูก (bone mineral density) ที่ลดลงอย่างมีนัยสำคัญ [9, 24, 25, 28, 29] แม้ว่ารายงานส่วนใหญ่จะแสดงให้เห็นว่า ความหนาแน่นกระดูกจะกลับ

เข้าสู่ค่าปกติภายหลังการหย่านม [2] แต่ก็มิได้หมายความว่าภาวะดังกล่าวจะไม่สร้างความเสียหายให้แก่กระดูกในระยะยาว ทั้งนี้เพราะ

1. ไม่มีข้อมูลซึ่งแสดงการเปลี่ยนแปลงทาง microstructure ของกระดูกและคุณสมบัติทางฟิสิกส์ เช่น ความแข็ง และความเปราะ การศึกษาส่วนใหญ่ทำในมนุษย์ โดยใช้เทคนิควิจัยแบบ non-invasive เพื่อให้แม่และเด็กปลอดภัย ค่าที่พอจะวัดได้จึงเป็นเพียงค่าความหนาแน่นกระดูกเท่านั้น แต่ค่าความหนาแน่นกระดูกมีความหมายน้อยมากในการใช้เพื่อประเมินความแข็งแรงของโครงสร้างกระดูก และความเสี่ยงที่จะเกิดกระดูกพรุนหรือกระดูกหักในอนาคต เนื่องจากต้องมีข้อมูลทาง microstructure มาประกอบด้วย โดยทั่วไปต้องใช้เทคนิคที่ซับซ้อนกว่า และ invasive มากกว่า เช่น เทคนิค bone histomorphometry (ซึ่งต้องมีการทำ bone biopsy และต้องใช้ผู้เชี่ยวชาญในการแปลผล) หรือ micro-computed tomography (μ CT ซึ่งมีค่าใช้จ่ายสูง) อนึ่งยังไม่มีการศึกษาตัวแปรเหล่านี้ในสัตว์ทดลองส่วนหนึ่งอาจเป็นเพราะสัตว์ทดลองที่ตั้งท้องมีราคาสูงมากในต่างประเทศ

2. ไม่มีงานวิจัยที่ติดตามการเปลี่ยนแปลงของกระดูกตั้งแต่เริ่มตั้งครรภ์ ให้นม จนกระทั่งเข้าสู่ระยะหมดประจำเดือน และวัยชรา ซึ่งเป็นช่วงเสี่ยงที่จะเกิดภาวะกระดูกพรุน และกระดูกหัก

แม้ว่ากระดูกบางจะเป็นภาวะที่พบได้ทั่วไปในระหว่างตั้งครรภ์และให้นม แต่สาเหตุที่ทำให้เกิดภาวะดังกล่าวยังไม่เป็นที่ทราบกันดีนัก งานวิจัยในหนูที่ไม่ได้ตั้งท้องพบว่า การฉีดฮอร์โมนโปรแลคตินจะทำให้มีการสร้างและสลายกระดูกเกิดขึ้นอย่างรวดเร็ว (bone turnover rate สูง) [21, 22] เพื่อให้มีแคลเซียมอิสระ (free ionized calcium) ออกมาในเลือดมากขึ้น และหากแคลเซียมที่ร่างกายได้รับจากอาหารไม่เพียงพอ การสลายกระดูกก็จะเกิดเร็วขึ้น ทำให้กระดูกบางซึ่งคล้ายกับที่เกิดในหนูที่ตั้งท้องและให้นม ดังนั้นจึงมีความเป็นไปได้ว่า โปรแลคตินจะเป็นสัญญาณกระตุ้นให้ร่างกายสลายแคลเซียมออกจากกระดูกเพื่อนำไปให้ทารกในครรภ์ หรือนำไปสร้างน้ำนม อย่างไรก็ตาม ยังไม่มีการพิสูจน์บทบาทนี้ของโปรแลคตินอย่างเป็นระบบ

นอกจากผลที่กระดูกแล้ว โปรแลคตินยังมีผลต่อลำไส้เล็กส่วนต้น (duodenum) ของหนูที่ไม่ได้ตั้งท้อง โดยจะกระตุ้นการดูดซึมแคลเซียมเข้าสู่ร่างกายด้วยกลไกแบบ transcellular, solvent drag-induced และ paracellular passive [7] ซึ่งอาจเป็นกระบวนการหนึ่งที่โปรแลคตินพยายามเพิ่มแคลเซียมอิสระให้กับร่างกาย แต่บทบาทเหล่านี้ยังไม่เคยได้รับการพิสูจน์ในหนูที่ตั้งท้องและให้นม อย่างไรก็ตาม การที่โปรแลคตินสามารถกระตุ้นการดูดซึมแคลเซียม และกระตุ้นวงจรการสร้างและสลายกระดูกทำให้เกิดสมมติฐานว่า หากเราให้แคลเซียมเสริมอย่างเพียงพอ แคลเซียมอาจเข้าสู่ร่างกายในปริมาณที่มากขึ้น และใช้กลไกการสร้างกระดูกที่ได้รับการกระตุ้นจากโปรแลคติน นำแคลเซียมเหล่านั้นมาเสริมสร้างความแข็งแรงของกระดูกได้ ผลคือร่างกายไม่เพียงแต่ปราศจากภาวะกระดูกบางเท่านั้น แต่กระดูกยังอาจแข็งแรงมากขึ้นอีกด้วย

แม้ว่าหลักการของการให้แคลเซียมเสริมอาจไม่ยุ่งยาก แต่การให้แคลเซียมเสริม (ไม่ว่าจะอยู่ในรูปอาหารแคลเซียมสูง หรือยาเม็ดแคลเซียม) โดยไม่พิจารณาถึงปริมาณ เวลา และรูปแบบของแคลเซียม ทำให้เกิดความสับสนเปลือง และอาจไม่ได้ผล ทั้งนี้เพราะ

1. แคลเซียมเป็นสารที่ดูดซึมได้น้อย โดยดูดซึมได้ประมาณร้อยละ 20–30 ของปริมาณที่รับประทาน

2. แคลเซียมแต่ละรูป เช่น calcium carbonate และ calcium citrate มีการแตกตัวไม่เท่ากัน [11] ทำให้ปริมาณที่ดูดซึมต่างกัน และแคลเซียมบางรูปยังมีราคาแพง และจะสิ้นเปลืองมากถ้าต้องกินในระยะเวลาสั้นๆ

มีรายงานจำนวนมากแสดงให้เห็นว่า การให้แคลเซียมเสริมช่วยป้องกันภาวะกระดูกบางระหว่างตั้งครรภ์ได้ แต่ไม่สามารถป้องกันภาวะกระดูกบางในระหว่างให้นม [27] เมื่อนำระดับโพรแลคตินในเลือดมารวมพิจารณา จะพบว่าระดับของโพรแลคตินมีความสัมพันธ์กับความต้องการแคลเซียมของร่างกาย เช่น ในระหว่างให้นม จะมีโพรแลคตินสูงที่สุด ซึ่งอาจเป็นเพราะต้องใช้โพรแลคตินเพื่อเร่งการดูดซึมแคลเซียมและสร้างน้ำนมทดแทน ดังนั้น หากจะมีการให้แคลเซียมเสริม ควรให้ในขณะที่มีระดับโพรแลคตินสูงที่สุด นั่นคือ 15–30 นาทีหลังให้นม อย่างไรก็ตาม ควรมีการทำวิจัยที่เป็นระบบเพื่อหาช่วงเวลาที่เหมาะสม ตลอดจนรูปแบบของแคลเซียมที่จะให้เสริม เพื่อให้ได้ผลคุ้มค่าใช้จ่ายมากที่สุด

อวัยวะลำไส้เล็กส่วนต้น (duodenum) เป็นเนื้อเยื่อที่เหมาะสมต่อการศึกษาดูดซึมแคลเซียมของลำไส้ [6] เนื่องจาก

1. ใช้กลไกการดูดซึมแคลเซียมทุกแบบ ซึ่งแทบจะไม่มีรายงานในลำไส้เล็กส่วนอื่น
2. เป็นลำไส้เล็กส่วนที่มีการดูดซึมแคลเซียมแบบ active transport มากที่สุด
3. เป็นตำแหน่งที่ตอบสนองต่อฮอร์โมนควบคุมสมดุลแคลเซียมมากที่สุด

อย่างไรก็ตาม แม้ว่าคณะผู้วิจัยจะใช้ลำไส้เล็กส่วนต้นเป็นต้นแบบ (model) ในการศึกษาผลของฮอร์โมน เช่น โพรแลคติน และ วิตามินดี และผลของพยาธิสภาพ เช่น ภาวะเลือดเป็นกรด (acidosis) [8] ที่มีต่อการดูดซึมแคลเซียมในลำไส้มานานกว่า 10 ปี แต่คณะผู้วิจัยก็ได้สังเกตเห็นปัญหาสำคัญซึ่งอาจกระทบต่อการนำเอาความรู้ดังกล่าวไปใช้ ทั้งนี้เพราะ ลำไส้เล็กส่วนต้นทั้งของมนุษย์และหนูค่อนข้างสั้น และอาหารจะผ่านลำไส้เล็กส่วนนี้อย่างรวดเร็ว ลำไส้เล็กส่วนต้นจึงอาจไม่ใช่ต้นแบบที่ดีสำหรับการศึกษากการให้แคลเซียมเสริมเพื่อการนำไปใช้ทางคลินิก

เมื่อพิจารณาลำไส้ส่วนที่เหลืออีก 6 ส่วน คือ proximal jejunum, distal jejunum, ileum, cecum, proximal colon และ distal colon จะพบว่า ลำไส้ใหญ่ส่วนซีคัมมีความน่าสนใจที่สุด เนื่องจาก

1. ลำไส้เล็กส่วนอื่นมักมีการขับแคลเซียมด้วย ทำให้การดูดซึมแคลเซียมสุทธิลดลง [15–19]
2. โพรแลคตินไม่มีผลกระตุ้นการดูดซึมแคลเซียมในลำไส้ใหญ่ส่วนโคลอน (เป็นการวิจัยในหนูที่ไม่ได้ตั้งท้อง) [20]

3. กากอาหารจะถูกเก็บอยู่ในกระเปาะลำไส้ใหญ่ส่วนซีคัมนาน ทำให้มีเวลาในการดูดซึม โดยเฉพาะสารที่ดูดซึมยากอย่างแคลเซียม

4. เคยมีรายงานว่าลำไส้ใหญ่ส่วนซีคัมมีอัตราการดูดซึมแคลเซียมแบบ active transport สูงสุด เมื่อเทียบกับลำไส้ทุกส่วน โดยมีอัตราการดูดซึมสูงกว่าลำไส้เล็กส่วนต้นถึง 9 เท่า [18]

อย่างไรก็ดี กลไกการดูดซึมแคลเซียมในลำไส้ใหญ่ส่วนซีคัมภายใต้การควบคุมของโพรแลคติน ยังไม่มีผู้ศึกษา และจำเป็นต้องได้รับการศึกษาอย่างละเอียดทั้งในหนูท้องและหนูปกติ การเข้าใจกลไกการดูดซึมมีความสำคัญเป็นอย่างยิ่งต่อการให้แคลเซียมเสริม ตัวอย่างเช่น ถ้าการขนส่งเป็นกลไกแบบ paracellular อาจพิจารณาให้แคลเซียมเสริมพร้อมกับสารอาหารที่กระตุ้นกลไกนี้ เช่น กลูโคส หรือ difructose anhydride [26] หรือถ้าต้องการให้แคลเซียมดูดซึมด้วยกลไก paracellular passive ยานั้น ต้องแตกตัวในลำไส้เล็กและให้แคลเซียมอิสระเกิน 5 mM [8] เป็นต้น

นอกจากเนื้อเยื่อลำไส้ของหนูแล้ว แผ่นเซลล์เยื่อบุผิวลำไส้เพาะเลี้ยง เช่น Caco-2 monolayer ก็มีประโยชน์อย่างมากต่อการศึกษากลไกการทำงานของโพรแลคตินในระดับเซลล์และโมเลกุล เนื่องจากแผ่นเซลล์เพาะเลี้ยงประกอบด้วยเซลล์ชนิดเดียว จึงทำให้ผลการวิจัยที่ได้มีค่าความแปรปรวนน้อยกว่ามาก

7.3 ความสำคัญของปัญหา

ที่ผ่านมามหาวิทยาลัยของโพรแลคตินต่อเมตาบอลิซึมของแคลเซียมได้รับการศึกษาในเชิงลึกในฐานะเป็นสารที่มีศักยภาพในการพัฒนาให้เป็นยาเพื่อช่วยบรรเทาภาวะกระดูกบางและกระดูกพรุน และช่วยกระตุ้นการดูดซึมแคลเซียมในวัยหลังหมดประจำเดือนและในวัยสูงอายุ อย่างไรก็ตาม ในงานวิจัยเรื่องนี้ คณะผู้วิจัยได้สร้างมุมมองใหม่ซึ่งมีศักยภาพในการพัฒนาเพื่อนำไปใช้ในอีกลักษณะหนึ่ง กล่าวคือ ในระหว่างตั้งครรภ์และให้นม ร่างกายจะมีระดับของโพรแลคตินสูงมากอยู่แล้ว หากแพทย์หรือสัตวแพทย์ได้ใช้ประโยชน์จากระดับของโพรแลคตินที่สูงนี้อย่างเต็มที่ ก็อาจสามารถป้องกันการเสื่อมลงของกระดูกในช่วงเวลาดังกล่าวได้ แม้จะยังไม่มีหลักฐานยืนยันชัดเจนว่า ภาวะกระดูกบางจากการตั้งครรภ์และให้นมจะทำให้เกิดโรคกระดูกพรุนเมื่อถึงวัยสูงอายุมากขึ้นหรือไม่ แต่การป้องกันหรือลดความเสี่ยงเพื่อไม่ให้เกิดภาวะกระดูกบางขึ้นเลย น่าจะเป็นการรักษาที่มีประสิทธิภาพสูงสุด และช่วยลดภาระค่าใช้จ่ายของผู้ป่วยในระยะยาว

อย่างไรก็ตาม ยังมีความจำเป็นต้องสร้างองค์ความรู้ใหม่ต่อยอดความรู้เดิมที่มีอยู่ โดยเฉพาะอย่างยิ่ง

1. การค้นหาตำแหน่งออกฤทธิ์ของโพรแลคตินที่ให้ประสิทธิภาพสูงสุดในการดูดซึมแคลเซียม ซึ่งคณะผู้วิจัยตั้งสมมติฐานว่า ลำไส้ใหญ่ส่วนซีคัมน่าจะมีความสำคัญทัดเทียมหรือมากกว่าลำไส้เล็กส่วนต้น
2. การพิสูจน์ว่าโพรแลคตินเป็นฮอร์โมนหลักที่ใช้เพิ่มการสร้างและการสลายกระดูก ตลอดจนใช้เพิ่มการดูดซึมแคลเซียมในระหว่างตั้งครรภ์และให้นม
3. การพิสูจน์ว่าโพรแลคตินที่สูงขึ้นมีผลอย่างไรต่อ microstructure ของกระดูก โดยใช้เทคนิคที่สามารถตรวจสอบโครงสร้างของกระดูกในระดับจุลภาค เช่น bone histomorphometry
4. การพิสูจน์ว่าการให้แคลเซียมเสริมในช่วงที่ระดับโพรแลคตินสูงมาก ๆ เช่นระหว่างให้นม จะช่วยลดผลกระทบที่มีต่อกระดูกหรือไม่

อนึ่ง ยังมีความจำเป็นต้องสร้างองค์ความรู้ด้านกลไกการดูดซึมแคลเซียมของลำไส้ในระดับเซลล์ และโมเลกุลเพิ่มเติมอีกด้วย เนื่องจากมีรายงานว่า โปรตีนขนส่งแคลเซียมที่ปรากฏอยู่ในตำราทั่วไปอาจไม่ใช่โปรตีนขนส่งแคลเซียมที่มีความสำคัญทางสรีรวิทยา ซึ่งทำให้โมเดลแบบจำลองของการดูดซึมแคลเซียมที่มีอยู่ในปัจจุบันขาดความชัดเจน และส่งผลต่อการแปลผลข้อมูลจากการวิจัย

8. วัตถุประสงค์

วัตถุประสงค์แบ่งเป็น 5 ส่วนตามปัญหาที่ทำการวิจัย ดังนี้

1. ศึกษากลไกการดูดซึมแคลเซียมในลำไส้ใหญ่ส่วนซีคัม ทั้งในภาวะปกติและในภาวะที่มีโปรแลคตินในเลือดสูงในหนูที่ไม่ได้ตั้งท้อง
2. ศึกษาผลของโปรแลคตินที่สูงขึ้นในระหว่างตั้งท้องและให้นมที่มีต่ออัตราการดูดซึมแคลเซียมของลำไส้ และศึกษาตำแหน่งออกฤทธิ์ของโปรแลคตินที่ให้ประสิทธิภาพสูงสุดในการดูดซึมแคลเซียม โดยเฉพาะที่ลำไส้เล็กส่วนต้นและลำไส้ใหญ่ส่วนซีคัม
3. ศึกษาว่าโปรแลคตินเป็นฮอร์โมนหลักที่ใช้เพิ่มการสร้างและการสลายกระดูก (high bone turnover) ในระหว่างตั้งครรภ์และให้นมหรือไม่
4. ศึกษาว่าโปรแลคตินที่สูงขึ้นส่งผลเสียต่อ microstructure ของกระดูกหรือไม่ และอย่างไร
5. พิสูจน์ว่าการให้แคลเซียมเสริมในช่วงที่ระดับโปรแลคตินสูงมากๆ เช่น ขณะให้นม จะช่วยลดผลกระทบที่มีต่อกระดูกหรือไม่

9. ระเบียบวิธีวิจัย

งานวิจัยชุดนี้ส่วนใหญ่ดำเนินการที่ห้องปฏิบัติการของภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ซึ่งเป็นสถานที่วิจัยหลักสำหรับวัดการดูดซึมแคลเซียม นอกจากนี้ยังมีการใช้ศูนย์เครื่องมือรวม (Central Instrument Facility: CIF) และห้องปฏิบัติการของเครือข่ายวิจัยด้านแคลเซียมและกระดูก คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

งานวิจัยนี้มีการใช้หนูขาว *Rattus norvegicus* สายพันธุ์ Sprague-Dawley จากสำนักสัตว์ทดลองแห่งชาติ ซึ่งได้รับการพิสูจน์แล้วว่า มีสายพันธุ์ที่ได้มาตรฐานระดับสากล และมีกลไกการดูดซึมแคลเซียมในลำไส้ใกล้เคียงกับที่พบในมนุษย์ [14] โครงการนี้ได้ผ่านความเห็นชอบจากคณะกรรมการจริยธรรมการใช้สัตว์ทดลองของคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล เป็นที่เรียบร้อยแล้ว

การวิจัยแบ่งเป็น 4 ส่วน (phase) ซึ่งสรุปได้ดังนี้

ส่วนที่ 1

ศึกษากลไกการดูดซึมแคลเซียมในลำไส้ใหญ่ส่วนซีคัม ทั้งในภาวะปกติและภาวะที่มีโปรแลคตินในเลือดสูงในหนูที่ไม่ได้ตั้งท้อง

สมมติฐาน โพรแลคตินกระตุ้นการดูดซึมแคลเซียมในลำไส้ใหญ่ส่วนซีคัมโดยมีอัตราการดูดซึมสูงกว่าที่พบในลำไส้เล็กส่วนต้น

สัตว์ทดลอง การทดลองนี้ใช้ลำไส้เล็กส่วนต้นและลำไส้ใหญ่ส่วนซีคัมของหนู Sprague-Dawley เพศเมียอายุ 8 สัปดาห์ที่ไม่ได้ตั้งท้อง โดยแบ่งหนูออกเป็น 3 กลุ่มหลัก คือกลุ่มควบคุม กลุ่มที่ได้รับฮอร์โมนโพรแลคติน และกลุ่มที่ได้รับ inhibitor ต่างๆ ซึ่งสามารถยับยั้งการทำงานของโพรแลคติน

ตัวแปรที่วัด มี 3 ตัวแปรหลัก คือ

1. อัตราการขนส่งแคลเซียมผ่านเยื่อผนังลำไส้
2. ตัวแปรทางไฟฟ้า ซึ่งจะบอกคุณสมบัติของเยื่อผนังลำไส้
3. Paracellular selectivity ซึ่งประกอบด้วย charge และ size selectivity

งานวิจัยในส่วนนี้ยังรวมถึงการศึกษากลไกการดูดซึมแคลเซียมของลำไส้ในระดับเซลล์และโมเลกุลในลำไส้ส่วนต่างๆ และ Caco-2 monolayer ด้วย

เทคนิค Ussing chamber ที่ใช้วัดตัวแปรทั้ง 3 ประเภทตลอดจนเครื่องมือและชุดซอฟต์แวร์ต่างๆ ได้รับการประดิษฐ์และพัฒนาขึ้นในห้องปฏิบัติการ ณ ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล โดย หัวหน้าโครงการและทีมวิศวกรของหน่วยห้องปฏิบัติการอเนกประสงค์ (MDL) คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ตั้งแต่ปี พ.ศ. 2542 โดยอิงตามทฤษฎีของ ศาสตราจารย์ Hans H. Ussing [30] เทคนิคและเครื่องมือที่ใช้ในปัจจุบันมีข้อดีกว่าผลิตภัณฑ์ที่ขายในต่างประเทศ คือ

1. มีปริมาตรน้อย คือ 6 mL ทำให้ประหยัดสารกัมมันตรังสี ฮอร์โมน และ inhibitor ที่ใช้
2. มีระบบที่ลดการปนเปื้อนของสารกัมมันตรังสี
3. มีระบบควบคุมความดันย่อยของก๊าซ (partial pressure)
4. มีราคาถูกกว่าผลิตภัณฑ์ของต่างประเทศประมาณ 10 เท่า

ส่วนที่ 2

ศึกษาผลของโพรแลคตินที่สูงขึ้นในระหว่างตั้งท้อง ให้นม และกำลังให้นม (ดูดนม) ที่มีต่ออัตราการดูดซึมแคลเซียมของลำไส้

สมมติฐาน โพรแลคตินที่สูงขึ้นในระหว่างตั้งท้องและให้นมกระตุ้นการดูดซึมแคลเซียมของลำไส้ ทำให้มีการเปลี่ยน baseline ของอัตราการดูดซึม เมื่อมีการดูดนมจะทำให้มีอัตราการดูดซึมแคลเซียมสูงขึ้นกว่าค่า baseline เป็นระยะๆ (ตัวอย่างเช่น ถ้าในหนูปกติมีอัตราการขนส่งแคลเซียมในลำไส้เล็กส่วนต้น 20 nmol/h/cm^2 เมื่อตั้งท้องและให้นมอาจมี baseline สูงขึ้นเป็น 40 nmol/h/cm^2 แต่ค่าเหล่านี้จะคงที่ทราบเท่าที่ระดับโพรแลคตินยังสูงคงที่ตลอด แต่เมื่อมีการดูดนม จะทำให้มีระดับโพรแลคตินสูงขึ้นเป็นระยะๆ และคาดว่าจะทำให้อัตราการขนส่งแคลเซียมเพิ่มขึ้นอีกเป็นระยะๆ ในสัดส่วนเดียวกันด้วย)

สัตว์ทดลอง การทดลองนี้ใช้ลำไส้เล็กส่วนต้นและลำไส้ใหญ่ส่วนซีคัมของหนู Sprague-Dawley ที่อยู่ในระยะตั้งท้อง (pregnancy) ระยะให้นม (lactation) และกำลังให้นม (suckling) สัตว์บางกลุ่มจะ

ได้รับสาร bromocriptine ซึ่งจะยับยั้งการหลั่งโพรแลคติน [5, 10] และบางกลุ่มจะได้ bromocriptine ร่วมกับฮอร์โมนโพรแลคตินสังเคราะห์ทดแทน

ตัวแปรที่วัด มี 3 ตัวแปรหลัก ดังที่กล่าวแล้วใน ระยะที่ 1

เทคนิค ใช้เทคนิค Ussing chamber

ส่วนที่ 3

ศึกษาว่าโพรแลคตินเป็นฮอร์โมนหลักที่ใช้เพิ่มการสร้างและการสลายกระดูก (high bone turnover) ในระหว่างตั้งครรภ์และให้นมหรือไม่ และมีผลต่อ microstructure ของกระดูกอย่างไร

สมมติฐาน โพรแลคตินที่สูงขึ้นในระหว่างตั้งครรภ์และให้นมกระตุ้นการสร้างและการสลายกระดูก และเปลี่ยน microstructure ของกระดูก

สัตว์ทดลอง การทดลองนี้ใช้กระดูกของหนู Sprague-Dawley ที่อยู่ในระยะตั้งครรภ์ (pregnancy) ระยะให้นม (lactation) และกำลังให้นม (suckling) สัตว์บางกลุ่มจะได้รับสาร bromocriptine ซึ่งจะยับยั้งการหลั่งโพรแลคตินจากต่อมใต้สมอง และบางกลุ่มจะได้ bromocriptine ร่วมกับฮอร์โมนโพรแลคตินสังเคราะห์ทดแทน

ตัวแปรที่วัด มี 3 ตัวแปรหลัก คือ

1. ความหนาแน่นกระดูก
2. ปริมาณแคลเซียมสุทธิในกระดูก
3. โครงสร้างทางจุลภาคของกระดูก

เทคนิค การวัดความหนาแน่นกระดูก ปริมาณแคลเซียมสุทธิในกระดูก และโครงสร้างของกระดูกในระดับจุลภาค ใช้เทคนิค dual energy X-ray densitometry (DXA), atomic absorption spectrophotometry และ bone histomorphometry ตามลำดับ

ส่วนที่ 4

ศึกษาว่าการให้แคลเซียมเสริมในช่วงที่ระดับโพรแลคตินสูงมากๆ จะช่วยลดผลกระทบที่มีต่อ microstructure ของกระดูกหรือไม่

สมมติฐาน การให้แคลเซียมเสริมในขณะที่มีระดับโพรแลคตินในเลือดสูง เพิ่มปริมาณแคลเซียมที่ดูดซึมเข้าสู่ร่างกายและช่วยยับยั้งการสลายกระดูก

สัตว์ทดลอง การทดลองนี้ใช้ลำไส้และกระดูกของหนู Sprague-Dawley ที่อยู่ในระยะตั้งครรภ์ (pregnancy) ระยะให้นม (lactation) และกำลังให้นม (suckling) สัตว์บางกลุ่มจะได้รับแคลเซียมเสริม 30 นาที่ก่อนให้นม (pre-suckling oral calcium supplementation)

10. สรุปประโยชน์ที่ได้จากงานวิจัย

ผลงานวิจัยจากโครงการนี้ได้ลงตีพิมพ์ในวารสารวิชาการระดับนานาชาติจำนวน 16 เรื่อง และมีผลกระทบใน 3 ระดับ ดังนี้

1. ระดับวิทยาศาสตร์พื้นฐาน

1.1 ทำให้ทราบว่านอกเหนือจากลำไส้เล็กส่วนต้นแล้ว ลำไส้ใหญ่ส่วนซีคัมซึ่งมีอัตราการดูดซึมแคลเซียมสูงกว่ามีกลไกการดูดซึมแคลเซียมอย่างไร องค์ความรู้พื้นฐานนี้มีประโยชน์ต่อสาขาวิชาสรีรวิทยาเป็นอย่างยิ่ง เพราะผลลัพธ์ที่ได้คือ ต้นแบบ (model) ของการขนส่งแคลเซียมในลำไส้ส่วนนี้ ซึ่งสามารถนำไปใช้ศึกษาผลของยา สมุนไพร หรือฮอร์โมนชนิดอื่นๆ ที่มีผลต่อการดูดซึมแคลเซียมได้ ปัจจุบันได้มีการใช้ต้นแบบนี้ขยายงานวิจัยไปสู่ปัญหาอื่น เช่น ภาวะเลือดเป็นกรด และภาวะขาดวิตามินดี ทำให้ผลิตงานวิจัยได้กว้างขวางขึ้น

1.2 ได้องค์ความรู้ใหม่ด้านสรีรวิทยาระบบต่อมไร้ท่อ โดยแสดงให้เห็นว่าโปรแลคตินสามารถกระตุ้นการดูดซึมแคลเซียมในลำไส้เล็กส่วนต้นและลำไส้ใหญ่ส่วนซีคัมได้ ทั้งในหนูปกติและหนูที่ตั้งท้อง

1.3 ได้หลักฐานยืนยันว่า ภาวะกระดูกบางในระหว่างให้นมเป็นผลมาจากฮอร์โมนโปรแลคตินที่สูงขึ้น และยังทำให้ทราบว่า ในระหว่างให้นมนั้นเกิดความเสียหายใน microstructure ของกระดูกค่อนข้างมาก ข้อมูลนี้ยังช่วยสนับสนุนว่า การศึกษากระดูกโดยปราศจากข้อมูลทาง microstructure (ใช้ X-ray เพียงอย่างเดียว) อาจทำให้การแปลผลการวิจัยคลาดเคลื่อนอีกด้วย

2. ระดับคลินิก

2.1 ข้อมูลเรื่องกลไกการดูดซึมแคลเซียมในลำไส้ที่ได้ อาจนำไปใช้พัฒนางานวิจัยเพื่อการพัฒนาและอาหารเสริมได้ เช่น อาจมีการพัฒนาเวชภัณฑ์เสริมแคลเซียมที่ให้ร่วมกับน้ำตาลบางชนิดเพื่อกระตุ้นการดูดซึมแคลเซียม (กลไกการดูดซึมแคลเซียมบางกลไกต้องอาศัยน้ำตาลโมเลกุลเดี่ยวเป็นตัวช่วย)

2.2 ได้ข้อมูลว่า ถ้าให้แคลเซียมเสริมในรูปแบบและในช่วงเวลาที่เหมาะสม จะช่วยป้องกันภาวะกระดูกบางในระหว่างตั้งครรภ์และให้นมได้ แม้ว่าผลที่ได้จากงานวิจัยเรื่องนี้จะยังไม่สามารถนำไปใช้กับหญิงตั้งครรภ์ หรือในอุตสาหกรรมโคนม (วัวนมที่กำลังให้น้ำนมอาจเกิดภาวะแคลเซียมต่ำจนเป็นอันตรายได้ [13, 23]) ได้โดยตรง แต่ความรู้ที่ได้ก็เป็นก้าวสำคัญในการวางแผนการวิจัยในมนุษย์ และในสัตว์เศรษฐกิจอื่นๆ ต่อไป

3. ระดับนโยบาย

ข้อมูลบางส่วนที่ได้จากงานวิจัยเรื่องนี้ หากได้รับการพิสูจน์ในมนุษย์ว่าเป็นจริงแล้ว อาจสามารถต่อยอดนำไปใช้ในระดับนโยบายได้ โดยเฉพาะถ้าการให้แคลเซียมเสริมสามารถป้องกันภาวะกระดูกบางได้ เพราะปัจจุบันมนุษย์อายุยืนขึ้น ทำให้พบโรคที่เกิดขึ้นในวัยชรา เช่น โรคกระดูก

พรุนมากขึ้น ประกอบกับค่าใช้จ่ายทางการแพทย์ที่มีแนวโน้มจะสูงขึ้นเรื่อยๆ แนวคิดเรื่องการรักษาโรคด้วยการป้องกันการเกิดโรคจึงน่าจะเป็นนโยบายทางสาธารณสุขที่มีประสิทธิภาพ และให้ประสิทธิผลสูงสุด ตัวอย่างเช่น อาจมีการรณรงค์ให้แม่ดื่มนมแคลเซียมสูงหนึ่งแก้ว หรือรับประทานแคลเซียมเสริม 30 นาทีก่อนให้นมลูก เพื่อให้โพรแลคตินที่จะหลั่งออกมามากขึ้นในขณะที่ให้นม กระตุ้นลำไส้ให้ดูดซึมแคลเซียมเข้าสู่ร่างกายเพิ่มขึ้น ผลคือร่างกายจะได้ไม่ต้องสลายกระดูกมากเกินไป และอาจช่วยชะลอภาวะกระดูกบางหรือกระดูกพรุนที่อาจเกิดขึ้นภายหลังได้

11. เอกสารอ้างอิง

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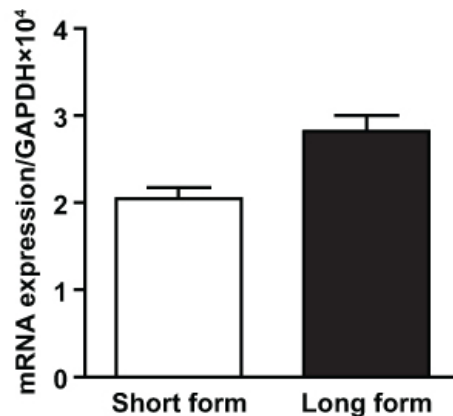
เนื้อหางานวิจัย

โครงการนี้เริ่มดำเนินการตั้งแต่ เดือนตุลาคม 2550 (เริ่มดำเนินการทันทีหลังส่งข้อเสนอทุน) และได้สิ้นสุดเดือนเมษายน 2554 เนื้อหางานวิจัยมีดังนี้

1. กลไกการดูดซึมแคลเซียมในลำไส้ใหญ่ส่วนซีคัม (cecum)

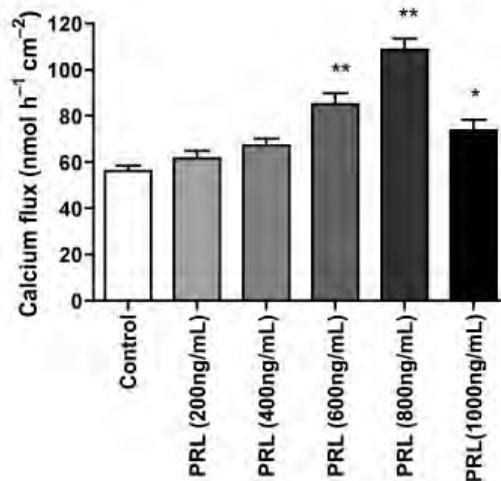
ลำไส้ใหญ่ส่วนซีคัมเป็นส่วนที่มีอัตราการดูดซึมแคลเซียมสูงสุด เมื่อเปรียบเทียบกับทางเดินอาหารส่วนอื่น ๆ อย่างไรก็ตามการดูดซึมแคลเซียมในลำไส้ส่วนนี้ยังมีผู้ศึกษาน้อย และผลของโพรแลคตินต่อลำไส้ใหญ่ส่วนซีคัมก็ยังไม่มีการศึกษาอย่างเป็นระบบ

จากการศึกษาด้วยเทคนิค real-time PCR คณะผู้วิจัยพบว่า เซลล์เยื่อบุของลำไส้ใหญ่ส่วนซีคัมสร้าง messenger RNA (mRNA) ของ prolactin receptor ทั้งแบบ short form และ long form (ภาพที่ 1) ซึ่งบ่งชี้ว่า ลำไส้ส่วนนี้สามารถตอบสนองต่อโพรแลคตินได้



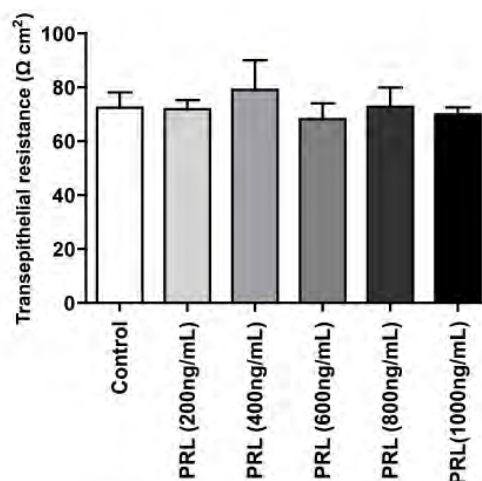
ภาพที่ 1 การแสดงออกของยีน prolactin receptor ซึ่งมีอยู่ 2 ประเภท คือ short form และ long form ในลำไส้ใหญ่ส่วนซีคัม จากการวิจัยพบว่า ลำไส้ใหญ่ส่วนซีคัมมีปริมาณ long form มากกว่า short form (n = 8)

จากการศึกษาอัตราการขนส่งแคลเซียมด้วย Ussing chamber technique พบว่า ฮอร์โมนโพรแลคตินสามารถเพิ่มอัตราการขนส่งแคลเซียมในลำไส้ใหญ่ส่วนซีคัมได้แบบ dose-dependent และเป็น bell-shaped โดยความเข้มข้นโพรแลคตินที่กระตุ้นให้เกิดการขนส่งแคลเซียมได้ดีที่สุด คือ 800 ng/mL (ภาพที่ 2) ที่การตอบสนองเป็นแบบ bell-shaped เนื่องจากโพรแลคติน 1 ตัว จะจับกับตัวรับ 2 ตัว (receptor dimerization) หากความเข้มข้นของโพรแลคตินมากเกินไปอาจทำให้ receptor dimerization เกิดขึ้นไม่ได้ เพราะโพรแลคตินจะจับกับตัวรับแบบ 1:1 แทน อนึ่ง ความเข้มข้นของโพรแลคตินที่ทำให้เกิดอัตราการดูดซึมแคลเซียมสูงสุด คือ 800 ng/mL ซึ่งเป็นความเข้มข้นที่อาจพบได้ในเลือดของแม่หนูที่กำลังให้ลูกดูดนม



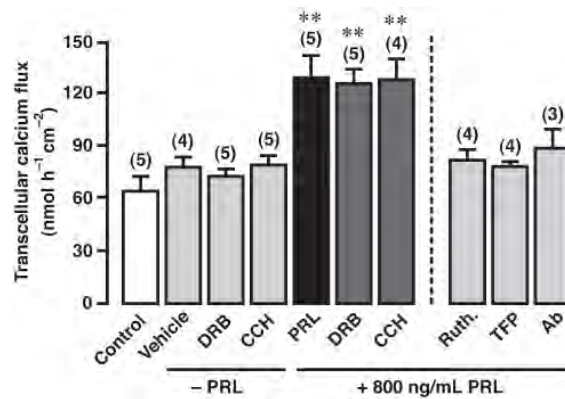
ภาพที่ 2 อัตราการขนส่งแคลเซียมในลำไส้ใหญ่ส่วนซีคัมเมื่อให้ลำไส้สัมผัสฮอร์โมนโพรแลคตินที่ความเข้มข้นต่าง ๆ นาน 60 นาที ** $P < 0.01$, * $P < 0.05$ เปรียบเทียบกับกลุ่มควบคุม ($n = 8-10$)

อย่างไรก็ตาม ฮอร์โมนนี้ไม่เปลี่ยนค่าความต้านทานของเนื้อเยื่อ ซึ่งเป็นตัวแปรตัวหนึ่งที่แสดงถึงการยอมให้สารผ่านช่องระหว่างเซลล์อย่างไม่เฉพาะเจาะจง หรือ non-selective (ภาพที่ 3) ผลการวิจัยแสดงให้เห็นว่า ฮอร์โมนโพรแลคตินไม่ทำให้แคลเซียมรั่วผ่านช่องระหว่างเซลล์แบบ non-selective (แต่ไม่ได้หมายความว่าแคลเซียมจะไม่ผ่านช่องระหว่างเซลล์แบบ charge-selective)



ภาพที่ 3 ค่าความต้านทาน (transepithelial resistance) ของลำไส้ใหญ่ส่วนซีคัมหลังสัมผัสฮอร์โมนโพรแลคตินที่ความเข้มข้นต่าง ๆ นาน 60 นาที ($n = 8-10$)

การศึกษากลไกการทำงานในระดับเซลล์โดยใช้ตัวยับยั้งประเภทต่าง ๆ บ่งชี้ว่า การออกฤทธิ์ของฮอร์โมนโพรแลคตินเป็นแบบ non-genomic เพราะไม่สามารถยับยั้งได้ด้วยตัวยับยั้ง gene transcription (DRB) และ protein synthesis (CCH) ดังภาพที่ 4 อย่างไรก็ตาม สารที่ลดการขนส่งแคลเซียมผ่าน brush-border membrane (ruthenium red; Ruth.) และ basolateral membrane (TFP) ตลอดจนแอนติบอดีที่ลดการทำงานของตัวรับโพรแลคติน (Ab) สามารถยับยั้งผลของโพรแลคตินได้

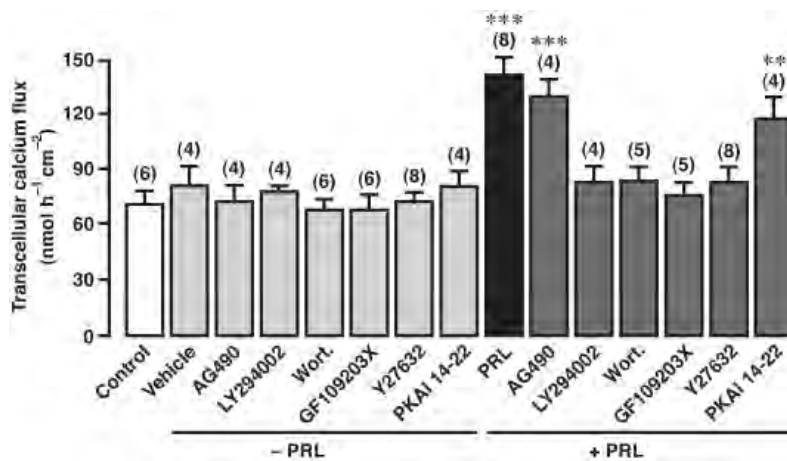


ภาพที่ 4 ผลของตัวยับยั้งประเภทต่าง ๆ ต่อการออกฤทธิ์ของฮอร์โมนโพรแลคตินเพื่อกระตุ้นการขนส่งแคลเซียมแบบผ่านเซลล์ ตัวเลขในวงเล็บแสดงจำนวนสัตว์ทดลอง ** $P < 0.01$ เปรียบเทียบกับกลุ่มควบคุม

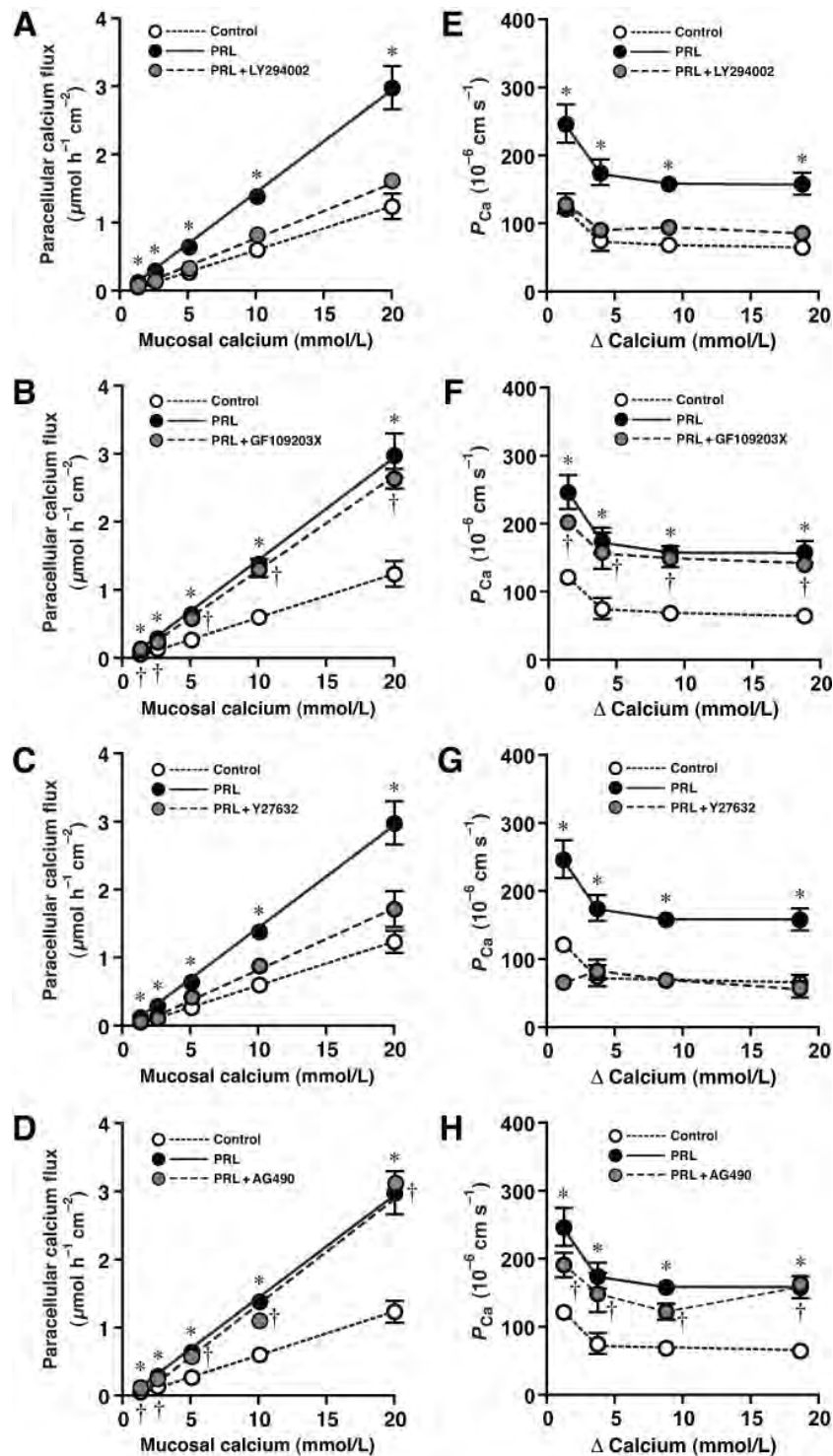
นอกจากนี้คณะผู้วิจัยยังพบว่า ผลของโพรแลคตินที่ล้าใส่ให้ใหญ่ส่วนซีกมจำเป็นต้องใช้โปรตีนเพื่อส่งสัญญาณภายในเซลล์จำนวนหนึ่ง ดังนี้

- การกระตุ้นการขนส่งแบบผ่านเซลล์ต้องใช้ PI3K, PKC และ ROCK (ภาพที่ 5)
- การกระตุ้นการขนส่งแบบผ่านช่องระหว่างเซลล์ต้องใช้ PI3K และ ROCK (ภาพที่ 6)

ตัวยับยั้งที่ใช้ไม่มีผลต่อการดูดซึมแคลเซียมของซีกมในภาวะปกติ หรืออีกนัยหนึ่งคือ ตัวยับยั้งเหล่านี้มีผลต่อการดูดซึมแคลเซียมที่ถูกกระตุ้นโดยโพรแลคตินเท่านั้น



ภาพที่ 5 ผลของตัวยับยั้งสำหรับ Janus kinase (AG490), PI3K (LY294002 และ wortmannin), PKC (GF109203X), ROCK (Y27632) และ protein kinase A (PKAI 14-22) ต่อการออกฤทธิ์ของโพรแลคตินที่กระตุ้นการขนส่งแคลเซียมแบบผ่านเซลล์ สาร vehicle ที่ใช้เพื่อละลายสารยับยั้งคือ 0.3% DMSO "-PRL" หมายถึง ซีกมที่ไม่สัมผัสกับโพรแลคติน "+PRL" หมายถึง ซีกมที่สัมผัสกับ 800 ng/mL PRL โดยตรงตลอด 80 นาทีใน Ussing chamber ตัวเลขในวงเล็บแสดงจำนวนสัตว์ทดลอง ** $P < 0.01$, *** $P < 0.001$ เปรียบเทียบกับกลุ่มควบคุม



ภาพที่ 6 ผลของตัวยับยั้งสำหรับ Janus kinase (AG490), PI3K (LY294002), PKC (GF109203X) และ ROCK (Y27632) ต่อการออกฤทธิ์ของโพรแลคตินที่กระตุ้นการขนส่งแคลเซียมแบบผ่านช่องระหว่างเซลล์ และคุณสมบัติการยอมให้แคลเซียมผ่านช่องระหว่างเซลล์ (P_{Ca}) ของลำไส้ใหญ่ส่วนซีคัม ($n = 4-5$) * $P < 0.01$ กลุ่ม PRL เปรียบเทียบกับกลุ่มควบคุม † $P < 0.01$ กลุ่ม PRL+ตัวยับยั้ง เปรียบเทียบกับกลุ่มควบคุม

2. ผลของโปรแลคตินที่สูงขึ้นในระหว่างตั้งท้องและให้นม

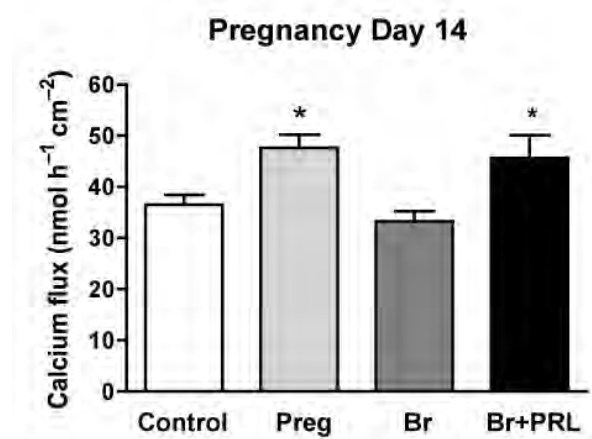
จากงานวิจัยที่ผ่านมาทำให้ทราบว่า โปรแลคตินซึ่งเป็นฮอร์โมนมีความเข้มข้นในเลือดสูงขึ้นในขณะตั้งท้องและให้นมสามารถกระตุ้นการดูดซึมแคลเซียมได้จากทั้งลำไส้เล็กส่วนต้น (duodenum) และจากลำไส้ใหญ่ส่วนซีกัม ข้อมูลนี้สนับสนุนสมมติฐานที่ว่า โปรแลคตินเป็นฮอร์โมนที่ใช้เพิ่มการดูดซึมแคลเซียมในระหว่างตั้งครรภ์และให้นม ทำให้สมดุลแคลเซียมของแม่ไม่ติดลบมากนัก

จากการใช้เทคนิค Ussing chamber วัดอัตราการขนส่งแคลเซียมในลำไส้เล็กส่วนต้น ทางคณะผู้วิจัยพบว่า โปรแลคตินเพิ่มอัตราการดูดซึมแคลเซียมในหนูที่ตั้งท้องวันที่ 14 (ภาพที่ 7) และให้นมวันที่ 14 ได้ (ภาพที่ 8) การทดลองในหนูท้องตามภาพที่ 7 แบ่งออกเป็น 4 กลุ่มคือ

1. Age-matched controls เป็นหนูปกติที่ไม่ตั้งท้อง
2. Pregnant rats (Preg) เป็นหนูที่ตั้งท้องวันที่ 14
3. Pregnant rats ที่ได้รับการฉีดยา bromocriptine (Br) ซึ่งเป็นยาที่ยับยั้งการหลั่งโปรแลคตินจากต่อมใต้สมอง นาน 7 วัน ก่อนการวัดอัตราการขนส่งแคลเซียม

4. Pregnant rats ที่ได้รับ Br และฉีดโปรแลคติน (PRL) ทดแทนนาน 7 วัน

งานวิจัยในหนูท้องแสดงให้เห็นอย่างชัดเจนว่า โปรแลคตินเป็นฮอร์โมนหลักที่ใช้กระตุ้นการขนส่งแคลเซียมของลำไส้เล็กส่วนต้นของหนูที่ตั้งท้อง

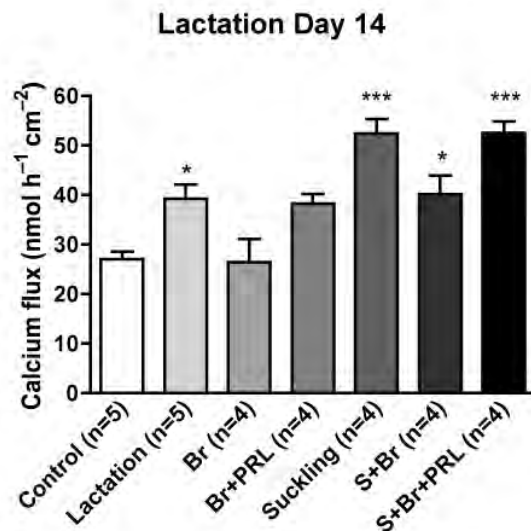


ภาพที่ 7 อัตราการขนส่งแคลเซียมในลำไส้เล็กส่วนต้นของหนูที่ตั้งท้อง 14 วัน * $P < 0.05$ เปรียบเทียบกับกลุ่มควบคุม ($n = 8-10$); Preg = pregnancy, Br = bromocriptine-treated rat

การทดลองในหนูให้นมตามภาพที่ 8 แบ่งออกเป็น 7 กลุ่มคือ

1. Age-matched controls เป็นหนูปกติที่ไม่ตั้งท้อง (และไม่ให้นม)
2. Lactation เป็นหนูที่ให้นมวันที่ 14 ซึ่งมีระดับโปรแลคตินในเลือดเฉลี่ยประมาณ 200–300 ng/mL
3. Lactating rats ที่ได้รับการฉีดยา bromocriptine (Br) ซึ่งเป็นยาที่ยับยั้งการหลั่งโปรแลคตินจากต่อมใต้สมอง นาน 7 วัน ก่อนการวัดอัตราการขนส่งแคลเซียม
4. Lactating rats ที่ได้รับ Br และฉีดโปรแลคติน (PRL) ทดแทนนาน 7 วัน

5. Lactating rats ที่ให้ลูกดูดนม (suckling) การดูดนมเป็นการเพิ่มระดับโพรแลคตินในแม่หนูแบบทันทีทันใด (acute) ให้สูงกว่าเดิมอีกประมาณ 2 เท่า (~400–650 ng/mL)
6. Lactating rats ที่ให้ลูกดูดนม (S) และฉีด Br แบบ single dose ก่อนการดูดนม 15 นาที
7. Lactating rats ที่ให้ลูกดูดนม (S) และฉีด Br และ PRL แบบ single dose ก่อนการดูดนม 15 นาที



ภาพที่ 8 อัตราการขนส่งแคลเซียมในลำไส้เล็กส่วนต้นของหนูที่ให้นมวันที่ 14 *** $P < 0.001$, * $P < 0.05$ เปรียบเทียบกับกลุ่มควบคุม; S = suckling rat, Br = bromocriptine-treated rat

การวิจัยในหนูให้ผลสรุปได้ว่า

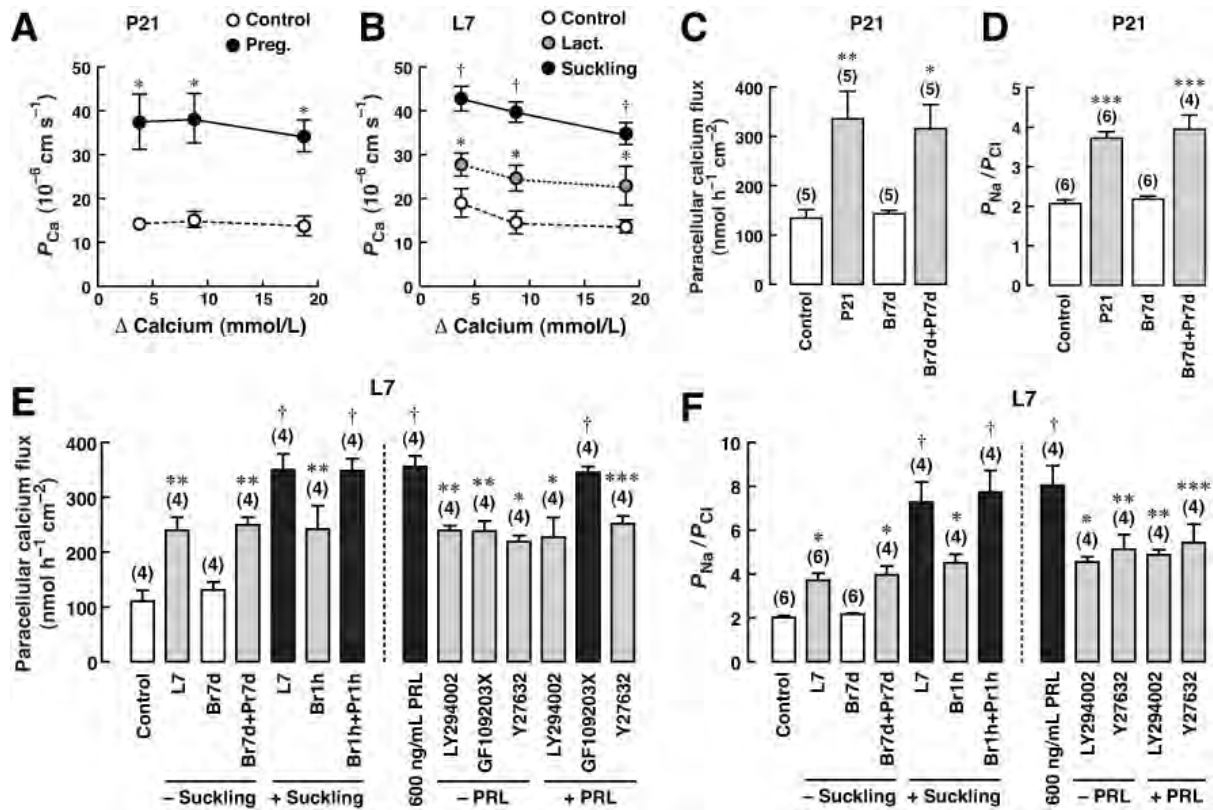
1. ผลของโพรแลคตินมี 2 แบบ คือ ผลแบบ chronic ซึ่งต้องสัมผัสโพรแลคตินเป็นเวลานาน (หมายถึงช่วง lactation ปกติ ที่มีระดับโพรแลคตินในเลือดสูงเฉลี่ย 200–300 ng/mL) และผลแบบ acute ซึ่งต้องสัมผัสโพรแลคตินที่มีความเข้มข้นสูงมาก ๆ ในช่วงเวลาสั้น ๆ ระหว่าง suckling ซึ่งมีโพรแลคติน ~650 ng/mL

2. ผลของโพรแลคตินแบบ acute เป็นผลชั่วคราว ซึ่งสามารถยับยั้งได้ด้วย single-dose bromocriptine แต่ single-dose bromocriptine ไม่สามารถยับยั้งผลแบบ chronic ได้ ผลแบบ acute จะเกิดขึ้นหลังโพรแลคตินเพิ่มขึ้นในเลือดจากการดูดนมได้ประมาณ 30 นาที ซึ่งทำให้คาดการณ์ได้ว่า หากให้แม่หนูกินแคลเซียมเสริมในช่วง 15–30 นาทีก่อนให้นมลูก น่าจะทำให้มีปริมาณแคลเซียมเข้าสู่ร่างกายสูงสุด

ผลแบบ chronic คาดว่าเป็นการปรับตัวของเซลล์เยื่อบุลำไส้ในระดับ molecular และ genomic ซึ่งจำเป็นต้องศึกษาถึงกลไกโดยใช้เทคนิคทาง bioinformatics และ molecular biology

อนึ่ง การศึกษาคุณสมบัติการยอมให้ผ่าน (permeability) ของผนังลำไส้ ซึ่งเป็นตัวแปรที่ทำให้ทราบว่าจะแคลเซียมผ่านผนังลำไส้ด้วยกลไกใด โดยอาศัยเทคนิค dilution potential และ permeability measurement ที่ได้พัฒนาขึ้นภายในห้องปฏิบัติการของคณะผู้วิจัย ได้แสดงให้เห็นว่า โพรแลคตินทำให้

ช่องระหว่างเซลล์ยอมให้อิออนประจุบวก (รวมถึงแคลเซียม) ผ่านได้มากยิ่งขึ้น โดยเฉพาะในช่วงหลังให้ลูกดูดนม และเป็นผลให้มีการขนส่งแคลเซียมผ่านช่องระหว่างเซลล์เพิ่มมากขึ้น (ภาพที่ 9)

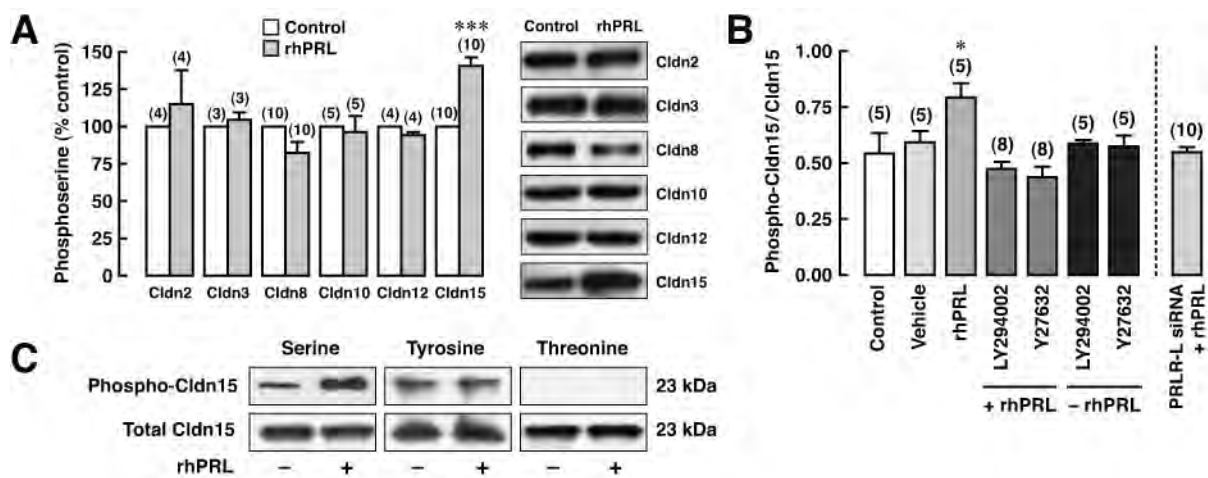


ภาพที่ 9 (A-B) การยอมให้แคลเซียมผ่านช่องระหว่างเซลล์ (P_{Ca}) ในลำไส้เล็กส่วนต้นของหนูที่ท้อง 21 วัน (P21) และในนม 7 วัน (L7) (C-D) อัตราการขนส่งแคลเซียมผ่านช่องระหว่างเซลล์ และการยอมให้ประจุบวกผ่านช่องระหว่างเซลล์ (P_{Na}/P_{Cl}) ในลำไส้ของหนูท้อง 21 วัน (E-F) อัตราการขนส่งแคลเซียมผ่านช่องระหว่างเซลล์ และการยอมให้ประจุบวกผ่านช่องระหว่างเซลล์ในลำไส้ของหนูให้นม 7 วัน ผลของโปรแลคตินบางส่วนต้องอาศัยโปรตีน PI3K, PKC และ ROCK *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ เปรียบเทียบกับกลุ่มควบคุม † $P < 0.05$ เปรียบเทียบกับกลุ่ม L7

นอกจากนี้คณะผู้วิจัยได้ดำเนินการวิจัยเพิ่มเติมเพื่อศึกษาว่า

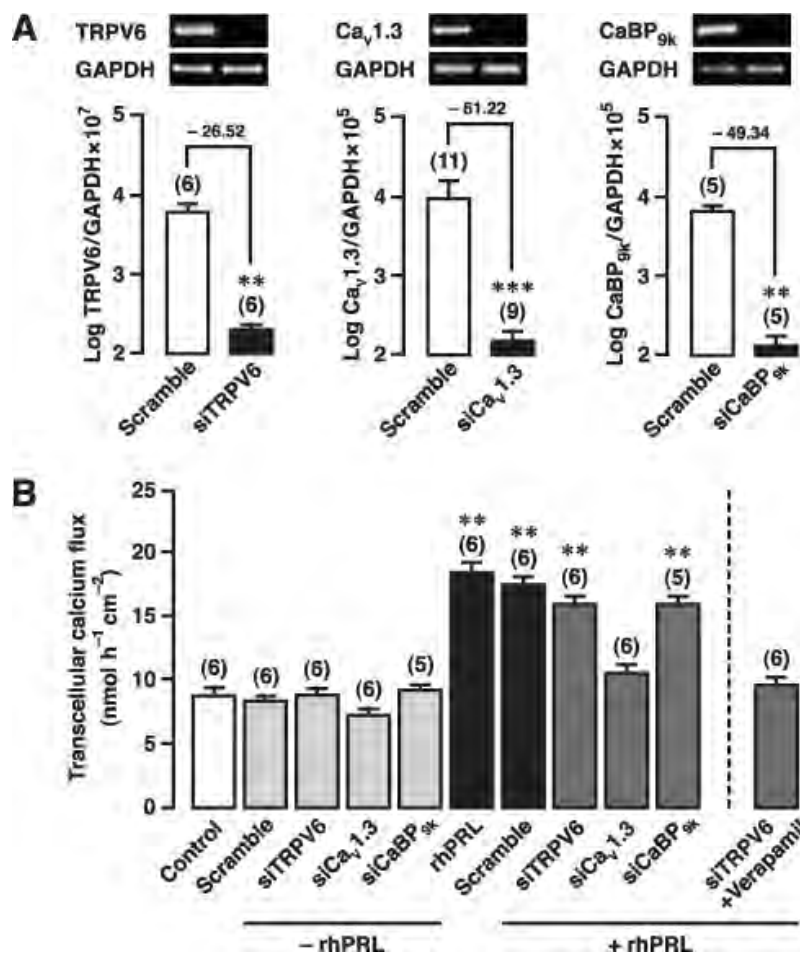
1. โปรแลคตินเพิ่มการขนส่งแคลเซียมแบบผ่านช่องระหว่างเซลล์ได้อย่างไร โดยมุ่งศึกษาโปรตีนในกลุ่ม "คลอดิน" (claudin) ซึ่งมีคุณสมบัติช่วยคัดแยกประจุที่ช่องระหว่างเซลล์ จากการวิจัยพบว่าอัตราการขนส่งแคลเซียมผ่านช่องระหว่างเซลล์ที่เพิ่มสูงขึ้นน่าจะเกี่ยวข้องกับการเติมหมู่ฟอสเฟต (phosphorylation) ให้กับโปรตีน claudin-15 (ภาพที่ 10) ผลงานวิจัยยังแสดงว่า การเติมหมู่ฟอสเฟตนี้

จำเป็นต้องอาศัยการทำงานของเอนไซม์ PI3K และ ROCK ซึ่งสอดคล้องกับผลการวิจัยที่ได้จากการศึกษาในลำไส้ส่วนซีคัม



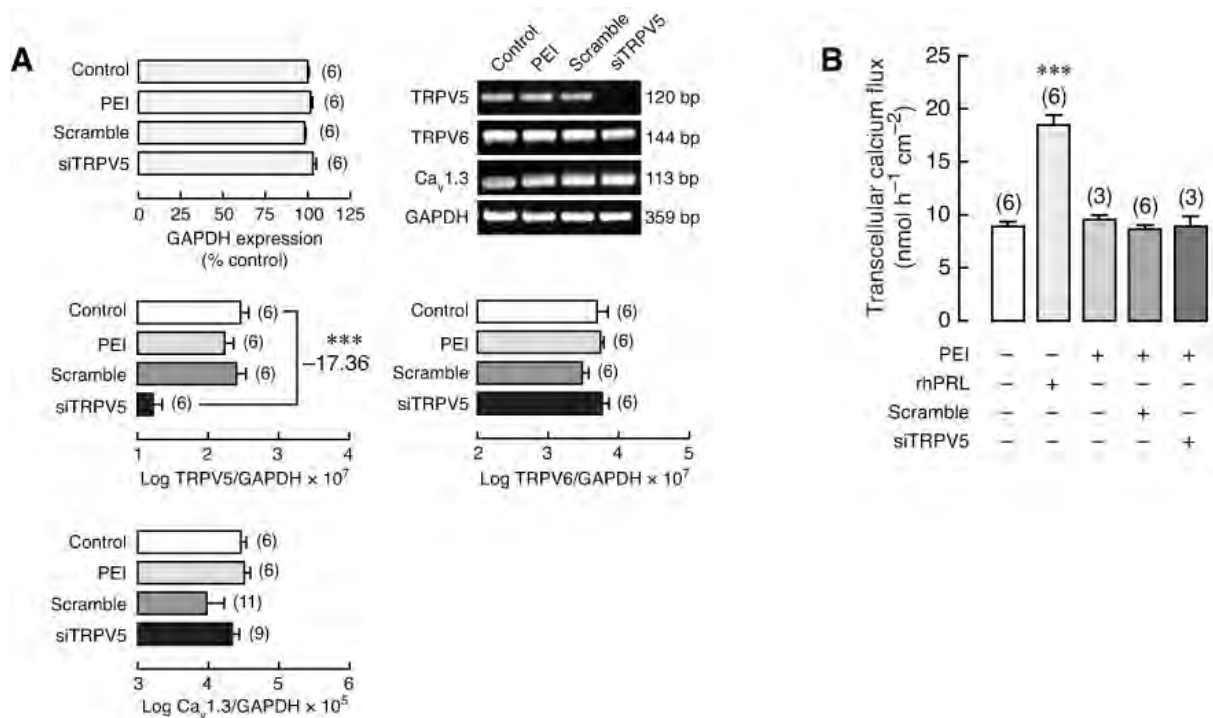
ภาพที่ 10 (A) แสดงระดับของ phosphorylated serine ของ claudin-15 ที่เพิ่มขึ้นโดยโพรแลคตินในเซลล์ Caco-2 (B) การเติมหมู่ฟอสเฟตที่ claudin-15 สามารถยับยั้งได้ด้วยสารที่ยับยั้งการทำงานของ PI3K และ ROCK สอดคล้องกับข้อมูลที่พบในลำไส้ใหญ่ส่วนซีคัม (C) แสดงให้เห็นว่าโพรแลคตินมีผลเพิ่มระดับของ phosphorylated serine ของ claudin-15 เท่านั้น แต่กลับไม่มีผลต่อระดับของ phosphorylated tyrosine และ threonine *** $P < 0.001$, * $P < 0.05$ เปรียบเทียบกับกลุ่มควบคุม

2. โพรแลคตินเพิ่มการขนส่งแคลเซียมแบบผ่านเซลล์ได้อย่างไร การศึกษาโดยใช้เทคนิค microarray แสดงให้เห็นว่า โพรแลคตินไม่น่าจะใช้โปรตีนขนส่งแคลเซียมชนิดปกติ (เช่น TRPV5 หรือ TRPV6) การวิจัยนำร่องโดยใช้เซลล์เพาะเลี้ยง Caco-2 พบว่า โพรแลคตินกระตุ้นการขนส่งแคลเซียมแบบผ่านเซลล์โดยอาศัยโปรตีนขนส่งแคลเซียมชื่อ L-type voltage-gated calcium channel $Ca_v1.3$ งานวิจัยในส่วนนี้ได้รับเงินทุนสนับสนุนเพิ่มเติมบางส่วนจากโครงการปริญญาเอกกาญจนาภิเษกของนายณรงค์ฤทธิ์ ทองอ่อน และทุนเมธีวิจัยอาวุโส สกว. ของ ศาสตราจารย์นันทิพย์ กฤษณามระ การต่อยอดงานวิจัยในส่วนนี้โดยใช้เทคนิค small interfering RNA (siRNA) พบว่า calcium channel ชนิด $Ca_v1.3$ มีความสำคัญต่อการทำงานของโพรแลคติน กล่าวคือ ผลของโพรแลคตินที่เพิ่มอัตราการขนส่งแคลเซียมจะไม่เกิดขึ้นหาก mRNA ของ $Ca_v1.3$ ถูกทำลาย (ภาพที่ 11)



ภาพที่ 11 (A) แสดงให้เห็นว่าการ knockdown ด้วย siRNA ที่จำเพาะต่อ TRPV6, $Ca_v1.3$ และ calbindin- D_{9k} ทำให้ระดับ mRNA ของยีนเหล่านั้นลดลง (B) อัตราการขนส่งแคลเซียมโดย Caco-2 monolayer (เป็นเซลล์ลำไส้เพาะเลี้ยงที่มีคุณสมบัติคล้ายลำไส้จริง) จากการกระตุ้นด้วยโพรแลคตินจะไม่เพิ่มขึ้นหลังการ knockdown ด้วย siRNA ที่จำเพาะต่อ $Ca_v1.3$ *** $P < 0.001$, ** $P < 0.01$ เปรียบเทียบกับกลุ่ม scramble (เป็นกลุ่มที่ให้เซลล์สัมผัสกับ siRNA ที่ไม่จับกับสาย mRNA ใด ๆ ภายในเซลล์)

3. การศึกษาโดยใช้เทคนิค siRNA ในเซลล์เยื่อบุเพาะเลี้ยง Caco-2 ยังช่วยยืนยันว่า ผลเฉียบพลันของโพรแลคตินอาศัย $Ca_v1.3$ เท่านั้น ไม่จำเป็นต้องใช้ TRPV5 และ TRPV6 ทั้งนี้เพราะโพรแลคตินสามารถกระตุ้นการขนส่งแคลเซียมได้แม้เซลล์จะไม่มีโปรตีน TRPV5 และ/หรือ TRPV6 แต่การขนส่งแคลเซียมไม่อาจเกิดขึ้นได้หากปราศจาก $Ca_v1.3$ (ภาพที่ 12-13)

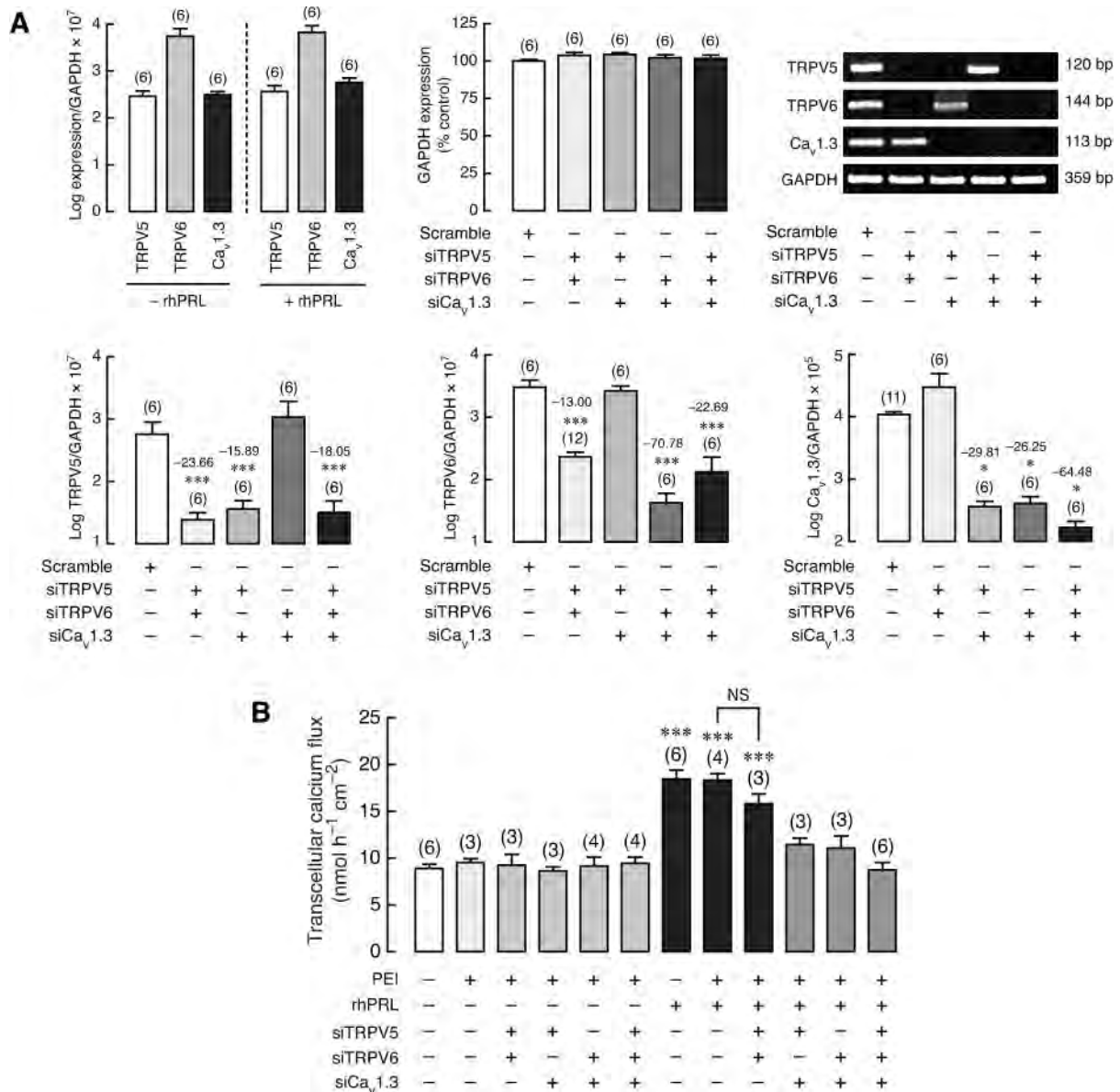


ภาพที่ 12 (A) แสดงให้เห็นว่า การแสดงออกของยีน GAPDH (housekeeping gene) เท่ากันในทุกกลุ่ม การทดลอง ส่วน siRNA ที่จำเพาะต่อ TRPV5 ลดการแสดงออกของ TRPV5 เท่านั้น แต่ไม่มีผลต่อ TRPV6 และ Ca_v1.3 (B) โพรแลคตินขนาด 600 ng/mL สามารถกระตุ้นการขนส่งแคลเซียมได้แม้เซลล์ จะไม่มี TRPV5 ซึ่งยืนยันว่าโปรตีนขนส่งแคลเซียมชนิดนี้ไม่จำเป็นต่อการทำงานแบบเฉียบพลันของ โพรแลคติน; PEI เป็น transfectant ที่ใช้นำ siRNA เข้าสู่เซลล์ *** $P < 0.001$ เปรียบเทียบกับกลุ่ม Control ตัวเลขในวงเล็บแสดงจำนวน Caco-2 monolayer ที่ใช้

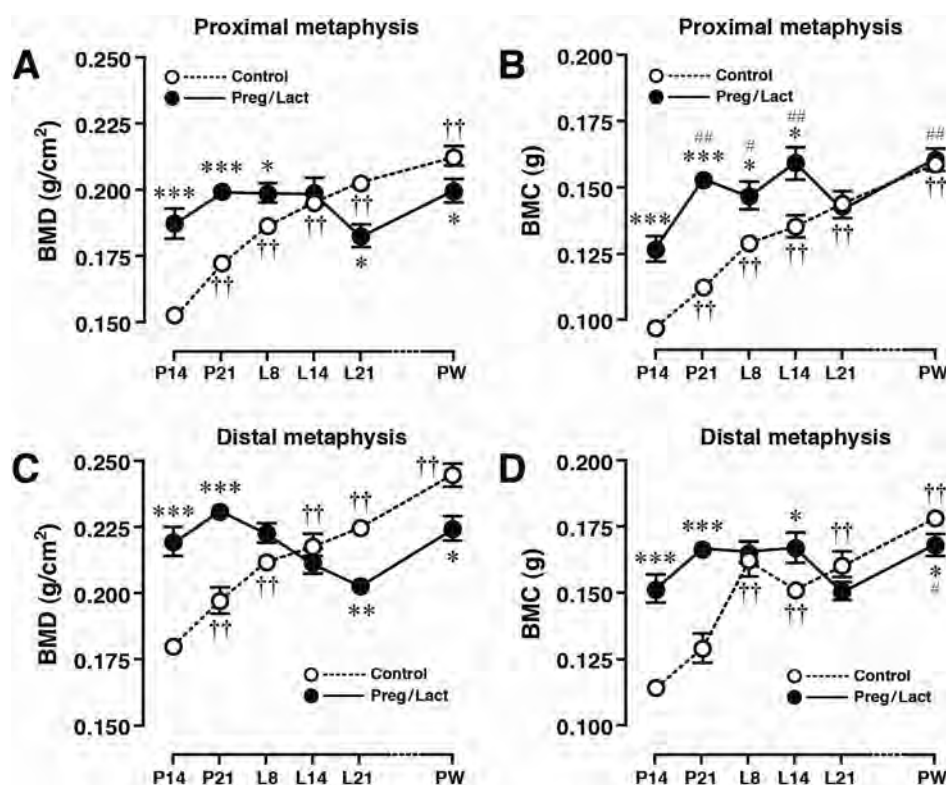
3. โพรแลคตินเป็นฮอร์โมนหลักที่ใช้เพิ่มการสร้างและการสลายกระดูก (high bone turnover) ในระหว่างตั้งครรภ์และให้นม

งานวิจัยนี้แบ่งออกเป็น 2 ส่วน คือ

1. การวัดความหนาแน่นของกระดูกโดยใช้เทคนิค dual energy X-ray absorptiometry [ใช้วัด bone mineral density (BMD) และ bone mineral content (BMC)] โดยศึกษาในหนูที่ตั้งท้องและ ให้นม ผลการวิจัยโดยใช้เครื่องวัดความหนาแน่นกระดูกบ่งชี้ว่า มวลกระดูกจะเพิ่มขึ้นในระหว่างตั้งท้อง แต่กลับลดลงในช่วงปลายของการให้นม (ภาพที่ 14) สาเหตุที่มวลกระดูกเพิ่มขึ้นในระหว่างตั้งท้องอาจ เป็นเพราะร่างกายต้องการสะสมแคลเซียมให้มากพอเพื่อนำมาใช้สร้างน้ำนมในระหว่างให้นมลูก



ภาพที่ 13 (A) เซลล์ Caco-2 ไม่เพิ่มการสังเคราะห์ mRNA ของ TRPV5, TRPV6 และ $Ca_v1.3$ หลังสัมผัสกับ 600 ng/mL recombinant human prolactin (rhPRL) นาน 1 ชั่วโมง นอกจากนี้ การแสดงออกของ GAPDH ไม่เปลี่ยนแปลงภายหลังได้รับ siRNA ชนิดต่าง ๆ ซึ่ง siRNA แต่ละชนิดจะมีผลต่อยีนที่เป็นเป้าหมายเท่านั้น **(B)** เซลล์ที่ปราศจาก TRPV5 และ/หรือ TRPV6 ยังคงมีอัตราการขนส่งแคลเซียมสูงขึ้นภายหลังได้รับโพรแลคติน ส่วนเซลล์ที่ปราศจาก $Ca_v1.3$ ไม่สามารถเพิ่มการขนส่งแคลเซียมได้ * $P < 0.05$, *** $P < 0.001$ เปรียบเทียบกับกลุ่ม Control หรือ Scramble ตัวเลขในวงเล็บแสดงจำนวน Caco-2 monolayer ที่ใช้

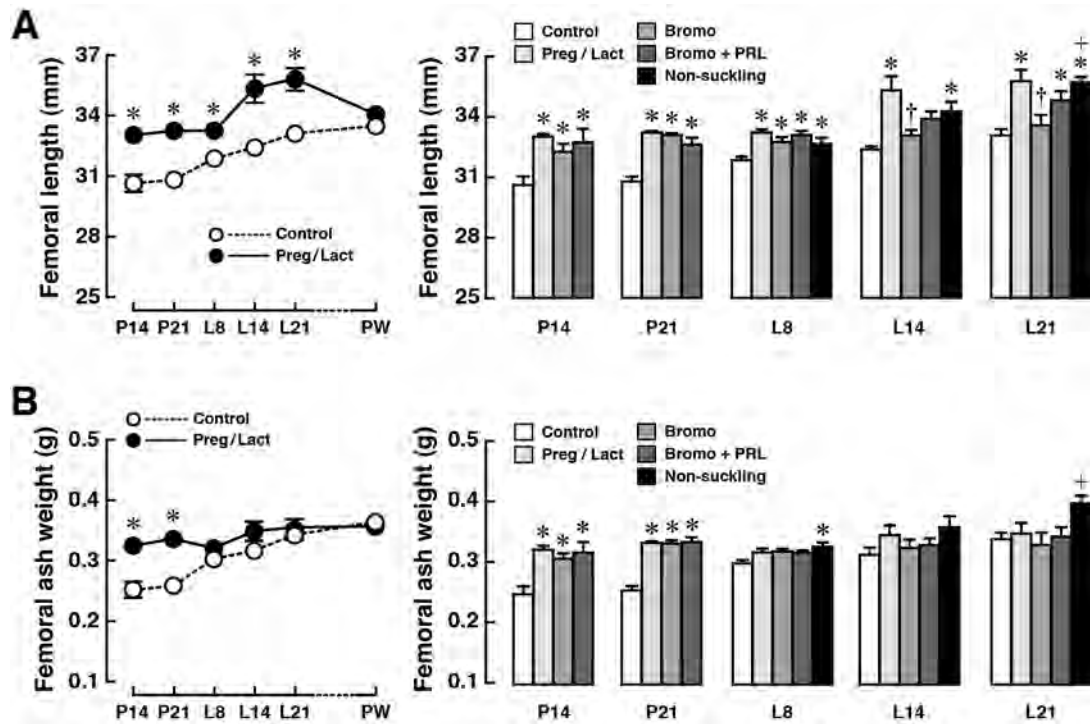


ภาพที่ 14 การเปลี่ยนแปลง bone mineral density (BMD) และ bone mineral content (BMC) ของแม่หนูในระหว่างตั้งท้อง (P) 14 และ 21 วัน, ระหว่างให้นม (L) 8, 14 และ 21 วัน และ 15 วันหลังหย่านม (PW) ค่า BMD และ BMC ของหนูกลุ่มควบคุมจะเพิ่มขึ้นตามอายุ *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ เปรียบเทียบกับกลุ่มควบคุม (age-matched control) †† $P < 0.01$ เปรียบเทียบกับกลุ่มควบคุมที่ P14

2. การศึกษาอัตราการสร้างและสลายกระดูกด้วยเทคนิค bone histomorphometry และ atomic absorption spectrophotometry พบว่า (จำนวนสัตว์ทดลอง 6–10 ตัว/กลุ่ม)

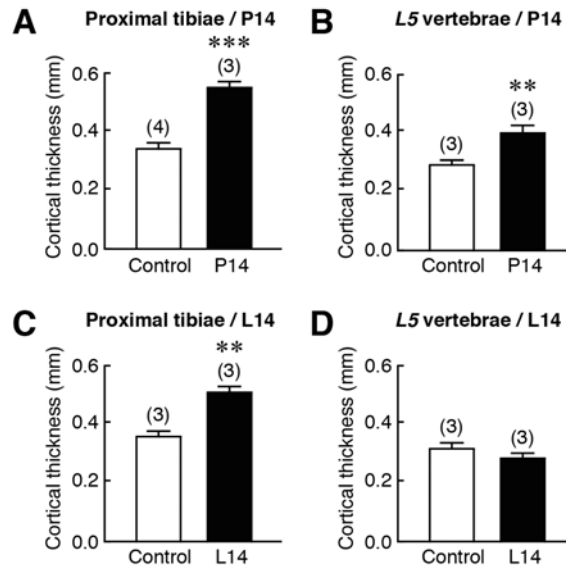
- ค่า total calcium content และ total magnesium content ของกระดูกขา มีค่าคงที่ตลอดช่วงการตั้งท้องและให้นม

- กระดูกต้นขา (femur) ของหนูที่ท้องและให้นมมีความยาวเพิ่มขึ้น (ภาพที่ 15) ซึ่งอาจเป็นเพราะมีการสะสมแคลเซียมที่กระดูกเพิ่มขึ้น และ/หรือมีการกระตุ้นการทำงานของ growth plate ซึ่งเป็นโครงสร้างที่มีความสำคัญต่อ endochondral bone formation และ bone elongation (โปรดดูรายละเอียดเพิ่มเติมในข้อ 7)

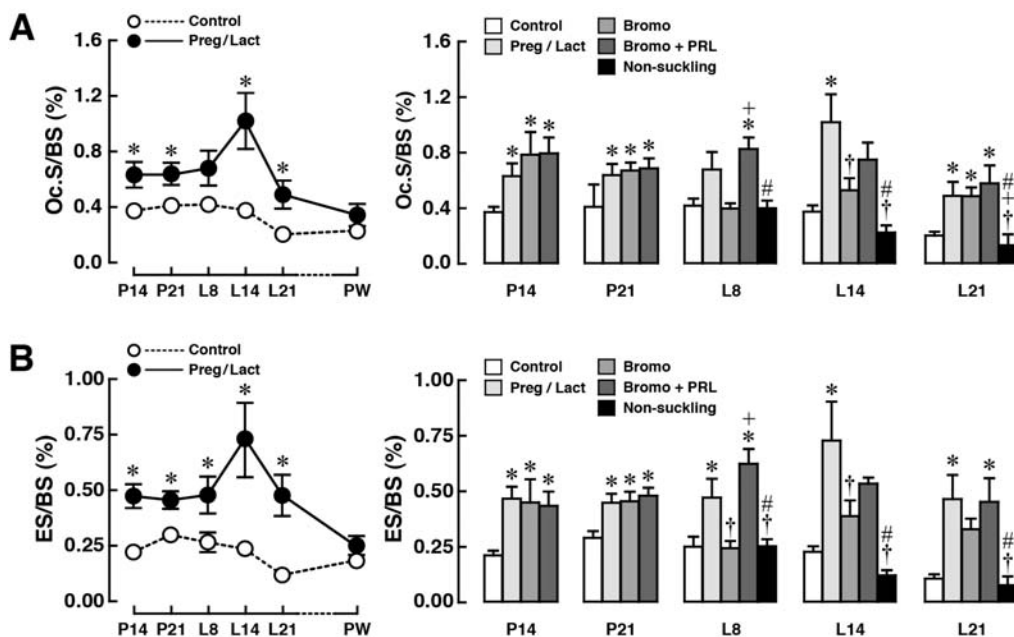


ภาพที่ 15 การเปลี่ยนแปลงความยาวกระดูกต้นขาและน้ำหนักกระดูกต้นขาของแม่หนูในระหว่างตั้งท้อง (P) 14 และ 21 วัน, ระหว่างให้นม (L) 8, 14 และ 21 วัน และ 15 วันหลังหย่านม (PW) แม่หนูบางกลุ่มจะได้รับการฉีดยา bromocriptine เพื่อลดการหลั่งโพรแลคติน และ/หรือได้รับการฉีดโพรแลคตินเสริม กลุ่ม Non-suckling หมายถึงกลุ่มแม่หนูที่นำลูกหนูออกไปตั้งแต่หลังคลอดเพื่อไม่ให้เกิดการดูดนมเกิดขึ้น * $P < 0.05$ เปรียบเทียบกับกลุ่มควบคุม (age-matched control) [†] $P < 0.05$ เปรียบเทียบกับกลุ่ม Preg/Lact ⁺ $P < 0.05$ เปรียบเทียบกับกลุ่ม Bromo

- ค่า cortical thickness ของกระดูก tibia จะเพิ่มขึ้นในขณะตั้งท้อง และหนาขึ้นต่อเนื่องจนถึงระยะให้นม (ภาพที่ 16) ซึ่งสันนิษฐานว่าเป็นกลไกที่ช่วยเสริมความแข็งแรงของกระดูกเพื่อไม่ให้กระดูกของแม่หักจากการสูญเสียแคลเซียมจำนวนมากจาก trabeculae ของกระดูก
- ค่า bone volume ของกระดูก trabeculae จะค่อนข้างคงที่ในขณะตั้งท้อง แต่กลับลดลงอย่างรวดเร็วในขณะให้นม ซึ่งส่วนหนึ่งเป็นผลมาจากการทำงานของโพรแลคติน ซึ่งมีผลกระตุ้นการสลายกระดูกโดยเซลล์สลายกระดูก (osteoclast) (ภาพที่ 17)

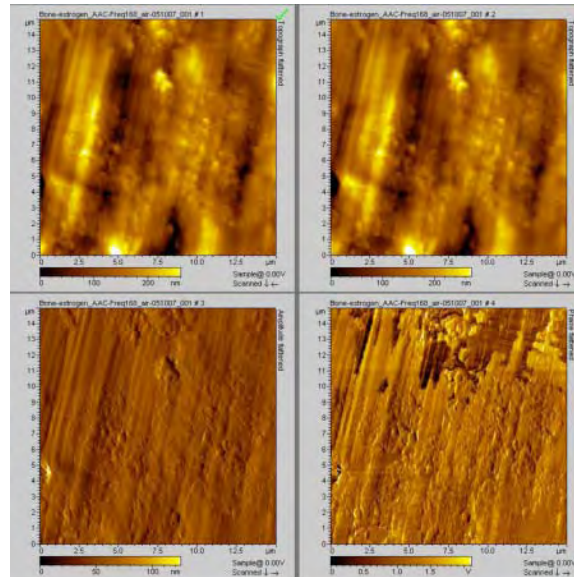


ภาพที่ 16 ความหนาของ cortex ของกระดูก tibia และ L5 vertebra ในหนูที่ท้องและให้นม *** $P < 0.001$, ** $P < 0.01$ เปรียบเทียบกับกลุ่มควบคุม



ภาพที่ 17 ตัวแปรที่บ่งชี้ถึงการทำงานของเซลล์สลายกระดูก [osteoclast surface (Oc.S/BS) และ eroded surface (ES/BS)] ของแม่หนูในระหว่างตั้งท้อง (P) 14 และ 21 วัน, ระหว่างให้นม (L) 8, 14 และ 21 วัน และ 15 วันหลังการหย่านม (PW) แม่หนูบางกลุ่มจะได้รับการฉีดยา bromocriptine เพื่อลดการหลั่งโพรแลคติน และ/หรือ ได้รับการฉีดโพรแลคตินเสริม กลุ่ม Non-suckling หมายถึงกลุ่มแม่หนูที่นำลูกหนูออกไปตั้งแต่หลังคลอดเพื่อไม่ให้เกิดการดูดนมเกิดขึ้น * $P < 0.05$ เปรียบเทียบกับกลุ่มควบคุม (age-matched control) [†] $P < 0.05$ เปรียบเทียบกับกลุ่ม Preg/Lact ⁺ $P < 0.05$ เปรียบเทียบกับกลุ่ม Bromo [#] $P < 0.05$ เปรียบเทียบกับกลุ่ม Bromo+PRL

นอกจากนี้ คณะผู้วิจัยได้ทำการวิจัยนำร่องเพื่อทดสอบการตรวจพื้นผิวกระดูกในระดับนาโนโดยใช้เทคนิค atomic force microscopy (AFM) โดยใช้กระดูกของหนูที่ขาดฮอร์โมน estrogen (ภาพที่ 18) อย่างไรก็ตาม ข้อมูลที่ได้มีความแปรปรวนสูงมากเนื่องจากกระดูกเป็นวัสดุที่ไม่เป็นเนื้อเดียว และขั้นตอนการตัดกระดูกทำให้มีวัสดุชนิดอื่นปะปนอยู่ในเนื้อกระดูก (เช่น methyl methacrylate resin) ซึ่งรบกวนการวัดในระดับนาโนด้วยเทคนิค AFM และ nanoindentation



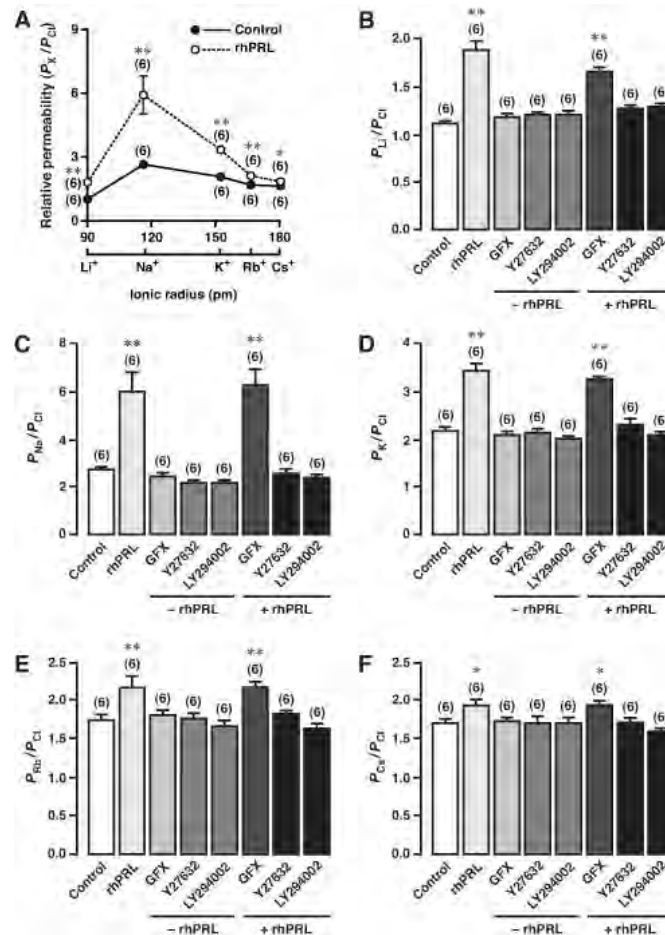
ภาพที่ 18 พื้นผิวของกระดูกในระดับนาโนเมื่อศึกษาด้วยเทคนิค atomic force microscopy ตัวอย่างกระดูกได้จากหนูที่ขาดฮอร์โมน estrogen จากการตัดรังไขนาน 4 สัปดาห์

4. การพัฒนาเทคนิค dilution potential และการวัดคุณสมบัติการยอมให้ผ่าน (permeability measurement)

การพัฒนาเทคนิค dilution potential และ permeability measurement เริ่มดำเนินการตั้งแต่วันที่ 1 ตุลาคม 2550 ใช้เวลาดำเนินการ 6 เดือน เทคนิค dilution potential ใช้ครั้งแรกในห้องปฏิบัติการของคณะผู้วิจัยเพื่อศึกษาการขนส่งแคลเซียมในลำไส้ขณะเกิดภาวะเลือดเป็นกรด (โครงการ MRG4980003) แต่เทคนิคในขณะนั้นยังมีความแม่นยำต่ำและสิ้นเปลืองสัตว์ทดลองจำนวนมาก

เทคนิคทั้งสองชนิดใช้ในการตรวจสอบว่า สารที่สนใจ (เช่น ฮอร์โมนโพรแลคติน) เพิ่มการขนส่งแคลเซียม หรือสารมีประจุอื่น ๆ ผ่านช่องระหว่างเซลล์แบบ charge-selective หรือไม่ เนื่องจากการพัฒนาเทคนิคโดยใช้ลำไส้ของหนูที่ตั้งท้องหรือให้นมมีค่าใช้จ่ายสูงมาก ดังนั้นคณะผู้วิจัยจึงพัฒนาเทคนิคดังกล่าวในเซลล์ลำไส้เพาะเลี้ยง Caco-2 และลำไส้เล็กส่วนต้นของหนูปกติที่สัมผัสวิตามินดี (ซึ่งเป็นฮอร์โมนหลักที่กระตุ้นการดูดซึมแคลเซียมที่ลำไส้เล็กส่วนต้น) ปัจจุบันเทคนิคนี้มีความแม่นยำสูงและได้ผลรวดเร็ว (การวัดเนื้อเยื่อ 1 ชิ้น ใช้เวลาประมาณ 30 นาที) ผลการวิจัยมีดังต่อไปนี้

1. โพรแลคตินเพิ่ม permeability ของลำไส้ต่อ monovalent ion หลายชนิด และบางชนิดมีขนาดใกล้เคียงกับแคลเซียม (ภาพที่ 19) ซึ่งแสดงว่าโพรแลคตินสามารถเปลี่ยน charge selectivity ของลำไส้ได้



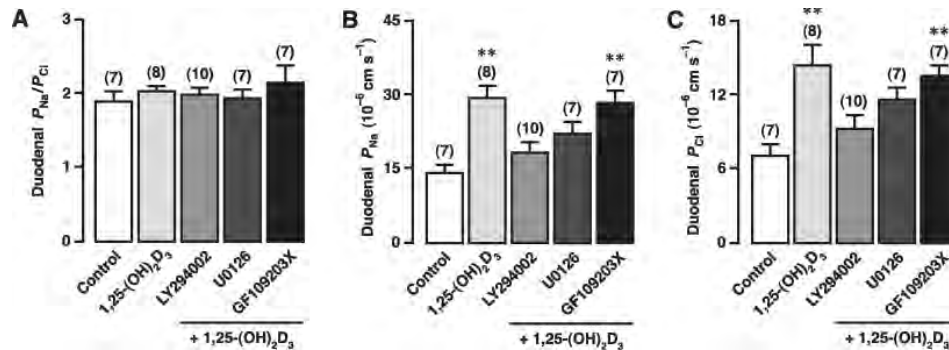
ภาพที่ 19 Permeability (P) ของแผ่นเซลล์เพาะเลี้ยง Caco-2 ต่อ monovalent ion ชนิดต่าง ๆ หลังการสัมผัสโพรแลคติน และ/หรือสัมผัสตัวยับยั้ง signal transduction ของโพรแลคติน ตัวเลขในวงเล็บคือจำนวนการทดลอง ** $P < 0.01$, * $P < 0.05$ เปรียบเทียบกับกลุ่มควบคุม LY294002 เป็นตัวยับยั้ง PI3K, Y27632 เป็นตัวยับยั้ง ROCK, GF109203X (GFX) เป็นตัวยับยั้ง PKC แบบ non-specific

งานวิจัยส่วนนี้ได้ตีพิมพ์ในวารสารระดับนานาชาติจำนวน 1 เรื่องคือ

Thongon N, Nakkrasae L, Thongbunchoo J, Krishnamra N, Charoenphandhu N.

Prolactin stimulates transepithelial calcium transport and modulates paracellular permselectivity in Caco-2 monolayer: Mediation by PKC and ROCK pathways. American Journal of Physiology – Cell Physiology 2008;294(5):C1158–C1168.

บางส่วนของผลงานตีพิมพ์เรื่องนี้ได้รับการสนับสนุนจากทุนเมธีวิจัยอาวุโส ของ ศาสตราจารย์เกียรติคุณ กฤษณามระ (เฉพาะการศึกษาโดยใช้ inhibitor ต่าง ๆ) และทุน Postdoctoral fellowship จากมหาวิทยาลัยมหิดล ของ ดร. ละเอียด นาคกระแสร (การศึกษาด้วยเทคนิค siRNA)



ภาพที่ 20 Permeability (P) ของเยื่อบุลำไส้เล็กส่วนต้นต่อโซเดียมและคลอไรด์ ภายหลังได้สัมผัสวิตามินดี และ/หรือสัมผัสตัวยับยั้ง signal transduction ของวิตามินดี ตัวเลขในวงเล็บคือจำนวนการทดลอง ** $P < 0.01$ เปรียบเทียบกับกลุ่มควบคุม LY294002 เป็นตัวยับยั้ง PI3K, U0126 เป็นตัวยับยั้ง MEK, GF109203X เป็นตัวยับยั้ง PKC แบบ non-specific

2. วิตามินดีเพิ่ม permeability ของทั้งไอออนประจุบวกและลบในสัดส่วนที่เท่ากัน (ภาพที่ 20) ซึ่งแปลผลได้ว่า วิตามินดีเพิ่มการขนส่งแคลเซียมผ่านช่องระหว่างเซลล์ได้แบบไม่จำเพาะเจาะจง งานวิจัยส่วนนี้ได้ตีพิมพ์ในวารสารระดับนานาชาติจำนวน 1 เรื่อง คือ

Tudpor K, Teerapornpuntakit J, Jantarajit W, Krishnamra N, Charoenphandhu N. 1,25-dihydroxyvitamin D₃ rapidly stimulates the solvent drag-induced paracellular calcium transport in the duodenum of female rats. Journal of Physiological Sciences 2008;58(5):297–307.

ผลงานตีพิมพ์ทั้งสองเรื่องยังแสดงถึงประโยชน์ของเทคนิค dilution potential เพื่อศึกษาคุณสมบัติการยอมให้ประจุผ่าน (charge-selective property) ซึ่งได้รับการยอมรับในระดับสากล

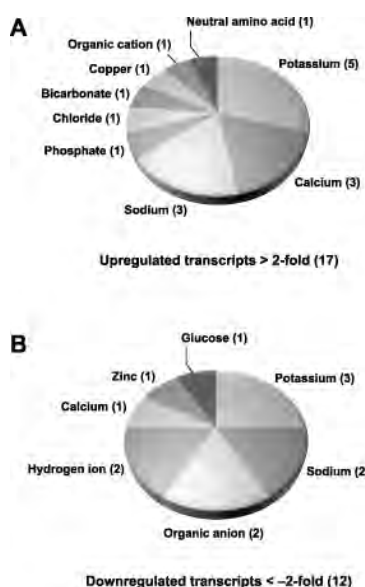
สรุป ปัจจุบันทางคณะผู้วิจัยได้พัฒนาเทคนิค dilution potential เพื่อตรวจวัด charge-selective property ของผนังเยื่อบุ จนได้เทคนิคมีความแม่นยำสูง

5. ผลของโปรแลคตินต่อการแสดงออกของยีนที่เกี่ยวข้องกับการขนส่งไอออนและสารอาหารประเภทต่าง ๆ ในลำไส้เล็กส่วนต้น

จากการวิจัยในหนูท้องและให้นมทำให้เกิดสมมติฐานว่า ผลเรื้อรัง (chronic) ของโปรแลคตินทำให้ลำไส้ปรับตัวในระดับ molecular และ genomic เพื่อให้ได้ข้อมูลเกี่ยวกับยีนที่มีการแสดงออกเพิ่มขึ้นในขณะที่มีโปรแลคตินในเลือดสูง คณะผู้วิจัยจึงได้ใช้เทคนิค microarray จากบริษัท Illumina ซึ่งมีความแม่นยำสูง เพื่อศึกษา transcriptome responses ของเซลล์ลำไส้ต่อฮอร์โมนโปรแลคติน งานวิจัยนี้ได้รับการสนับสนุนวิจัยบางส่วน จากทุนเมธีวิจัยอาวุโสของ ศาสตราจารย์เกียรติคุณ กฤษณามระ, จากคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล (ทุนสนับสนุนการวิจัยเพิ่มเติมของ นพ. นรุตพล เจริญพันธุ์) และจากบัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล

ผลการวิจัยพบว่า โปรแลคตินเพิ่มการแสดงออกของยีนจำนวน 321 ชนิดและลดการแสดงออก 241 ชนิด และมียีนจำนวนหนึ่งที่เป็นยีนที่เกี่ยวข้องกับการขนส่งไอออนและสารอาหาร ซึ่งรวมถึงแคลเซียมด้วย (ภาพที่ 21) งานวิจัยส่วนนี้ได้ตีพิมพ์ในวารสารระดับนานาชาติจำนวน 1 เรื่อง คือ

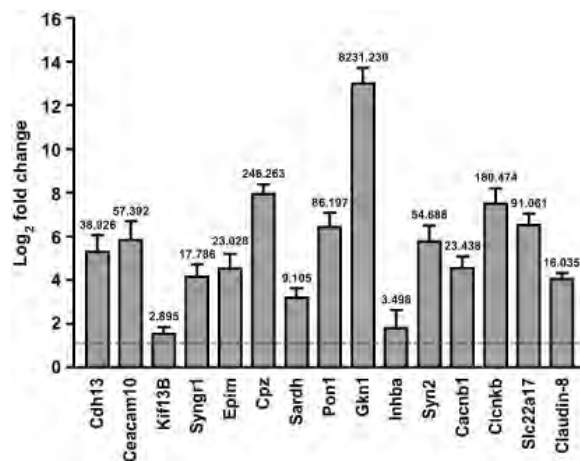
Charoenphandhu N, Wongdee K, Teerapornpundakit J, Thongchote K, Krishnamra N. Transcriptome responses of duodenal epithelial cells to prolactin in pituitary-grafted rats. *Molecular and Cellular Endocrinology* 2008;296:41–52.



ภาพที่ 21 ยีนที่เกี่ยวข้องกับการขนส่งไอออนและสารอาหารที่มีการแสดงออกเพิ่มขึ้น (A) และลดลง (B) ในขณะที่มีฮอร์โมนโปรแลคตินในเลือดสูง คณะผู้วิจัยจำลองภาวะโปรแลคตินในเลือดสูงโดยการทำ pituitary transplantation นาน 4 สัปดาห์ หนูที่ได้รับการผ่าตัดทำ transplantation เป็นหนูเพศเมียปกติที่ไม่ได้ตั้งท้อง ยีนขนส่งแคลเซียมที่เปลี่ยนแปลงส่วนหนึ่ง คือ voltage-gated calcium channels ตัวเลขในวงเล็บแสดงจำนวนยีนที่ตรวจพบการเปลี่ยนแปลงด้วยเทคนิค microarray

เนื่องจาก microarray เป็นเทคนิคทาง bioinformatics ที่ศึกษายีนประมาณ 20,000 ยีนพร้อม ๆ กัน จึงจำเป็นต้องสุ่มยีนจำนวน 15–20 ยีนออกมา เพื่อมาทดสอบด้วย real-time PCR ซึ่งจะช่วยยืนยันว่าผลจาก microarray ที่ทำมีความน่าเชื่อถือหรือไม่ primer ที่ได้พัฒนาขึ้น ตลอดจนสารเคมีชนิดพิเศษ และสารทางพันธุกรรมสำหรับการทำ real-time PCR ส่วนหนึ่ง ได้นำไปใช้ยืนยันผลของ microarray ในกรณีศึกษาอื่นด้วย ได้แก่

1. ผลของภาวะเลือดเป็นกรดเรื้อรังต่อเซลล์ลำไส้เล็กส่วนต้น ซึ่งเป็นการศึกษาในโครงการ MRG4980001 ซึ่งในขณะที่ปิดโครงการนั้น (เดือนมกราคม 2551) ยังไม่ได้ตีพิมพ์ผลงานในส่วนนี้ เพราะยังขาดการยืนยันผล microarray ด้วยเทคนิค real-time PCR ที่มีความครบถ้วน ตัวอย่างของยีนที่ศึกษาได้แสดงในภาพที่ 22

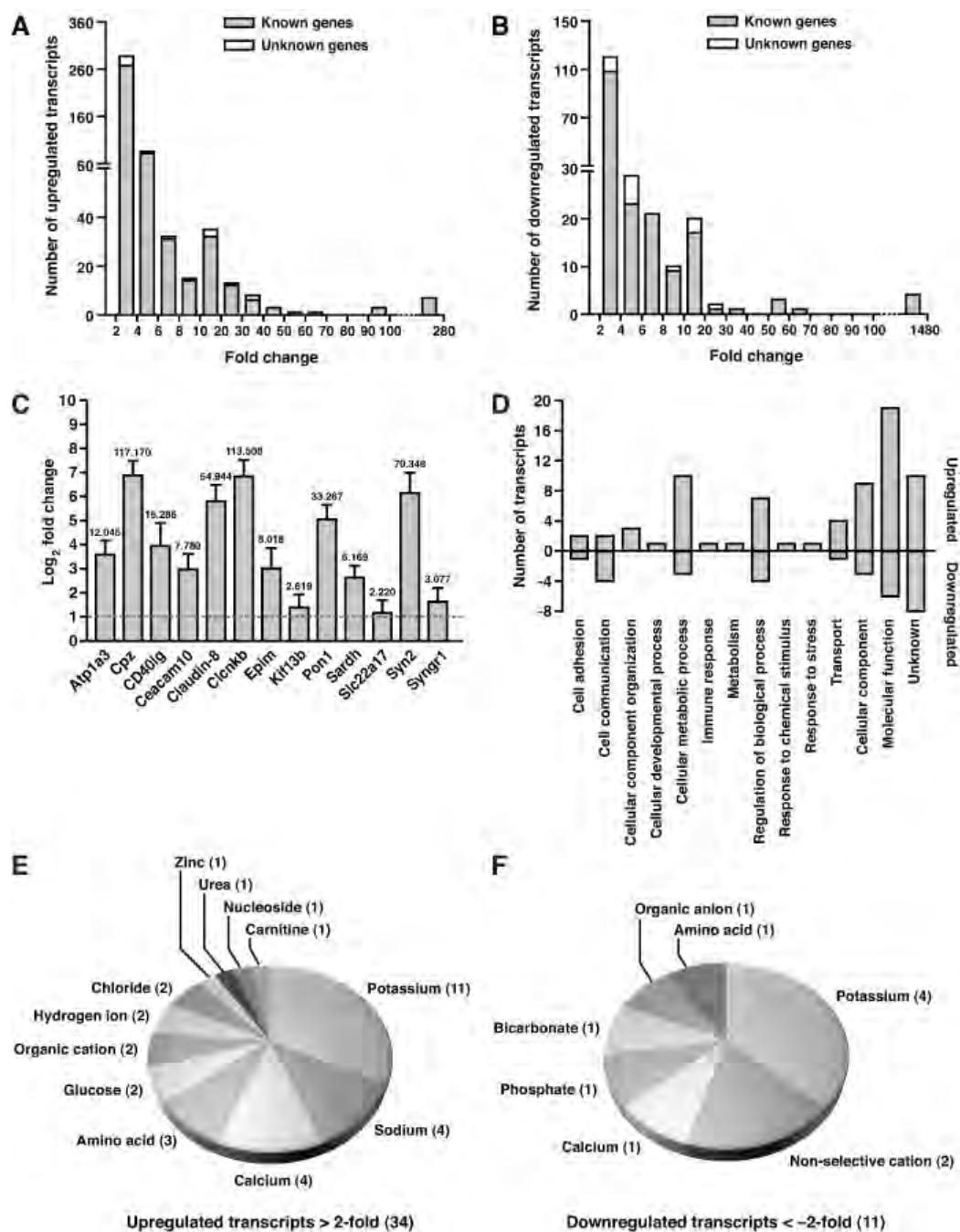


ภาพที่ 22 ยีนของเซลล์ลำไส้เล็กส่วนต้นที่มีการแสดงออกมากขึ้นหลังเกิดภาวะเลือดเป็นกรดแบบเมตาบอลิก นาน 21 วัน (chronic metabolic acidosis) ตัวเลขที่อยู่เหนือกราฟคือ จำนวนเท่าของการเปลี่ยนแปลง เส้นประแสดงระดับการเปลี่ยนแปลงของยีนที่เพิ่มขึ้น 2 เท่า

ปัจจุบันงานวิจัยส่วนนี้ได้ตีพิมพ์ในวารสารระดับนานาชาติจำนวน 1 เรื่อง คือ

Wongdee K, Teerapornpuntakit J, Riengrojpitak S, Krishnamra N, Charoenphandhu N. Gene expression profile of duodenal epithelial cells in response to chronic metabolic acidosis. Molecular and Cellular Biochemistry 2009;321(1–2):173–188.

2. ผลของการออกกำลังกายต่อเซลล์ลำไส้เล็กส่วนต้น ซึ่งพบว่าการออกกำลังกายด้วยวิธีการว่ายน้ำแบบทนทาน (endurance swimming) นาน 2 สัปดาห์ เพิ่มการแสดงออกของยีนที่เกี่ยวข้องกับการขนส่งไอออนและสารอาหารจำนวนมาก ดังแสดงในภาพที่ 23

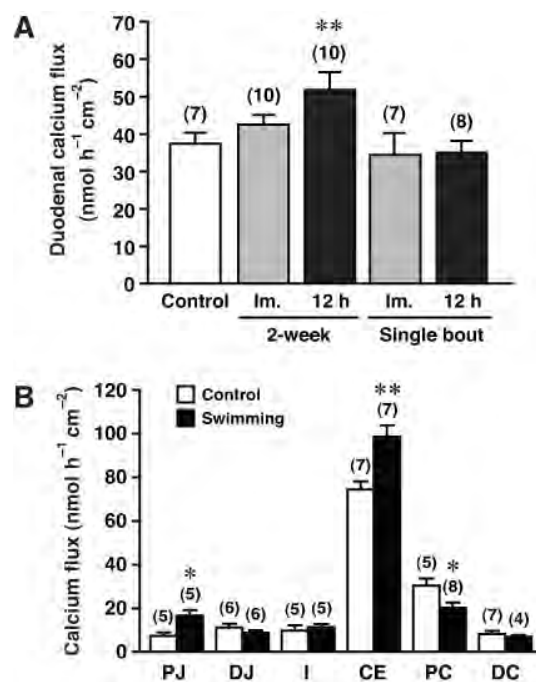


ภาพที่ 23 จำนวนและชนิดของยีนที่เพิ่มหรือลดในลำไส้เล็กส่วนต้นของหนู ภายใต้การออกกำลังกายด้วยวิธีว่ายน้ำนาน 2 สัปดาห์ ข้อมูลในภาพ A, B, D, E และ F ได้จากการวิเคราะห์ด้วย microarray ส่วนข้อมูลในภาพ C ได้จาก real-time PCR ซึ่งใช้ยืนยันผลของ microarray ตัวเลขในวงเล็บของภาพ E และ F คือจำนวนยีน

นอกจากนี้ทางคณะผู้วิจัยยังได้ศึกษาอัตราการดูดซึมแคลเซียมที่เพิ่มขึ้นภายหลังการว่ายน้ำอีกด้วย จากเหตุผลดังนี้คือ

- การว่ายน้ำเป็นการออกกำลังกายที่มีประสิทธิภาพสูง สามารถปฏิบัติได้ง่าย และมีความเป็นไปได้ว่าอาจเสริมฤทธิ์ของฮอร์โมนโพรแลคตินได้หากนำมาใช้กับผู้ที่อยู่ในระหว่างให้นมบุตร
- ผลจากการศึกษาด้วย microarray พบว่าเซลล์ลำไส้สังเคราะห์สารพันธุกรรมที่เกี่ยวข้องกับการดูดซึมแคลเซียมเพิ่มมากขึ้นหลังการฝึกว่ายน้ำ
- คณะผู้วิจัยมีเทคนิคการวัดอัตราการขนส่งแคลเซียม (ที่พัฒนาขึ้นเพื่อใช้วัดผลของโพรแลคติน) และเครื่องมือวัดคุณสมบัติทางไฟฟ้าของแผ่นเยื่อเรียบร้อยแล้ว

ผลการวิจัยพบว่าการฝึกว่ายน้ำนาน 2 สัปดาห์สามารถเพิ่มอัตราการขนส่งแคลเซียมในลำไส้เล็กส่วนต้น (duodenum; D) ส่วนต้นของลำไส้เล็กส่วนกลาง (proximal jejunum; PJ) และลำไส้ใหญ่ส่วนซีคัม (cecum; CE) ได้ แต่กลับลดอัตราการดูดซึมในลำไส้ใหญ่โคลอนส่วนต้น (proximal colon; PC) เล็กน้อย ซึ่งไม่กระทบต่อภาพรวมของการดูดซึมแคลเซียมที่เพิ่มขึ้นมากนัก ส่วนอัตราการดูดซึมแคลเซียมใน distal jejunum (DJ), ileum (I) และ distal colon (DC) ไม่เปลี่ยนแปลง (ภาพที่ 24)



ภาพที่ 24 ผลของการฝึกว่ายน้ำนาน 2 สัปดาห์ต่ออัตราการขนส่งแคลเซียมในลำไส้ส่วนต่าง ๆ การว่ายน้ำเพียงครั้งเดียว (single bout) ไม่สามารถกระตุ้นการขนส่งแคลเซียมได้ "Im." หมายถึงการวัดอัตราการขนส่งทันทีหลังว่ายน้ำ "12 h" หมายถึงการวัดหลังพักนาน 12 ชั่วโมง การพักช่วยให้ระบบไหลเวียนและระบบประสาทอัตโนมัติกลับมาทำงานปกติหลังออกกำลังกาย ตัวเลขในวงเล็บแสดงจำนวนสัตว์ทดลอง * $P < 0.05$, ** $P < 0.01$ เทียบกับกลุ่มควบคุม

ปัจจุบันงานวิจัยส่วนนี้ได้ตีพิมพ์ในวารสารระดับนานาชาติจำนวน 1 เรื่อง คือ

Teerapornpuntakit J, Dorkkam N, Wongdee K, Krishnamra N, Charoenphandhu N. Endurance swimming stimulates transepithelial calcium transport and alters the expression of genes related to calcium absorption in the intestine of rats. American Journal of Physiology Endocrinology and Metabolism 2009;296(4):E775–E786.

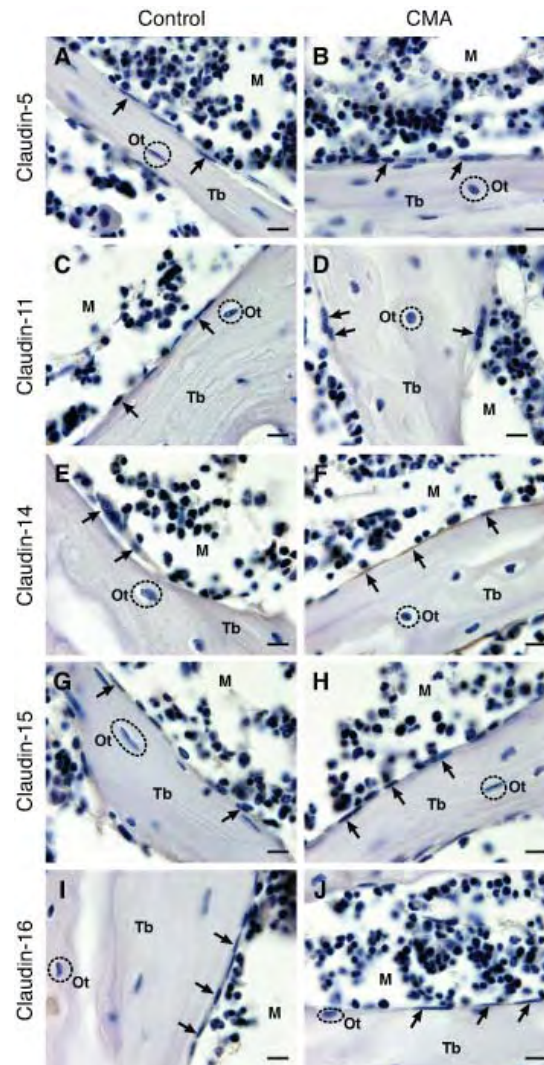
สรุปงานวิเคราะห์ข้อมูล microarray มีความสำคัญต่อการวิจัยด้านการดูดซึมแคลเซียมของลำไส้เพราะทำให้ทราบว่า calcium channel ชนิดที่มีรายงานในวารสารวิชาการและตำราทั่วไป น่าจะมีความสำคัญต่อการขนส่งแคลเซียมในขณะให้นม และอาจจำเป็นต้องทำวิจัยเพิ่มเติมเพื่อพิสูจน์ว่า ยาที่ยับยั้ง $Ca_v1.3$ (ซึ่งมักเป็นยาในกลุ่มยาลดความดัน) มีผลต่อการดูดซึมแคลเซียมจากอาหารโดยเฉพาะในระหว่างให้นมบุตรหรือไม่

6. การพัฒนาเทคนิค immunohistochemistry สำหรับการตรวจวัดการแสดงออกของโปรตีนในกระดูก

การวิจัยในสัตว์ทดลองที่ผ่านมาประสบกับคำถามที่สำคัญคำถามหนึ่ง คือ กระดูกของหนูที่ท้องและให้นมสามารถตอบสนองต่อโพรแลคตินได้โดยตรงหรือไม่ การศึกษา prolactin receptor protein expression ในกระดูกด้วยเทคนิค immunohistochemistry สามารถตอบคำถามนี้ได้ อย่างไรก็ตามเทคนิคนี้จำเป็นต้องตัดกระดูกให้เป็นแผ่นบาง ๆ มีความหนา 7 ไมโครเมตร ซึ่งทำได้ยากเนื่องจากกระดูกเป็นเนื้อเยื่อที่แข็ง การตัดให้มีความบางสม่ำเสมอต้องละลายแคลเซียมและแร่ธาตุอื่น ๆ ออกจากกระดูกทั้งหมด (decalcification) ด้วยสารละลาย EDTA buffer ซึ่งอาจใช้เวลาหลายสัปดาห์ และกระบวนการ decalcification สามารถทำลายโปรตีนของเซลล์ ซึ่งอาจรบกวนการวิเคราะห์ด้วยเทคนิค immunohistochemistry ทั่วไป

เพื่อลดข้อจำกัดนี้ ผู้วิจัยได้พัฒนาและปรับปรุงเทคนิค immunohistochemistry สำหรับกระดูกขึ้น โดยเริ่มทำวิจัยนำร่องด้วยการใช้เทคนิคที่พัฒนาขึ้นใหม่นี้ศึกษา claudin expression ใน bone-lining cell ก่อน ผลการวิจัยพบว่า bone-lining cell สามารถสร้างโปรตีนในกลุ่ม claudin หลายชนิด เช่น claudin-5, claudin-11, claudin-14, claudin-15 และ claudin-16 (ภาพที่ 25) ซึ่งคาดว่าจะมีส่วนสำคัญต่อการควบคุมการปลดปล่อยแคลเซียมออกจากผิวกระดูก ปริมาณโปรตีนเหล่านี้จะเปลี่ยนแปลงได้ขณะเกิดภาวะเลือดเป็นกรด (chronic metabolic acidosis; CMA) ซึ่งเป็นภาวะที่เร่งการปลดปล่อยแคลเซียมออกจากผิวกระดูก ปัจจุบันงานวิจัยส่วนนี้ได้ตีพิมพ์ในวารสารระดับนานาชาติจำนวน 1 เรื่อง คือ

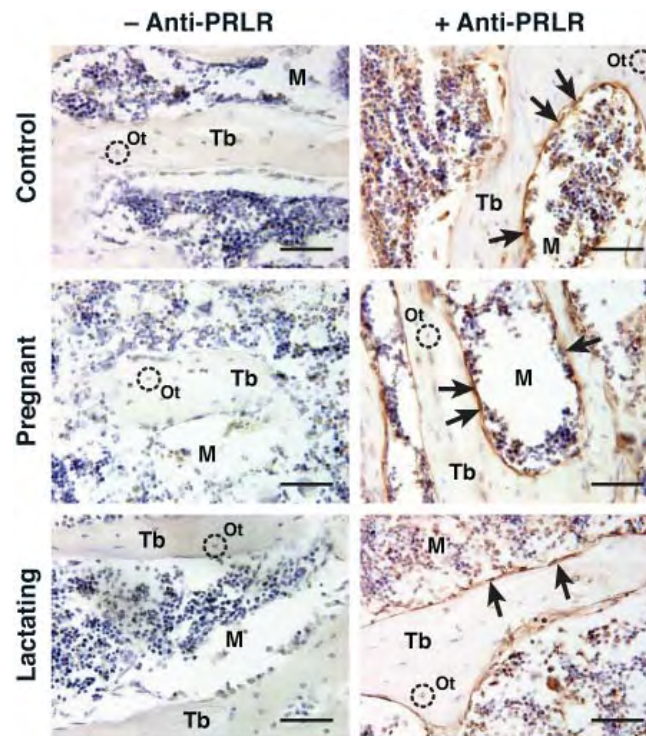
Wongdee K, Riengrojpitak S, Krishnamra N, Charoenphandhu N. Claudin expression in the bone-lining cells of female rats exposed to long-standing acidemia. *Experimental and Molecular Pathology* 2010;88(2):305–310.



ภาพที่ 25 การแสดงออกของโปรตีน claudin-5, -11, -14, -15 และ -16 ใน bone-lining cell (ลูกศร) ของกระดูก tibia ที่ได้จากหนูปกติ (control) และหนูที่ถูกเหนี่ยวนำให้เกิดภาวะเลือดเป็นกรด (CMA) การศึกษานี้ใช้เทคนิค immunohistochemistry ซึ่งได้รับการพัฒนาและปรับปรุงให้เหมาะสมต่อการศึกษาการแสดงออกของโปรตีนในตัวอย่างเนื้อเยื่อชนิด paraffin-embedded decalcified tibial section (Ot = osteocyte; Tb = bone trabecula; M = marrow cavity)

เมื่อคณะผู้วิจัยได้นำเทคนิค immunohistochemistry ที่ได้พัฒนาขึ้น มาใช้ศึกษาการแสดงออกของ prolactin receptor ในกระดูก ผลการวิจัยพบว่า เซลล์สร้างกระดูก (osteoblast) มี prolactin

receptor (ภาพที่ 26) ซึ่งเป็นการยืนยันโดยอาศัยผลการทดลองว่า เซลล์ของกระดูกสามารถตอบสนองต่อฮอร์โมนโพรแลคตินได้โดยตรง prolactin receptor เหล่านี้มีอยู่ในเซลล์กระดูกของหนูปกติ หนูที่ตั้งท้อง ตลอดจนหนูที่อยู่ในระหว่างให้นม นอกจากนี้ยังพบ prolactin receptor ในเซลล์กระดูกอ่อนของ growth plate อีกด้วย ซึ่งแสดงให้เห็นว่า growth plate ก็สามารถตอบสนองต่อโพรแลคตินได้โดยตรงเช่นกัน



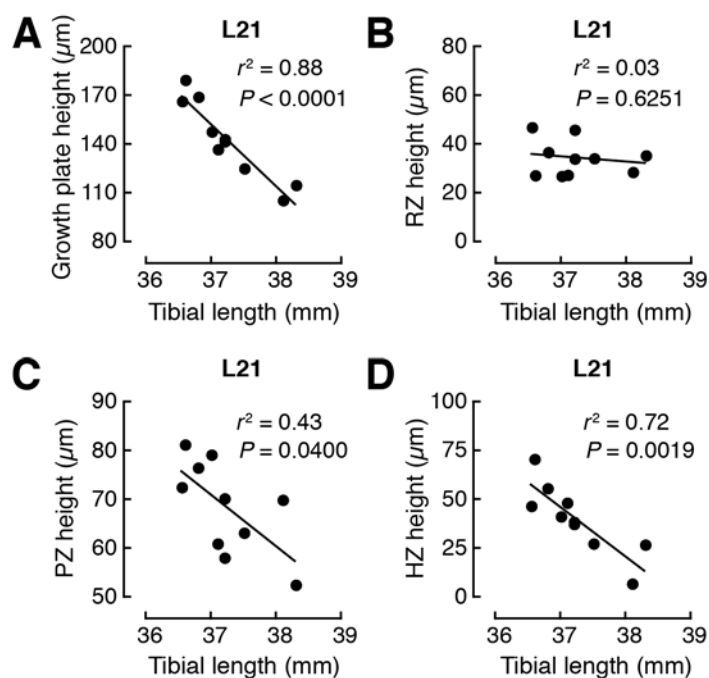
ภาพที่ 26 การแสดงออกของ prolactin receptor protein ในเซลล์สร้างกระดูกของหนูกลุ่มควบคุม หนูท้อง และหนูให้นม รูปด้านซ้ายเป็นตัวอย่างเนื้อเยื่อที่เป็น negative control (ไม่ได้ incubate กับ prolactin receptor antibody; -Anti-PRLR) ส่วนรูปทางขวาเป็นตัวอย่างเนื้อเยื่อที่มี prolactin receptor antibody (+Anti-PRLR) การศึกษาใช้เทคนิค immunohistochemistry ซึ่งได้รับการพัฒนาและปรับปรุงให้เหมาะสมต่อการศึกษการแสดงออกของโปรตีนในตัวอย่างเนื้อเยื่อชนิด paraffin-embedded decalcified tibial section (Ot = osteocyte; Tb = bone trabecula; M = marrow cavity)

สรุป ผู้วิจัยได้พัฒนาและปรับปรุงเทคนิค immunohistochemistry ให้เหมาะสมต่อการศึกษการแสดงออกของโปรตีนในตัวอย่างเนื้อเยื่อชนิด paraffin-embedded decalcified bone section และได้แสดงให้เห็นว่า เซลล์สร้างกระดูกมี prolactin receptor ซึ่งยืนยันว่ากระดูกสามารถตอบสนองต่อโพรแลคตินได้โดยตรง

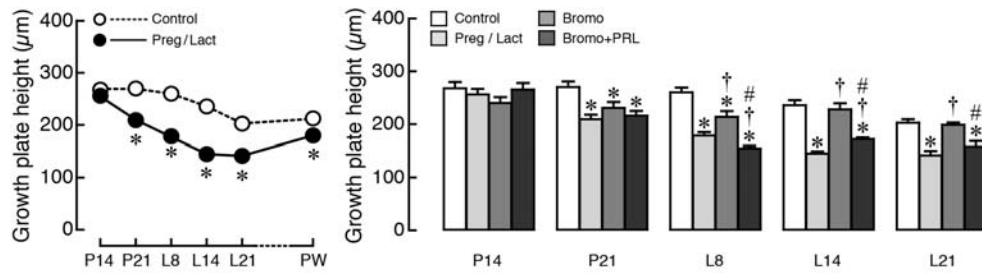
7. การศึกษาผลของโปรแลคตินต่อ growth plate cartilage ของแม่หนู

การพัฒนาเทคนิค immunohistochemistry สำหรับตรวจวัดการแสดงออกของโปรตีนในกระดูก ทำให้คณะผู้วิจัยพบว่า นอกจากเซลล์สร้างกระดูก (osteoblast) แล้ว เซลล์กระดูกอ่อน (chondrocyte) ที่อยู่ในบริเวณ growth plate ของกระดูก tibia ของแม่หนูที่กำลังตั้งท้องและให้นมก็มี prolactin receptor protein เช่นเดียวกัน จึงมีความเป็นไปได้ว่า growth plate cartilage ของแม่หนูอาจตอบสนองต่อฮอร์โมนโปรแลคตินที่เพิ่มสูงขึ้นในเลือดได้ เนื่องจากการทำงานของ growth plate มีความสัมพันธ์อย่างใกล้ชิดกับความยาวของกระดูก (ซึ่งพบว่าความยาวกระดูก tibia จะเพิ่มขึ้นในระหว่างตั้งท้อง) และมีความเป็นไปได้ว่า ความสูงของ growth plate อาจเปลี่ยนแปลงไปในระหว่างตั้งท้องและให้นม

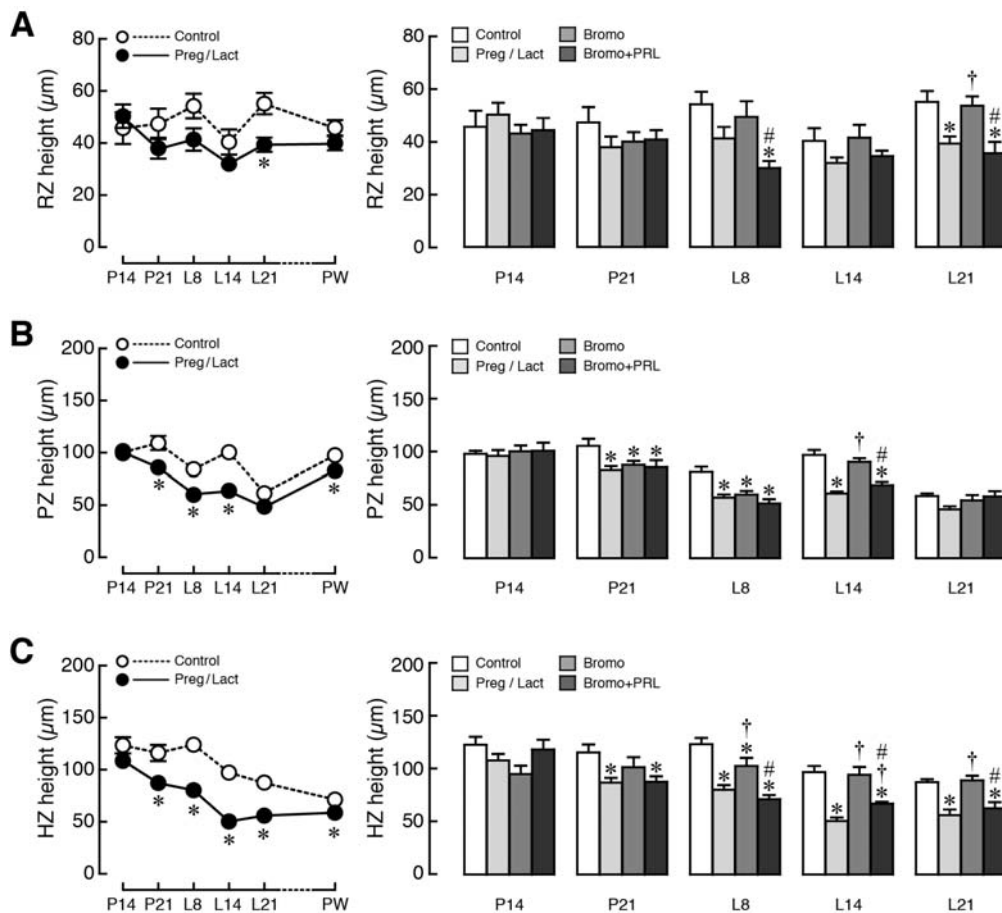
ผลการวิจัยในหนูที่ตั้งท้องและให้นมยืนยันว่า ความยาวของกระดูก tibia ที่เพิ่มขึ้นสัมพันธ์กับความสูงของ growth plate ที่ลดลง (ภาพที่ 27) โดยเฉพาะความสูงของ proliferating zone และ hypertrophic zone ซึ่งอาจเกิดจากการที่เซลล์กระดูกอ่อนในโซนเหล่านี้พัฒนาไปเป็นเนื้อกระดูกมากขึ้น จึงทำให้กระดูกยาวขึ้นแต่ความสูงของ growth plate กลับลดลง การวิจัยในแม่หนูให้นมที่ได้รับการฉีดยา bromocriptine i.p. เพื่อยับยั้งการหลั่งฮอร์โมนโปรแลคตินจากต่อมใต้สมอง และ/หรือ การฉีดฮอร์โมนโปรแลคตินแสดงให้เห็นว่า ฮอร์โมนโปรแลคตินมีผลต่อเซลล์กระดูกอ่อนบริเวณ growth plate และมีส่วนทำให้ความสูงของ growth plate เปลี่ยนแปลงไปในระหว่างให้นม (ภาพที่ 28 และ 29)



ภาพที่ 27 (A–D) ความสัมพันธ์ (correlation) ระหว่างความยาวของกระดูก tibia ของหนูที่กำลังตั้งท้องและให้นมกับความสูงของ growth plate และโซนต่างๆ ของ growth plate [ได้แก่ residual zone (RZ), proliferating zone (PZ) และ hypertrophic zone (HZ)]



ภาพที่ 28 การเปลี่ยนแปลงของความสูงของ growth plate cartilage ในแมหนูที่กำลังตั้งท้อง 14 วัน (P14) และ 21 วัน (P21), แมหนูที่กำลังให้นมวันที่ 8 (L8), 14 (L14) และ 21 (L21) และวันที่ 15 หลังหย่านม (PW) แมหนูบางกลุ่มได้รับยา bromocriptine i.p. (Bromo) นาน 7 วัน เพื่อยับยั้งการหลั่งโพรแลคตินจากต่อมใต้สมอง หรือได้รับทั้ง bromocriptine และฉีดฮอร์โมนโพรแลคตินเสริม (Bromo+PRL) หนูตัวเมียในกลุ่มควบคุมมีอายุเท่ากับแมหนูในช่วงเวลาที่ศึกษา * $P < 0.05$ เทียบกับกลุ่มควบคุม † $P < 0.05$ เทียบกับกลุ่ม Preg/Lact # $P < 0.05$ เทียบกับกลุ่ม Bromo



ภาพที่ 29 การเปลี่ยนแปลงของความสูงของโซนต่าง ๆ ใน growth plate cartilage [ได้แก่ residual zone (RZ), proliferating zone (PZ) และ hypertrophic zone (HZ)] ในแมหนูที่กำลังตั้งท้อง 14 วัน

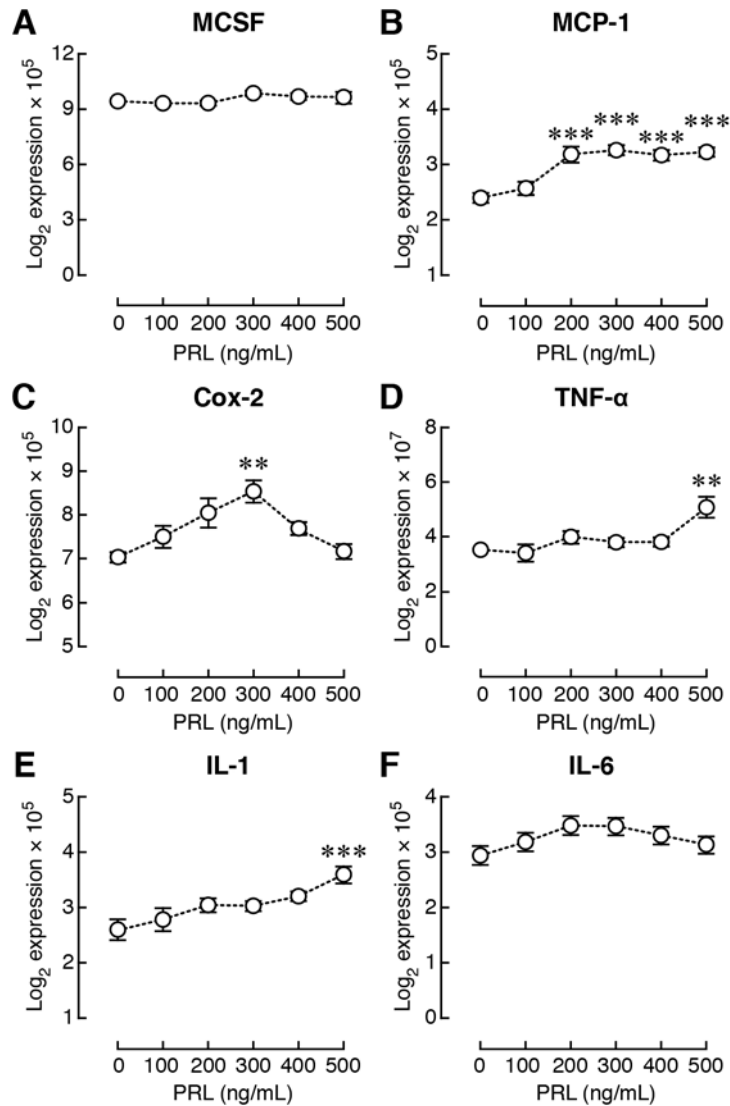
(P14) และ 21 วัน (P21), แม่หนูที่กำลังให้นมวันที่ 8 (L8), 14 (L14) และ 21 (L21) และวันที่ 15 หลังหย่านม (PW) แม่หนูบางกลุ่มได้รับยา bromocriptine i.p. (Bromo) นาน 7 วัน เพื่อยับยั้งการหลั่งโพรแลคตินจากต่อมใต้สมอง หรือได้รับทั้ง bromocriptine และฉีดฮอร์โมนโพรแลคตินเสริม (Bromo+PRL) หนูตัวเมียในกลุ่มควบคุมมีอายุเท่ากับแม่หนูในช่วงเวลาที่ศึกษา * $P < 0.05$ เทียบกับกลุ่มควบคุม [†] $P < 0.05$ เทียบกับกลุ่ม Preg/Lact [#] $P < 0.05$ เทียบกับกลุ่ม Bromo

สรุป โพรแลคตินมีผลต่อการทำงานของเซลล์กระดูกอ่อนบริเวณ growth plate ของแม่หนูที่กำลังให้นม ซึ่งอาจมีความสำคัญต่อการสร้างกระดูกจากกระดูกอ่อน (endochondral bone formation) และการสะสมแคลเซียมในกระดูก แคลเซียมที่นำมาสะสมในระหว่างการสร้างกระดูกแบบนี้ จะทำให้มีแคลเซียมเก็บไว้ในกระดูกอย่างเพียงพอเพื่อให้โพรแลคตินสลายไปใช้ในภายหลัง กล่าวคือ โพรแลคตินอาจกระตุ้นทั้งการสร้างและสลายกระดูก (increased bone turnover) ทั้งนี้เพื่อให้แม่มีแคลเซียมเก็บสะสมไว้อย่างเพียงพอ และยังสามารถสลายกระดูกนำไปใช้สร้างน้ำนมได้ตลอดเวลาอีกด้วย

8. การศึกษาผลของโพรแลคตินต่อการสร้าง osteoclastogenic cytokines ของเซลล์สร้างกระดูก

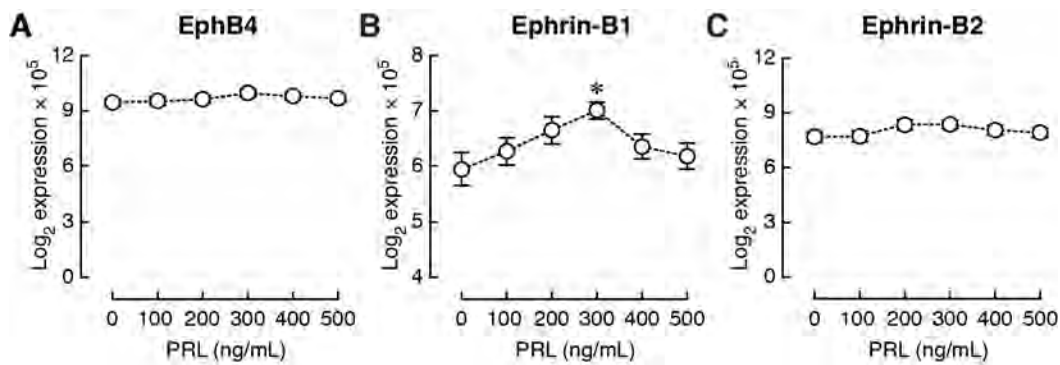
เนื่องจากแม่หนูที่กำลังให้นมลูกมีอัตราการสลายกระดูกที่สูงมากตามที่รายงานแล้วข้างต้น จึงมีความเป็นไปได้ว่า โพรแลคตินอาจกระตุ้นให้มีการสร้างไซโตไคน์อื่น ๆ ที่สามารถกระตุ้นเซลล์สลายกระดูก (osteoclastogenic cytokines) เช่น monocyte chemotactic protein (MCP)-1, interleukin (IL)-1, IL-6 และ tumor necrosis factor (TNF)- α เป็นต้น และเซลล์ที่สามารถสร้างไซโตไคน์เหล่านี้กลับเป็นเซลล์สร้างกระดูก (เซลล์สร้างกระดูก หรือ osteoblast นอกจากจะมีหน้าที่สร้างกระดูกแล้ว ยังสามารถหลั่งไซโตไคน์เพื่อกระตุ้นการสลายกระดูกได้ด้วย) คณะผู้วิจัยจึงดำเนินการทดลองชุดนี้เพื่อศึกษาผลของโพรแลคตินต่อการสร้าง osteoclastogenic cytokines ของเซลล์สร้างกระดูกชนิด UMR106 ซึ่งเป็น osteoblastic cell line จากหนู

ผลการวิจัยแสดงในภาพที่ 30 ซึ่งพบว่า ฮอร์โมนโพรแลคตินสามารถเพิ่มการสร้าง mRNA ของไซโตไคน์หลายชนิด ได้แก่ MCP-1, TNF- α และ IL-1 จากเซลล์สร้างกระดูกเพื่อใช้กระตุ้นเซลล์สลายกระดูก แต่ไม่มีผลต่อการสร้าง macrophage colony-stimulating factor (MCSF) และ IL-6 นอกจากนี้ โพรแลคตินยังสามารถเพิ่ม mRNA ของ cyclooxygenase (Cox)-2 ได้ด้วย โดยทั่วไป Cox-2 จะทำให้มีการสร้างสารเคมีประเภท prostaglandins มากขึ้น สารเคมีประเภท prostaglandins นี้สามารถกระตุ้นการทำงานของเซลล์สลายกระดูกได้



ภาพที่ 30 (A–F) ผลของโปรแลคติน (PRL) ที่ความเข้มข้นต่าง ๆ กันต่อ mRNA expression ของ MCSF, MCP-1, Cox-2, TNF- α , IL-1 และ IL-6 ใน UMR106 osteoblast-like cell เซลล์ที่ศึกษาสัมพันธ์กับฮอร์โมนโปรแลคตินนาน 48 ชั่วโมงก่อนสกัด mRNA ** $P < 0.01$, *** $P < 0.001$ เทียบกับกลุ่มควบคุม (0 ng/mL PRL)

อนึ่ง โปรแลคตินยังสามารถเพิ่ม mRNA ของ Ephrin-B1 ในเซลล์สร้างกระดูกได้อีกด้วย (ภาพที่ 31) โดยระบบ Ephrin-Eph นี้เป็นส่วนสำคัญที่ทำให้เซลล์สร้างกระดูกและเซลล์สลายกระดูกมาเชื่อมต่อกัน และแลกเปลี่ยนข้อมูลซึ่งกันและกัน การกระตุ้นการทำงานของระบบนี้จึงอาจนำไปสู่การกระตุ้นหรือยับยั้งการสลายกระดูกได้



ภาพที่ 31 (A–C) ผลของโปรแลคติน (PRL) ที่ความเข้มข้นต่าง ๆ กันต่อ mRNA expression ของ EphB4, Ephrin-B1 และ Ephrin-B2 ใน UMR106 osteoblast-like cell เซลล์ที่ศึกษาสัมพันธ์กับฮอร์โมนโปรแลคตินนาน 48 ชั่วโมงก่อนสกัด mRNA * $P < 0.05$ เทียบกับกลุ่มควบคุม (0 ng/mL PRL)

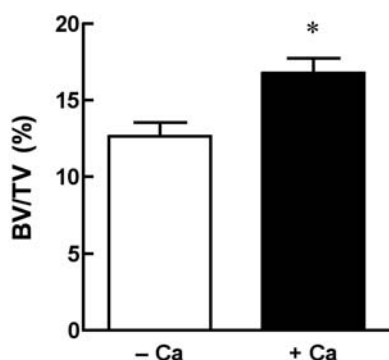
สรุป ผลการทดลองแบบ in vitro ในเซลล์สร้างกระดูกเพาะเลี้ยงชนิด UMR106 ทำให้ได้ข้อสรุปว่า โปรแลคตินอาจกระตุ้นการสลายกระดูกของแม่หนูผ่านการสร้างไซโตไคน์และสารเคมีหลายชนิดจากเซลล์สร้างกระดูก และยังแสดงให้เห็นว่า การกระตุ้นเซลล์สร้างกระดูกอาจนำไปสู่การสลายกระดูกได้ ทั้งนี้ขึ้นอยู่กับไซโตไคน์ที่เซลล์สร้างกระดูกผลิตขึ้น ในกรณีนี้โปรแลคตินกระตุ้นการสร้าง MCP-1, TNF- α และ IL-1 ซึ่งเป็นไซโตไคน์ที่สามารถกระตุ้นการทำงานของเซลล์สลายกระดูกได้ จึงทำให้มีการสลายกระดูกเพิ่มมากขึ้น

9. ผลการให้แคลเซียมเสริมทางปากต่อปริมาตรกระดูกของแม่หนูที่กำลังให้นม

เนื่องจากผลการวิจัยที่ได้ยืนยันว่า อัตราการดูดซึมแคลเซียมของลำไส้จะเพิ่มขึ้นอย่างมากในระหว่างให้นม ซึ่งเป็นผลโดยตรงจากฮอร์โมนโปรแลคตินที่เพิ่มสูงขึ้นในเลือดของแม่ ฉะนั้นการให้แคลเซียมเสริมทางปากในระหว่างให้นมจึงน่าจะช่วยให้ร่างกายแม่ได้รับปริมาณแคลเซียมเข้าสู่ร่างกายเพิ่มขึ้นไปด้วย สมมติฐานนี้ได้รับการตีพิมพ์ในวารสาร Trends in Endocrinology & Metabolism (Impact Factor 2009 = 6.562)

คณะผู้วิจัยได้ดำเนินการวิจัยเพื่อพิสูจน์ว่า การให้แคลเซียมเสริมทางปากสามารถป้องกันภาวะกระดูกบาง (osteopenia) จากการให้นมได้ โดยได้ป้อนแคลเซียมเสริมให้กับแม่หนูที่กำลังให้นมลูกทุกวันจนกระทั่งหย่านม จากนั้นทำการตรวจวัดปริมาตรกระดูก (tibial trabeculae) ของแม่หนูด้วยเทคนิค bone histomorphometry

ผลการวิจัยในแม่หนูที่กำลังให้นมแสดงให้เห็นว่า การให้ CaCl₂ เสริมทางปาก 30 นาทีก่อนให้นมลูก นาน 2 สัปดาห์ สามารถชะลอการสูญเสียมวลกระดูกในแม่หนูที่กำลังให้นมได้ (ภาพที่ 32) และปริมาณแคลเซียมที่เหมาะสม คือ 6 mg per kilogram body weight per dose โดยให้วันละ 3–4 ครั้ง อย่างไรก็ตาม ยังมีความจำเป็นต้องดำเนินการวิจัยในมนุษย์เพิ่มเติมเพื่อยืนยันปริมาณแคลเซียมที่จะให้เสริม



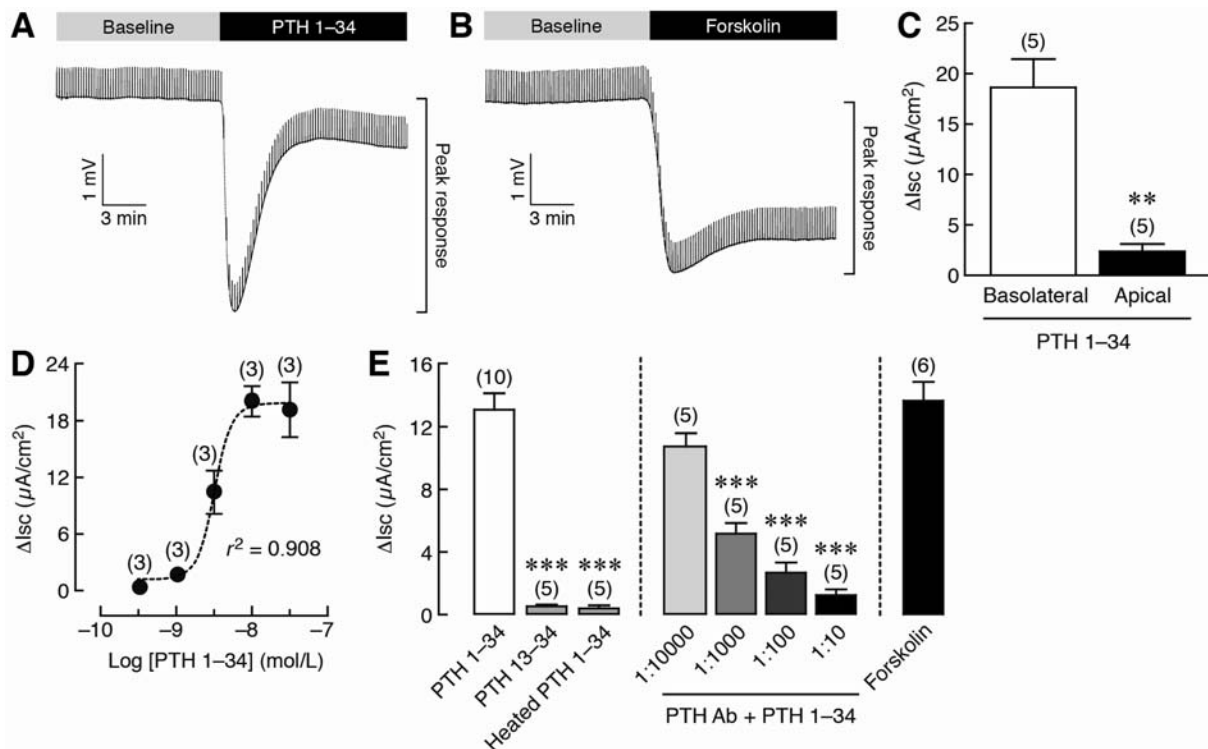
ภาพที่ 32 Bone volume (BV)/tissue volume (TV) ของแม่หนูที่กำลังให้นมที่ได้รับ (+ Ca; n = 4) และไม่ได้รับ (- Ca; n = 14) CaCl_2 เสริมทางปาก 30 นาทีก่อนให้นมลูก นาน 2 สัปดาห์ ปริมาณแคลเซียมที่ได้รับคือ 6 mg per kilogram body weight per dose * $P < 0.05$ เทียบกับกลุ่ม - Ca

10. การสร้างผลกระทบ (impact) ต่อการวิจัยด้านแคลเซียมและกระดูกในวงกว้าง

1. การศึกษา 3 สภาวะหลักที่กระตุ้นการขนส่งแคลเซียม (ได้แก่ ภาวะโพรแลคตินในเลือดสูง ภาวะเลือดเป็นกรด และการว่ายน้ำ) ด้วยเทคนิค microarray มีความสำคัญยิ่งต่อการวิจัยด้านแคลเซียมและกระดูก เพราะได้ช่วยยืนยันว่า โปรตีนขนส่งแคลเซียมในลำไส้เล็กไม่ได้มีแค่ TRPV5, TRPV6, calbindin, NCX1 และ PMCA อย่างที่เข้าใจกัน แต่ได้แสดงให้เห็นว่า โปรตีนขนส่งชนิด voltage-gated calcium channel (เช่น $\text{Ca}_v1.3$) ก็มีบทบาทเด่นเช่นกัน โดยเฉพาะการเป็นช่องทางขนส่งแคลเซียมเข้าสู่เซลล์หลังการกระตุ้นด้วยฮอร์โมนโพรแลคติน

2. เทคนิคการวัด paracellular calcium transport และ dilution potential ที่พัฒนาขึ้น ได้แสดงคุณสมบัติที่โดดเด่นประการหนึ่งของช่องระหว่างเซลล์ ที่เรียกว่า rectification คุณสมบัตินี้ช่วยยืนยันว่า เซลล์สามารถควบคุม charge-selective property ได้ กล่าวคือ ช่องระหว่างเซลล์ไม่ได้เป็นเพียงช่องโหล่งธรรมดา แต่เป็นช่องที่สามารถปรับคุณสมบัติได้ตามความต้องการของเซลล์ (หรือร่างกาย) และมีแนวโน้มว่าฮอร์โมนโพรแลคตินสามารถปรับคุณสมบัติ rectification นี้ได้แบบ non-genomic

3. เทคนิคการวัดค่าตัวแปรทางไฟฟ้า เช่นค่า short circuit current (Isc) และค่า transepithelial potential difference (PD) ที่ได้พัฒนาให้มีความละเอียดแม่นยำมากขึ้น ได้นำไปใช้ศึกษาผลของ parathyroid hormone (PTH; ซึ่งเป็นฮอร์โมนควบคุมสมดุลแคลเซียมที่สำคัญตัวหนึ่ง) ต่อการขนส่งไอออนผ่าน intestinal cell monolayer ทำให้ทราบว่า PTH มีผลโดยตรงต่อเซลล์เยื่อบุลำไส้ และสามารถกระตุ้นการขนส่งไอออนประจุลบ เช่น ไบคาร์บอเนต ออกจากเซลล์แบบ active transport ได้ (ภาพที่ 33) ข้อมูลที่ได้มีความสำคัญและอาจส่งผลกระทบในวงกว้าง เนื่องจากเป็นครั้งแรกที่มีการรายงาน direct ion regulatory function ของ PTH ในเซลล์ลำไส้ (ในตำราแพทย์ทั่วไปจะกล่าวว่า PTH ไม่มีผลโดยตรงต่อเซลล์ลำไส้)



ภาพที่ 33 ผลของ recombinant parathyroid hormone (PTH 1-34) ต่อการขนส่งไอออนประจุลบในแผ่นเซลล์เยื่อบุผิวลำไส้เพาะเลี้ยง Caco-2 monolayer ตัวแปรทางไฟฟ้าชนิด short circuit current (Isc) เป็นตัวแปรที่แสดงถึงอัตราการขนส่งไอออนผ่าน monolayer (**A** และ **B**) transepithelial potential difference ที่เปลี่ยนแปลงหลังให้ Caco-2 monolayer สัมผัสกับ PTH และ forskolin (ซึ่งเป็นสารที่กระตุ้นการสร้าง cAMP ภายในเซลล์ เพื่อเลียนแบบผลของ PTH) (**C**) ผลของ PTH ต่อ Isc ที่เปลี่ยนแปลงไป เมื่อให้เซลล์สัมผัสกับ PTH ทางด้าน basolateral หรือ apical (**D**) การศึกษา dose response โดยให้เซลล์สัมผัสกับ PTH ความเข้มข้นต่าง ๆ ทางด้าน basolateral (**E**) ผลของ PTH ถูกยับยั้งด้วย inactive PTH analog (PTH 13-34), heated PTH และแอนติบอดี (Ab) ต่อ PTH ** $P < 0.01$, *** $P < 0.001$ เทียบกับ white bar ทางด้านซ้าย

Output ที่ได้จากโครงการ

1. ผลงานวิจัยที่ได้ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ จำนวน 16 เรื่อง โดยมีหัวหน้าโครงการเป็น **corresponding author** ทั้ง 16 เรื่อง ดังนี้

ผลงานตีพิมพ์	First author	Corresponding author	Impact factor 2009
1. Thongon N, Nakkrasae L, Thongbunchoo J, Krishnamra N, Charoenphandhu N . Prolactin stimulates transepithelial calcium transport and modulates paracellular permselectivity in Caco-2 monolayer: Mediation by PKC and ROCK pathways. American Journal of Physiology – Cell Physiology 2008;294(5):C1158–C1168.	–	✓	4.013
2. Tudpor K, Teerapornpuntakit J, Jantarajit W, Krishnamra N, Charoenphandhu N . 1,25-dihydroxyvitamin D ₃ rapidly stimulates the solvent drag-induced paracellular calcium transport in the duodenum of female rats. Journal of Physiological Sciences 2008;58(5):297–307.	–	✓	1.125
3. Charoenphandhu N , Wongdee K, Teerapornpuntakit J, Thongchote K, Krishnamra N. Transcriptome responses of duodenal epithelial cells to prolactin in pituitary-grafted rats. Molecular and Cellular Endocrinology 2008;296:41–52.	✓	✓	3.503
4. Wongdee K, Teerapornpuntakit J, Riengrojpitak S, Krishnamra N, Charoenphandhu N . Gene expression profile of duodenal epithelial cells in response to chronic metabolic acidosis. Molecular and Cellular Biochemistry 2009;321(1–2):173–188.	–	✓	1.896
5. Teerapornpuntakit J, Dorkkam N, Wongdee K, Krishnamra N, Charoenphandhu N . Endurance swimming stimulates transepithelial calcium transport and alters the expression of genes related to calcium absorption in the intestine of rats. American Journal of Physiology – Endocrinology and Metabolism 2009;296(4):E775–E786.	–	✓	4.395
6. Charoenphandhu N , Nakkrasae L, Kraidith K, Teerapornpuntakit J, Thongchote K, Thongon N, Krishnamra N. Two-step stimulation of intestinal Ca ²⁺ absorption during lactation by long-term prolactin exposure and suckling-induced prolactin surge. American Journal of Physiology – Endocrinology and Metabolism 2009;297(3):E609–E619.	✓	✓	4.395
7. Kraidith K, Jantarajit W, Teerapornpuntakit J, Nakkrasae L, Krishnamra N, Charoenphandhu N . Direct stimulation of the transcellular and paracellular calcium transport in the rat cecum by prolactin. Pflügers Archiv – European Journal of Physiology 2009;458(5):993–1005.	–	✓	3.695

8. Thongon N, Nakkrasae L, Thongbunchoo J, Krishnamra N, Charoenphandhu N . Enhancement of calcium transport in Caco-2 monolayer through PKC ζ -dependent Ca $_v$ 1.3-mediated transcellular and rectifying paracellular pathways by prolactin. American Journal of Physiology – Cell Physiology 2009;296(6):C1373–C1382.	–	✓	4.013
9. Suntornsarattoon P, Wongdee K, Krishnamra N, Charoenphandhu N . Femoral bone mineral density and bone mineral content in bromocriptine-treated pregnant and lactating rats. Journal of Physiological Sciences 2010;60(1):1–8.	–	✓	1.125
10. Nakkrasae L, Thongon N, Thongbunchoo J, Krishnamra N, Charoenphandhu N . Transepithelial calcium transport in prolactin-exposed intestine-like Caco-2 monolayer after combinatorial knockdown of TRPV5, TRPV6 and Ca $_v$ 1.3. Journal of Physiological Sciences 2010;60(1):9–17.	–	✓	1.125
11. Wongdee K, Riengrojpitak S, Krishnamra N, Charoenphandhu N . Claudin expression in the bone-lining cells of female rats exposed to long-standing acidemia. Experimental and Molecular Pathology 2010;88(2):305–310.	–	✓	2.056
12. Charoenphandhu N , Wongdee K, Krishnamra N. Is prolactin the cardinal calciotropic maternal hormone? Trends in Endocrinology and Metabolism 2010;21(7):395–401.	✓	✓	6.562
13. Suntornsarattoon P, Wongdee K, Goswami S, Krishnamra N, Charoenphandhu N . Bone modeling in bromocriptine-treated pregnant and lactating rats: possible osteoregulatory role of prolactin in lactation. American Journal of Physiology – Endocrinology and Metabolism 2010;299(3):E426–E436.	–	✓	4.395
14. Suntornsarattoon P, Wongdee K, Krishnamra N, Charoenphandhu N . Possible chondroregulatory role of prolactin on the tibial growth plate of lactating rats. Histochemistry and Cell Biology 2010;134(5):483–491.	–	✓	3.021
15. Wongdee K, Tulalamba W, Thongbunchoo J, Krishnamra N, Charoenphandhu N . Prolactin alters the mRNA expression of osteoblast-derived osteoclastogenic factors in osteoblast-like UMR106 cells. Molecular and Cellular Biochemistry 2011;349(1–2):195–204.	–	✓	1.896
16. Laohapitakworn S, Thongbunchoo J, Nakkrasae L, Krishnamra N, Charoenphandhu N . Parathyroid hormone (PTH) rapidly enhances CFTR-mediated HCO $_3^-$ secretion in intestinal epithelium-like Caco-2 monolayer: a novel ion regulatory action of PTH. American Journal of Physiology – Cell Physiology 2011 (in press).	–	✓	4.013

Reprint ของผลงานตีพิมพ์ได้แนบมาในภาคผนวก 2

2. การเผยแพร่ข้อมูลเพื่อประชาชนทั่วไปผ่านสื่อ จำนวน 7 ครั้ง

- 2.1 หัวข้อข่าวเรื่อง "ตอบแทนพระคุณแม่...ห่างไกลโรคกระดูกพรุน"
ผ่านสำนักข่าวแห่งชาติ กรมประชาสัมพันธ์ วันที่ 7 สิงหาคม 2552
- 2.2 หัวข้อข่าวเรื่อง "วิจัยเด่นแนะแม่ดื่มนมก่อนให้นมลูก"
หนังสือพิมพ์ไทยโพสต์ ฉบับวันที่ 7 สิงหาคม 2552
- 2.3 หัวข้อข่าวเรื่อง "เสริมแคลเซียมแม่ก่อนให้ลูกดื่มนม"
หนังสือพิมพ์ไทยรัฐ ฉบับวันที่ 15 สิงหาคม 2552
- 2.4 หัวข้อข่าวเรื่อง "ดื่มนมเพิ่มแคลเซียมก่อนให้นมลูก"
หนังสือพิมพ์เดลินิวส์ ฉบับวันที่ 18 สิงหาคม 2552
- 2.5 หัวข้อข่าวเรื่อง "แนะแม่ดื่มนมก่อนให้นมลูก เสริม-ชะลอกระดูกพรุน"
หนังสือพิมพ์โลกวันนี้ ฉบับวันที่ 18 สิงหาคม 2552
- 2.6 หัวข้อข่าวเรื่อง "แนะแม่ดื่มนมก่อนให้นมลูก เพื่อกระดูกที่แข็งแรง"
วารสาร Thai Nursing Time ฉบับเดือน กันยายน 2552
- 2.7 รายการวิจัยไทยคิด (ของ สกว.) ตอน “แคลเซียม” มิตรแท้ กระดูกและฟัน
สถานที่เผยแพร่ ทีวี่ไทย วันที่ 31 มกราคม 2553

มีสำเนาบทความหรือหลักฐานแนบในภาคผนวก 3

3. การได้รับเชิญเป็นวิทยากร (invited speaker) จำนวน 3 ครั้ง

3.1 **Charoenphandhu N.** “Multidisciplinary research on bone and calcium metabolism” at the 34th Congress on Science and Technology of Thailand (STT 34), Bangkok, Thailand, October 31–November 2, 2008.

3.2 **Charoenphandhu N.** “Multidisciplinary research on bone and calcium metabolism: From molecular to clinical study” at the 2nd Biochemistry and Molecular Biology (BMB) Conference, Khon Kaen, Thailand, May 7–8, 2009

3.3 **Charoenphandhu N.** "Molecular mechanism of the prolactin-enhanced intestinal Ca^{2+} absorption: potential application for the prevention of maternal osteopenia" at the 3rd Biochemistry and Molecular Biology (BMB) International Conference, Chiang Mai, Thailand, April 6–8, 2011.

มีบทคัดย่อแนบในภาคผนวก 4

4. การนำเสนอผลงานระดับชาติ จำนวน 6 เรื่อง

4.1 Suntornsaratoon P, Krishnamra N, **Charoenphandhu N.** Bromocriptine can prevent lactation-induced bone loss in femoral metaphyses: Dual-energy X-ray absorptiometry study, at the 39th Physiological Society of Thailand's Annual Conference, Chonburi, May 5–8, 2010.

4.2 Kraidith K, Krishnamra N, **Charoenphandhu N.** A longitudinal study of the prolactin-stimulated transepithelial calcium transport in the duodenum of pregnant and lactating rats, at the 39th Physiological Society of Thailand's Annual Conference, Chonburi, May 5–8, 2010.

4.3 **Charoenphandhu N**, Kraidith K, Nakkrasae L, Thongon N, Krishnamra N. Prolactin-stimulated calcium absorption in the duodenal epithelium of lactating rats and intestine-like Caco-2 monolayer, การประชุม "นักวิจัยรุ่นใหม่...พบ...เมธีวิจัยอาวุโส สกว." ระหว่างวันที่ 15–17 ตุลาคม 2552 ณ โรงแรมฮอลิเดย์อินน์ รีสอร์ท รีเจนท์ บีช ชะอำ จังหวัดเพชรบุรี

4.4 **Charoenphandhu N**, Kraidith K, Nakkrasae L, Jantarajit W, Krishnamra N. Stimulation of cecal calcium transport in female rats by prolactin, การประชุม "นักวิจัยรุ่นใหม่...พบ...เมธีวิจัยอาวุโส สกว." ระหว่างวันที่ 14–16 ตุลาคม 2553 ณ โรงแรมฮอลิเดย์อินน์ รีสอร์ท รีเจนท์ บีช ชะอำ จังหวัดเพชรบุรี

4.5 Suntornsaratoon P, Krishnamra N, **Charoenphandhu N.** Possible osteoregulatory role of prolactin in lactation, at the Graduate Research Exposition 2010, Faculty of Science, Mahidol University, Bangkok, Thailand, October 27, 2010.

4.6 Laohapitakworn S, Krishnamra N, **Charoenphandhu N.** Parathyroid hormone induces HCO_3^- secretion in intestinal cell line Caco-2, at the Graduate Research Exposition 2010, Faculty of Science, Mahidol University, Bangkok, Thailand, October 27, 2010.

มีบทคัดย่อแนบในภาคผนวก 4

5. การนำเสนอผลงานระดับนานาชาติ จำนวน 2 เรื่อง

5.1 **Charoenphandhu N**, Kraidith K, Thongon N, Nakkrasae L, Krishnamra N. Prolactin-enhanced calcium absorption in the duodenum of lactating rats is mediated by the phosphoinositide 3-kinase (PI3K) pathway, at the 36th International Congress of Physiological Sciences (IUPS 2009), Kyoto, Japan, July 27–August 1, 2009.

5.2 Kraidith K, Krishnamra N, **Charoenphandhu N**. Prolactin stimulates transepithelial calcium transport in the cecum of female rats, at the 36th International Congress of Physiological Sciences (IUPS 2009), Kyoto, Japan, July 27-August 1, 2009.

มีบทคัดย่อแนบในภาคผนวก 4

6. การผลิตกำลังคนด้านการวิจัย (เฉพาะที่สำเร็จการศึกษาแล้ว) จำนวน 4 คน

ระดับ	ชื่อ-นามสกุล	รหัสนักศึกษา	สาขาวิชา
ปริญญาเอก	นายศุภฤกษ์ เลหาพิทักษ์วร	4702094 SCMM/D	เวชศาสตร์ระดับโมเลกุล
ปริญญาโท	นางสาวปาหั่น สุนทรสารทูล	4936449 SCPS/M	สรีรวิทยา
ปริญญาโท	นางสาวกมลชนก ไกรดิษฐ์	4936450 SCPS/M	สรีรวิทยา
ปริญญาโท	นางสาวนิธิตา ดอกคำ	4936073 SCEP/M	สรีรวิทยาของการออกกำลังกาย

7. รางวัลที่หัวหน้าโครงการวิจัยได้รับ จำนวน 4 รางวัล

ลำดับที่	ปี ค.ศ.	รางวัล
1	2009	Travel Grant Award, The 36 th International Congress of Physiological Sciences, Kyoto, Japan, awarded by the American Physiological Society (APS) and the International Union of Physiological Sciences (IUPS)
2	2010	The Major Advisor's Role in the Honorable Mention for Thesis Award 2009 (Master Level in Health Science) from the Faculty of Graduate Studies, Mahidol University, Thailand

3	2011	The Major Advisor's Role in the Honorable Mention for Thesis Award 2010 (Master Level in Health Science) from the Faculty of Graduate Studies, Mahidol University, Thailand
4	2011	Young BMB Award from the Biochemistry and Molecular Biology Section, The Science Society of Thailand

มีหลักฐานแนบในภาคผนวก 5

ภาคผนวก 1

บทความสำหรับการเผยแพร่

บทความเผยแพร่

แคลเซียมเป็นธาตุอาหารสารพัดประโยชน์ชนิดหนึ่ง เพราะแคลเซียมมีความจำเป็นต่อการทำงานของร่างกายทุกระบบ ไม่ว่าจะเป็นระบบไหลเวียนโลหิต การทำงานของสมอง และสร้างความแข็งแรงให้กับกระดูก ในแต่ละวันเราได้รับแคลเซียมจากอาหาร เช่น ผลิตภัณฑ์จากนม กุ้งแห้งตัวเล็ก ปลาเล็กปลาน้อย ถั่วแดง และงาดำ เป็นต้น เพื่อชดเชยกับที่สูญเสียจากร่างกายทางปัสสาวะ แต่ “แม่” ของเรานั้นต้องเสียแคลเซียมในปริมาณมากเพื่อการเจริญเติบโตของลูกในครรภ์ก่อนที่ลูกจะเกิดเสียอีก นอกจากนี้ “แม่” ยังต้องเสียแคลเซียมอีกมากมายทางน้ำนมเพื่อเสริมสร้างให้ลูกเติบโตขึ้นอย่างสมบูรณ์ และแข็งแรงโดยเฉพาะในช่วงขวบปีแรก

เมื่อเวลาผ่านไป ขณะที่ลูกเติบโตและ “แม่” สูงวัยขึ้นและกำลังก้าวเข้าสู่ช่วงชีวิตในวัยผู้สูงอายุ จากปัจจัยทางพันธุกรรมและการขาดฮอร์โมนเพศหญิงหลังหมดประจำเดือนทำให้ “แม่” ณ เวลานั้นต้องเผชิญกับการสูญเสียแคลเซียมจำนวนมากจากกระดูก ซึ่งอาจนำไปสู่โรคกระดูกพรุนในที่สุด เหตุการณ์นี้เป็นผลร่วมกันระหว่างความเสื่อมที่เกิดขึ้นเองตามธรรมชาติและจากปัจจัยร่วมมากมายที่เป็นตัวเสริมเร่งให้เกิดภาวะกระดูกพรุนได้รวดเร็วมากยิ่งขึ้น เช่น การขาดการออกกำลังกาย การรับประทานอาหารที่มีแคลเซียมต่ำ ตลอดจนพยาธิสภาพบางประการ ได้แก่ ภาวะเลือดเป็นกรดเรื้อรัง เนื่องจากบางชนิด และโรคทางไต เป็นต้น ภาวะกระดูกพรุนนี้เป็นเหตุให้คุณภาพชีวิตของ “แม่” ลดลง แต่ก็ก็เป็นภาวะที่สามารถชะลอหรือบรรเทาได้หากได้รับการป้องกันและรักษาอย่างถูกวิธี

โดยทั่วไปการรักษาโรคกระดูกพรุนสามารถทำได้ด้วยการใช้ยาหรือฮอร์โมน แต่การชะลอการเกิดหรือการป้องกันจะเป็นวิธีที่มีประสิทธิภาพกว่าการรักษาหลังเกิดโรคแล้ว ปัจจุบันมีอยู่หลายวิธีที่มีรายงานทางวิชาการว่าสามารถชะลอหรือลดความรุนแรงของโรคนี้ได้ อาทิ การออกกำลังกายที่มีความเหมาะสม และการให้แคลเซียมรับประทานเสริม การชักชวนให้แม่ออกกำลังกายสม่ำเสมอและรับประทานอาหารแคลเซียมสูงจะเป็นประโยชน์ต่อสุขภาพกระดูกของแม่ในระยะยาว อย่างไรก็ตาม ในทางวิชาการยังมีข้อจำกัดสำหรับการช่วยให้กระดูกของ “แม่” แข็งแรงอยู่ 2 ประการดังนี้คือ

1. การออกกำลังกายที่มีแรงกระทำต่อกระดูกและข้อสูง อาจทำให้เกิดการบาดเจ็บของข้อต่อหรือทำให้ข้อเสื่อมได้ ฉะนั้นจึงมีการตั้งสมมติฐานว่าการออกกำลังกายอาจต้องเป็นแบบแรงกระแทกต่ำ (non-impact exercise) เช่น การว่ายน้ำ ที่ลดการบาดเจ็บของข้อต่อได้ แต่องค์ความรู้เรื่องผลของการออกกำลังกายแบบแรงกระแทกต่ำต่อการสร้างความแข็งแรงของกระดูกมีอยู่น้อยมาก งานวิจัยส่วนใหญ่เน้นที่การเพิ่มความแข็งแรงของกระดูกในคนอายุน้อยหรือในนักกีฬา ซึ่งส่วนใหญ่เป็นการออกกำลังกายแบบมีแรงกระแทกสูง เช่น การวิ่ง หรือกระโดด

2. ประสิทธิภาพของกระบวนการดูดซึมแคลเซียมจากอาหารเข้าสู่ร่างกายจะลดลงตามอายุของ “แม่” ที่เพิ่มขึ้น ดังนั้นแคลเซียมจากอาหารแต่เพียงอย่างเดียวอาจไม่เพียงพอต่อความต้องการของร่างกาย จึงมีความจำเป็นต้องรับประทานแคลเซียมเสริม ซึ่งแม้ว่าจะทำให้ร่างกายได้รับแคลเซียมมากขึ้น แต่ร่างกายก็ไม่สามารถดูดซึมแคลเซียมเข้าสู่ร่างกายได้ทั้งหมด การเพิ่มประสิทธิภาพการดูดซึม

แคลเซียมของร่างกายตลอดจนการพัฒนารูปแบบแคลเซียมที่จะเข้าสู่ร่างกายได้ดีจำเป็นต้องมีองค์ความรู้ด้านกลไกการดูดซึมแคลเซียมเข้าสู่ร่างกาย องค์ความรู้ดังกล่าวนี้เป็นที่ทราบและยอมรับมากกว่าหนึ่งทศวรรษ อย่างไรก็ตาม ผลการวิจัยในปัจจุบันที่ใช้เทคนิคอันทันสมัยมากขึ้นบ่งชี้ว่า องค์ความรู้นี้มีความคลาดเคลื่อนค่อนข้างมาก ดังนั้นนักวิจัยด้านแคลเซียมและกระดูกจำเป็นต้องศึกษาค้นคว้าเพิ่มเติมเพื่อพัฒนาองค์ความรู้ที่ถูกต้องขึ้นมาใหม่ สำหรับการต่อยอดความรู้เพื่อเพิ่มประสิทธิภาพการดูดซึมแคลเซียม

ปัจจุบัน งานวิจัยส่วนหนึ่งซึ่งได้รับการสนับสนุนทุนวิจัยจากทางคณะวิทยาศาสตร์มหาวิทยาลัยมหิดล และสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) แสดงให้เห็นว่า มีความเป็นไปได้ที่จะป้องกันการสูญเสียมวลกระดูกของแม่ในระหว่างเลี้ยงบุตรด้วยน้ำนมโดยให้แม่รับประทานแคลเซียมเสริมในปริมาณและช่วงเวลาที่เหมาะสม คือ ประมาณ 6 มิลลิกรัมต่อน้ำหนักตัว 1 กิโลกรัม ราว 30 นาที ก่อนให้นมลูก วันละ 3-4 ครั้ง ทั้งนี้เพราะในระหว่างให้นมบุตรจะมีฮอร์โมนที่อยู่ในกระแสเลือดหลายชนิด เช่น โพรแลคติน ที่สามารถเพิ่มประสิทธิภาพการดูดซึมแคลเซียมจากอาหารที่รับประทานเข้าสู่ร่างกายได้ในอัตราที่สูงขึ้นเป็นเท่าตัว ซึ่งจะช่วยลดอัตราการสลายกระดูกของแม่เพื่อนำแคลเซียมออกมาใช้ลงได้ และเป็นที่เชื่อกันว่ากระบวนการนี้จะได้อีกวิธีการหนึ่งที่จะช่วยให้ “แม่” ผู้เป็นที่รักของลูกทุก ๆ คนมีกระดูกที่สมบูรณ์แข็งแรงได้ไปจนสูงวัย

Prolactin stimulates transepithelial calcium transport and modulates paracellular permselectivity in Caco-2 monolayer: mediation by PKC and ROCK pathways

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Thongon N, Nakkrasae L-I, Thongbunchoo J, Krishnamra N, Charoenphandhu N. Prolactin stimulates transepithelial calcium transport and modulates paracellular permselectivity in Caco-2 monolayer: mediation by PKC and ROCK pathways. *Am J Physiol Cell Physiol* 294: C1158–C1168, 2008. First published March 19, 2008; doi:10.1152/ajpcell.00020.2008.—Prolactin (PRL) was previously demonstrated to rapidly enhance calcium absorption in rat duodenum and the intestine-like Caco-2 monolayer. However, its mechanism was not completely understood. Here, we investigated nongenomic effects of PRL on the transepithelial calcium transport and paracellular permselectivity in the Caco-2 monolayer by Ussing chamber technique. PRL increased the transcellular and paracellular calcium fluxes and paracellular calcium permeability within 60 min after exposure but decreased the transepithelial resistance of the monolayer. The effects of PRL could not be inhibited by RNA polymerase II inhibitor (5,6-dichloro-1- β -D-ribofimbimazole), confirming that PRL actions were nongenomic. Exposure to protein kinase C (PKC) or RhoA-associated coiled-coil forming kinase (ROCK) inhibitors (GF-109203X and Y-27632, respectively) abolished the stimulatory effect of PRL on transcellular calcium transport, whereas ROCK inhibitor, but not PKC inhibitor, diminished the PRL effect on paracellular calcium transport. Knockdown of the long isoform of PRL receptor (PRLR-L) also prevented the enhancement of calcium transport by PRL. In addition, PRL markedly increased paracellular sodium permeability and the permeability ratio of sodium to chloride, which are indicators of the paracellular charge-selective property and are known to be associated with the enhanced paracellular calcium transport. The permeability of other cations in the alkali metal series was also increased by PRL, and such increases were abolished by ROCK inhibitor. It could be concluded that PRL stimulated transepithelial calcium transport through PRLR-L and increased paracellular permeability to cations in the Caco-2 monolayer. These nongenomic actions of PRL were mediated by the PKC and ROCK signaling pathways.

charge selectivity; dilution potential; paracellular transport; phosphoinositide 3-kinase; protein kinase C; long form of prolactin receptor; RhoA; short interference RNA; transcellular transport

DURING PREGNANCY and lactation, prolactin (PRL) is a calcium-regulating hormone that mitigates negative calcium balance by increasing intestinal calcium absorption (10). In vivo studies in normal rats suggested that PRL stimulated both transcellular active and paracellular passive calcium transport in the duodenum and proximal jejunum (10, 33). Exposure to high physiological levels of PRL of 400–600 ng/ml, which are compa-

rable to the levels attained during lactation and suckling (2), rapidly enhanced transepithelial calcium transport in isolated duodenal epithelium and intestine-like Caco-2 monolayer in a dose-dependent manner (25). However, the signaling pathways of PRL in the intestinal absorptive cells and the detailed mechanisms of the PRL-enhanced calcium transport were not completely understood.

Calcium traverses the intestinal epithelium via both transcellular and paracellular pathways (22). Transcellular active calcium transport is a metabolically energized process, consisting of apical calcium entry via the transient receptor potential vanilloid family Ca^{2+} channel 6 (TRPV6), cytoplasmic calcium translocation in a calbindin-D_{9K}-bound form, and basolateral calcium extrusion via the plasma membrane Ca^{2+} -ATPase (PMCA) (22). On the other hand, paracellular passive calcium transport is dependent on the transepithelial calcium gradient and transepithelial resistance (TER) and is absent when both sides of the epithelium contain equal calcium concentration (12, 29). In normal intestinal epithelia, movement of ions and nutrients across the paracellular pathway is generally regulated by the size- and charge-selective properties of the tight junction, which contains several charge-selective claudin proteins arranged in arrays of channellike barriers (32, 49, 54). Several mediators, e.g., phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and RhoA-associated coiled-coil forming kinase (ROCK), can modulate permselectivity of the paracellular pathway (5, 15, 25, 47).

Our recent investigation (25) demonstrated that the Caco-2 monolayer, which strongly expressed short and long isoforms of the PRL receptor (PRLR-S and -L, respectively), responded to PRL by increasing both transcellular and paracellular calcium transport. However, little was known regarding the effect of PRL on the paracellular permselectivity and electrical properties of the epithelium. Moreover, it was not known whether PRLR-S or PRLR-L was responsible for PRL signaling in the intestinal absorptive cells, but there was a report that in mammary epithelia PRL augmented galactopoiesis and electrolyte transport through PRLR-L, whereas PRLR-S silenced those actions (3, 6, 24, 42). The signaling pathway of PRL in Caco-2 cells, in contrast to mammary epithelial cells, may be nongenomic since the actions were observed very rapidly within 60 min after PRL exposure (25). Interestingly, in Caco-2 cells and duodenal epithelium, the nongenomic actions

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of PRL were mediated by the PI3K pathway, but not the putative Janus kinase (JAK)2 or mitogen-activated protein kinase (MAPK) pathways (25). PI3K is the ultimate upstream kinase to several downstream targets, including PKC and ROCK, which are known to modulate transepithelial calcium transport and increase paracellular permeability, respectively (4, 21, 28, 36, 47). Thus PKC and ROCK pathways may mediate the actions of PRL.

In the present study, we used Caco-2 cells to demonstrate PRL-enhanced calcium absorption. Despite being human colorectal adenocarcinoma cells, confluent Caco-2 monolayers have been used widely in the studies of calcium and drug absorption because they have functional similarity to the small intestine, including the presence of brush border, expression of sucrase-isomaltase enzymes, and expression of the transcellular calcium transporters and charge-selective paracellular proteins, e.g., TRPV6, calbindin-D_{9K}, PMCA, and claudin-1, -2, -3, and -5 (37–39, 57, 58). The Caco-2 monolayer also responds to PRL in a dose-dependent manner, with a maximal effective concentration of 600 ng/ml, similar to that seen in duodenal epithelium (25). However, the normal paracellular passive calcium transport and cationic permselectivity of the Caco-2 monolayer as well as its response to PRL by altering paracellular calcium transport have not been fully characterized.

Therefore, the objectives of this study were 1) to demonstrate that the rapid stimulatory effects of PRL on transepithelial calcium transport in the Caco-2 monolayer are non-genomic, 2) to show that PRL signaling involves the PKC and ROCK pathways, 3) to elucidate the normal characteristics of the paracellular calcium transport and cationic permselectivity of the Caco-2 monolayer, as well as the effects of PRL on these parameters, and 4) to demonstrate that PRLR-L is required for the PRL-enhanced calcium absorption.

MATERIALS AND METHODS

Cell culture. Caco-2 cells [American Type Culture Collection (ATCC) no. HTB-37] were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 15% fetal bovine serum (FBS; GIBCO, Grand Island, NY), 1% L-glutamine (GIBCO), 1% nonessential amino acid (Sigma), and 100 U/ml penicillin-streptomycin. Cells were propagated in 75-cm² T flasks (Corning) under a humidified atmosphere containing 5% CO₂ at 37°C and subcultured as described in the ATCC protocol. Confluent Caco-2 monolayers were prepared by seeding cells on polyester Snapwell inserts with 12-mm diameter and 0.4-μm pore size (catalog no. 3801; Corning) at 5 × 10⁵ cells/well. Culture medium was changed daily after 48 h of seeding. Monolayers were incubated at 37°C for 14 days in a humidified atmosphere containing 5% CO₂.

On the experimental day, the Snapwell was mounted in a modified Ussing chamber with an exposed surface area of 1.13 cm² to measure electrical parameters, calcium fluxes, and/or ion permeability, as previously described (25). The monolayer was incubated for 20 min in the chamber before the 60-min experiment was carried out.

Bathing solution. The bathing solution for the Ussing chamber experiments contained (in mmol/l) 118 NaCl, 4.7 KCl, 1.1 MgCl₂, 1.25 CaCl₂, 23 NaHCO₃, 12 D-glucose, and 2 mannitol (all purchased from Sigma). The solution, continuously gassed with humidified 5% CO₂ in 95% O₂, was maintained at 37°C and pH 7.4 and had an osmolality of 290–293 mosmol/kgH₂O as measured by a freezing point-based osmometer (model 3320; Advanced Instruments, Norwood, MA). Water used in the present work had a resistance >18.3 MΩ·cm and a free ionized calcium concentration <2.5 nmol/l.

Short interference RNA transfection. Two short interference RNA (siRNA) sequences targeted for human PRLR-L, i.e., 5'-GGGC-UAAUGCAUGGUGACCTT-3' and 5'-GGUCACCAUGCUAAGC-CCTT-3', scrambled siRNAs, and the transfection reagent kit were supplied by Ambion (Austin, TX). Caco-2 cells were seeded in Snapwells at 5 × 10⁵ cells/well for 12 days. Thereafter, in vitro transfection was performed with the 1:400 siPORT amine transfection reagent (Ambion) according to the manufacturer's instruction. The siRNAs were added in the culture medium to obtain a final concentration of 1 nmol/l. Control cells were treated with siPORT without siRNA. After a 48-h transfection, the PRL-stimulated calcium transport across the PRLR-L knockdown monolayer was measured. The siRNA efficiency was determined by quantitative real-time PCR (qRT-PCR). This PRLR-L knockdown study was approved by the Institutional Biosafety Committee (IBC) of the Faculty of Science, Mahidol University.

mRNA isolation, qRT-PCR, and sequencing. With the use of TRIzol reagent (Invitrogen, Carlsbad, CA), total RNA was prepared from the PRLR-L knockdown Caco-2 cells as previously described (13). One microgram of the total RNA was reverse-transcribed with the iScript kit (Bio-Rad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control gene to check the consistency of reverse transcription (% coefficient of variation <1%; n = 20). Sense and antisense primers of PRLRs and GAPDH were designed by OLIGO 6 (Molecular Biology Insights, Cascade, CO) and Primer Validator 1.4 (Naratt Software, Bangkok, Thailand), as shown in Table 1. The amplification reaction using real-time PCR (model MiniOpticon; Bio-Rad) was performed with the iQ SYBR Green SuperMix (Bio-Rad). Relative expression of PRLR over GAPDH was calculated from the threshold cycle (C_t) values by the 2^{-ΔC_t} method. After qRT-PCR, the PCR products were also visualized on a 1.5% agarose gel stained with 1.0 μg/ml ethidium bromide. Thereafter, all PCR products were extracted with the HiYield Gel/PCR DNA Extraction kit (Real Biotech, Taipei, Taiwan) and were sequenced with the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Measurement of electrical parameters. Three electrical parameters, i.e., potential difference (PD), short-circuit current (I_{sc}), and TER, were determined as previously described (12). In brief, a pair of Ag/AgCl electrodes connected to agar bridges (3.0 mol/l KCl per 4 g% agar) was located near each surface of the mounted Snapwell for measurement of PD. The other ends of the PD-sensing electrodes were connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL). Another pair of Ag/AgCl electrodes connected in series to the EVC-4000 current-generating unit was placed at the end of each hemichamber to supply I_{sc}. TER and conductance (G; G = 1/TER) were calculated from Ohm's equation.

Measurement of calcium, mannitol, and polyethylene glycol fluxes. Calcium transport across Caco-2 monolayer was determined by the method of Charoenphandhu et al. (12). After 20-min incubation, the Ussing chamber was filled with fresh bathing solution containing

Table 1. *Homo sapiens* oligonucleotide sequences used in qRT-PCR experiments

Name	Accession No.	Primer (forward/reverse)	Product Length, bp
PRLR-S	AF416619	5'-GGTGACCCCTTGATGTTG-3' 5'-TTCTGGTATATGCTCTTCAGC-3'	145
PRLR-L	NM_000949	5'-ACTTGCTCTTTCTCCAG-3' 5'-TCCCTCAAGAATACTAAGCAG-3'	100
GAPDH	NM_002046	5'-CTGGTAAAGTGGATATTGTTG-3' 5'-GAGGCTGTTGTCTACTTCTC-3'	359

qRT-PCR, quantitative RT-PCR; PRLR-S, short isoform of prolactin receptor; PRLR-L, long isoform of prolactin receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

$^{45}\text{CaCl}_2$ (final specific activity of 500 mCi/mol; Amersham, Little Chalfont, UK). Radioactivity of ^{45}Ca was analyzed with a liquid scintillation spectrophotometer (model Tri-Carb 3100 TR; Perkin-Elmer, Shelton, CT). Total calcium concentration was analyzed by atomic absorption spectroscopy (model SpectrAA-300; Varian Techtron, Victoria, Australia). Unidirectional flux ($J_{\text{H} \rightarrow \text{C}}$, $\text{nmol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$) of calcium from the hot side (H) to the cold side (C) was calculated with Eqs. 1 and 2.

$$J_{\text{H} \rightarrow \text{C}} = R_{\text{H} \rightarrow \text{C}} / (S_{\text{H}} \times A) \quad (1)$$

$$S_{\text{H}} = C_{\text{H}} / C_{\text{T}} \quad (2)$$

where $R_{\text{H} \rightarrow \text{C}}$ is the rate of tracer appearance in the cold side (cpm/h); S_{H} is the specific activity in the hot side (cpm/nmol); A is the surface area of the Snapwell (cm^2); C_{H} is the mean radioactivity in the hot side (cpm); and C_{T} is the total calcium in the hot side (nmol).

Calcium fluxes in the absence of calcium concentration gradient (i.e., bathing solution in both hemichambers contained equal calcium concentration) represented the transcellular active calcium transport (12). The calcium gradient-dependent paracellular passive transport was studied by determining calcium fluxes in the presence of varying apical calcium concentrations (12), i.e., 1.25, 2.5, 5, 10, 20, 40, and 80 mmol/l.

In some experiments, the Caco-2 monolayer was bathed on both sides with solution containing 1 mmol/l mannitol and 1 mmol/l polyethylene glycol (PEG). Paracellular markers [^3H]mannitol (Amersham; molecular weight 180; molecular radius ~ 350 pm) and [^{14}C]PEG (Amersham; molecular weight 4,000; molecular radius ~ 2.5 nm) were added in the bathing solution to obtain final specific activities of 750 and 500 mCi/mol, respectively. Transepithelial mannitol flux and PEG flux were then measured.

In an Ussing chamber, the Caco-2 monolayer was directly exposed on the basolateral side for 60 min to 600 ng/ml recombinant human PRL (rhPRL) (purity $>97\%$; catalog no. 682-PL; R&D Systems, Minneapolis, MN), which is the maximal effective concentration reported by Jantarajit et al. (25), or rhPRL plus inhibitors, which were an RNA polymerase II inhibitor [50 $\mu\text{mol/l}$ 5,6-dichloro-1- β -D-ribo-benzimidazole (DRB); Calbiochem, La Jolla, CA], a pan-specific PKC inhibitor (0.8 or 1 $\mu\text{mol/l}$ GF-109203X; A. G. Scientific, San Diego, CA), and a selective ROCK inhibitor (1 $\mu\text{mol/l}$ Y-27632; Calbiochem).

Permeability measurement. Permeability of sodium (P_{Na}) and chloride (P_{Cl}) were determined by the dilution potential technique, modified from the methods of Kahle et al. (26) and Hou et al. (23). In brief, Caco-2 monolayer was equilibrated for 20 min in normal bathing solution containing 145 mmol/l NaCl before the apical solution was replaced with 72.5 mmol/l NaCl-containing solution. Osmolality was maintained by an equivalent amount of mannitol. Changes in the electrical parameters before and after solution replacement were recorded until stable. The ion permeability ratio ($P_{\text{Na}}/P_{\text{Cl}}$) was calculated from the dilution potential (V_{δ}) with Eq. 3 (45)

$$V_{\delta} = \frac{RT}{F} \ln \frac{P_{\text{Na}} C_{\text{a}} + P_{\text{Cl}} C_{\text{b}}}{P_{\text{Na}} C_{\text{b}} + P_{\text{Cl}} C_{\text{a}}} \quad (3)$$

where P_{Na} is the absolute permeability of sodium; P_{Cl} is the absolute permeability of chloride; C_{a} is the apical NaCl concentration; C_{b} is the basolateral NaCl concentration; and R , T , and F are the gas constant, temperature, and the Faraday constant.

When given $\rho = P_{\text{Na}}/P_{\text{Cl}}$, $\phi = C_{\text{b}}/C_{\text{a}}$, and $v = FV_{\delta}/RT$, Eq. 3 is rewritten as

$$\rho = (\phi - e^v) / (\phi e^v - 1) \quad (4)$$

According to the Kimizuka-Koketsu equation (31),

$$G = \frac{F^2}{RT} (P_{\text{Na}} C_{\text{b}} + P_{\text{Cl}} C_{\text{a}}) \quad (5)$$

where G is the transepithelial conductance and P_{Na} and P_{Cl} are calculated from G and ρ by using Eqs. 6 and 7, respectively.

$$P_{\text{Na}} = \frac{GRT}{C_{\text{a}} F^2} \times \frac{\rho}{1 + \rho} \quad (6)$$

$$P_{\text{Cl}} = P_{\text{Na}} / \rho \quad (7)$$

To study the permeability ratios of metal ions in the Group 1 series (alkali metals), i.e., $P_{\text{X}}/P_{\text{Cl}}$ (X^+ is Li^+ , Na^+ , K^+ , Rb^+ , or Cs^+ in the form of chloride salt; all purchased from Sigma), Na^+ as the primary conductor in the bathing solution was substituted with X^+ , while pH was maintained with 10 mmol/l HEPES (Sigma). The solution used was titrated with 1 mol/l mannitol until the osmolality of 290 mosmol/kgH₂O was obtained. During the experimental period, the Caco-2 monolayer was equilibrated for 20 min in normal bathing solution containing 145 mmol/l NaCl before the apical solution was replaced with 100 mmol/l X^+ -containing solution. Thereafter, the concentration of X^+ was diluted to 50 mmol/l to create diffusion (dilution) potential. $P_{\text{X}}/P_{\text{Cl}}$ was calculated as described previously (45).

Calcium permeability (P_{Ca}) of the Caco-2 monolayer via the paracellular pathway was calculated from Eq. 8 (12)

$$P_{\text{Ca}} = J_{\text{Ca}} / \Delta C \quad (8)$$

where J_{Ca} is the paracellular passive calcium flux and ΔC is the difference between the apical and basolateral calcium concentrations.

In some experiments, the monolayer was exposed to 1 $\mu\text{mol/l}$ GF-109203X, 1 $\mu\text{mol/l}$ Y-27632, or 75 $\mu\text{mol/l}$ LY-294002 (PI3K inhibitor; Tocris Bioscience, Bristol, UK).

Statistical analyses. Results are expressed as means $\pm$ SE. Two sets of data were compared with the unpaired Student's *t*-test. Multiple comparisons were performed by one-way analysis of variance (ANOVA) with Dunnett's posttest. Linear regression with slope analysis was performed to obtain the apical calcium concentration-calcium flux and TER relationships. Nonlinear regression was performed with the one-phase exponential decay equation to demonstrate the Δ calcium-calcium permeability relationship as previously described (12). The level of significance for all statistical tests was $P < 0.05$. Data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA).

RESULTS

Nongenomic action of PRL on transcellular active calcium transport involves PKC and ROCK pathways. As shown in Fig. 1, 600 ng/ml rhPRL significantly stimulated the transcellular active calcium transport in Caco-2 monolayer from the control value of 9.22 ± 0.48 ($n = 10$) to 18.04 ± 0.51 $\text{nmol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$ ($n = 12$, $P < 0.01$). PRL concurrently decreased the TER of the monolayer by $\sim 30\%$ (Table 2). Here, 50 $\mu\text{mol/l}$ DRB, a classic RNA polymerase II inhibitor, was used to inhibit gene transcription. DRB has been used to demonstrate nongenomic effects of several hormones, including vitamin D (43). Since the effect of PRL occurred within 60 min and was not inhibited by DRB, PRL exerted its rapid action via nongenomic signaling pathway(s).

In the presence of 0.8 $\mu\text{mol/l}$ GF-109203X (PKC inhibitor) and 1 $\mu\text{mol/l}$ Y-27632 (ROCK inhibitor), the effect of PRL on the transcellular active calcium transport was diminished (Fig. 1), while only Y-27632 inhibited the action of PRL on the TER (Table 2), suggesting that both PKC and ROCK pathways were involved in the signal transduction of PRL. A higher concentration of GF-109203X, i.e., 1 $\mu\text{mol/l}$, was used to confirm that

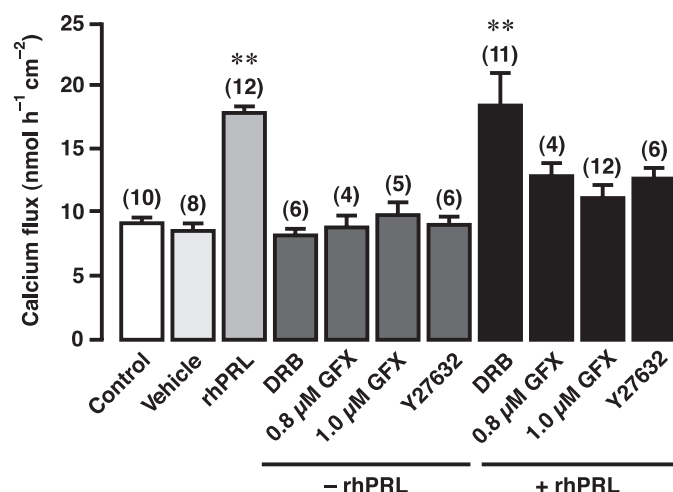


Fig. 1. Transcellular active calcium fluxes in Caco-2 monolayer exposed to RNA polymerase II inhibitor (50 $\mu\text{mol/l}$ 5,6-dichloro-1- β -D-ribozimidazole; DRB), protein kinase C (PKC) inhibitor (0.8 and 1 $\mu\text{mol/l}$ GF-109203X; GFX), or RhoA-associated coiled-coil forming kinase (ROCK) inhibitor (1 $\mu\text{mol/l}$ Y-27632) in the presence (+rhPRL) or absence (-rhPRL) of 600 ng/ml recombinant human prolactin (rhPRL). DMSO 0.3% (vol/vol) was used as vehicle for preparation of inhibitors. $^{**}P < 0.01$ compared with control group. Numbers in parentheses represent the number of independent Snapwells.

inhibition of PKC completely abolished the effect of PRL on the transcellular calcium transport. Neither inhibitor alone nor 0.3% (vol/vol) DMSO, a vehicle for the preparation of inhibitors, had an effect on the transcellular active calcium flux or TER (Fig. 1).

PRL stimulates paracellular passive calcium transport via ROCK pathway. The relationship between apical calcium concentrations (C_{Ca}), which created the transepithelial calcium gradient ($\Delta\text{calcium}$), and calcium flux (J_{Ca}) in Caco-2 monolayer shows linearity ($r^2 = 0.964$, Fig. 2, A and B). J_{Ca} under this condition represents the $\Delta\text{calcium}$ gradient-dependent paracellular passive transport. After exposure to rhPRL, J_{Ca} at all C_{Ca} (1.25–80 mmol/l) were significantly increased compared with their respective controls ($r^2 = 0.956$, $P < 0.01$). The slope, but not the y-intercept, of the PRL-exposed graph is higher than that of the control, i.e., 3.76 ± 0.13 vs. $6.41 \pm 0.24 \times 10^{-3}$ cm/h ($P < 0.001$). Interestingly, incubation of Caco-2 monolayer with rhPRL plus 1 $\mu\text{mol/l}$ GF-109203X did not affect the paracellular passive calcium transport (Fig. 2A), whereas Y-27632 completely abolished the effect of PRL on this mode of calcium transport (Fig. 2B). In addition, rhPRL increased the paracellular P_{Ca} , and this effect was abolished by Y-27632 but not 1 $\mu\text{mol/l}$ GF-109203X (Fig. 2, C and D).

Since tight junctions of several epithelia, as an array of channellike structures, show a conductance block after exposure to divalent cations, including calcium (49), we measured the TER of the monolayer in the presence of transepithelial calcium gradient. As depicted in Fig. 3, increases in apical C_{Ca} led to an increase in TER with linear correlation ($r^2 = 0.882$). Exposure to rhPRL, on the other hand, decreased the TER of the monolayer ($P < 0.01$) compared with the corresponding control values at the respective C_{Ca} . However, the TER of the PRL-exposed monolayer was still increased with the apical C_{Ca} ($r^2 = 0.842$). The slopes of both regression lines are significantly different, i.e., 6.71 ± 0.43 vs. 3.67 ± 0.28

$\Omega \cdot \text{cm}^2 \cdot \text{l} \cdot \text{mmol}^{-1}$ ($P < 0.001$). A reduction in TER after PRL exposure explained the PRL-stimulated paracellular passive calcium transport.

The ROCK inhibitor (Fig. 3B), but not the PKC inhibitor (Fig. 3A), abolished the effect of PRL on the apical calcium-induced alteration in TER. Since we recently reported (25) that PI3K inhibitor (75 $\mu\text{mol/l}$ LY-294002) also compromised the paracellular passive calcium transport in Caco-2 monolayer, the effect of this inhibitor on the apical calcium-induced alteration in TER was also examined. Figure 3C shows that PI3K inhibitor, similar to ROCK inhibitor, totally abolished the effect of PRL on TER. Inhibitor alone or 0.3% (vol/vol) DMSO had no effect on the paracellular passive calcium transport and did not alter TER (data not shown).

PRL alters charge-selective property of paracellular pathway via ROCK pathway. Increased paracellular transport of calcium is usually associated with alterations in the size- and charge-selective properties of the tight junction, which is the principal barrier for paracellular ion transport (53, 54). However, in the present study, transepithelial fluxes of mannitol and PEG-4000, which are indicators of the widening of the tight junction or change in size selectivity (50), were not affected by PRL (Table 3), suggesting that charge selectivity rather than size selectivity was the determinant of the paracellular transport in the presence of PRL.

The paracellular charge selectivity is determined by P_{Na} , P_{Cl} , and P_{Na}/P_{Cl} . By using the dilution potential technique, we found that rhPRL increased P_{Na} (Fig. 4A), but not P_{Cl} (Fig. 4B), of Caco-2 monolayer from the control value of 7.81 ± 0.32 ($n = 8$) to $10.96 \pm 0.42 \times 10^{-6}$ cm/s ($n = 8$, $P < 0.01$), thereby increasing P_{Na}/P_{Cl} from 3.73 ± 0.23 ($n = 8$) to 8.07 ± 0.82 ($n = 8$, $P < 0.01$) (Fig. 4C). The effects of PRL on P_{Na} and P_{Na}/P_{Cl} were completely abolished by Y-27632, but not by 1 $\mu\text{mol/l}$ GF-109203X. Inhibitor alone or vehicle had no effect on the charge selectivity.

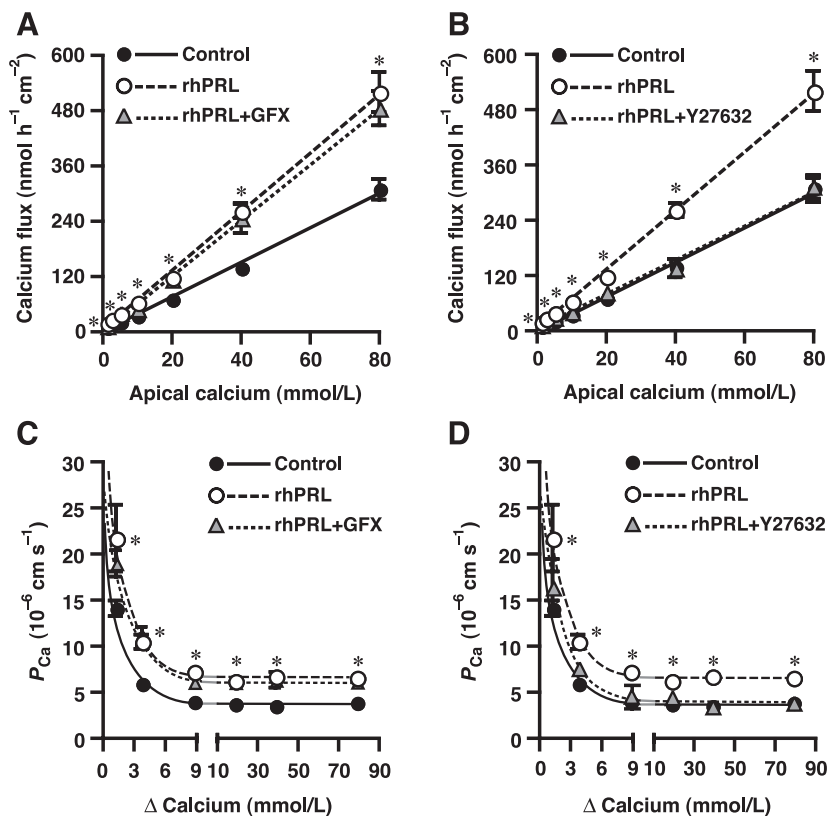
Furthermore, we investigated permselectivity of the paracellular pathway to other cations in the alkali metal series with

Table 2. Epithelial electrical parameters for Caco-2 monolayer

Condition	n	PD, mV	I_{sc} , $\mu\text{A}/\text{cm}^2$	TER, $\Omega \cdot \text{cm}^2$
Control	15	0.89 ± 0.12	2.53 ± 0.40	368.17 ± 11.44
Vehicle	10	0.95 ± 0.12	2.40 ± 0.31	381.67 ± 9.11
600 ng/ml rhPRL	15	0.94 ± 0.06	3.67 ± 0.27	$255.67 \pm 7.10^*$
Inhibitors				
50 $\mu\text{mol/l}$ DRB	12	0.81 ± 0.11	2.17 ± 0.27	371.53 ± 23.49
1 $\mu\text{mol/l}$ GF-109203X	13	0.95 ± 0.18	2.62 ± 0.47	348.97 ± 19.80
1 $\mu\text{mol/l}$ Y-27632	14	0.63 ± 0.08	2.00 ± 0.30	373.81 ± 18.55
600 ng/ml rhPRL +				
50 $\mu\text{mol/l}$ DRB	14	0.96 ± 0.14	3.71 ± 0.53	$261.71 \pm 11.52^*$
1 $\mu\text{mol/l}$ GF-109203X	15	0.79 ± 0.15	3.07 ± 0.51	$249.44 \pm 13.64^*$
1 $\mu\text{mol/l}$ Y-27632	14	0.85 ± 0.10	2.29 ± 0.28	377.38 ± 18.54

Values are means $\pm$ SE for transepithelial potential difference (PD), short-circuit current (I_{sc}), and transepithelial resistance (TER) in Caco-2 monolayer directly exposed to recombinant human prolactin (rhPRL), RNA polymerase II inhibitor [5,6-dichloro-1- β -D-ribozimidazole (DRB)], protein kinase C (PKC) inhibitor (GF-109203X), RhoA-associated coiled-coil forming kinase (ROCK) inhibitor (Y-27632), or rhPRL plus inhibitors. DMSO 0.3% (vol/vol) was used as vehicle for preparation of inhibitors. The monolayer was bathed on both sides with 1.25 mmol/l calcium-containing solution. The apical side was negative with respect to the basolateral side. $^*P < 0.01$ vs. control group.

Fig. 2. Gradient-dependent paracellular passive calcium fluxes (A and B) and calcium permeability (P_{Ca} ; C and D) in Caco-2 monolayer directly exposed to 600 ng/ml rhPRL, PKC inhibitor (1 μ M GFX), or ROCK inhibitor (1 μ M Y-27632). The apical bathing solution contained a calcium concentration of 1.25, 2.5, 5, 10, 20, 40, or 80 mmol/l. Inhibitors alone or vehicle (DMSO) had no effect on either calcium flux or permeability (data not shown). This experiment was performed on 210 independent Snapwell setups ($n = 5$ per value of apical calcium concentration). Control and rhPRL data in A and C were reused in panels B and D, respectively, for better presentation. * $P < 0.01$, control vs. rhPRL.



ionic radii ranging from 90 to 181 pm (ionic radii of Na⁺ and Ca²⁺ are 114 and 116 pm, respectively). Under normal conditions, the Caco-2 monolayer showed relative permeability to alkali metal ions as follows: $P_{Na} > P_K > P_{Rb} > P_{Cs} > P_{Li}$ (Fig. 5A). rhPRL also increased P_X/P_{Cl} (where X⁺ is Li⁺, K⁺, Rb⁺, or Cs⁺) of all other alkali metal ions (Fig. 5, B, D–F), i.e., P_{Li}/P_{Cl} from 1.09 ± 0.02 to 1.86 ± 0.09 ($n = 6$, $P < 0.01$), P_K/P_{Cl} from 2.14 ± 0.07 to 3.41 ± 0.17 ($n = 6$, $P < 0.01$), P_{Rb}/P_{Cl} from 1.75 ± 0.07 to 2.18 ± 0.15 ($n = 6$, $P < 0.01$), and P_{Cs}/P_{Cl} from 1.70 ± 0.03 to 1.91 ± 0.08 ($n = 6$, $P < 0.05$). The effect of PRL on P_X/P_{Cl} was diminished by Y-27632 and LY-294002, but not by 1 μ M GF-109203X. Therefore, the PRL-induced alteration in the

cationic permselectivity in Caco-2 monolayer involved the ROCK pathway as well as the PI3K pathway.

In another series of experiments, we investigated whether the TER-reducing action of PRL could be applied to other cations of variable sizes. The monolayer was bathed on both sides with solution containing the alkali metal ion of interest, while TER was measured simultaneously. Under control conditions, the TER-ionic radius graph showed a V-shaped relationship, with the lowest TER (or the highest conductance) when Na⁺ was used as the primary conductor (Fig. 6). Exposure to PRL significantly decreased TER (or increased ion conductance) no matter which alkali ion was used as the primary conductor. Similar to the permeability studies, this

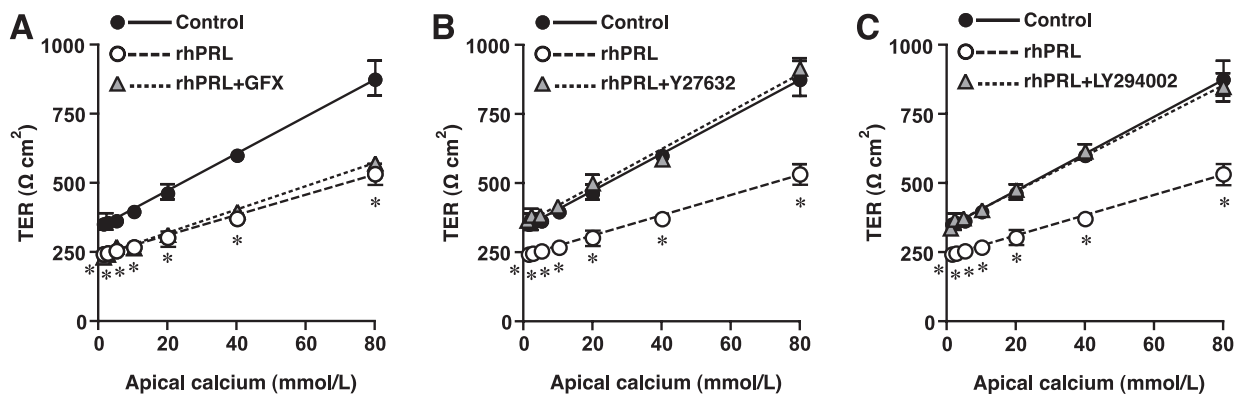


Fig. 3. Effect of 600 ng/ml rhPRL on the relationship between apical calcium concentration and transepithelial resistance (TER) in Caco-2 monolayer ($n = 5$ per value of apical calcium concentration) incubated with PKC inhibitor (1 μ M GFX; A), ROCK inhibitor (1 μ M Y-27632; B), or phosphoinositide 3-kinase (PI3K) inhibitor (75 μ M LY-294002; C). Inhibitors alone or vehicle (DMSO) had no effect on TER (data not shown). Control and rhPRL data in A were reused in B and C for better comparison. * $P < 0.01$, control vs. rhPRL.

Table 3. *Transepithelial mannitol and PEG-4000 fluxes*

Condition	n	Mannitol, $\text{nmol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$	PEG-4000, $\text{nmol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$
Control	7	5.60 ± 0.27	3.89 ± 0.58
600 ng/ml rhPRL	7	5.36 ± 0.34	3.96 ± 0.28

Values are mean $\pm$ SE transepithelial fluxes. Caco-2 monolayer was bathed with ^3H -labeled mannitol- and ^{14}C -labeled polyethylene glycol (PEG)-containing solution, with or without 600 ng/ml rhPRL. Both mannitol and PEG-4000 are exclusively transported via the paracellular channel and can be used to demonstrate the widening of the tight junction or change in size selectivity.

effect of PRL was abolished by Y-27632 (Fig. 6B) and LY-294002 (Fig. 6C), but not by $1 \mu\text{mol/l}$ GF-109203X (Fig. 6A).

PRL exerts its stimulatory action on transepithelial calcium transport via PRLR-L. Since PRL normally uses PRLR-L to exert its actions, especially on the mammary gland epithelia (3), we investigated whether PRLR-L is required for the action of PRL in Caco-2 cells with a PRLR-L siRNA knockdown technique. After 48-h incubation with PRLR-L siRNA, expression of PRLR-L mRNA in Caco-2 cells was abolished ($n = 5$, $P < 0.01$) (Fig. 7, A and C), while the expression of PRLR-S was unaltered (Fig. 7, A and B). The siPORT used as a transfection reagent had no effect on the expression of PRLR-L or PRLR-S. Amplicon sequencing confirmed the results of qRT-PCR.

In the Ussing chamber experiments (Fig. 8), the PRLR-L knockdown monolayer (siPORT+, siRNA+) exhibited transcellular active calcium flux and electrical properties comparable to those of the control (siPORT-, siRNA-) or siPORT-treated (siPORT+, siRNA-) monolayers (Fig. 8 and Table 4). Similar to the effect of PRL on the normal Caco-2 monolayer in Fig. 1, rhPRL enhanced the transcellular active calcium flux of the siPORT-treated monolayer (siPORT+, siRNA-, rhPRL-) from 9.25 ± 1.08 ($n = 5$) to 17.05 ± 0.65 $\text{nmol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$ (siPORT+, siRNA-, rhPRL+) ($n = 5$, $P < 0.01$). The TER of the siPORT-treated monolayer was also decreased by PRL (Table 4). After incubation with PRLR-L siRNA, the PRL-enhanced calcium transport and the PRL-altered TER in Caco-2 monolayer were completely reverted to basal levels. These findings indicated that PRL exerted its stimulatory action on calcium transport in the Caco-2 monolayer via PRLR-L.

DISCUSSION

PRL has been shown to exert both genomic and nongenomic actions on duodenum and proximal jejunum (25, 50, 51). In the present study, the effect of PRL on transepithelial calcium transport in Caco-2 monolayer was nongenomic, since the responses to PRL could be observed within 60 min and were not diminished by $50 \mu\text{mol/l}$ DRB, which is an inhibitor of gene transcription (43). We also demonstrated that the nongenomic action of PRL was mediated by the PKC and ROCK pathways.

The Caco-2 monolayer is a suitable model for investigating PRL action on transcellular active calcium transport because Caco-2 cells possess functional PRLR and transcellular calcium transporters (19, 25). In the absence of transepithelial calcium gradient, electrogenic transcellular active calcium transport involves the functions of TRPV6, calbindin- $\text{D}_{9\text{k}}$, and PMCA (22). We previously showed (11) that PRL stimulated both apical calcium uptake and activity of the basolateral

PMCA of purified duodenal membrane vesicles. The present results confirmed that transcellular calcium transport was increased by twofold after exposure to rhPRL, consistent with findings in the duodenal epithelium (25).

Interestingly, the TER of Caco-2 monolayer was decreased by PRL, similar to that observed after exposure to vitamin D, which is an important regulator of calcium absorption (14). Since the PD of the PRL-treated monolayer was not changed, it was conceivable that PRL did not interfere with the electrogenic transcellular sodium transport via Na^+/K^+ -ATPases, which, in the absence of chemical gradient and diffusion

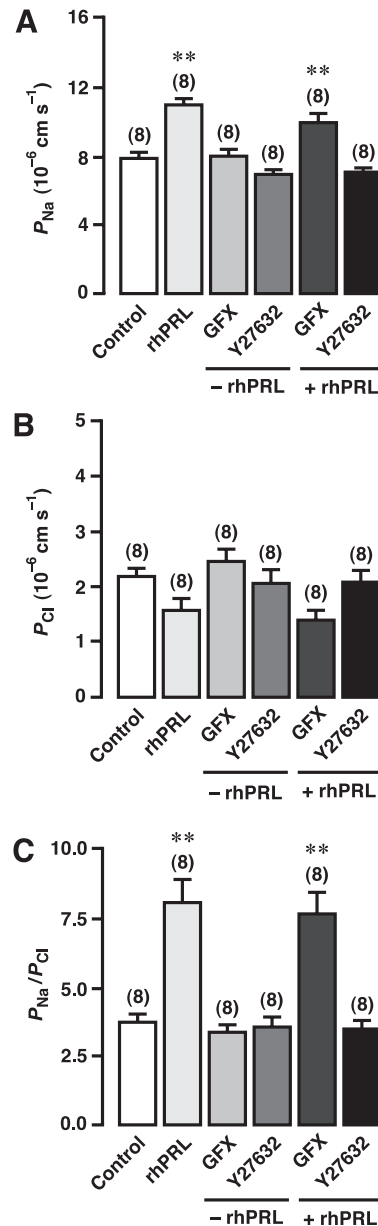


Fig. 4. Effects of rhPRL on sodium permeability (P_{Na} ; A), chloride permeability (P_{Cl} ; B), and $P_{\text{Na}}/P_{\text{Cl}}$ (C), which indicate charge-selective property of Caco-2 monolayer. Cells were incubated with PKC inhibitor ($1 \mu\text{mol/l}$ GFX) or ROCK inhibitor ($1 \mu\text{mol/l}$ Y-27632) in the presence (+rhPRL) or absence (-rhPRL) of 600 ng/ml rhPRL. DMSO 0.3% (vol/vol) used as vehicle had no effect on these parameters (data not shown). ** $P < 0.01$ compared with control group. Numbers in parentheses represent the number of independent Snapwells.

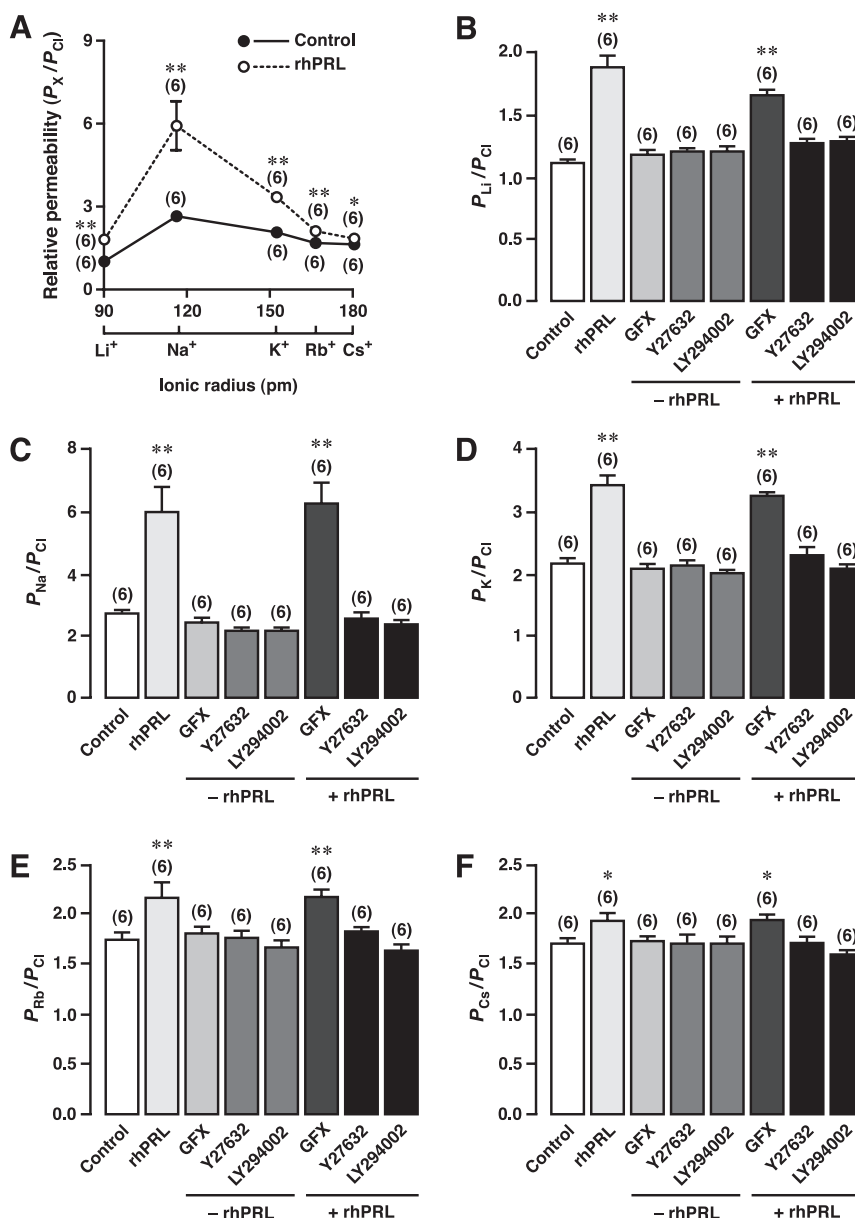


Fig. 5. A: effect of 600 ng/ml rhPRL on the relative paracellular permeability of the alkali metal ions over chloride (P_X/P_{Cl} ; X = Li⁺, Na⁺, K⁺, Rb⁺, or Cs⁺) plotted against their ionic radii. B–F: P_X/P_{Cl} of Caco-2 monolayer incubated with PKC inhibitor (1 μ Mol/l GFX), ROCK inhibitor (1 μ Mol/l Y-27632), or PI3K inhibitor (75 μ Mol/l LY-294002) in the presence (+rhPRL) or absence (–rhPRL) of 600 ng/ml rhPRL. DMSO 0.3% (vol/vol) used as vehicle had no effect on P_X/P_{Cl} (data not shown). * $P < 0.05$, ** $P < 0.01$ compared with control group. Numbers in parentheses represent the number of independent Snapwells.

potential, contributed up to 95% of the PD (46). Although PRL increased the electrogenic transcellular calcium transport, changes in the PD should be very small and not detectable by the Ussing technique. The PD change ($V_{\Delta, Ca}$) due to the PRL-enhanced transcellular calcium transport, if present, could be estimated from the TER and the difference ($J_{\Delta, Ca}$) between the control ($9.22 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$) and PRL-exposed ($18.04 \text{ nmol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$) fluxes, i.e., $V_{\Delta, Ca} = J_{\Delta, Ca} z F \times \text{TER}$ ($z = +2$, $F = 96,485.34 \text{ C/mol}$, $\text{TER} = 255.67 \Omega \cdot \text{cm}^2$). Hence $V_{\Delta, Ca}$ was $120.88 \mu\text{V}$, which could be considered negligible. When the monolayer was short-circuited, the external current (I_{sc}) moved across the monolayer via the low-resistance pathway, i.e., the paracellular pathway (20). Therefore, a decrease in TER that was calculated from PD and I_{sc} in the presence of PRL must have resulted from the PRL-induced increase in paracellular permeability, and thus enhanced paracellular calcium transport was anticipated.

Little is currently known regarding the paracellular calcium transport in Caco-2 monolayers. Generally, the paracellular passive calcium transport is more physiologically important since it is achieved at a small cost of energy and is nonsaturable (i.e., a graph of apical calcium vs. calcium flux shows linearity) (29). This transport mechanism, which occurs along the entire length of the small intestine, becomes significant when luminal calcium concentration exceeds 5 mmol/l, which is an average luminal calcium concentration in the rat duodenum (12, 55). When the apical calcium concentration was 20 mmol/l, the transcellular calcium flux contributed $<10\%$ of the total flux (Fig. 2). Nevertheless, transcellular calcium transport is still important during increased calcium demand, e.g., during pregnancy, lactation, and low calcium intake (8, 10). Here we found that PRL enhanced both transcellular and paracellular calcium transport as well as calcium permeability. The PRL-stimulated paracellular calcium flux at 5 mmol/l apical calcium was

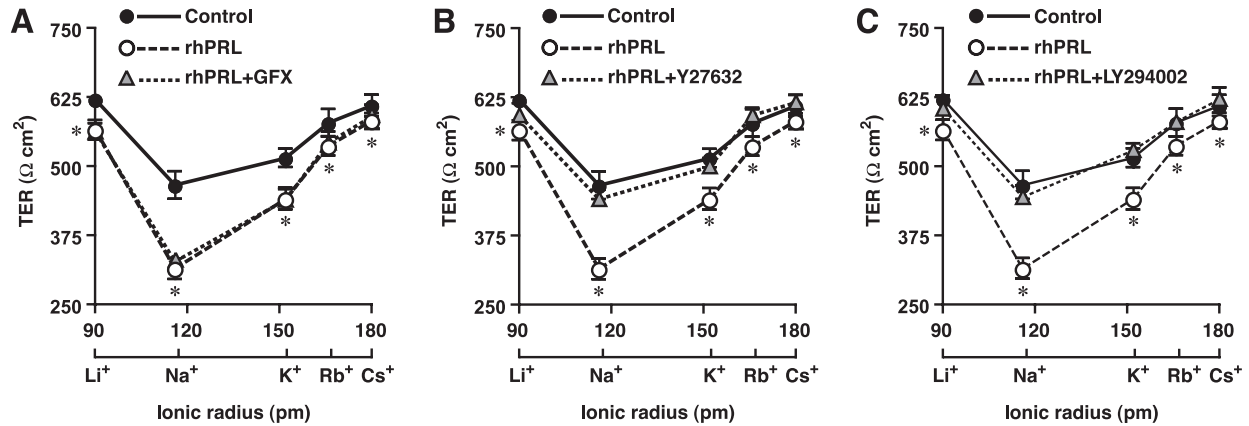


Fig. 6. Alterations of TER after apical Na^+ being substituted with other alkali metal ions. Caco-2 monolayers ($n = 6$ per studied ion) were directly incubated with 600 ng/ml rhPRL with or without PKC inhibitor (1 $\mu\text{mol/l}$ GFX; A), ROCK inhibitor (1 $\mu\text{mol/l}$ Y-27632; B), or PI3K inhibitor (75 $\mu\text{mol/l}$ LY-294002; C). TER values of the monolayer exposed to inhibitors alone or vehicle (DMSO) are comparable to those of control (data not shown). Control and rhPRL data in A were reused in B and C. * $P < 0.01$, control vs. rhPRL.

~2.1-fold higher than that at 1.25 mmol/l calcium in the transcellular study, implicating the physiological significance of the paracellular transport. Moreover, as seen in Fig. 3, an increase in the apical calcium concentration also led to a divalent ion-induced conductance block, i.e., a linear increase in TER, as seen with many conventional ion channels (30, 40). Conductance block is probably caused by binding of calcium to the fixed negative charges of the paracellular proteins (49). A similar effect of high apical calcium has been reported in other epithelia, e.g., MDCK-II monolayer (49). After exposure to

PRL, the increase in TER was attenuated, i.e., the paracellular conductance was increased, thus explaining increases in the paracellular calcium flux, calcium permeability, and slope of the apical calcium vs. paracellular calcium flux relationship.

Paracellular conductance and transport of ions can be regulated by changing the size- and/or charge-selective property of the paracellular pathway (32, 54). These properties are determined by different mechanisms (32). Paracellular size restriction, which is between 350 and 540 pm in the Caco-2 monolayer (9), is compromised by the myosin light chain kinase-mediated contraction of the perijunctional actomyosin ring complex and cytoskeletal arrangement (52). However, previous investigation using cytochalasin E, which widens the tight junction by disrupting actin polymerization, suggested that PRL-induced increase in duodenal calcium flux did not involve size selectivity of the paracellular pathway (50). This was confirmed by the present finding of no change in mannitol and PEG fluxes after exposure to PRL. Therefore, it appeared that

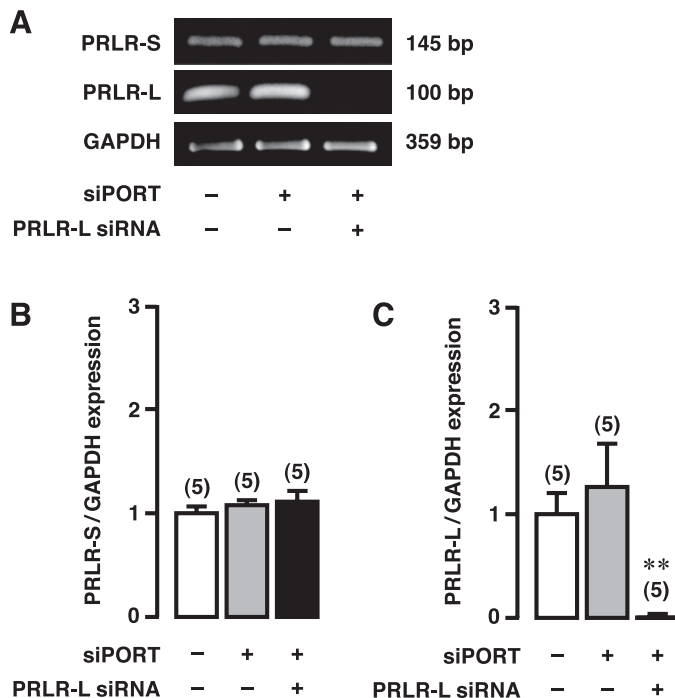


Fig. 7. Representative electrophoretic image (A) and quantitative real-time PCR (B and C) demonstrate expression of short (PRLR-S) and long (PRLR-L) forms of PRL receptor in Caco-2 monolayer incubated for 48 h with siPORT (transfection agent) or siPORT + 1 nmol/l PRLR-L short interference RNA (siRNA). The absence of PRLR-L expression confirms the success of PRLR-L knockdown. ** $P < 0.01$ compared with control group (-siPORT/-siRNA). Numbers in parentheses represent the number of independent Snapwells.

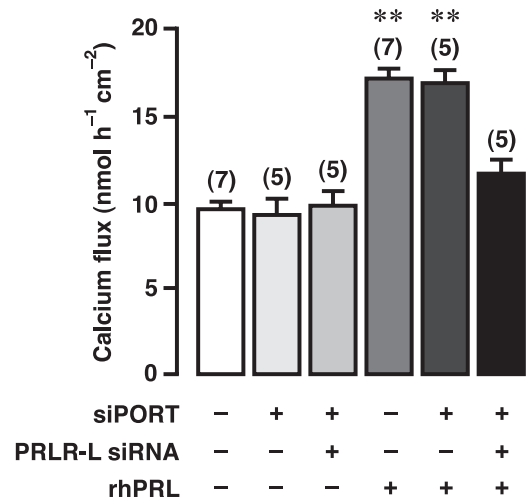


Fig. 8. Transcellular active calcium fluxes in Caco-2 monolayer exposed to 600 ng/ml rhPRL, siPORT (transfection agent), and/or 1 nmol/l siRNA targeting PRLR-L. ** $P < 0.01$ compared with control group (-siPORT, -siRNA, -rhPRL). Numbers in parentheses represent the number of independent Snapwells.

Table 4. Epithelial electrical parameters for PRLR-L knockdown experiment

Condition	n	PD, mV	I_{sc} , $\mu A/cm^2$	TER, $\Omega \cdot cm^2$
Control	7	0.74 ± 0.10	2.00 ± 0.31	395.24 ± 11.90
siPORT	5	0.64 ± 0.12	2.00 ± 0.32	390.00 ± 29.15
siPORT+siRNA	5	0.80 ± 0.13	2.00 ± 0.32	400.00 ± 15.81
rhPRL	7	0.83 ± 0.06	3.14 ± 0.26	$258.10 \pm 12.12^*$
rhPRL+siPORT	5	0.72 ± 0.19	3.40 ± 0.68	$206.67 \pm 22.11^*$
rhPRL+siPORT+siRNA	5	0.88 ± 0.13	2.40 ± 0.40	376.67 ± 24.49

Values are means $\pm$ SE for transepithelial PD, I_{sc} , and TER in Caco-2 monolayer exposed to 600 ng/ml rhPRL, siPORT (transfection agent), and/or 1 nmol/l short interference RNA (siRNA) targeting PRLR-L. The monolayer was bathed on both sides with 1.25 mmol/l calcium-containing solution. The apical side was negative with respect to the basolateral side. $^*P < 0.01$ vs. control group.

the epithelial charge selectivity, but not the size selectivity, regulated the intestinal paracellular calcium transport. Generally, charge selectivity is defined by fixed negative or positive charges on the extracellular loops of tight junction proteins of the claudin family, which provide a hindrance for the permeation of ions with the opposite charge (32). Expressions of some claudins are associated with increased calcium permeability. For example, claudin-16 is widely recognized to be important for paracellular calcium and magnesium reabsorption in the thick ascending limb of the loop of Henle (17). Expression of claudin-3 in the duodenum is under the regulation of vitamin D (34), a hormone that also stimulates paracellular calcium transport (14). However, it is not known whether PRL alters the functions or localization of claudins in the Caco-2 monolayer and intestinal epithelia.

To access the charge-selective property of the Caco-2 monolayer, we used P_{Na}/P_{Cl} as an indicator. Under control conditions, a P_{Na}/P_{Cl} of 3.73 is comparable to those previously reported in Caco-2 monolayer (9) and small intestine (12, 41), indicating that these epithelia are more permeable to cations than anions. In contrast, normal colonic epithelium is more permeable to anions (16). It is noteworthy that the paracellular selectivity causes sodium and chloride movement to deviate from the mobility ratio of ~ 0.6 in free solution (20, 41). In addition, permeabilities of other cations in the alkali metal series, i.e., Li^+ , K^+ , Rb^+ , and Cs^+ , are also greater than that of Cl^- , and comply with Eisenman sequence VII (i.e., $P_{Na} > P_K > P_{Rb} > P_{Cs} > P_{Li}$) (for review, see Ref. 18). Similar to that reported in other epithelia (49), the conductance (reciprocal of TER) of the paracellular pathway was greatest when NaCl was used as the primary conductor, as seen in the ionic radius vs. TER curve, which showed a V-shaped relationship (Fig. 6). Such patterns were not altered by PRL. Although the paracellular pathway of the Caco-2 monolayer manifests a selective property similar to that of typical ion channels, whereby an ion current is not simply determined by the ionic or hydrated size of the ion, PRL enhanced the paracellular permeability of both sodium and calcium, which are of comparable ionic sizes. Our findings that PRL increased P_{Na} and permeability to other alkali metal ions, but not P_{Cl} , suggested that PRL could increase the activity of the paracellular proteins with fixed negative charges by an unknown mechanism, thereby enhancing paracellular conductance, calcium permeability, and paracellular calcium transport.

PRL exerts its functions by binding to the membrane-bound PRLRs with a ratio of PRL to PRLRs of 1:2 (3). Two isoforms of PRLRs, i.e., PRLR-S and -L, have been identified in intestinal absorptive cells and Caco-2 cells (25). Investigations in mammary epithelia demonstrated that signal transduction of PRL was transferred by PRLR-L, whereas PRLR-S silenced PRL actions because it lacks the cytoplasmic tails required to activate downstream pathways (3, 6, 24, 42). On the other hand, PRLR-S may transmit PRL signals in the liver and endothelial cells (24, 44). In the PRLR-L knockdown Caco-2 monolayer, which expressed a normal level of PRLR-S, the effects of PRL on transcellular calcium transport and TER were totally abolished, indicating that actions of PRL are primarily mediated by PRLR-L.

Despite strong evidence pertaining to PRL-stimulated intestinal calcium absorption (10), little is known regarding PRL signal transduction in the intestinal epithelia and the Caco-2 monolayer. In mammary epithelia, PRL binding to PRLRs triggers dimerization of PRLRs, leading to activation of the putative JAK2 signaling (3). However, our recent investigation (25) elucidated that the PI3K pathway, but not the JAK2 pathway, is involved in the PRL-enhanced transcellular and paracellular calcium transport. The present data clearly showed that the PI3K pathway was involved in the rapid effects of PRL on the paracellular permeability to alkali metal ions and TER. Signal transduction of PRL via PI3K has also been reported in liver, pancreas, and T-lymphoma Nb2 cells (1, 7, 56). The PI3K pathway covers a large number of downstream targets, including PKC and ROCK (21). The Rho-related pathway was found to be associated with PRL signaling in endothelial cells (35), while the PKC pathway mediated PRL actions in cholangiocytes (48), adrenocortical cells (27), and the intestine, in which PKC enhanced intestinal calcium transport (4). Activation of the ROCK pathway increased paracellular permeability in several epithelia, such as brain endothelium and renal proximal tubule (28, 47). PKC-ROCK cross talk could also affect paracellular permeability in endothelial cells (47). Therefore, in the present study we focused on the PKC- and ROCK-mediated PRL actions in Caco-2 cells and were able to demonstrate that the ROCK pathway mediated PRL actions on transcellular and paracellular calcium transport, calcium permeability, charge selectivity, and TER. On the other hand, inhibition of the PKC pathway could only diminish transcellular calcium transport. Nevertheless, further investigations are required to demonstrate the detailed molecular mechanisms of PKC- and ROCK-mediated PRL actions in Caco-2 monolayer.

In conclusion, we provide evidence, for the first time, that PRL exerts its nongenomic stimulatory action on transcellular active calcium transport in the Caco-2 monolayer via the PKC and ROCK pathways. PRL also increased paracellular permeability and decreased TER via the ROCK pathway, thereby enhancing paracellular passive calcium transport. Moreover, PRL-induced increase in paracellular transport was by altering epithelial charge selectivity rather than size selectivity. The actions of PRL were mediated by the long isoform of PRLR. Our findings elaborate possible mechanisms of the PRL-stimulated intestinal calcium transport, which has long been reported in the rat (10).

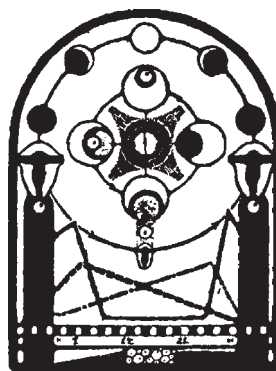
GRANTS

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Transcriptome responses of duodenal epithelial cells to prolactin in pituitary-grafted rats

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ABSTRACT

Chronic prolactin (PRL) exposure can affect several functions of duodenal epithelia, especially those associated with fluid and electrolyte transport. However, little is known regarding its molecular mechanism. To identify PRL-regulated genes, microarray analysis was performed on RNA samples from duodenal epithelial cells of anterior pituitary (AP)-grafted hyperprolactinemic rats. Herein, we identified 321 transcripts upregulated and 241 transcripts downregulated after 4 weeks of AP transplantation. Results from real-time PCR analyses of 15 selected genes were consistent with the microarray results. Gene ontology analysis demonstrated pleiotropic effects of PRL on several cellular processes, including cellular metabolic process, cell communication and cell adhesion. Interestingly, 17 upregulated transcripts and 12 downregulated transcripts are involved in the transport of ions and nutrients, e.g., Ca^{2+} , Na^+ , K^+ , Cl^- and glucose, thus agreeing with the established action of PRL on electrolyte homeostasis. The present results provided fundamental information for further investigations on mechanism of PRL actions in the intestine.

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1. Introduction

Prolactin (PRL) is one of the most versatile hormones with respect to its array of actions in vertebrates. The more than 300 PRL actions can be classified into 5 categories, i.e., water and electrolyte balance, growth and development, brain and behavior, reproduction, and immunoregulation (Bole-Feyssot et al., 1998). PRL receptors (PRLR) are widely expressed in tissues from all 3 germ layers including the digestive tract where PRL exerts 3 major actions, i.e., ion and nutrient absorption, epithelial cell proliferation, and gastrointestinal motility (Charoenphandhu and Krishnamra, 2007; Bujanover et al., 2002; Chen et al., 1997). Most effects of PRL on the intestinal epithelial cells are genomic and are dependent on de novo gene transcription (Bole-Feyssot et al., 1998). However, little is known regarding the PRL-regulated intestinal genes.

Salient actions of PRL on the intestinal epithelial cells are related to ion transport. Duodenum appears to be the most responsive intestinal segment to PRL (Charoenphandhu and Krishnamra, 2007). In pregnant and lactation rats, PRL acts as a Ca^{2+} -regulating

hormone that enhances the duodenal Ca^{2+} absorption, thus mitigating massive Ca^{2+} loss from bone for fetal growth and galactopoiesis (Charoenphandhu and Krishnamra, 2007; Boass et al., 1992). Long-term exposure to hyperprolactinemia induced by 4-week anterior pituitary (AP) transplantation was found to increase the transcellular Ca^{2+} transport in rat duodenum by ~2-fold (Tudpor et al., 2005). The direct stimulatory action of PRL on Ca^{2+} absorption has been confirmed by investigations using ex vivo duodenal tissue and the intestinal epithelium-like Caco-2 monolayer (Jantarajit et al., 2007; Thongon et al., 2008). In addition to Ca^{2+} , exposure to PRL for 2 days also increased the intestinal transport of water, Na^+ , K^+ and Cl^- as well as nutrient molecules, such as glucose and neutral amino acids (e.g., glycine and proline) (Mainoya, 1975a,d). Thus, we hypothesized that a number of duodenal transporter genes involved in ion and nutrient transport may be upregulated in response to long-term hyperprolactinemia.

Besides the transport activities, growth and maturation of the intestinal mucosa is influenced by PRL. In the jejunum of 2-week-old rats, PRL induced proliferation of epithelial cells and ~3-fold increases in maltase and alkaline phosphatase activities (Bujanover et al., 2002). PRL also led to mucosal hypertrophy in pregnant and lactating rats (Mainoya, 1978). Moreover, an increase in the plasma PRL level was reported to stimulate proliferation of the colonic epithelial cells, and could be an unfavorable prognostic

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factor in patients with colonic carcinoma (Bhatavdekar et al., 1995). Regarding PRL effect on the cellular metabolism, PRL increased the activities of UDP-glucuronosyltransferase and glutathione S-transferase in the jejunal epithelial cells of lactating rats (Luquita et al., 1999). Therefore, PRL appeared to exert a variety of actions beyond the scope of ion or nutrient transport, and considerable genomic response of the intestinal epithelial cells to long-term PRL exposure, which could be determined by a genome-wide expression study, was anticipated.

To avoid chronic stress from daily handling and PRL injection, hyperprolactinemia in the present study was induced by AP transplantation. Within 15 days after transplanting two extra AP glands from donors under the renal capsule of the recipient, continuous secretion of PRL from the AP allografts in the absence of the hypothalamic dopaminergic inhibition resulted in a sustained increase in the plasma PRL level (Charoenphandhu et al., 2007; Adler et al., 1989). Four weeks after transplantation, the plasma PRL levels are known to be maintained within the range of 90–100 ng/mL (Piyabhan et al., 2000), which are comparable to the levels attained during pregnancy in humans and rats (Boass et al., 1992). Other AP hormones were not secreted from the grafts due to the absence of their respective trophic factors from the hypothalamus.

The principal objective of this study was to analyze the profile of PRL-regulated genes in the duodenal epithelial cells by using microarray and quantitative real-time PCR (qRT-PCR) techniques. Prior to the profile study, expression of PRLR was determined to show that the duodenal tissue was a target of PRL, and whether hyperprolactinemia induced the downregulation of PRLR. The Illumina's high-performance BeadArray™ technology was used in our microarray study because it has been reported to exhibit high selectivity and sensitivity in gene expression profiling as a result of the high redundancy of each probe built in the bead array (Kuhn et al., 2004; Yan et al., 2008).

2. Materials and methods

2.1. Animals

Eighteen female Sprague–Dawley rats (10-week-old, weighing 200–220 g), were obtained from the National Laboratory Animal Centre, Thailand. They were individually placed in hanging stainless steel cages, fed standard pellets (Perfect Companion, Bangkok, Thailand) and distilled water *ad libitum* under 12/12 h light/dark cycle. Room temperature was controlled at 23–25 °C, and the relative humidity was about 50–60%. After 7-day acclimatization period, rats were randomly divided into two groups, i.e., sham-operated ($n=6$) and AP-grafted groups ($n=4$). Eight rats acted as donors for AP transplantation. This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University, Bangkok, Thailand.

2.2. Anterior pituitary (AP) transplantation

The procedure was modified from the methods of Adler et al. (1989) and Charoenphandhu et al. (2007). In brief, a 1.0 cm paracostal incision was made to expose the left renal capsule of the recipient rat. Two 10-week-old donors were then decapitated to collect the pituitary glands which were immediately inserted into the prepared renal capsule of the recipient rat (i.e., 2 glands/rat) and finally covered with the renal fascia. Muscle and skin were sutured with sterile silk 3/0, and cleaned with 70% ethanol and povidone-iodine. Wound dressing was performed daily. Sham operation consisted of exposure of the left kidney and a gentle touch of the renal fascia with forceps. Visual examination of the well-vascularized hypophyseal graft and immunohistochemical staining for PRL production were performed at the end of the experiments to confirm successful AP transplantation (Charoenphandhu et al., 2007). At 4 weeks after transplantation, plasma PRL levels have been known to increase to 90–100 ng/mL, which are comparable to the levels during pregnancy (Boass et al., 1992).

2.3. Tissue preparation

A median laparotomy was performed under 50 mg/kg sodium pentobarbitone i.p. (Abbott, North Chicago, IL, USA) anesthesia. The hypophyseal grafts were gently

dissected from the perirenal tissue for immunohistochemical analysis. The duodenal segment (10 cm) was removed and cut longitudinally along the radix mesenterii to expose the mucosa. Duodenal epithelial cells were collected by scraping the mucosal surface with an ice-cold glass slide, and homogenized at 4 °C with a glass-Teflon Potter-Elvehjem tissue grinder (Wheaton, Millville, NJ, USA) as previously described (Jantarajit et al., 2007). Gene expression profile of each duodenal sample was analyzed separately by microarray.

2.4. Immunohistochemistry for PRL production

Imaging technique was performed to evaluate the viability of AP grafts (e.g., no sign of inflammation or graft rejection) as well as PRL production. Paraffin-embedded 4.0- μ m sections were used to detect PRL production in the hypophyseal grafts. After blocking endogenous peroxidase activity and non-specific background with 3% H₂O₂ and 3% horse serum (Sigma, St. Louis, MO, USA), respectively, the sections were incubated for 60 min with 1:300 PRL polyclonal antibody (Dako, Carpinteria, CA, USA) prior to 10-min incubation with biotin-conjugated anti-rabbit secondary antibody and peroxidase-conjugated streptavidin (Dako). The chromogenic reaction was carried out with 3,3'-diaminobenzidine (Dako) to produce a brownish product. The slides were finally counterstained with hematoxylin (Sigma) for 5 min. The normal pituitary gland and perirenal fat pad were used as positive and negative controls, respectively. Hematoxylin–eosin (H&E) staining was also performed to identify structures of the graft. Images were acquired under light microscope (model BX51 TRF; Olympus, Tokyo, Japan).

2.5. Total RNA and cDNA preparation

By using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), the total RNA was prepared from duodenal epithelial cells, as previously described (Thongon et al., 2008). The total RNA was treated with RQ1 DNase (Promega, Madison, WI, USA), and later purified with RNeasy Mini kit (Qiagen, Valencia, CA, USA). Purity of the total RNA was determined by the ratio of absorbance readings at 260 and 280 nm, the ratio of which fell in the range of 1.8–2.0. Integrity of RNA was analyzed by denaturing agarose gel electrophoresis with the 28S rRNA band appearing approximately twice as intense as the 18S rRNA band. Purified total RNA samples were later used for both microarray and qRT-PCR studies. As for qRT-PCR, 1 μ g total RNA was reverse-transcribed with the oligo-dT₁₅ primer and the iScript kit (Bio-rad, Hercules, CA, USA) to cDNA by a thermal cycler (model MyCycler; Bio-rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control gene to check the consistency of the reverse transcription (percent coefficient of variation <10%, $n=10$).

2.6. Microarray

Illumina TotalPrep RNA amplification/in vitro transcription kit (catalog no. IL1791; Ambion, Austin, TX, USA) was used to amplify and generate biotinylated cRNA from 500 ng total RNA for hybridization with Illumina BeadChip array. After purification, biotinylated cRNA was quantified with NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). 750 ng of biotinylated cRNA from each duodenal sample was separately hybridized on the RatRef-12 Expression BeadChip (binary manifest file version V1.0.R0.11222119.A; Illumina, San Diego, CA, USA). Each RatRef-12 BeadChip is constructed with BeadArray™ technology, and contains 12 whole-genome gene expression arrays, thus allowing 12 independent samples to be hybridized to a single chip. Each array, which can probe 21,910 genes, consists of 22,523 oligonucleotide probes (50-base gene-specific sequence and 29-base address sequence per probe) selected primarily from the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database (release 16; <http://www.ncbi.nlm.nih.gov/RefSeq>). After incubation at 58 °C for 20 h, the BeadChip was washed, blocked and incubated at room temperature with streptavidin–Cy3-containing solution (Illumina) according to the manufacturer's instruction. Fluorescent signals were scanned with BeadStation 500GX Genetic Analysis System (Illumina). Image registration and data extraction were automated with BeadStudio 3.1.3 (Illumina).

2.7. Quantitative real-time PCR (qRT-PCR) and sequencing

Primers used in the present study were designed by OLIGO 6 (Molecular Biology Insights, Cascade, CO, USA) and Primer Validator 1.4 (Naratt Software, Bangkok, Thailand), as shown in Table 1. qRT-PCR and melting curve analyses were performed by Bio-rad MiniOpticon with the iQ SYBR Green SuperMix (Bio-rad) as previously described (Thongon et al., 2008). Gene expression levels were normalized to GAPDH expression. The PCR products were also visualized on 1.5% agarose gel stained with 1.0 μ g/mL ethidium bromide under a UV transilluminator (Alpha Innotech, San Leandro, CA, USA). After electrophoresis, all PCR products were extracted by the HiYield Gel/PCR DNA Extraction kit (Real Biotech Corporation, Taipei, Taiwan), and were sequenced by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). qRT-PCR experiments were performed in triplicate.

Table 1*Rattus norvegicus* oligonucleotide sequences used in qRT-PCR experiments.

Gene	Accession no.	Primer (forward/reverse)	Product length (bp)
<i>Prolactin receptor</i>			
Short isoform	NM.012630	5'-TTCTACCACCATCGCAAC-3'; 5'-CTGATCTCGTTTGTCATTGAG-3'	120
Long isoform	NM.001034111	5'-TCAAGCAACCGCAGACTC-3'; 5'-CAGTTTAGCCAATCGTTCCA-3'	107
<i>Cell adhesion (GO:0007155)</i>			
Ceacam10	NM.173339	5'-TCTCCTTTGATTGCTCTTCT-3'; 5'-CTCTGGGGTTCACGAGTG-3'	75
<i>Cellular component organization and biogenesis (GO:0016043)</i>			
Syngn1	NM.019166	5'-TCGGCTCCATTGTGAATGAG-3'; 5'-CTTTCTTGGCGTCCTTGACA-3'	192
<i>Cellular developmental process (GO:0048869)</i>			
Epim	NM.012748	5'-TCCGTAGATTACGTGGAGCA-3'; 5'-AGGACAGCAATGACAGCCA-3'	119
<i>Cellular metabolic process (GO:0044237)</i>			
Cpz	NM.031766	5'-GGATGCGGCTATAACCAT-3'; 5'-TCCAGGAGAAAGTGCAGCA-3'	114
Kynu	NM.053902	5'-GAGGAGTCGTCTGTGACAAG-3'; 5'-GCAGTAAGCAGTCTGATGAAC-3'	106
Sardh	NM.053664	5'-TTGCTAGGAATCTCCAGGA-3'; 5'-TGGAACTCTTCTCAGTGGTG-3'	194
<i>Developmental process (GO:0032502)</i>			
Dbn1	NM.031024	5'-ACCTGTCTGCTTCTCTACCA-3'; 5'-CTAAGCTACAATCTGGTCTGGG-3'	160
<i>Metabolism (GO:0008152)</i>			
Pon1	NM.032077	5'-TTGAATGAGAAGGAGCCAGC-3'; 5'-TAAACACCTCCACGGTGA-3'	166
<i>Regulation of biological process (GO:0050789)</i>			
CD40lg	NM.053353	5'-TGGAAAATGGGAGACAAC-3'; 5'-CAGAGGCTGACGATGAAT-3'	118
Gkn1	NM.198972	5'-GACAGCAATGTAGACGGAAGT-3'; 5'-TTTTCATAGTCCCAGAGGC-3'	119
Inhba	NM.017128	5'-GAATGAATCATGGAGCAGACC-3'; 5'-TGCTGAAACAGACGGATGGT-3'	195
Syn2	NM.019159	5'-GTAATGGCAATTGCAGTAGTCC-3'; 5'-AACATGCCGTCAGCACTGAG-3'	181
<i>Transport (GO:0006810)</i>			
Cacnb1	NM.017346	5'-AGGACTTCTGCACATCAAGG-3'; 5'-TTGTACCTGACTTGCTGGAG-3'	175
Clcnkb	NM.173103	5'-CTCCACATTACCTCTCCC-3'; 5'-GCAGCAGACAACCTAGAAAG-3'	104
Slc22a17	NM.177421	5'-GTCGTGGGATTGACTGCTGA-3'; 5'-CATCAGGTAGACCAAGGTCA-3'	146
<i>Housekeeping gene</i>			
GAPDH	NM.017008	5'-AGTCTACTGGCGTCTTTCAC-3'; 5'-TCATATTCTCGTGTTTCAC-3'	133

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; full names of other genes are presented in Tables 2 and 3. Gene ontology (GO) identifiers are presented in parentheses.

2.8. Data analysis

Unless otherwise specified, differential gene expression of microarray and qRT-PCR were considered statistically significant when there was a 2-fold or greater difference in expression between the sham-operated and AP-grafted groups (Sigala et al., 2008). Microarray data were analyzed by BeadStudio Gene Expression Module 3.2.6 and Genome Viewer 3.2.7 (Illumina). The Illumina's Cubic-Spline normalization algorithm was applied to adjust sample signals to minimize the effects of variation arising from non-biological factors. False discovery checks were performed with the "compute false discovery rate" function of the BeadStudio. Comparison between two groups of samples (sham-operated vs. AP-grafted) was done with the *t*-test differential expression algorithm. $P < 0.05$ was used as the criterion for statistical significance. Gene Ontology (GO) analysis for characterizing the biological properties of gene products was performed according to the GO Consortium's instruction at the GO website (<http://www.geneontology.org>). The used GO search engines were AmiGO (<http://amigo.geneontology.org>), High-Throughput GoMiner (<http://discover.nci.nih.gov/gominer>), and Ensembl (<http://www.ensembl.org>). Gene symbol and full name of gene can be searched from the Rat Genome Database (<http://rgd.mcw.edu>). The qRT-PCR results compared by using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) are expressed as $\log_2$ means $\pm$ S.E. Graphics were performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Immunohistochemical analysis showed PRL production in the hypophyseal allograft

At 4 weeks after AP transplantation, the two implanted glands were removed from the perirenal connective tissues, and were analyzed for their viability and function histologically and immunohistochemically. The excised grafts were highly vascularized reddish-gray tissue with an average diameter of 1.5–2.5 mm ($n = 4$). H&E staining demonstrated a group of cells of varying size, arranged in irregular cords and clusters (Fig. 1A), agreeing with

the histological morphology of normal pituitary gland. Numerous thin-walled sinusoidal networks with richly fenestrated endothelia were seen between the clusters of cells. Immunohistochemical analysis revealed the presence of PRL inside the pars distalis of the implanted glands (Fig. 1B and C), similar to that seen in normal pituitary gland (Fig. 1D). The adjacent fat tissues, fascia, and pars intermedia (negative control) did not show PRL immunoreactivity. Active signs of microvascular endothelial damage and lymphoid proliferation were not seen, suggesting the absence of graft rejection and allograft vasculopathy. The results corroborated that the AP-grafted rats possessed two healthy ectopic pituitary glands which actively produced extra PRL.

3.2. Chronic PRL exposure downregulated PRLR in the duodenal epithelial cells

PRLR mRNA expression was demonstrated by PCR. Since chronic exposure to PRL did not change GAPDH expression (Fig. 2A), mRNA expression levels were first normalized by GAPDH expression. In the present study, both short (-S) and long (-L) isoforms of PRLRs were strongly expressed in the duodenal epithelial cells (Fig. 2B), suggesting that these cells could be target of PRL action. The electrophoretic bands obtained were of expected sizes (Fig. 2B), and amplicon sequencing confirmed qRT-PCR findings. Since prolonged exposure to a high level of PRL could downregulate its receptor (Bowen et al., 2000; Swaminathan et al., 2008), a study of PRLR expression after AP transplantation was performed. qRT-PCR demonstrated (Fig. 2C) that the duodenal epithelial cells from AP-grafted rats showed a decrease in PRLR-L expression by -2.146 -fold ($P < 0.05$). In contrast, the expression levels of PRLR-S in both sham-operated and AP-grafted rats were comparable.

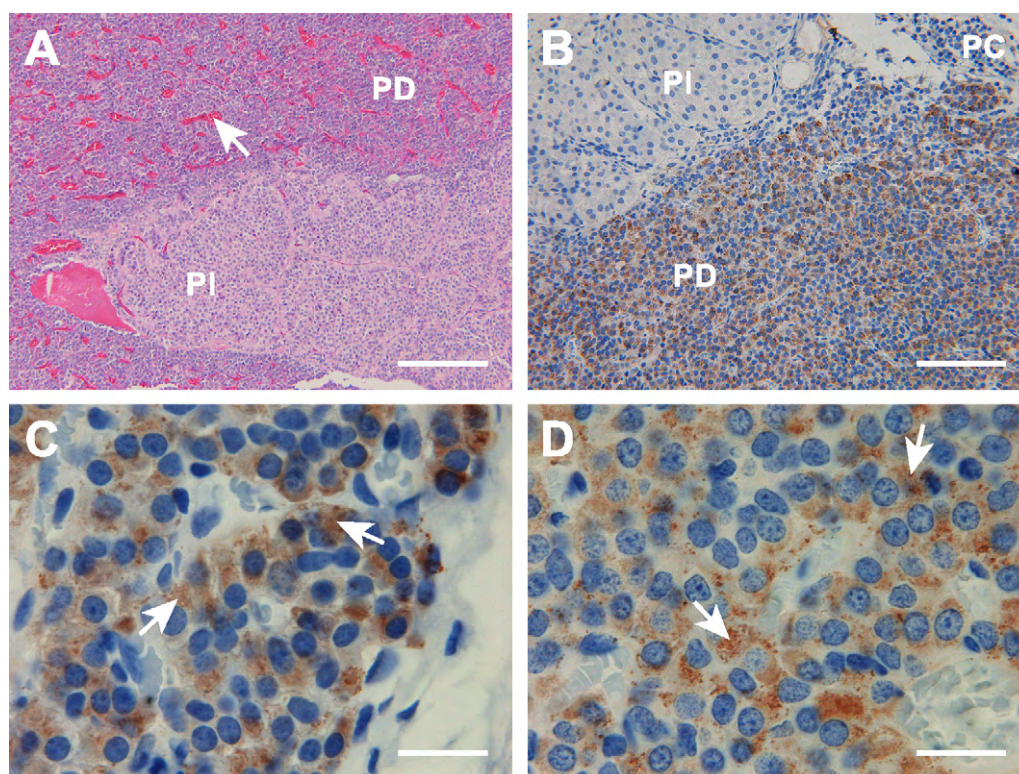


Fig. 1. Representative data of the immunohistochemical analysis for PRL production ($n=4-6$). (A) Hematoxylin–eosin (H&E) staining of the pars distalis (PD) and pars intermedia (PI) of a hypophyseal allograft dissected from a 4-week AP-grafted rat. The arrow indicates a thin-walled sinusoid filled with numerous erythrocytes. Scale bar, 200 μm . (B) Immunohistochemical staining of an implanted pituitary gland with anti-PRL antibody. The PD is strongly labelled with brownish products of peroxidase, whereas the PI used as a negative control is not stained. The perirenal connective tissues (PC) do not show PRL immunoreactivity. Scale bar, 100 μm . (C) PRL-producing cells (arrows) in a hypophyseal allograft. Scale bar, 20 μm . (D) PRL-producing cells (arrows) in a normal pituitary gland, which is used as a positive control. This gland was dissected from the pituitary fossa of a sham-operated rat. Scale bar, 20 μm .

3.3. Microarray study revealed transcriptome responses of the duodenal epithelial cells after 4 weeks of AP transplantation

Among the 21,910 genes, the microarray analysis detected a total of 321 genes upregulated by more than 2-fold (287 known genes and 34 unknown genes) and 241 genes downregulated by lower than -2 -fold (210 known genes and 31 unknown genes) in the duodenal epithelial cells of AP-grafted rats (Fig. 3A and B, and Supplemental Tables S1 and S2). All known genes which were upregulated by more than 10-fold or downregulated by less than -10 -fold are listed in order of fold change in Tables 2 and 3, respectively. As shown in Fig. 3, analysis of the distribution of fold changes demonstrated that one half of the PRL-altered genes were increased in the range of 2- to 4-fold (156 known genes and 25 unknown genes), or were decreased in the range of -2 to -4 -fold (111 known genes and 18 unknown genes). There were 8 upregulated genes, i.e., *Kynu*, *Clcnkb*, *Kcnab2*, *Rup2*, *Clstn2*, *Prlpd*, *Sardh* and *Epim*, and 4 downregulated genes, i.e., *Apoa2*, *S100a9*, *Mfap4* and *Grik3*, the expression levels of which were changed by a factor of 100 or higher (Tables 2 and 3).

Furthermore, we searched the GO and Ensembl databases to obtain functional classification of each PRL-altered gene. Only the genes changed by more than 10-fold or less than -10 -fold were categorized into 14 major GO groups, each with unique GO identifier, as depicted in Fig. 4A. There were a total of 49 annotated genes in the upregulated set, and 37 annotated genes in the downregulated set (Table 4). The upregulated genes (Fig. 4B) were predominantly involved in the molecular function (11 genes, 20.75% of >10 -fold upregulated genes), cellular metabolic process (10 genes, 18.87%), regulation of biological process (7 genes, 13.21%), transport (5

genes, 9.43%) and cellular component (4 genes, 7.55%). Within the downregulated group (Fig. 4C), the majority of genes were involved in the regulation of biological process (8 genes, 20% of <-10 -fold downregulated genes), cell communication (7 genes, 17.50%) and molecular function (6 genes, 15%).

Since the effects of PRL on ion and nutrient transport have been well established in the intestine, especially in the duodenum (Charoenphandhu and Krishnamra, 2007; Mainoya, 1975a,b), we further analyzed all upregulated and downregulated genes in the transport category (GO identifier 0006810). The results showed that PRL changed the expression of several genes involved in the transport of Ca^{2+} , Na^{+} , K^{+} , Cl^{-} , H^{+} , Zn^{2+} , Cu^{2+} , HCO_3^{-} , phosphate, glucose, neutral amino acid, organic cation, and organic anion (Tables 5 and 6). Interestingly, the majority of the PRL-altered transporter genes were involved in K^{+} transport (Fig. 5A and B), i.e., 5 upregulated transcripts (29.41% of upregulated transcripts in the transport category) and 3 downregulated transcripts (25%). PRL also altered the expression of two genes which are subunits of $\text{Na}^{+}/\text{K}^{+}$ -ATPase, a known target protein of PRL (Lalonde et al., 1992; Charoenphandhu et al., 2006), i.e., *Atp1a2* and *Atp1a3* by 2.023- and -7.244 -fold (Tables 5 and 6), respectively.

Moreover, PRL increased the expression of several duodenal genes reported to be involved in ion and nutrient transport in the intestine and/or the total body water and electrolyte homeostasis, such as *Klk7*, *Avp*, *Npb*, *Kng1*, *Ren1*, *Trpc2*, *Npy2r*, *Nppc* and *Gcgr* (Supplemental Table S1), while decreasing the expression of others, such as *Npy1r*, *Npy5r*, *Chrm1*, *P2ry12*, *Galr3*, *Hrh2*, *Npr2* and *Drd3* (Supplemental Table S2). PRL also upregulated *Pthr1* encoding parathyroid hormone receptor 1 by 2.015-fold, and downregulated *Calca* encoding calcitonin/calcitonin-related polypeptide

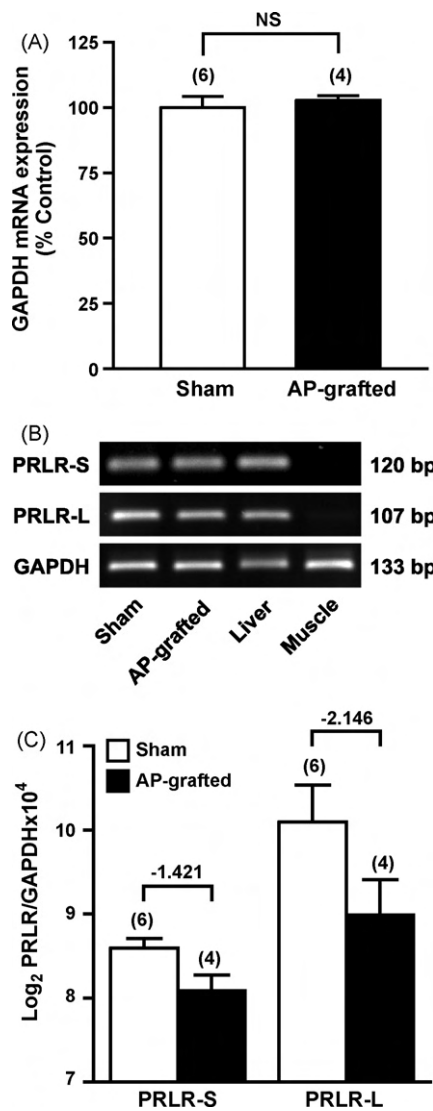


Fig. 2. (A) Expression of GAPDH, the housekeeping gene used for normalization in the qRT-PCR study, in the duodenal epithelial cells of sham-operated (Sham) and AP-grafted rats. Results were obtained from qRT-PCR and expressed as means $\pm$ S.E. NS, not significant. (B) Representative electrophoretic bands of short (-S) and long (-L) isoforms of PRLR in the duodenal epithelial cells of Sham and AP-grafted rats. Liver and gastrocnemius muscle from a Sham rat were used as positive and negative controls, respectively. Data were obtained from conventional RT-PCR at 36 cycles. (C) Expression of PRLR-S and -L in the duodenal epithelial cells of Sham and AP-grafted rats as determined by qRT-PCR. GAPDH is a housekeeping gene used for normalization. Results are expressed as log₂ means $\pm$ S.E. Fold changes of PRLR-S and PRLR-L expression are presented above their respective columns. Numbers in parentheses represent the number of animals.

α by -2.300 -fold, both of which have been known to regulate intestinal Ca^{2+} transport and/or Ca^{2+} homeostasis.

Finally, we used qRT-PCR to verify changes in gene expression demonstrated by microarray. We selected genes to be tested by qRT-PCR according to their biological functions, i.e., 1–3 genes per each category (Table 1). As shown in Fig. 6, the qRT-PCR results of 15 studied transcripts were all consistent with the microarray results in response to hyperprolactinemia.

4. Discussion

In the present study, we demonstrated, for the first time, the transcriptome responses of the duodenal epithelial cells to hyper-

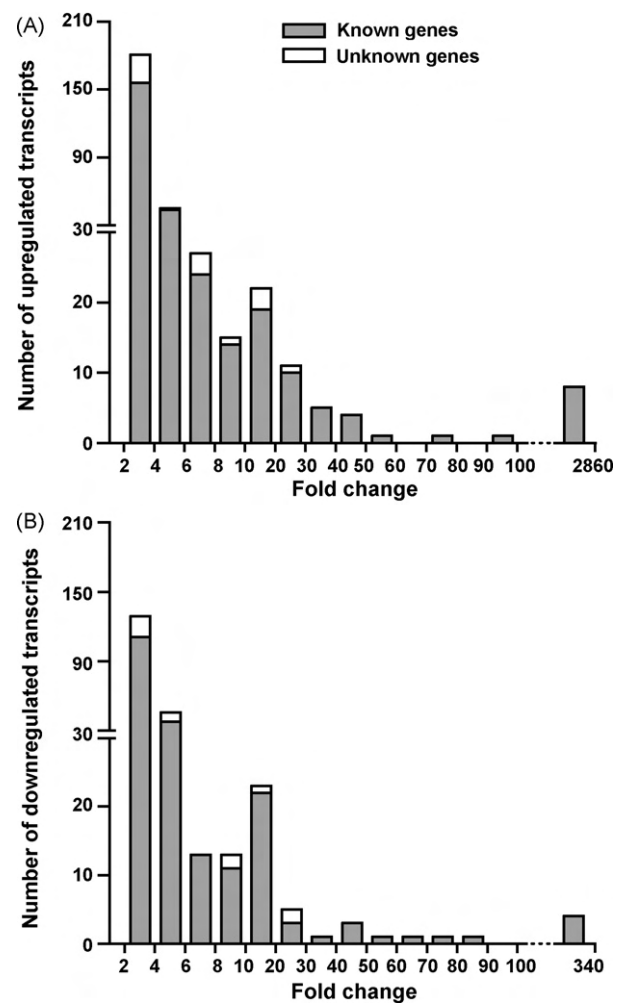


Fig. 3. Distribution of fold changes of genes upregulated (A) and downregulated (B) in the duodenal epithelial cells of AP-grafted rats as determined by microarray. This data set includes transcripts with expression levels changed by more than 2-fold or less than -2 -fold.

prolactinemia using the highly sensitive Illumina microarray. There was a good quantitative correlation between the fold changes determined by microarray and qRT-PCR. The present results supported the hypothesis that PRL could exert its actions on the duodenal epithelium in a genomic manner. Gene ontology analysis also suggested pleiotropic actions of PRL on a number of cellular processes, especially the transport function, thus confirming the previous studies both in vitro at the tissue level and in vivo that PRL regulated the body electrolyte homeostasis by controlling the rates of intestinal ion transport. Although the samples were collected by scraping and may contain other mucosal cells, e.g., goblet cells, there has been no report on the effect of PRL on other mucosal cell types. It was, therefore, implicated that our results represented the expression profile of the duodenal absorptive cells.

The presence of PRLR in the duodenal epithelial cells indicated that these cells could be direct targets of PRL actions. Our results were consistent with several previous investigations which showed PRLR expression in the duodenum at both transcriptional and translational levels (Jantarajit et al., 2007). However, nothing is known pertaining duodenal PRLR expression under either physiological (e.g., pregnancy and lactation) or pathological (e.g., prolactinoma) hyperprolactinemia. As demonstrated by our qRT-PCR, chronic exposure to PRL could downregulate duodenal PRLR, similar to that typically seen in other cells, such as luteal cells and human

Table 2
Known genes upregulated in the duodenal epithelial cells of AP-grafted rats.

Gene name	Gene symbol	Accession no.	Fold change
Kynureninase (L-kynurenine hydrolase)	Kynu	NM.053902	2859.417
Chloride channel Kb	Clcnkb	NM.173103	397.970
Potassium voltage-gated channel, shaker-related subfamily, beta member 2	Kcnab2	NM.017304	213.821
Urinary protein 2	Rup2	NM.001034950	147.546
Calsyntenin 2	Clstn2	NM.134377	136.802
Prolactin-like protein D	Prldp	NM.022537	134.864
Sarcosine dehydrogenase	Sardh	NM.053664	123.675
Epimorphin	Epim	NM.012748	100.643
Pyrimidinergic receptor P2Y, G-protein coupled, 6	P2ry6	NM.057124	93.969
Carboxypeptidase Z	Cpz	NM.031766	75.040
Solute carrier family 38, member 5	Slc38a5	NM.138854	52.576
Toll-like receptor 4	Tlr4	NM.019178	46.702
Cholinergic receptor, muscarinic 2	Chrm2	NM.031016	42.014
Paraoxonase 1	Pon1	NM.032077	41.760
Fatty acid binding protein 3	Fabp3	NM.024162	40.893
Insulin 2	Ins2	NM.019130	39.318
Protease, serine, 2	Prss2	NM.012729	39.121
CEA-related cell adhesion molecule 10	Ceacam10	NM.173339	34.794
Kallikrein 7	Klk7	NM.012593	33.569
Mitogen-activated protein kinase 15	Mapk15	NM.173331	32.832
Drebrin 1	Dbrn1	NM.031024	28.679
Synapsin II, transcript variant 2	Syn2	NM.019159	28.674
Pregnancy upregulated non-ubiquitously expressed CaM kinase	Pnck	NM.017275	27.032
Synaptogyrin 1	Syng1	NM.019166	27.020
GTP binding protein 3	Gtpbp3	NM.001011919	25.783
Sex hormone binding globulin	Shbg	NM.012650	22.897
Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein	Nfatc2ip	NM.001007692	22.859
Syncollin	Sycn	NM.139086	22.518
Formin binding protein 1	Fbnp1	NM.138914	22.349
Unc-5 homolog A (<i>C. elegans</i>)	Unc5a	NM.022206	21.680
Parvalbumin	Pvalb	NM.022499	17.373
Zinc finger protein 382	Znf382	NM.144749	16.306
Hemochromatosis type 2 (juvenile) homolog (human)	Hfe2	NM.001012080	16.123
FSH primary response 1	Fshprh1	NM.012955	15.476
GATA binding protein 3	Gata3	NM.133293	15.366
Sodium channel, voltage-gated, type I, alpha	Scn1a	NM.030875	14.246
MAD homolog 9 (<i>Drosophila</i>)	Smad9	NM.138872	14.104
Growth arrest specific 8	Gas8	NM.001039030	13.856
Calcium channel, voltage-dependent, beta 1 subunit	Cacnb1	NM.017346	12.633
CD40 ligand	Cd40lg	NM.053353	12.457
Zona pellucida glycoprotein 4	Zp4	NM.172330	12.451
Insulin 1	Ins1	NM.019129	12.398
Protamine 3	Prm3	NM.001002855	12.356
Outer dense fiber of sperm tails 4	Odf4	NM.001007670	11.871
FYVE, RhoGEF and PH domain containing 1	Fgd1	NM.001037546	11.434
Mitochondrial hepatocellular carcinoma-downregulated carrier protein	Hdmcp	NM.001001509	11.245
Calpain 6	Capn6	NM.031808	10.646
Calpain 3	Capn3	NM.017117	10.388
SH3-domain GRB2-like 3	Sh3gl3	NM.031238	10.101

Results are expressed as fold difference in signal intensity. This data set includes mRNAs whose levels changed by more than 10-fold in response to hyperprolactinemia. The complete list of the upregulated transcripts (more than 2-fold) is presented as [supplemental data \(Table S1\)](#).

embryo kidney 293T cells (Bowen et al., 2000; Swaminathan et al., 2008). However, despite the downregulation of PRLR, the duodenum appeared to be responsive to PRL since the stimulatory effect of PRLR on the transepithelial Ca^{2+} absorption in the duodenum of AP-grafted rats was still detectable (Tudpor et al., 2005). Moreover, similar to the results in luteal cells (Bowen et al., 2000), chronic PRL exposure predominantly downregulated PRLR-L, but not PRLR-S. The exact explanation of the differential PRLR isoform expression is not fully understood. Generally, PRLR-S and -L are expressed as cell surface receptors, and both isoforms possess comparable binding affinities (Bole-Feysot et al., 1998). It is suggested that PRL exerts its actions through PRLR-L, whereas PRLR-S does not mediate the transcriptional activation (Lesueur et al., 1991), or may even block the signal transduction of PRLR-L (Hu et al., 2001). Nevertheless, overexpression of PRLR-S completely rescued mammapoiesis in the heterozygous PRLR (PRLR^{+/-}) mice (Binart et al., 2003), indicating possible role of PRLR-S in the mammary gland in vivo. In the

intestine, known direct PRL actions are presumably mediated by PRLR-L, especially those involved in water, electrolyte and nutrient absorption (Thongon et al., 2008). The absence of change in the duodenal PRLR-S expression after AP transplantation suggested that PRL exerted its actions predominantly via PRLR-L.

Microarray analysis revealed several PRL-regulated genes in the transport category. Functions of PRL as an osmoregulatory and calciotropic hormone are conserved throughout the evolution from fishes to mammals (Sakamoto and McCormick, 2006; Charoenphandhu and Krishnamra, 2007; Norris, 2007). In rats, PRL has long been known to stimulate Ca^{2+} , Na^{+} and Cl^{-} transport in the duodenum (Tudpor et al., 2005; Mainoya, 1975d), and its Ca^{2+} -regulating action becomes more significant during pregnancy and lactation (Boass et al., 1992). Herein, we found that PRL altered the expression of $\text{Na}^{+}/\text{K}^{+}$ -ATPase α polypeptides Atp1a2 and Atp1a3, which are subunits of $\text{Na}^{+}/\text{K}^{+}$ -ATPase essential for the transcellular Na^{+} transport and the solvent drag-induced paracellular transport

Table 3

Known genes downregulated in the duodenal epithelial cells of AP-grafted rats.

Gene name	Gene symbol	Accession no.	Fold change
Apolipoprotein A-II	Apoa2	NM.013112	–333.239
S100 calcium binding protein A9 (calgranulin B)	S100a9	NM.053587	–184.269
Microfibrillar-associated protein 4	Mfap4	NM.001034124	–132.304
Glutamate receptor, ionotropic, kainate 3	Grik3	NM.181373	–103.219
Kinesin 13B	Kif13B	NM.213626	–81.392
Inhibin beta-A	Inhba	NM.017128	–75.430
Phosphate cytidyltransferase 1, choline, beta isoform	Pcyt1b	NM.173151	–69.764
Kringle containing transmembrane protein 1	Kremen1	NM.053649	–55.434
Dyslexia susceptibility 1 candidate 1 homolog (human)	Dyx1c1	NM.001007010	–44.282
Potassium inwardly rectifying channel, subfamily J, member 16	Kcnj16	NM.053314	–43.750
Mast cell protease 8	Mcpt8	NM.021598	–43.715
Connector enhancer of kinase suppressor of Ras 2	Cnksr2	NM.021686	–36.672
Ataxin 1	Atxn1	NM.012726	–24.906
Olfactory receptor 1078	Olr1078	NM.207597	–21.566
Dynein, axonemal, light intermediate polypeptide 1	Dnali1	NM.001031647	–21.532
Patched homolog 1 (<i>Drosophila</i>)	Ptch1	NM.053566	–19.512
T-complex protein 11 (mouse)	Tcp11	NM.001007695	–19.249
Sterol O-acyltransferase 1	Soat1	NM.031118	–19.244
Neurogenic differentiation 3	Neurod3	NM.019207	–18.431
Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites	Srms	NM.001011961	–16.639
p21 (CDKN1A)-activated kinase 3	Pak3	NM.019210	–15.416
Taste receptor, type 2, member 10	Tas2r10	NM.023995	–14.844
PR-Vbeta1	VCS-beta1	NM.001017497	–14.458
Vomerolnasal 1 receptor, B9	V1rb9	NM.173297	–13.607
CNDP dipeptidase 2 (metallopeptidase M20 family)	Cndp2	NM.001010920	–13.014
Solute carrier family 9, member 4	Slc9a4	NM.173098	–12.871
Succinate receptor 1	Sucnr1	NM.001001518	–12.769
Ly49 inhibitory receptor 5	Ly49i5	NM.001009501	–12.573
Tumor necrosis factor receptor superfamily, member 8	Tnfrsf8	NM.019135	–12.454
Surfactant associated protein D	Sftpd	NM.012878	–12.059
Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 4	Gabra4	NM.080587	–12.047
Profilin 2	Pfn2	NM.030873	–11.924
Prostaglandin D receptor	Ptgdr	NM.022241	–11.867
Erythrocyte protein band 4.1-like 1, transcript variant 2	Epb4.1l1	NM.021681	–11.439
G protein-coupled receptor 44	Gpr44	NM.001012070	–11.342
Trace-amine-associated receptor 7e	Taar7e	NM.175590	–10.730
Inter-alpha trypsin inhibitor, heavy chain 3	Itih3	NM.017351	–10.660

Results are expressed as fold difference in signal intensity. This data set includes mRNAs whose levels changed by less than –10-fold in response to hyperprolactinemia. The complete list of the downregulated transcripts (lower than –2-fold) is presented as [supplemental data \(Table S2\)](#).

of ions and nutrients (Charoenphandhu et al., 2006; Tanrattana et al., 2004). Na^+/K^+ -ATPase is a principal target protein of PRL in several tissues of vertebrates, e.g., branchial epithelium of marine teleost (Pickford et al., 1970), ascending limb and distal convoluted tubule of rat kidneys (Laborde et al., 1992), and duodenal epithelium of rats (Charoenphandhu et al., 2006). Furthermore, PRL changed the expression of the voltage-dependent Ca_v channel (Ca_v) subunits. Since the transepithelial Ca^{2+} flux exhibited a good correlation with L-type Ca_v activity and the L-type Ca_v blocker verapamil could block intestinal Ca^{2+} absorption (Morgan et al., 2007; Pento and Johnson, 1983), the altered Ca_v expression could explain the PRL-enhanced transepithelial calcium transport. In addition to Ca^{2+} , Na^+ and Cl^- , some investigators demonstrated a stimulatory effect of PRL on the intestinal transport of K^+ , glucose and neutral amino acids (Mainoya, 1975a,b,c). The effect of PRL on K^+ transport is indeed predominant in the colon where it inhibits the Ca^{2+} -dependent K^+ secretion (Puntheeranurak et al., 2007). Physiological significance of the PRL-regulated K^+ transport in the rat duodenum is currently not known, but it may be essential for maternal K^+ homeostasis since the expression of eight K^+ -related genes were altered by PRL. Furthermore, the PRL-induced downregulation of Zn^{2+} transporter Slc30a3 supported the previous finding reported by Travaglini and co-workers (1991) that hyperprolactinemia from prolactinoma led to a decrease in serum Zn^{2+} levels, which could be normalized by administration of bromocriptine, an inhibitor of pituitary PRL secretion. As for the intestinal transport of Cu^{2+} , HCO_3^- , phosphate, organic cations and organic anions, the effect

of PRL on their transporters have never been demonstrated functionally either in vitro or in vivo, although the expression of the related genes were altered quite significantly by PRL.

Moreover, PRL also upregulated several genes known to regulate the intestinal ion transport and/or total body fluid and electrolyte homeostasis, e.g., kallikrein 7 (Klk7), arginine vasopressin (Avp), neuropeptide B (Npb), kininogen 1 (Kng1), renin 1 (Ren1), transient receptor potential cation channel (Trpc2), neuropeptide Y receptors (Npy1r, Npy2r and Npy5r), natriuretic peptide precursor type C (Nppc), glucagon receptor (Gcgr), muscarinic cholinergic receptor 1 (Chrm1), purinergic receptors (P2rx2 and P2ry12), galanin receptor 3 (Galr3), histamine receptor H2 (Hrh2), natriuretic peptide receptor 2 (Npr2) and dopamine receptor D3 (Drd3) (Antunes-Rodrigues et al., 2004; Cox, 2007; Hirota and McKay, 2006; Kinoshita et al., 2006; Margolius et al., 1985; Norris, 2007; Schultheiss et al., 2006; Walling et al., 1977; Zhang et al., 2007). Avp encoding arginine vasopressin not only plays a major role in osmoregulation, but also increases the duodenal motility (Li et al., 2007a). Thus, Avp upregulation could partially explain the PRL-induced increase in the gastrointestinal emptying time during lactation (Chen et al., 1997). Regarding Klk7 as a member of the kallikrein-kinin system, products of this system were found to stimulate Na^+ transport across the basolateral membrane of the intestinal absorptive cells (Moriwaki and Fujimori, 1979). Some natriuretic peptide-related genes, e.g., natriuretic peptide precursor type C (Nppc) and natriuretic peptide receptor 2 (Npr2), as well as renin-angiotensin system-related genes, e.g., renin (Ren1), are widely accepted as regulators of water

Table 4
Identification of PRL-altered transcripts in each gene ontology category.

Upregulated transcripts	Downregulated transcripts
GO:000815 Biological process	GO:000815 Biological process
GO:0007155 Cell adhesion	GO:0007155 Cell adhesion
Ceacam10 (34.794)	Mfap4 (–132.304)
GO:0007154 Cell communication	GO:0007154 Cell communication
Clstn2 (136.802)	Grik3 (–103.219)
Chrm2 (42.014)	Cnksr2 (–36.672)
Fshprh1 (15.476)	Olr1078 (–21.566)
	Pak3 (–15.416)
	Sucnr1 (–12.769)
	Gabra4 (–12.047)
	Taar7e (–10.730)
GO:0016043 Cellular component organization and biogenesis	GO:0016043 Cellular component organization and biogenesis
Syngn1 (27.020)	Kif13B (–81.392)
Hdmcp (11.245)	
GO:0048869 Cellular developmental process	
Epim (100.643)	
GO:0044237 Cellular metabolic process	GO:0044237 Cellular metabolic process
Carbohydrate metabolism	Lipid metabolism
Ins2 (39.318)	Apoa2 (–333.239)
Ins1 (12.398)	Pcyt1b (–69.764)
Lipid metabolism	Soat1 (–19.244)
Fabp3 (40.893)	Amine metabolism
Nucleic acid metabolism	Itih3 (–10.660)
Gtpbp3 (25.783)	
Protein metabolism	
Kynu (2859.417)	
Sardh (123.675)	
Cpz (75.040)	
Mapk15 (32.832)	
Pnck (27.032)	
Capn3 (10.388)	
GO:0032502 Developmental process	GO:0032502 Developmental process
Dbn1 (28.679)	Kremen1 (–55.434)
	Sftpd (–12.059)
	Ptgdr (–11.867)
GO:0008152 Metabolism	
Pon1 (41.760)	
GO:0050789 Regulation of biological process	GO:0050789 Regulation of biological process
Syn2 (28.674)	S100a9 (–184.269)
Nfatc2ip (22.859)	Inhba (–75.430)
Syncn (22.518)	Atxn1 (–24.906)
Znf382 (16.306)	Ptch1 (–19.512)
Smad9 (14.104)	Neurod3 (–18.431)
Cd40lg (12.457)	Tnfrsf8 (–12.454)
Fgd1 (11.434)	Pfn2 (–11.924)
	Gpr44 (–11.342)
GO:0042221 Response to chemical stimulus	
Prss2 (39.121)	
GO:0006950 Response to stress	
Tlr4 (46.702)	
GO:0005575 Cellular component	GO:0005575 Cellular component
Hfe2 (16.123)	Mcpt8 (–43.715)
Gas8 (13.856)	Dnali1 (–21.532)
Prm3 (12.356)	VCS-beta1 (–14.458)
Odf4 (11.871)	
GO:0003674 Molecular function	GO:0003674 Molecular function
Calcium ion binding	Protein binding
Pvalb (17.373)	Epb4.1l1 (–11.439)
Protein binding	Peptidase activity
Shbg (22.897)	Cndp2 (–13.014)
Fnbp1 (22.349)	Receptor activity
Zp4 (12.451)	Tcp11 (–19.249)
Sh3gl3 (10.101)	Tas2r10 (–14.844)
Hormone activity	V1rb9 (–13.607)
Prlpd (134.864)	Ly49i5 (–12.573)
Peptidase activity	
Klk7 (33.569)	
Capn6 (10.646)	
Receptor activity	
P2ry6 (93.969)	
Unc5a (21.680)	
Transcription factor activity	
Gata3 (15.366)	
Unknown	Unknown

Table 4 (Continued)

Upregulated transcripts		Downregulated transcripts	
Rup2	(147.546)	Dyx1c1	(−44.282)
MGC108778	(23.930)	RN.13478	(−22.425)
LOC363483	(19.150)	LOC312102	(−20.541)
LOC497899	(18.844)	Srms	(−16.639)
RN.37544	(17.404)	RN.3886	(−12.456)

Numbers in parentheses represent fold changes of corresponding transcripts. Each category is accompanied by a unique GO identifier supplied by the GO search engines. Within each category, transcripts are listed in order of fold change. This data set includes mRNAs with the expression levels changed by more than 10-fold or less than −10-fold in response to hyperprolactinemia.

Table 5

PRL-upregulated transcripts in the transport category (GO:0006810).

Gene name	Gene symbol	Accession no.	Fold change
<i>Calcium transport</i>			
Calcium channel, voltage-dependent, beta 1 subunit	Cacnb1	NM.017346	12.633
Calcium channel, voltage-dependent, T type, alpha 1H subunit	Cacna1h	NM.153814	8.392
Calcium channel, voltage-dependent, T type, alpha 1G subunit	Cacna1g	NM.031601	7.017
<i>Phosphate transport</i>			
Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	Slc17a7	NM.053859	3.582
<i>Sodium transport</i>			
Sodium channel, voltage-gated, type I, alpha	Scn1a	NM.030875	14.246
Amiloride-sensitive cation channel 3	Accn3	NM.173135	2.084
ATPase, Na ⁺ /K ⁺ transporting, alpha 2 polypeptide	Atp1a2	NM.012505	2.023
<i>Chloride transport</i>			
Chloride channel Kb	Clcnkb	NM.173103	397.970
<i>Potassium transport</i>			
Potassium voltage-gated channel, shaker-related subfamily, beta member 2	Kcnab2	NM.017304	213.821
Potassium voltage-gated channel, Isk-related subfamily, member 1	Kcne1	NM.012973	5.314
Potassium inwardly rectifying channel, subfamily J, member 5	Kcnj5	NM.017297	2.547
Potassium voltage-gated channel, subfamily H (eag-related), member 2	Kcnh2	NM.053949	2.209
Potassium voltage-gated channel, subfamily Q, member 3	Kcnq3	NM.031597	2.049
<i>Bicarbonate transport</i>			
Solute carrier family 4, sodium bicarbonate cotransporter, member 9	Slc4a9	NM.152938	2.353
<i>Copper transport</i>			
ATPase, Cu ²⁺ transporting, beta polypeptide	Atp7b	NM.012511	4.160
<i>Organic cation transport</i>			
Solute carrier family 22 (organic cation transporter), member 17	Slc22a17	NM.177421	2.646
<i>Neutral amino acid transport</i>			
Solute carrier family 38, member 5	Slc38a5	NM.138854	52.576

Results are expressed as fold difference in signal intensity. This data set includes mRNAs with the expression levels changed by more than 2-fold in response to hyperprolactinemia.

Table 6

PRL-downregulated transcripts in the transport category (GO:0006810).

Gene name	Gene symbol	Accession no.	Fold change
<i>Calcium transport</i>			
Calcium channel, voltage-dependent, L type, alpha 1E subunit	Cacna1e	NM.019294	−2.424
<i>Sodium transport</i>			
Solute carrier family 9, member 4	Slc9a4	NM.173098	−12.871
ATPase, Na ⁺ /K ⁺ transporting, alpha 3 polypeptide	Atp1a3	NM.012506	−7.244
<i>Potassium transport</i>			
Potassium inwardly rectifying channel, subfamily J, member 16	Kcnj16	NM.053314	−43.750
Potassium voltage-gated channel, subfamily H (eag-related), member 1	Kcnh1	NM.031742	−2.297
Potassium voltage-gated channel, subfamily G, member 3, transcript variant 1	Kcng3	NM.133426	−2.061
<i>Hydrogen ion transport</i>			
ATPase, H ⁺ transporting, V0 subunit D, isoform 2	Atp6v0d2	NM.001011972	−4.735
ATPase, H ⁺ transporting, V1 subunit G isoform 2	Atp6v1g2	NM.212490	−3.151
<i>Zinc transport</i>			
Solute carrier family 30 (zinc transporter), member 3	Slc30a3	NM.001013243	−2.737
<i>Organic anion transport</i>			
Solute carrier organic anion transporter family, member 6c1	Slco6c1	NM.173338	−2.767
Kidney specific organic anion transporter	Slc21a4	NM.030837	−2.473
<i>Glucose transport</i>			
Solute carrier family 2 (facilitated glucose transporter), member 4	Slc2a4	NM.012751	−2.137

Results are expressed as fold difference in signal intensity. This data set includes mRNAs with the expression levels changed by lower than −2-fold in response to hyperprolactinemia.

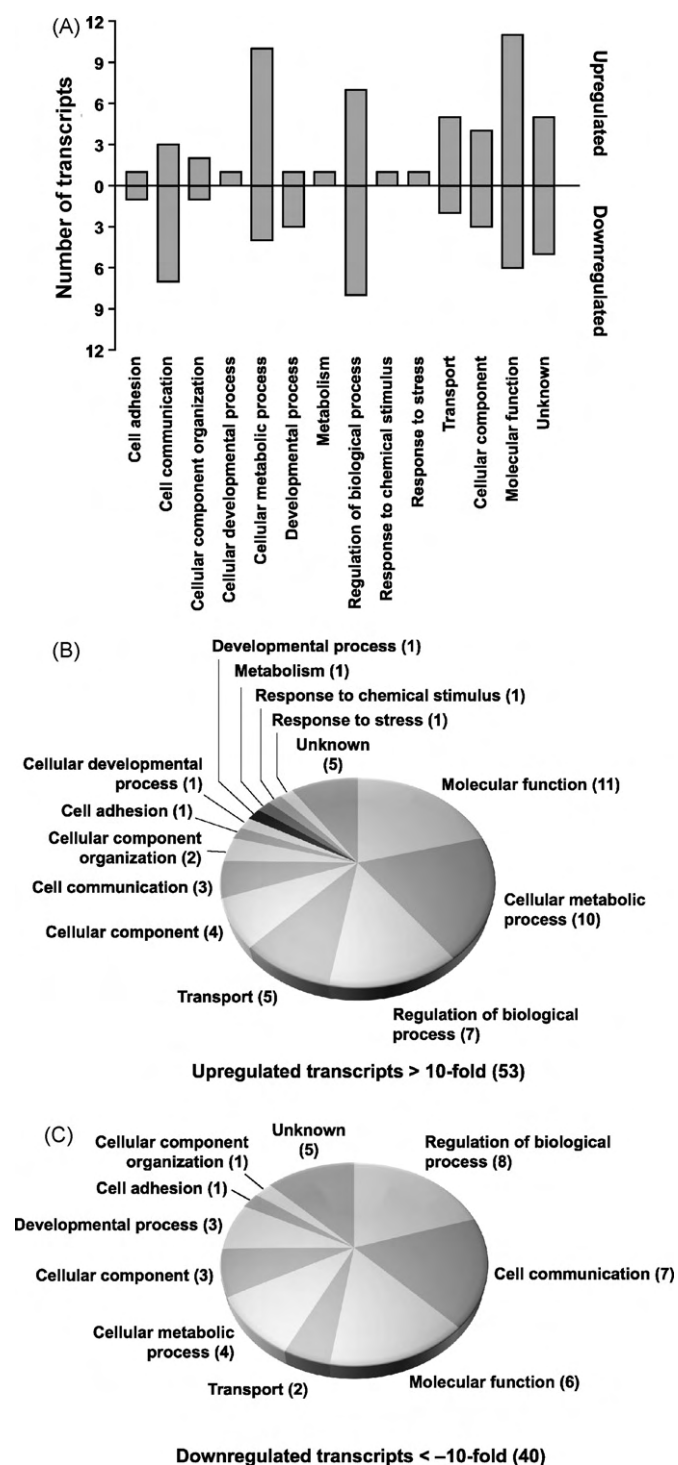


Fig. 4. (A) Distribution of genes in each gene ontology category. Columns above the abscissa represent transcripts that were upregulated at 4 weeks after AP transplantation, whereas columns below the abscissa represent downregulated transcripts. All transcripts were functionally classified according to known cellular functions by using GO search engines. (B) A pie chart shows proportional distribution of upregulated genes in each gene ontology category. (C) A pie chart shows proportional distribution of downregulated genes in each category. Numbers in parentheses represent the number of upregulated or downregulated transcripts. This data set includes transcripts whose levels changed by more than 10-fold or less than -10-fold.

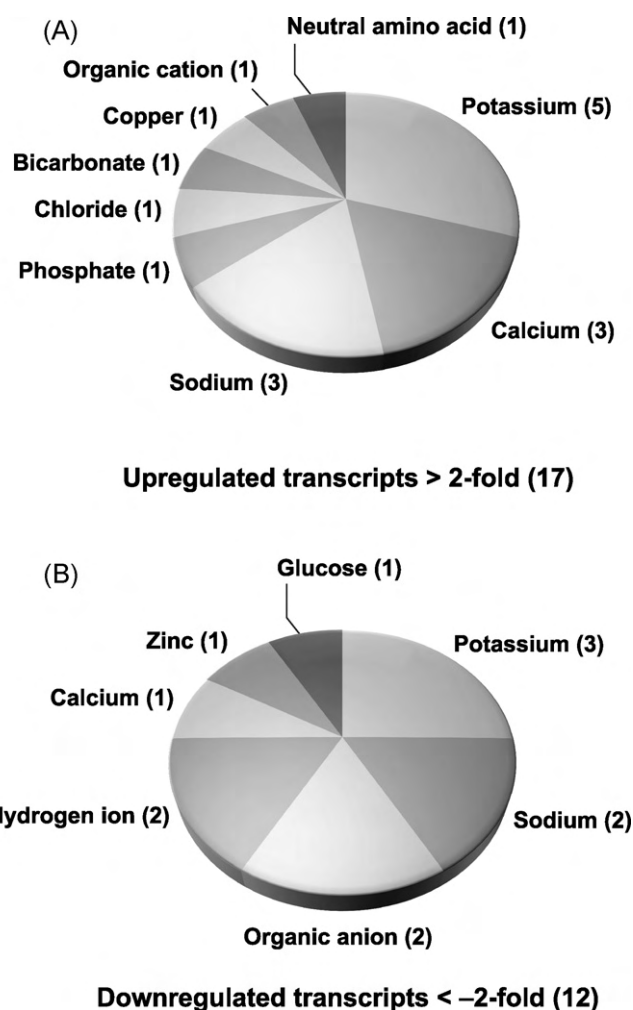


Fig. 5. Pie charts show proportional distributions of upregulated (A) and downregulated (B) genes in the transport category (GO:0006810). The transcripts were functionally classified according to the transported ions or substances. Numbers in parentheses represent the number of upregulated or downregulated transcripts. This data set includes transcripts with expression levels changed by more than 2-fold or less than -2-fold.

and Na^+ balance (Antunes-Rodrigues et al., 2004), whereas the muscarinic cholinergic receptors (Chrm) and neuropeptide Y receptors (e.g., Npy1r, Npy2r and Npy5r) are capable of modulating intestinal fluid secretion (Cox, 2007; Hirota and McKay, 2006). Downregulation of galanin receptor 3 (Galr3) possibly affected the intestinal transport activity, since galanin could increase the active Na^+ and Cl^- absorption in the rabbit ileum (Homaidan et al., 1994).

Of interest is the finding of the PRL-induced upregulation of Pthr1 encoding parathyroid hormone receptor 1 and the downregulation of Calca encoding calcitonin/calcitonin-related polypeptide α , a hypocalcemic hormone. Although the actions of both proteins on the duodenal epithelium have never been reported, we postulate that such actions might be part of the indirect mechanism by which PRL regulates the duodenal Ca^{2+} absorption and/or Ca^{2+} balance. Nemere and Norman (1986) previously reported that parathyroid hormone 1–34, which could bind to Pthr1, significantly stimulated Ca^{2+} transport in the perfused, isolated duodenal loops.

Besides the genes related to electrolyte homeostasis, PRL also altered a number of genes in other categories, including cell adhesion, cell communication, cellular developmental process, and cellular metabolic process, as shown in Table 4. Although most PRL-altered genes are of unknown functions in the intestine,

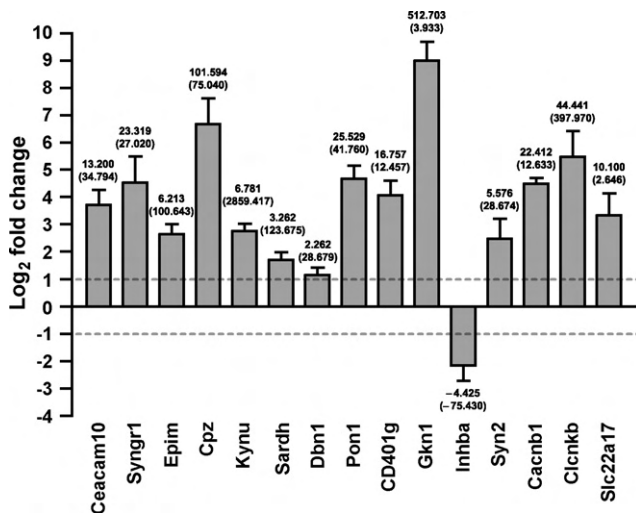


Fig. 6. Fold changes in the expression levels of 15 transcripts selected from various GO categories in the duodenal epithelial cells of AP-grafted rats ($n=4$) vs. sham-operated rats ($n=6$). Gene expression was normalized by GAPDH. Twofold upregulation or downregulation are indicated by the two dashed lines. Fold change value of each gene from qRT-PCR is presented on its respective column. Corresponding microarray data are given in parentheses. Full names of genes are shown in Tables 2 and 3.

alteration of some transcripts, such as epimorphin (Epim), zinc finger protein 382 (Znf382), kringle-containing transmembrane protein 1 (Kremen1), purinergic receptor (P2rx2), and insulin (Ins1 and Ins2), might explain PRL actions on the intestinal cell proliferation and maturation (Coutinho-Silva et al., 2005; Gebelein et al., 1998; Iizuka et al., 2007; Ménard et al., 1999). Mainoya (1978) demonstrated that, during pregnancy and lactation, the intestinal weight of rats was markedly increased, and such effect could be abolished by bromocriptine. Hence, the lactation-induced intestinal hypertrophy could be attributed to the proliferative effect of PRL. In contrast, Muller and Dowling (1981) could not detect intestinal hypertrophy in hyperprolactinemic rats with serum PRL of 870 ng/mL. This discrepancy could be due to the typical biphasic action of PRL, in which excess PRL molecules bind to PRLRs as non-functional 1:1 PRL-PRLR complex instead of a functional 1:2 complex (Fuh et al., 1993). To induce intestinal hypertrophy, we speculate that PRL may do so by increasing the expression of a morphogenic protein epimorphin (Epim) or purinergic receptor P2X (P2rx2), both of which have been reported to enhance survival of intestinal epithelial cells (Iizuka et al., 2007; Coutinho-Silva et al., 2005). Alternatively, PRL may induce production of certain local growth factors, such as insulin (Ins1 and Ins2), which can stimulate proliferation of jejunal epithelial cells (Ménard et al., 1999). Other upregulated transcripts in the regulation of biological process and molecular function categories, e.g., mothers against decapentaplegic homolog 9 (Smad9), also appear to be essential for growth and development of the intestinal epithelium.

PRL is also involved in the biotransformation of certain exogenous and endogenous compounds, e.g., *p*-nitrophenol and androgenic steroids (Luquita et al., 1999). Since the intestinal epithelia are normally exposed to drugs and toxic substances, an elevated plasma PRL in lactating animals may help neutralize these chemicals at the absorptive sites, thereby reducing the amount of their toxic metabolites in milk. It is possible that the upregulation of UDP-glucuronosyltransferase (Udpgr2) gene of the cellular metabolic process category (Supplemental Tables S1) could be important for the PRL-regulated phase II biotransformation of drugs and steroid hormones during lactation. Luquita and co-workers (1999) demonstrated that 1–3 mg/kg/day PRL administration

over 4 days increased UDP-glucuronosyltransferase activity in the jejunum of ovariectomized rats. Consistent with a previous report on a high activity of an antioxidative protein paraoxonase in lactating animals (Turk et al., 2005), paraoxonase (Pon1) expression was increased in our microarray study by ~41-fold (Table 2). In addition, other genes, e.g., nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein (Nfatc2ip) and GATA binding protein 3 (Gata3), may also function in the modulation of the immunoprotective or barrier function of the intestinal epithelium (Li et al., 2007b; Kiwamoto et al., 2006).

In summary, the present study demonstrated that PRL altered the expression of a number of genes related to fluid and electrolyte homeostasis (especially for Ca^{2+} , Na^{+} , K^{+} and Cl^{-} transport) and cell proliferation, both of which are well-established functions of PRL in the intestine. However, the precise functions of these PRL-regulated genes in the duodenal epithelial cells as well as the association between PRL and the functions of those genes are currently unknown. Our results thus provide direction for further investigations into the mechanism of PRL actions in the intestine during pregnancy and lactation.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2008.09.025.

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Gene expression profile of duodenal epithelial cells in response to chronic metabolic acidosis

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Abstract Chronic metabolic acidosis (CMA) affects ion transport, permeability, and metabolism of the intestinal absorptive cells. Most effects of CMA on the intestine are long-term adaptations at genomic level. To identify the CMA-regulated genes, the Illumina's microarray featuring high-performance BeadArray technology was performed on RNA samples from the rat duodenal epithelial cells exposed to long-standing acidemia. After 21 days of CMA, we found 423 transcripts upregulated and 261 transcripts downregulated. Gene ontology analysis suggested effects of CMA on cellular processes, such as cell adhesion, proliferation, fuel metabolism, and biotransformation. Interestingly, 27 upregulated transcripts (e.g., *Aqp1*, *Cacnb1*, *Atp1a2*, *Kcnab2*, and *Slc2a1*) and 13 downregulated transcripts (e.g., *Slc17a7*, *Slc9a4*, and *Slc30a3*) are involved in the absorption of water, ions, and nutrients. Some upregulated genes, such as *Slc38a5* and *Slc1a7* encoding glutamine transporters, may be parts of the total body adaptation to alleviate negative nitrogen balance. Therefore, the present results provided a novel genome-

wide information for further investigations of the mechanism of CMA effect on the intestine.

Keywords Acidemia · Duodenum · Gene ontology · Microarray · NH_4Cl · Real-time PCR

Introduction

Chronic metabolic acidosis (CMA), a common problem seen in chronic kidney diseases due to the reduced capacity of the kidney to synthesize ammonia and excrete hydrogen ions (as in renal failure) or continuous loss of alkali (as in distal renal tubular acidosis), is associated with many metabolic derangements, including massive bone loss, nephrocalcinosis, hypotension, altered blood flow to visceral organs, and multiple-organ damage [1]. CMA also affected the intestine whereby its metabolic and barrier functions were impaired [2]. On the other hand, a number of investigations suggested that CMA could induce long-term adaptations at molecular or genomic levels in the intestinal epithelial cells to provide the body with the much needed essential nutrients to alleviate metabolic derangement [3, 4]. Therefore, the genome-wide microarray study should be useful for demonstrating the genetic response of the intestinal epithelial cells to CMA.

Changes in the transepithelial transport of water, major elements, and nutrients, such as Na^+ , Cl^- , K^+ , HCO_3^- , Ca^{2+} , inorganic phosphate (P_i), and amino acids, have been well described as the hallmark of CMA-induced changes in the intestinal functions [4–8]. For instance, a long-term decrease in arterial pH has been reported to markedly increase Na^+ , Cl^- , and water absorption in the ileum and decrease K^+ absorption in the jejunum [7, 9]. CMA also increased HCO_3^- absorption in the proximal colon and

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decreased HCO_3^- secretion in the distal colon [6]. We previously demonstrated in the rat duodenum that 21-day NH_4Cl -induced CMA increased the mRNA expression of important genes involved in Ca^{2+} transport, e.g., those coding for transient receptor potential vanilloid family Ca^{2+} channels and plasma membrane Ca^{2+} -ATPase, thereby stimulating Ca^{2+} absorption presumably to supply more Ca^{2+} to mitigate the CMA-induced negative calcium balance and osteopenia [4]. Regarding phosphate metabolism, acidosis stimulated the Na^+ -dependent phosphate absorption and expression of the Na^+/P_i -cotransporters in the small intestine, which helped prevent excessive liberation of phosphate from bone during CMA [10]. In addition, CMA enhanced the intestinal absorption of glutamine which could protect the mucosa from acid-induced injury [3, 11].

As for the barrier functions of the intestinal epithelium, CMA led to a sizable hyperpermeability to hydrophilic macromolecules with a decrease in the transepithelial resistance (TER), in part, due to an impairment of ATP biosynthesis caused by CMA interfering with the functions of several enzymes, such as phosphofructokinase [2, 12, 13]. Studies in rat intestinal epithelial cell line IEC-6 exposed to lipopolysaccharide confirmed that defective epithelial barrier directly resulted from the cytoplasmic acidification as a consequence of extracellular acidosis [14]. We further demonstrated in the rat duodenum that 21-day CMA increased the expression of several tight junction proteins of the claudin family, which are known to regulate the paracellular permeability [15]. CMA has also been reported to alter other cellular processes known to affect survival of the intestinal epithelial cells, such as cell proliferation, apoptosis, lipid peroxidation, and activity of inducible nitric oxide synthase [13, 16].

The objective of the present study was to analyze the profile of CMA-altered genes in the duodenal epithelial cells by using microarray and quantitative real-time PCR (qRT-PCR) techniques. The duodenum was used because it is the important site of ion and nutrient absorption, and the duodenal epithelial cells evidently respond to CMA by changing the expression of several transporter genes [4, 15]. Herein, the Illumina's microarray featuring the high-performance BeadArray technology was used, since this platform exhibits high selectivity and sensitivity in gene expression profiling as a result of the high redundancy of each probe built in the bead array [17].

Materials and methods

Animals

Ten female Sprague-Dawley rats, weighing 180–200 g (10-week-old), were obtained from the National Laboratory

Animal Centre, Thailand. They were individually placed in hanging stainless steel cages, fed standard pellets (Perfect Companion, Bangkok, Thailand), and distilled water ad libitum under 12/12 h light/dark cycle. Room temperature was controlled at 23–25°C, and the relative humidity was about 50–60%. After 7-day acclimatization, rats were randomly divided into two groups, i.e., control ($n = 6$) and CMA groups ($n = 4$). To induce CMA, rats were supplied ad libitum for 21 days with drinking water containing 1.5% w/v NH_4Cl (Sigma, St. Louis, MO, USA) to induce an acid-loading acidemia [4, 10, 15]. Food and water intake were recorded daily. This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University, Bangkok, Thailand.

Tissue preparation

A median laparotomy was performed under 50 mg/kg sodium pentobarbitone i.p. (Abbott, North Chicago, IL, USA) anesthesia. A duodenal segment (10 cm) was removed and cut longitudinally along the radix mesenterii to expose the mucosal surface. The tissue was rinsed with cold phosphate buffered saline pH 7.4 to remove the luminal contents. Duodenal epithelial cells were collected by scraping the mucosa with an ice-cold glass slide and homogenized at 4°C with a glass-Teflon Potter-Elvehjem tissue grinder (Wheaton, Millville, NJ, USA) as previously described [15]. Gene expression profile of each duodenal sample was analyzed separately by microarray.

Arterial blood gas analysis

Heparinized arterial blood sample (5 ml) was collected from the left ventricle. Blood gas analysis for plasma pH, partial pressure of O_2 and CO_2 , and HCO_3^- concentration was performed to confirm the success of the acidosis induction by using an automated analyzer (model Ultra C, Nova Biomedical, Waltham, MA, USA).

Total RNA and cDNA preparation

By using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was prepared from duodenal epithelial cells, as previously described [18]. The total RNA was treated with RQ1 DNase (Promega, Madison, WI, USA) and subsequently purified with RNeasy Mini kit (Qiagen, Valencia, CA, USA). Purity of the total RNA was determined by the ratio of absorbance readings at 260 and 280 nm, the ratio of which was greater than 1.8. Integrity of RNA was analyzed by denaturing agarose gel electrophoresis with the 28S rRNA band appearing approximately twice as intense as the 18S rRNA band. Purified total RNA samples were later used for both microarray and qRT-PCR

analyses. As for qRT-PCR, 1 µg total RNA was reverse-transcribed with the oligo-dT₂₀ primer and the iScript kit (Bio-rad, Hercules, CA, USA) by using a Bio-rad MyCycler. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control gene to check the consistency of the reverse transcription (percent coefficient of variation < 10%, $n = 10$).

Microarray

Illumina TotalPrep RNA amplification kit (catalog no. IL1791; Ambion, Austin, TX, USA) was used to amplify and generate biotinylated cRNA from 500 ng total RNA for hybridization with Illumina BeadChip array. After the in vitro transcription and purification, biotinylated cRNA was quantified with a NanoDrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Of biotinylated cRNA from each duodenal sample, 750 ng was separately hybridized on the RatRef-12 Expression BeadChip (binary manifest file version V1_0_R0_11222119_A; Illumina, San Diego, CA, USA). Each RatRef-12 BeadChip is constructed with BeadArrayTM technology [17] and contains 12 whole-genome gene expression arrays, thus allowing 12 independent samples to be hybridized to a single chip. Each array which can probe 21,910 genes consists of 22,523 oligonucleotide probes (50-base gene-specific sequence and 29-base address sequence per probe) selected primarily from the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database release 16 (<http://www.ncbi.nlm.nih.gov/RefSeq>). Negative controls that define the system background consist of oligonucleotide probes which have no corresponding targets in the rat genomes. After incubation at 58°C for 20 h with cRNA, the BeadChip was washed, blocked, and incubated at room temperature with streptavidin-Cy3-containing solution (Illumina) according to the manufacturer's manual. Fluorescent signals were scanned with BeadStation 500GX Genetic Analysis System (Illumina). Image registration and data extraction were automated with BeadStudio 3.1.1 (Illumina).

Quantitative real-time PCR (qRT-PCR) and sequencing

Primers used in the present study were designed by OLIGO 6 (Molecular Biology Insights, Cascade, CO, USA) and Primer Validator 1.4 (Naratt Software, Bangkok, Thailand), as shown in Table 1. qRT-PCR and melting curve analysis were performed in triplicate by using Bio-rad MiniOpticon with the iQ SYBR Green SuperMix (Bio-rad) as previously described [18]. Gene expression levels were normalized by GAPDH expression. The PCR products were also visualized on a 1.5% agarose gel stained with 1.0 µg/ml ethidium bromide under ultraviolet

transilluminator (Alpha Innotech, San Leandro, CA, USA). After electrophoresis, the PCR products were extracted by the HiYield Gel/PCR DNA Extraction kit (Real Biotech Corporation, Taipei, Taiwan) and were sequenced by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Data analyses

Differential gene expression of microarray and qRT-PCR was considered statistically significant, when there was a twofold or greater difference in expression between the control and CMA groups [19]. Microarray data were analyzed by BeadStudio Gene Expression Module 3.2.3 (Illumina). The Illumina's Cubic-Spline normalization algorithm was applied to adjust sample signals to minimize the effects of variation arising from non-biological factors. Comparisons between samples from control and CMA rats were done with the *t*-test differential expression algorithm. Gene ontology (GO) analysis for characterizing the biological functions of gene products was performed according to the GO Consortium's instruction at the GO website (<http://www.geneontology.org>). GO search engines used in the present study were AmiGO (<http://amigo.geneontology.org>) and Ensembl (<http://www.ensembl.org>). Description of the GO category names as well as corresponding GO identifiers are available at the GO download page (<http://www.geneontology.org/GO.downloads.shtml>). Gene symbol and full name of gene can be searched from the Rat Genome Database (<http://rgd.mcw.edu>). The qRT-PCR results compared by using $2^{-\Delta\Delta C_T}$ method [20] are expressed as log₂ means ± SE. All graphics were obtained using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Results

Oral administration of 1.5% NH₄Cl for 21 days resulted in sustained CMA

CMA was induced by continuous acid loading with 1.5% NH₄Cl given ad libitum in drinking water [4, 10, 15]. Plasma pH of CMA rats was significantly lower than that of control rats, i.e., 7.27 ± 0.02 ($n = 4$) vs. 7.37 ± 0.04 ($n = 6$, $P < 0.05$), respectively, with a decrease in plasma HCO₃⁻ concentration. Partial pressure of O₂ was not altered. Daily water intake was more than 60 ml/day, suggesting that the CMA rats consumed sufficient amount of 1.5% NH₄Cl-containing water to induce sustained acidemia throughout the experimental period. The present blood gas profile agreed with typical metabolic response to CMA as reported previously [4].

Table 1 *Rattus norvegicus* oligonucleotide sequences used in qRT-PCR experiments

Gene	Accession no.	Primer (forward/reverse)	Product length (bp)
<i>Cell adhesion (GO:0007155)</i>			
Cdh13	NM_138889	5'-CCGAAGATTGCCGTTTACAG-3' 5'-AGGAGGAAGGAAGATGCTGTC-3'	124
Ceacam10	NM_173339	5'-TCTCCTTTGATTGCTCTTCCT-3' 5'-CTCTGGGGTTCACGAGTG-3'	75
<i>Cellular component organization and biogenesis (GO:0016043)</i>			
Kif13B	NM_213626	5'-CAGCAATGACCCAATGCA-3' 5'-TCCACACCTCGACAATTCTG-3'	159
Syngri1	NM_019166	5'-TCGGCTCCATTGTGAATGAG-3' 5'-CTTCTTGCGGTCCTTGACA-3'	192
<i>Cellular developmental process (GO:0048869)</i>			
Epim	NM_012748	5'-TCCGTAGATTACGTGGAGCA-3' 5'-AGGACAGCAATGACAGCCA-3'	119
<i>Cellular metabolic process (GO:0044237)</i>			
Cpz	NM_031766	5'-GGATGCGGCCTATAACCAT-3' 5'-TCCAGGAGAAAAGTGCAGCA-3'	114
Sardh	NM_053664	5'-TTGCTAGGAATCTCCCAGGA-3' 5'-TGGAACACTCTTCTCAGTGGTG-3'	194
<i>Metabolism (GO:0008152)</i>			
Pon1	NM_032077	5'-TTGAATGAGAAGGAGCCAGC-3' 5'-TAAACACCTCCACGGTGGA-3'	166
<i>Regulation of biological process (GO:0050789)</i>			
Gkn1	NM_198972	5'-GACAGCAATGTAGACGGAAGT-3' 5'-TTTTTCATAGTCCCAGAGGC-3'	119
Inhba	NM_017128	5'-GAATGAACTCATGGAGCAGACC-3' 5'-TGCTGAAACAGACGGATGGT-3'	195
Syn2	NM_019159	5'-GTAATGGCATTGCAGTAGGTCC-3' 5'-AACATGCCGTCAGCACTGAG-3'	181
<i>Transport (GO:0006810)</i>			
Cacnb1	NM_017346	5'-AGGACTTCCTGCACATCAAGG-3' 5'-TTGTACCTGACTTGCTGGAG-3'	175
Clnkb	NM_173103	5'-CTCCACATTACCTCTCCC-3' 5'-GCAGCAGACAACCCTAGAAG-3'	104
Slc22a17	NM_177421	5'-GTCGTGGGATTGTACTGCTGA-3' 5'-CATCAGGTAGACACCAAGGTCA-3'	146
<i>Paracellular protein</i>			
Claudin-8	NM_001037774	5'-GCTGGAATCATCTTCTTCAT-3' 5'-CATCCACCAGCGGGTTGTAG-3'	100
<i>Housekeeping gene</i>			
GAPDH	NM_017008	5'-AGTCTACTGGCGTCTTCAC-3' 5'-TCATATTTCTCGTGGTTCAC-3'	133

GAPDH glyceraldehyde-3-phosphate dehydrogenase; full names of other genes are presented in Tables 2, 3, or Supplemental Table S1. Gene ontology (GO) identifiers are presented in parentheses

CMA led to genetic responses in the duodenal epithelial cells

After a long-term exposure to plasma acidic pH, the duodenal epithelial cells increased expression of 423 genes and

decreased expression of 261 genes greater than twofold. As depicted in Fig. 1, 88.42% (374 genes) and 85.06% (222 genes) of the CMA-altered transcripts were upregulated and downregulated in the range of 2- to 10-fold, respectively. Transcripts with the expression levels changed by

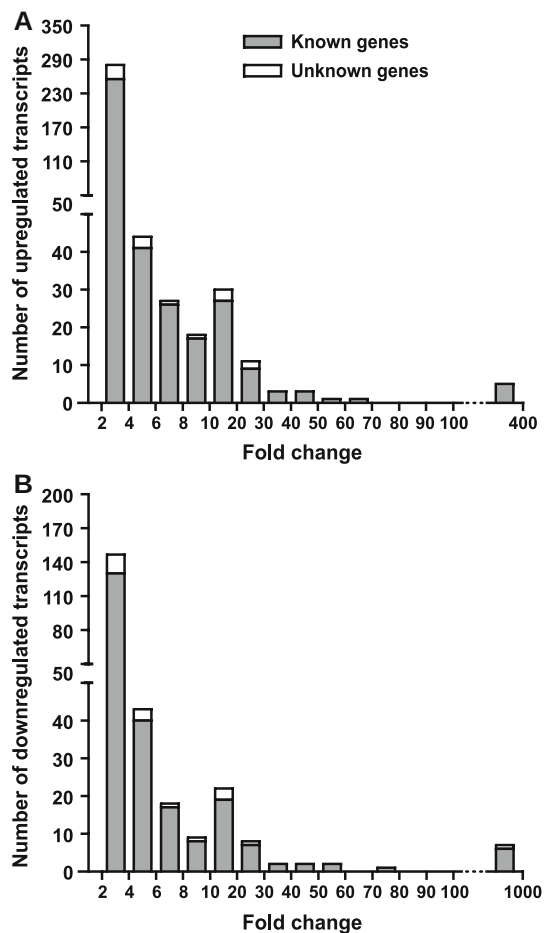


Fig. 1 Distribution of fold changes of upregulated (a) and downregulated transcripts (b) in the duodenal epithelial cells of CMA rats as determined by microarray. This data set includes transcripts with expression levels changed by greater than twofold. The complete lists of CMA-altered transcripts are presented in Supplemental Tables S1 and S2

greater than 10-fold are shown in Tables 2 and 3, while the complete lists of the CMA-altered transcripts are shown in Supplemental Tables S1 and S2. CMA markedly upregulated five known genes by greater than 100-fold, i.e., chloride channel Kb (Clcnkb), voltage-gated potassium channel β_2 (Kcnab2), prolactin-like protein D (Prld), calsyntenin 2 (Clstn2), and carcinoembryonic antigen (CEA)-related cell adhesion molecule 10 (Ceacam10), while downregulated six known genes by greater than 100-fold, i.e., L-kynurenine hydrolase (Kynu), apolipoprotein A-II (Apoa2), urinary protein 2 (Rup2), calgranulin B (S100a9), microfibrillar-associated protein 4 (Mfap4), and kainate glutamate receptor 3 (Grik3).

Approximately 90% of the CMA-altered transcripts are genes of “known” biological functions (Fig. 1) which have been listed on both Ensembl and NCBI databases. However, the majority of these known genes are of unknown

functions in the duodenal epithelial cells. GO analysis indicated that CMA altered several functions of the duodenal epithelial cells, such as cell adhesion, cell communication, and ion transport (Fig. 2a, Table 4). As shown in Fig. 2b, c, most CMA-upregulated genes were involved in molecular function (22.45%), cellular metabolic process (16.33%), and regulation of biological process (12.24%), while most downregulated genes were related to molecular function (30.77%), cell communication (15.38%), regulation of biological process (15.38%), and cellular component (12.82%).

Since previous studies demonstrated that acidosis predominantly affected the transport functions of the intestinal epithelium [4, 5, 7, 11, 21], we further performed GO analysis on genes in the transport category (GO identifier 0006810). Interestingly, there were 24 transporter genes upregulated (~6% of 423 CMA-upregulated genes) and 12 genes downregulated (~5% of 261 CMA-downregulated genes) after exposure to 21-day acidemia (Fig. 3a, b). Those genes were involved in the transport of several solute species, i.e., Na^+ , K^+ , Ca^{2+} , Cu^{2+} , Zn^{2+} , H^+ , Cl^- , I^- , phosphate, organic cations, organic anions, amino acids, and glucose (Tables 5, 6). Eight transcripts in the transport category have been known to encode K^+ channels (6 genes upregulated and 2 genes downregulated). Seventeen transcripts are members of the solute carrier (Slc) family. The fold differences in the expression levels of most transporter genes were in the range of 2- to 10-fold.

In addition to the GO analysis, we also reviewed the functions of all CMA-altered transcripts based upon the information available on the PubMed (<http://www.pubmed.gov>) and found that there were a number of CMA-altered transcripts known to be directly related to the intestinal ion transport or total body fluid homeostasis. Those transcripts included arginine vasopressin (Avp), natriuretic peptide receptor 2 (Npr2), angiotensin II receptor type 1 (Agtr1a), neuropeptide Y receptor Y1, Y2, and Y5 (Npy1r, Npy2r, and Npy5r), stanniocalcin 1 (Stc1), gastrin (Gast), cholecystokinin B receptor (Cckbr), somatostatin receptor 5 (Sstr5), and kallikrein 7 (klk7) [22–27]. We also found three transcripts of aquaporins which are water channels, i.e., Aqp1, Aqp4, and Aqp5, upregulated by 2.076-, 2.107-, and 2.259-fold, respectively, and Aqp6 downregulated by 2.079-fold.

Finally, qRT-PCR was used to verify changes in gene expression as demonstrated by microarray. We selected genes on the basis of their biological functions, i.e., 1–3 genes per GO category, to be tested by qRT-PCR (Table 1). Since CMA did not alter GAPDH mRNA expression (Fig. 4a), gene expression levels were first normalized by GAPDH expression. As demonstrated in Fig. 4b, the qRT-PCR results of 14 studied transcripts were all consistent with the microarray results in response to

Table 2 Known genes upregulated in the duodenal epithelial cells of CMA rats

Gene name	Gene symbol	Accession no.	Fold change
Chloride channel Kb	Clcnkb	NM_173103	378.960
Potassium voltage-gated channel, shaker-related subfamily, beta member 2	Kcnab2	NM_017304	244.220
Prolactin-like protein D	Prldp	NM_022537	223.402
Calsyntenin 2	Clstn2	NM_134377	204.541
CEA-related cell adhesion molecule 10	Ceacam10	NM_173339	107.481
Fatty acid binding protein 3	Fabp3	NM_024162	63.198
Epimorphin	Epim	NM_012748	51.529
Membrane-associated DHHC26 zinc finger protein	LOC300323	NM_001039333	49.350
Paraoxonase 1	Pon1	NM_032077	43.874
Synapsin II, transcript variant 2	Syn2	NM_019159	41.228
Inhibin beta-A	Inhba	NM_017128	32.610
FSH primary response 1	Fshprh1	NM_012955	31.003
Kinesin 13B	kif13B	NM_213626	30.842
Sex hormone binding globulin	Shbg	NM_012650	29.623
Toll-like receptor 4	Tlr4	NM_019178	26.338
Sarcosine dehydrogenase	Sardh	NM_053664	26.270
Guanine nucleotide binding protein, alpha 12	Gna12	NM_031034	23.751
Mitochondrial hepatocellular carcinoma-downregulated carrier protein	Hdmcp	NM_001001509	22.826
Synaptogyrin 1	Syng1	NM_019166	22.665
C1q and tumor necrosis factor related protein 5	C1qtnf5	NM_001012123	22.055
Gastrokine 1	Gkn1	NM_198972	21.347
NHL repeat containing 1	Nhlrc1	NM_199236	20.161
Ly6-C antigen	Ly6c	NM_020103	19.693
Sterol O-acyltransferase 1	Soat1	NM_031118	19.360
Regulating synaptic membrane exocytosis 2, transcript variant 1	Rims2	NM_053945	18.322
Unc-5 homolog A (<i>C. elegans</i>)	Unc5a	NM_022206	17.632
Dynein, axonemal, light intermediate polypeptide 1	Dnali1	NM_001031647	16.200
GTP binding protein 3	Gtpbp3	NM_001011919	15.966
Calcium channel, voltage-dependent, beta 1 subunit	Cacnb1	NM_017346	15.544
Endothelial cell-specific molecule 1	Esm1	NM_022604	15.350
Synaptotagmin-like 1	Syt11	NM_001025651	13.993
Tumor protein D52-like 2	Tpd52l2	NM_198744	13.805
Corticotropin releasing hormone receptor 1	Crhr1	NM_030999	13.489
ELAV (embryonic lethal, abnormal vision, <i>Drosophila</i>)-like 3 (Hu antigen C)	Elavl3	NM_172324	13.362
Arginine vasopressin	Avp	NM_016992	12.942
Solute carrier family 22 (organic cation transporter), member 17	Slc22a17	NM_177421	12.648
Cadherin 13	Cdh13	NM_138889	12.238
Complement component factor H	Cfh	NM_130409	12.142
Pregnancy upregulated non-ubiquitously expressed CaM kinase	Pnck	NM_017275	11.612
Fc receptor, IgE, high affinity I, alpha polypeptide	Fcer1a	NM_012724	11.329
Guanylate cyclase 1, soluble, beta 3	Gucylb3	NM_012769	11.250
Phosphatidylserine-specific phospholipase A1	Pspla1	NM_138882	11.221
Zona pellucida glycoprotein 4	Zp4	NM_172330	11.106
Natriuretic peptide receptor 2	Npr2	NM_053838	11.076
Trefoil factor 1	Tff1	NM_057129	11.066
Mitogen-activated protein kinase 15	Mapk15	NM_173331	10.955
Retinoic acid early transcript 1L	Raet1l	NM_001013063	10.527
Chemokine (C-X-C motif) receptor 4	Cxcr4	NM_022205	10.198
ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1, 3)-N-acetylglactosaminide alpha-2,6-sialyltransferase 3	St6galnac3	NM_019123	10.131

Results are presented as fold difference in signal intensity. This data set includes mRNAs whose levels changed by greater than 10-fold in response to CMA. The complete list of upregulated transcripts is presented as supplemental data (Table S1)

Table 3 Known genes downregulated in the duodenal epithelial cells of CMA rats

Gene name	Gene symbol	Accession no.	Fold change
Kynureninase (L-kynurenine hydrolase)	Kynu	NM_053902	−901.355
Apolipoprotein A-II	Apoa2	NM_013112	−745.193
Urinary protein 2	Rup2	NM_001034950	−245.631
S100 calcium binding protein A9 (calgranulin B)	S100a9	NM_053587	−205.159
Microfibrillar-associated protein 4	Mfap4	NM_001034124	−145.392
Glutamate receptor, ionotropic, kainate 3	Grik3	NM_181373	−124.661
Mast cell protease 8	Mcpt8	NM_021598	−77.942
Kringle containing transmembrane protein 1	Kremen1	NM_053649	−56.059
Trace-amine-associated receptor 6	Taar6	NM_175584	−50.427
Dyslexia susceptibility 1 candidate 1 homolog (human)	Dyx1c1	NM_001007010	−45.170
Pyrimidinergic receptor P2Y, G-protein coupled, 6	P2ry6	NM_057124	−43.521
Phosphate cytidylyltransferase 1, choline, beta isoform	Pcyt1b	NM_173151	−37.309
Vomerolnasal 1 receptor, G6	Vlrg6	NM_001008957	−31.762
Connector enhancer of kinase suppressor of Ras 2	Cnksr2	NM_021686	−28.351
Tumor necrosis factor receptor superfamily, member 8	Tnfrsf8	NM_019135	−26.822
T-complex protein 11 (mouse)	Tcp11	NM_001007695	−26.240
Drebrin 1	Dbn1	NM_031024	−26.023
GATA binding protein 3	Gata3	NM_133293	−22.270
Ataxin 1	Atxn1	NM_012726	−20.662
Olfactory receptor 1687	Olr1687	NM_001000508	−20.230
Prostaglandin D receptor	Ptgdr	NM_022241	−19.862
Neurogenic differentiation 3	Neurod3	NM_019207	−19.665
Solute carrier family 9, member 4	Slc9a4	NM_173098	−18.241
Taste receptor, type 2, member 10	Tas2r10	NM_023995	−16.209
BMP/retinoic acid-inducible neural-specific protein 2	Brinp2	NM_173115	−15.652
Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	Gcnt2	NM_001001511	−15.366
Myosin, heavy polypeptide 3, skeletal muscle, embryonic	Myh3	NM_012604	−14.843
Ubiquitin-like, containing PHD and RING finger domains, 1 (mapped)	Uhrf1_mapped	NM_001008882	−14.770
Retina and anterior neural fold homeobox	Rax	NM_053678	−13.712
Prolactin-like protein 1	Prloi	NM_031316	−13.607
FXFD domain-containing ion transport regulator 7	Fxyd7	NM_022008	−13.150
PR-Vbeta1	VCS-beta1	NM_001017497	−12.924
Ly49 inhibitory receptor 5	Ly49i5	NM_001009501	−11.943
Surfactant associated protein D	Sftpd	NM_012878	−11.905
GTPase activating protein testicular GAP1	tGap1	NM_001007635	−11.780
RT1 class I, M1, gene 2	RT1-M1-2	NM_001008849	−11.625
Aldehyde dehydrogenase family 6, subfamily A1	Aldh6a1	NM_031057	−10.890
Prolactin-like protein-F beta	LOC306929	NM_001008341	−10.228
Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 4	Gabra4	NM_080587	−10.221

Results are presented as fold difference in signal intensity. This data set includes mRNAs whose levels changed by greater than 10-fold in response to CMA. The complete list of downregulated transcripts is presented as supplemental data (Table S2)

21-day CMA. The amplicon sequencing confirmed our qRT-PCR results. In addition, we also investigated the expression of claudin-8, a gene coding for a tight junction protein which has been reported to regulate epithelial permeability and transepithelial ion transport across the

paracellular space [28, 29]. The qRT-PCR result showed that CMA upregulated claudin-8 expression by 16.035-fold (Fig. 4b), thus agreeing with our previous report using the semi-quantitative PCR on the duodenal absorptive cells of CMA rats [15].

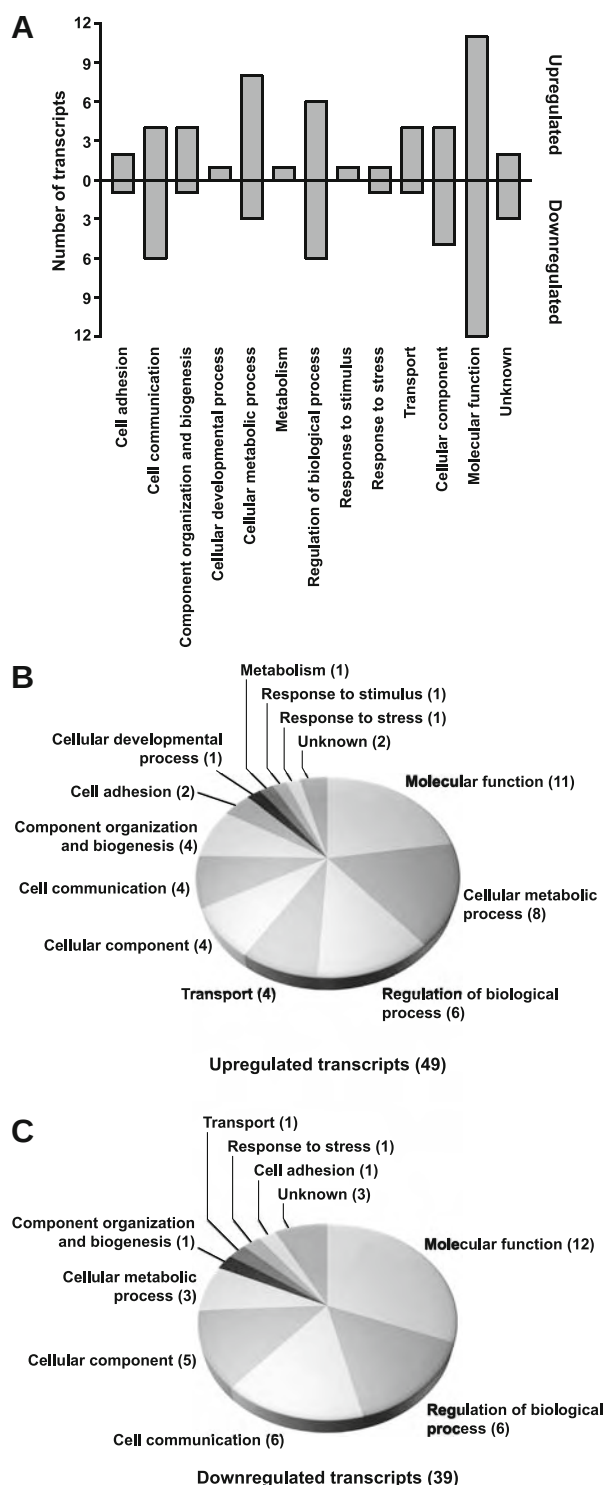


Fig. 2 **a** Distribution of genes in each gene ontology category. Columns above the *abscissa* represent transcripts that were upregulated by CMA, whereas columns below the *abscissa* represent downregulated transcripts. **b** A pie chart shows proportional distribution of upregulated genes in each gene ontology category. **c** A pie chart shows proportional distribution of downregulated genes in each category. Numbers in parentheses represent the number of transcripts. This data set includes transcripts with expression levels changed by greater than 10-fold

Discussion

CMA causes a number of metabolic disturbances to several organs, e.g., heart, kidney, and bone. In response to these disturbances, the body produces compensatory mechanisms to restore homeostasis, such as hyperventilation to get rid of CO_2 , renal elimination of acid equivalents, and liberation of phosphate from bone [30]. Intestinal epithelial cells also exhibit compensatory response to CMA, for example, by changing the rates of ion transport, enhancing glutamine absorption, and increasing paracellular permeability [2–4, 11]. Similar to other tissues, the intestinal cell responses to CMA usually require a long-term exposure to acidemia and are therefore postulated to occur at the genomic level [4]. In the present study, we identified 684 duodenal genes with their expression levels changed after 21 days of CMA. We also showed a good quantitative correlation between the fold changes determined by microarray and qRT-PCR. Although the majority of the CMA-altered genes could be categorized by GO search engines, the exact functions of these genes in the duodenal epithelium are still not known.

Nutrient absorption and regulation of fluid and electrolyte homeostasis are the hallmark of intestinal functions. Previous *in vivo* and *in vitro* investigations demonstrated that CMA could modulate the transepithelial transport of several ions, especially Na^+ , K^+ , Ca^{2+} , and phosphate [4, 9, 10, 31]. Feldman and Charney [9] showed an increase in the serosa-to-mucosa K^+ transport, which is normally dependent on the expression of K^+ channels on the apical membrane [32], in the jejunal and ileal epithelia of acidotic rats. Thus, the present report on the CMA-altered expression of K^+ channels or carriers, e.g., *Kcnab2* and *Kcne2*, agreed with the results from *in vivo* studies. The *Kcnab2* and *Kcne2* encoding voltage-gated K^+ channels may also play an important role in the regulation of membrane potential, which is important for the Na^+ -dependent absorption of glucose, amino acids, and long-chain fatty acids in the small intestine [33]. Upregulation of the expression of Na^+ transporters was consistent with the previous finding that CMA could alter Na^+/K^+ -ATPase mRNA expression and also explained the CMA-modulated intestinal sodium absorption *in vivo* [9]. Moreover, CMA changed the expression of the voltage-dependent Ca^{2+} channel (Ca_v) subunits, e.g., *Cacnb1*. Since Ca_v activity was strongly correlated with the transepithelial Ca^{2+} flux and the L-type Ca_v blockers, such as nifedipine and verapamil, could block the intestinal Ca^{2+} absorption [34, 35], an increase in the Ca_v expression or change in Ca_v isoform expression could explain the CMA-enhanced transepithelial Ca^{2+} transport. Furthermore, the CMA-stimulated Ca^{2+} absorption could be partially explained by

Table 4 Identification of CMA-altered transcripts in each gene ontology category

Upregulated transcripts		Downregulated transcripts	
<i>GO:000815 Biological process</i>		<i>GO:000815 Biological process</i>	
<i>GO:0007155 Cell adhesion</i>		<i>GO:0007155 Cell adhesion</i>	
Ceacam10	(107.481)	Mfap4	(−145.392)
Cdh13	(12.238)		
<i>GO:0007154 Cell communication</i>		<i>GO:0007154 Cell communication</i>	
Clstn2	(204.541)	Grik3	(−124.661)
Fshprh1	(31.003)	Kremen1	(−56.059)
Gna12	(23.751)	Taar6	(−50.427)
Crhr1	(13.489)	Cnksr2	(−28.351)
		Olrl687	(−20.230)
		Gabra4	(−10.221)
<i>GO:0016043 Cellular component organization and biogenesis</i>		<i>GO:0016043 Cellular component organization and biogenesis</i>	
Kif13B	(30.842)	Dbn1	(−26.023)
Hdmcp	(22.826)		
Syng1	(22.665)		
Rims2	(18.322)		
<i>GO:0048869 Cellular developmental process</i>		<i>GO:0048869 Cellular developmental process</i>	
Epim	(51.529)		
<i>GO:0044237 Cellular metabolic process</i>		<i>GO:0044237 Cellular metabolic process</i>	
<i>Lipid metabolism</i>		<i>Lipid metabolism</i>	
Fabp3	(63.198)	Apoa2	(−745.193)
Soat1	(19.360)	Pcyt1b	(−37.309)
Pspla1	(11.221)		
<i>Nucleic acid metabolism</i>		<i>Amine metabolism</i>	
Gtpbp3	(15.966)	Kynu	(−901.355)
Npr2	(11.076)		
<i>Protein metabolism</i>			
Sardh	(26.270)		
Pnck	(11.612)		
Mapk15	(10.955)		
<i>GO:0008152 Metabolism</i>			
Pon1	(43.874)		
<i>GO:0050789 Regulation of biological process</i>		<i>GO:0050789 Regulation of biological process</i>	
Syn2	(41.228)	S100a9	(−205.159)
Inhba	(32.610)	Tnfrsf8	(−26.822)
Gkn1	(21.347)	Atxn1	(−20.662)
Nhlrc1	(20.161)	Neurod3	(−19.665)
Esm1	(15.350)	Brinp2	(−15.652)
Cfh	(12.142)	Fxyd7	(−13.150)
<i>GO:0050896 Response to stimulus</i>			
Tff1	(11.066)		
<i>GO:0006950 Response to stress</i>		<i>GO:0006950 Response to stress</i>	
Tlr4	(26.338)	Sftpd	(−11.905)
<i>GO:0005575 Cellular component</i>		<i>GO:0005575 Cellular component</i>	
C1qtnf5	(22.055)	Mcpt8	(−77.942)
Dnali1	(16.200)	Vlrg6	(−31.762)
Zp4	(11.106)	Myh3	(−14.843)
Raet11	(10.527)	VCS-beta1	(−12.924)

Table 4 continued

Upregulated transcripts		Downregulated transcripts	
<i>GO:0003674 Molecular function</i>		<i>GO:0003674 Molecular function</i>	
Catalytic activity		tGap1	(−11.780)
Gucy1b3	(11.250)	Dyx1c1	(−45.170)
St6galnac3	(10.131)	Gcnt2	(−15.366)
Chemokine receptor activity		Aldh6a1	(−10.890)
Cxcr4	(10.198)	Hormone activity	
Hormone activity		Pr1pi	(−13.607)
Pr1pd	(223.402)	LOC306929	(−10.228)
Avp	(12.942)	Receptor activity	
Immunoglobulin binding		P2ry6	(−43.521)
Fcer1a	(11.329)	Tcp11	(−26.240)
Nucleic acid binding		Ptldr	(−19.862)
Elavl3	(13.362)	Tas2r10	(−16.209)
Protein binding		Ly49i5	(−11.943)
Shbg	(29.623)	Transcription factor activity	
Sytl1	(13.993)	Gata3	(−22.270)
Tpd52l2	(13.805)	Rax	(−13.712)
Receptor activity			
Unc5a	(17.632)		
<i>Unknown</i>		<i>Unknown</i>	
LOC300323	(43.350)	Rup2	(−245.631)
Ly6c	(19.693)	Uhrf1_mapped	(−14.770)
		RT1-M1-2	(−11.625)

Numbers in parentheses represent fold changes of the corresponding transcripts. Each category is accompanied by a unique GO identifier supplied by the GO search engines. In each category, transcripts are listed in order of fold change. This data set includes mRNAs with the expression levels changed by greater than 10-fold in response to CMA

the upregulation of Pth1r and downregulation of Calca genes coding for parathyroid hormone (PTH) receptor 1 and a hypocalcemic hormone calcitonin/calcitonin-related polypeptide α , respectively, because both PTH and calcitonin have been reported to directly modulate Ca^{2+} transport in the small intestine [36, 37]. It is possible that the CMA-induced change in the expression of Ca^{2+} transporters and stimulation of Ca^{2+} absorption are parts of a compensatory response to mitigate negative calcium balance during CMA.

CMA also modulates the intestinal phosphate absorption, but the findings are still controversial. Borowitz et al. showed by using in vivo recirculating perfusion technique that acidemia decreased Na^{+} -dependent phosphate transport in the rat jejunum [31], whereas Stauber et al. reported the CMA-stimulated phosphate uptake in the brush border membrane vesicles prepared from the whole small intestine as well as an increase in the Na^{+}/P_i -cotransporter IIb (Slc34a2) protein expression in the ileum, but not in the jejunum [10]. Here, we found a

downregulation of the Na^{+}/P_i -cotransporter Slc17a7 after 21-day CMA, implicating a decrease in phosphate transport in the duodenum. Although the exact explanation for this discrepancy is currently not known, we speculate that the segmental heterogeneity of the intestinal phosphate absorption and transporter localization could be the main determinants of intestinal response to CMA. In addition, CMA altered expression of transporters for Cu^{2+} , Zn^{2+} , H^{+} , Cl^{-} , I^{-} , organic cations, and organic anions; however, effects of CMA on the transport of these ions remain to be elucidated.

Regarding the transporters that contribute to the regulation of extracellular or intracellular pH, although some investigators previously demonstrated changes in the intestinal HCO_3^{-} absorption and NHE activity by acidosis [6, 38], we did not observe any change in the expression of either HCO_3^{-} transporters (usually members of the solute carrier family 4 or Slc4) or NHE 2 and 3 (Slc9 members) greater than twofold. It was possible that mild acidemia ($\sim \text{pH}$ 7.3) as in the present study, unlike

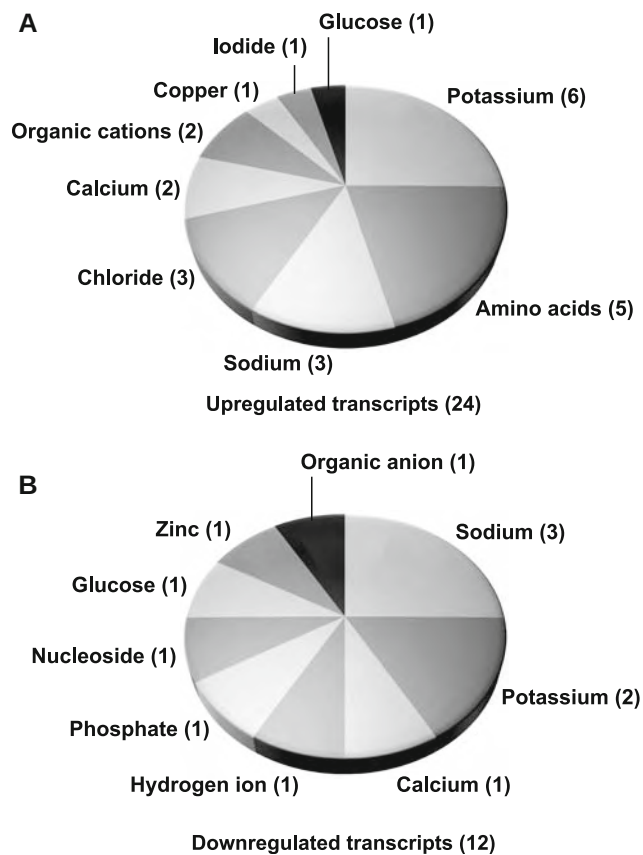


Fig. 3 Pie charts show proportional distributions of upregulated (a) and downregulated (b) transcripts in the transport category (GO:0006810). The transcripts were functionally classified according to the transported ions, nutrients, or substances. Numbers in parentheses represent the number of transcripts. This data set includes transcripts with expression levels changed by greater than twofold

profound acidemia (pH 7.1–7.2) in previous studies [10], was unable to alter expression of those transporters. Alternately, such changes in the expression, if present, may only occur during the early acidotic period, since the previous reports on the CMA-modulated HCO_3^- absorption and NHE activity were evident in less than 10 days [6, 38]. Nevertheless, the present 21-day CMA showed tendency to increase expression of the Na^+ -coupled HCO_3^- transporter Slc4a10 by 1.557-fold while significantly downregulated mRNA expression of NHE4 (Slc9a4) by 18.241-fold. We speculate that changes in the expression of NHE and HCO_3^- transporter expression may reduce severity of the intracellular acidosis and help replenish the decreased plasma HCO_3^- concentration during CMA, respectively.

Interestingly, we found upregulation of two neutral amino acid transporters, i.e., the system N transporter Slc38a5 and the Na^+ -dependent transporter Slc1a7, both of which are capable of transporting L-glutamine [39, 40].

Glutamine is the most abundant amino acid in the body and plays a crucial role in inter-organ nitrogen transfer during CMA-induced hypercatabolic states, such as sepsis, shock, and diabetes mellitus [1, 11, 41]. Glutamine is also a major source of energy for intestinal epithelial cells and helps maintain the epithelial integrity [42, 43]. Several investigations demonstrated that CMA significantly stimulated intestinal glutamine absorption [3, 11], thus agreeing with our microarray results. In addition to glutamine, CMA upregulated mRNA expression of Slc6a13 and Slc6a15, both of which are members of the Na^+ -dependent neutral amino acid transporter B⁰AT2 system [44, 45]. As a result, the CMA-induced increases in the intestinal absorption of leucine, isoleucine, valine, proline, and methionine are anticipated [44, 45]. Furthermore, CMA may modulate intestinal glucose metabolism or transport, since upregulation of Slc2a1, known as the facilitated glucose transporter 1 (GLUT1), was demonstrated.

CMA also altered expression of other duodenal genes known to regulate water and electrolyte homeostasis, e.g., aquaporins (Aqp1, Aqp4, Aqp5, and Aqp6), arginine vasopressin (Avp), natriuretic peptide receptor (Npr2), angiotensin II receptor (Agtr1a), neuropeptide Y receptors (Npy1r, Npy2r and Npy5r), stanniocalcin 1 (Stc1), gastrin (Gast), cholecystokinin B receptor (Cckbr), somatostatin receptor 5 (Sstr5), and kallikrein 7 (klk7) [22–27, 46]. Aquaporins are widely expressed along the entire length of the small intestine [46] and are likely to play a role in the transcellular water absorption [47]. Our microarray results on the CMA-regulated aquaporin expression were consistent with the previous report that an acidotic arterial pH markedly enhanced water absorption in the rat ileum [8]. Since vasopressin expression was upregulated and vasopressin was known to inhibit electrogenic Na^+ absorption and stimulate electrogenic K^+ and Cl^- secretion in the distal colon of guinea pig [48], it is possible that CMA-induced vasopressin expression could directly regulate duodenal ion transport in an autocrine or paracrine manner similar to that seen in the rat choroid plexus epithelium [49]. The roles of gastrin–cholecystokinin–somatostatin, neuropeptide Y, and kallikrein–kinin systems in the regulation of intestinal ion absorption and secretion, despite having been reported in other conditions, have not been investigated in CMA.

In addition to the transcellular transport, CMA has been known to increase paracellular permeability of both intact intestinal tissue and Caco-2 intestinal-like monolayer to small charged and neutral hydrophilic molecules [2]. The intestinal permeability as well as the integrity are maintained, in part, by cell proliferation and cell differentiation. A number of CMA-regulated genes, such as Gkn1, Inhba, trefoil factors (Tff1 and Tff2), chemokines (Cxcr and Ccl), interleukins (Il1 and Il6), Bcl2, Insr, and insulin-like

Table 5 CMA-upregulated transcripts in the transport category (GO:0006810)

Gene name	Gene symbol	Accession no.	Fold change
<i>Calcium transport</i>			
Calcium channel, voltage-dependent, beta 1 subunit	Cacnb1	NM_017346	15.544
Calcium channel, voltage-dependent, gamma subunit 8	Cacng8	NM_080696	5.180
<i>Sodium transport</i>			
Sodium channel, voltage-gated, type II, beta	Scn2b	NM_012877	4.737
Sodium channel, voltage-gated, type I, alpha	Scn1a	NM_030875	3.567
ATPase, Na ⁺ /K ⁺ transporting, alpha 2 polypeptide	Atp1a2	NM_012505	2.266
<i>Chloride transport</i>			
Chloride channel Kb	Clcnkb	NM_173103	378.960
Chloride intracellular channel 4	Clic4	NM_031818	3.570
Putative chloride channel 4-2	Clcn4-2	NM_022198	2.205
<i>Potassium transport</i>			
Potassium voltage-gated channel, shaker-related subfamily, beta member 2	Kcnab2	NM_017304	244.220
Potassium voltage-gated channel, Isk-related family, member 2	Kcne2	NM_133603	5.360
Potassium inwardly rectifying channel, subfamily J, member 5	Kcnj5	NM_017297	4.931
Potassium inwardly rectifying channel, subfamily J, member 8	Kcnj8	NM_017099	4.814
Solute carrier family 12, member 4	Slc12a4	NM_019229	3.786
Potassium voltage-gated channel, Shaw-related subfamily, member 3	Kcnc3	NM_053997	2.006
<i>Copper transport</i>			
ATPase, Cu ²⁺ transporting, beta polypeptide	Atp7b	NM_012511	2.348
<i>Iodide transport</i>			
Solute carrier family 5 (sodium iodide symporter), member 5	Slc5a5	NM_052983	2.300
<i>Organic cation transport</i>			
Solute carrier family 22 (organic cation transporter), member 17	Slc22a17	NM_177421	12.648
Solute carrier family 22 (organic cation transporter), member 2	Slc22a2	NM_031584	2.161
<i>Glucose transport</i>			
Solute carrier family 2 (facilitated glucose transporter), member 1	Slc2a1	NM_138827	2.007
<i>Amino acid transport</i>			
Solute carrier family 38, member 5	Slc38a5	NM_138854	7.697
Solute carrier family 6 (neurotransmitter transporter), member 15	Slc6a15	NM_172321	3.724
Solute carrier family 1, member 7	Slc1a7	NM_153723	3.514
Solute carrier family 6 (neurotransmitter transporter, GABA), member 13	Slc6a13	NM_133623	3.371
Solute carrier family 1 (glial high affinity glutamate transporter), member 3	Slc1a3	NM_019225	2.237

Results are expressed as fold difference in signal intensity. This data set includes mRNAs with the expression levels changed by greater than twofold in response to CMA

growth factor-related genes (Igfbp3 and Igfbp7), are important for the regulation of cell proliferation and apoptosis [50–55]. The product of Gkn1 gene, i.e., antrum mucosal protein-18 (also known as gastrophilin 1), is a potent mitogen which can stimulate proliferation of the intestinal epithelial cells [51]. On the other hand, trefoil factors can prevent cell death. It was evident that Tff1 exerted an antiapoptotic effect on the intestinal epithelial cells, perhaps in an autocrine or paracrine manner [52]. CMA may also retain epithelial integrity by increasing the expression of a morphogenic protein epimorphin (Epim), which has been reported to enhance survival of the

intestinal epithelial cells [56], or by modulating cell adhesion through alteration of adhesion molecule gene expression, e.g., Ceacam10 and cadherin (Cdh13 and Cdh16). Recently, we demonstrated that 21-day CMA upregulated several tight junction-related genes, including claudins which are known to regulate cell adhesion, epithelial permeability, and paracellular ion transport [15]. In the present study, by using qRT-PCR we confirmed that CMA significantly increased claudin-8 mRNA expression by ~16-fold.

Besides the absorptive function, the intestine plays important roles in biotransformation and elimination of

Table 6 CMA-downregulated transcripts in the transport category (GO:0006810)

Gene name	Gene symbol	Accession no.	Fold change
<i>Calcium transport</i>			
Transient receptor potential cation channel, subfamily C, member 4, transcript variant 1	Trpc4	NM_053434	−3.505
<i>Phosphate transport</i>			
Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	Slc17a7	NM_053859	−2.822
<i>Sodium transport</i>			
Solute carrier family 9, member 4	Slc9a4	NM_173098	−18.241
ATPase, Na ⁺ /K ⁺ transporting, alpha 3 polypeptide	Atp1a3	NM_012506	−8.939
Solute carrier family 12, member 1	Slc12a1	NM_019134	−2.730
<i>Potassium transport</i>			
Potassium voltage-gated channel, subfamily H (eag-related), member 1	Kcnh1	NM_031742	−2.994
Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	Kcna1	NM_031828	−2.472
<i>Hydrogen ion transport</i>			
ATPase, H ⁺ transporting, V1 subunit G isoform 2	Atp6v1g2	NM_212490	−2.231
<i>Organic anion transport</i>			
Solute carrier organic anion transporter family, member 6c1	Slco6c1	NM_173338	−2.097
<i>Zinc transport</i>			
Solute carrier family 30 (zinc transporter), member 3	Slc30a3	NM_001013243	−4.445
<i>Glucose transport</i>			
Solute carrier family 2 (facilitated glucose transporter), member 4	Slc2a4	NM_012751	−2.213
<i>Nucleoside transport</i>			
Solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	Slc28a3	NM_080908	−2.045

Results are expressed as fold difference in signal intensity. This data set includes mRNAs with the expression levels changed by greater than twofold in response to CMA

toxic metabolites. Since CMA can interfere with ATP production and induce mild injury through nitric oxide and interleukins [16], CMA is usually associated with high-circulating toxic substances generated during metabolic disturbance of the body. It is likely that toxic metabolites from anaerobic stress are released from the intestinal tissues as well. Expressions of 6-phosphofructo-2-kinase (Pfkfb) 1 and 2, the glycolytic enzymes, and interleukins (Il1a and Il6) were changed in this microarray study. Therefore, CMA is likely to alter expression of genes related to detoxification and biotransformation in the intestinal epithelial cells. We found that there were changes in the expression of phase I biotransformation genes coding heme-containing monooxygenases of the cytochrome P450 (Cyp) family, such as Cyp2b3, Cyp2c13, Cyp2f2, Cyp3a11, Cyp11a1, Cyp20a1, and Cyp26b1 (Supplemental Tables S1 and S2), and aldehyde oxidase (Aox1), all of which can metabolize multiple pharmaceutical compounds, toxins, retinoids, or lipid derivatives [57–60]. Uridine 5'-diphospho-glucuronosyltransferase (Udpgr2) upregulated by CMA are essential for glucuronidation (phase II biotransformation) of various chemical agents to increase their water solubility, allowing them to be eliminated via the kidney [58]. Some upregulated genes, e.g., paraoxonase

(Pon1), can act as an antioxidant and may help alleviate oxidative stress which occurs during acidosis [61, 62]. Gkn1, Tff1, and Tff2 may play important roles in the maintenance of mucosal integrity and mediating repair process after exposure to hazardous conditions [51, 52, 63].

In conclusion, our investigation provided evidence, for the first time, by using microarray and qRT-PCR that long-term exposure to acidemia pH 7.27 could lead to genetic responses of the duodenal epithelial cells by altering expression of 684 genes. Although CMA was likely to disturb functions of duodenal cells, e.g., ATP biosynthesis, it also induced compensatory adaptations at the genomic level to help alleviate a plethora of multiple-organ metabolic dysregulation and imbalance. For example, the CMA-induced negative calcium and nitrogen balance could be palliated by enhancing intestinal calcium and glutamine absorption, respectively, through the upregulation of the responsible transporters [4, 11]. Thus, the present results are fundamental for further investigations into the mechanism of CMA-induced adaptation or injury of the intestinal epithelial cells as well as into the intestinal responses to long-standing acidemia associated with certain medical conditions, such as renal tubular acidosis, renal failure, and sepsis.

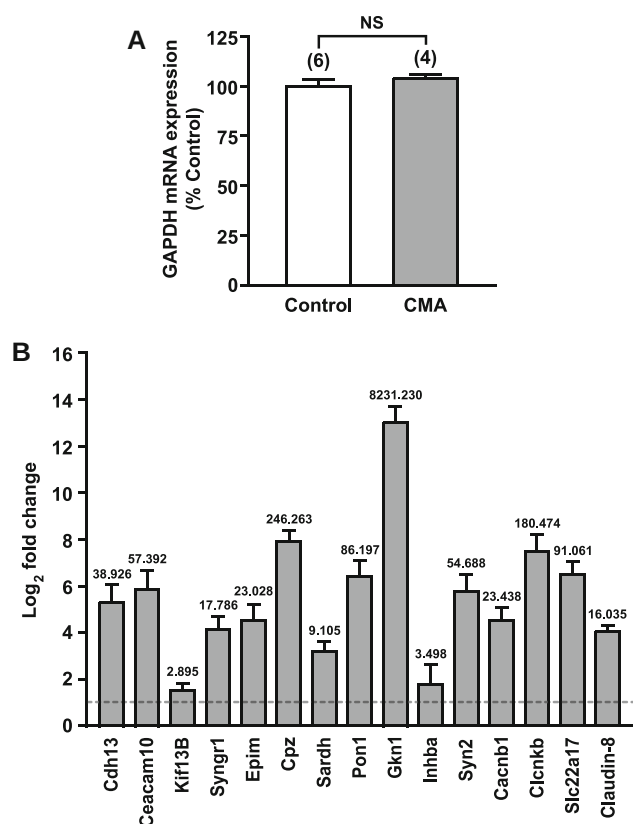


Fig. 4 **a** Expression of GAPDH, the housekeeping gene used for normalization in the qRT-PCR study, in the duodenal epithelial cells of CMA and control rats. Results were obtained from qRT-PCR and expressed as means $\pm$ SE. Numbers in parentheses represent the number of animals. *NS* not significant. **b** Fold changes in the expression levels of claudin-8 and 14 transcripts selected from various GO categories in the duodenal epithelial cells of CMA rats ($n = 4$) versus control rats ($n = 6$), i.e., cell adhesion (Cdh13 and Ceacam10), cellular component organization and biogenesis (Kif13B and Syng1), cellular developmental process (Epim), cellular metabolic process (Cpz and Srdh), metabolism (Pon1), regulation of biological process (Gkn1, Inhba, and Syn2), and transport (Cacnb1, Clcnkb, and Slc22a17). Gene expression was normalized by GAPDH. Twofold upregulation is indicated by the *dashed line*. The fold change of each gene is presented above its respective *bar*. Full names of genes are shown in Tables 2 and 3

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Endurance swimming stimulates transepithelial calcium transport and alters the expression of genes related to calcium absorption in the intestine of rats

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Teerapornpuntakit J, Dorkkam N, Wongdee K, Krishnamra N, Charoenphandhu N. Endurance swimming stimulates transepithelial calcium transport and alters the expression of genes related to calcium absorption in the intestine of rats. *Am J Physiol Endocrinol Metab* 296: E775–E786, 2009. First published January 27, 2009; doi:10.1152/ajpendo.90904.2008.—Endurance impact exercise, e.g., running, is known to enhance the intestinal calcium absorption. However, nonimpact exercise, e.g., swimming, is more appropriate for osteoporotic patients with cardiovascular diseases or disorders of bone and joint, but the effect of swimming on the intestinal calcium transport was unknown. This study, therefore, aimed to investigate the transepithelial calcium transport and the expression of related genes in the intestine of rats trained to swim nonstop 1 h/day, 5 days/wk for 2 wk. We found that endurance swimming stimulated calcium transport in the duodenum, proximal jejunum, and cecum, while decreasing that in the proximal colon. Swimming affected neither the transepithelial potential difference nor resistance. As demonstrated by real-time PCR, the small intestine, especially the duodenum, responded to swimming by upregulating a number of genes related to the transcellular calcium transport, i.e., TRPV5, TRPV6, calbindin-D_{9k}, PMCA_{1b}, and NCX1, and the paracellular calcium transport, i.e., ZO-1, ZO-2, ZO-3, cingulin, occludin, and claudins, as well as nuclear receptor of 1,25(OH)₂D₃. In contrast, swimming downregulated those genes in the colon. Microarray analysis showed that swimming also altered the expression of duodenal genes related to the transport of several ions and nutrients, e.g., Na⁺, K⁺, Cl[−], glucose, and amino acids. In conclusion, endurance swimming enhanced intestinal calcium absorption, in part, by upregulating the calcium transporter genes. The present microarray study also provided relevant information for further investigations into the intestinal nutrient and electrolyte transport during nonimpact exercise.

gene ontology; microarray; nonimpact exercise; real-time polymerase chain reaction; Ussing chamber

ENDURANCE WEIGHT-BEARING or impact exercise with moderate intensity such as running is known to benefit calcium metabolism by increasing bone formation rate, bone mineral density (BMD), and bone strength and by decreasing urinary calcium excretion, thereby inducing positive calcium balance in trained animals (22, 26). These beneficial effects of endurance running are contributed, in part, by the enhanced intestinal calcium absorption (58). However, little is known regarding the mechanism by which exercise affects the intestinal calcium transport.

In contrast to running, swimming is an unloaded nonimpact exercise that is suitable for several groups of patients, such as

cardiovascular, postmenopausal, osteoporotic patients, and patients, who need to prevent high blood pressure or impact that can cause bone and joint injuries (17, 38, 43, 49). Although the effect of swimming on bone metabolism may not be as obvious as seen in running, it appears to increase bone mass, particularly that of the lower extremities in young female swimmers (15). Long-term swimming with moderate intensity is suggested to have a rehabilitative effect on bone in postmenopausal women, since it can increase BMD in estrogen-deficient aging animals (38). Hart et al. (22) also demonstrated a greater bone mass, BMD, and bone strength in ovariectomized swimming rats. However, the effect of endurance swimming on the intestinal calcium absorption has never been reported.

Calcium traverses the intestinal epithelium through the transcellular and paracellular pathways (24). The transcellular calcium transport is a 1,25(OH)₂D₃-dependent three-step process consisting of 1) calcium entry from the luminal side via the transient receptor potential vanilloid family calcium channels (TRPV) 5 and 6, 2) cytoplasmic calcium translocation in a calbindin-D_{9k}-bound form, and 3) basolateral calcium extrusion via the plasma membrane Ca²⁺-ATPase (PMCA_{1b}) and Na⁺/Ca²⁺ exchanger (NCX1; Ref. 24). On the other hand, the paracellular calcium transport is regulated by the tight junction that acts as a size and charge-selective barrier for ion movement (52). It has been shown that some transmembrane tight junction proteins of the claudin family, e.g., claudin-2, -3, and -12, which polymerize to form ion-selective paracellular channels, can regulate the transepithelial calcium transport (8, 18). Interestingly, the expression of claudin-3 was found to be 1,25(OH)₂D₃ dependent (32). Another transmembrane protein of tight junction, occludin, is also important for the maintenance of epithelial integrity (52). A number of cytoplasmic tight junction proteins, e.g., zonula occludens proteins (ZO)-1, -2, and -3 and cingulin, could also regulate the expression, distribution, and functions of claudins (21, 52). Moreover, the paracellular calcium transport may occur with the solvent drag, which is driven by the paracellular standing sodium gradient created by Na⁺-K⁺-ATPase (7). Generally, the transcellular calcium transport is mostly confined to the duodenum, proximal jejunum, and cecum, whereas the paracellular mechanism is found in all segments (16, 29). The expression pattern of those calcium transport-related genes has been, however, intensively studied in the duodenum but not in the distal segments.

Previous investigations (8, 47, 51) demonstrated that the enhanced intestinal calcium absorption in several physiological and pathological conditions, such as exposure to 1,25(OH)₂D₃,

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estrogen supplementation, or chronic metabolic acidosis, was usually correlated with upregulation of genes related to trans-cellular and/or paracellular calcium transport. Thus the enhanced calcium absorption associated with endurance swimming may also result from the altered expression of these genes. In addition, endurance exercise was known to induce transport of other ions and long-term metabolic changes in the intestinal epithelial cells (3, 19, 33, 53). Thus a genome-wide study using microarray should help identify other intestinal genes that were affected by endurance swimming.

Therefore, the objectives of the present study were 1) to study mRNA expression of genes related to calcium absorption along the whole length of the intestine, 2) to demonstrate the effect of endurance swimming on the intestinal calcium absorption as well as on the plasma calcium and inorganic phosphate concentrations, 3) to investigate the effects of swimming on the expression of genes related to calcium absorption, and 4) to identify other swimming-altered intestinal genes by using microarray technique.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats, weighing 180–200 g (10-wk-old), were obtained from the National Laboratory Animal Centre (Thailand). They were housed in the laboratory animal husbandry unit for at least 7 days before the experiments under 12:12-h light-dark cycle and were fed regular pellets, containing 1.0% calcium and 0.9% phosphorus (Perfect Companion, Bangkok, Thailand), and distilled water ad libitum. The room had a temperature of 20–25°C, humidity of 50–60%, and average illuminance of 150–200 lux in the daytime. Food intake was recorded daily. This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University (Bangkok, Thailand). All animals were cared for in accordance with the principles and guidelines of the American Physiological Society's "Guiding Principles in the Care and Use of Animals."

Swimming protocol. The present swimming protocol known to be a nonimpact endurance exercise with moderate intensity was modified from the methods of Elhaïmeur et al. (14) and the American Physiological Society's "Resource Book for the Design of Animal Exercise Protocols." In brief, rats were randomly divided into two groups. The swimming group was assigned to perform endurance swimming for 2 wk, while the age-matched controls remained sedentary in a swimming pool filled with tap water to a depth of 5 cm. Swimming rats were initially trained for a week until they could swim nonstop 1 h/day. Swimming frequency was 5 days/wk. Four rats concurrently swam in a glass swimming pool with a dimension of $0.8 \times 0.8 \times 0.8$ m³. Each rat swam in a space of $0.4 \times 0.4 \times 0.8$ m³ filled with 50 cm of tap water. The water temperature was maintained at 30–32°C. Body weight was recorded before training and then every swimming day for both control and swimming groups. Twelve hours after the last swimming session, animals were killed for determination of calcium flux or gene expression. To confirm that changes in calcium transport observed in the swimming group were due to the 2-wk training, calcium fluxes were determined in the age-matched untrained rats subjected to a single-bout swimming. In some experiments, the transepithelial calcium fluxes were immediately determined after the last swimming session (i.e., without 12-h rest).

Tissue preparation. A median laparotomy was performed while the animals were under 50 mg/kg pentobarbitone sodium intraperitoneal (Abbott, North Chicago, IL) anesthesia. Intestinal segments, including duodenum (10 cm), proximal and distal jejunum (10 cm each), ileum (8 cm), cecum (4 cm), and proximal and distal colon (8 cm each), were removed, rinsed in an ice-cold bathing solution, and cut longitudinally along the radix mesenterii to expose the mucosa. The

duodenal segment was then mounted in a modified Ussing chamber to measure calcium flux as described previously (28). The tissue was incubated for 20 min in the chamber before the 60-min calcium flux measurement was carried out. As for the quantitative real-time PCR (qRT-PCR) and microarray studies, intestinal epithelial cells were collected by scraping the mucosal surface once with an ice-cold glass slide (10, 28). Gastrocnemius muscle was collected for analysis of citrate synthase activity. Femora were dissected for determination of BMD and bone mineral content (BMC). Arterial blood sample (5 ml) was drawn from the left ventricle. Wet and dry heart weights were also determined.

Measurement of BMD and BMC. Femoral BMD and BMC were assessed by dual-energy X-ray absorptiometer (model Lunar PIXImus2; GE Medical Systems, Madison, WI), operated with a software version 2.10. The dual-energy supply was 80/35 kVp at 500 μ A.

Bathing solution. The bathing solution, continuously gassed with humidified 5% CO₂-95% O₂, contained (in mmol/l) the following: 118 NaCl, 4.7 KCl, 1.1 MgCl₂, 1.25 CaCl₂, 23 NaHCO₃, 12 D-glucose, and 2 mannitol (all purchased from Sigma). The solution was maintained at 37°C (pH 7.4) and had an osmolality of 290–292 mosmol/kgH₂O as measured by a freezing point-based osmometer (model 3320; Advanced Instruments, Norwood, MA). Water used in calcium flux measurement had a resistance >18.3 M Ω cm and a free-ionized calcium concentration <2.5 nmol/l.

Electrical measurement. Three electrical parameters, i.e., potential difference (PD), short-circuit current (I_{sc}), and transepithelial resistance (TER), were determined as previously described (28). A pair of Ag/AgCl electrodes connected to agar bridges (3.0 mol/l KCl per 4 g% agar) was located near each surface of the mounted duodenal tissue for measurement of PD. The other ends of the PD-sensing electrodes were connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL) and finally to a PowerLab 4/30 operated with a software Chart 5.2.2 for Mac OS X (ADInstruments; Colorado Springs, CO). Another pair of Ag/AgCl electrodes was placed close to the other end of the hemichamber to supply I_{sc} , which was also measured by a PowerLab 4/30 connected in series to the EVC-4000 current-generating unit (World Precision Instruments). TER was calculated by Ohm's equation.

Calcium flux measurement. Transepithelial calcium fluxes were determined by the modified method of Charoenphandhu et al. (8). Although we measured calcium fluxes in both small and large intestine, the duodenum was of interest because it is the most efficient site for calcium absorption (16, 29). After a 20-min incubation in the Ussing chamber, the tissue was bathed on both sides with fresh bathing solution. One side was ⁴⁵CaCl₂-containing bathing solution (initial amount of 5 mCi/ml, final specific activity of ~450–500 mCi/mol, Amersham, Buckinghamshire, UK; cold calcium concentration of 1.25 mmol/l). The transepithelial calcium flux ($J_{H \rightarrow C}$) from the hot side (H) to the cold side (C) was calculated by Equations 1 and 2.

$$J_{H \rightarrow C} = R_{H \rightarrow C} / (S_H \times A) \quad (1)$$

$$S_H = C_H / C_{tot} \quad (2)$$

where $R_{H \rightarrow C}$ is the rate of tracer appearance in the cold side (cpm/h); S_H is the specific activity in the hot side (cpm/nmol); A is the surface area of the tissue (0.69 cm²); C_H is the mean radioactivity in the hot side (cpm); and C_{tot} is the total calcium in the hot side (nmol). Radioactivity of ⁴⁵Ca was analyzed by liquid scintillation spectrophotometry (model Tri-Carb 3100TR; Perkin-Elmer, Boston, MA). Total calcium concentration of the bathing solution was analyzed by atomic absorption spectrophotometry (model SpectraAA-300; Varian Techtron, Springvale, Australia).

Total RNA and cDNA preparation. With the use of TRIzol reagent (Invitrogen, Carlsbad, CA), the total RNA was prepared from intestinal epithelial cells, as previously described (10, 28). The total RNA was treated with RQ1 DNase (Promega, Madison, WI) and later

purified with RNeasy Mini kit (Qiagen, Valencia, CA). The purity of the total RNA was determined by the ratio of absorbance readings at 260 and 280 nm, the ratio of which fell in the range of 1.8–2.0. Integrity of RNA was analyzed by denaturing agarose gel electrophoresis with the 28S-rRNA band appearing approximately twice as intense as the 18S-rRNA band. The total RNA samples were used for both qRT-PCR and microarray. As for qRT-PCR, 1 µg total RNA was reverse-transcribed with oligo-dT₂₀ primer and iScript kit (Bio-Rad, Hercules, CA) by a thermal cycler (model MyCycler; Bio-Rad). GAPDH, a housekeeping gene, served as a control gene to check the consistency of the reverse transcription (percent coefficient of variation <5%; *n* = 30).

qRT-PCR and sequencing. Primers used in the present study (Supplemental Table S1; supplemental data for this article are available online at the *Am J Physiol Endocrinol Metab* website.) were designed by OLIGO 6 (Molecular Biology Insights, Cascade, CO) and Primer Validator 1.4 (Naratt Software, Bangkok, Thailand) or as previously used by Charoenphandhu et al. (8). qRT-PCR and melting curve analyses were performed by Bio-Rad MiniOpticon with iQ SYBR Green SuperMix (Bio-Rad). Gene expression levels were first normalized by GAPDH expression. The PCR products were also visualized on a 1.5% agarose gel stained with 1.0 µg/ml ethidium bromide under a ultraviolet transilluminator (Alpha Innotech, San Leandro, CA). After electrophoresis, all PCR products were extracted by the HiYield Gel/PCR DNA extraction kit (Real Biotech, Taipei, Taiwan) and were sequenced by the ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). qRT-PCR experiments were performed in triplicate.

Microarray. The total RNA samples were obtained from six swimming rats and six age-matched control rats. The Illumina's high-performance BeadArray technology was used in the present microarray study because of the reported high selectivity and sensitivity in gene expression profiling as a result of the high redundancy of each probe built in the BeadArray (31). As for the protocol, Illumina TotalPrep RNA amplification/in vitro transcription kit (catalog no. IL1791; Ambion, Austin, TX) was used to amplify and generate biotinylated cRNA from 500 ng total RNA from duodenal scraping for hybridization with Illumina BeadChip array. After purification, biotinylated duodenal cRNA was quantified with NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA); 750 ng of biotinylated cRNA from each duodenal sample were individually hybridized on the RatRef-12 Expression BeadChip (binary manifest file version V1_0_R0_11222119_A; Illumina, San Diego, CA). Each BeadChip constructed with BeadArray technology contains 12 whole-genome gene expression arrays, thus allowing 12 independent samples to be hybridized to a single chip. Each array, which can probe 21,910 genes, consists of 22,523 oligonucleotide probes (50-base gene-specific sequence and 29-base address sequence per probe) selected primarily from the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database (release 16; <http://www.ncbi.nlm.nih.gov/RefSeq>). After incubation at 58°C for 20 h, the BeadChip was washed, blocked, and incubated at room temperature with streptavidin-Cy3-containing solution (Illumina) according to the manufacturer's instruction. Fluorescent signals were scanned with BeadStation 500GX Genetic Analysis System (Illumina). Image registration and data extraction were automatic with BeadStudio 3.1.1 (Illumina). Complete details of the microarray data can be found in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>) under Accession No. GSE13499.

Citrate synthase activity assay. Gastrocnemius muscles from 2-wk swimming rats were collected at 12 h posttraining. To determined citrate synthase activity, muscles were homogenized in ice-cold homogenizing buffer (pH 7.4) containing 20 mmol/l HEPES, 0.25 mol/l sucrose, and 1 mmol/l EDTA, as previously described (48). Immediately before assay, 50 µl homogenate were diluted with 1.2 ml of 100 mmol/l Tris buffer (pH 8.0) containing 3 mmol/l acetyl CoA, and 1 mmol/l 5,5'-dithiobis-2-nitrobenzoic acid (all purchased from Sigma).

To determine the basal activity, the diluted homogenate was read for the optical density change at 412 nm every 30 s for 4 min at 25°C by a spectrophotometer (model UV-2550; Shimadzu, Kyoto, Japan). The reaction was started by adding oxaloacetate (Sigma) dissolved in 100 mmol/l Tris buffer and 100 mmol/l K₂HPO₄ (3:1) to obtain a final concentration of 6.5 mmol/l.

Arterial blood gas analysis and blood chemistry. Blood gas analysis was performed with an automated equipment (model Ultra C; Nova Biomedical, Waltham, MA) to obtain plasma pH, partial pressure of O₂, partial pressure of CO₂, and HCO₃⁻ concentration. Total plasma calcium and inorganic phosphate were determined by modified o-cresolphthalein complexone and phosphomolybdate-based kits, respectively, by using Dimension RxL analyzer (Dade Behring, Marburg, Germany). Free-ionized calcium was measured by ion-selective electrode (model Stat Profile CCX; Nova Biomedical) under an anaerobic environment. Plasma lactate concentration was analyzed by the lactate oxidase technique with Stat Profile CCX system (Nova Biomedical).

Data analysis. Body weight, heart weight, blood chemistry, muscle enzyme activity, and calcium flux are expressed as means ± SE. Unless otherwise specified, comparisons between two groups were performed by Student's *t*-test, while multiple comparisons were performed by one-way ANOVA with Dunnett's posttest. The level of significance for all statistical tests was *P* < 0.05. Data were analyzed by GraphPad Prism 5 (GraphPad Software, San Diego, CA). Differential gene expression of microarray and qRT-PCR was considered significant when there was a twofold or greater difference in expression between the control and swimming groups (9). Microarray data were analyzed by BeadStudio Gene Expression Module 3.2.3 and Genome Viewer 3.1.7 (Illumina). False discovery checks were performed with the "compute false discovery rate" function of the BeadStudio. The Illumina's Cubic-Spline normalization algorithm was applied to adjust sample signals to minimize the effects of variation arising from nonbiological factors. Comparison between two groups of samples (control vs. swimming) was done with the *t*-test differential expression algorithm (detection *P* value < 0.05). Gene Ontology (GO) analysis for characterizing the biological properties of gene products was performed with AmiGO (<http://amigo.geneontology.org>) according to the GO Consortium's instruction at the GO website (<http://www.geneontology.org>). The results of qRT-PCR were compared using 2^{-ΔΔC_T} method (34) and expressed as log₂ means ± SE. All graphics were obtained using GraphPad Prism 5.

RESULTS

Cardiac hypertrophy and the enhanced citrate synthase activity confirmed successful exercise training. After 2 wk of swimming, the wet heart weight, dry heart weight, wet heart weight per body weight, and dry heart weight per body weight were significantly increased (Fig. 1, A and D). Swimming also increased the citrate synthase activity in gastrocnemius muscle by ~40% (Fig. 1E) but did not change plasma lactate concentration (Table 1). Swimming rats manifested normal plasma concentrations of calcium, phosphate, and HCO₃⁻ as well as normal plasma pH and arterial blood gas (Table 1). Arterial blood gas and blood chemistry determined immediately after the last training session (Table 1) and 12 h posttraining (data not shown) were not different. Our results indicated that the present training was a nonimpact endurance exercise with moderate intensity (36). However, a 2-wk period may be too short to demonstrate the beneficial effect of swimming on bone, since femoral BMD and BMC of swimming rats were not changed (Supplemental Fig. S1).

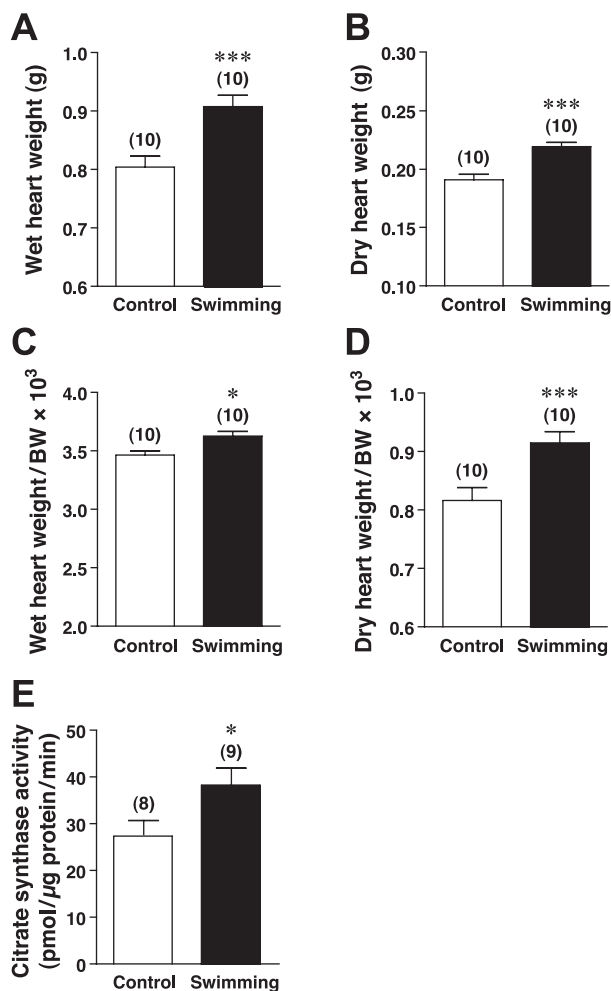


Fig. 1. Wet heart weight (A), dry heart weight (B), wet heart weight per body weight (BW; C), dry heart weight per BW (D), and citrate synthase activity (E) in the age-matched control and swimming rats. * $P < 0.05$, *** $P < 0.001$, compared with the control group. Numbers in parentheses represent number of experimental animals.

Endurance swimming stimulated the transepithelial calcium transport in the duodenum, proximal jejunum, and cecum. In the Ussing chamber study, the duodenum of swimming rats that had a 12-h rest before the calcium flux determination showed an increase in the transepithelial calcium transport by

Table 1. Arterial blood gas and blood chemistry after 2 wk of swimming

Parameters	Experimental Groups	
	Age-matched control (n = 9)	2 wk swimming (n = 9)
Total plasma calcium, mmol/l	2.68 ± 0.07	2.77 ± 0.04
Ionized calcium, mmol/l	1.17 ± 0.06	1.21 ± 0.03
Inorganic phosphate, mmol/l	2.79 ± 0.08	3.07 ± 0.07
Plasma pH	7.39 ± 0.05	7.33 ± 0.02
HCO ₃ ⁻ , mmol/l	35.43 ± 2.66	34.93 ± 1.55
Partial pressure O ₂ , kPa	9.66 ± 0.43	8.76 ± 0.43
Partial pressure CO ₂ , kPa	2.73 ± 0.39	2.80 ± 0.26
Lactate, mmol/l	3.23 ± 0.65	1.87 ± 0.35

Values are means ± SE. All parameters were immediately determined after the last swimming session.

~39% (Fig. 2A). However, when calcium flux was measured immediately after swimming, there was no change in the transepithelial calcium transport (Fig. 2A). In addition, endurance swimming did not affect the electrical parameters, namely potential difference, short-circuit current, and TER, in the duodenal epithelium (Table 2). Besides duodenum, endurance swimming (with 12-h rest) significantly increased the mucosa-to-serosa calcium flux in the proximal jejunum and cecum by ~120 and ~32%, respectively, whereas it decreased that in the proximal colon by ~34% (Fig. 2B). Single-bout swimming in untrained rats changed neither calcium flux (Fig. 2A) nor electrical parameters (Table 2).

Epithelial cells of all intestinal segments expressed essential genes required for calcium absorption. Since endurance swimming significantly altered the intestinal calcium absorption, we further investigated the expression of the genes associated with calcium transport in the entire length of the intestine. Before the differential expression study in swimming rats, the normal profiles of calcium transporter genes were demonstrated. The results showed that all intestinal segments, i.e., duodenum, proximal and distal jejunum, ileum, cecum, and proximal and distal colon ($n = 10$ each), strongly expressed the genes involved in the transcellular calcium transport, i.e., TRPV5, TRPV6, calbindin-D_{9k}, PMCA_{1b}, and NCX1 (Fig. 3A), as well as those possibly involved in the paracellular ion transport, i.e., Na⁺-K⁺-ATPase (β₁-subunit), ZO-1, ZO-2, ZO-3, cingulin, occludin, and claudin-2, -3, and -12 (Fig. 3B). A classical

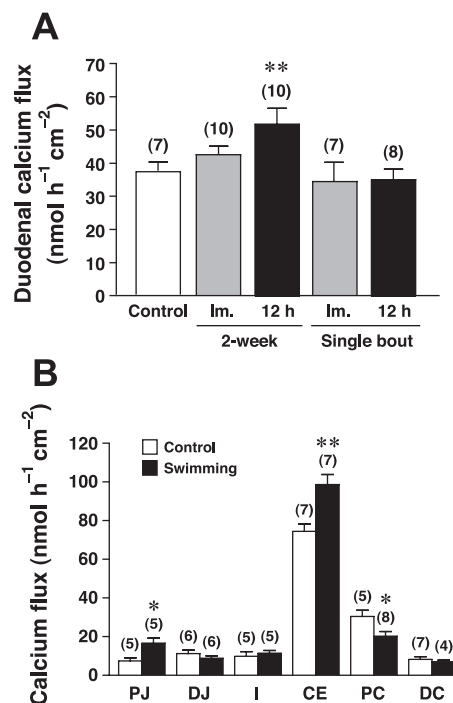


Fig. 2. A: duodenal calcium transport in the control, 2-wk swimming, and single-bout swimming rats. All rats were age matched. Calcium fluxes were measured immediately after swimming (Im.) or 12 h postswimming. B: mucosa-to-serosa calcium fluxes determined at 12 h postswimming in the proximal (PJ) and distal jejunum (DJ), ileum (I), cecum (CE), and proximal (PC) and distal colon (DC). Since the intestinal tissue was bathed on both sides with physiological bathing solution containing 1.25 mmol/l calcium, the observed calcium fluxes represented the active calcium transport. * $P < 0.05$, ** $P < 0.01$, compared with the control group. Numbers in parentheses represent the number of tissues.

Table 2. *Electrical parameters of the duodenal epithelium*

Experimental groups	n	Electrical parameters		
		PD, mV	I_{sc} , $\mu\text{A}/\text{cm}^2$	TER, $\Omega \text{ cm}^2$
Age-matched control	9	5.39 $\pm$ 0.50	49.96 $\pm$ 7.07	121.36 $\pm$ 13.54
2-wk (immediate)	7	6.74 $\pm$ 0.19	66.52 $\pm$ 5.26	105.23 $\pm$ 8.23
2-wk (12 h)	9	6.42 $\pm$ 0.29	64.86 $\pm$ 5.48	102.97 $\pm$ 6.94
Single-bout (immediate)	7	6.08 $\pm$ 0.73	88.64 $\pm$ 19.76	83.07 $\pm$ 16.44
Single-bout (12 h)	8	7.04 $\pm$ 0.61	76.26 $\pm$ 10.19	101.02 $\pm$ 22.82

Values are means $\pm$ SE. Epithelial electrical parameters, i.e., transepithelial potential difference (PD), short-circuit current (I_{sc}), and transepithelial resistance (TER), of the duodenal tissues from the control, 2-wk trained, and single-bout swimming rats. Apical side was negative with respect to the basolateral side. Electrical parameters were measured immediately after swimming or 12 h postswimming.

nuclear receptor for 1,25(OH) $_2$ D $_3$, i.e., VDR, was also expressed along the whole length of the intestine (Fig. 3A). Interestingly, expressions of some calcium transporter genes, i.e., TRPV5, TRPV6, calbindin-D $_{9k}$, and PMCA $_{1b}$, were high-est in the cecum (Fig. 3A).

Endurance swimming differentially changed the expression of calcium transporter genes in the small and large intestine. Expression of calcium transporter genes in swimming rats was demonstrated by qRT-PCR. Since 2 wk of swimming did not change GAPDH expression (Fig. 4A), mRNA expression levels were first normalized by GAPDH expression. As seen in Fig. 4, B and G, the small intestine ($n = 10$) responded to endurance swimming by upregulating VDR and the transcellular genes, i.e., TRPV5, TRPV6, calbindin-D $_{9k}$, PMCA $_{1b}$, and NCX1, whereas the large intestine ($n = 10$) showed no response or responded by decreasing those transcripts. Regarding the paracellular genes, the expression of Na $^+$ -K $^+$ -ATPase (β_1 -subunit) and claudin-12 was modestly changed after swimming (Fig. 5, A and J). The expression of ZO-1, ZO-2, ZO-3, cingulin, and occludin was markedly upregulated in the duodenum but was downregulated in the colon (Fig. 5, B-F). Interestingly, claudin-2 was upregulated in most intestinal segments, while claudin-3 was upregulated in the duodenum, ileum, and cecum but was downregulated in the colon. The present data also indicated that the proximal parts of the small intestine, which are generally the efficient sites for calcium absorption (16, 29), responded well to endurance swimming by increasing the expression levels of calcium transporter genes.

Microarray study revealed transcriptome responses of the duodenal epithelial cells after 2 wk of endurance swimming. Besides calcium absorption, endurance exercise has been reported to affect absorption of other electrolytes, nutrients, and water as well as intestinal permeability, presumably at the genomic level (3, 19, 33, 53). Therefore, the genome-wide microarray study was performed in duodenal cells from control ($n = 6$) and swimming rats ($n = 6$) to demonstrate transcriptome responses after swimming. Among the 21,910 genes, microarray analysis detected a total of 490 genes being up-regulated by more than twofold (459 known genes and 31 unknown genes) and 211 genes downregulated by lower than -2 -fold (188 known genes and 23 unknown genes) in the duodenal epithelial cells of swimming rats (Fig. 6, A and B; Supplemental Tables S2 and S3). Analysis of the distribution of fold changes demonstrated that $\sim 60\%$ of the swimming-altered transcripts were increased in the range of two- to

fourfold (267 known genes and 20 unknown genes) or were decreased in the range of -2 - to -4 -fold (108 known genes and 12 unknown genes). To verify the microarray results, 13 known genes with sequences available in the NCBI database were selected to be tested by qRT-PCR according to their biological functions as defined in GO categories, i.e., one to three genes per each category (Supplemental Table S1). As shown in Fig. 6C, the qRT-PCR results of all transcripts agreed with the microarray results in response to swimming.

GO analyses of the genes changed by >10 -fold further showed that there were a total of 61 annotated genes in the upregulated set and 22 annotated genes in the downregulated set (Fig. 6D). The upregulated genes were predominantly involved in the molecular function (19 genes, 26.76% of >10 -fold upregulated genes), cellular metabolic process (10 genes, 14.08%), cellular component (9 genes, 12.68%), regulation of biological process (7 genes, 9.86%), and transport (4 genes, 5.63%). Within the downregulated group, the majority of genes was involved in the molecular function (6 genes, 20.00% of less than -10 -fold downregulated genes), cell communication (4 genes, 13.33%), and regulation of biological process (4 genes, 13.33%). The gene names in each category are presented in Supplemental Table S4.

Further analysis of all upregulated and downregulated genes in the transport category (GO identifier 0006810) demonstrated that endurance swimming changed the expression of several genes involved in the transport of ions, such as Ca $^{2+}$, Na $^+$, K $^+$, Cl $^-$, and Zn $^{2+}$, and nutrients, such as glucose and amino acids (Fig. 6, E and F). The majority of the swimming-altered transporter genes were related to K $^+$ transport (Supplemental Tables S5 and S6), i.e., 11 upregulated transcripts (32.35% of upregulated transcripts in the transport category) and 4 down-regulated transcripts (36.36%). Endurance swimming also increased the expression of some voltage-gated calcium channels, e.g., Cacna1h, Cacng8, and Cacnb3. In addition, a number of the swimming-altered transcripts (Supplemental Tables S2 and S3) were involved in water and electrolyte homeostasis, such as cholinergic receptor (Chrm2), kallikrein-kinin (Klk7 and Kng1), neuropeptide Y (Npy, Npy1r, and Npy2r), aquaporin (Aqp1 and Aqp2), renin (Ren1), arginine vasopressin (Avp), and galanin receptor (Galr3), as well as epithelial permeability and integrity, such as claudin-8 (Cldn8) and epimorphin (Epim; Refs. 1, 2, 12, 23, 27, 35, 50).

DISCUSSION

How the intestinal calcium absorption was affected by exercise has so far received little attention, although it is the sole pathway to provide calcium for bone mineralization (24). In the present study, we demonstrated, for the first time, that swimming stimulated the intestinal calcium transport in female rats and altered the expression of several genes related to the intestinal calcium absorption. The complete expression profiles of calcium transporter genes along the entire length of the intestine as well as the transcriptome responses of the duodenal epithelial cells to swimming were elucidated. Our swimming protocol was considered an endurance nonimpact exercise with moderate intensity from its ability to induce cardiac hypertrophy and enhance muscle enzyme activity without elevation of plasma lactate concentration (36).

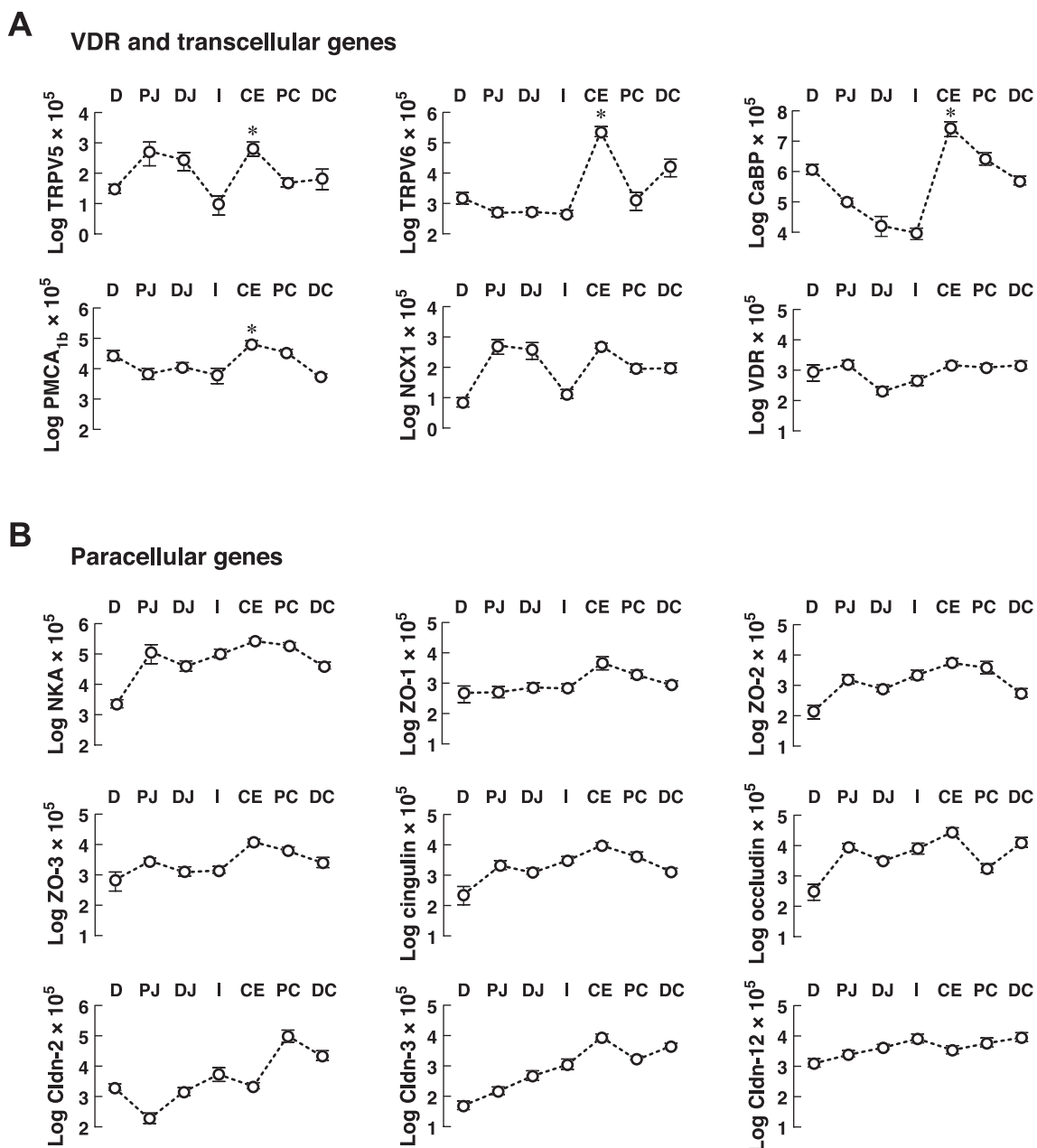


Fig. 3. Expression of nuclear vitamin D receptor (A; VDR) and genes related to the transcellular calcium transport, i.e., transient receptor potential vanilloid family calcium channels 5 and 6 (TRPV5 and TRPV6), calcium-binding protein calbindin- D_{9k} (CaBP), plasma membrane Ca^{2+} -ATPase (PMCA $_{1b}$), and $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (NCX1), and genes (B) related to the paracellular ion transport, i.e., β_1 -subunit of Na^{+} - K^{+} -ATPase (NKA), zonula occludens (ZO)-1, ZO-2, ZO-3, cingulin, occludin, and claudin-2, -3, and -12, in the intestinal epithelial cells of normal rats. Seven intestinal segments, i.e., duodenum (D), proximal and distal jejunum, ileum, cecum, and proximal and distal colon (PJ, DJ, I, CE, PC, and DC, respectively), were used in this study ($n = 10$ per intestinal segment). Data were obtained from quantitative real-time PCR (qRT-PCR). GAPDH is a housekeeping gene used for normalization. Results are expressed as log means $\pm$ SE. * $P < 0.05$, cecum vs. duodenum (Mann-Whitney test).

Swimming-enhanced calcium absorption has been anticipated, since endurance exercise is widely known to increase bone formation and elevate the plasma level of $1,25(\text{OH})_2\text{D}_3$, a classical stimulatory hormone for intestinal calcium transport (22, 57). However, previous studies of physical activity and intestinal calcium absorption usually used impact exercise, such as running, as a model. By using a flat-bed treadmill exercise, Yeh et al. (58) found that the exercise-trained female rats had a higher rate of duodenal calcium absorption than the sedentary controls. In well-trained male athletes orally admin-

istered with stable strontium as a marker of calcium absorption, Zittermann et al. (61) demonstrated increases in the fractional calcium absorption and serum $1,25(\text{OH})_2\text{D}_3$ level after a short-term moderate exercise bout. Another study (60) in male athletes with a minimum of 8 h/wk of endurance sport activities confirmed a 4% increase in the fractional strontium absorption compared with the age-matched sedentary controls. In contrast, immobilization by sciatic denervation in female rats led to a decrease in the duodenal calcium absorption, especially the passive components (58). A report (42) in paraplegic rats

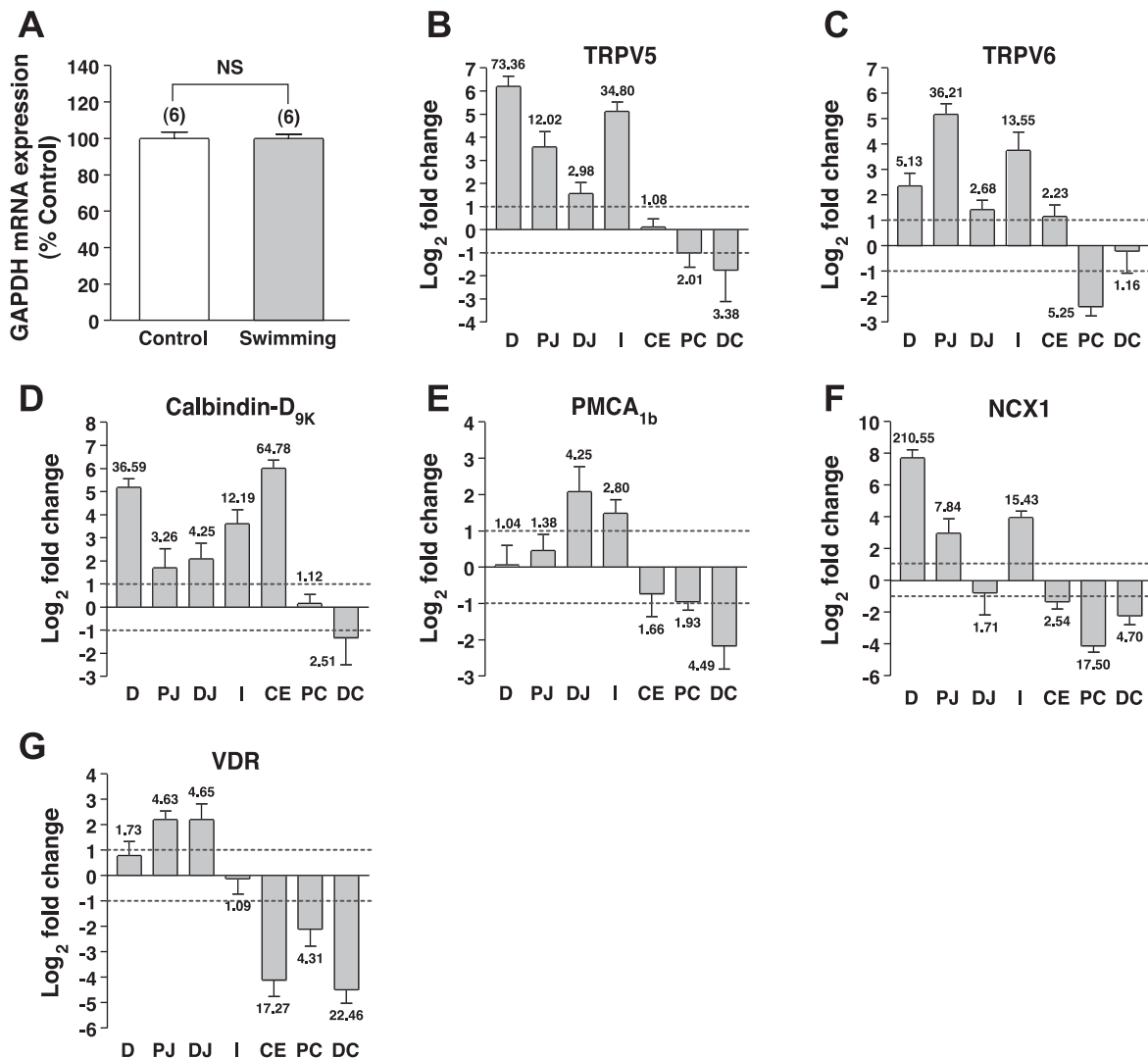


Fig. 4. A: expression of GAPDH mRNA in the duodenal epithelial cells of the control and swimming rats. Numbers in parentheses represent number of experimental animals. NS, not significant. B–G: fold changes in the expression levels of the VDR gene and transcellular genes, i.e., TRPV5, TRPV6, calbindin-D_{9k}, PMCA_{1b}, and NCX1, in the epithelial cells collected from the duodenum, proximal and distal jejunum, ileum, cecum, and proximal and distal colon of swimming ($n = 10$) vs. age-matched control rats ($n = 10$). Gene expression was first normalized by GAPDH. Results are expressed as log₂ means $\pm$ SE. Twofold upregulation or downregulation are indicated by dashed lines. Fold change value of each gene from qRT-PCR is presented on its respective column.

showed increases in fecal calcium and phosphorus contents, presumably as a result of a decrease in the intestinal absorption of these elements. The present study demonstrated that swimming could also enhance the intestinal calcium absorption, similar to that seen during impact exercise. Although the stimulatory effect of swimming on calcium absorption was clearly observed at 2 wk, no change in BMD and BMC was observed in 2-wk-swimming rats (Supplemental Fig. S1). Since Hart et al. (22) were able to demonstrate positive bone response after 12 wk of swimming, it was likely that a detectable change in bone may require a longer than the 2-wk exercise training period.

It was noted that the enhanced calcium absorption was not observed immediately after exercise. This phenomenon could be explained by the fact that exercise normally suppressed the absorptive functions of the intestine by a number of mechanisms, e.g., modulation of autonomic outflow and reduction in mucosal blood supply (3), and such effects appeared to persist

even in the removed tissue. On the other hand, an increase in calcium absorption at 12 h postswimming in 2-wk-trained rats and the absence of such a finding in single-bout swimming rats strongly suggested that exercise induced a long-lasting adaptation in the intestinal epithelial cells at molecular or genomic levels, presumably by upregulating the calcium transporter genes.

Before the investigation of gene expression in swimming, the complete expression profiles of calcium transporter genes in the small and large intestine were determined. Although calcium absorption occurs along the whole length of the intestine, most previous investigations focused on the expression of genes related to the transcellular calcium transport in the duodenum because it is the important segment for calcium absorption (29). Here, our expression profiles of transcellular genes in the duodenum were consistent with the previous reports by Müller et al. (41) and Barley et al. (4). Interestingly, expression of critical transcellular genes, e.g., TRPV6, calbindin-D_{9k}, and PMCA_{1b}, was highest in the cecum, the segment

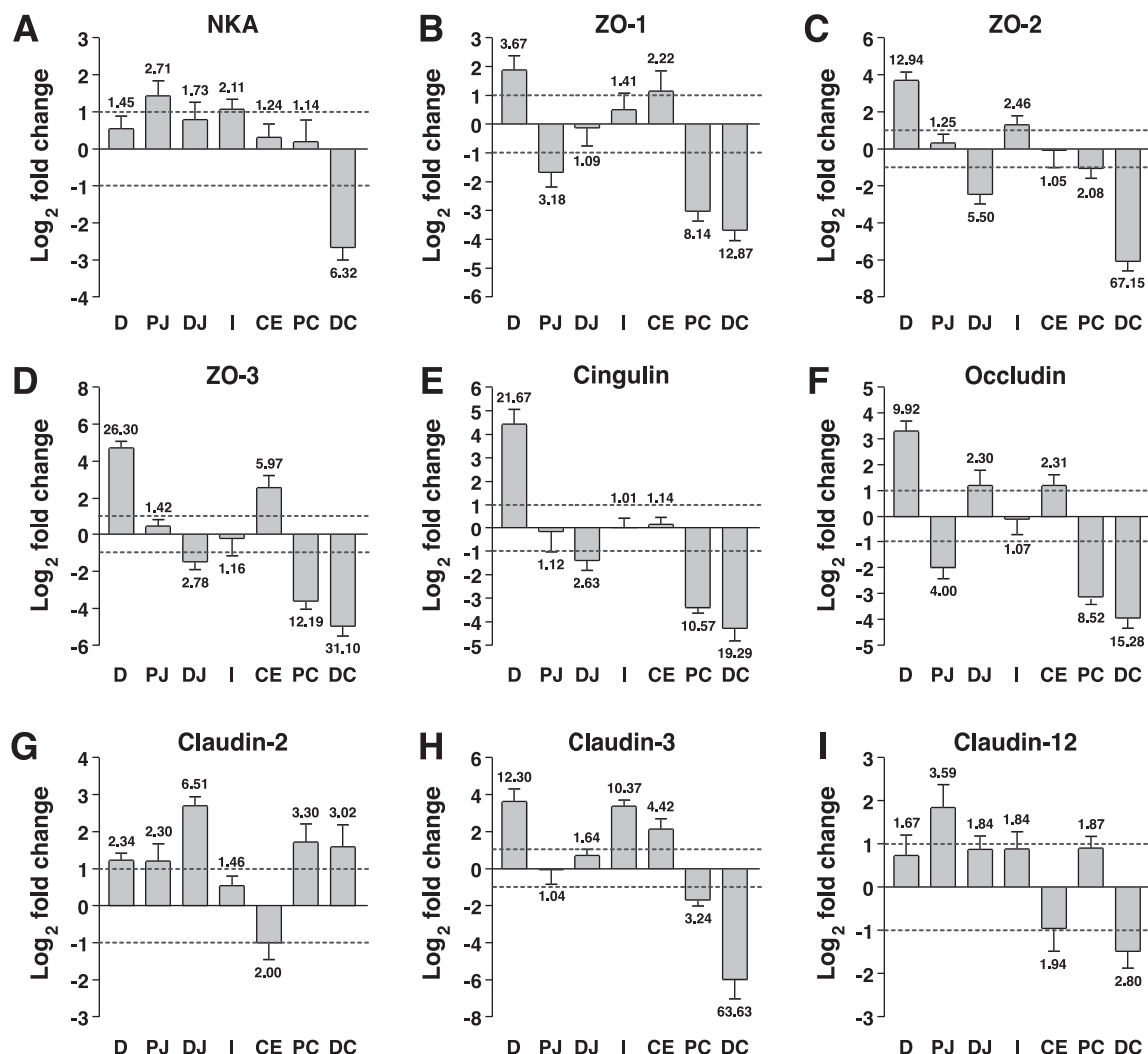


Fig. 5. A–I: fold changes in the expression levels of the paracellular genes, i.e., β_1 -subunit of $\text{Na}^+\text{-K}^+$ -ATPase (NKA), ZO-1, ZO-2, ZO-3, cingulin, occludin, and claudin-2, -3, and -12, in the epithelial cells collected from the duodenum, proximal and distal jejunum, ileum, cecum, and proximal and distal colon of swimming ($n = 10$) vs. age-matched control rats ($n = 10$). Gene expression was first normalized by GAPDH. Results are expressed as $\log_2$ means $\pm$ SE. Twofold upregulation or downregulation are indicated by dashed lines. Fold change value of each gene from qRT-PCR is presented on its respective column.

with the highest rate of calcium absorption (30). Moreover, the distal jejunum and ileum also expressed all transcellular genes, even though they predominantly transport calcium via the paracellular passive mechanism (16, 29). Regarding the paracellular genes, all intestinal segments strongly expressed $\text{Na}^+\text{-K}^+$ -ATPase and the relevant tight junction genes. It has been shown that $\text{Na}^+\text{-K}^+$ -ATPases lining the paracellular membranes were essential for generation of solvent drag, which was a driving force for the paracellular ion transport, especially in the proximal small intestine (7). Claudin-2 and -12 created

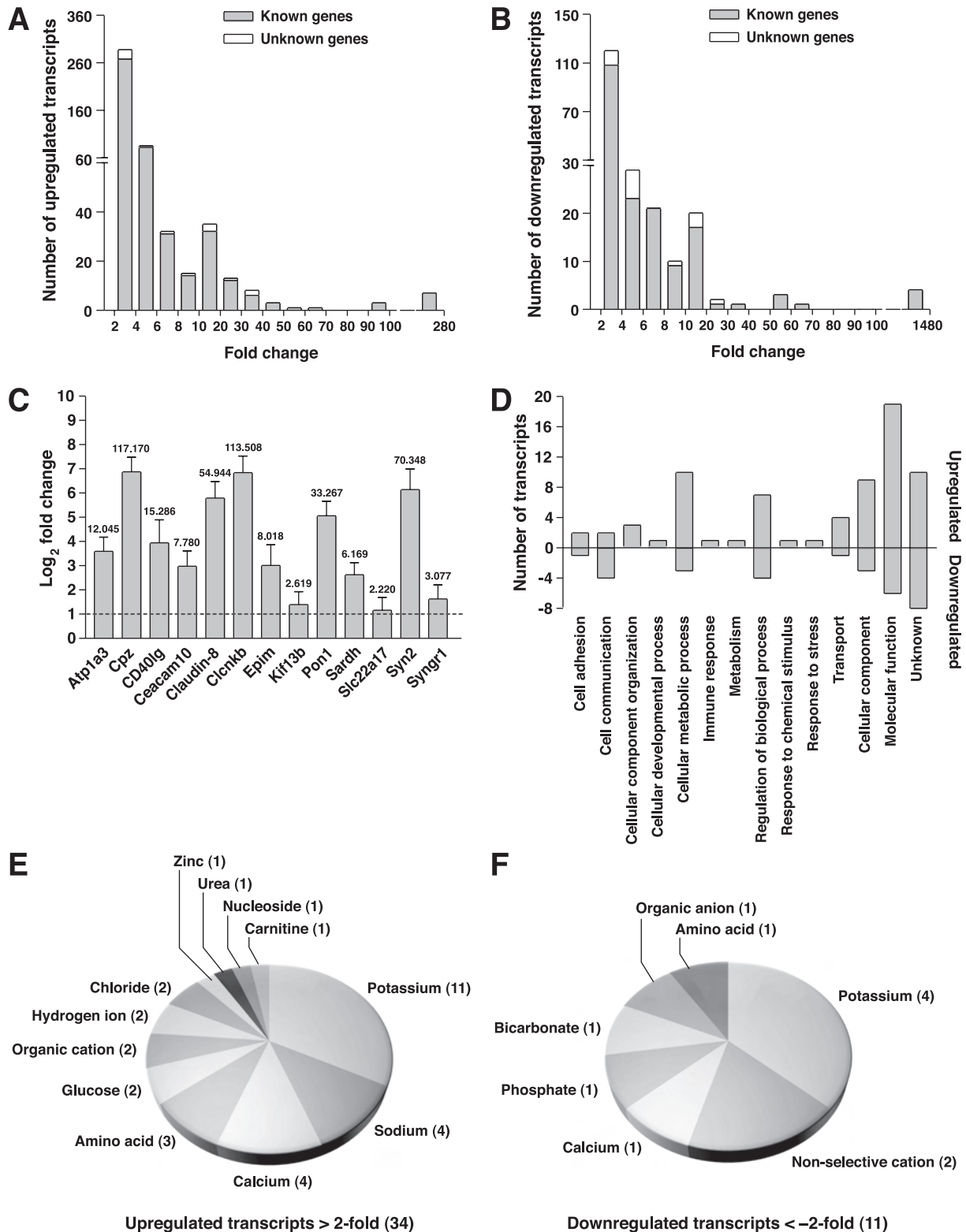
cation-selective tight junctional pores for the paracellular calcium movement (18). Expression of these paracellular genes was found to increase in conditions known to enhance the paracellular calcium transport, such as chronic metabolic acidosis (8, 10). Thus the present expression profiling suggested that both small and large intestine expressed important genes required for the transcellular and paracellular calcium transport.

Up until now, nothing has been known regarding changes in the expression of the calcium transporter genes in the intestine of exercise-trained rats. Studies (46) in immobilized rats

Fig. 6. A–B: distribution of fold changes of genes upregulated and downregulated in the duodenal epithelial cells of swimming rats as determined by microarray. This data set includes transcripts with expression levels changed by more than 2-fold or less than -2 -fold. C: fold changes in expression levels of 13 transcripts selected from various Gene Ontology (GO) categories in the duodenal epithelial cells of swimming rats ($n = 6$) vs. age-matched control rats ($n = 6$) as determined by qRT-PCR. Gene expression was normalized by GAPDH. Twofold change is indicated by the dashed line. Fold change value of each gene is presented on its respective column. D: distribution of genes in each GO category. Columns above abscissa represent transcripts that were upregulated after 2 wk of swimming, whereas columns below abscissa represent downregulated transcripts. All transcripts were functionally classified according to known cellular functions by using GO search engine. This data set includes transcripts with expression levels changed by more than 10-fold or less than -10 -fold. E–F: pie charts show proportional distributions of upregulated and downregulated transcripts in the transport category (GO:0006810). Transcripts were functionally classified according to the transported ions, nutrients, or substances. Numbers in parentheses represent the number of upregulated or downregulated transcripts. This data set includes transcripts with expression levels changed by more than 2-fold or less than -2 -fold after swimming.

showed decreases in the mRNA expression of TRPV5, TRPV6, calbindin-D_{9k}, but not PMCA_{1b} in the rat duodenum, with no change in the colon. Herein, we provided evidence that swimming, in contrast to immobilization, upregulated several intestinal genes for the transcellular calcium transport in the proximal small intestine and cecum, the important sites for active

calcium absorption. Of interest was the ~210-fold upregulation of NCX1 mRNA in the duodenum, which probably contributed substantially to the observed swimming-stimulated transcellular calcium transport, despite the fact that NCX1 transport capacity is ~20% of PMCA_{1b} (55). It was possible that PMCA_{1b} expression was near maximum under normal condi-



tions so that to increase calcium extrusion, the duodenal cells of swimming rats markedly upregulated NCX1. Nevertheless, the paracellular calcium transport rather than the transcellular transport is predominant in the distal jejunum and ileum (16); thus it was not a surprise to see no change in the calcium flux after swimming despite the upregulated transcellular genes. Although the ileum could also transport calcium via the active mechanism, the calcium movement proceeds at a very slow rate (16, 54). The active transport in the ileum may have a considerable contribution to the overall calcium absorption because of its length and the relatively long transit time of the luminal content (54). As for the colon, the exact reason as to why transcellular genes and calcium fluxes were decreased is not known. However, such changes should not have much impact on the overall calcium absorption because of the relatively low fractional calcium absorption in the colonic segment (16).

Of interest was also the swimming-induced upregulation of the voltage-gated calcium channel (Ca_v) subunits as shown in the microarray data. Because of a strong correlation between transcellular calcium flux and L-type Ca_v activity (40) as well as inhibition of the intestinal calcium absorption by the L-type Ca_v blocker verapamil (45), the altered Ca_v expression, in addition to TRPV5 and TRPV6, could partially explain the swimming-enhanced duodenal calcium transport. Upregulation of parvalbumin by approximately sixfold (Supplemental Table S2) may also help facilitate the cytoplasmic calcium translocation in the duodenal cells, similar to calbindin- D_{9k} (6). Relative contributions of Ca_v and parvalbumin to the total calcium absorption remain to be investigated. It was speculated that their roles may become more significant when TRPV6 and/or calbindin- D_{9k} are absent or saturated as in the TRPV6/calbindin- D_{9k} double knockout mice in which active calcium transport occurred at a normal rate (5).

Besides the transcellular genes, swimming also altered the expression of paracellular genes in several intestinal segments (Fig. 5). Expression of claudin-2, -3, and -12, which could form cation-selective paracellular pores (18, 52), was increased in many segments of the small intestine, suggesting an enhanced paracellular calcium transport in these parts. The absence of changes in TER after swimming further evinced that claudins probably create calcium-selective pores without formation of nonselective paracellular pores or widening of the tight junction that usually leads to the alteration in the trans-epithelial potential difference and a reduction in TER (20). Upregulation of other tight junction genes, e.g., ZOs, cingulin, and occludin, which could regulate the ion-selective functions of claudins (21, 52), was predominantly observed in the duodenum, whereas the colon manifested downregulation of these genes. Since Na^+/K^+ -ATPase expression was slightly changed (Fig. 5), it was postulated that swimming did not alter the solvent drag-induced calcium transport in the small intestine.

The mechanism of swimming-altered gene expression is currently unknown, but it might be mediated by $1,25(\text{OH})_2\text{D}_3$, as the plasma $1,25(\text{OH})_2\text{D}_3$ concentration is typically elevated after endurance exercise (57, 58, 60). In contrast, immobilization was associated with suppressed $1,25(\text{OH})_2\text{D}_3$ synthesis (46). Expression of several genes, such as TRPV6, calbindin- D_{9k} , PMCA $_{1b}$, and claudin-3, is also $1,25(\text{OH})_2\text{D}_3$ dependent (32, 51). In the present study, the expression patterns of those genes in swimming rats were consistent with that of VDR.

In addition to calcium absorption, microarray analysis revealed that swimming altered a number of intestinal functions, including cell communication, metabolism, and transport of several ions and nutrients, e.g., Na^+ , K^+ , Cl^- , and glucose. It is not known as to why the majority of upregulated transporters was related to K^+ transport. However, previous investigation suggested that the voltage-gated K^+ channels regulated membrane potential, which was, in turn, important for the Na^+ -dependent absorption of glucose and amino acids in the small intestine (37). Although the physiological functions of most swimming-altered genes have never been investigated, a number of genes are possibly involved in water and electrolyte homeostasis, such as cholinergic receptor, kallikrein-kinin, neuropeptide Y, aquaporin, renin, vasopressin, and galanin receptor (2, 12, 23, 25, 35, 50). These genes could partially explain changes in water and electrolyte metabolism during exercise training (3). Upregulation of claudin-8, known to act as a paracellular cation barrier to Na^+ , K^+ , H^+ , and NH_4^+ , as well as a regulator of epithelial integrity (1), may be a part of the compensatory change to prevent allergen leakage and intestinal hyperpermeability during exercise, as previously reported in humans and dogs (13, 33). Furthermore, the epithelial integrity may be upheld by upregulating genes of local factors which could stimulate growth and differentiation of the intestinal epithelial cells, such as epimorphin and insulin (Supplemental Table S2; Refs. 27, 39). Similar to the effects of exercise on biotransformation in hepatocytes (59), swimming increased transcripts of certain intestinal genes, e.g., UDP-glucuronosyltransferase (Udpgrt2), glutathione S-transferase (Gst2), and paraoxonase (Pon1), which could neutralize xenobiotics and protect the body from toxic substances (11, 44, 56).

In conclusion, we demonstrated that swimming significantly stimulated calcium absorption in the duodenum, proximal jejunum, and cecum of female rats, presumably by upregulating crucial genes for transcellular and paracellular calcium transport, such as TRPV6, calbindin- D_{9k} , PMCA $_{1b}$, NCX1, and claudin-2. Swimming also altered the expression of genes pertaining to transport of ions and nutrients as well as water homeostasis, although further investigation is required to determine their physiological significances. Our findings concerning the swimming-enhanced calcium absorption thus help elucidate the physiological adaptation of calcium metabolism during nonimpact exercise, which should benefit cardiovascular, postmenopausal, and osteoporotic patients. The microarray data also provide direction for further investigations in the regulation of intestinal permeability and electrolyte homeostasis during exercise.

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Two-step stimulation of intestinal Ca^{2+} absorption during lactation by long-term prolactin exposure and suckling-induced prolactin surge

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Charoenphandhu N, Nakkrasae L, Kraidith K, Teerapornpuntakit J, Thongchote K, Thongon N, Krishnamra N. Two-step stimulation of intestinal Ca^{2+} absorption during lactation by long-term prolactin exposure and suckling-induced prolactin surge. *Am J Physiol Endocrinol Metab* 297: E609–E619, 2009. First published June 30, 2009; doi:10.1152/ajpendo.00347.2009.—During pregnancy and lactation, the enhanced intestinal Ca^{2+} absorption serves to provide Ca^{2+} for fetal development and lactogenesis; however, the responsible hormone and its mechanisms remain elusive. We elucidated herein that prolactin (PRL) markedly stimulated the transcellular and paracellular Ca^{2+} transport in the duodenum of pregnant and lactating rats as well as in Caco-2 monolayer in a two-step manner. Specifically, a long-term exposure to PRL in pregnancy and lactation induced an adaptation in duodenal cells at genomic levels by upregulating the expression of genes related to transcellular transport, e.g., TRPV5/6 and calbindin- D_{9k} , and the paracellular transport, e.g., claudin-3, thereby raising Ca^{2+} absorption rate to a new “baseline” (Step 1). During suckling, PRL surge further increased Ca^{2+} absorption to a higher level (Step 2) in a nongenomic manner to match Ca^{2+} loss in milk. PRL-enhanced apical Ca^{2+} uptake was responsible for the increased transcellular transport, whereas PRL-enhanced paracellular transport required claudin-15, which regulated epithelial cation selectivity and paracellular Ca^{2+} movement. Such nongenomic PRL actions were mediated by phosphoinositide 3-kinase, protein kinase C, and RhoA-associated coiled-coil-forming kinase pathways. In conclusion, two-step stimulation of intestinal Ca^{2+} absorption resulted from long-term PRL exposure, which upregulated Ca^{2+} transporter genes to elevate the transport baseline, and the suckling-induced transient PRL surge, which further increased Ca^{2+} transport to the maximal capacity. The present findings also suggested that Ca^{2+} supplementation at 15–30 min prior to breastfeeding may best benefit the lactating mother, since more Ca^{2+} could be absorbed as a result of the suckling-induced PRL surge.

calcium transport; lactating rats; pituitary transplantation; pregnancy; small interfering RNA

A TREMENDOUS AMOUNT of maternal Ca^{2+} (>200 mg/day) is lost during pregnancy and lactation for fetal growth and milk production, thereby leading to severe negative Ca^{2+} balance and progressive osteopenia in mothers (12, 29). Therefore, adequate oral Ca^{2+} intake is of critical importance for the maintenance of fetomaternal bone health (29, 30). How intestinal Ca^{2+} absorption is regulated during these reproductive periods is still elusive, but it is known to be $1,25(\text{OH})_2\text{D}_3$ independent (16, 26). Our previous investigations in nulliparous rats and Caco-2 monolayer indicated that the lactogenic

hormone prolactin (PRL) markedly enhanced the transepithelial Ca^{2+} transport through signaling pathways involving phosphoinositide 3-kinase (PI3K) as well as two serine/threonine kinases, namely protein kinase C (PKC) and RhoA-associated coiled-coil-forming kinase (ROCK) (20, 34, 35). Despite the markedly elevated plasma PRL levels during pregnancy (75–100 ng/ml), lactation (200–300 ng/ml), and suckling (400–650 ng/ml), i.e., >10 times the normal levels (7–10 ng/ml), physiological significances of the PRL-enhanced intestinal Ca^{2+} absorption in pregnancy and lactation have never been elucidated.

Generally, Ca^{2+} traverses the intestinal epithelium via transcellular and paracellular pathways (14). Transcellular active Ca^{2+} transport is a three-step metabolically energized process consisting of apical Ca^{2+} entry via the transient receptor potential vanilloid family Ca^{2+} channels (TRPV) 5/6 and L-type voltage-dependent Ca^{2+} channels (Ca_v), cytoplasmic Ca^{2+} translocation in a calbindin- D_{9k} -bound form, and basolateral Ca^{2+} extrusion via plasma membrane Ca^{2+} -ATPase (PMCA_{1b}) (18, 35). In contrast, the paracellular passive Ca^{2+} transport, which is dependent on the transepithelial Ca^{2+} gradient, does not occur when both sides of the epithelium contain equal Ca^{2+} concentration (14, 35) but may increase with a decrease in transepithelial resistance. Paracellular ion movement is normally regulated by the size- and charge-selective properties of the tight junction, which contains several charge-selective claudin proteins arranged in arrays of channel-like barriers (38). A number of claudins, such as claudin-2, -3, -8, -10, -12, and -15, were found to be cation selective (1, 15, 39, 40). Several mediators, e.g., PKA, PKC, PI3K, and ROCK, can modulate paracellular permselectivity in part via claudin phosphorylation (2, 11, 19, 31, 34).

Although PRL was reported to stimulate both transcellular and paracellular Ca^{2+} transport in ex vivo duodenal tissues (7), there was a disparity among results obtained from different models of PRL exposure. In the 4-wk anterior pituitary (AP)-grafted rats with sustained plasma PRL of 90–100 ng/ml comparable with the levels attained during pregnancy, the marked increase in duodenal Ca^{2+} transport was long lasting and was observed in PRL-free solution (36). In contrast, ex vivo duodenal tissue exhibited an abrupt increase in Ca^{2+} transport only when directly exposed to a much higher PRL concentration (>400 ng/ml) comparable with the levels of suckling-induced PRL surge (20). It is thus hypothesized that a modest hyperprolactinemia during pregnancy and lactation leads to a long-lasting adaptation in the intestinal absorptive cells to enhance Ca^{2+} absorption, whereas PRL surge after

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intermittent suckling rapidly but transiently induces a further increase in Ca²⁺ absorption.

Therefore, the objectives of the present study were 1) to elucidate the physiological significance of PRL in the regulation of intestinal Ca²⁺ absorption during pregnancy, lactation, and suckling and 2) to demonstrate possible mechanisms and signaling pathways of the PRL-enhanced intestinal Ca²⁺ transport. Duodenum was used in this study because it is the efficient site for both transcellular and paracellular Ca²⁺ transport (18). In some experiments, human colorectal adenocarcinoma Caco-2 monolayer, a standard model for Ca²⁺ absorption study, was used since it can be genetically manipulated and has functional properties similar to the small intestinal cells (6, 44, 45).

MATERIALS AND METHODS

Animals. Pregnant and age-matched nulliparous Sprague-Dawley rats were obtained from the National Laboratory Animal Centre (Nakhon Pathom, Thailand). They were housed in the husbandry unit for ≥ 7 days prior to the experiments and were fed standard pellets and distilled water ad libitum. After delivery, the litter size was adjusted to 8 pups/dam. On the experimental day, pups were separated from the dam (–suckling) for 2 h before Ca²⁺ flux study. As for the suckling group (+suckling), pups were returned to the dam after 2-h separation, and suckling was allowed for 30 min before Ca²⁺ flux study. Milk volume was calculated from the weight of pups before and after suckling and milk density of 1.61 g/ml. In some experiments, dams were injected daily with bromocriptine and/or PRL subcutaneously (purified from ovine pituitary gland, catalog no. L6520; Sigma) for 7 days or given single injections 1 h prior to the experiment. This study was approved by the Animal Care and Use Committee of the Faculty of Science, Mahidol University.

Cell culture. Caco-2 cells (American Type Culture Collection no. HTB-37) were grown in Dulbecco's modified Eagle medium (Sigma) supplemented with 15% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acid, 100 U/ml penicillin-streptomycin, and 0.25 μ l/ml amphotericin B. Confluent Caco-2 monolayers were prepared by seeding cells (5×10^5 cells/cm²) on polyester Snapwell with 12-mm diameter and 0.4- μ m pore size (Corning). Culture medium was changed daily after 48 h of seeding. Monolayers were incubated at 37°C for 14 days in a humidified atmosphere containing 5% CO₂. To investigate PRL effects on Ca²⁺ transport, Caco-2 monolayer was exposed to 600 ng/ml recombinant human PRL (rhPRL; purity >97%, catalog no. 682-PL; R & D Systems), the most effective concentration reported by Jantarajit et al. (20).

Small interfering RNA transfection. Small interfering RNA (siRNA) oligonucleotides targeted for long isoform of human PRL receptor (PRLR-L; 5'-GGGCUAUAGCAUGGUGACCUU-3' and 5'-GGUCACCAUGCUAUAGCCCUU-3') and claudin-15 (5'-GCAAAUACGGCAGAAACGCUU-3' and 5'-UUCGUUUUAGC-CGUCUUUGCG-3') were designed by siRNA Target Designer 1.51 and synthesized by T7 RiboMax Express RNAi System (Promega). Scramble siRNA (5'-GGCGCAAUAAAGCAAGACC-3' and 5'-GGUCUUGCUUUUAGGCGCC-3'), which had no homology to any other genes, was used as a negative control. As described previously (35), Caco-2 cells were first plated on Snapwell at 5×10^5 cells/cm². On day 12 after seeding, in vitro transfection was performed with 10 μ g/ml polyethyleneimine (PEI) and 1 μ mol/ml siRNA. On day 14 (i.e., 48 h after transfection), siRNA-treated monolayers were used for the experiments. Efficiency of siRNA was evaluated by quantitative real-time PCR (qRT-PCR). The knockdown protocol was approved by the Institutional Biosafety Committee of Mahidol University.

AP transplantation. As described previously (10), two 10-wk-old donor rats were decapitated to collect the pituitary glands, which were immediately transplanted under the renal capsule of a recipient rat

(i.e., 2 glands/rat). Sham operation consisted of exposure of the left kidney and a gentle touch of the renal fascia with forceps. Visual examination of the well-vascularized hypophyseal graft and immunohistochemical staining for PRL production were performed at the end

Table 1. Oligonucleotide sequences used in PCR study

Gene (Accession No.)	Primer	Product Length, bp
<i>Homo sapiens</i>		
PRLR-S (AF416619)		145
Forward	5'-GGTGACCCCTTGATGTTG-3'	
Reverse	5'-TTCTGGTATATGCTCTTCAGC-3'	
PRLR-L (NM_000949)		100
Forward	5'-ACTTGCTCTTTCTCCAG-3'	
Reverse	5'-TCCCTCAAGAATACTAAGCAG-3'	
Claudin-2 (NM_020384)		102
Forward	5'-CTCTTCAGGCGTAATGGA-3'	
Reverse	5'-CTTGGTGCTATGCTCTTC-3'	
Claudin-3 (NM_001306)		121
Forward	5'-CTGCTCTGCTGCTCGTGT-3'	
Reverse	5'-TAGTCCCTGCGGTCGTAGC-3'	
Claudin-8 (NM_199328)		97
Forward	5'-TGGTGCTGATTGTTGGAGGAG-3'	
Reverse	5'-GTTGTGCGATGGGAAGGTATC-3'	
Claudin-10 (NM_182848)		179
Forward	5'-CTCTGGTGTCTGGTGTGCG-3'	
Reverse	5'-GATAGTAAATGCGCTCGG-3'	
Claudin-12 (NM_012129)		174
Forward	5'-GCCCCATATACAATCTCTTAG-3'	
Reverse	5'-GTAAAGCCATACCTTACTTC-3'	
Claudin-15 (NM_014343)		90
Forward	5'-AAGTGGAGACGGACCTGAGC-3'	
Reverse	5'-GCGGCTAAGGAGGGTGT-3'	
GAPDH (NM_002046)		359
Forward	5'-CTGGTAAAGTGGATATTGTTG-3'	
Reverse	5'-GAGGCTGTTGTCTACTTCTC-3'	
<i>Rattus norvegicus</i>		
PRLR-S (NM_012630)		120
Forward	5'-TTCTACCACCATCGCAAC-3'	
Reverse	5'-CTGATCTCGTTTGTCTATTGAG-3'	
PRLR-L (NM_001034111)		107
Forward	5'-TCAAGCAACCGCAGACTC-3'	
Reverse	5'-CAGTTTAGCCAATCGTTCCA-3'	
PRL (NM_012629)		171
Forward	5'-CAAACCTTCTGTTCTGCC-3'	
Reverse	5'-CAGCATCTTGGACATACTG-3'	
TRPV5 (NM_053787)		163
Forward	5'-CTTACGGGTTGAACACCACCA-3'	
Reverse	5'-TTGCAGAACACAGAGCCCTCA-3'	
TRPV6 (NM_053686)		80
Forward	5'-ATCCGCGCGTATGCGAC-3'	
Reverse	5'-AGTTTTTCTGCTCACTGTTTTTGG-3'	
Calbindin-D _{9k} (X_16635)		174
Forward	5'-CCCGAAGAAATGAAGAGCATTTT-3'	
Reverse	5'-TTCTCCATCACCGTTCTTATCCA-3'	
PMCA _{1b} (NM_053311)		109
Forward	5'-CGCCATCTTCTGCACAATT-3'	
Reverse	5'-CAGCCATTGTTCTATTGAAAGTTC-3'	
ZO-1 (XM_218747)		270
Forward	5'-GTATCCGATTGTTGTGTTCC-3'	
Reverse	5'-TCACTTGTAGCACCATCCGC-3'	
Occludin (NM_031329)		188
Forward	5'-CACGTTCCGACCAATGC-3'	
Reverse	5'-CCCGTTCCATAGGCTC-3'	
Claudin-3 (NM_031700)		246
Forward	5'-GCACCCACCAAGATCTCTA-3'	
Reverse	5'-AGGCTGTCTGCTCTTCCA-3'	
Claudin-15 (XM_222085)		330
Forward	5'-GCTGTGCCACCGACTCCC-3'	
Reverse	5'-CAGAGCCAGTTTCACTTTG-3'	
GAPDH (NM_017008)		133
Forward	5'-AGTCTACTGGCGTCTTTCAC-3'	
Reverse	5'-TCATATTTCTCGTGGTTCAC-3'	

PRLR-S and -L, short and long isoforms, respectively, of prolactin receptor; PRL, prolactin; TRPV5 and -6, transient receptor potential vanilloid family Ca²⁺ channels 5 and 6, respectively; PMCA_{1b}, plasma membrane Ca²⁺-ATPase isoform 1b.

of the experiments to confirm successful AP transplantation (10). At 4 wk after transplantation, plasma PRL levels are known to increase to 90–100 ng/ml (28).

Surgery and tissue collection. A median laparotomy was performed under 50 mg/kg ip pentobarbitone sodium (Abbott) anesthesia. Duodenal segment was removed and cut longitudinally to expose the mucosa. Duodenal segment was then mounted in a modified Ussing chamber to measure Ca^{2+} fluxes, as described previously (20). The tissue was incubated for 20 min in the chamber before the 60-min experiment. For mRNA and protein expression studies, epithelial cells were collected by scraping the mucosal surface with an ice-cold glass slide (10). Arterial blood (5 ml) was also collected by cardiac puncture.

Determination of serum PRL. Serum PRL concentrations were determined by rat PRL enzyme immunoassay kit (catalog no. A05101; SPI Bio, Massy Cedex, France) according to the manufacturer's instruction. The sensitivity of the assay was 0.4 ng/ml, and the intra- and interassay coefficients of variation were 10 and 15%, respectively.

RNA isolation, PCR, and sequencing. By using TRIzol reagent (Invitrogen), total RNA was prepared according to the method of Chaoenphandhu et al. (10). One microgram of total RNA was reverse-transcribed with oligo(dT)₂₀ primer and iScript kit (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control gene to check the consistency of reverse transcription. Primers are presented in Table 1 (9, 20, 34). Amplification reaction using conventional thermal cycler was performed with GoTaq Green Master Mix (Promega), whereas qRT-PCR using real-time PCR (model MiniOpticon; Bio-Rad) was performed with iQ SYBR Green SuperMix (Bio-Rad) according to the manufacturers' instructions. Relative expression was calculated from the threshold cycle (C_T) values by using the $2^{-\Delta\Delta C_T}$ method, and twofold change was considered significant (10). PCR products were also visualized on a 1.5% agarose gel stained with 1.0 $\mu\text{g}/\text{ml}$ ethidium bromide under a UV transilluminator (Alpha Innotech). After electrophoresis, PCR products were extracted by HiYield Gel/PCR DNA Extraction kit (Real Biotech, Taipei, Taiwan) and were sequenced by ABI Prism 3100 Genetic Analyzer (Applied Biosystems). qRT-PCR experiments were performed in triplicate.

Immunoprecipitation. Cell lysate was sonicated and incubated at 4°C for 20 min. Thereafter, lysate was incubated for 2 h at 4°C on a rocking platform with 1:1,000 rabbit polyclonal antibodies against claudin-2, -3, -8, -10, -12, or -15 (Santa Cruz Biotechnology). The mixture was then incubated at 4°C for 2 h with EZview Red protein-A affinity gel (Sigma). Beads were washed three times and resuspended in 50 μl of buffer containing 20% glycerol, 10% SDS, 1% bromophenol blue, 4% 2-mercaptoethanol, and 0.5 mol/l Tris·HCl, pH 6.8. Eluted proteins were subjected to Western blot analysis.

Western blot analysis. As described previously (20), 100- μg proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham) by electroblotting. Membranes were blocked at 25°C for 4 h with 5% nonfat milk and were probed overnight at 4°C with 1:1,000 rabbit polyclonal antibodies (Santa Cruz Biotechnology) raised against the conserved extracellular domain of PRLR, claudins, or phosphorylated amino acid residues (i.e., phosphorylated serine, threonine, and tyrosine). Membranes were also reprobed with 1:5,000 anti- β -actin monoclonal or 1:1,000 anti-claudin polyclonal antibodies (Santa Cruz Biotechnology). After 2-h incubation at 25°C with 1:2,000 secondary antibodies (Santa Cruz Biotechnology), blots were visualized by enhanced chemiluminescence kit (Amersham).

Confocal laser-scanning microscopy. Caco-2 cells were plated on coverslips in six-well culture plates (10^5 cells/ cm^2) and maintained for 12 days. On day 12, cells were transfected with claudin-15 siRNA. After 48-h incubation, the monolayer was fixed with 100% ethanol for 20 min at -20°C. After blocking nonspecific bindings, cells were then incubated overnight at 4°C with 1:20 rabbit anti-occludin or claudin-15 polyclonal antibodies (Santa Cruz Biotechnology). They were later rinsed with phosphate-buffered saline (PBS) with Tween-20, pH 7.4, prior to 3-h incubation with 1:300 Alexa fluor 488-conjugated secondary antibody (Molecular Probes) at room temperature. For negative controls, cells were incubated with 3% bovine serum albumin in PBS instead of specific antibody. Images were captured with a FV1000 confocal laser-scanning microscope (Olympus).

Bathing solution for Ussing chamber study. The bathing solution, continuously gassed with humidified 5% CO_2 in 95% O_2 , contained (in mmol/l) 118 NaCl, 4.7 KCl, 1.1 MgCl_2 , 1.25 CaCl_2 , 23 NaHCO_3 ,

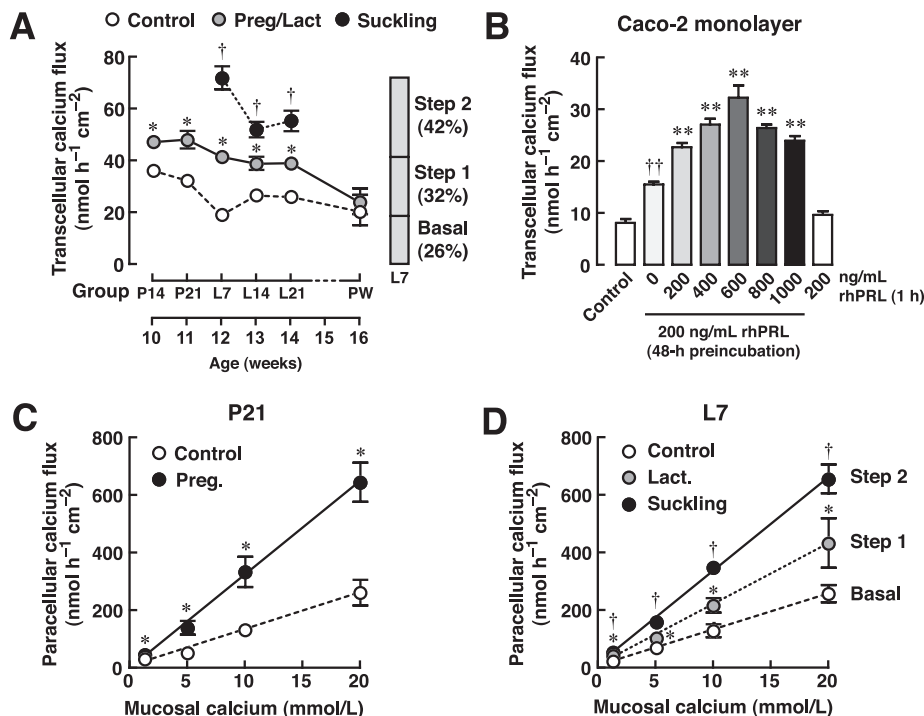


Fig. 1. A: transcellular Ca^{2+} transport in the duodenum of pregnant/lactating, suckling, and age-matched control rats ($n = 5-9$). Ca^{2+} flux was studied at midpregnancy (P14), late pregnancy (P21), early lactation (L7), midlactation (L14), late lactation (L21), and 15-day postweaning (PW). Two-step stimulation of the transcellular Ca^{2+} transport and the %contribution in L7 rats are depicted. * $P < 0.05$ compared with control group; † $P < 0.05$ compared with corresponding lactating group. B: transcellular Ca^{2+} transport in 200 ng/ml recombinant human prolactin (rhPRL)-preincubated Caco-2 monolayer after acute exposure (1 h) to 200–1,000 ng/ml rhPRL ($n = 3-6$). Preincubation with 200 ng/ml rhPRL for 48 h was to represent long-term PRL effects in lactation, whereas acute PRL exposure was to mimic PRL surge during suckling. †† $P < 0.01$ compared with control group; ** $P < 0.01$ compared with preincubated group without acute PRL treatment. C and D: paracellular Ca^{2+} transport in the duodenum of P21, L7, and L7 + suckling rats ($n = 5-6$ /each Ca^{2+} concentration). Ca^{2+} flux was measured in the presence of transepithelial Ca^{2+} gradient (i.e., various mucosal Ca^{2+} concentrations). Two-step stimulation was demonstrated during lactation. * $P < 0.05$ compared with control group; † $P < 0.05$ compared with corresponding lactating group.

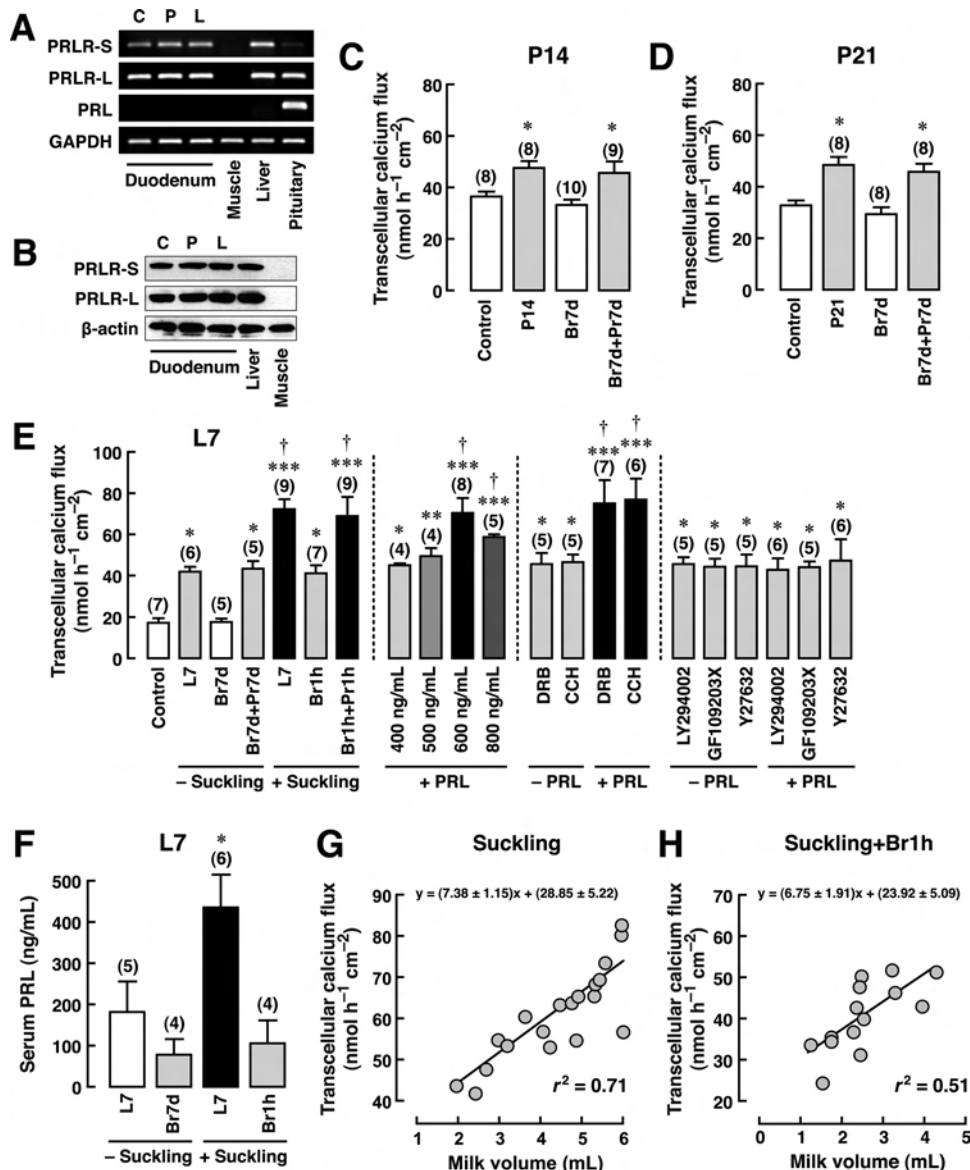
12 D-glucose, and 2 mannitol (Sigma). The solution was maintained at 37°C, pH 7.4, and had an osmolality of 290–295 mmol/kg H_2O . In some experiments, PRL or inhibitors of gene transcription [RNA polymerase II inhibitor; 50 $\mu\text{mol/l}$ 5,6-dichloro-1- β -D-ribobenzimidazole (DRB); Calbiochem], protein synthesis [70 $\mu\text{mol/l}$ cycloheximide (CCH); Sigma], PI3K (75 $\mu\text{mol/l}$ LY294002; Tocris), PKC (panspecific inhibitor; 1 $\mu\text{mol/l}$ GF109203X; AG Scientific), or ROCK (1 $\mu\text{mol/l}$ Y27632; Calbiochem) were added in the bathing solution.

Electrical measurement. Potential difference (PD), short-circuit current (Isc), and transepithelial resistance (TER) were determined as previously described (20). Briefly, a pair of Ag/AgCl electrodes connected to agar bridges (3.0 mol/l KCl per 4 g% agar) was located near each surface of the mounted tissue or Snapwell for measurement of PD. The other ends of the PD-sensing electrodes were connected to a preamplifier (model EVC-4000; World Precision Instruments) and finally to PowerLab 4/30 (AD Instruments). Another pair of Ag/AgCl electrodes was placed at the end of each hemichamber to supply Isc, which was also measured by a PowerLab 4/30 connected in series to EVC-4000 current-generating unit. TER and conductance were calculated by Ohm's equation.

Calcium flux measurement. Ca^{2+} flux was determined by the method of Charoenphandhu et al. (9). Briefly, after 20-min incubation, Ussing chamber was filled with fresh bathing solution. One side was $^{45}\text{CaCl}_2$ -containing bathing solution (initial amount of 5 mCi/ml, final specific activity of ~ 450 –500 mCi/mol; Amersham). ^{45}Ca radioactivity was analyzed by a liquid scintillation spectrophotometer (model Tri-Carb 3100; Packard Instruments). Total Ca^{2+} concentration of the bathing solution was analyzed by an atomic absorption spectrophotometer (model SpectraAA-300; Varian Techtron, Springvale, Australia). Ca^{2+} fluxes in the absence of Ca^{2+} concentration gradient (i.e., bathing solution in both hemichambers contained equal Ca^{2+} concentration) represented the active Ca^{2+} transport. The Ca^{2+} gradient-dependent paracellular passive fluxes were measured by determining Ca^{2+} fluxes in the presence of varying apical Ca^{2+} concentrations (9).

Permeability measurement. Permeability of sodium (P_{Na}) and chloride (P_{Cl}), which were indicative of charge selectivity, were measured by the dilution potential technique (34). In brief, duodenal epithelium or Caco-2 monolayer was equilibrated for 20 min in normal bathing solution containing 145 mmol/l NaCl before the apical solution was substituted with 72.5 mmol/l NaCl. Osmolality was maintained by an

Fig. 2. Representative images of mRNA ($n = 3$; A) and protein expression ($n = 3$; B) of short (PRLR-S) and long (PRLR-L) isoforms of PRL receptors (PRLR) and PRL (mRNA only) in the duodenum of nonmated control (C), pregnant (P), and lactating (L) rats. GAPDH was a housekeeping gene for PCR study, whereas β -actin was a housekeeping gene for Western blot analysis. Liver and muscle served as positive and negative controls, respectively, for PRLR expression. Pituitary gland was a positive control for PRL expression. C and D: transcellular Ca^{2+} transport in P14 and P21 rats administered with bromocriptine (Br7d) with or without PRL supplementation (Pr7d) for 7 days. * $P < 0.05$ compared with control group. Numbers of animals are shown in parentheses. E: transcellular Ca^{2+} transport in L7 rats with (+suckling) or without suckling (–suckling). Bromocriptine injection 1 h (Br1h) prior to Ca^{2+} flux study was to abolish the suckling-induced PRL surge, which was mimicked by concurrent PRL supplementation (Pr1h). In some experiments, duodenal tissues of L7 rats (–suckling) were acutely exposed to 400–800 ng/ml PRL in the bathing solution, which resembled acute exposure during PRL surge. To demonstrate PRL-signaling pathways, PRL and inhibitors of gene transcription [50 $\mu\text{mol/l}$ 5,6-dichloro-1- β -D-ribobenzimidazole (DRB)], protein biosynthesis [70 $\mu\text{mol/l}$ cycloheximide (CCH)], phosphoinositide 3-kinase (PI3K; 75 $\mu\text{mol/l}$ LY-294002), PKC (1 $\mu\text{mol/l}$ GF-109203X), or RhoA-associated coiled-coil-forming kinase (ROCK; 1 $\mu\text{mol/l}$ Y-27632) were added in the solution. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with nonmated control group (Basal Ca^{2+} flux). † $P < 0.05$ compared with L7 –suckling (Step 1 Ca^{2+} flux). F: serum PRL levels in L7 rats. PRL surge was determined at 30 min postsuckling. * $P < 0.05$ compared with L7 –suckling group. G and H: correlation between Ca^{2+} fluxes and milk volume in suckling rats. Data were pooled from L7, L14, and L21 rats with (total animals = 14) or without (total animals = 19) Br1h administration.



equivalent amount of mannitol. Changes in the PD before and after fluid replacement (i.e., dilution potential) were recorded every 10 s until stable. $P_{\text{Na}}/P_{\text{Cl}}$ was calculated from the dilution potential by using the Goldman-Hodgkin-Katz equation, whereas P_{Na} and P_{Cl} were calculated from the transepithelial conductance and $P_{\text{Na}}/P_{\text{Cl}}$ by using Kimizuka-Koketsu equations (34). Permeability of Ca^{2+} (P_{Ca}) was calculated from the paracellular Ca^{2+} flux and the difference between apical and basolateral Ca^{2+} concentrations, as described previously (34).

^{45}Ca uptake study. Caco-2 cells were seeded on Snapwells for 12 days. On day 12, *in vitro* transfection was performed with 10 $\mu\text{g}/\text{ml}$ PEI and 1 nmol/ml claudin-15 siRNA. Control cells were treated only with PEI. After 48-h incubation, the monolayer was exposed to 600 ng/ml rhPRL for 1 h and then bathed on the apical side with bathing solution containing $16,000 \text{ counts} \cdot \text{min}^{-1} \cdot 100 \mu\text{l}^{-1}$ $^{45}\text{CaCl}_2$ for 2 min before harvesting. ^{45}Ca radioactivity was analyzed by liquid scintillation spectrophotometer.

Statistical analysis. Results are expressed as means $\pm$ SE. Two sets of data were compared using the unpaired Student *t*-test. One-way analysis of variance with Dunnett's multiple comparison test was used for multiple sets of data. Linear regression with slope analysis was performed for correlation study. The level of significance was $P < 0.05$. Data were analyzed by GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

RESULTS

Two-step stimulation of duodenal Ca^{2+} absorption during pregnancy and lactation. Transepithelial Ca^{2+} transport was investigated in primiparous rats at different reproductive

phases, i.e., midpregnancy (day 14; P14), late pregnancy (day 21; P21), early lactation (day 7; L7), midlactation (day 14; L14), late lactation (day 21; L21), and 15-day postweaning (PW), as well as in their age-matched nulliparous controls. Lactating rats were separated from pups for 2 h prior to Ca^{2+} flux study. As demonstrated by Ussing chamber technique (Fig. 1A), the transcellular Ca^{2+} transport was increased significantly in pregnancy and lactation, but not PW, to a new "baseline" level (Step 1). Thirty-minute suckling further increased the transcellular Ca^{2+} transport to a new level above the elevated baseline (Step 2). In L7 rats, Step 1 and Step 2 Ca^{2+} fluxes contributed ~ 32 and $\sim 42\%$, respectively, to the total transcellular Ca^{2+} flux (Fig. 1A). Similarly, Caco-2 monolayer preincubated with 200 ng/ml rhPRL (dissolved in culture medium) for 48 h to represent long-term PRL exposure later exhibited 90% increase in the transcellular Ca^{2+} transport, although bathing solution in Ussing chamber contained no rhPRL (Fig. 1B). Acute exposure of preincubated Caco-2 monolayer to 200–1,000 ng/ml rhPRL (dissolved in bathing solution) further enhanced the transcellular Ca^{2+} transport in a dose-response manner. However, without 48-h preincubation, 200 ng/ml rhPRL in bathing solution was unable to stimulate Ca^{2+} transport (Fig. 1B).

The paracellular Ca^{2+} transport was also enhanced in P21 rats (Fig. 1C). Furthermore, consistent with the transcellular

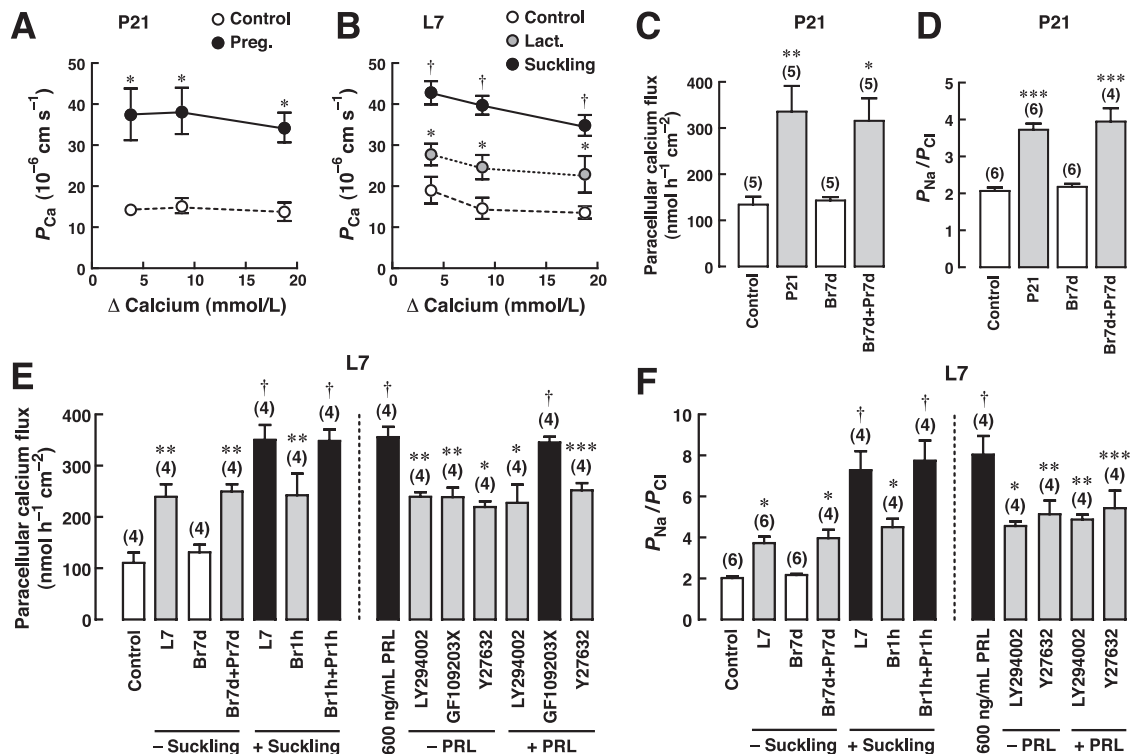


Fig. 3. A and B: permeability of Ca^{2+} (P_{Ca}) in the duodenum of pregnant, lactating, and suckling rats ($n = 5$ –6/each Ca^{2+} concentration) plotted against Ca^{2+} concentration differences between apical and basolateral compartments ($\Delta \text{calcium}$). C: paracellular Ca^{2+} fluxes measured in the presence of 10 mmol/l apical Ca^{2+} in the duodenum of P21 rats. D: permeability of sodium/chloride ($P_{\text{Na}}/P_{\text{Cl}}$) as an indicator of charge selectivity in the duodenum of P21 rats. E and F: paracellular duodenal Ca^{2+} transport and $P_{\text{Na}}/P_{\text{Cl}}$ in L7 rats with or without suckling. In some experiments, L7 rats were administered with Br7d or Br1h to suppress chronic PRL secretion and suckling-induced PRL surge, respectively. PRL signalings were investigated by exposing 600 ng/ml PRL-treated L7 duodenal tissues to various inhibitors. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with control group (Basal). † $P < 0.05$ compared with L7 –suckling (Step 1). Numbers in parentheses represent the number of rats.

transport, the two-step stimulation of the paracellular Ca^{2+} transport was similarly observed in L7 rats (Fig. 1D).

PRL as a regulator of transcellular Ca^{2+} transport during pregnancy and lactation. To find out whether PRL was the key regulator of the two-step increase in Ca^{2+} transport in lactating animals, the presence of PRLR was first investigated in the duodenal epithelial cells of P21, L7, and control rats. By using conventional PCR (Fig. 2A) and Western blot analysis (Fig. 2B), short (PRLR-S) and long (PRLR-L) isoforms of PRLR were identified, confirming the duodenum as a target of PRL. Figure 2A also shows that duodenal cells did not express endogenous PRL; i.e., the observed PRL effects were due to circulating PRL.

In P14 and P21 rats, 7-day administration of $4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ sc bromocriptine (Br7d), an inhibitor of pituitary PRL secretion, completely abolished the pregnancy-enhanced transcellular Ca^{2+} transport (Step 1), whereas exogenous PRL supplementation [$0.6 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ sc for 7 days (Pr7d)] totally reversed the bromocriptine effects (Fig. 2, C and D). Inhibition of Step 1 Ca^{2+} transport by Br7d was also observed in L7 (Fig. 2E), L14, and L21 rats (data not shown). Interestingly, in L7 rats with suckling, a single dose of 4 mg/kg bromocriptine administered 1 h before suckling (Br1h) completely abolished PRL surge (Fig. 2F) as well as the Step 2 transcellular Ca^{2+} transport (Fig. 2E), which was restored by a single dose of PRL [0.6 mg/kg sc (Pr1h)]. In addition, there was a strong correlation between the volume of milk ingested by pups and Ca^{2+} absorption during suckling (Fig. 2G). Such correlation became less significant after Br1h administration (Fig. 2H), suggesting that PRL surge may be the signal to match Ca^{2+} absorption with Ca^{2+} loss in milk.

The enhanced Ca^{2+} transport by the suckling-induced PRL surge could be mimicked in vitro by incubating an ex vivo duodenal tissue from L7 rats in bathing solution containing 600 and 800 but not 400 and 500 ng/ml PRL (Fig. 2E). The acute stimulatory effect of 600 ng/ml PRL was not abolished by inhibitors of gene transcription ($50 \text{ } \mu\text{mol/l}$ DRB) or protein biosynthesis ($70 \text{ } \mu\text{mol/l}$ cycloheximide) (Fig. 2E) but was completely abolished by inhibitors

of PI3K ($75 \text{ } \mu\text{mol/l}$ LY-294002), PKC ($1 \text{ } \mu\text{mol/l}$ GF-109203X), and ROCK ($1 \text{ } \mu\text{mol/l}$ Y-27632). The present findings suggested that the acute stimulatory effect of PRL surge on the transcellular Ca^{2+} transport (Step 2) in lactating rats was mediated by nongenomic signaling pathways involving PI3K, PKC, and ROCK.

PRL as a regulator of paracellular Ca^{2+} transport during pregnancy and lactation. In addition to transcellular Ca^{2+} transport, pregnancy and lactation increased paracellular Ca^{2+} permeability (P_{Ca} ; Fig. 3, A and B), whereas it decreased the TER (Supplemental Fig. S1, A and B; Supplemental Material for this article is available at the *AJP-Endocrinology and Metabolism* web site) of the duodenal epithelium. Suckling further increased P_{Ca} above the baseline level in lactation (Fig. 3B). The findings that decreases in TER that accompanied the increased Ca^{2+} transport were abolished by Br7d and then restored by Pr7d (Supplemental Fig. S1, A and B) strongly suggested that PRL was responsible for these responses. Further studies in P21 rats demonstrated that long-term PRL exposure markedly stimulated the gradient-dependent paracellular passive Ca^{2+} flux (Fig. 3C) in part by increasing the cation selectivity of the paracellular space, as indicated by the permeability ratio of sodium/chloride ($P_{\text{Na}}/P_{\text{Cl}}$) (Fig. 3D). The PRL-induced increase in $P_{\text{Na}}/P_{\text{Cl}}$ in pregnancy resulted from an increase in P_{Na} , without a change in P_{Cl} (Supplemental Fig. S1, C and D).

Long-term PRL exposure during lactation also stimulated the paracellular Ca^{2+} transport (Fig. 3E) and increased $P_{\text{Na}}/P_{\text{Cl}}$ (Fig. 3F) by raising P_{Na} (Supplemental Fig. S1E), without affecting P_{Cl} (Supplemental Fig. S1F). Suckling-induced PRL surge further increased the paracellular Ca^{2+} flux, $P_{\text{Na}}/P_{\text{Cl}}$, and P_{Na} above the baseline levels in lactation. Acute effects of direct exposure to 600 ng/ml PRL, which mimicked the suckling-induced PRL surge, were totally diminished by inhibitors of PI3K and ROCK, but not by PKC inhibitor (Fig. 3, E and F, and Supplemental Fig. S1, E and F). These findings corroborated that the acute effects of PRL surge on the paracellular Ca^{2+} transport (Step 2) and charge selectivity in lactating rats were mediated by nongenomic PI3K and ROCK pathways.

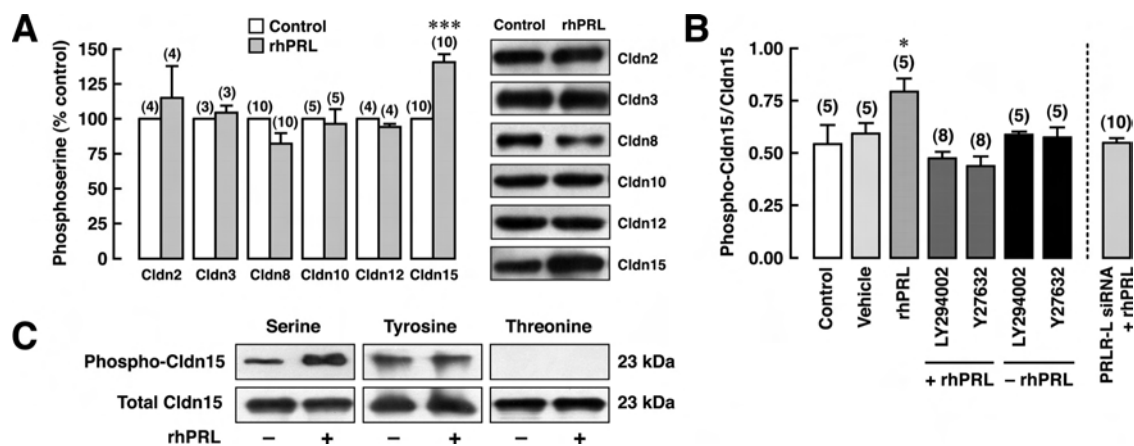


Fig. 4. A: phosphorylated claudin (Cldn)-2, -3, -8, -10, -12, and -15 at serine residues in 600 ng/ml rhPRL-treated Caco-2 monolayers, as demonstrated by immunoprecipitation. Representative electrophoretic images were also depicted. *** $P < 0.001$ compared with its respective control. Numbers in parentheses represent the number of independent monolayers. B: serine-phosphorylated Cldn-15 levels normalized with total claudin-15 in 600 ng/ml rhPRL-treated native and PRLR-L knockdown Caco-2 monolayers. In some experiments, $75 \text{ } \mu\text{mol/l}$ LY-294002 or $1 \text{ } \mu\text{mol/l}$ Y-27632 was added in the medium to demonstrate PRL-signaling pathways. * $P < 0.05$ compared with control group. C: representative bands of phosphorylated Cldn-15 at serine and tyrosine residues in rhPRL-treated Caco-2 monolayers ($n = 5$), as demonstrated by immunoprecipitation. Cldn-15 did not exhibit threonine phosphorylation.

Claudin-15 was essential for the PRL-stimulated paracellular Ca^{2+} transport. Since PRL augmented the paracellular Ca^{2+} transport by altering the charge selectivity of duodenal epithelium, it was possible that PRL may have changed the properties of certain charge-selective proteins of the claudin family. Generally, alterations of claudin functions are regulated by phosphorylation (11, 19). Therefore, we determined the amount of phosphorylated claudins in 600 ng/ml rhPRL-exposed Caco-2 monolayer by immunoprecipitation technique. The results showed that PRL induced serine phosphorylation of claudin-15 but not claudin-2, -3, -8, -10, or -12 (Fig. 4A). Serine phosphorylation of claudin-15 was prevented by PI3K and ROCK inhibitors as well as PRLR-L knockdown using siRNA (Fig. 4B). PRL was without effect on tyrosine or threonine phosphorylation of claudin-15 (Fig. 4C). PRLR-L siRNA did not interfere with PRLR-S or claudin-15 expression (Fig. 5A).

To verify the involvement of claudin-15 in the PRL-stimulated paracellular Ca^{2+} transport, Caco-2 monolayer was sub-

jected to claudin-15 siRNA transfection. Claudin-15 knockdown markedly reduced mRNA expression, protein expression, and membrane localization of claudin-15 (Fig. 5, B–F), as demonstrated by qRT-PCR, Western blot analysis, and confocal laser-scanning microscopic techniques, respectively. Claudin-15 siRNA did not alter the expression of claudin-2, -3, -8, -10, -12, or occludin (Fig. 5, C–F). Scrambled siRNA and transfecting agent (10 $\mu\text{g}/\text{ml}$ PEI) had no effect on claudin-15 expression (Fig. 5B). Using chamber study of normal Caco-2 monolayer showed that 600 ng/ml rhPRL markedly increased the paracellular Ca^{2+} transport (Fig. 6A) and P_{Ca} (Fig. 6B), whereas it decreased TER (Fig. 6C), similar to what was observed in the duodenum of lactating rats. This PRL-enhanced paracellular Ca^{2+} transport could be explained by a large increase in cation selectivity ($P_{\text{Na}}/P_{\text{Cl}}$ and P_{Na}) and a slight decrease in P_{Cl} (Fig. 6D and Supplemental Fig. S2, A and B). Moreover, PRL effects on the paracellular Ca^{2+} flux, P_{Ca} , TER, $P_{\text{Na}}/P_{\text{Cl}}$, and P_{Na} were diminished after claudin-15 knockdown (Fig. 6, A–D, and Supplemental Fig. S2A). Since

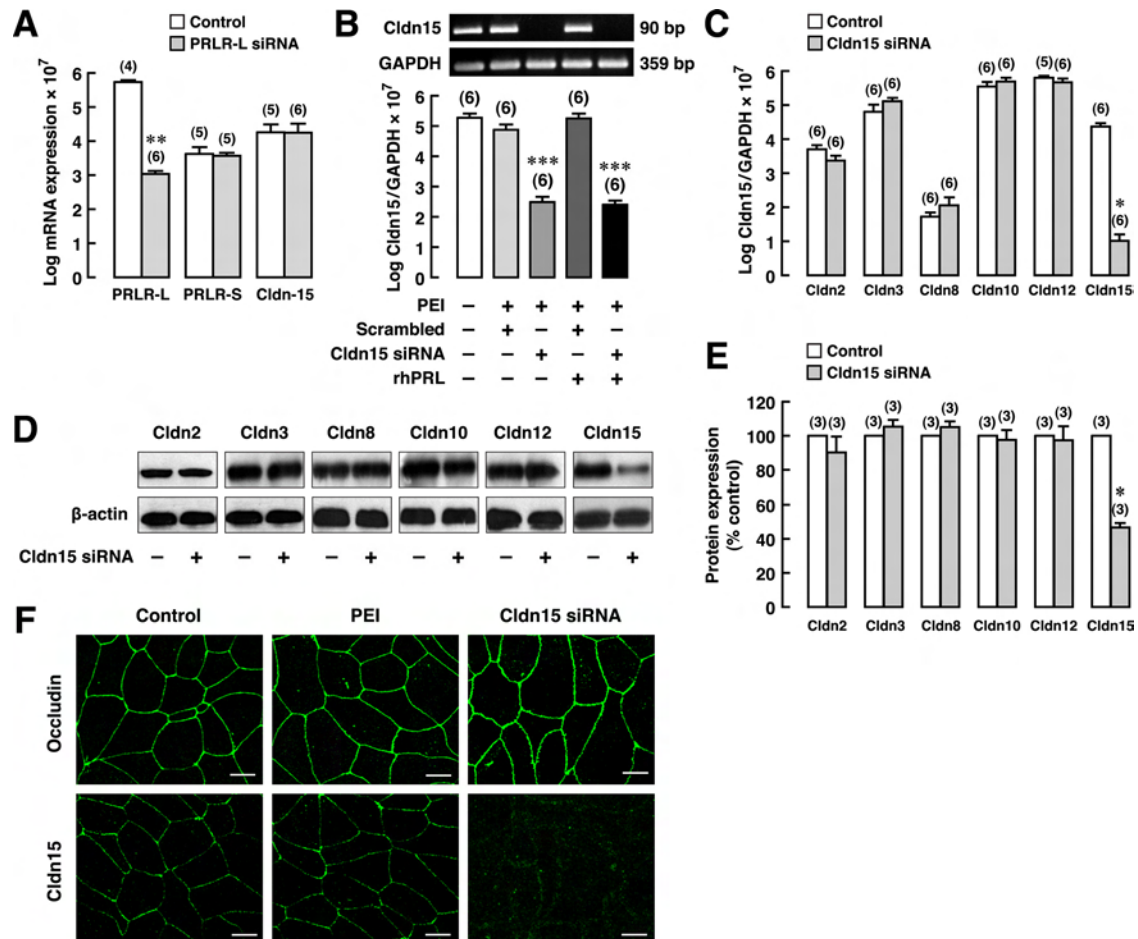


Fig. 5. A: expression of PRLR-L, PRLR-S, and Cldn-15 in PRLR-L knockdown Caco-2 cells, as demonstrated by quantitative real-time PCR (qRT-PCR). $^{**}P < 0.01$, small interfering RNA (siRNA) vs. control [polyethyleneimine (PEI) treated]. Numbers in parentheses represent the number of independent samples. B: Cldn-15 expression normalized by GAPDH expression in Cldn-15 siRNA-treated Caco-2 monolayers. Some monolayers were also exposed to 600 ng/ml rhPRL for 1 h before qRT-PCR study. Representative electrophoretic bands of Cldn-15 from conventional PCR were also illustrated along with those of GAPDH (36 cycles). $^{***}P < 0.001$ compared with control (open bar). C–E: mRNA and protein expression of Cldn-2, -3, -8, -10, -12, and -15, as demonstrated by qRT-PCR and Western blot analysis, respectively, in Cldn-15 siRNA-treated Caco-2 monolayers. Cldn protein expression was normalized by β -actin expression. $^{*}P < 0.05$ compared with corresponding control. F: localization of tight junction protein occludin and Cldn-15 in control (no treatment), PEI-treated, and Cldn-15 siRNA-treated Caco-2 monolayers, as demonstrated by confocal laser-scanning microscopic technique. Cldn-15 fluorescent signals were detected on the lateral membrane of control and PEI-treated monolayers but not in Cldn-15 siRNA-treated monolayer. Cldn-15 knockdown had no effect on occludin localization. Scale bars, 10 μm .

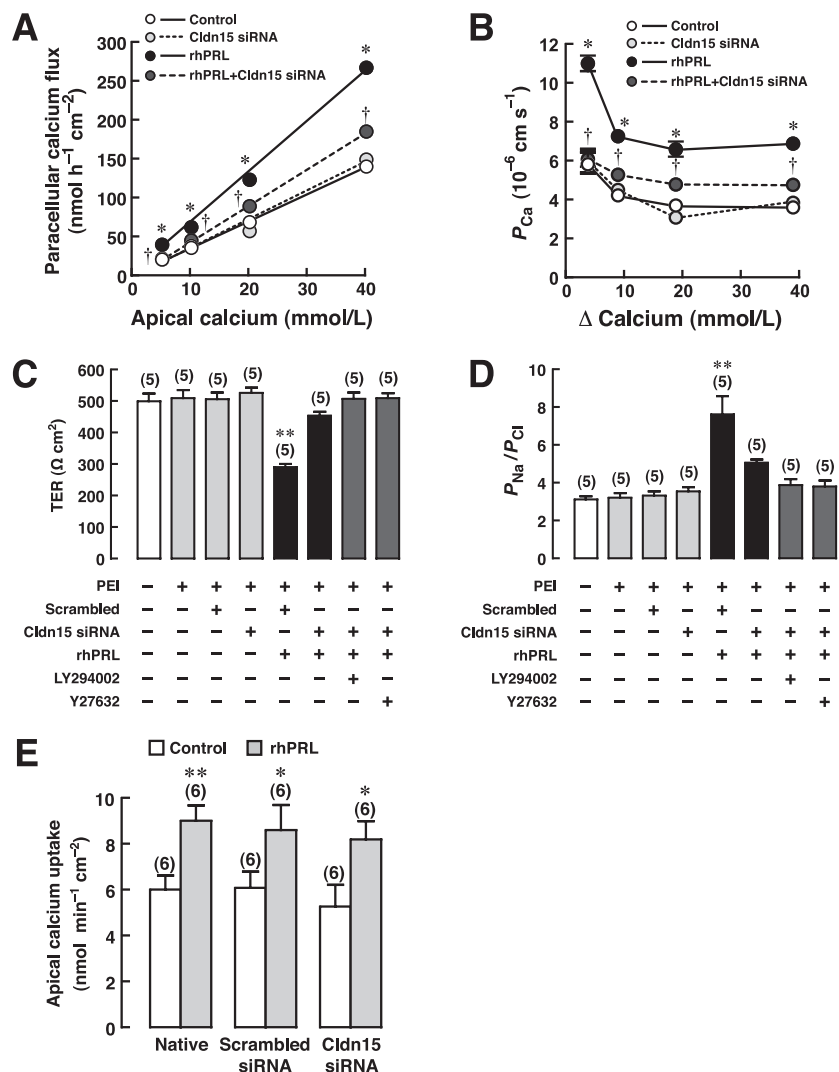


Fig. 6. A and B: paracellular Ca^{2+} transport and P_{Ca} in native and Cldn-15 knockdown Caco-2 monolayers directly exposed to 600 ng/ml rhPRL ($n = 5$ /each Ca^{2+} concentration). * $P < 0.05$ rhPRL vs. control; † $P < 0.05$, rhPRL vs. rhPRL + Cldn-15 siRNA. C and D: transepithelial resistance (TER) and $P_{\text{Na}}/P_{\text{Cl}}$ of native and Cldn-15 knockdown Caco-2 monolayers directly exposed to rhPRL or rhPRL plus inhibitors. ** $P < 0.01$, rhPRL vs. control (open bar). E: ^{45}Ca uptake at 2 min in native (PEI-treated) and Cldn-15 knockdown Caco-2 monolayers directly exposed to rhPRL. * $P < 0.05$ and ** $P < 0.01$ compared with its respective control group. Numbers in parentheses represent the number of independent experiments.

PI3K and ROCK inhibitors did not further increase TER (Fig. 6C) or further decrease $P_{\text{Na}}/P_{\text{Cl}}$ and P_{Na} (Fig. 6D and Supplemental Fig. S2A), the cation-selective claudin-15 might be a target of both mediators.

PRL stimulated apical Ca^{2+} uptake. To determine the effect of PRL on Ca^{2+} entry, which is the first step of the transcellular Ca^{2+} transport, ^{45}Ca uptake study was performed in Caco-2 monolayer. Two minutes after ^{45}Ca -containing medium was added to the apical compartment, the PRL-stimulated Ca^{2+} uptake of comparable rates was observed in both native and claudin-15 knockdown Caco-2 monolayers (Fig. 6E), suggesting that acute PRL exposure stimulated the transcellular Ca^{2+} transport by enhancing apical Ca^{2+} uptake.

Long-term PRL exposure altered the expression of genes related to Ca^{2+} transport and permselectivity. Besides non-genomic actions induced by transient PRL surge during suckling, chronic PRL exposure during pregnancy and lactation may induce long-lasting adaptations to enhance Ca^{2+} absorption, e.g., upregulation of certain Ca^{2+} transporter genes. Thus, the Step 1 Ca^{2+} transport was completely diminished by Br7d but not by Br1h (Fig. 2E). This hypothesis was confirmed by using nulliparous rats transplanted with two extra AP glands under the renal fascia. In the absence of hypothalamic inhibi-

tion, the AP grafts continuously released PRL but not other AP hormones that, in contrast, required hypothalamic stimulation. The AP-grafted rats were hyperprolactinemic with reported plasma PRL levels of ~90–100 ng/ml, comparable with the levels attained during pregnancy (28). PRL synthesis in AP grafts was confirmed by immunohistochemistry (Fig. 7, A and B).

Four weeks after AP transplantation, hyperprolactinemia led to upregulation of several genes involved in Ca^{2+} transport, i.e., TRPV5, TRPV6, calbindin-D_{9k}, and claudin-3 (9, 18), whereas the tight junction genes ZO-1 and occludin were downregulated (Fig. 7C).

DISCUSSION

The question of what factor enhances the intestinal Ca^{2+} absorption to mitigate negative Ca^{2+} balance and optimize fetomaternal Ca^{2+} homeostasis in pregnancy and lactation has remained enigmatic for decades. In humans, mothers lose ~200–300 and ~300–400 mg of Ca^{2+} each day during pregnancy and lactation, respectively, thereby leading to an ~10% decrease in total bone mass (29). Rodents with a litter of 8–10 pups may lose up to 20% of their skeletal mass in milk

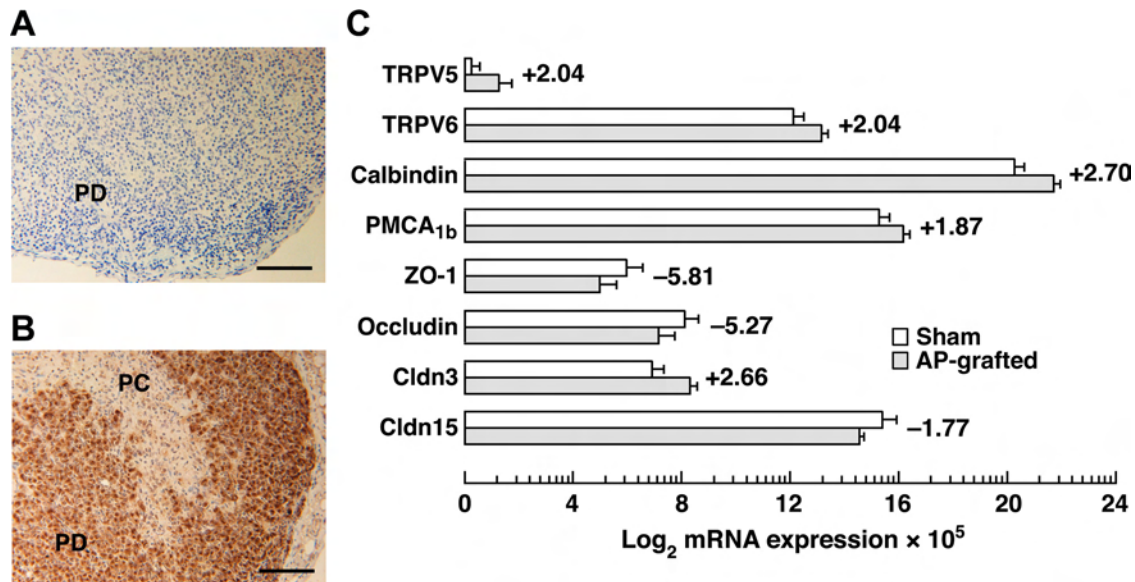


Fig. 7. Representative photomicrographic images of a pituitary graft dissected from a 4-wk anterior pituitary (AP) rat ($n = 10$). A: control image for PRL immunohistochemical analysis; i.e., the 4- μm paraffin-embedded section was incubated with secondary antibody in the absence of anti-PRL primary antibody. Active signs of microvascular endothelial damage and lymphoid proliferation were not seen, suggesting no graft rejection and allograft vasculopathy. B: immunohistochemical staining of an implanted pituitary gland with anti-PRL antibody. The pars distalis (PD) is strongly labeled with brownish products of peroxidase, whereas the perirenal connective tissues (PC) do not show PRL immunoreactivity. Scale bars, 100 μm . C: expression of genes related to Ca^{2+} transport and paracellular permeability in duodenal epithelial cells of 4-wk hyperprolactinemic AP-grafted rats ($n = 7$ –10) vs. age-matched sham rats ($n = 7$ –10), as demonstrated by qRT-PCR ($\log_2$ means $\pm$ SE $\times 10^5$). Fold change value of each gene is presented on its respective columns. + and –, upregulation and downregulation, respectively. TRPV5 and -6, transient receptor potential vanilloid family Ca^{2+} channels 5 and 6, respectively; PMCA_{1b}, plasma Ca^{2+} ATPase isoform 1b.

production (27). With such high Ca^{2+} loss, it is surprising that intestinal Ca^{2+} absorption during pregnancy and lactation is $1,25(\text{OH})_2\text{D}_3$ independent and does not correlate with the levels of other Ca^{2+} -regulating hormones, namely parathyroid hormone (PTH), PTH-related peptide, calcitonin, and estrogen (14, 29). Herein, we elucidated for the first time that PRL is a novel Ca^{2+} -regulating hormone that induces a two-step stimulation of Ca^{2+} absorption during pregnancy, lactation, and suckling. Therefore, PRL is essentially a regulator of Ca^{2+} balance by controlling both input (i.e., provision of Ca^{2+}) and output (i.e., lactogenesis) of Ca^{2+} in these reproductive phases.

Long-term PRL exposure over a period of several days led to an adaptive elevation of the basal intestinal Ca^{2+} transport to a new baseline (Step 1). This adaptation occurred at genomic levels through alteration of gene expression and was abolished by Br7d but not Br1h. In suckling rats (+suckling), Br1h could only decrease Step 2 Ca^{2+} transport to Step 1 level without further decreasing Step 1 Ca^{2+} transport (Fig. 2E). van Cromphaut et al. (37) previously showed that upregulation of TRPV6, calbindin-D_{9k}, and PMCA_{1b} could account for the enhanced transcellular Ca^{2+} absorption in the duodenum of pregnant and lactating mice. Similarly, the present findings showed PRL-induced upregulation of TRPV5, TRPV6, and calbindin-D_{9k} in the duodenal cells of AP-grafted rats, which was consistent with an earlier report of a 25% upregulation of duodenal calbindin-D_{9k} by 9-day administration of PRL (5). Moreover, our previous genome-wide study in the rat duodenum by microarray indicated the PRL-induced increases in the expression of other Ca^{2+} transporter genes, namely Ca_v and parvalbumin, which may also contribute to the enhanced transcellular Ca^{2+} absorption (4, 10). Upregulation of these Ca^{2+} transporter genes, i.e., TRPV5, TRPV6, and Ca_v for apical

Ca^{2+} uptake or calbindin-D_{9k} and parvalbumin for cytoplasmic diffusion, stressed the importance of adequate Ca^{2+} supply for fetal development and lactogenesis. Such redundancy also explains why TRPV6/calbindin-D_{9k} double-knockout mice were normocalcemic and still exhibited transcellular active Ca^{2+} absorption (3).

The Step 1 enhancement of the paracellular Ca^{2+} absorption appeared to involve the PRL-induced downregulation of occludin and ZO-1, both of which are important for the tight junction assembly, size-selective paracellular permeability, and maintenance of transepithelial resistance (13, 41). A decrease in transepithelial resistance after long-term PRL exposure in P21 and L7 rats (Supplemental Fig. S1, A and B) suggested possible enlargement of paracellular pores for passage of small solutes. In addition to the size selectivity, long-term PRL exposure also altered the charge selectivity of the paracellular barriers to favor cations over anions (i.e., an increase in $P_{\text{Na}}/P_{\text{Cl}}$; Fig. 3, D and F), which was consistent with the PRL-induced upregulation of cation-selective tight junction gene claudin-3. Previous investigations provided evidence that duodenal claudin-3 expression was $1,25(\text{OH})_2\text{D}_3$ dependent (23), and its upregulation was associated with conditions of increased intestinal Ca^{2+} absorption, e.g., chronic metabolic acidosis (9). Although PRL augmented both transcellular and paracellular components of Step 1 Ca^{2+} absorption, the latter may be of greater physiological significance since, under normal luminal Ca^{2+} concentration of ~ 2 –6 mmol/l (42), the paracellular Ca^{2+} flux was about three times greater than the transcellular flux (i.e., ~ 150 vs. ~ 50 nmol $\cdot$ h⁻¹ $\cdot$ cm⁻²). Nevertheless, transcellular active Ca^{2+} transport may become significant in low dietary Ca^{2+} intake condition.

The present results (Fig. 2), together with previous findings by Tudpor et al. (36), suggested that chronic PRL effects on the intestinal Ca^{2+} absorption (Step 1) were seen at plasma PRL concentrations ~ 100 – 200 ng/ml, as in pregnancy and lactation. In contrast, the acute stimulatory effect on Ca^{2+} absorption required ~ 400 ng/ml PRL, as attained during the suckling-induced PRL surge (Fig. 2F). This acute effect, which was superimposed on chronic PRL action, was responsible for the Step 2 transcellular and paracellular Ca^{2+} transport and could also be mimicked in vitro in Caco-2 monolayer (Fig. 1B) and in 600–800 ng/ml PRL-exposed duodenum of L7 rats (Fig. 2E). Moreover, the significant correlation between the suckling-enhanced transcellular Ca^{2+} flux and milk volume was suggestive of the orchestrating role of PRL in matching Ca^{2+} absorption to Ca^{2+} loss in milk.

Regarding the mechanisms of acute PRL action, it has been reported that PRL stimulated PMCA activity in purified basolateral membrane of the rat duodenum, thereby enhancing Ca^{2+} extrusion (8). Moreover, consistent with the previous report (35), the Step 2 transcellular Ca^{2+} transport also resulted from the PRL-enhanced apical Ca^{2+} entry, particularly via $\text{Ca}_v1.3$. However, further in vivo investigation is required to confirm that the mechanisms by which PRL enhanced apical Ca^{2+} entry in the Step 1 and Step 2 transcellular Ca^{2+} transport were different, i.e., TRPV5/6 for Step 1 and $\text{Ca}_v1.3$ for Step 2. On the other hand, the Step 2 paracellular Ca^{2+} transport was likely to be dependent on phosphorylation of certain charge-selective claudins. Ikari et al. (19) revealed that serine phosphorylation of the kidney-specific claudin-16 by protein kinase A was crucial for Mg^{2+} reabsorption in the thick ascending limb of the loop of Henle. Although a number of claudins, e.g., claudin-3, -8, and -10, were cation selective (1, 39, 40), and some, e.g., claudin-2 and -12, were required for the $1,25(\text{OH})_2\text{D}_3$ -stimulated Ca^{2+} absorption (15), only claudin-15 was essential for the PRL-induced increases in the paracellular Ca^{2+} transport, cation selectivity, and Ca^{2+} permeability as well as the decrease in transepithelial resistance (Fig. 6, A–D). Since claudin-15 possesses several negatively charged amino acids on the first extracellular loop for cation permeability (39), it was not surprising to find that P_{Na} , but not P_{Cl} , was markedly increased by PRL. The significance of claudin-15 was demonstrated recently in claudin-15-knockout mice that exhibited a decrease in the paracellular ion permeability and enlargement of the small intestine (33). Since claudin-15 mRNA was not upregulated in the long-term PRL-exposed duodenal epithelial cells, claudin-15 may be important only for the Step 2 paracellular Ca^{2+} transport or nongenomic PRL action. Alternatively, long-term exposure to PRL may increase protein expression and/or activity of claudin-15 without an increase in claudin-15 mRNA expression.

Since we found previously that PRLR-L knockdown diminished the transcellular Ca^{2+} transport in Caco-2 monolayer (34), and PRLR-L knockdown in this study completely abolished the PRL-induced claudin-15 phosphorylation, it was likely that the PRL-enhanced Ca^{2+} absorption was mediated by PRLR-L despite the fact that both PRLR-L and -S were expressed in duodenal epithelial cells of pregnant and lactating rats and in Caco-2 monolayer. This was consistent with the PRL-induced increases in lactogenesis and electrolyte transport in mammary epithelial cells (25). Although PRL actions in

Step 1 Ca^{2+} absorption involved de novo gene transcription and those in Step 2 were, in contrast, nongenomic, their signal transduction should be quite similar since the signals presumably originated from the same receptor isoform. Step 2 transcellular Ca^{2+} absorption was mediated by PI3K, PKC, and ROCK, whereas claudin-15 phosphorylation and paracellular Ca^{2+} transport were dependent only on PI3K and ROCK, similar to that in the duodenum of nulliparous rat (20). However, PI3K, PKC, and ROCK are closely associated since both PKC and ROCK are downstream from PI3K (17). The ROCK-related pathway of PRL signaling was also reported in endothelial cells (24), whereas the PKC pathway mediated PRL actions in adrenocortical cells (21) and cholangiocytes (32). In the small intestine, PKC activation is known to be important for both apical Ca^{2+} entry and basolateral Ca^{2+} extrusion (22), presumably via phosphorylation of TRPV6 and PMCA, respectively. Interestingly, PRL signaling in the small intestine was independent of Janus kinase 2, an important mediator of PRL in mammary epithelial cells (43).

In conclusion, we elucidated the complexity of PRL-induced stimulation of intestinal Ca^{2+} absorption in pregnancy and lactation and demonstrated a two-step stimulation in suckling. Long-term PRL exposure led to adaptations of intestinal cells at genomic levels to upregulate several Ca^{2+} transport genes, thereby raising the “baseline” Ca^{2+} absorption (Step 1). During suckling, transient PRL surge further increased Ca^{2+} absorption (Step 2) within 30 min to the maximal capacity, which apparently matched Ca^{2+} loss in milk. The PRL-stimulated Step 2 Ca^{2+} transport occurred via both transcellular and paracellular pathways in a PI3K/PKC/ROCK-dependent manner in part by increasing apical Ca^{2+} uptake and claudin-15 phosphorylation, respectively. Thus, the present findings provide significant fundamental knowledge for physiological, nutritional, and medical scientists to gain more insight into Ca^{2+} homeostasis in the reproductive periods and for further development of Ca^{2+} -fortified diet to help alleviate negative Ca^{2+} balance. The finding that suckling-induced PRL surge considerably increases the efficiency of intestinal Ca^{2+} absorption may provide direction for health policy. For example, Ca^{2+} supplementation at 15–30 min prior to breastfeeding may best benefit fetal and maternal Ca^{2+} metabolism while reducing the incidence of pregnancy-induced osteoporosis.

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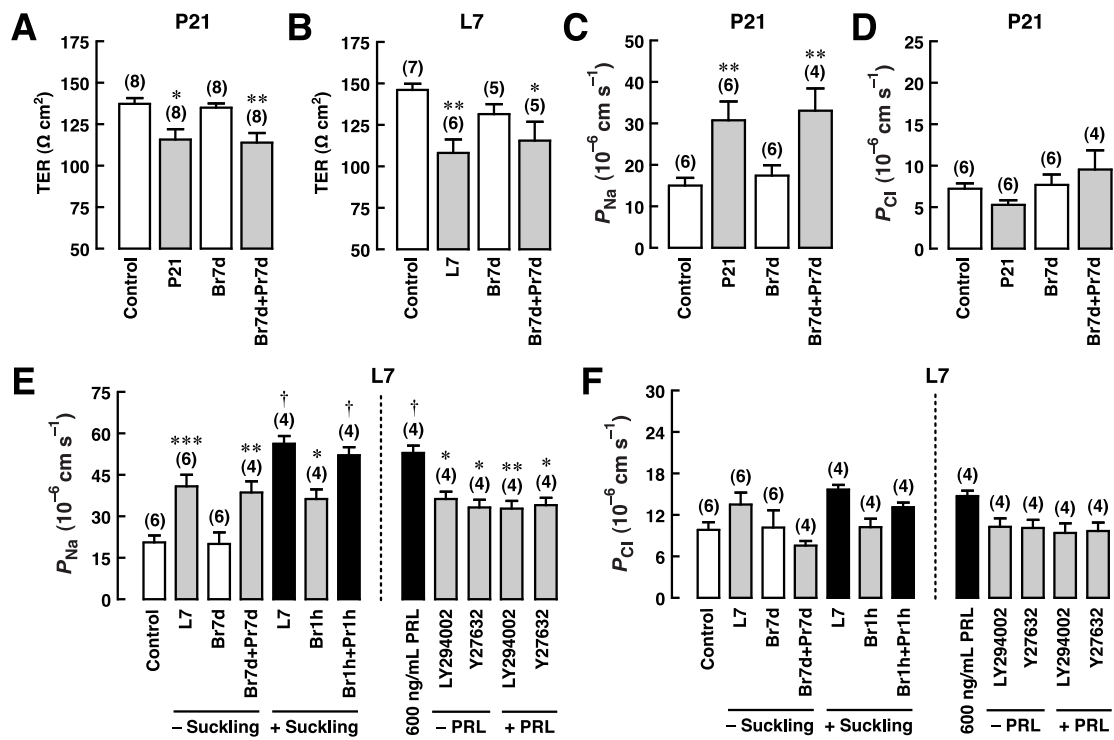


Figure S1: Charoenphandhu et al.

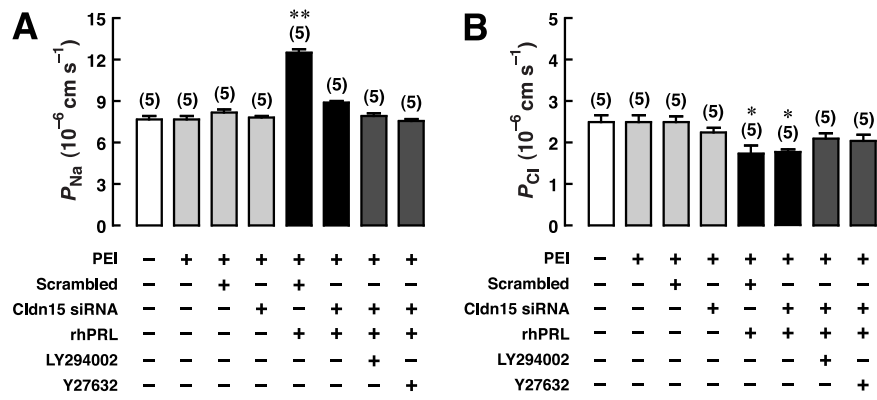


Figure S2: Charoenphandhu et al.

Legends to supplemental figures

Figure S1. (A–B) Transepithelial resistance (TER) in the duodenum of P21 and L7 rats. (C–F) P_{Na} and P_{Cl} in the duodenum of P21, L7 and suckling rats. Rats were administered with Br7d or Br1h to suppress chronic PRL secretion (with or without Pr7d supplementation) and suckling-induced PRL surge (with or without Pr1h supplementation), respectively. PRL signalings were investigated by exposing 600 ng/mL PRL-treated L7 duodenal tissues to various inhibitors. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control group. [†] $P < 0.05$ compared with L7 without suckling. Numbers in parentheses represent the number of rats.

Figure S2. (A–B) P_{Na} and P_{Cl} in claudin-15 siRNA-treated Caco-2 monolayers directly exposed to 600 ng/mL rhPRL. In some experiments, claudin-15 knockdown monolayers were also incubated with rhPRL plus 75 $\mu\text{mol/L}$ LY294002 or 1 $\mu\text{mol/L}$ Y27632. * $P < 0.05$, ** $P < 0.01$ compared with control (white column). Numbers in parentheses represent the number of independent Snapwells.

Direct stimulation of the transcellular and paracellular calcium transport in the rat cecum by prolactin

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Abstract Prolactin (PRL) is reported to stimulate calcium absorption in the rat's small intestine. However, little is known regarding its effects on the cecum, a part of the large intestine with the highest rate of intestinal calcium transport. We demonstrated herein by quantitative real-time polymerase chain reaction and Western blot analysis that the cecum could be a target organ of PRL since cecal epithelial cells strongly expressed PRL receptors. In Ussing chamber experiments, PRL enhanced the transcellular cecal calcium absorption in a biphasic dose–response manner. PRL also increased the paracellular calcium permeability and passive calcium transport in the cecum, which could be explained by the PRL-induced decrease in transepithelial resistance and increase in cation selectivity of the cecal epithelium. PRL actions in the cecum were abolished by inhibitors of phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and RhoA-associated coiled-coil forming kinase (ROCK), but not inhibitors of gene transcription and protein biosynthesis. In conclusion, PRL directly enhanced the transcellular and paracellular calcium transport in the rat

cecum through the nongenomic signaling pathways involving PI3K, PKC, and ROCK.

Keywords Calcium absorption · Dilution potential · Paracellular transport · Prolactin receptor · Transcellular transport · Ussing chamber

Introduction

During pregnancy and lactation, prolactin (PRL) serves as a calcium-regulating hormone which markedly stimulates intestinal calcium absorption to help alleviate negative calcium balance due to massive calcium loss for fetal growth and milk production [5]. The PRL-enhanced calcium absorption has been intensively investigated in the small intestine [6, 25], especially in the duodenum, where the transcellular active calcium transport is prominent [11, 29]. However, the effects of PRL on the large intestine as well as its mechanisms were largely unknown.

It is widely known that, in monogastric herbivores, rats and humans, the cecum is for absorption of fluids and salts and is the site for luminal microfloral production of short-chain fatty acids, folate, and vitamin K [16, 32, 33]. Studies on the intestinal calcium absorption demonstrated that, besides the duodenum, the proximal part of the large intestine, particularly the cecum, is another important site for the transcellular active calcium transport [40]. Moreover, experiments in rats showed that when compared to other intestinal segments, cecum has the highest rate of calcium transport [28, 39]. Under normal conditions, it was suggested that the cecal epithelium absorbs a significant amount of free calcium which is released during microbial fermentation of the cecal contents through production of short-chain fatty acids and other acidic molecules, such as lactic acid, butyric acid, and

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succinic acid, as well as calcium released from the degradation of dietary fiber [10, 36]. Accumulation of free calcium in the cecal lumen may also be enough to increase the paracellular passive calcium transport [28]. Since PRL plays an important role in supplying additional calcium to the body, we postulated that it may increase the cecal calcium absorption via both transcellular and paracellular routes as seen in the small intestine [5].

Generally, the transcellular calcium transport is a three-step metabolically energized process, consisting of the apical uptake via the transient receptor potential vanilloid family calcium channel 5 and 6 (TRPV5/6) and L-type calcium channel $\text{Ca}_v1.3$, cytoplasmic translocation in a calbindin- D_{9k} -bound form, and the basolateral extrusion via the plasma membrane Ca^{2+} -ATPase 1b (PMCA1b) and $\text{Na}^+/\text{Ca}^{2+}$ exchanger 1 (NCX1) [20, 30, 49]. In contrast, the paracellular passive calcium transport is dependent on the transepithelial calcium gradient and is absent when both sides of the epithelium have equal calcium concentration [29]. Paracellular calcium movement is regulated by the tight junction, which contains several charge-selective proteins, particularly claudin-2, claudin-3, and claudin-12, arranged in the arrays of channel-like paracellular pores [14, 30]. Besides, the integrity of the tight junction is regulated by tight junction proteins, such as zonula occludens-1 (ZO-1) and occludin [50].

It is apparent that the paracellular calcium transport is predominant in the small intestine [10, 29]. However, when calcium demand is markedly increased, such as during pregnancy and lactation, contribution from the transcellular transport component to the total calcium absorption becomes more significant, in part, as a result of the stimulatory action of PRL [3, 5]. Recently, we demonstrated in the duodenum and Caco-2 monolayer that PRL enhanced both transcellular active and paracellular passive calcium transport through the phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and RhoA-associated coiled-coil forming kinase (ROCK) pathways [25, 48, 49]. However, PRL signaling in the large intestine, including the cecum, had never been investigated.

Therefore, the objectives of the present study were (a) to demonstrate that the cecum was a target organ of PRL, (b) to investigate the effects of PRL on the cecal calcium transport, and (c) to investigate the mechanisms of the PRL-enhanced calcium absorption in the cecum.

Materials and methods

Animals

Female Sprague–Dawley rats (8 weeks old, weighing 180–200 g) were obtained from the National Laboratory Animal

Centre, Thailand. They were placed in hanging stainless steel cages, fed with standard pellets containing 1% wt/wt calcium and 100 IU vitamin D per 100 g of diet (Perfect Companion, Bangkok, Thailand), and provided with distilled water ad libitum under 12:12 h light/dark cycle. The room had temperature of 20–25°C, humidity of 50–60%, and average illuminance of 150–200 lux in the daytime. Body weight and food intake were recorded daily. This study has been approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University, Bangkok, Thailand.

Tissue preparation

After 7-day acclimatization, the rat was anesthetized by administering 50 mg/kg sodium pentobarbitone i.p. (Abbott, North Chicago, IL, USA). Thereafter, median laparotomy was performed. Duodenum (10 cm), proximal and distal jejunum (10 cm each), ileum (8 cm), cecum (4 cm), and proximal and distal colon (8 cm each) were removed and cut longitudinally to expose the mucosa. In the calcium transport experiments, the muscularis proper was stripped off before the tissue was mounted in a modified Ussing chamber as described previously [25]. The tissue was incubated for 20 min in the chamber before the 60-min experiment was carried out. As for the mRNA and protein expression studies, epithelial cells were collected by scraping the mucosal surface with an ice-cold glass slide [8].

Total RNA preparation and cDNA synthesis

Total RNA was extracted from mucosal scrapings by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified with RNeasy Mini kit (Qiagen, Valencia, CA, USA). Purity and integrity of RNA were determined by 260/280 nm absorbance and denaturing agarose gel electrophoresis, respectively [8]. One microgram of the total RNA was then reverse-transcribed to cDNA with the oligo-dT₂₀ primer and the iScript kit (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control gene to check the consistency of the reverse transcription.

Quantitative real-time PCR and sequencing

Primers used in this study were shown in Table 1. Quantitative real-time polymerase chain reaction (qRT-PCR) and melting curve analysis were performed by the Bio-rad MiniOpticon with the iQ SYBR Green SuperMix (Bio-rad) as previously described [8]. Relative expression of PRL receptor (PRLR) over GAPDH was calculated from the threshold cycle (C_t) values by using $2^{-\Delta C_t}$ method. The

Table 1 *Rattus norvegicus* oligonucleotide sequences used in qRT-PCR experiments

Name	Accession no.	Primer (forward/reverse)	Product length (bp)
Prolactin receptors			
PRLR-S	NM_012630	5'-TTCTACCACCATCGCAAC-3' 5'-CTGATCTCGTTTGTCATTGAG-3'	120
PRLR-L	NM_001034111	5'-TCAAGCAACCGCAGACTC-3' 5'-CAGTTTAGCCAATCGTTCCA-3'	107
Transcellular genes			
TRPV5	NM_053787	5'-CTTACGGGTGAACACCACCA-3' 5'-TTGCAGAACCACAGAGCCTCTA-3'	163
TRPV6	NM_053686	5'-ATCCGCCGCTATGCAC-3' 5'-AGTTTTTCTGGTCACTGTTTTTG-3'	80
Ca _v 1.3	NM_017298	5'-TCAGCGTCAGTGTGTGGAATA-3' 5'-CGAAAGGCGAGGAGTTTCA-3'	110
Calbindin-D _{9k}	X_16635	5'-CCCGAAGAAATGAAGAGCATTTT-3' 5'-TTCTCCATCACCGTTCTTATCCA-3'	174
PMCA _{1b}	NM_053311	5'-CGCCATCTTCTGCACAATT-3' 5'-CAGCCATTGTTCTATTGAAAGTTC-3'	109
NCX1	NM_019268	5'-GTTGTGTTTCGCTTGGGTTGC-3' 5'-CGTGGGAGTTGACTACTTTC-3'	163
Paracellular genes			
Claudin-2	NM_001106846	5'-GCTGCTGAGGGTAGAATGA-3' 5'-GCTCGCTTGATAAGTGTC-3'	107
Claudin-3	NM_031700	5'-GCACCCACCAAGATCCTCTA-3' 5'-AGGCTGTCTGTCTCTTCCA-3'	246
Claudin-12	XM_001067932	5'-CCTTCAAGTCTTCGGTGCC-3' 5'-CAGGAGGATGGGAGTACAG-3'	312
ZO-1	XM_218747	5'-GTATCCGATTGTTGTGTTCC-3' 5'-TCACTTGTAGCACCATCCGC-3'	270
Occludin	NM_031329	5'-CACGTTTCGACCAATGC-3' 5'-CCCGTTCCATAGGCTC-3'	188
Housekeeping gene			
GAPDH	NM_017008	5'-AGTCTACTGGCGTCTTCAC-3' 5'-TCATATTCTCGTGGTTCAC-3'	133

PRLR-S short isoform of prolactin receptor, *PRLR-L* long isoform of prolactin receptor, *TRPV5* transient receptor potential vanilloid family Ca²⁺ channel 5, *TRPV6* transient receptor potential vanilloid family Ca²⁺ channel 6, *Ca_v1.3* voltage-dependent L-type Ca²⁺ channel 1.3, *PMCA_{1b}* plasma membrane Ca²⁺-ATPase isoform 1b, *NCX1* Na⁺/Ca²⁺ exchanger 1, *ZO-1* zonula occludens-1, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

PCR products were also visualized on a 1.5% agarose gel stained with 1.0 µg/mL ethidium bromide under a UV transilluminator (Alpha Innotech, San Leandro, CA, USA). After electrophoresis, all PCR products were purified from a gel by the HiYield Gel/PCR DNA Extraction kit (Real Biotech Corporation, Taipei, Taiwan) and sequenced by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Western blot analysis

As previously described [25], scraped mucosal cells were lysed in lysis buffer (0.5 mmol/L Tris pH 7.5, 1.5 mol/L NaCl, 10% NP-40, 5% DOC, 10 mmol/L Na ethylenediaminetetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/mL pepstatin A; all purchased from Sigma, St. Louis, MO, USA). Lysates were sonicated at 4°C and centrifuged at 20,000×g for 10 min. One hundred micrograms proteins were separated by sodium dodecyl sulfate polyacrylamide

gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK) by electroblotting. Membranes were blocked at 25°C for 4 h with 5% nonfat milk and were probed overnight at 4°C with 1:500 rabbit antirat PRLR polyclonal antibodies (catalog no. sc-30225; Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against the conserved extracellular domain. Membranes were later reprobed with 1:5,000 mouse antirat β-actin monoclonal antibodies (catalog no. sc-47778; Santa Cruz). After 2-h incubation at 25°C with 1:20,000 goat antirabbit (catalog no. sc-2004) or antimouse (catalog no. sc-2005) secondary antibodies (Santa Cruz), blots were visualized by enhanced chemiluminescence kit (Amersham).

Bathing solution for Ussing chamber study

The bathing solution continuously gassed with humidified 5% CO₂ in 95% O₂, contained (in mmol/L) 118 NaCl, 4.7 KCl, 1.1 MgCl₂, 1.25 CaCl₂, 23 NaHCO₃, 12 D-glucose, and 2 mannitol (all purchased from Sigma). The solution

was maintained at 37°C, pH 7.4, and had an osmolality of 290–293 mmol kg⁻¹ water as measured by a freezing point-based osmometer (model 3320; Advanced Instruments, Norwood, MA, USA).

Measurement of epithelial electrical parameters

Three electrical parameters, i.e., potential difference (PD), short-circuit current (*I*_{sc}), and transepithelial resistance (TER), were determined according to the method of Charoenphandhu et al. [6]. In brief, a pair of Ag/AgCl electrodes connected to agar bridges was placed near each surface of the mounted tissue for PD measurement. The other ends of the PD-sensing electrodes were connected to the EVC-4000 pre-amplifier (World Precision Instruments, Sarasota, FL, USA) and finally to a PowerLab 4/30 operated with the software Chart 5.2.2 for Mac OS X (ADInstruments, Colorado Springs, CO, USA). Another pair of Ag/AgCl electrodes was placed at the end of each hemichamber to supply external current, which was also measured by a PowerLab 4/30 connected in series to the EVC-4000 current-generating unit. TER and conductance (*G*; *G*=1/TER) were calculated by Ohm's equation. Fluid resistance was automatically subtracted by the EVC-4000 system. In some experiments, *I*_{sc} was continuously applied to nullify PD, i.e., the experiments were performed under short-circuit condition. Unless otherwise specified, the experiments were normally performed under open-circuit condition since the absence of PD did not significantly affect calcium flux (Supplemental Figure S1). In other words, the voltage-dependent calcium transport in the cecum could be considered negligible, similar to that observed in the duodenum [6].

Measurement of calcium flux

Transepithelial calcium flux was determined by the method of Charoenphandhu et al. [6]. In brief, after 20-min incubation, Ussing chamber was filled on one side with ⁴⁵CaCl₂-containing bathing solution (specific activity of 500 mCi/mol; Amersham). Unidirectional flux (*J*_{H→C}) from the hot side (H) to the cold side (C) was calculated with Eqs. 1 and 2.

$$J_{H \rightarrow C} = R_{H \rightarrow C} / (S_H \times A) \quad (1)$$

$$S_H = C_H / C_T \quad (2)$$

where *R*_{H→C} was the rate of tracer appearance in the cold side (counts per minute per hour), *S*_H was the specific activity in the hot side (counts per minute per nanomole) and did not vary with time (a constant value), *A* was the surface area of the tissue (0.69 cm²), *C*_H was the radioactivity in the hot side (counts per minute), and *C*_T was the total calcium in the hot side (nanomole).

Radioactivity of ⁴⁵Ca in counts per minute was analyzed by liquid scintillation spectrophotometry (model Tri-Carb 3100; Packard Instruments, Meriden, CT, USA). Total calcium concentration in the hot side was determined by atomic absorption spectrophotometry (model SpectrAA-300; Varian Techtron, Springvale, Australia).

Calcium fluxes in the absence of transepithelial calcium gradient, i.e., bathing solution in both hemichambers containing equal calcium concentration, represented the active calcium transport [6]. The calcium gradient-dependent paracellular passive fluxes were measured by determining the calcium fluxes in the presence of varying apical calcium concentrations [48], i.e., 1.25, 2.5, 5, 10, and 20 mmol/L (*n*=4 per each concentration).

Unless otherwise specified, the measured calcium fluxes represented calcium transport in mucosa-to-serosa direction. The serosa-to-mucosa calcium flux in the cecum was relatively small and had no significant influence on the net calcium transport (Supplemental Figure S2). In addition, PRL did not enhance calcium transport in serosa-to-mucosa direction (Supplemental Figure S2).

Determination of the epithelial permeability and Na⁺/Cl⁻ selectivity

Permeability of sodium (*P*_{Na}) and chloride (*P*_{Cl}) which are indicative of the epithelial charge selectivity were measured by the dilution potential technique [21, 26]. The mounted cecum was equilibrated for 20 min in bathing solution containing 145 mmol/L NaCl before the apical solution was replaced with 72.5 mmol/L NaCl-containing solution. Osmolality of the solution was maintained by an equivalent amount of mannitol. Changes in the electrical parameters before and after fluid replacement were continuously recorded by PowerLab 4/30 until stable. The ion permeability ratio (*P*_{Na}/*P*_{Cl}) was calculated from the dilution potential (*V*_δ) using the modified Goldman–Hodgkin–Katz equation (Eq. 3).

$$\rho = (\phi - e^v) / (\phi e^v - 1) \quad (3)$$

where $\rho = P_{Na}/P_{Cl}$; $\phi = C_b/C_a$; $v = FV_\delta/RT$; *P*_{Na} was the absolute permeability of sodium; *P*_{Cl} was the absolute permeability of chloride; *C*_a was the mucosal NaCl concentration; *C*_b was the serosal NaCl concentration; and *R*, *T*, and *F* had their conventional meanings.

Thereafter, *P*_{Na} and *P*_{Cl} were calculated from the conductance (*G*) and *P*_{Na}/*P*_{Cl} using Kimizuka–Koketsu equations [31], as follows:

$$P_{Na} = \frac{GRT}{C_a F^2} \times \frac{\rho}{1 + \rho} \quad (4)$$

$$P_{Cl} = P_{Na} / \rho \quad (5)$$

The paracellular calcium permeability (P_{Ca}) was calculated from Eq. 6 [48].

$$P_{Ca} = J_{Ca} / \Delta C \quad (6)$$

where J_{Ca} was the paracellular passive calcium flux and ΔC was the difference between the apical and basolateral calcium concentrations.

Experimental protocol

To demonstrate the effects of PRL on calcium transport, electrical parameters, and epithelial permeability, the mounted cecal tissue was directly exposed on the serosal side to 200, 400, 600, 800, or 1,000 ng/mL PRL (catalog no. L6520; Sigma) during the 80-min experiment. In some experiments, the most efficient dose of PRL (i.e., 800 ng/mL) plus RNA polymerase II inhibitor (50 μ mol/L 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB); Calbiochem, San Diego, CA, USA), protein synthesis inhibitor (70 μ mol/L cycloheximide (CCH);

Sigma), Janus kinase (JAK) 2 inhibitor (50 μ mol/L AG490; Calbiochem), PI3K inhibitors (75 μ mol/L LY294002 or 200 nmol/L wortmannin; Tocris Bioscience, Bristol, UK), pan-specific PKC inhibitor (1 μ mol/L GF109203X; A.G. Scientific, San Diego, CA, USA), selective ROCK inhibitor (1 μ mol/L Y27632; Calbiochem), or myristoylated protein kinase A inhibitor 14–22 amide (10 μ mol/L PKAI 14–22; Calbiochem) were added to the serosal solution to identify possible PRL signaling pathways. Wortmannin was more potent but less specific than LY294002 [18, 23]. PKAI 14–22 was first dissolved in distilled water, while other inhibitors were dissolved in DMSO (Sigma). Concentration of dimethyl sulfoxide (DMSO) in the bathing solution was 0.3% vol/vol, which did not affect the viability of cells [6]. To verify that PRL was able to stimulate the transcellular calcium transport, the cecal tissue was directly exposed to inhibitors of apical calcium uptake (50 μ mol/L ruthenium red; Sigma) or calmodulin-dependent PMCA (100 μ mol/L trifluoperazine; TFP; Sigma). Polyclonal antibody against PRLR (1 μ g/mL; catalog no. sc-30225; Santa Cruz) was directly

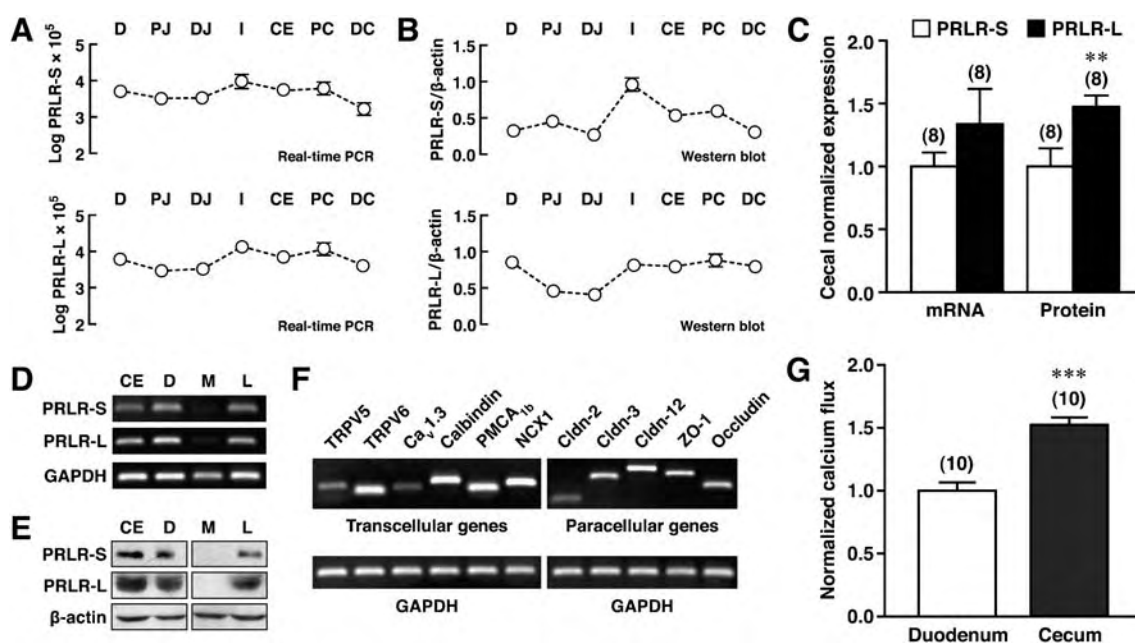


Fig. 1 **a, b** Expression of short (-S) and long (-L) isoforms of PRLRs in the duodenum (D), proximal (PJ) and distal jejunum (DJ), ileum (I), cecum (CE), and proximal (PC) and distal colon (DC) as demonstrated by qRT-PCR (**a**; $n=8$ per segment) and Western blot analysis (**b**; $n=8$ per segment). qRT-PCR data expressed as log means $\pm$ SE were normalized by GAPDH expression, while PRLR protein expression was normalized by β -actin expression. **c** Normalized expression of PRLR-S and PRLR-L in the cecum of female rats. PRLR-L expression appears as fold difference from PRLR-S expression, while setting PRLR-S expression as 1. ** $p<0.01$ PRLR-S expression vs. PRLR-L expression. **d** Representative electrophoretic bands of PRLR-S and PRLR-L mRNAs in duodenum (D) and cecum

(CE). Gastrocnemius muscle (M) and liver (L) were used as negative and positive controls, respectively. **e** Representative bands of PRLR-S and PRLR-L proteins in duodenum (D) and cecum (CE). Gastrocnemius muscle (M) and liver (L) were negative and positive controls, respectively. **f** Electrophoretic bands of genes related to the transcellular and paracellular calcium transport in the cecum ($n=10$). GAPDH was used as a housekeeping gene. **g** Normalized transepithelial calcium fluxes in the duodenum and cecum as determined by Ussing chamber technique. Calcium concentration on both sides was 1.25 mmol/L. *** $p<0.001$ cecal flux vs. duodenal flux. Numbers in parentheses (**c** and **g**) represent the number of experimental animals

added in bathing solution to confirm that PRLR mediated PRL actions.

Statistical analysis

Results are expressed as means $\pm$ standard error (SE). Two sets of data were compared by using the unpaired Student's *t* test. Multiple comparisons were performed by one-way analysis of variance with Dunnett's multiple comparison test. Linear regression was used to demonstrate the relationships between mucosal calcium and paracellular calcium flux, and mucosal calcium and TER. The level of significance for all statistical tests was $p < 0.05$. All data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA, USA).

Results

Cecal epithelial cells expressed PRLRs and essential calcium transporters

As shown in Fig. 1a, b, both short (-S) and long (-L) isoforms of PRLR mRNAs and proteins were expressed in the duodenum, proximal and distal jejunum, ileum, cecum, and proximal and distal colon of rats. In the cecum (Fig. 1c–e), PRLR-L was more abundant than PRLR-S. Although calcium absorption has been demonstrated in cecum [28], the expression of calcium transport genes has not been completely investigated in cecal epithelial cells. Therefore, we used PCR to show that cecal cells strongly expressed all essential genes required for the transcellular calcium transport, i.e., TRPV5, TRPV6, $\text{Ca}_v1.3$, calbindin- D_{9k} , PMCA $_{1b}$, and NCX1, and for the paracellular calcium transport, i.e., claudin-2, claudin-3, claudin-12, ZO-1, and occludin (Fig. 1f). Thus, the results suggested that the cecal epithelium of female rats could absorb calcium via both transcellular and paracellular pathways. Calcium transport in the cecum may be of importance since the cecal calcium flux was ~ 1.5 -fold higher than that in the duodenum even in the absence of calcium concentration gradient (Fig. 1g).

PRL stimulated the cecal calcium transport in a biphasic dose–response manner

Dose–response study demonstrated that high physiological concentrations of PRL of 600 and 800 ng/mL, which were comparable to the levels observed during suckling in lactating rats [1], markedly enhanced the transcellular calcium transport by ~ 1.5 - and ~ 2 -fold, respectively, while 200 and 400 ng/mL PRL were without effects (Fig. 2a). Interestingly, the pathological concentration of 1,000 ng/mL,

as seen in prolactinomas, only slightly increased calcium transport in the cecum (Fig. 2a). Since such PRL effect was not observed in the presence of apical calcium uptake inhibitor (50 $\mu\text{mol/L}$ ruthenium red) or basolateral calcium extrusion inhibitor (100 $\mu\text{mol/L}$ trifluoperazine), the PRL-stimulated calcium transport in this experiment was confirmed to occur via the transcellular pathway (Fig. 2b). The fact that the PRL-enhanced cecal calcium transport occurred rapidly within 60 min after exposure and was not abolished by inhibitors of gene transcription (50 $\mu\text{mol/L}$ DRB) or protein biosynthesis (70 $\mu\text{mol/L}$ cycloheximide; Fig. 2b), suggesting that the stimulatory actions of PRL was exerted via the nongenomic signaling pathways. Direct exposure to DRB and cycloheximide alone did not affect the basal rate of the transcellular

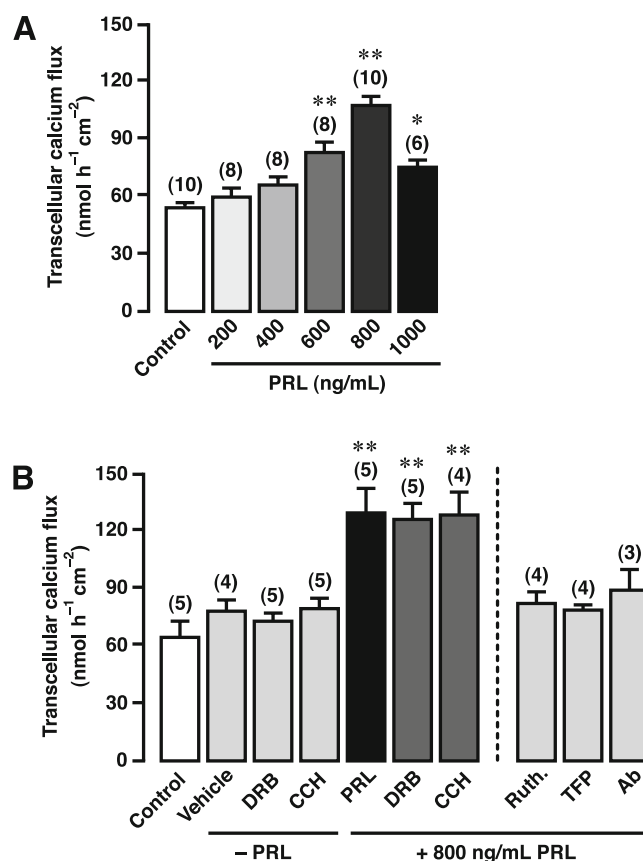


Fig. 2 **a** Dose-dependent effect of PRL on the transcellular calcium transport in the cecum. **b** Transcellular calcium transport in the cecum directly exposed to inhibitors of RNA polymerase II (50 $\mu\text{mol/L}$ DRB), protein synthesis (70 $\mu\text{mol/L}$ CCH), apical calcium uptake (50 $\mu\text{mol/L}$ ruthenium red (*Ruth.*); apical side), basolateral calcium extrusion (100 $\mu\text{mol/L}$ trifluoperazine (*TFP*); basolateral side), or 1 $\mu\text{g/mL}$ PRLR antibody (*Ab*; basolateral side) with (+PRL) and without (–PRL) 800 ng/mL PRL (maximal effective concentration). DMSO was a vehicle for preparation of DRB and CCH. Numbers in parentheses represent the number of animals. * $p < 0.05$, ** $p < 0.01$ compared with its respective control group

Table 2 Effects of PRL on epithelial electrical parameters

Condition	Number	Electrical parameters		
		PD (mV)	Isc (μAcm^{-2})	TER ($\Omega\text{ cm}^2$)
Control	7	5.43 $\pm$ 0.67	55.23 $\pm$ 4.15	102.90 $\pm$ 11.53
PRL				
200 ng/mL	5	3.67 $\pm$ 0.39	52.20 $\pm$ 5.50	71.90 $\pm$ 3.39
400 ng/mL	4	4.22 $\pm$ 0.69	55.21 $\pm$ 6.29	79.02 $\pm$ 11.04
600 ng/mL	4	3.97 $\pm$ 0.72	57.37 $\pm$ 8.06	68.23 $\pm$ 5.93*
800 ng/mL	8	4.26 $\pm$ 0.18	59.52 $\pm$ 1.22	69.41 $\pm$ 3.27*
1,000 ng/mL	5	4.02 $\pm$ 0.56	58.84 $\pm$ 9.52	69.91 $\pm$ 2.65*

Values are means $\pm$ SE. Cecum was directly exposed to 200, 400, 600, 800, or 1,000 ng/mL PRL. Mounted tissue was bathed on both sides with 1.25 mmol/L calcium-containing solution. The mucosal side was negative with respect to the serosal side. The experiments were performed under open-circuit condition interrupted by measuring the short-circuit current

* $p < 0.05$ compared with the control group

calcium transport (Fig. 2b). PRL-stimulated calcium transport was absent when the cecum was continuously exposed to 1 $\mu\text{g/mL}$ PRLR antibody, indicating that PRLR mediated PRL actions in this intestinal segment (Fig. 2b). Moreover, the decrease in TER by 600, 800, and 1,000 ng/mL PRL (Table 2) suggested the PRL-induced increase in paracellular permeability. Since 800 ng/mL was the maximal effective concentration of PRL for stimulation of calcium transport, this concentration was used in the subsequent experiments.

The PRL-enhanced transcellular calcium transport in cecum was mediated by PI3K, PKC, and ROCK signaling pathways

Previous investigations showed that PRL signaling in mammary epithelium was mediated by JAK2, whereas that in the duodenum and Caco-2 monolayer was mediated by PI3K, PKC, and ROCK [25, 48]. Moreover, an increase in intestinal calcium absorption via the transcellular route was also reported to be protein kinase A (PKA)-dependent [9, 30]. Herein, we found that the PRL-enhanced transcellular calcium transport in cecum was completely abolished by inhibitors of PI3K (75 $\mu\text{mol/L}$ LY294002 and 200 nmol/L wortmannin), PKC (1 $\mu\text{mol/L}$ GF109203X), and ROCK (1 $\mu\text{mol/L}$ Y27632), but not inhibitors of JAK2 (50 $\mu\text{mol/L}$ AG490) and PKA (10 $\mu\text{mol/L}$ PKAI 14–22; Fig. 3). Exposure to inhibitors alone did not affect the calcium flux. The data, therefore, indicated that PRL exerted its stimulatory actions on the cecal calcium transport via the PI3K, PKC, and ROCK signaling pathways.

PRL increased the paracellular calcium transport and calcium permeability in cecum via PI3K and ROCK pathways

Paracellular calcium transport and calcium permeability were evaluated in the presence of the transepithelial calcium gradient. Under normal conditions (Fig. 4a–d), the paracellular cecal calcium transport increased linearly with the mucosal calcium concentration ($r^2 = 0.86$). After exposure to PRL (Fig. 4a–d), the paracellular calcium

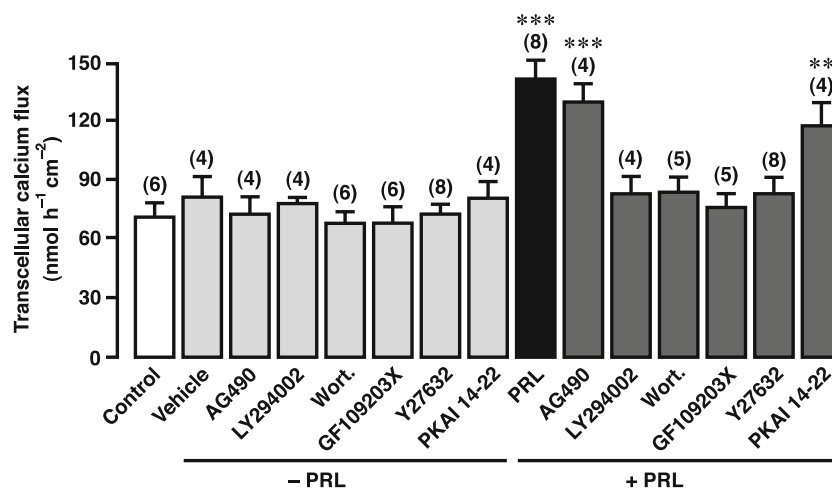


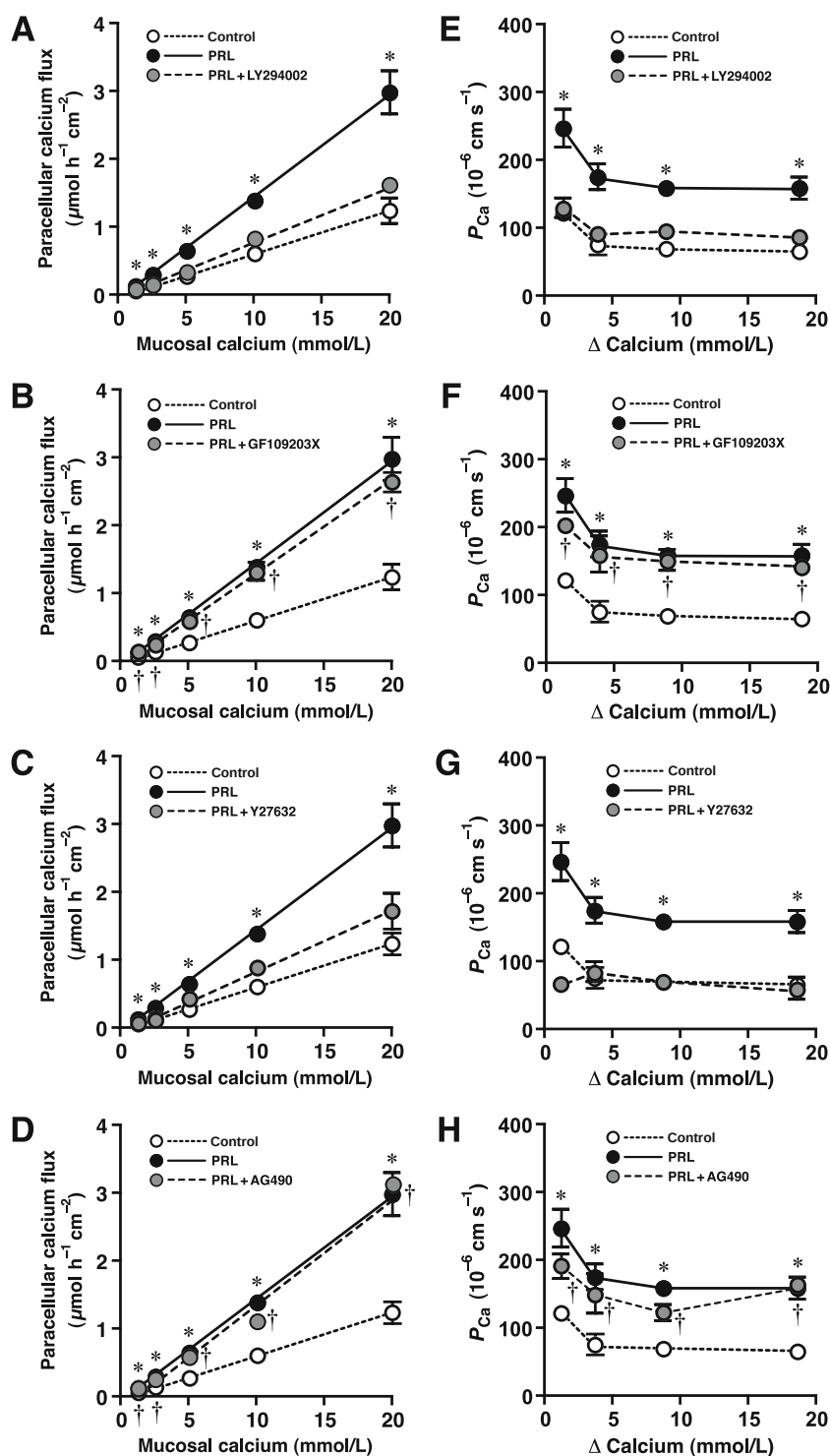
Fig. 3 Transcellular calcium transport in the cecum exposed to JAK2 inhibitor (50 $\mu\text{mol/L}$ AG490), PI3K inhibitors (75 $\mu\text{mol/L}$ LY294002 and 200 nmol/L wortmannin), PKC inhibitor (1 $\mu\text{mol/L}$ GF109203X), ROCK inhibitor (1 $\mu\text{mol/L}$ Y27632), or myristoylated PKA inhibitor 14–22 amide (10 $\mu\text{mol/L}$ PKAI 14–22) with (+PRL) and without

(-PRL) 800 ng/mL PRL. PKAI 14–22 was dissolved in water, whereas other inhibitors were dissolved in DMSO (vehicle). All experiments were performed under open-circuit condition. Numbers in parentheses represent the number of animals. ** $p < 0.01$, *** $p < 0.001$ compared with the control group

fluxes were markedly enhanced, and the correlation between mucosal calcium concentrations and calcium fluxes still showed linearity ($r^2=0.93$). The slopes of the regression lines were significantly increased by 2.4-fold, i.e., from the control value of 63.06 ± 5.95 to $152.62 \pm 9.67 \text{ cm h}^{-1}$ ($p < 0.001$). PRL also increased calcium

permeability of the cecal epithelium as demonstrated in Fig. 4e–h. The PRL-induced increases in the paracellular calcium transport and calcium permeability in the cecum were totally abolished by inhibitors of PI3K (Fig. 4a, e) and ROCK (Fig. 4c, g), but not by PKC inhibitor (Fig. 4b, f) or JAK2 inhibitor (Fig. 4d, h).

Fig. 4 **a–d** Paracellular calcium transport and **e–h** calcium permeability (P_{Ca}) in the cecum directly exposed to 800 ng/mL PRL in the presence and absence of PI3K inhibitor (75 $\mu\text{mol/L}$ LY294002), PKC inhibitor (1 $\mu\text{mol/L}$ GF109203X), ROCK inhibitor (1 $\mu\text{mol/L}$ Y27632), or JAK2 inhibitor (50 $\mu\text{mol/L}$ AG490). The mucosal side was bathed with solution containing various calcium concentrations, i.e., 1.25, 2.5, 5, 10, or 20 mmol/L ($n=4$ per each concentration; total 120 setups used), whereas the serosal solution had calcium concentration of 1.25 mmol/L. The same values of the control and PRL-treated groups are presented in **a–d** and **e–h** with those of the inhibitor-treated groups for clear comparisons. All experiments were performed under open-circuit condition. * $p < 0.001$ PRL vs. control. † $p < 0.001$ PRL+inhibitor vs. control



As seen in Fig. 5, TER values of normal cecal epithelia were linearly increased with mucosal calcium concentration ($r^2=0.99$) as a result of the calcium-induced conductance block of the paracellular pores, similar to that reported previously [47]. Hence, I_{sc} but not PD was gradually decreased when mucosal calcium concentration was increased (data not shown). In 800 ng/mL PRL-treated cecal epithelia, although the relationship between mucosal calcium and TER still showed linearity ($r^2=0.99$), PRL markedly reduced TER, and the slopes were reduced from the control value of 119.59 ± 3.03 to 71.01 ± 1.77 $\text{k}\Omega \text{ cm}^2 \text{ L mmol}^{-1}$ ($p<0.001$). Such PRL effects on TER were abolished by PI3K inhibitor (Fig. 5a) and ROCK inhibitor (Fig. 5c), but not by PKC inhibitor (Fig. 5b) or JAK2 inhibitor (Fig. 5d). Inhibitors alone were without effects. The results, therefore, suggested that PRL enhanced the paracellular cecal calcium transport by increasing the epithelial cation selectivity through PI3K and ROCK pathways.

PRL altered Na^+/Cl^- -selective property of the cecal epithelium

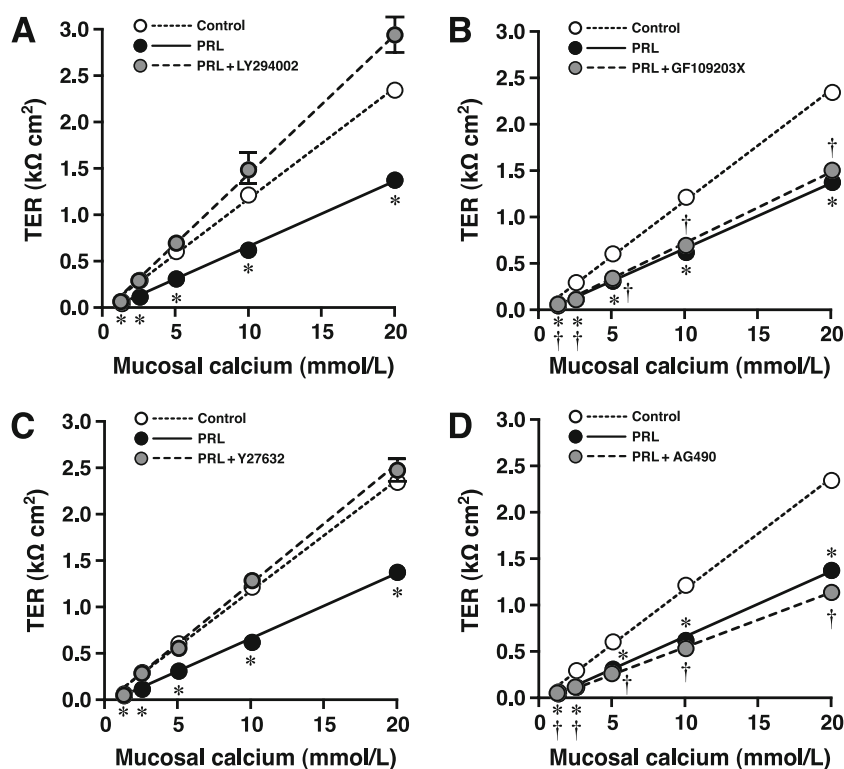
Since the paracellular calcium transport and epithelial calcium permeability could be increased by altering the charge-selective property of cecal epithelium, we determined the absolute permeability to sodium (P_{Na}) and chloride (P_{Cl}) as well as the $P_{\text{Na}}/P_{\text{Cl}}$ ratio, all of which are indicative of the paracellular charge selectivity [21, 47]. Under normal conditions (Fig. 6), the cecal epithelium exhibited a cation selectivity with P_{Na} being greater than

P_{Cl} ($p<0.001$), and $P_{\text{Na}}/P_{\text{Cl}}$ was ~ 2.0 . After exposure to 800 ng/mL PRL, P_{Na} was markedly increased by ~ 2.6 -fold (Fig. 6a) without any change in P_{Cl} (Fig. 6b), thereby raising $P_{\text{Na}}/P_{\text{Cl}}$ (Fig. 6c). The increases in P_{Na} and $P_{\text{Na}}/P_{\text{Cl}}$, similar to the increase in paracellular calcium transport, were diminished by PI3K and ROCK inhibitors, but not by PKC inhibitor (Fig. 6a, c). Inhibitors alone were without effects. The results, therefore, suggested that PRL enhanced the paracellular cecal calcium transport by increasing the epithelial cation selectivity through PI3K and ROCK pathways.

Discussion

In the present study, we demonstrated, for the first time, that PRL stimulated the transcellular and paracellular calcium transport in rat cecum. The presence of PRLRs in cecal epithelial cells and the ability of PRLR antibody to block PRL-stimulated calcium transport confirmed direct action of PRL on this intestinal segment. It was evident that the PRL-enhanced transcellular cecal calcium transport was dependent on PI3K, PKC, and ROCK, while the enhanced paracellular calcium transport required PI3K and ROCK, but not PKC. Such PRL signalings were nongenomic, since they were observed within 60 min after PRL exposure, and were not abolished by inhibitors of gene transcription (DRB) or protein biosynthesis (cycloheximide).

Fig. 5 Effect of 800 ng/mL PRL on the relationship between mucosal calcium concentration and transepithelial resistance (TER) of cecal epithelium ($n=4$ per each concentration) incubated with **a** PI3K inhibitor (75 $\mu\text{mol/L}$ LY294002), **b** PKC inhibitor (1 $\mu\text{mol/L}$ GF109203X), **c** ROCK inhibitor (1 $\mu\text{mol/L}$ Y27632), or **d** JAK2 inhibitor (50 $\mu\text{mol/L}$ AG490). The same control and PRL data are presented in **a–d** with those of the inhibitor-treated groups for clear comparisons. * $p<0.001$ PRL vs. control. † $p<0.001$ PRL+inhibitor vs. control



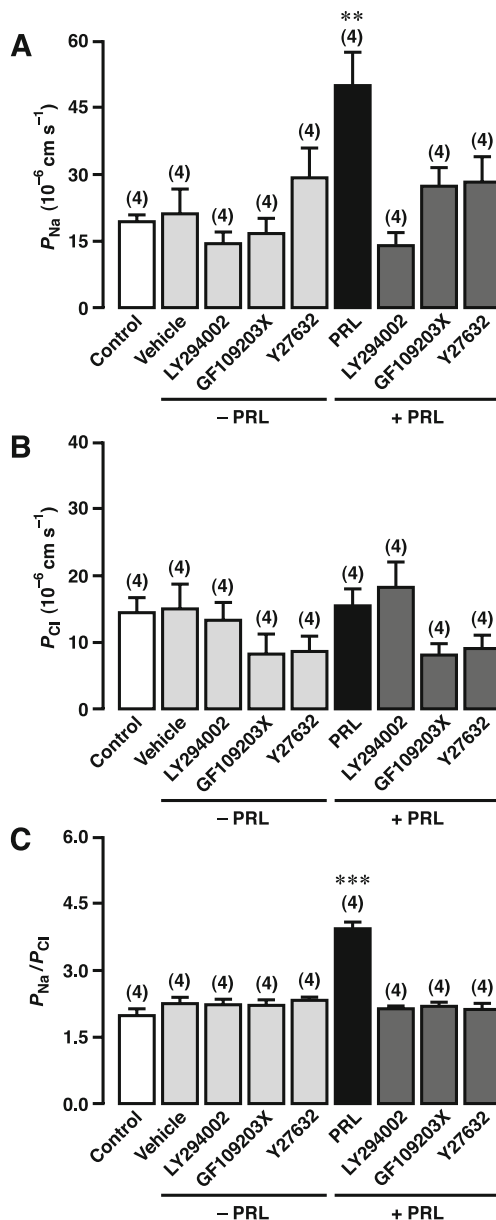


Fig. 6 **a** P_{Na} , **b** P_{Cl} , and **c** P_{Na}/P_{Cl} of the cecal epithelia exposed to PI3K inhibitor (75 $\mu\text{mol/L}$ LY294002), PKC inhibitor (1 $\mu\text{mol/L}$ GF109203X), or ROCK inhibitor (1 $\mu\text{mol/L}$ Y27632) with (+PRL) and without (–PRL) 800 ng/mL PRL. DMSO was used as vehicle for inhibitor preparation. Numbers in parentheses represent the number of animals. ** $p < 0.01$, *** $p < 0.001$ compared with the control group

Although most recent investigations have focused on calcium transport in the small intestine, the proximal large intestine, particularly the cecum, is a highly efficient site for calcium absorption [39]. Karch and Feldmeier reported that the rat cecum, as compared to other intestinal segments, had the highest rate of calcium absorption [28]. The present calcium flux study confirmed that the cecal calcium transport was greater than that in the duodenum by ~1.5-fold. Besides, the cecum which strongly expresses transcellular calcium

transporters (Fig. 1f) is another site with sizable transcellular calcium absorption [28]. In some mammals, e.g., mole rats, the transcellular active calcium transport exclusively occurs in the proximal large intestine [41].

Physiological significance of the cecum in an overall calcium absorption was questionable because calcium may be present in the cecal lumen as a water-insoluble complex with indigestible luminal content [12]. Cecectomy also did not affect the fractional calcium absorption in 1,25(OH)₂D₃-repleted rats [4]. However, other studies showed that microbial fermentation in the cecum could liberate calcium from the indigestible fibers [10, 51]. Moreover, short-chain fatty acid as well as small organic acids, e.g., propionic acid, succinic acid, and lactic acid, produced during fermentation decreased the luminal pH, which in turn released calcium from the complexes [10, 36, 38]. Cecal calcium absorption becomes salient when hindgut fermentors, such as humans and rats, ingest naturally occurring resistant starch (e.g., oatmeal and pearl barley), a fermentable carbohydrate that benefits healthy individuals as well as diabetic patients [44, 51].

The aforementioned evidence also collectively suggests that the cecum has a high capacity to supply additional calcium to the body when needed, for examples, in pregnancy and lactation [42]. Coincidentally, PRL is a hormone with markedly elevated levels during these periods, and we have previously shown that it was an important hormone to enhance calcium absorption, especially in the duodenum and proximal jejunum [5]. However, PRL was without effect on calcium transport in the distal large intestine although it could regulate water absorption and inhibit the calcium-dependent Cl[–] and K⁺ secretion in the colon [34, 43]. In the present study, the cecum, which expressed essential genes for the transcellular and paracellular calcium transport, responded to PRL by increasing calcium fluxes via both transcellular and paracellular pathways, similar to that seen in the duodenum [25]. Calcium fluxes observed in the 800 ng/mL PRL-treated cecum was greater than that in the duodenum exposed to the same PRL concentration by 2-fold, i.e., ~120 nmol h^{–1} cm^{–2} in the cecum compared to ~60 nmol h^{–1} cm^{–2} reported in the duodenum [25]. In the presence of ~5–20 mmol/L transepithelial calcium gradient, this PRL concentration markedly increased the amount of calcium transported by cecal epithelium from a nanomole range to micromole range. These information collectively strengthened the physiological significance of the cecal calcium absorption during hyperprolactinemic states, such as in lactating animals.

Nevertheless, nothing was known pertaining the detailed mechanisms by which PRL enhanced cecal calcium transport. The PRL-stimulated transcellular calcium absorption in the duodenum was explained by increases in the brush-border calcium uptake and PMCA activity [7]. In the

cecum, the PRL effect was also abolished by inhibitors of apical calcium uptake (ruthenium red) or basolateral calcium extrusion (trifluoperazine). Regarding the cecal paracellular calcium transport, the PRL-induced increase in calcium movement through the paracellular space was explained by a decrease in TER (Fig. 5) as well as an increase in the epithelial cation selectivity, as indicated by the increased P_{Na} and P_{Na}/P_{Cl} (Fig. 6), similar to that observed in Caco-2 monolayer [48]. The P_{Na}/P_{Cl} value of the control groups, which increased from the mobility ratio of Na^+ and Cl^- of ~ 0.6 in free solution to ~ 2.0 [17], also suggested that, under normal conditions, the cecal epithelium naturally prefers paracellular movement of cations more than anions. Generally, tight junction proteins of the claudin family undergo polymerization to form charge-selective paracellular pores [15]. Thus, the epithelial charge selectivity is determined by the fixed negative or positive charges on the extracellular loops of claudins which impede the movement of ions with the opposite charge [50]. Claudin-2, claudin-3, and claudin-12, which are also expressed in cecal epithelial cells, have been suggested to regulate the paracellular calcium transport [14, 35]. Our recent preliminary study in Caco-2 monolayer showed that an increase in cation selectivity observed after the PRL-induced claudin phosphorylation could increase the paracellular calcium movement (Nakkrasae et al. 2009, unpublished observation). However, further experiment is required to demonstrate that the PRL-induced increases in paracellular cecal calcium transport and cation selectivity are directly mediated by claudin phosphorylation.

PRL signaling pathways in the intestinal epithelial cells have been studied in the duodenum and Caco-2 intestinal-like cells [25, 48], while that in the cecum was unknown. Since cecal epithelial cells predominantly expressed PRLR-L, it was likely that PRL exerted its functions through PRLR-L, similar to that reported in Caco-2 cells [48]. In contrast, as PRLR-S lacks the cytoplasmic tails required to activate the intracellular downstream pathways, PRLR-S may act as the dominant-negative molecules to silence or reduce PRL actions [2]. Interestingly, the typical biphasic response to PRL was also demonstrated in the cecum, i.e., in contrast to the lower effective doses (600 and 800 ng/mL), 1,000 ng/mL PRL only slightly increased calcium transport. This phenomenon has been explained by inability of PRLRs to form functional dimers. At extremely high PRL concentrations, most available PRLRs were occupied as nonfunctional 1:1 complexes instead of 1:2 functional complexes needed to induce signal transduction [13, 22].

The principal PRL signaling pathways found in the cecum, i.e., PI3K, PKC, and ROCK, were consistent with those reported in duodenal epithelial cells and Caco-2 monolayer [25, 48]. Although JAK2 usually mediates PRL signals in several tissues, e.g., mammary epithelium [24],

and several factors, e.g., $1,25(OH)_2D_3$, could increase calcium uptake through PKA [30], the PRL effects herein appeared to be independent of both JAK2 and PKA. Generally, PI3K is the most upstream enzyme of several kinase targets, including PKC and ROCK known to modulate the transepithelial calcium transport and paracellular permeability [19, 27, 37, 46]. In the cecum, PKC was likely to be more important in the transcellular calcium transport than the paracellular transport, presumably by its ability to augment the basolateral calcium efflux [30]. In contrast, ROCK was essential for both transport mechanisms. It was noteworthy that activation of ROCK is commonly required to increase paracellular permeability or decrease TER in several epithelia, e.g., brain endothelium, renal proximal tubule, and human colonic epithelial monolayer [27, 45, 46]. Hence, PRL might use this common pathway to decrease TER, and increase cation selectivity and paracellular calcium permeability, thereby enhancing the paracellular calcium transport.

Finally, it could be concluded that the rat cecum was a target tissue of PRL, in which it directly stimulated the transcellular and paracellular calcium transport via the non-genomic signaling pathways. The downstream pathways for PRL signaling involved PI3K, ROCK, and PKC, the last of which mainly mediated the signal for the PRL-enhanced transcellular calcium transport. The present investigation, therefore, elaborated possible physiological roles of PRL in the cecum and provided more complete understanding of the effects of PRL on calcium homeostasis. Since the rate of cecal calcium absorption is subjected to effects of certain dietary components, e.g., resistant carbohydrates, we speculate that such nutraceuticals could be useful for enhancement of calcium absorption during lactation, when the plasma PRL surge is in the range of ~ 650 – 800 ng/mL [1].

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Conflict of interest The authors have no conflict of interest.

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Enhancement of calcium transport in Caco-2 monolayer through PKC ζ -dependent Ca v 1.3-mediated transcellular and rectifying paracellular pathways by prolactin

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Thongon N, Nakkrasae L, Thongbunchoo J, Krishnamra N, Charoenphandhu N. Enhancement of calcium transport in Caco-2 monolayer through PKC ζ -dependent Ca v 1.3-mediated transcellular and rectifying paracellular pathways by prolactin. *Am J Physiol Cell Physiol* 296: C1373–C1382, 2009. First published April 1, 2009; doi:10.1152/ajpcell.00053.2009.—Previous investigations suggested that prolactin (PRL) stimulated the intestinal calcium absorption through phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and RhoA-associated coiled-coil forming kinase (ROCK) signaling pathways. However, little was known regarding its detailed mechanisms for the stimulation of transcellular and voltage-dependent paracellular calcium transport. By using Ussing chamber technique, we found that the PRL-induced increase in the transcellular calcium flux and decrease in transepithelial resistance of intestinal-like Caco-2 monolayer were not abolished by inhibitors of gene transcription and protein biosynthesis. The PRL-stimulated transcellular calcium transport was completely inhibited by the L-type calcium channel blockers (nifedipine and verapamil) and plasma membrane Ca $^{2+}$ -ATPase (PMCA) inhibitor (trifluoperazine) as well as small interfering RNA targeting voltage-dependent L-type calcium channel Ca v 1.3, but not TRPV6 or calbindin-D $_{9k}$. As demonstrated by ^{45}Ca uptake study, PI3K and PKC, but not ROCK, were essential for the PRL-enhanced apical calcium entry. In addition, PRL was unable to enhance the transcellular calcium transport after PKC ζ knockdown or exposure to inhibitors of PKC ζ , but not of PKC α , PKC β , PKC ϵ , PKC μ , or protein kinase A. Voltage-clamping experiments further showed that PRL markedly stimulated the voltage-dependent calcium transport and removed the paracellular rectification. Such PRL effects on paracellular transport were completely abolished by inhibitors of PI3K (LY-294002) and ROCK (Y-27632). It could be concluded that the PRL-stimulated transcellular calcium transport in Caco-2 monolayer was mediated by Ca v 1.3 and PMCA, presumably through PI3K and PKC ζ pathways, while the enhanced voltage-dependent calcium transport occurred through PI3K and ROCK pathways.

calcium uptake; protein kinase C; rectification; small interfering RNA; voltage-dependent calcium transport

PROLACTIN (PRL) IS A CRUCIAL calcium-regulating hormone in pregnant and lactating animals since it increases intestinal calcium absorption, renal calcium reabsorption, and bone turnover ultimately to supply more calcium for fetal growth and milk production (4, 5). In vivo studies in rats suggested that PRL could stimulate transcellular and paracellular calcium absorption in several intestinal segments, particularly in the

duodenum and proximal jejunum in which PRL receptors (PRLR) are strongly expressed (20, 25, 26). Although direct stimulatory actions of PRL on intestinal calcium transport has been demonstrated, little is currently known regarding the detailed mechanisms of PRL in intestinal epithelial cells.

Under normal conditions, calcium predominantly traverses the intestinal epithelium via the paracellular pathway, especially after a calcium-enriched meal that favors the calcium gradient-dependent paracellular calcium transport, whereas the transcellular calcium transport becomes more significant during high calcium demand, such as pregnancy and lactation (5, 23). A number of calcium transport proteins are essential for the transcellular calcium transfer, namely, transient receptor potential vanilloid family calcium channel (TRPV)-6 and voltage-dependent L-type calcium channel Ca v 1.3 for the apical calcium entry, calcium-binding protein (CaBP) calbindin-D $_{9k}$ for cytoplasmic translocation, and plasma membrane Ca $^{2+}$ -ATPase (PMCA) isoform 1b and Na $^{+}$ /Ca $^{2+}$ exchanger (NCX)-1 for basolateral extrusion (18, 34, 35). It was apparent that, under the influence of various hormones and physiological conditions, different transport proteins may be recruited to augment calcium absorption. For example, L-type calcium channels were responsible for the enhanced transcellular calcium transport in response to parathyroid hormone-related peptide and luminal glucose (34, 51), while TRPV6 was required for the 1,25(OH) $_2$ D $_3$ - and 17 β -estradiol-stimulated calcium absorption (23, 46). Regarding the PRL-stimulated calcium transport, it was not known which transporter proteins were involved.

The paracellular calcium transport driven by an electrochemical gradient (i.e., calcium gradient and voltage difference), on the other hand, is regulated by several tight junction proteins, e.g., claudin-2 and -12, which polymerize to form an array of channel-like paracellular pores (15, 43, 47). Thus, the paracellular channels appeared to exhibit charge and size selectivity, voltage response, and rectification similar to those found in typical ion channels (12, 30, 41, 43). Physiological significance of the voltage-dependent calcium transport in the small intestine is controversial, but it may be of greater importance when the transepithelial potential difference (PD) is increased by some nutrients, such as glucose, short-chain fatty acid, phenylalanine, cysteine, and proline (14, 40, 48). Recently, PRL has been shown to modify some properties of the paracellular channel, i.e., charge selectivity and calcium permeability, which led to an enhancement of the calcium gradient-dependent paracellular calcium transport (45). The effects of PRL on other paracellular properties, such as response to

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voltage change and rectification, or the voltage-dependent calcium transport remained unknown. However, they could not be demonstrated in the presence of calcium gradient because high calcium concentration occluded the paracellular pores (known as the paracellular conductance block), which in turn altered the transepithelial resistance (TER) (43, 45). Such paracellular properties should be validated by a voltage-clamp study in an absence of calcium gradient.

In addition to the calcium transport mechanism, the signaling pathways of PRL were also elusive. In the rat duodenum and intestinal epithelium-like Caco-2 monolayer, phosphoinositide 3-kinase (PI3K) was required for PRL signaling (20, 45). Further investigations in Caco-2 cells suggested that the PRL-stimulated transcellular and gradient-dependent paracellular calcium transport relied on protein kinase C (PKC) and RhoA-associated coiled-coil forming kinase (ROCK) (45), but which PKC isozyme was involved was not known.

Therefore, the objectives of the present study were 1) to identify the transcellular transporters and PKC isozyme that were essential for the PRL-stimulated transcellular calcium transport, 2) to investigate the paracellular properties altered by PRL, and 3) to demonstrate the effects of PRL on the voltage-dependent calcium transport as well as the responsible signaling pathways. The human colorectal adenocarcinoma Caco-2 cells were used in this study because they have functional similarities to the small intestinal cells, including the presence of brush border, expression of sucrase-isomaltase, and expression of the transcellular calcium transporters and charge-selective paracellular proteins, e.g., TRPV6, PMCA, claudin-1, -2, -3, and -5 (32, 37, 38, 50, 52). Caco-2 monolayer was also a standard model for calcium absorption study and was responsive to PRL (20, 45).

MATERIALS AND METHODS

Cell culture. Caco-2 intestinal cells [no. HTB-37, American Type Culture Collection (ATCC)] were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 15% fetal bovine serum (Gibco, Grand Island, NY), 1% L-glutamine (Gibco), 1% nonessential amino acid (Sigma), 100 U/ml penicillin/streptomycin (Sigma), and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B (Sigma). Cells were propagated in 75- cm^2 T flask (Corning, NY) under a humidified atmosphere containing 5% CO_2 at 37°C and were subcultured as described in the ATCC protocol. Confluent Caco-2 monolayers were prepared by seeding cells (5.0×10^5 cells/ cm^2) on polyester Snapwell with 12-mm diameter and 0.4- μm pore size (Corning). Culture medium was changed daily after 48 h of seeding. On day 14 after

seeding, the Snapwell was mounted in a modified Ussing chamber with an exposed surface area of 1.13 cm^2 to measure the electrical parameters and calcium fluxes, as previously described (45). The monolayer was equilibrated for 20 min in the chamber before the 60-min experiment was performed.

Small interfering RNA transfection. Small interfering RNA (siRNA) oligonucleotides targeted for human TRPV6 (5'-GGGAAACACAGU-GUUACAC-3' and 5'-GTGUAACACUGUGUUUCCC-3'), $\text{Ca}_v1.3$ (5'-GAGCACC UUUGACAAUUUC-3' and 5'-AAGAAUUGUCAAG-GTGCUC-3'), calbindin- D_{9k} (5'-GAUGAUCUCUUUCAAGAAC-3' and 5'-GUUCUUGAAAGAGAUAUC-3'), and PKC $_{\zeta}$ (5'-GACGACAA-GAACGAGGACG-3' and 5'-CUGCUGUUCUUGCUCCUGCAA-3') were designed by siRNA Target Designer (version 1.51; Promega, Madison, WI), and synthesized by T7 RiboMax Express RNAi System (Promega) according to the manufacturer's instruction. Scramble siRNA that had no homology to any other genes was used as a negative control. As previously described (45), Caco-2 cells were first plated on Snapwell at 5.0×10^5 cells/ cm^2 . At day 12 after seeding, in vitro transfection was performed with 10 $\mu\text{g}/\text{ml}$ polyethylenimine and 1 $\mu\text{mol}/\text{ml}$ siRNA molecules. At day 14 (i.e., 48 h after transfection), transepithelial calcium flux was determined. Efficiency of siRNA was evaluated by quantitative real-time PCR (qRT-PCR). The knockdown protocol was approved by the Institutional Biosafety Committee (IBC) of Mahidol University.

mRNA isolation, quantitative real-time PCR, and sequencing. By using TRIzol reagent (Invitrogen, Carlsbad, CA), total RNA was prepared from Caco-2 cells, as previously described (45). One microgram of total RNA was reverse-transcribed with iScript kit (Bio-Rad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping gene, served as a control gene to check the consistency of the reverse transcription. Sense and antisense primers used for qRT-PCR are presented in Table 1. The amplification reaction using real-time PCR (model MiniOpticon; Bio-Rad) was performed with iQ SYBR Green SuperMix (Bio-Rad). Relative expression of studied genes over GAPDH was calculated from the threshold cycle (C_t) values by using $2^{-\Delta C_t}$ method. After qRT-PCR, the PCR products were also visualized on a 1.5% agarose gel stained with 1.0 $\mu\text{g}/\text{ml}$ ethidium bromide. Thereafter, all PCR products were extracted by the HiYield Gel/PCR DNA Extraction kit (Real Biotech, Taipei, Taiwan) and were sequenced by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Bathing solution. The bathing solution for Ussing chamber experiments contained (in mmol/l) 118 NaCl, 4.7 KCl, 1.1 MgCl_2 , 1.25 CaCl_2 , 23 NaHCO_3 , 12 D-glucose, and 2 mannitol (all purchased from Sigma). The solution, continuously gassed with humidified 5% CO_2 in 95% O_2 , was maintained at 37°C, pH 7.4, and had an osmolality of 290–293 mmol/kg water as measured by a freezing point-based osmometer (model 3320; Advanced Instruments, Norwood, MA).

Table 1. *Homo sapiens* oligonucleotide sequences used in the quantitative real-time PCR experiments

Name	Accession No.	Primer (forward/reverse)	Product Length, bp
TRPV6	AF365928	5'-TCTGACTGCGTGTCTCAC-3' 5'-ACATTCTCTGGCGTTCAT-3'	144
$\text{Ca}_v1.3$	EU363339	5'-TGATCCAAGTGGAGCAGTCA-3' 5'-GTGTGAAAGTCCGCTAGGAGA-3'	113
Calbindin- D_{9k}	L13220	5'-TCTCCTGAGGAAGTGAAGAGG-3' 5'-GGGAATTCAGCCTGAATCAA-3'	106
PKC $_{\zeta}$	NM_002744	5'-TAATCAGAGTCATCGGGCG-3' 5'-TCTGTCTGTACCCAGTCAA-3'	138
GAPDH	NM_002046	5'-CTGGTAAAGTGGATATTGTTG-3' 5'-GAGGCTGTTGTCTACTTCTC-3'	359

TRPV6, transient receptor potential vanilloid family Ca^{2+} channel 6; $\text{Ca}_v1.3$, voltage-dependent L-type Ca^{2+} channel 1.3; PKC $_{\zeta}$, ζ -isozyme of protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

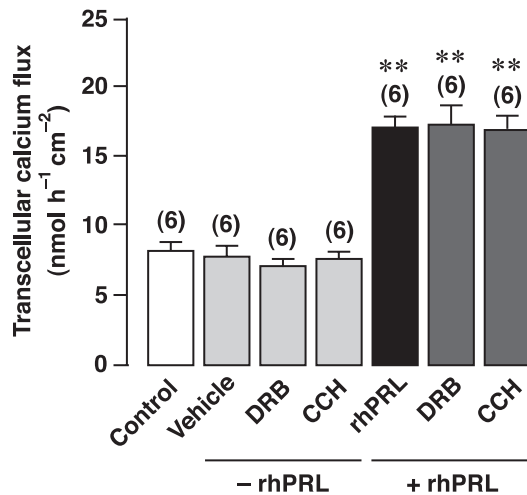


Fig. 1. Transcellular calcium transport in Caco-2 monolayers directly exposed to RNA polymerase II inhibitor [50 $\mu\text{mol/l}$ 5,6-dichloro-1- β -D-ribobenzimidazole (DRB); inhibitor of gene transcription] or protein synthesis inhibitor (70 $\mu\text{mol/l}$ cycloheximide; CCH) in the presence (+rhPRL) and absence (–rhPRL) of 600 ng/ml recombinant human prolactin (rhPRL). DMSO was used as vehicle for inhibitor preparation. Numbers in parentheses represent the number of independent Snapwells. ** $P < 0.01$ compared with the control group.

Distilled water used in the present work had a resistance $>18.3 \text{ M}\Omega \cdot \text{cm}$ and free-ionized calcium $<2.5 \text{ nmol/l}$.

Measurement of electrical parameters. Electrical parameters, i.e., potential (voltage) difference (PD), short-circuit current (I_{sc}), and TER, were determined as described previously (7, 20). In brief, a pair of Ag/AgCl electrodes connected to agar bridges (3.0 mol/l KCl per 4 g% agar) was located near each surface of the mounted Snapwell for measurement of PD. The other ends of the PD-sensing electrodes were connected to a preamplifier (model EVC-4000; World Precision Instruments, Sarasota, FL). Another pair of Ag/AgCl electrodes connected in series to the EVC-4000 current-generating unit was placed at the end of each hemichamber to supply I_{sc} or external current for voltage clamping. TER was calculated from Ohm's equation. Fluid resistance was automatically subtracted by the EVC-4000 system.

Calcium flux measurement. Calcium fluxes were determined by modified method of Charoenphandhu et al. (7). After a 20-min incubation in Ussing chamber, the bathing solution was changed to a fresh one. The solution on one side contained ^{45}Ca (initial specific activity of 5 mCi/ml, final specific activity of ~ 450 –500 mCi/mol; Amersham, Buckinghamshire, UK). Samples were collected from the chamber to calculate the unidirectional flux ($J_{\text{H} \rightarrow \text{C}}$) from the hot side (H) to the cold side (C), as follows:

$$J_{\text{H} \rightarrow \text{C}} = R_{\text{H} \rightarrow \text{C}} / (S_{\text{H}} \times A)$$

$$S_{\text{H}} = C_{^{45}\text{Ca}} / C_{\text{T}}$$

where $R_{\text{H} \rightarrow \text{C}}$ was the rate of tracer appearance in the cold side (cpm/h), S_{H} was the specific activity in the hot side (cpm/nmol), A was the surface area of Snapwell (cm^2), $C_{^{45}\text{Ca}}$ was a mean radioactivity in the hot side (cpm), and C_{T} was the total calcium in the hot side (nmol).

Radioactivity of ^{45}Ca in counts per minute (cpm) was analyzed by liquid scintillation spectrophotometer (model Tri-Carb 3100; Packard, Meriden, CT). Total calcium concentration in the hot side was determined by atomic absorption spectrophotometry (model SpectrAA-300; Varian Techtron, Springvale, Victoria, Australia). In the absence of transepithelial calcium gradient, i.e., bathing solution in both hemichambers contained equal calcium concen-

tration of 1.25 mmol/l, the measured calcium fluxes represented the transcellular active calcium transport (45).

As for the experimental protocol, Caco-2 monolayer was directly incubated on the basolateral side for 60 min with 600 ng/ml recombinant human PRL (rhPRL) (purity $>97\%$; catalog no. 682-PL; R&D Systems, Minneapolis, MN), which is the maximal effective concentration reported by Jantarajit et al. (20). In some experiments, the monolayers were also exposed to PKC activator [100 nmol/l phorbol-12-myristate-13-acetate (PMA), Calbiochem, San Diego, CA], or inhibitors for RNA polymerase II [50 $\mu\text{mol/l}$ 5,6-dichloro-1- β -D-ribobenzimidazole (DRB), Calbiochem], protein biosynthesis (70 $\mu\text{mol/l}$ cycloheximide, Sigma), L-type calcium channel (1, 5, or 10 $\mu\text{mol/l}$ nifedipine, or 100 nmol/l verapamil; Sigma), calmodulin-dependent PMCA (100 $\mu\text{mol/l}$ trifluoperazine, Sigma), $\text{PKC}_{\alpha/\beta}$ (5 $\mu\text{mol/l}$ Gö-6976, Calbiochem), PKC_{ζ} (40 $\mu\text{mol/l}$ myristoylated PKC_{ζ} pseudosubstrate peptide; Calbiochem), PKC_{θ} (30 $\mu\text{mol/l}$ myristoylated PKC_{θ} pseudosubstrate peptide; Calbiochem), PKC_{ϵ} (4 $\mu\text{mol/l}$ PKC_{ϵ} translocation inhibitor peptide; Calbiochem), or PKA (10 $\mu\text{mol/l}$ myristoylated PKA inhibitor 14–22 amide; Calbiochem).

Voltage clamping. Calcium fluxes in clamped preparations represented the voltage-dependent calcium transport through the paracellular route (22). Transepithelial potential differences across the con-

Table 2. Epithelial electrical parameters of Caco-2 monolayers

Condition	n	Electrical Parameter		
		PD, mV	I_{sc} , $\mu\text{A}/\text{cm}^2$	TER, $\Omega \cdot \text{cm}^2$
Control	15	1.01 ± 0.12	2.73 ± 0.70	376.67 ± 37.56
Vehicle	10	0.94 ± 0.32	2.60 ± 0.84	386.67 ± 35.83
600 ng/ml rhPRL	15	0.88 ± 0.19	3.47 ± 0.92	$253.22 \pm 28.01^*$
Inhibitors				
50 $\mu\text{mol/l}$ DRB	12	1.03 ± 0.28	2.50 ± 0.67	418.75 ± 82.28
70 $\mu\text{mol/l}$ CCH	13	1.03 ± 0.23	2.62 ± 0.51	396.15 ± 55.76
10 $\mu\text{mol/l}$ Nifedipine	14	1.17 ± 0.33	3.00 ± 0.88	395.48 ± 48.35
100 nmol/l Verapamil	10	0.95 ± 0.22	2.50 ± 0.53	381.67 ± 46.78
100 $\mu\text{mol/l}$ TFP	10	0.96 ± 0.28	2.40 ± 0.70	405.00 ± 66.23
5 $\mu\text{mol/l}$ Gö-6976	6	1.27 ± 0.19	3.17 ± 0.40	394.44 ± 15.47
40 $\mu\text{mol/l}$ myr- PKC_{ζ}	6	1.28 ± 0.14	3.50 ± 0.43	371.39 ± 15.09
30 $\mu\text{mol/l}$ myr- PKC_{θ}	6	1.08 ± 0.12	2.67 ± 0.33	430.56 ± 23.34
4 $\mu\text{mol/l}$ trans- PKC_{ϵ}	6	1.03 ± 0.11	2.50 ± 0.22	413.89 ± 23.37
100 nmol/l PMA	4	1.08 ± 0.13	2.25 ± 0.25	408.33 ± 49.30
10 $\mu\text{mol/l}$ PKAI 14-22	4	1.00 ± 0.12	2.50 ± 0.58	408.35 ± 48.11
600 ng/ml rhPRL +				
50 $\mu\text{mol/l}$ DRB	12	0.83 ± 0.31	3.33 ± 1.03	$241.39 \pm 40.14^*$
70 $\mu\text{mol/l}$ CCH	13	0.89 ± 0.27	3.46 ± 0.88	$256.28 \pm 36.57^*$
10 $\mu\text{mol/l}$ Nifedipine	14	0.77 ± 0.22	3.14 ± 0.77	$243.45 \pm 29.63^*$
100 nmol/l Verapamil	10	0.92 ± 0.40	3.67 ± 1.37	$245.83 \pm 34.86^*$
100 $\mu\text{mol/l}$ TFP	10	0.87 ± 0.16	3.50 ± 0.55	$248.61 \pm 35.13^*$
5 $\mu\text{mol/l}$ Gö-6976	6	1.02 ± 0.21	3.83 ± 0.98	$268.06 \pm 19.62^*$
40 $\mu\text{mol/l}$ myr- PKC_{ζ}	6	1.00 ± 0.19	4.50 ± 0.89	$225.00 \pm 06.80^*$
30 $\mu\text{mol/l}$ myr- PKC_{θ}	6	1.13 ± 0.20	4.33 ± 0.71	$259.80 \pm 08.52^*$
4 $\mu\text{mol/l}$ trans- PKC_{ϵ}	6	0.93 ± 0.19	3.50 ± 1.05	$276.94 \pm 49.89^*$
100 nmol/l PMA	4	1.00 ± 0.21	4.00 ± 0.82	$250.00 \pm 10.21^*$
10 $\mu\text{mol/l}$ PKAI 14-22	4	0.95 ± 0.13	3.75 ± 0.50	$254.17 \pm 22.05^*$

Values are means $\pm$ SE. Caco-2 monolayer was exposed to recombinant human prolactin (rhPRL), RNA polymerase II inhibitor [5,6-dichloro-1- β -D-ribobenzimidazole (DRB)], protein synthesis inhibitor [cycloheximide (CCH)], L-type calcium channel blockers (nifedipine and verapamil), plasma membrane Ca^{2+} -ATPase (PMCA) inhibitor [trifluoperazine (TFP)], PKC inhibitors [Gö-6976 for $\text{PKC}_{\alpha/\beta}$, myristoylated (myr) pseudosubstrates for $\text{PKC}_{\zeta/\theta}$ and translocation (trans) inhibitor peptide for PKC_{ϵ}], PKC activator (PMA), myristoylated PKA inhibitor 14-22 amide (PKAI 14-22), or rhPRL plus inhibitors. DMSO (0.3% vol/vol) was used as vehicle for preparation of inhibitors. Mounted monolayer was bathed on both sides with 1.25 mmol/l calcium-containing solution. The apical side was negative with respect to the basolateral side. PD, potential difference; I_{sc} , short-circuit current; TER, transepithelial resistance. * $P < 0.01$ compared with the control group.

trol and 600 ng/ml rhPRL-treated monolayers were clamped to -25 , -10 , -5 , 0 , $+5$, $+10$, or $+25$ mV (the serosal side was considered zero) during 60-min experiment by using EVC-4000 system. Trans-epithelial calcium fluxes in apical-to-basolateral (A-to-B) and basolateral-to-apical (B-to-A) directions were determined. Net calcium flux was a subtraction of B-to-A flux from A-to-B flux. In some experiments, Caco-2 monolayers ($n = 5$ per each voltage; total 280 Snapwells used) were exposed to 600 ng/ml rhPRL plus PI3K inhibitor (75 $\mu\text{mol/l}$ LY-294002; Tocris Bioscience, Bristol, UK), pan-specific PKC inhibitor (1 $\mu\text{mol/l}$ GF-109203X; AG Scientific, San Diego, CA), or ROCK inhibitor (1 $\mu\text{mol/l}$ Y-27632; Calbiochem).

Calcium uptake study. The calcium uptake protocol was modified from the method of Charoenphandhu et al. (6). In brief, Caco-2 cells (1.0×10^6 cells/cm²) were propagated in 6-well plates for 3 days. Confluent Caco-2 cells were then incubated with 600 ng/ml rhPRL for 60 min until ^{45}Ca was added to obtain a final specific activity of ~ 500 mCi/mol. After exactly 1-, 3-, 6-, 9-, 12-, 20-, 30-, or 40-min exposure to ^{45}Ca ($n = 5$ per each time interval; total 225 setups used; cells for 0 min were not incubated with ^{45}Ca), cells were immediately washed in ice-cold ^{45}Ca -free solution to stop the physiological processes, placed in 5 mmol/l EGTA-containing solution to eliminate the adsorbed extracellular calcium, centrifuged at 550 g for 5 min, and finally lysed by 30% vol/vol Triton X-100. Radioactivity of ^{45}Ca in the homogenate was analyzed by a liquid scintillation spectrophotometer (Packard). Cellular protein was determined by bicinchoninic acid method using a commercial kit (Sigma). In some experiments, 600

ng/ml rhPRL-treated cells were also incubated with 75 $\mu\text{mol/l}$ LY-294002, 1 $\mu\text{mol/l}$ GF-109203X, or 1 $\mu\text{mol/l}$ Y-27632.

Statistical analysis. Results are expressed as means $\pm$ SE. Two sets of data were compared using the unpaired Student's t -test. One-way analysis of variance with Dunnett's multiple-comparison test was used for multiple sets of data. The level of significance for all statistical tests was $P < 0.05$. The curves of calcium uptake vs. time and of calcium flux vs. voltage were obtained using one-phase association and second-order polynomial equations, respectively (36). Data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA).

RESULTS

PRL signaling in Caco-2 monolayer was nongenomic and protein synthesis independent. Calcium flux studies using Ussing chamber technique demonstrated that 600 ng/ml rhPRL significantly increased the transcellular calcium transport by ~ 2.13 -fold (Fig. 1), while decreasing TER in Caco-2 monolayer (Table 2). PRL effects occurred within 60 min after treatment. Exposure to PRL plus inhibitors of de novo gene transcription (50 $\mu\text{mol/l}$ DRB) or protein biosynthesis (70 $\mu\text{mol/l}$ cycloheximide) had no effect on the transcellular calcium transport (Fig. 1). DRB or cycloheximide alone were without effect on the basal calcium transport (Fig. 1) and

Fig. 2. A: expression of transient receptor potential vanilloid family calcium channel 6 (TRPV6), voltage-dependent L-type Ca^{2+} channel 1.3 ($\text{Ca}_v1.3$) and calbindin- D_{9k} (CaBP $_{9k}$) in Caco-2 cells transfected with scramble small interfering RNA (siRNA; negative control) or siRNA targeting (si) TRPV6, $\text{Ca}_v1.3$, or CaBP $_{9k}$ mRNAs. Quantitative real-time PCR (qRT-PCR) results are expressed as log means $\pm$ SE. Representative electrophoretic bands and fold differences between the expression in control and knockdown cells (conventional PCR, 36 cycles) are also presented along with qRT-PCR results. GAPDH was used for normalization. $**P < 0.01$, $***P < 0.001$ compared with scramble. B: transcellular calcium transport in TRPV6, $\text{Ca}_v1.3$, and CaBP $_{9k}$ knockdown Caco-2 monolayers with (+rhPRL) or without (−rhPRL) 600 ng/ml rhPRL in the basolateral solution. In some experiments, TRPV6 knockdown monolayers were exposed to 600 ng/ml rhPRL plus 100 nmol/l verapamil ($\text{Ca}_v1.3$ blocker; on the apical side). Control group denotes normal Caco-2 monolayers (without siRNA transfection). $**P < 0.01$ compared with the control group. Numbers in parentheses represent the number of independent Snapwells.

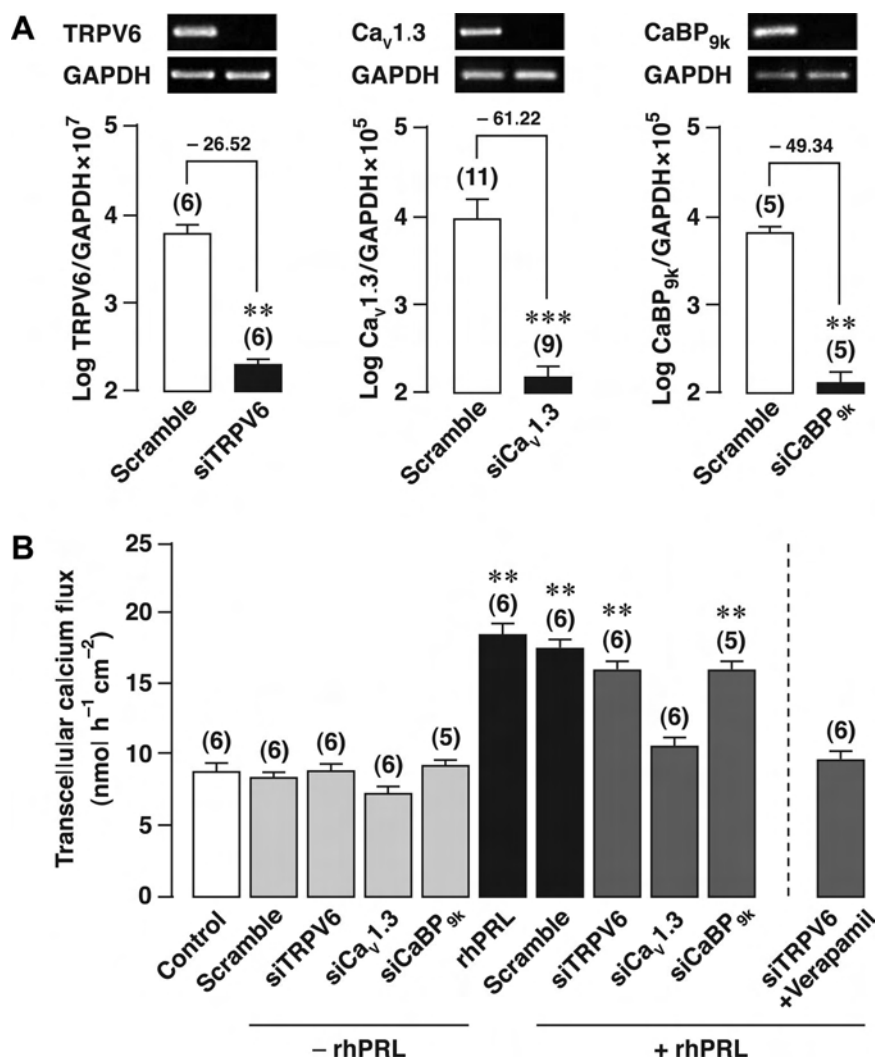


Table 3. Epithelial electrical parameters of knockdown *Caco-2* monolayers

Condition	n	Electrical Parameter		
		PD, mV	I_{sc} , $\mu\text{A}/\text{cm}^2$	TER, $\Omega \cdot \text{cm}^2$
Control	6	0.98 ± 0.24	2.33 ± 0.52	427.78 ± 89.24
Scramble	6	0.88 ± 0.54	2.17 ± 1.17	409.72 ± 66.34
siTRPV6	6	0.98 ± 0.43	2.33 ± 0.82	416.67 ± 75.28
si $\text{Ca}_v1.3$	6	0.90 ± 0.30	2.60 ± 0.24	391.67 ± 49.16
siCaBP _{9k}	5	1.10 ± 0.23	3.17 ± 0.75	351.38 ± 35.13
siPKC ζ	3	0.87 ± 0.21	2.33 ± 0.58	372.22 ± 25.46
600 ng/ml rhPRL	6	0.84 ± 0.11	3.17 ± 0.98	$248.89 \pm 27.22^*$
600 ng/ml rhPRL +				
Scramble	6	0.75 ± 0.58	2.83 ± 1.60	$250.00 \pm 49.55^*$
siTRPV6	6	0.82 ± 0.37	3.17 ± 1.17	$252.22 \pm 35.07^*$
si $\text{Ca}_v1.3$	6	0.93 ± 0.62	3.67 ± 1.86	$243.06 \pm 33.51^*$
siCaBP _{9k}	5	1.00 ± 0.28	4.17 ± 1.17	$242.22 \pm 34.10^*$
siTRPV6 + verapamil	6	0.87 ± 0.27	3.83 ± 0.98	$225.28 \pm 31.10^*$
siPKC ζ	3	0.80 ± 0.30	3.33 ± 0.58	$236.11 \pm 60.28^*$

Values are means $\pm$ SE. *Caco-2* cells were transfected with small interfering (si)RNAs targeting (si) TRPV6, $\text{Ca}_v1.3$, calbindin-D_{9k} (CaBP_{9k}), or PKC ζ . Mounted monolayer was bathed on both sides with 1.25 mmol/l calcium-containing solution. The apical side was negative with respect to the basolateral side. In some experiments, TRPV6-knockdown monolayers were incubated with 100 nmol/l verapamil, which inhibited $\text{Ca}_v1.3$ activity. * $P < 0.01$ compared with the control group.

electrical parameters (Table 2). The effect of DRB were consistent with that reported previously (45). The data indicated that PRL exerted stimulatory effects on the transcellular calcium transport via nongenomic, protein synthesis-independent signaling pathways.

PRL increased transcellular calcium transport via L-type calcium channel $\text{Ca}_v1.3$ and PMCA. In the transcellular calcium transport, calcium has been reported to traverse the apical membrane through TRPV6 and $\text{Ca}_v1.3$ before binding to calbindin-D_{9k} for cytoplasmic translocation to the basolateral side (18, 31, 34). We therefore generated siRNA-transfected *Caco-2* monolayers to verify the significance of each putative protein in the PRL-stimulated calcium transport. We show in Fig. 2 that knockdown of TRPV6, $\text{Ca}_v1.3$, and calbindin-D_{9k} markedly reduced mRNA expressions by 26.52-, 61.22-, and 49.34-fold, respectively, with no significant effect on the basal

calcium transport or electrical parameters (Table 3). Interestingly, the PRL-enhanced calcium transport was completely abolished in $\text{Ca}_v1.3$ knockdown monolayer, and not in the TRPV6 and calbindin-D_{9k} knockdown monolayers (Fig. 2B). Exposure to 1, 5, and 10 $\mu\text{mol/l}$ nifedipine, a classical L-type calcium channel blocker (dihydropyridine group), on the apical side, but not the basolateral side, diminished the PRL-stimulated transcellular calcium transport in a concentration-dependent manner (Fig. 3). Similar inhibition was observed after incubating *Caco-2* monolayer on the apical side with another L-type calcium channel blocker, verapamil (phenylalkylamine group) (Fig. 3). The involvement of $\text{Ca}_v1.3$ in the calcium transport response to PRL was supported by the finding that 100 nmol/l verapamil could prevent the PRL-stimulated calcium transport in TRPV6 knockdown monolayer (Fig. 2B). The results, therefore, indicated that $\text{Ca}_v1.3$ at the apical membrane was required for calcium entry during the PRL-stimulated transcellular calcium transport. Basolateral calcium extrusion under PRL stimulation was likely to be mediated by PMCA rather than NCX1, since calcium flux was totally abolished by a calmodulin-dependent PMCA inhibitor trifluoperazine (Fig. 3).

PRL stimulated apical calcium uptake via the PKC signaling pathway. Although $\text{Ca}_v1.3$, a channel for calcium entry, was required for PRL actions, the effects of PRL on apical calcium uptake in *Caco-2* cells as well as responsible signaling pathways were not known. Herein, the calcium uptake study showed that, under normal conditions, the relationship between calcium uptake and time complied with the one-phase association equation ($r^2 = 0.95$), and calcium accumulation in *Caco-2* cells ascended to the maximal value ($Y_{\max}$) of ~ 15 nmol/ μg protein with a rate constant of $\sim 0.15 \text{ min}^{-1}$ (Fig. 4). Exposure to 600 ng/ml rhPRL did not change $Y_{\max}$ value. However, the rate constant that represented the rate of calcium uptake was increased in PRL-treated cells by approximately twofold (Fig. 4D).

To demonstrate the PRL signaling pathways for stimulation of apical calcium uptake, *Caco-2* cells were incubated with inhibitors for PI3K, PKC, and ROCK, all of which have been reported to mediate PRL actions in intestinal epithelial cells (20, 45). The results showed that PI3K inhibitor (LY-294002) and panspecific PKC inhibitor (GF-109203X), but not ROCK

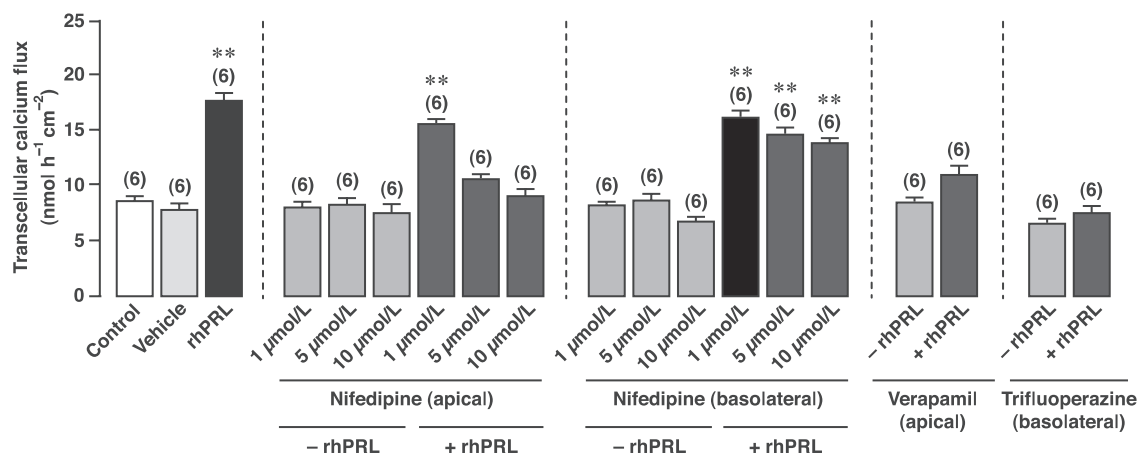
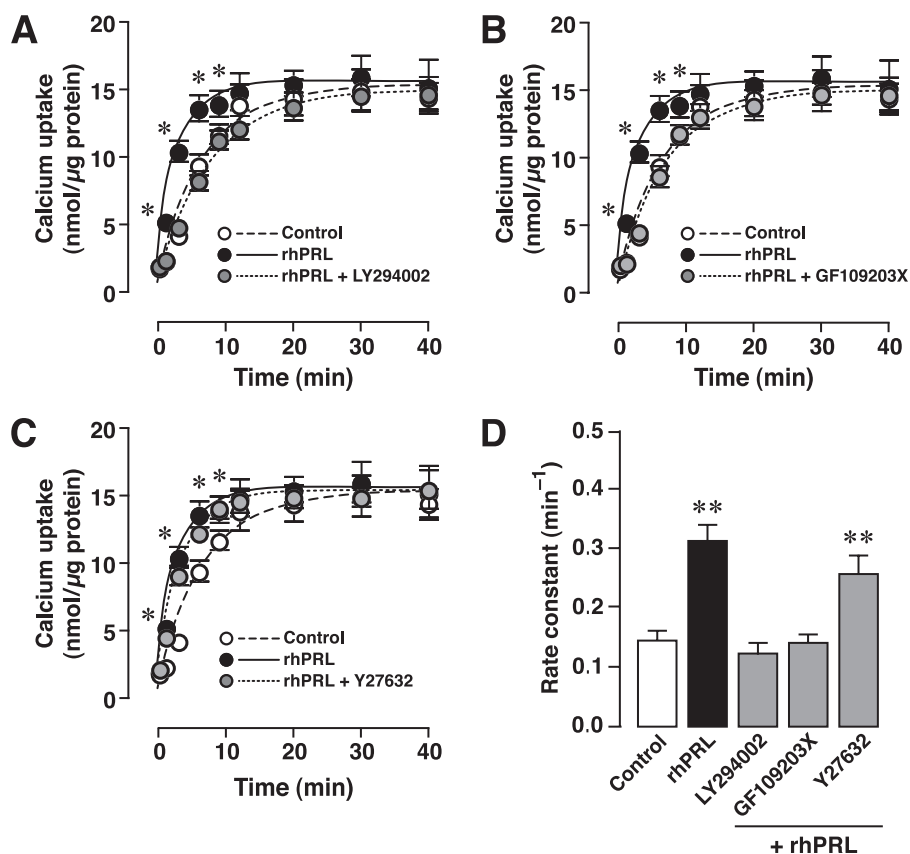


Fig. 3. Transcellular calcium transport in *Caco-2* monolayers directly exposed on the apical side to L-type calcium channel blockers (1, 5, and 10 $\mu\text{mol/l}$ nifedipine or 100 nmol/l verapamil), or on the basolateral side to various concentrations of nifedipine or plasma membrane Ca^{2+} -ATPase (PMCA) inhibitor (100 $\mu\text{mol/l}$ trifluoperazine) in the presence (+rhPRL) and absence (-rhPRL) of 600 ng/ml rhPRL. DMSO was used as vehicle for inhibitor preparation. Numbers in parentheses represent the number of independent Snapwells. ** $P < 0.01$ compared with the control group.

Fig. 4. Calcium uptake in Caco-2 cells ($n = 5$ per each time interval; total 225 Snapwells used) treated with 600 ng/ml rhPRL, or rhPRL plus phosphoinositide 3-kinase (PI3K) inhibitor (75 $\mu\text{mol/l}$ LY-294002) (A), panspecific PKC inhibitor (1 $\mu\text{mol/l}$ GF-109203X) (B), or RhoA-associated coiled-coil forming kinase (ROCK) inhibitor (1 $\mu\text{mol/l}$ Y-27632) (C). Cells were incubated in ^{45}Ca -containing solution for 0, 1, 3, 6, 9, 12, 20, 30, or 40 min. Control and rhPRL data in A were reused in B and C for better comparison. * $P < 0.01$ rhPRL vs. control. D: rate constant, which is indicative of the rate of calcium uptake, in Caco-2 cells exposed to rhPRL or rhPRL plus inhibitors. Data were obtained from the one-phase association curves in A–C. ** $P < 0.01$ compared with the control group.



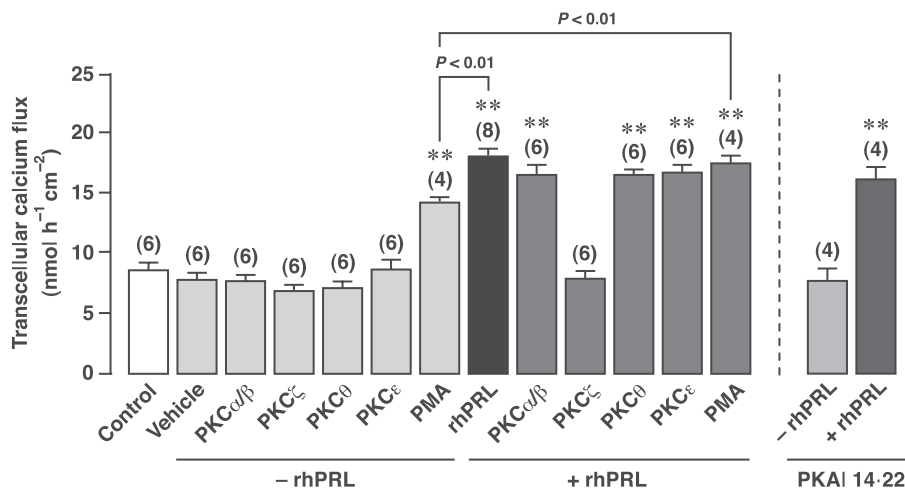
inhibitor (Y-27632), could abolish the effect of PRL on calcium uptake (Fig. 4).

The effect of PRL on the transcellular calcium transport was mediated by PKC_ζ . Since the calcium uptake study showed that PKC was required for the PRL-stimulated transcellular calcium transport, we proceeded to identify the responsible PKC isozyme by exposing Caco-2 monolayer to various PKC isozyme inhibitors in the presence or absence of 600 ng/ml rhPRL. We demonstrated that PKC inhibitors alone had no effect on the basal calcium transport (Fig. 5). PKC_ζ inhibitor, but not inhibitors of $\text{PKC}_{\alpha/\beta}$ (Gö-6976), PKC_θ , or PKC_ϵ , abolished the PRL-enhanced transcellular calcium transport in

Caco-2 monolayer (Fig. 5). Since a high concentration of Gö-6976 (5 $\mu\text{mol/l}$) used in the present study also inhibited PKC_μ (16), it was likely that PRL action did not involve PKC_μ . A classical PKC activator, phorbol-12-myristate-13-acetate (PMA), which could not activate PKC_ζ , also itself stimulated the transcellular calcium transport but with a smaller magnitude than PRL (Fig. 5). Exposure to 600 ng/ml rhPRL further increased calcium transport in PMA-treated monolayer (Fig. 5). Neither PKC inhibitors nor PMA had effect on the PRL-induced decrease in TER (Table 2).

Since a number of hormones, such as parathyroid hormone and $1,25(\text{OH})_2\text{D}_3$, enhanced calcium transport through the

Fig. 5. Transcellular calcium transport in Caco-2 monolayers exposed to selective $\text{PKC}_{\alpha/\beta}$ inhibitor (5 $\mu\text{mol/l}$ Gö-6976), 40 $\mu\text{mol/l}$ myristoylated PKC_ζ pseudosubstrate peptide, 30 $\mu\text{mol/l}$ myristoylated PKC_θ pseudosubstrate peptide, 4 $\mu\text{mol/l}$ PKC_ϵ translocation inhibitor peptide, PKC activator (100 nmol/l phorbol-12-myristate-13-acetate; PMA), or 10 $\mu\text{mol/l}$ myristoylated PKA inhibitor 14-22 amide (PKAI 14-22), in the presence (+rhPRL) or absence (–rhPRL) of 600 ng/ml rhPRL. All cell-permeable inhibitor peptides were dissolved in water. Gö-6976 and PMA were dissolved in DMSO (vehicle). Numbers in parentheses represent the number of independent Snapwells. ** $P < 0.01$ compared with the control group.



transcellular route in a protein kinase A (PKA)-dependent manner (10, 11, 23), we examined a possibility of PKA-mediated PRL action. However, exposure to a cell-permeable PKA inhibitor, myristoylated PKA inhibitor 14-22 amide, did not prevent the PRL effect on the transcellular calcium transport (Fig. 5).

PKC ζ knockdown abolished the PRL-stimulated transcellular calcium transport. To confirm that PRL exerted its actions through PKC ζ , calcium fluxes were determined in PKC ζ knockdown Caco-2 monolayer, which manifested a decrease in PKC ζ mRNA expression by 68.86-fold (Fig. 6A). The results showed that 600 ng/ml rhPRL did not increase the transcellular calcium flux in PKC ζ knockdown monolayer (Fig. 6B), while neither scramble siRNA nor PKC ζ siRNA altered the basal calcium flux (Fig. 6B) or electrical parameters (Table 3). However, PKC ζ knockdown had no effect on the PRL-induced decrease in TER (Table 3).

PRL enhanced the voltage-dependent paracellular calcium via ROCK pathway. In addition to the transcellular pathway, PRL has been reported to augment calcium movement through the paracellular space. However, effects of PRL on the voltage-dependent paracellular calcium transport were not known. Under normal conditions, the voltage-clamp study revealed that the apical-to-basolateral (A-to-B; Fig. 7A) calcium fluxes were dramatically increased with positive apical voltage (basolateral voltage was zero), whereas the increase in basolateral-to-apical (B-to-A; Fig. 7B) calcium fluxes with negative apical voltage was comparatively much smaller. Given an equal driving force (i.e., transepithelial voltage) of opposite signs, the presence of unidirectional calcium fluxes being larger in one direction than the other suggested that the paracellular space of Caco-2 monolayer possessed a property known as rectification. This phenomenon, therefore, resulted in a large net calcium flux (equivalent to electrical current) at apical voltage positive and a relatively small net calcium flux at voltage negative (Fig. 7, C and F).

PRL-treated Caco-2 monolayer exhibited significant increases in A-to-B and B-to-A calcium fluxes at the apical voltage ranges of 0 to +25 mV and -25 to -5 mV, respectively, thereby increasing the net calcium fluxes (Fig. 7C). Interestingly, after PRL exposure, the B-to-A calcium flux vs. voltage curve nearly became a mirror image of the A-to-B curve. In other words, paracellular space of PRL-treated Caco-2 monolayer no longer exhibited rectification. Such PRL effects on the voltage-dependent paracellular calcium transport were completely abolished by 75 $\mu\text{mol/l}$ LY-294002 (Fig. 7, A-C) and 1 $\mu\text{mol/l}$ Y-27632 (Fig. 7, D-F), which were PI3K and ROCK inhibitors, respectively.

DISCUSSION

Although the cellular mechanism of PRL action was still not fully understood, a twofold increase in the intestinal calcium absorption both in vivo and in vitro induced by PRL suggested a significant role of this hormone as a regulator of calcium homeostasis in conditions of high circulating PRL levels, such as in pregnancy and lactation (5). In the present study, we provided further information on the cellular mechanism of PRL as well as the possible signaling pathways. Acting via PI3K and PKC ζ , PRL enhanced the transcellular active calcium transport by increasing calcium entry through $\text{Ca}_v1.3$ and the

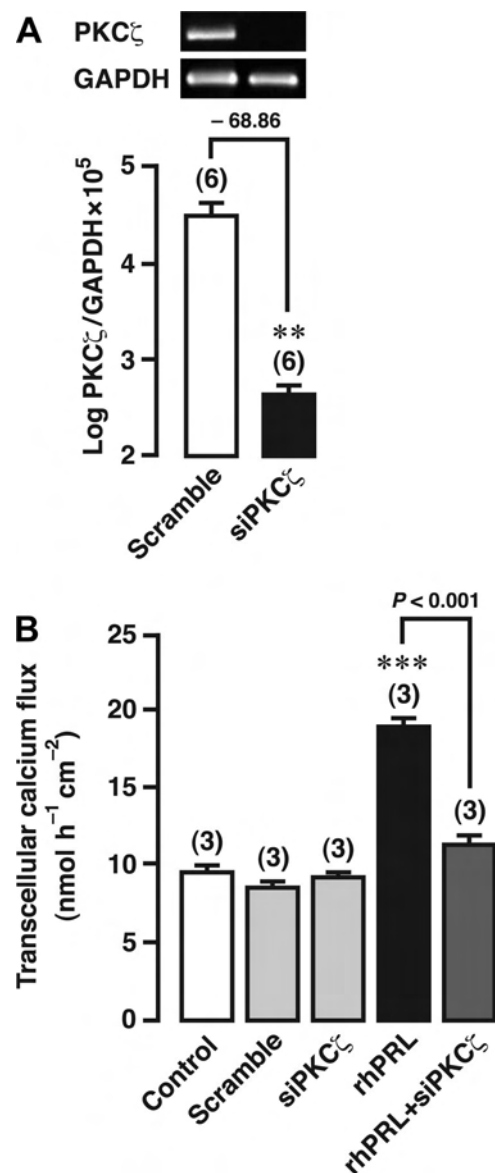


Fig. 6. A: expression of PKC ζ in Caco-2 cells transfected with scramble siRNA (negative control) or siRNA targeting PKC ζ . qRT-PCR results are expressed as log means $\pm$ SE. Representative electrophoretic bands and fold differences between the expression in control and knockdown cells (conventional PCR, 36 cycles) are also presented along with qRT-PCR results. GAPDH was used for normalization. ** $P < 0.01$ compared with scramble. B: transcellular calcium transport in PKC ζ knockdown Caco-2 monolayers exposed to 600 ng/ml rhPRL. The control group denotes normal Caco-2 monolayers (without siRNA transfection). *** $P < 0.001$ rhPRL vs. rhPRL + siPKC ζ . Numbers in parentheses represent the number of independent Snapwells.

basolateral calcium extrusion by PMCA. PRL also stimulated the voltage-dependent paracellular calcium transport through PI3K and ROCK.

The theoretical concept of the transcellular calcium transport in the intestine was recently challenged by the findings that calbindin-D $_{9k}$ knockout and TRPV6/calbindin-D $_{9k}$ double-knockout mice were normocalcemic and still exhibited active calcium absorption (1, 2, 27). We also found in this study that the rapid nongenomic effects of PRL on the transcellular active calcium transport in Caco-2 monolayer did not require the

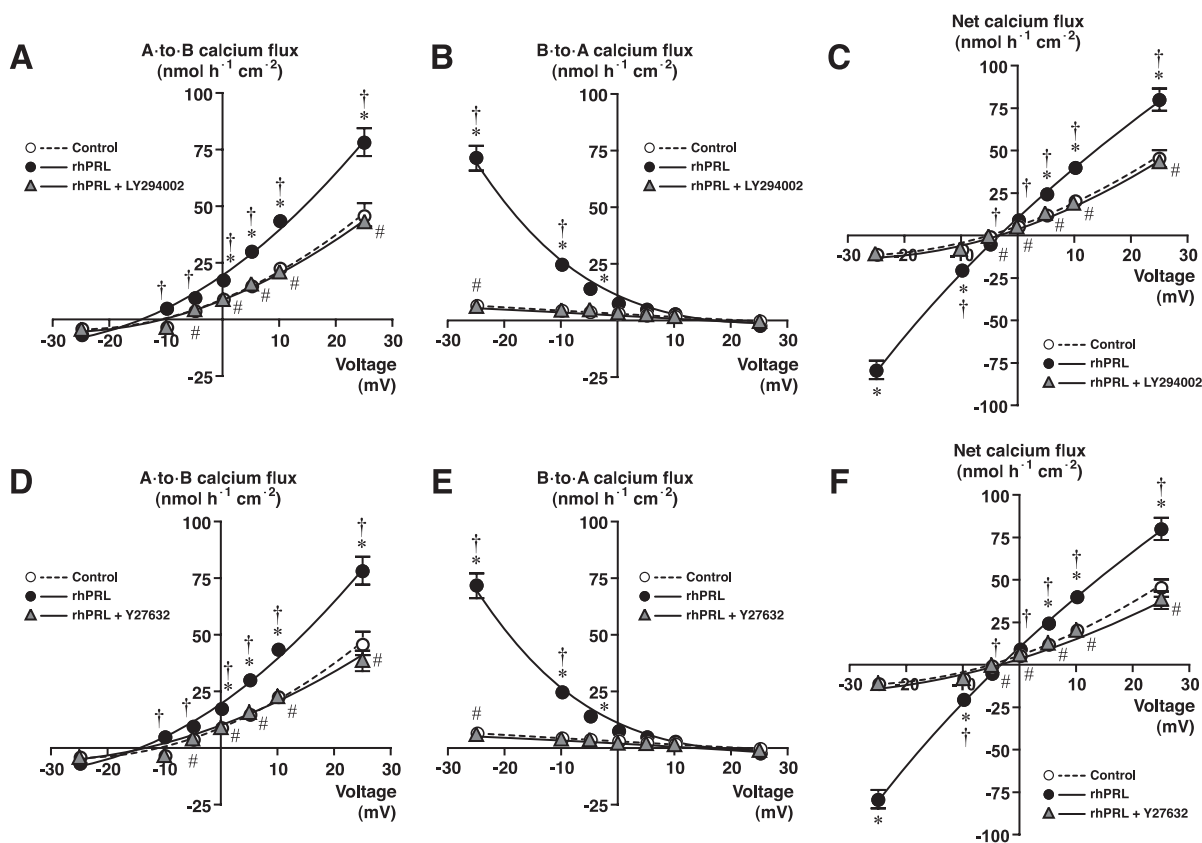


Fig. 7. Effects of PI3K inhibitor (75 $\mu\text{mol/l}$ LY-294002) (A–C) and ROCK inhibitor (1 $\mu\text{mol/l}$ Y-27632) (D–F) on the PRL-enhanced voltage-dependent paracellular calcium transport across Caco-2 monolayer ($n = 5$ per each voltage point; total 280 Snapwells used). The apical voltage was clamped to -25 , -10 , -5 , 0 , $+5$, $+10$, or $+25$ mV, with the basolateral side being a reference. Calcium fluxes in the apical-to-basolateral (A-to-B) (A and D) and basolateral-to-apical (B-to-A) (B and E) directions as well as net calcium fluxes (C and F) are presented. By using paired Snapwells, the net calcium flux was a subtraction of B-to-A flux from A-to-B flux. The mounted Snapwell was bathed on both sides with 1.25 mmol/l Ca^{2+} -containing solution. * $P < 0.01$ rhPRL vs. control. † $P < 0.01$, value of the rhPRL-treated group vs. value of the rhPRL-treated group at -25 mV (for A-to-B and net flux) or $+25$ mV (for B-to-A). # $P < 0.01$ value of the control group vs. value of the control group at -25 mV (for A-to-B and net flux) or $+25$ mV (for B-to-A). Values of the control and rhPRL-treated groups in D, E, and F are from those in A, B, and C, respectively.

presence of TRPV6 or calbindin-D_{9k}, although both transport proteins may contribute to the responses to other hormones, such as genomic 1,25(OH)₂D₃, 17 β -estradiol, and glucocorticoids (19, 46). It appeared that redundancy in the mechanisms underlying calcium absorption may involve other calcium channel(s), such as L-type and T-type calcium channels, which could provide alternative route for apical calcium entry (9). Supporting evidence was provided by recent reports that dihydropyridine (nifedipine)- and phenylalkylamine (verapamil)-sensitive L-type calcium channel $\text{Ca}_v1.3$ could mediate calcium entry for the transcellular calcium absorption, especially during ingestion of high-calcium diet (31, 34). Nifedipine and verapamil also inhibited the stimulatory effects of parathyroid hormone-related peptide, nongenomic 1,25(OH)₂D₃, and luminal glucose on intestinal calcium absorption (10, 34, 51). Our previous microarray study has identified several L-type calcium channels upregulated in the duodenal epithelial cells of the pituitary-grafted hyperprolactinemic rats (8). Here, the absence of PRL effect on calcium transport after $\text{Ca}_v1.3$ knockdown or nifedipine/verapamil exposure strongly suggested a physiological role of $\text{Ca}_v1.3$ during PRL stimulation.

Similar to the apical calcium entry, the cytoplasmic calcium translocation across the intestinal epithelial cells may be mediated by other calcium-binding proteins (CaBP) besides cal-

bindin-D_{9k}, e.g., calmodulin and parvalbumin, as well as vesicular calcium transport (3, 13). Interestingly, the duodenal epithelial cells of hyperprolactinemic rats were found to manifest a ~ 17 -fold increase in parvalbumin mRNA expression as demonstrated by microarray (8), but its physiological significance in calcium transport remains to be investigated. Regarding the basolateral calcium extrusion, ~ 80 – 85% of calcium efflux normally occurs through PMCA, whereas the remaining is performed by NCX1 (18, 49). PRL was previously found to increase PMCA activity in purified basolateral membrane vesicles from the duodenum (6); therefore, PMCA was likely to mediate the PRL-enhanced calcium transport.

As for the paracellular pathway, the present results together with that reported by Thongon et al. (45) suggest that PRL augmented the paracellular calcium absorption in a concentration- and voltage-dependent manner. In the study of passive calcium absorption, PRL was found to alter the charge-selective property of tight junction without changing the pore size, thereby increasing calcium permeability and calcium flux through the paracellular route (44, 45). Our present investigation (Fig. 7) further suggests that the paracellular pores for calcium transport are rectifiers. In other words, the paracellular pores can conduct much larger A-to-B calcium flux at apical voltages positive than B-to-A calcium flux at apical voltage

negative even in an absence of the transepithelial calcium gradient, rendering the degree of rectification $J_{i,+25}/J_{o,-25} = 46.23/6.95 \approx 7$ (where J represents calcium flux in $\text{nmol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$ at $+25$ mV or -25 mV; i and o denote A-to-B and B-to-A, respectively). The paracellular rectification could be explained by a model of a channel (or pore) with an energy barrier that impedes calcium movement in a B-to-A direction (for review see Ref. 41). Although the exact paracellular structures acting as the energy barrier have never been identified, we speculated that they might be tight junction proteins, claudins, which had their charge-selective extracellular loops protruding into the paracellular space (47). Several claudins, such as claudin-2, -3, and -12, are known to be involved in the intestinal calcium absorption (7, 15).

After exposure to PRL, the voltage-dependent calcium transport in both A-to-B and B-to-A directions were markedly increased, and the degree of rectification was reduced to ~ 1 , suggesting that an energy barrier or cation-selective barriers of the paracellular pores were removed by PRL. Changes in the paracellular barrier properties could have resulted from the PRL-induced claudin phosphorylation (L. Nakkrasae and N. Charoenphandhu, unpublished observation, 2009). PRL-enhanced voltage-dependent paracellular calcium transport may be of physiological importance, presumably when the intestinal absorptive cells were exposed to nutrients that could alter the transepithelial potential difference, e.g., glucose, short-chain fatty acid, and neutral amino acids (14, 40, 48). Although the modulation of potential difference could also facilitate calcium transport via the transcellular pathway (11), the transcellular calcium transport was much smaller in magnitude when compared with the paracellular calcium transport, particularly in Caco-2 monolayer (7, 45). However, there was a disparity regarding the magnitude of paracellular calcium flux between different models. For example, in perfused intestinal loop, nifedipine inhibited calcium absorption in the presence of 10 mmol/l luminal calcium with similar extent to 1.25 mmol/l calcium, suggesting that the majority of calcium flux in this model was Ca_v mediated rather than diffusive paracellular (35).

The action of PRL on calcium absorption involved the signaling molecules PI3K, PKC, and ROCK (20, 45). PI3K was likely to be the most upstream mediator, which transmitted PRL signals to PKC and ROCK for the transcellular and paracellular calcium transport, respectively (17, 21, 29, 45). In some cells, e.g., L6 muscle cells, PKC_ζ is a key molecule within the PI3K signal cascade (39). PKC is a large family of serine/threonine kinases, consisting of ~ 12 members (33). Some PKC isozymes, e.g., PKC_α , PKC_β , and PKC_θ , but not PKC_ζ , are activated by diacylglycerol (DAG), rendering them sensitive to DAG analog phorbol ester (i.e., PMA) (33). Several investigators provided evidence that activities of the transcellular calcium transport proteins TRPV6, $\text{Ca}_v1.3$, and PMCA could be modulated by PKA and/or PKC, especially $\text{PKC}_{\beta\text{II}}$ (23, 24, 34). However, PKA and phorbol-sensitive PKCs (e.g., PKC_α , PKC_β , and PKC_θ) were not involved in PRL signaling, since PRL actions were blocked only by an inhibitor or siRNA of PKC_ζ , which is a phorbol-insensitive isozyme (33). The presence of the additive effect of PRL in PMA-treated monolayer also suggested that PRL signaled through another signaling pathway that was not phorbol-sensitive PKCs. The absence of PRL-PMA synergism or truly additive effect, on the other

hand, probably indicated that PKC_ζ and phorbol-sensitive PKCs shared some target proteins.

In addition to the calcium gradient-dependent calcium transport, PRL exerted its stimulatory effects on the voltage-dependent calcium transport via ROCK pathway. PRL was previously reported to use the Rho-associated pathway in endothelial cells (28), in which a GTP-bound RhoA activated the serine/threonine kinase ROCK. In T84 colonic adenocarcinoma cell monolayer, the presence of Rho/ROCK activities was essential for a decrease in TER and disassembly of apical junctional complex triggered by extracellular calcium depletion (42). However, it is currently unknown as to why the PRL-induced decrease in TER, which was closely related to the increased paracellular permeability, was not abolished by LY-294002 and Y-27632. It was possible that PRL may have other signaling molecules for modulation of paracellular properties. PKC_ζ might be not involved in the PRL-enhanced calcium transport via the paracellular pathway because PKC_ζ inhibitor was without effect on the PRL-induced decrease in TER.

In conclusion, we demonstrated that PRL stimulated the transcellular active calcium transport via $\text{Ca}_v1.3$ and PMCA, but not TRPV6, calbindin- D_{9k} , or NCX1. Such PRL action was mediated by PI3K and phorbol-insensitive PKC_ζ in a non-genomic manner. Moreover, PRL removed the paracellular rectification and enhanced the voltage-dependent paracellular calcium transport via the PI3K and ROCK pathways. The present findings provide more detailed information on the cellular mechanisms of PRL in the intestinal epithelial cells, which could be used to explain how PRL enhances calcium absorption in pregnant and lactating animals.

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Femoral bone mineral density and bone mineral content in bromocriptine-treated pregnant and lactating rats

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Abstract Since hyperprolactinemia was found to induce osteopenia in the metaphysis of long bone in non-mated female rats, pregnant and lactating rats with sustainedly high plasma prolactin (PRL) levels might also exhibit some changes in their long bones. We performed a longitudinal study in pregnant, lactating and post-weaning rats, using dual-energy X-ray absorptiometry to demonstrate site-specific changes (i.e., metaphysis vs. diaphysis) in femoral bone mineral density (BMD) and content (BMC). The results showed that femoral metaphyseal BMD and BMC were higher when compared to their age-matched controls during pregnancy, before decreasing in late lactation and post-weaning. On the other hand, femoral diaphyseal BMC increased during pregnancy, early lactating and mid-lactating periods without change during late lactation and post-weaning. After 7 days of bromocriptine administration which inhibited endogenous PRL secretion, the lactation-induced increases in BMC during early and mid-lactating periods were abolished. Moreover, a decrease in metaphyseal BMD during late lactation was restored to the control levels by bromocriptine. However, bromocriptine

did not antagonize the pregnancy-induced increases in BMD and BMC. It could be concluded that the effect of PRL on bone was variable during the reproductive periods. While having no effect on femoral BMD and BMC during pregnancy, PRL was responsible for bone gain in early and mid-lactating periods, but induced bone loss during late lactating period.

Keywords Bone density · Dual-energy X-ray absorptiometry (DXA) · Femur · Lactation · Pregnancy

Introduction

Previous investigations in non-mated female rats demonstrated that short-term exposure to high plasma prolactin (PRL) levels induced by 4 weeks of anterior pituitary transplantation (AP) led to net bone gain, while prolonged PRL exposure longer than 4 weeks gradually led to net bone loss [1, 2]. Such hyperprolactinemic effects appeared to be observed primarily at the trabecular sites, e.g., vertebrae and sternum, but not the cortical sites, e.g., tibiae and femora [3, 4]. However, by using dual-energy X-ray absorptiometric (DXA) analysis on specific regions of rat femur, we found that prolactin could decrease bone mineral density (BMD) and content (BMC) of the femoral metaphysis, which was predominantly trabecular bone, but not diaphysis which was predominantly cortical bone [5]. Thus, it was possible that elevated plasma PRL levels during pregnancy (~ 75 – 100 ng/mL) and lactation (~ 200 – 300 ng/mL), i.e., physiological hyperprolactinemia, could alter BMD and BMC of the long bone.

Reports on site-specific changes in BMD and BMC (metaphysis vs. diaphysis) of long bone during pregnancy and lactation in rats were controversial. Zeni and co-workers

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[6] reported that *in situ* BMDs of the proximal and distal tibiae were not changed on the first day postpartum, but were decreased at the end of lactation. Similarly, single-photon absorptiometric analysis revealed greater lactation-induced bone loss in the femoral metaphysis than in the diaphysis [7]. However, densitometric changes at mid-pregnancy and mid-lactation, which have high rates of maternal calcium loss, were presently not known. Nishiwaki and co-workers [8] demonstrated decreases in BMDs in the distal femur, lumbar spine and caudal spine (i.e., trabecular sites) at week 2–3 of lactation, but changes in cortical BMDs were not studied. Thus, a complete longitudinal densitometric study was first performed to demonstrate changes in site-specific femoral BMD and BMC during these reproductive periods.

To investigate the effects of endogenous PRL on BMD and BMC during pregnancy and lactation, an inhibitor of pituitary PRL secretion, bromocriptine, was administered to the animals. Bromocriptine is a dopaminergic D2 receptor agonist which mimics the hypothalamic PRL-inhibitory factor dopamine, thereby suppressing PRL release from the pituitary lactotrophs [9, 10]. Since osteoblasts were found to directly respond to several monoamines, e.g., serotonergic and adrenergic agonists [11], expression of D2 receptors, which have two isoforms known as long and short isoforms, was also determined to exclude possible direct actions of bromocriptine on osteoblasts.

Materials and methods

Animals

Non-mated (nulliparous) and pregnant (primiparous) Sprague–Dawley rats (8-week-old, weighing 210–220 g), were obtained from the Animal Centre of Thailand, Salaya, Thailand. They were placed in hanging stainless steel cages, fed standard chow containing 1% w/w calcium and 4,000 IU/kg vitamin D (Perfect Companion, Bangkok, Thailand), and distilled water *ad libitum* under 12:12 h light:dark cycle. Room temperature was controlled at 23–25°C, and the relative humidity was about 50–60%. Body weight and food intake were recorded daily. After delivery, the litter size was adjusted to eight pups per dam. Animals were cared for in accordance with the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences”. This study has been approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Science, Mahidol University.

Experimental design

Rats were divided into 6 groups, i.e., 14-day pregnancy (P14; mid-pregnancy), 21-day pregnancy (P21; late

pregnancy), 8-day lactation (L8; early lactation), 14-day lactation (L14; mid-lactation), 21-day lactation (L21; late lactation), and 15-day post-weaning (PW), with ages of 10, 11, 12, 13, 14, and 16 weeks, respectively. In some experiments, prior to the removal of femora, pregnant and lactating rats were administered for 7 days with 4 mg/kg per day bromocriptine *s.c.*, which have been known to diminish plasma PRL in pregnant and lactating rats [1, 9]. Thus, each group consisted of vehicle-treated mated (pregnant or lactating) rats, bromocriptine-treated mated rats, and vehicle-treated age-matched non-mated control rats. Bromocriptine (Sigma, St. Louis, MO, USA) was first dissolved in a mixture (vehicle) of tartaric acid, absolute ethanol, and normal saline, as described previously [12]. After the rats were killed, BMD and BMC of proximal metaphysis, distal metaphysis (trabecular sites), and diaphysis (cortical site) of the femur were determined.

Primary osteoblast culture

As described previously [13], tibiae were dissected from a 9-week-old non-mated female rat by sterile surgical technique. After removing the connective tissues and marrow cells, the bones were cut into small dice, and incubated on a shaker (60 cycles/min) for 2 h at 37°C in a 25-cm² T-flask (Corning, NY, USA) containing DMEM supplemented with 100 U/mL penicillin/streptomycin and 2 mg/mL collagenase (all purchased from Sigma). Thereafter, bone dice were washed with DMEM, and then cultured in DMEM supplemented with 15% FBS, 100 U/mL penicillin/streptomycin, 100 µg/mL ascorbate-2-phosphate, and 0.5 mM sodium pyruvate (Sigma) at 37°C with 5% CO₂. Osteoblasts proliferated and migrated from bone dice into the media within 3 days. Media were changed daily until day 20 for expression study. Rat osteoblast-like UMR-106 cells [American Type Culture Collection (ATCC) no. CRL-1661] were cultured as described by the ATCC’s instruction.

Polymerase chain reaction (PCR) and sequencing

Total RNA was prepared from primary rat osteoblasts, UMR-106 cells, pituitary glands, gastrocnemius muscle (negative control), and brain (positive control) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified with RNeasy Mini kit (Qiagen, Valencia, CA, USA). 1 µg of the total RNA was reverse-transcribed with oligo-dT₂₀ primer and iScript kit (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control gene to check the consistency of reverse transcription. Conventional PCR was performed for 36 cycles with the GoTaq Green Master Mix (Promega, Madison, WI, USA) and Bio-rad

MyCycler. Oligonucleotide sequences used as primers were 5'-CAGTCGAGCTTTCAGAGCCAA-3' and 5'-TCC ATTCTCCGCCTGTTCA-3' for long isoform of rat D2 receptor (amplicon 129 bp), 5'-TATGGCTTGAAGAG CCGTG-3' and 5'-TGTCTGCCTTCCCTTCTGA-3' for short isoform of rat D2 receptor (amplicon 134 bp), and 5'-AGTCTACTGGCGTCTTCAC-3' and 5'-TCATATTTTC TCGTGGTTCAC-3' for GAPDH (amplicon 133 bp). PCR products were visualized on a 1.5% agarose gel stained with 1.0 µg/mL ethidium bromide under a UV transilluminator (Alpha Innotech, San Leandro, CA, USA). After electrophoresis, PCR products were purified by the HiYield Gel/PCR DNA Extraction kit (Real Biotech Corporation, Taipei, Taiwan), and were sequenced by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

BMD and BMC measurements

Site-specific BMD and BMC were determined by the modified methods of Binkley et al. [14] and Thongchote et al. [5]. Femora of pregnant and lactating rats were removed and cleaned of adhesive tissue. Femoral BMD and BMC were assessed by a dual-energy X-ray absorptiometer (model Lunar PIXImus2; GE Medical Systems, Madison, WI, USA), operated with software version 2.10. The dual-energy supply was 80/35 kVp at 500 µA. The regions of interest (ROI) for femoral metaphysis included the proximal and distal 8 mm of the femur, whereas the ROI of femoral diaphysis included the middle part of the femur between its 8-mm ends. The machine was calibrated daily using a phantom provided by the manufacturer. The interassay coefficient of variation was less than 0.3%.

Statistical analysis

Results are expressed as mean $\pm$ SE. Two-group and multiple comparisons were performed by Student's *t* test and one-way ANOVA with Newman-Keuls post-test, respectively. The level of significance for all statistical tests was $P < 0.05$. Data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA, USA).

Results

Prior to investigating the effects of PRL on site-specific BMD and BMC of femora of pregnant and lactating rats, expression of dopaminergic D2 receptor was determined. As shown in Fig. 1, long isoform D2 receptor expression was observed only in pituitary gland and brain (positive control), but not in primary rat osteoblasts, osteoblast-like

UMR-106 cells, or gastrocnemius muscle (negative control). Short isoform of D2 receptor mRNAs were not expressed in osteoblasts. Thus, the results indicated that bromocriptine had no direct action on osteoblasts.

Densitometric analysis in non-mated control rats showed that femoral BMD and BMC of both trabecular sites (proximal and distal metaphyses) and cortical site (diaphysis) increased with age (Figs. 2, 3). When compared to non-mated control, BMD of proximal femoral metaphysis in the P14, P21, and L8 groups were significantly higher, while that of the L21 and PW groups were lower (Fig. 2a). BMC of the proximal metaphysis was also higher during pregnancy (P14 and P21), early lactation (L8), and mid-lactation (L14), without any difference from control thereafter (Fig. 2b). Similarly, BMD of distal femoral metaphysis in the P14 and P21 groups were markedly elevated when compared to non-mated control, and became lower in the L21 and PW groups (Fig. 2c). BMC of distal femoral metaphysis was higher in the P14, P21, and L14 groups, and later became lower in the PW group (Fig. 2d). Neither BMD nor BMC of distal metaphysis in the L8 group was different from the control values. As for the cortical site, when compared to control, significantly higher BMD was found in the P14, P21, and L14 groups (Fig. 3a), while higher diaphyseal BMC was found in the P14, P21, L8, and L14 groups (Fig. 3b).

To demonstrate the effects of PRL on femoral BMD and BMC in pregnant and lactating rats, bromocriptine was administered for 7 days prior to removal of the femora. As shown in Fig. 4, suppression of PRL secretion had no effect on the pregnancy-induced increases in BMD and BMC in the P14 and P21 groups. On the other hand, during lactation, an absence of PRL abolished increases in metaphyseal and diaphyseal BMC, but not BMD, in the L8 and L14 groups (Fig. 5a–d). In the L21 group, absence of PRL secretion also prevented a decrease in BMD and restored it to the control levels (Fig. 5e), without effect on BMC (Fig. 5f).

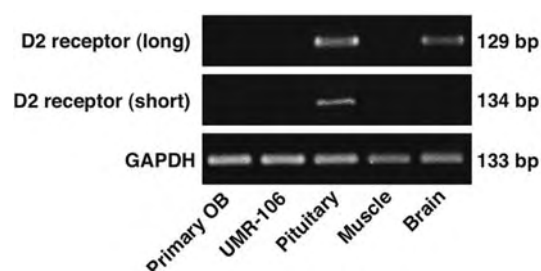


Fig. 1 Expression of long and short isoforms of subtype 2 dopamine (D2) receptor mRNAs in primary rat osteoblasts (OB), osteoblast-like UMR-106 cells, and pituitary gland. Gastrocnemius muscle and brain were used as negative and positive controls, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a housekeeping gene. This result was a representative of three independent repeats

Fig. 2 BMD and BMC of **a–b** proximal and **c–d** distal femoral metaphyses in mated (pregnant, lactating, and post-weaning) and non-mated age-matched control rats ($n = 7–9$ per group).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with its respective age-matched control group. †† $P < 0.01$ compared with age-matched control of P14 group. # $P < 0.05$, ## $P < 0.01$ compared with the P14 group. P14 and P21 designate 14- and 21-day pregnant rats, while L8, L14, and L21 designate 8-, 14-, and 21-day lactating rats, respectively. PW indicates 15-day post-weaning rats

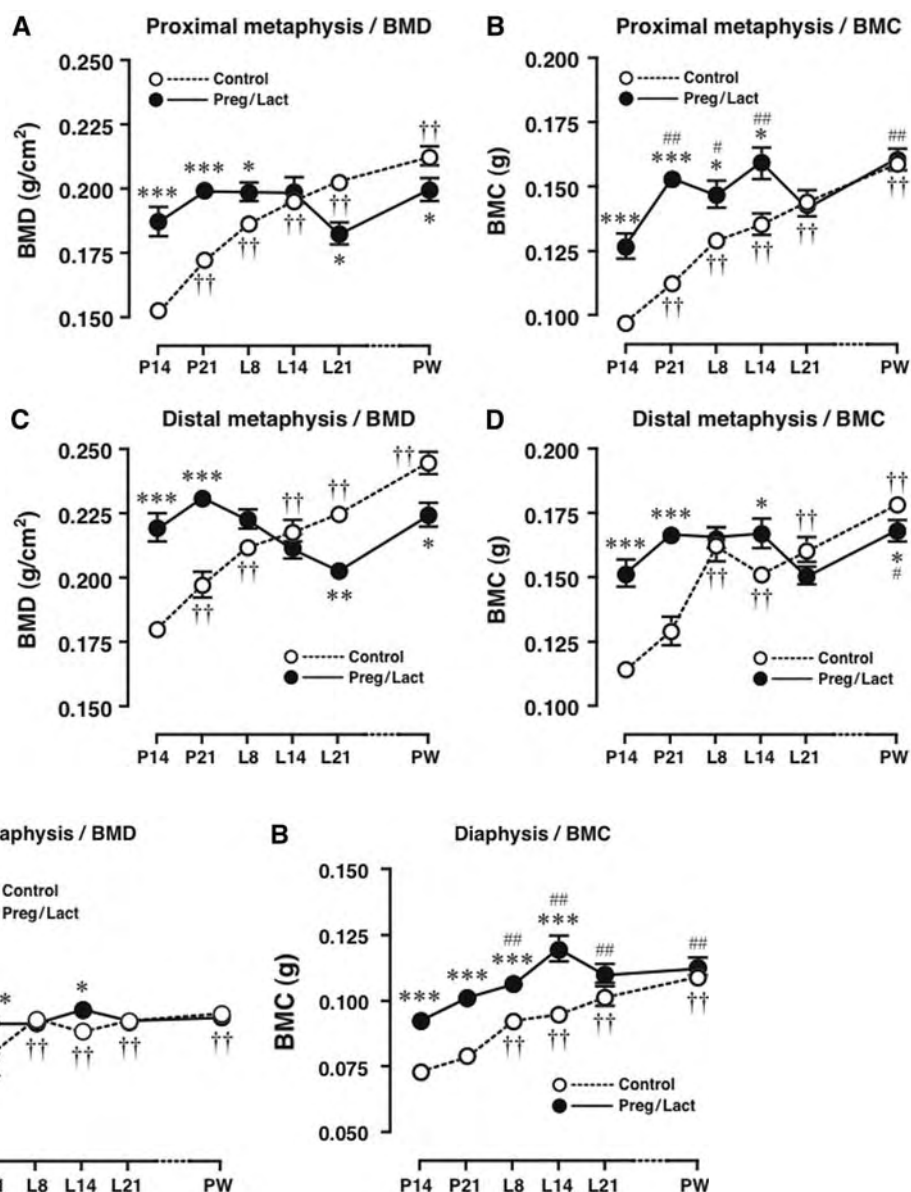


Fig. 3 **a** BMD and **b** BMC of femoral diaphyses in mated (pregnant, lactating, and post-weaning) and non-mated age-matched control rats ($n = 7–9$ per group). * $P < 0.05$, *** $P < 0.001$ compared with its respective age-matched control group. †† $P < 0.01$ compared with

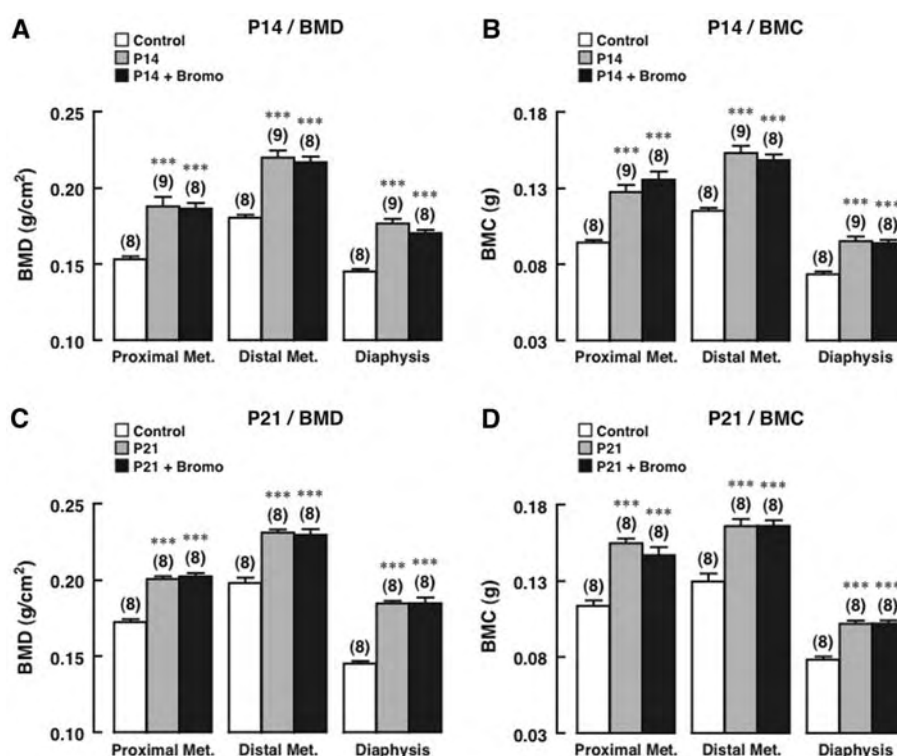
age-matched control of P14 group. ## $P < 0.01$ compared with P14 group. P14 and P21 designate 14- and 21-day pregnant rats, while L8, L14, and L21 designate 8-, 14-, and 21-day lactating rats, respectively. PW indicates 15-day post-weaning rats

Discussion

Little is currently known regarding the effects of hyperprolactinemia during pregnancy and lactation on the long bone. In the present study, we demonstrated that BMD and BMC of both trabecular and cortical parts of the rat femur were significantly increased during pregnancy until mid-lactation (L14). During late lactation (L21) and 15-day post-weaning, BMD of trabecular bone, but not cortical bone, was decreased. Furthermore, effects of PRL on bone during these reproductive periods were evaluated by administration of a selective D2

receptor agonist bromocriptine. The results suggested that PRL secreted from the pituitary gland was responsible for BMD and BMC changes during lactation, but not pregnancy. Daily administration of 4 mg/kg per day bromocriptine for 7 days was recently shown to decrease serum PRL of L7 rats by greater than 50% [15]. Moreover, in L7 rats with suckling, a single dose of bromocriptine injection markedly decrease serum PRL level from ~430 to ~100 ng/mL [15]. Since direct effect of bromocriptine on bone has never been reported, and osteoblasts did not express D2 receptor (Fig. 1), the bromocriptine effects observed in the present study

Fig. 4 BMD and BMC of proximal and distal femoral metaphyses (*Met.*) and femoral diaphyses in **a,b** 14-day pregnant rats (*P14*), and **c,d** 21-day pregnant rats (*P21*) treated with bromocriptine (*Bromo*). *** $P < 0.001$ compared with vehicle-treated age-matched control group. Numbers in parentheses represent the number of animals in each group



should occur through suppression of pituitary PRL release.

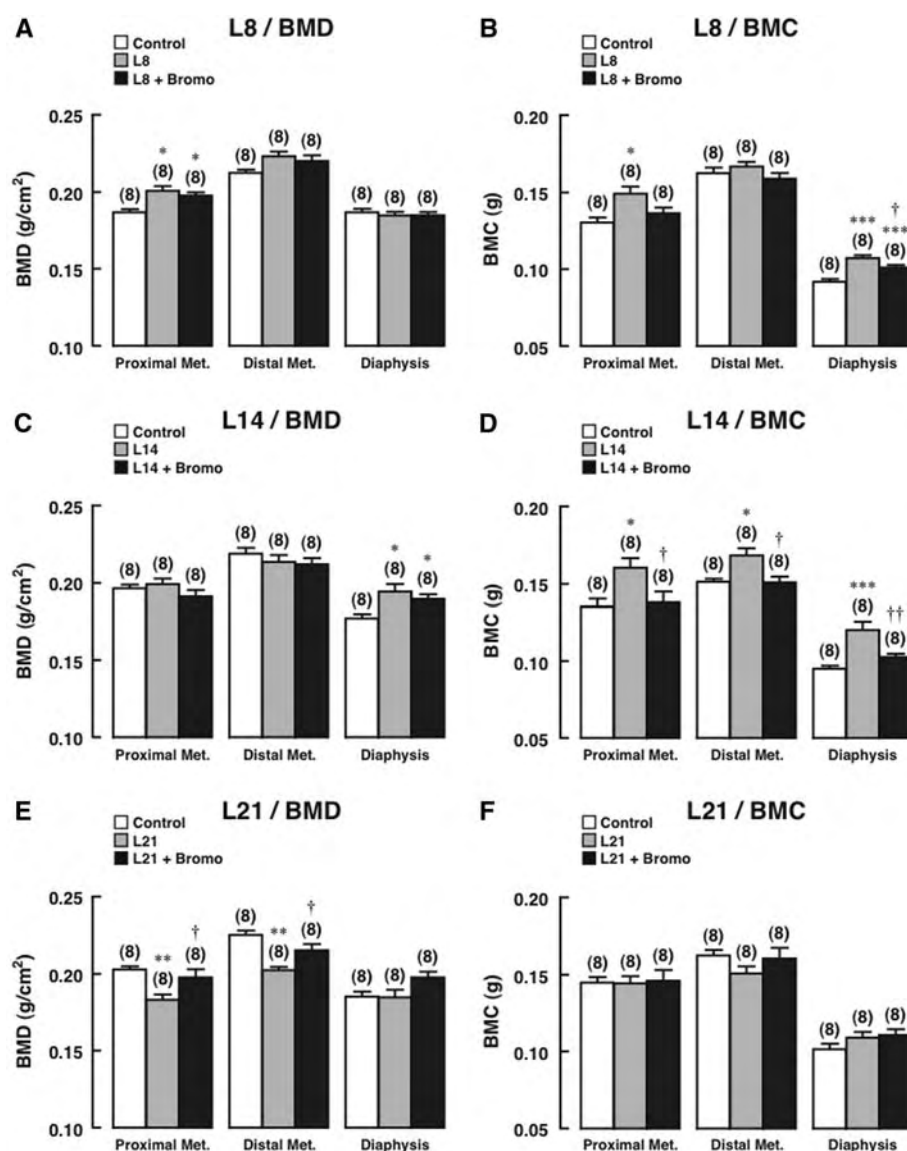
In general, fetal development during pregnancy leads to a massive drain on maternal calcium pool [16]. In humans, ~200–300 mg/day calcium was lost during the second and third trimester to maintain bone calcium accretion in the fetus [17]. However, changes in bone density during pregnancy remained controversial. Several investigators showed that there was no change in BMD in either pregnant women or rats [6, 16, 18]. In contrast, Gonen and co-workers [19] reported increased BMD of lumbar vertebrae, femur and tibia in pregnant rats, whereas others reported pregnancy-induced osteoporosis in humans [20, 21]. Such controversy could partly be due to the fact that an *in situ* densitometric measurement in living subjects is not sensitive enough to detect a slight change in bone density. Herein, by using *ex vivo* densitometric measurement, BMD and BMC of pregnant rats were found to increase at both femoral metaphysis and diaphysis. Despite the lack of evidence for the underlying mechanism, we speculated that the enhanced intestinal calcium absorption, in part, due to elevated serum PRL, placental lactogen and/or 1,25(OH)₂D₃ levels, was responsible for supplying calcium both for fetuses, and for maternal trabecular and cortical bone calcium accretion [15, 22, 23], thereby leading to increases in ash weight, calcium content, and cross-sectional area of femur [1, 24]. Changes in the size and cross-sectional area of femur without proportional change in the total calcium content partially explained why BMC

changes occurred in an absence of BMD change. Increased bone calcium content during pregnancy, as indicated by BMC (Figs. 2, 3), may also provide a reserved calcium pool for later use in milk production.

During lactation, an increase in BMC persisted until mid-lactation, presumably due to the lactation-induced increase in intestinal calcium absorption and renal calcium reabsorption [15, 16, 25]. PRL surge during suckling (~400–650 ng/mL) may further contribute to the elevated BMC, since short-term hyperprolactinemia of 2–4 weeks could increase bone calcium content in the rat femur, and further augment calcium absorption in the small intestine [1, 15, 26]. Besides metaphysis, PRL also had a positive effect on the femoral diaphysis of L8 and L14 rats, despite having no effect on the cortical sites in non-mated rats [4]. Although diaphyseal bone gain could be due to periosteal or endosteal bone formation, little is known regarding the direct effect of PRL on both processes in early lactation. We speculated that PRL did affect cortical bone but only in rats fed high-calcium diet [4], and perhaps in rats with enhanced intestinal calcium absorption. Bone histomorphometric study in hyperprolactinemic rats as well as in early lactating rats should reveal the effect of PRL on endosteal and periosteal calcium accretion.

When breastfeeding was prolonged, ongoing calcium loss in milk production eventually culminated in osteopenia in late lactation (L21), which was still detected at day 15 post-weaning. In exclusively breastfeeding women, calcium loss during this period can be as high as

Fig. 5 BMD and BMC of proximal and distal femoral metaphyses (*Met.*) and femoral diaphyses in **a,b** 8-day lactating rats (*L8*), **c,d** 14-day lactating rats (*L14*), and **e,f** 21-day lactating rats (*L21*) treated with bromocriptine (*Bromo*). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with vehicle-treated age-matched control group. † $P < 0.05$, †† $P < 0.01$ compared with its respective vehicle-treated lactating group. Numbers in parentheses represent the number of animals in each group



400 mg/day [17], while lactating rats lose 20–30% of their skeletal mass over 21 days of lactation [27, 28]. Interestingly, the observed bone loss only at trabecular site (metaphysis) was similar to that previously reported in rat vertebrae and distal femur [8]. Predominant bone loss in metaphyses may be due to the fact that the trabecular sites have more surface areas for bone resorption than the cortical sites. Several weeks after weaning, bone mass appeared to be completely restored to the normal level [16], presumably due to an increase in osteoclast apoptosis and decreased expression of osteoblast-derived bone resorption mediator, receptor activator of nuclear factor κ B ligand (RANKL) [29].

A number of hormones act in concert to regulate bone resorption process during lactation. For example, parathyroid hormone-related protein (PTHrP) produced by the mammary gland is able to stimulate bone resorption and

mobilize bone calcium for lactogenesis, whereas calcitonin helps to limit bone loss during lactation [16, 30]. In the present study, a decrease in metaphyseal BMD during late lactation was likely to be due to a lactogenic hormone PRL. Although PRL could induce a net bone gain in young growing rats or after short-term exposure in adults, chronic hyperprolactinemia longer than 5 weeks may lead to progressive trabecular osteopenia [2]. In non-mated female rats, chronic hyperprolactinemia markedly decreased trabecular bone volume and trabecular number, while enhancing bone turnover [2].

Since high plasma PRL levels suppressed ovarian estrogen synthesis [31–33], hyperprolactinemia-induced osteopenia was previously thought to be due to estrogen depletion. However, the presence of PRL receptors in primary rat osteoblasts and osteoblast-like MG-63 cells indicated direct PRL action on bone [2, 13]. At the

molecular level, PRL upregulated RANKL and downregulated osteoprotegerin (OPG) in osteoblasts, thereby enhancing osteoclastogenesis and osteoclast-mediated bone resorption [2]. PRL also suppressed alkaline phosphatase activity and osteocalcin expression, both of which were important for the osteoblast-mediated bone formation [2]. Although the aforementioned mechanism is able to explain metaphyseal bone loss in rat femur during late lactation, the molecular mechanism by which PRL increased bone mass during early and mid-lactation is currently unknown. In addition to the PRL-enhanced intestinal calcium absorption to supply more calcium for bone formation [23], it was possible that, during these reproductive periods, PRL may upregulate OPG rather than RANKL in osteoblasts of lactating rats, similar to that observed in human fetal osteoblasts [34], thus in turn reducing osteoclast proliferation.

In conclusion, from pregnancy to mid-lactation, BMD and BMC of both femoral metaphysis and diaphysis were increased. On the other hand, net bone loss was observed only in the femoral metaphysis during late lactation and day 15 post-weaning. Such bone changes during lactation, but not pregnancy, were found to be under the regulation of PRL. Interestingly, in contrast to non-mated rats in which PRL predominantly affected trabecular sites [3, 4], PRL induced net bone gain in both trabecular and cortical parts of femur of L8 and L14 rats, presumably to expand the calcium storage pool. Therefore, the present findings, together with the facts that PRL is able to stimulate intestinal calcium absorption and renal calcium reabsorption [1, 15, 23], suggested that PRL orchestrated total body calcium metabolism during lactation to guarantee an adequate calcium supply for milk production.

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Conflict of interest statement The authors declare no conflicts of interest.

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Transepithelial calcium transport in prolactin-exposed intestine-like Caco-2 monolayer after combinatorial knockdown of TRPV5, TRPV6 and $\text{Ca}_v1.3$

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Abstract The milk-producing hormone prolactin (PRL) increases the transcellular intestinal calcium absorption by enhancing apical calcium uptake through voltage-dependent L-type calcium channel (Ca_v) 1.3. However, the redundancy of apical calcium channels raised the possibility that $\text{Ca}_v1.3$ may operate with other channels, especially transient receptor potential vanilloid family calcium channels (TRPV) 5 or 6, in an interdependent manner. Herein, TRPV5 knockdown (KD), TRPV5/TRPV6, TRPV5/ $\text{Ca}_v1.3$, and TRPV6/ $\text{Ca}_v1.3$ double KD, and TRPV5/TRPV6/ $\text{Ca}_v1.3$ triple KD Caco-2 monolayers were generated by transfecting cells with small interfering RNAs (siRNA). siRNAs downregulated only the target mRNAs, and did not induce compensatory upregulation of the remaining channels. After exposure to 600 ng/mL PRL, the transcellular calcium transport was increased by ~2-fold in scrambled siRNA-treated, TRPV5 KD and TRPV5/TRPV6 KD monolayers, but not in TRPV5/ $\text{Ca}_v1.3$, TRPV6/ $\text{Ca}_v1.3$ and TRPV5/TRPV6/ $\text{Ca}_v1.3$ KD monolayers. The results suggested that $\text{Ca}_v1.3$ was the sole apical channel responsible

for the PRL-stimulated transcellular calcium transport in intestine-like Caco-2 monolayer.

Keywords Calcium absorption · Small interfering RNA (siRNA) · Transcellular transport · Triple knockdown · Voltage-dependent calcium channel (Ca_v)

Introduction

Prolactin (PRL) has been shown to be a calcium-regulating hormone since it could stimulate transcellular calcium absorption in intestinal epithelium of rats as well as in intestine-like Caco-2 monolayers [1–4]. Transcellular calcium transport is crucial during high calcium demand, such as pregnancy, lactation, or inadequate calcium intake [5]. It is a three-step process consisting of (1) apical calcium entry via transient receptor potential vanilloid Ca^{2+} channels (TRPV) 5, TRPV6 and voltage-dependent L-type calcium channel (Ca_v) 1.3, (2) cytoplasmic diffusion in a calbindin- D_{9k} -bound form, and (3) basolateral extrusion via plasma membrane Ca^{2+} -ATPase (PMCA) and $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) [6–9]. In general, $\text{Ca}_v1.3$ mediates intestinal calcium absorption under certain conditions, e.g., in the presence of depolarization state induced by glucose absorption [7, 8].

Recently, PRL was found to rapidly enhance transcellular calcium absorption in a non-genomic manner via $\text{Ca}_v1.3$, but not TRPV6 [9]. Pituitary-grafted rats with hyperprolactinemia also exhibited upregulation of L-type calcium channels [10]. However, the redundancy of apical calcium channels raised the possibility that the PRL-stimulated transcellular calcium transport may still require the presence of TRPV5 and/or TRPV6. Alternatively, one channel may be upregulated to compensate for the absence of the others. For example, TRPV5 knockout mice with

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hypercalciuria responded to negative calcium balance by upregulating TRPV6 expression in the small intestine to increase calcium absorption [11]. Indeed, it was not known whether calcium entry through TRPV5 contributed to the PRL-stimulated calcium flux in the intestine. Since TRPV5 and TRPV6, known to share 75% homology at the amino acid level and exhibit a similar ion permeation sequence for divalent cations ($\text{Ca}^{2+} > \text{Sr}^{2+} \approx \text{Ba}^{2+} > \text{Mn}^{2+}$), were co-expressed in the intestinal epithelial cells [12, 13], it was necessary to investigate the participation of TRPV5 in the PRL-stimulated calcium transport. Furthermore, whether $\text{Ca}_v1.3$ is the sole calcium channel required for PRL action was still not known.

Therefore, the objectives of the present study were (1) to examine the involvement of TRPV5 in the PRL-stimulated calcium absorption in intestinal epithelial monolayer, (2) to investigate whether an absence of one calcium channel led to upregulation of the others, and (3) to provide evidence that $\text{Ca}_v1.3$ was the sole calcium channel which mediated the PRL-stimulated calcium transport. Human colorectal adenocarcinoma cell line, Caco-2 cells, were used in the present study as they are easy to manipulate genetically. Despite being derived from the colon and having relatively low basal calcium flux compared to the small intestine [14], confluent Caco-2 monolayer has been widely used in the studies of drugs or hormones that enhance calcium absorption since it possesses characteristics of the small intestine, including the presence of the brush border, expression of sucrase-isomaltase enzymes, and expression of the transcellular calcium transporters [15–17]. Moreover, Caco-2 monolayer also expresses functional PRL receptors and responds well to PRL by increasing transcellular calcium flux by ~ 2 -fold, similar to that observed in the rat duodenum [14].

Materials and methods

Cell culture

Caco-2 cells (ATCC No. HTB-37) were grown in Dulbecco's modified Eagle medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 15% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 1% L-glutamine (Gibco), 1% non-essential amino acid (Sigma), 100 U/mL penicillin/streptomycin (Sigma), and 0.25 $\mu\text{L}/\text{mL}$ amphotericin B (Sigma). Cells were later propagated in 25- cm^2 T-flask (Corning, NY, USA) under a humidified atmosphere containing 5% CO_2 at 37°C, and subcultured as described in the ATCC protocol. Confluent Caco-2 monolayers were prepared by seeding cells (5.0×10^5 cells/ cm^2) on a polyester Snapwell with 12 mm diameter and 0.4 μm pore size (Corning). Culture medium was changed daily after 48 h of seeding.

Small interfering RNA transfection

Small interfering RNA oligonucleotides targeted for human TRPV5, TRPV6, and $\text{Ca}_v1.3$ as well as scrambled sequences were designed by siRNA Target Designer (version 1.51; Promega, Madison, WI, USA), as shown in Table 1. All oligonucleotides were synthesized by T7 RiboMax Express RNAi System (Promega) according to the manufacturer's instruction. At day 12 after seeding of Caco-2 cells on a Snapwell, in vitro transfection was performed with 10 $\mu\text{g}/\text{mL}$ polyethyleneimine (PEI) and 1 $\mu\text{mol}/\text{mL}$ siRNA molecules to generate TRPV5 knock-down (KD), TRPV5/TRPV6, TRPV5/ $\text{Ca}_v1.3$, TRPV6/ $\text{Ca}_v1.3$ double KD, and TRPV5/TRPV6/ $\text{Ca}_v1.3$ triple KD monolayers. At day 14 (i.e., 48 h after transfection), the Snapwell was mounted in an Ussing chamber with an exposed surface area of 1.13 cm^2 to measure the electrical parameters and calcium fluxes, as previously described [18]. Efficiency of siRNA was evaluated by quantitative real-time PCR (qRT-PCR). Recent investigations showed that conventional siRNA KD successfully abolished the functions of TRPV5 and TRPV6 channels, as determined by patch-clamp technique [19, 20]. This KD protocol was approved by the Institutional Biosafety Committee of the Mahidol University.

Quantitative real-time PCR and sequencing

Expression levels of TRPV5, TRPV6 and $\text{Ca}_v1.3$ were quantified by a real-time PCR (model MiniOpticon; Bio-Rad) as described previously [9]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control for normalization. Sense and antisense primers used for qRT-PCR are presented in Table 2. PCR reaction was performed with iQ SYBR Green SuperMix (Bio-Rad). Relative expression of TRPV5, TRPV6 or $\text{Ca}_v1.3$ over GAPDH was calculated from the threshold

Table 1 siRNA oligonucleotides used in KD study

Targets	siRNA oligonucleotides
TRPV5 siRNA	5'-GGCACUUGAAUCUUGGACU-3'
	5'-AAAGUCCAAGAUUCAAGUGCC-3'
TRPV6 siRNA	5'-GGGAAACACAGUGUUACAC-3'
	5'-GTGUAACACUGUGUUUCCCC-3'
$\text{Ca}_v1.3$ siRNA	5'-GAGCACC UUUGACAAUUUC-3'
	5'-AAGAAAUUGUCAAGGTGCUC-3'
Scramble siRNA	5'-GGCGCAAUAAAGCAAGACC-3'
	5'-AAGGUCUUGCUUUAUUGCCC-3'

TRPV5/6 Transient receptor potential vanilloid family Ca^{2+} channels 5/6, $\text{Ca}_v1.3$ voltage-dependent L-type Ca^{2+} channel 1.3

Table 2 *Homo sapiens* oligonucleotide sequences used in the qRT-PCR experiments

	Name	Accession no.	Primer (forward/reverse)	Product length (bp)
<i>TRPV5/6</i> Transient receptor potential vanilloid family Ca^{2+} channels 5/6, <i>Ca_v1.3</i> voltage-dependent L-type Ca^{2+} channel 1.3, <i>GAPDH</i> glyceraldehyde-3-phosphate dehydrogenase	TRPV5	NM_019841	5'-CACTGTTATTGATGCACCTGC-3' 5'-CCATCATGGCGATGAACA-3'	120
	TRPV6	AF365928	5'-TCTGACTGCGTGTTCAC-3' 5'-ACATTCCTTGGCGTTCAT-3'	144
	Ca _v 1.3	NM_000720	5'-TGATCCAAGTGGAGCAGTCA-3' 5'-GTGTGAAAGTCCGGTAGGAGA-3'	113
	GAPDH	NM_002046	5'-CTGGTAAAGTGGATATTGTTG-3' 5'-GAGGCTGTTGTCATACTTCTC-3'	359

cycle (C_t) values by using $2^{\Delta C_t}$ method. Besides melting curve analysis, PCR products were also visualized on 1.5% agarose gel stained with 1.0 $\mu\text{g/mL}$ ethidium bromide. After electrophoresis, PCR bands were extracted by HiYield Gel/PCR DNA Extraction kit (Real Biotech Corporation, Taipei, Taiwan), and were sequenced by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). qRT-PCR experiments were performed in triplicate.

Bathing solution for Ussing chamber technique

The bathing solution for Ussing chamber experiments contained (in mmol/L) 118 NaCl, 4.7 KCl, 1.1 MgCl_2 , 1.25 CaCl_2 , 23 NaHCO_3 , 12 D-glucose and 2 mannitol (all purchased from Sigma). The solution was continuously gassed with humidified 5% CO_2 in 95% O_2 , maintained at 37°C, pH 7.4, and had an osmolality of 290–293 mmol/kg water. Distilled water used in the present work had a resistance higher than 18.3 $\text{M}\Omega\text{ cm}$ and a free ionized calcium concentration of <2.5 nmol/L.

Measurement of electrical parameters

Three electrical parameters, i.e., potential (voltage) difference (PD), short-circuit current (I_{sc}), and transepithelial resistance (TER), were determined as described previously [14]. In brief, a pair of Ag/AgCl electrodes connected to agar bridges (3.0 mol/L KCl per 4% agar) was located near each surface of the mounted Snapwell for measurement of PD. The other ends of the PD-sensing electrodes were connected to a pre-amplifier (model EVC-4000; World Precision Instruments, Sarasota, FL, USA). Another pair of Ag/AgCl electrodes connected in series to the EVC-4000 current-generating unit was placed at the end of each hemichamber to supply I_{sc} , which is external current to nullify PD. TER was calculated from Ohm's equation. Fluid resistance was automatically subtracted by the EVC-4000 system.

Calcium flux measurement

Calcium fluxes were determined by the method of Thongon et al. [9]. In brief, after a 20-min incubation in the Ussing chamber, the bathing solution was changed with a fresh one. The solution on one side contained ^{45}Ca (initial specific activity of 5 mCi/mL, final specific activity of $\sim 450\text{--}500$ mCi/mol; Amersham, Buckinghamshire, UK). Unidirectional flux of calcium ($J_{\text{H}\rightarrow\text{C}}$, $\text{nmol h}^{-1}\text{ cm}^{-2}$) from the hot side (H) to the cold side (C) was calculated with Eqs. 1 and 2.

$$J_{\text{H}\rightarrow\text{C}} = R_{\text{H}\rightarrow\text{C}} / (S_{\text{H}} \times A) \quad (1)$$

$$S_{\text{H}} = C_{\text{H}} / C_{\text{T}} \quad (2)$$

where $R_{\text{H}\rightarrow\text{C}}$ is the rate of tracer appearance in the cold side (cpm/h), S_{H} the specific activity in the hot side (cpm/nmol), A the surface area of Snapwell (cm^2), C_{H} is a radioactivity in the hot side (cpm), and C_{T} is the total calcium in the hot side (nmol). ^{45}Ca radioactivity in counts per minute (cpm) was analyzed by liquid scintillation spectrophotometer (model Tri-Carb 3100; Packard, Meriden, CT, USA). Total calcium concentration in the hot side was determined by atomic absorption spectrophotometer (model SpectrAA-300; Varian Techtron, Springvale, Australia). In the absence of transepithelial calcium gradient, i.e., bathing solution in both hemichambers contained equal calcium concentration of 1.25 mmol/L, the measured calcium fluxes represented the transcellular active calcium transport [9, 14]. In some experiments, Caco-2 monolayer was exposed on the basolateral side to 600 ng/mL recombinant human PRL (rhPRL; purity >97%; catalog no. 682-PL; R&D Systems, Minneapolis, MN, USA), which is the maximal effective concentration reported by Jantarajit et al. [14].

Statistic analysis

Unless otherwise specified, results are expressed as mean $\pm$ SE. Multiple sets of data were compared by one-way analysis of variance (ANOVA) with Dunnett's

multiple comparison test. The level of significance for all statistical tests was $P < 0.05$. Data were analyzed by GraphPad Prism (version 4.0 for Mac OS X; GraphPad Software, San Diego, CA, USA).

Results

Expression of TRPV5, TRPV6 and $\text{Ca}_v1.3$ in TRPV5 KD monolayer

Since it was not known whether TRPV5 mediated the PRL-stimulated transcellular calcium transport, we generated TRPV5 KD monolayers to demonstrate the significance of TRPV5 for such PRL action. After being treated with PEI, scrambled siRNA, or TRPV5 siRNA, Caco-2 cells exhibited a stable expression of GAPDH (Fig. 1a), indicating

that GAPDH could be used as a control transcript. mRNA expression of TRPV5, but not TRPV6 or $\text{Ca}_v1.3$, markedly decreased in TRPV5 KD cells by ~ 17 -fold (Fig. 1b–e). The results suggested that TRPV5 siRNA was effective in suppressing TRPV5 expression without causing compensatory upregulation of TRPV6 and $\text{Ca}_v1.3$.

PRL-enhanced calcium transport in TRPV5 KD monolayer

After being exposed to 600 ng/mL rhPRL, both control and TRPV5 KD monolayers manifested an approximately twofold increase in the transcellular calcium transport (Fig. 2). The basal calcium transport was not affected by transfecting agent PEI, scrambled siRNA, or TRPV5 siRNA. The results, therefore, suggested that TRPV5 may not be required for the PRL-enhanced transcellular calcium transport.

Expression of TRPV5, TRPV6 and $\text{Ca}_v1.3$ in Caco-2 monolayers after combinatorial KD

To exclude the possibility that, in response to PRL, the remaining two channels were upregulated to compensate for the absence of the other channels after single KD of TRPV5, TRPV6, or $\text{Ca}_v1.3$, we generated Caco-2 monolayers with double KD of TRPV5/TRPV6, TRPV5/ $\text{Ca}_v1.3$, or TRPV6/ $\text{Ca}_v1.3$, or triple KD of TRPV5/TRPV6/ $\text{Ca}_v1.3$. We first demonstrated that, in a control monolayer, exposure to 600 ng/mL rhPRL for 60 min did not alter TRPV5, TRPV6, and $\text{Ca}_v1.3$ expression (Fig. 3a). Moreover, GAPDH expression was stable in both double and triple

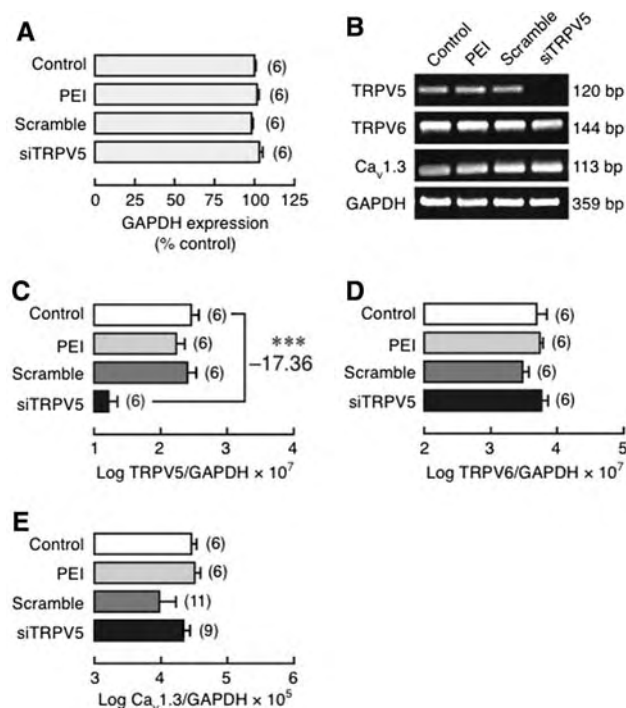


Fig. 1 **a** Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the control, PEI-treated, scrambled siRNA (Scramble)-transfected and TRPV5 siRNA (siTRPV5)-transfected Caco-2 monolayers. GAPDH expression of the control group was adjusted to 100%, while those of other groups were relative to the control group. **b** Representative electrophoretic bands of TRPV5, TRPV6 and $\text{Ca}_v1.3$ expression in the control, PEI, Scramble and siTRPV5 groups. **c–e** Expression of TRPV5, TRPV6 and $\text{Ca}_v1.3$ in TRPV5 KD Caco-2 monolayer as demonstrated by qRT-PCR. Expression levels were normalized by GAPDH expression. Values are presented as log mean $\pm$ SE. *** $P < 0.001$ compared with the control group. Value of fold difference was also presented along with the statistical symbol. Numbers in parentheses represent the number of independent experiments. Each experiment was performed in triplicate

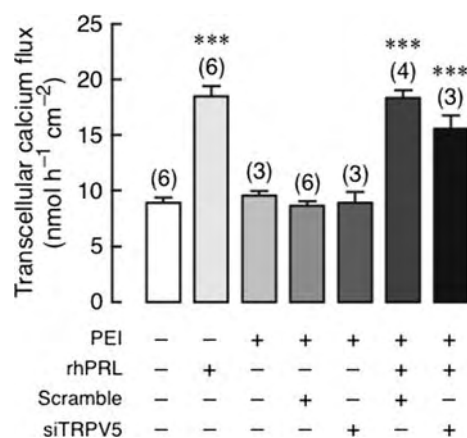


Fig. 2 Transcellular calcium transport in Caco-2 monolayer transfected with TRPV5 siRNA (siTRPV5) with or without 600 ng/mL rhPRL exposure. Scrambled siRNA was used as a negative control. Monolayer was bathed on both sides with 1.25 mmol/L calcium-containing solution. *** $P < 0.001$ compared with the control group. Numbers in parentheses represent the number of independent experiments

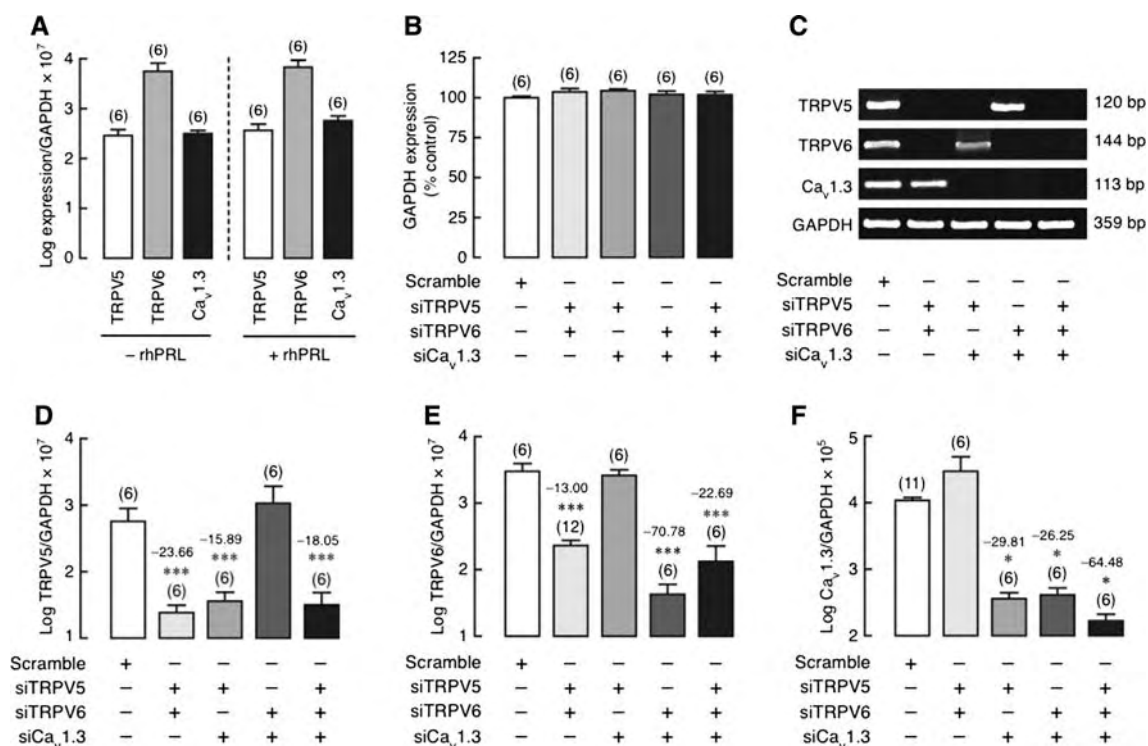


Fig. 3 **a** Expression of TRPV5, TRPV6, and Ca_v1.3 mRNA in Caco-2 monolayer directly exposed for 60 min to 600 ng/mL rhPRL. **b** GAPDH expression in TRPV5/TRPV6, TRPV5/Ca_v1.3, TRPV6/Ca_v1.3 double KD, and TRPV5/TRPV6/Ca_v1.3 triple KD monolayers. GAPDH expression in scrambled siRNA-treated group was adjusted to 100%. **c** Representative electrophoretic bands of TRPV5, TRPV6, and Ca_v1.3 mRNA expression in double and triple KD monolayers. **d–f** Expression of TRPV5, TRPV6, and Ca_v1.3 mRNA in double and triple KD Caco-2 monolayers as demonstrated by

qRT-PCR. Expression levels were normalized by GAPDH expression. Data are presented as log mean ± SE. **P* < 0.05, ****P* < 0.001 compared with the scrambled siRNA-treated group. Values of fold differences were also presented along with the statistical symbols. Numbers in parentheses represent the number of independent experiments. Each experiment was performed in triplicate. siTRPV5, siTRPV6, and siCa_v1.3 mean siRNAs against TRPV5, TRPV6, and Ca_v1.3 mRNA, respectively

KD monolayers (Fig. 3b). TRPV5 mRNA expressions in TRPV5/TRPV6, TRPV5/Ca_v1.3, and triple KD monolayers were decreased by ~24-, ~16-, and ~18-fold, respectively, whereas no significant change was found in TRPV6/Ca_v1.3 KD monolayer (Fig. 3c, d). Similarly, TRPV6 and Ca_v1.3 mRNA expressions were downregulated only in the presence of their target siRNA molecules (Fig. 3c, e, f). These findings indicated that there was no compensatory expression of the remaining channel when mRNAs of the two channels were disrupted. In other words, the lack of expression of one channel could not be rescued by expression of the others.

PRL-enhanced calcium transport in Caco-2 monolayers after combinatorial KD of TRPV5, TRPV6 and Ca_v1.3

In TRPV5/TRPV6 double KD monolayer, 600 ng/mL rhPRL was able to stimulate the transcellular calcium transport, similar to that observed in the control and PEI-treated monolayers (Fig. 4). However, in TRPV5/Ca_v1.3, TRPV6/Ca_v1.3 double KD, and triple KD monolayers, the

PRL-stimulated transcellular calcium transport was completely abolished (Fig. 4). Such combinatorial KD had no effect on the basal calcium transport, PD, *I*_{sc}, or the PRL-induced decrease in TER (Figs. 4, 5), the latter of which indirectly represented an increase in the paracellular permeability by PRL [9, 18, 21]. The results corroborated that Ca_v1.3 was the sole calcium channels required for the PRL-stimulated transcellular calcium transport across the intestinal epithelium.

Discussion

A significant amount of maternal calcium (~200–400 mg/day) is lost during pregnancy and lactation for fetal growth and milk production, thus resulting in severe negative calcium balance and osteopenia [22]. In lactation, PRL is not only an important hormone for lactogenesis but also for the regulation of overall calcium metabolism in pregnancy and lactation, in part, by stimulating renal calcium reabsorption and transcellular calcium absorption in the small

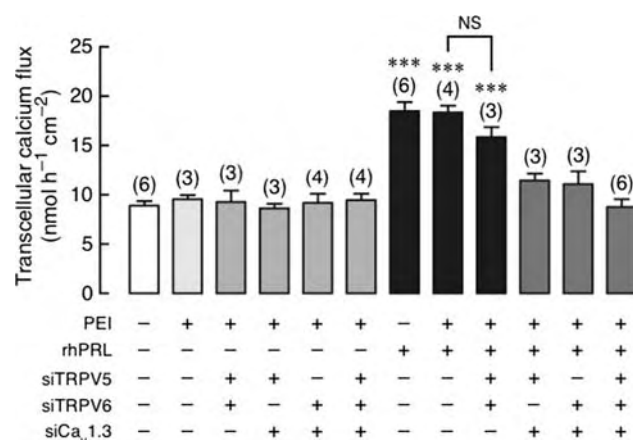


Fig. 4 Transcellular calcium transport in TRPV5/TRPV6, TRPV5/ $\text{Ca}_v1.3$, TRPV6/ $\text{Ca}_v1.3$ double KD, and TRPV5/TRPV6/ $\text{Ca}_v1.3$ triple KD monolayers directly exposed to 600 ng/mL rhPRL. PEI was a transfecting agent. Monolayer was bathed on both sides with 1.25 mmol/L calcium-containing solution. *** $P < 0.001$ compared with the scrambled siRNA-treated group (negative control). Numbers in parentheses represent the number of independent Snapwells. siTRPV5, siTRPV6, and siCa_v1.3 mean siRNAs against TRPV5, TRPV6, and Ca_v1.3 mRNA, respectively. NS Not significant

intestine, thereby ameliorating stress on calcium metabolism [2, 5]. Previous investigations in duodenal epithelial cells showed that PRL enhanced the transcellular calcium transport by increasing the apical (brush-border) calcium uptake [23]. The present study further demonstrated that Ca_v1.3 was the sole apical calcium channel for the PRL-stimulated transcellular calcium transport in Caco-2 monolayer, which is an accepted model for calcium absorption study [9, 15].

Three calcium channels, namely TRPV5, TRPV6, and Ca_v1.3, were co-expressed on the apical membrane of duodenal, jejunal, and colonic epithelial cells [24, 25]. For the past decade, TRPV6 has been postulated to be the principal calcium channel for apical calcium entry in the small intestine, whereas TRPV5, with its expression being much lower than that of TRPV6 in both duodenum [26] and Caco-2 cells (Fig. 3a), was thought to play a minor role. However, physiological significance of TRPV6 became doubtful when TRPV6 null mice were found to be normocalcemic and responded to 1,25(OH)₂D₃ by increasing transcellular calcium transport, similar to that observed in the wild-type mice [27, 28]. Moreover, Morgan and co-workers [8] suggested that TRPV5 and TRPV6 could not have been involved in the glucose-stimulated calcium absorption since both channels normally operated at negative membrane potentials below -90 mV (hyperpolarization), whereas postprandial glucose-rich luminal condition usually led to cell depolarization. Therefore, in the presence of luminal glucose, Ca_v1.3 must have been responsible for the intestinal calcium absorption [7, 8].

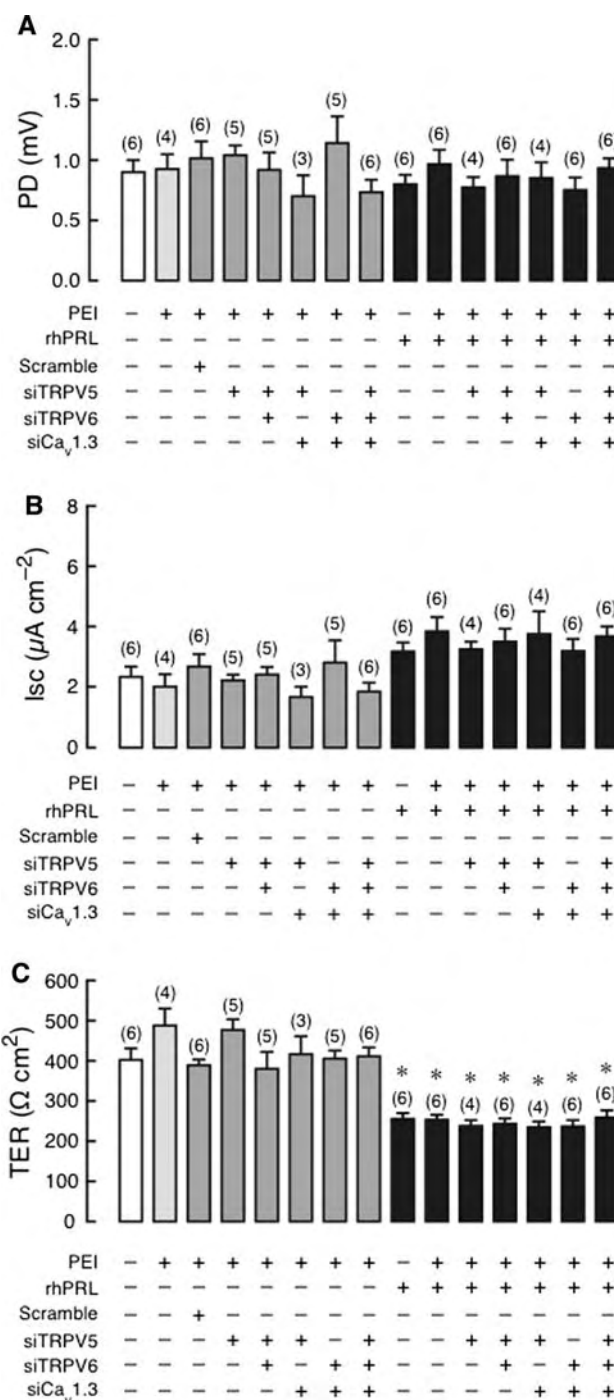


Fig. 5 **a** Transepithelial potential difference (PD), **b** short-circuit current (I_{sc}), and **c** transepithelial resistance (TER) in TRPV5 KD, TRPV5/TRPV6, TRPV5/ $\text{Ca}_v1.3$, TRPV6/ $\text{Ca}_v1.3$ double KD, and TRPV5/TRPV6/ $\text{Ca}_v1.3$ triple KD monolayers directly exposed to 600 ng/mL rhPRL. PEI was a transfecting agent. The apical side had negative voltage with respect to the basolateral side. * $P < 0.05$ compared with the scrambled siRNA-treated group. Numbers in parentheses represent the number of independent Snapwells. siTRPV5, siTRPV6, and siCa_v1.3 mean siRNAs against TRPV5, TRPV6, and Ca_v1.3 mRNA, respectively. The absence of changes in PRL actions in KD monolayers indicated that these calcium channels were not required for the PRL-induced increase in paracellular permeability

Nevertheless, intestinal TRPV5 and TRPV6 could be important for the enhancement of transcellular calcium transport in certain stimulating conditions, such as chronic metabolic acidosis, and long-term exposure to estrogen or $1,25(\text{OH})_2\text{D}_3$ [26, 29–31]. Indeed, TRPV6 expression was markedly upregulated after $1,25(\text{OH})_2\text{D}_3$ administration to vitamin D-deficient mice [32]. A number of hormones or local factors, such as parathyroid hormone, arginine vasopressin, prostaglandin E_2 , and calcitonin, were also found to activate TRPV5 and/or TRPV6 by raising intracellular cAMP or cGMP levels [33]. It is possible that TRPV5, TRPV6, and $\text{Ca}_v1.3$ may differentially respond to various stimuli or extracellular environments. In other words, these channels have their own unique properties and can function independently of each other. Such a hypothesis was confirmed by the present findings that neither TRPV5 nor $\text{Ca}_v1.3$ was upregulated when TRPV6 expression was suppressed by siRNA. Nevertheless, other alternative mechanisms for apical calcium entry might exist, since TRPV5/TRPV6/ $\text{Ca}_v1.3$ triple KD Caco-2 monolayer still exhibited transcellular calcium flux with a magnitude comparable to that of normal monolayer. Possible alternative pathways included the vesicular transport and voltage-dependent T-type calcium channels, the latter of which was reported to mediate the testosterone-stimulated calcium transport in renal tubular epithelium [34]. Alternatively, since siRNA-induced calcium channel KD did not totally suppress mRNA expression of target calcium channels (Fig. 3d–f), it was possible that a small number of calcium channels were sufficient to allow calcium entry under a non-stimulated condition. Feedback regulation of calcium influx could also explain the unaltered basal calcium transport after triple KD, which should lead to a decrease in the intracellular calcium concentration (Ca_i). Low Ca_i has been postulated to promote activities of TRPV5 and TRPV6 channels [35, 36], thereby leading to an increase in calcium influx.

Our previous investigation provided evidence that stimulatory effect of PRL on transepithelial calcium transport was abolished by $\text{Ca}_v1.3$ KD or L-type calcium channel blockers, nifedipine and verapamil, in the apical solution, but not in the basolateral solution [9]. $\text{Ca}_v1.3$ thus appeared to be responsible for apical calcium entry in PRL-exposed intestinal epithelium [9], but participation of TRPV5 and compensatory TRPV6 upregulation after $\text{Ca}_v1.3$ KD were still possible and were not excluded experimentally. Herein, we further elucidated that only $\text{Ca}_v1.3$ was involved in the PRL-stimulated transcellular calcium transport across the intestinal epithelium. Interdependent or interactive functions of TRPV5, TRPV6, and $\text{Ca}_v1.3$ in the presence of PRL could also be ruled out because enhanced transcellular calcium transport was still observed in TRPV5/TRPV6 double KD Caco-2 monolayer.

Molecular mechanism by which PRL stimulates intestinal calcium absorption is poorly understood. Recent studies in duodenal epithelium and Caco-2 monolayer substantiated pivotal roles of phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC), especially PKC_ζ isozyme, in the intracellular PRL signaling [14]. Panspecific PKC inhibitor (GF109203X) was shown to diminish the PRL-accelerated apical calcium uptake [9]. Although $\text{Ca}_v1.3$ could be phosphorylated by several PKC isozymes, e.g., PKC_β [7], it is not known whether PRL-activated PKC directly phosphorylated $\text{Ca}_v1.3$. Since Na^+ and glucose uptake through sodium-dependent glucose transporter 1 (SGLT1) at the apical membrane is coupled with $\text{Ca}_v1.3$ opening, which requires cell depolarization induced by SGLT1-mediated Na^+ entry [8, 37], it is also possible that PRL indirectly activates $\text{Ca}_v1.3$ by acting through SGLT1, known to be expressed in Caco-2 cells [38].

Besides the transcellular calcium transport, PRL could also stimulate calcium transport across the paracellular route [5]. The observed decrease in TER by PRL in the present study confirmed an increase in paracellular permeability, which could facilitate the paracellular calcium transport [9, 18, 21]. Due to its non-saturable passive nature, paracellular calcium transport could become substantial after ingestion of calcium-rich meals, or in the presence of a transepithelial calcium gradient [6]. Moreover, the presence of PRL-induced decrease in TER in TRPV5/TRPV6/ $\text{Ca}_v1.3$ KD monolayer suggested that the PRL-enhanced paracellular permeability did not involve the functions of these three calcium channels, although perijunctional cytoskeletal rearrangement, an initial step to increase paracellular permeability, may be controlled, in part, by apical calcium entry [37, 39].

In conclusion, we used siRNA-based combinatorial KD technique to elucidate that the apical calcium entry step in the PRL-stimulated transcellular calcium transport in the intestine-like Caco-2 monolayer occurred solely via $\text{Ca}_v1.3$. Although intestinal epithelial cells expressed several calcium channels, i.e., TRPV5, TRPV6, and $\text{Ca}_v1.3$, there was no redundancy in their functions, at least during PRL exposure, since the PRL-stimulated calcium transport was totally diminished after $\text{Ca}_v1.3$ siRNA transfection. The present results, therefore, provided corroborative evidence for better understanding of the cellular mechanism of PRL in intestinal epithelial cells, and could explain the mechanism by which PRL stimulated intestinal calcium absorption in pregnant and lactating animals.

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Conflict of interest statement The authors declare no conflict of interest.

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Claudin expression in the bone-lining cells of female rats exposed to long-standing acidemia

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ABSTRACT

Besides enhancing osteoclast-mediated bone resorption, chronic metabolic acidosis (CMA) induces mineral efflux across the epithelial-like bone membrane formed by bone-lining cells (inactive osteoblasts), possibly via the paracellular pathway. However, there was a compensatory mechanism that restricted bone loss in the late phase of CMA, and changes in the expression of claudins, which are tight junction proteins known to regulate epithelial barrier function, were therefore anticipated in bone-lining cells. Herein, primary rat osteoblasts were found to express several transcripts of claudins, i.e., claudin-5, -11, -14, -15 and -16. Their protein expressions in bone-lining cells were demonstrated by immunohistochemistry in decalcified tibial sections. After exposure to CMA induced by oral administration of 1.5% NH₄Cl for 21 days, expression of claudin-14, which normally seals the paracellular space and restricts ion movement, was increased, whereas that of claudin-15 and -16 which form pores for ion transport were decreased. Expressions of claudin-5 and -11 were not changed by CMA. In conclusion, the bone-lining cells of rats exposed to CMA for 21 days upregulated an ion-restrictive claudin (i.e., claudin-14), while downregulating ion-permeable claudins (i.e., claudin-15 and -16). These cellular responses might be parts of a compensatory mechanism accounting for deceleration of bone loss in late CMA.

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Introduction

Chronic metabolic acidosis (CMA) is a common acid-base disturbance caused by a reduction in capacity of the kidney to synthesize ammonia and excrete H⁺ (e.g., chronic renal failure), continuous loss of HCO₃⁻ (e.g., distal renal tubular acidosis), or accumulation of acidic substances. A number of studies showed that CMA affects bone metabolism by inducing physicochemical dissolution of bone minerals, suppressing osteoblast-mediated bone formation, and enhancing osteoclast-mediated bone resorption, thereby resulting in massive bone loss, hypercalciuria, nephrocalcinosis and negative calcium balance (Bushinsky and Lechleider, 1987; Dominguez and Raisz, 1979; Kraut et al., 1986; Krieger et al., 1992). However, our recent longitudinal study in rats exposed to CMA for 10 months suggested the existence of an unknown compensatory mechanism that decelerated bone loss and maintained bone mass during CMA (Assapun et al., 2009).

Bone calcium is present in the form of hydroxyapatite crystal, as well as free ions dissolved in the bone extracellular fluid (BECF), which is an intraosseous fluid compartment with different ion

compositions from plasma (e.g., high K⁺ concentration in BECF) (Green and Kleeman, 1991; Rubinacci et al., 2000). BECF is separated from the plasma by a “functional” epithelial-like bone membrane created by bone-lining cells (inactive osteoblasts), and its ion compositions were reported to be controlled by bone-lining cells (Bushinsky et al., 1989; Parfitt 1989; Soares et al., 1992; Trumbore et al., 1980). In acidosis, calcium is released from BECF by transporting across the bone membrane, presumably via the paracellular pathway (Bushinsky and Frick, 2000; Bushinsky, 2001; Marenzana et al., 2005). Besides calcium, the effluxes of other bone minerals, such as sodium, potassium and phosphate may also help buffer acid during CMA (Bergstrom and Wallace, 1954; Green and Kleeman, 1991).

In normal epithelia, the presence of tight junction is crucial for the fine-tuning of paracellular permeability, the maintenance of epithelial integrity, and the formation of paracellular barrier (Furuse and Tsukita, 2006; Tsukita et al., 2001). Since tight junctions are also present in osteoblasts (Hatakeyama et al., 2008; Prêle et al., 2003; Soares et al., 1992; Weinger and Holtrop, 1974; Wongdee et al., 2008), several investigations have suggested that bone-lining cells might regulate the paracellular ion exchange between the two fluid compartments, i.e., plasma and BECF, in an epithelial-like manner (Bushinsky et al., 1989; Hatakeyama et al., 2008; Rubinacci et al., 2000; Trumbore et al., 1980). However, it was still not known how ions were transported across the tight junction in CMA.

Abbreviations: BECF, bone extracellular fluid; CMA, chronic metabolic acidosis.

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In general, regulation of tight junction permeability for the paracellular transport of ions and small molecules across normal epithelia requires tight junction proteins of the claudin family, which consists of 24 members. Although claudins are predominantly localized in the tight junction, some claudins (e.g., claudin-3 and -7) can be found in other parts of the plasma membrane (Charoenphandhu et al., 2007; Holmes et al., 2006). Several investigators provided evidence that claudins could be divided into 2 subtypes, i.e., ion-restrictive and ion-permeable claudins (for review Anderson and Van Itallie, 2009). For example, claudin-5, -11 and -14 were demonstrated to seal the paracellular space and restrict paracellular ion movement (Ben-Yosef et al., 2003; Gow et al., 1999; Nitta et al., 2003), while claudin-15 and -16 assembled into a row of channel-like pores for paracellular ion transport (Tsukita et al., 2001; Van Itallie and Anderson, 2006). Interestingly, osteoblasts and bone-lining cells were also reported to express claudins, and their expressions were controlled by various humoral mediators, e.g., insulin-like growth factor I (Hatakeyama et al., 2008; Prêle et al., 2003; Wongdee et al., 2008). Since it was recently shown that long-term exposure to CMA eventually induced a compensatory response to limit bone mineral loss (Assapun et al., 2009), we hypothesized that expression of ion-restrictive claudins may be upregulated, whereas that of ion-permeable claudins were downregulated in the bone-lining cells of rats exposed to long-standing acidemia.

Materials and methods

Animals

Female Sprague–Dawley rats (8-week-old, weighing 180–220 g) were obtained from the National Animal Centre, Salaya, Thailand. They were housed in the laboratory animal husbandry unit under 12:12-h light–dark cycle, and fed regular chows (Perfect Companion, Bangkok, Thailand), and distilled water *ad libitum*. Room temperature was maintained at 20–25 °C, and relative humidity was 50–60%. After acclimatization for 7 days, the rats were randomly divided into two groups, i.e., age-matched control ($n=6$) and CMA groups ($n=4$). In CMA group, rats were provided *ad libitum* for 21 days with water containing 1.5% w/v NH_4Cl (Sigma, St Louis, MO, USA) to induce an acid-loading acidemia. Food and water intake were recorded daily. This study has been approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Science, Mahidol University, Bangkok, Thailand.

Arterial blood gas analysis

Heparinized arterial blood sample (5 mL) was collected from the left ventricle. Blood gas analysis of plasma pH, partial pressure of O_2 and CO_2 , and HCO_3^- concentration was performed to confirm the success of CMA induction by using an automated analyzer (model Ultra C; Nova Biomedical, Waltham, MA, USA).

Primary osteoblast culture

Under 50 mg/kg thiopental sodium i.p. anesthesia, tibiae were removed from a 9-week-old rat by sterile surgical technique, as previously described (Charoenphandhu et al., 2008; Wongdee et al., 2008). After the connective tissues and marrow cells were removed, bones were cut into small dice, and cultured in a 25-cm² T-flask (Corning, NY, USA) with DMEM supplemented with 15% fetal bovine serum, 100 U/mL penicillin-streptomycin, and 100 µg/mL ascorbate-2-phosphate (Sigma). Cells were incubated at 37 °C with 5% CO_2 . Osteoblasts proliferated and migrated from the bone dice into culture medium within 3 days. The medium was changed every 3 days. Six flasks of confluent cells from the same rat were pooled together for total RNA preparation. Five rats ($n=5$) were used in PCR studies.

Total RNA preparation and PCR

The total RNA samples were prepared from primary osteoblasts by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was then treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and subsequently purified by RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Purity of the total RNA was determined by the ratio of absorbance readings at 260 and 280 nm, the ratio of which was in the range of 1.8–2.0. One microgram of total RNA was reverse-transcribed with iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) to cDNA by a Bio-Rad MyCycler. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control gene to check the consistency of the reverse transcription. Primers used in the PCR studies are shown in Table 1. Conventional PCR was performed with GoTaq Green Master Mix (Promega) and Bio-Rad MyCycler. PCR products were collected at 36 cycles and visualized on 2% agarose gel stained with 1 µg/mL ethidium bromide (Sigma) under UV transilluminator (Alpha Innotech, San Leandro, USA). The important steps, including PCR amplification, cycle optimization and electrophoresis were performed in triplicate.

Bone preparation

As previously described by Wongdee et al. (2008), tibiae from normal and CMA rats were dissected, washed with 0.9% w/v NaCl and fixed at 4 °C overnight in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). Thereafter, they were washed with cold PBS pH 7.4 and decalcified in 20% w/v ethylenediaminetetraacetic acid (EDTA; Sigma) at 4 °C for 3 weeks. Decalcifying solution was replaced every 3 days. The success of decalcification was confirmed by dual-energy X-ray absorptiometry (model Lunar PIXImus2; GE Medical Systems, Madison, WI, USA). Finally, decalcified bones were embedded in paraffin, and cut into 7-µm sections by a microtome.

Immunohistochemistry

After deparaffinization in xylene and graded ethanol, sections were incubated at 37 °C for 30 min in antigen retrieval solution (0.01 mg/mL proteinase K, 50 mM Tris–HCl pH 7.5 and 5 mM EDTA). They were then incubated in 3% H_2O_2 for 1 h to reduce the endogenous peroxidase activity. Non-specific binding was blocked by incubating sections in 4% bovine serum albumin (BSA) in 0.1 M PBS for 2 h. After blocking, sections were incubated overnight at 4 °C with 1:200 anti-claudin-5, -11, -15, -16 (Santa Cruz Biotechnology, CA, USA), or -14 antibodies (Abcam, Cambridge, MA, USA). The sections were later washed with 0.1 M PBS, incubated for 1 h with biotinylated goat anti-rabbit antibody (Zymed, South San Francisco, CA, USA), and finally incubated for 1 h at room temperature with streptavidin-

Table 1

Rattus norvegicus oligonucleotide sequences used in the PCR experiments.

Gene	Accession no.	Primer (forward/reverse)	Product length (bp)
Claudin-5	NM_031701	5'-CGCTTGTGGCACTCTTGT-3' 5'-ACTCCGGACTACGATGTTG-3'	168
Claudin-11	NM_053457	5'-ATTGGCATCATCGTCACAAC-3' 5'-ATGTCCACCAGGGGCTTG-3'	158
Claudin-14	NM_001013429	5'-CTGTACTCTGGGCTTCATC-3' 5'-CACACATAGTCATTCAACCTG-3'	230
Claudin-15	XM_222085	5'-GCTGTGCCACCGACTCCC-3' 5'-CAGAGCCAGTTTCATACTTG-3'	330
Claudin-16	NM_131905	5'-ATCTTCTTCAGTACGCTGCC-3' 5'-CGATGAGTAATACGGTCCC-3'	372
GAPDH	NM_017008	5'-AGTCTACTGGCGTCTTCAC-3' 5'-TCATATTTCTCGTGGTTCAC-3'	133

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

conjugated horseradish peroxidase (HRP) solution (Zymed). As for claudin-16, the donkey anti-goat HRP-IgG antibody (Santa Cruz) was used instead of biotinylated antibody/streptavidin-HRP solution. Immunohistochemical reaction was developed with a chromogenic peroxidase substrate 3,3'-diaminobenzidine (DAB; Pierce, Rockford, IL, USA). The negative control sections were obtained by incubating the sections from the control or CMA rats in the absence of primary antibody. Sections were counterstained with hematoxylin and examined under a light microscope (model BX51TRF; Olympus, Tokyo, Japan) and Image-Pro Plus 5 (Media Cybernetics, Bethesda, MD, USA).

Measurement of signal intensity

Densitometric determination of immunohistochemical signals was modified from the method of [Lehr et al. \(1999\)](#). Selection of DAB-positive signals (brownish color) was performed with Adobe Photoshop 9 (Adobe System, San Jose, CA, USA) by filtering the specific color shade (in red-green-blue [RGB] mode) and color intensity in the regions of interest (ROI). The RGB color range was red = 142, green = 103 and blue = 65, and the fuzziness value was adjusted to 75%. All pixels with DAB-positive color on the ROI were automatically highlighted by the software. Pixel counting was performed by the Histogram command, which showed the numbers of the total and DAB-positive pixels in the ROI. Relative signal intensity was normalized to the negative control signals (i.e., signals from the negative control sections). The relative signal intensity was calculated as followed,

Relative intensity

$$= \frac{(\text{Positive pixels} / \text{Total pixels})_{\text{Age-matched control or CMA}}}{(\text{Positive pixels} / \text{Total pixels})_{\text{Negative control}}}$$

Statistical analysis

The results are expressed as means $\pm$ SE. Comparisons between CMA and control groups were determined by unpaired Student's *t*-test. The level of significance for all statistical tests was $p < 0.05$. Data were analyzed by GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

CMA was induced by continuous acid-loading with 1.5% NH_4Cl given in drinking water for 21 days. Plasma pH of CMA rats was significantly lower than that of control rats, i.e., 7.27 ± 0.02 ($n = 4$) vs. 7.37 ± 0.04 ($n = 6$, $p < 0.05$), respectively, with a decrease in plasma HCO_3^- concentration. Daily water intake was not less than 60 mL/day, suggesting that consumption of NH_4Cl -containing water in CMA rats was sufficient to induce sustained acidemia throughout the experimental period. The present blood gas profile represented a normal metabolic response to CMA, consistent with that reported previously by [Charoenphandhu et al. \(2007\)](#).

Prior to immunohistochemical localization of claudins, PCR experiments were performed to demonstrate that primary osteoblasts derived from the tibiae of adult female rats expressed claudins. As shown in [Fig. 1](#), primary rat osteoblasts strongly expressed claudin-5, -11, -14, -15 and -16 transcripts. Immunohistochemical analysis in tibial sections also confirmed that claudin-5, -11, -14, -15 and -16 proteins were expressed in the bone-lining cells (inactive or flat osteoblasts), but not in osteocytes or marrow cells ([Figs. 2A–J](#)). Morphology and localization of the present bone-lining cells were consistent with those described previously ([Weinger and Holtrop, 1974](#)). After exposure to long-standing acidemia, bone-lining cells upregulated claudin-14 expression by 1.62-fold, while downregulat-

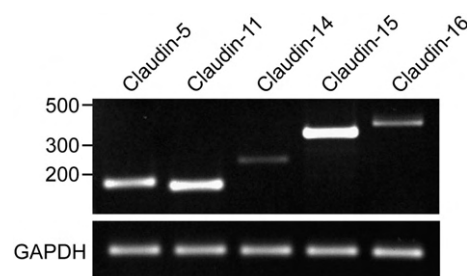


Fig. 1. Representative electrophoretic bands of claudin-5, -11, -14, -15 and -16 in primary rat osteoblasts ($n = 5$). Expression of GAPDH, a housekeeping gene, is presented along with the studied genes. Numbers on the left indicate the base pairs of PCR products.

ing expression of claudin-15 and -16 by 2.87- and 6.15-fold, respectively ([Figs. 2 and 3](#)). However, the expressions of claudin-5 and -11 were not changed by CMA ([Figs. 2 and 3](#)).

Discussion

During CMA, a number of compensatory responses are initiated to alleviate acidemia and negative calcium balance, e.g., increased intestinal calcium and phosphate absorption, and enhanced renal H^+ excretion and HCO_3^- reabsorption ([Rose and Post, 2001](#)). Bone also participates in the neutralization of H^+ by liberating calcium and other minerals, e.g., sodium, potassium, carbonate and phosphate, from BECF across bone membrane into the plasma ([Bergstrom and Wallace, 1954](#); [Bushinsky et al., 2003](#); [Green and Kleeman, 1991](#); [Rose and Post, 2001](#)), leading to reduced bone mineral content. This compartmentalization (i.e., BECF and plasma) is achieved by a layer of bone-lining cells ([Parfitt 1989](#); [Soares et al., 1992](#)). In addition, CMA also induced more calcium efflux from bone by enhancing the osteoclast-mediated bone resorption ([Assapun et al., 2009](#)). However, recent evidence showed that prolonged exposure to CMA appeared to induce a compensatory response in bone to restrict bone loss ([Assapun et al., 2009](#)). Specifically, a marked decrease in bone mass was observed only in the early phase of CMA. Thereafter, the rate of bone loss was gradually decreased ([Assapun et al., 2009](#)). Nevertheless, the exact mechanism responsible for this observation was unknown. It has been suggested that calcium efflux from BECF to plasma occurred through the paracellular pathway ([Marenzana et al., 2005](#)), perhaps across the tight junction formed by osteoblasts and bone-lining cells ([Soares et al., 1992](#); [Weinger and Holtrop, 1974](#)). Since the paracellular ion transport in normal epithelia could be regulated and restricted by certain tight junction proteins of the claudin family ([Anderson and Van Itallie, 2009](#)), the CMA-induced changes in claudin expression in bone-lining cells might be a part of the adaptive response of bone to minimize mineral loss. Indeed, the possibility of the CMA-induced upregulation of claudin expression in epithelial cells is not totally without suggestive evidence. Recently, 21-day CMA was found to increase the expression of claudin-2, -3, -6, -8, -11, -12, -14, -19 and -22 in the duodenal epithelial cells, which presumably contributed to the CMA-induced increase in the intestinal calcium absorption ([Charoenphandhu et al., 2006 and 2007](#)).

In the present study, we found that expression of claudin-14 was upregulated, while that of claudin-15 and -16 was downregulated in the bone-lining cells. The expression patterns of claudins generally vary among different epithelia or epithelial-like structure, i.e., they are the “fingerprint” of the epithelia. Most claudins increase the tightness of tight junction and create a barrier to restrict the paracellular ion transport (e.g., claudin-5, -11 and -14), whereas some claudins possess the ion-permeable property (e.g., claudin-15 and -16), which were essential in the formation of paracellular pores to facilitate ion movement ([Tsukita et al., 2001](#); [Anderson and Van Itallie, 2009](#)).

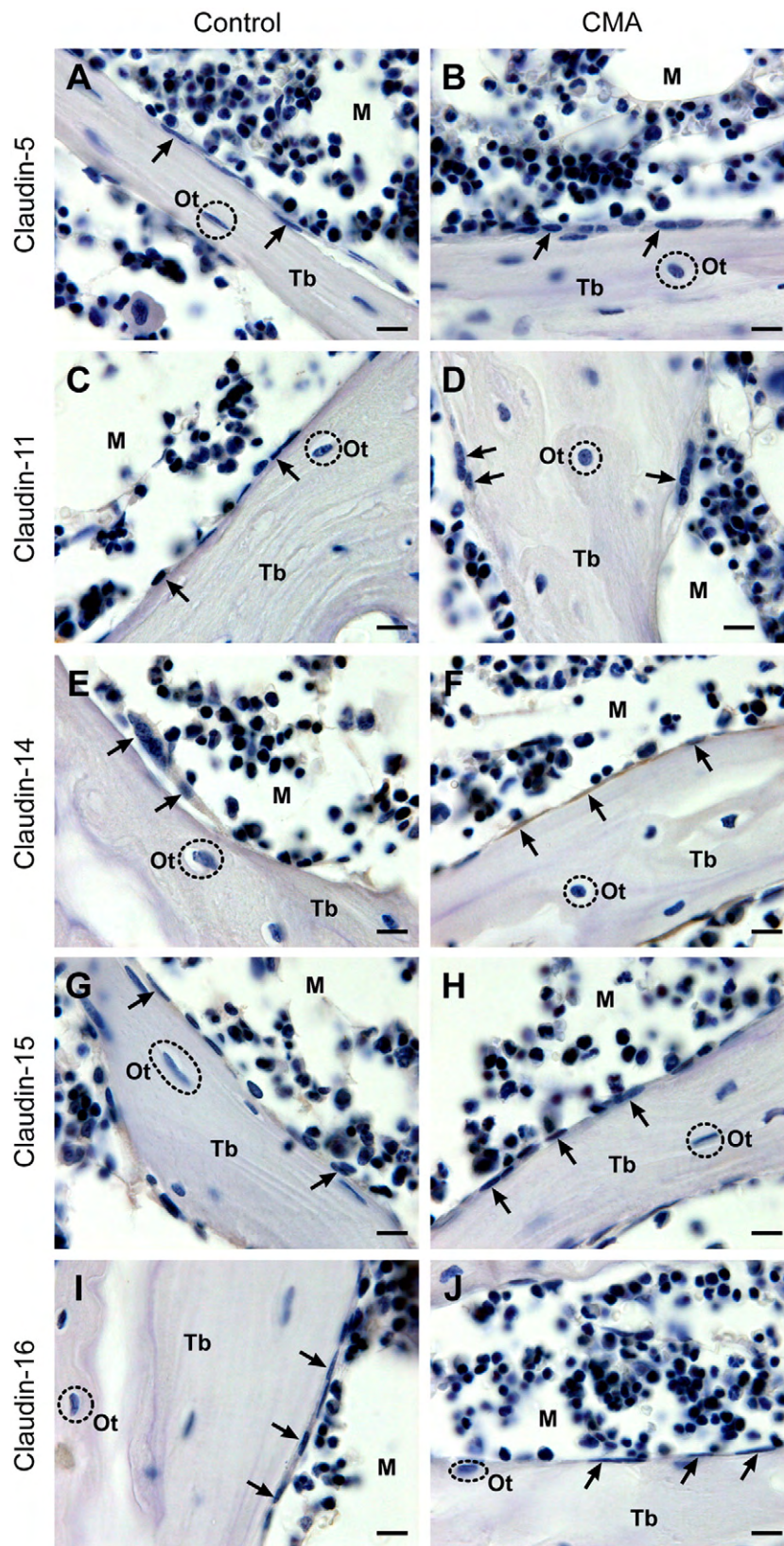


Fig. 2. (A–J) Immunohistochemical localization of claudin-5, -11, -14, -15 and -16 in paraffin-embedded decalcified tibial sections from control and CMA rats. The positive signals of claudins were seen in bone-lining cells (inactive osteoblasts; arrows), which covered bone trabeculae (Tb). No signal was detected in osteocytes (Ot) or bone marrow (M). Bars, 10 μ m.

For instance, claudin-5 is expressed in endothelial cells for the maintenance of blood-brain barrier to protect the brain from circulating neurotoxic substances (Nitta et al., 2003). Similarly,

claudin-11 is expressed in sertoli cells in the testis and forms the blood-testis barrier to prevent autoimmune responses against differentiated germ cells (Furuse and Tsukita, 2006). Nevertheless,

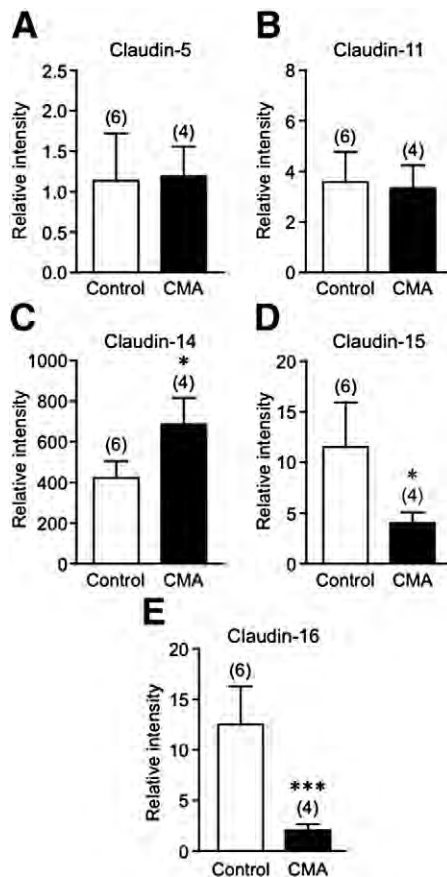


Fig. 3. (A–E) Relative signal intensity (arbitrary unit) of the expression of claudin-5, -11, -14, -15, and -16 in paraffin-embedded decalcified tibial sections from control and CMA rats. The intensities of the negative control sections were normalized to 1. Numbers in parentheses represent the number of animals in each group. * $p < 0.05$, and *** $p < 0.001$ compared with its respective control group.

expressions of claudin-5 and -11 in bone-lining cells were not changed by CMA.

Similar to claudin-5 and -11, claudin-14 also shows an ion-restrictive property. Under normal conditions, claudin-14 is expressed in several epithelia, e.g., the outer hair cells of the inner ear and intestinal epithelium of rodents (Ben-Yosef et al., 2003; Holmes et al., 2006; Charoenphandhu et al., 2007). In humans, mutations of claudin-14 may lead to deafness because its presence is essential for the maintenance of proper ion compositions of the endolymph and perilymph for sound transduction (Ben-Yosef et al., 2003). Overexpression of claudin-14 in Madin–Darby canine kidney-II monolayer led to an increase in the transepithelial resistance and a decrease in the paracellular cation permeability (Ben-Yosef et al., 2003). It was thus possible that upregulation of claudin-14 in bone-lining cells may enhance barrier function of the bone membrane to restrict mineral efflux from bone.

In contrast to claudin-14, claudin-15 and -16 have ion-permeable property, and can form the paracellular pores for cation transport (Van Itallie et al., 2003). Claudin-15 knockout mice manifested an enlargement of the proximal small intestine by ~2-fold with a decrease in the transepithelial ionic conductance (Tamura et al., 2008). Knockdown of claudin-15 also reduced cation permeability in the intestine-like Caco-2 monolayer (Charoenphandhu et al., 2009). Similarly, claudin-16 is involved in the regulation of the paracellular cation transport, especially calcium and magnesium reabsorption in the thick ascending limb of the loop of Henle (Simon et al., 1999). A strong evidence for the significance of claudin-16 is from its mutation that leads to a lethal inherited disease called familial hypomagnesemia with hypercalciuria and nephrocalcinosis. Since claudin-15 and

-16 normally enhance the paracellular cation transport, downregulation of both claudins in the present study suggested that mineral efflux across the bone membrane was reduced.

In conclusion, the bone-lining cells responded to CMA by upregulating claudin-14 and downregulating claudin-15 and -16, which could partially explain the previous *in vivo* findings that, in prolonged exposure to CMA, further bone loss was prevented, and bone mineral content was maintained at a subnormal level thereafter (Assapun et al., 2009). However, future investigation is required to demonstrate the molecular mechanisms of the CMA-induced changes in claudin expression in bone-lining cells in relation with changes in the rate of mineral efflux across the bone membrane in CMA rats.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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Is prolactin the cardinal calciotropic maternal hormone?

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To produce offspring, mothers require a large amount of calcium for fetal growth and milk production. Increased calcium demand leads to enhanced intestinal calcium absorption and stockpiling of bone calcium in pregnancy prior to demineralization in lactation. These coordinated events must be carefully organized by calciotropic hormone(s), but the classical hormones, namely 1,25-dihydroxyvitamin D₃, parathyroid hormone and calcitonin, do not appear to be responsible. Plasma prolactin (PRL) levels are elevated during pregnancy and, in view of the presence of PRL receptors in gut, bone and mammary glands, as well as recent evidence of the stimulatory effects of PRL on intestinal calcium transport, bone resorption and mammary calcium secretion, we postulate that PRL is the cardinal calciotropic hormone during pregnancy and lactation.

Stress on maternal calcium metabolism imposed by pregnancy and lactation

Women lose a huge amount of calcium, ~200–300 mg/day in the third trimester for fetal bone development and ~300–1000 mg/day during breastfeeding [1–3], and three maternal systems, namely intestine, bone and kidney (Figure 1), must work together in a highly coordinated manner under endocrine control to guarantee calcium adequacy for the developing fetus and newborn. Surprisingly, the principal calcium-regulating hormone in pregnancy and lactation remains a controversial issue. Several investigations have shown that neither bone mineral loss nor increased intestinal calcium absorption during these reproductive periods depend on ovarian or adrenal hormones [4]. Moreover, there is no strong correlation between maternal calcium demand and the major calcium-regulating hormones – 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], parathyroid hormone (PTH) and calcitonin [3,5,6]. In vitamin D receptor (VDR) knockout mice, expression of the transient receptor potential channel, subfamily V, member 6 (TRPV6), a calcium channel required for transcellular calcium absorption in the small intestine (Box 1), was still upregulated during pregnancy and lactation [7]. A recent study in pregnant VDR knockout mice confirmed that pregnancy stimulates duodenal calcium absorption in a VDR-independent manner [8]. Maternal calcium metabolism must therefore be regulated by some other hormone(s).

Evidence that supports calciotropic roles of PRL

Plasma levels of PRL, a 23 kDa polypeptide hormone secreted from the anterior pituitary gland, are strikingly elevated during pregnancy (75–100 ng/mL, compared to nonpregnant levels of 7–10 ng/mL) [5], to help maintain functional corpus luteum in pregnancy (for a review on PRL action, see Ref. [9]). To induce lactation, plasma PRL levels are elevated to 200–300 ng/mL, and transiently surge within 15 min of suckling to ~400–800 ng/mL, and these elevated levels last for 60–90 min [10]. Pregnancy and lactation are therefore considered to be physiological hyperprolactinemic states.

Why is PRL a strong candidate for the calcium-regulating hormone during these reproductive periods? In addition to its elevated plasma levels during pregnancy and lactation, PRL has been shown to have proto-osmoregulatory and hypercalcemic roles in fish, and increases branchial and intestinal calcium permeability while preventing excessive demineralization of bone and scale [11,12]. Indeed, on an evolutionary timescale, before PTH first appeared and fully functioned in amphibians, PRL and other PRL-like hormones (e.g. somatolactin) served as the major hypercalcemic hormones in fish [11]. Hypercalcemic activities of PRL are also evident in amphibians, reptiles and birds [11,13]. Although the lower vertebrates such as fishes and amphibians might have different modes of calcium homeostasis than mammals, this suggests a conserved role for PRL in calcium metabolism.

In mammals, PRL receptor (PRLR) expression in intestine, kidney and bone, the major calcium-handling organs, indicates a direct calciotropic action of PRL in these organs [14–16]. In fact, exogenous PRL administration stimulates intestinal calcium absorption in non-pregnant female rats [5] and increases intestinal calcium transport and bone calcium mobilization in vitamin D-deficient rats fed a low calcium diet [6]. PRLR knockout mice also manifest an impairment of bone development [17]. Moreover, in patients with PRL-producing tumors or pathological hyperprolactinemia induced by prolonged uses of antipsychotic drugs (e.g. risperidone and amisulpride, both of which antagonize the PRL-inhibiting factor dopamine), bone resorption is dramatically increased, resulting in low bone mass and overt osteoporosis [18,19].

Two-step stimulation of intestinal calcium absorption by PRL

PRL-stimulated maternal calcium absorption across the intestinal epithelium takes place by a two-step mechanism

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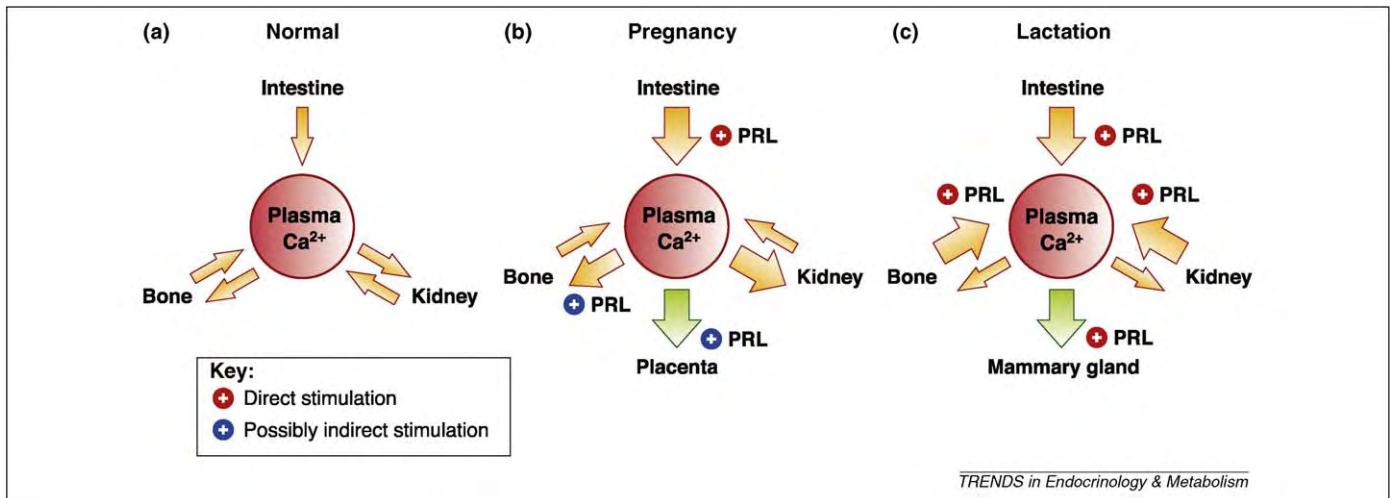


Figure 1. Hypothetical model for maternal total body calcium homeostasis as regulated by PRL. (a) Under normal conditions, bone formation and resorption are balanced, and renal calcium excretion is minimal. PRL acts as a coordinator to provide adequate calcium and to mobilize calcium for fetal bone development and milk production. (b) During pregnancy, PRL markedly enhances intestinal calcium absorption, that in turn indirectly increases maternal bone calcium accretion and placental calcium transfer [10,27]. If intestinal calcium absorption exceeds the body's requirement plus bone calcium accumulation, excess calcium will be excreted via the kidney, thereby leading to maternal hypercalciuria (also known as absorptive hypercalciuria) [29,33]. (c) In late lactation, PRL still enhances the intestinal calcium absorption to supply more calcium for milk production [10]. Adequate milk calcium supply is also achieved by PRL-enhanced renal calcium reabsorption and bone resorption, leading to maternal hypocalciuria and osteopenia, respectively [16,29,37]. Moreover, mammary calcium secretion has been found to be regulated by PRL [47].

(Figure 2) [10], similar to that which occurs in non-mated animals [6,14,20]. Specifically, sustained hyperprolactinemia of 75–100 ng/mL during pregnancy, or 200–300 ng/mL during lactation, induces a long-lasting adaptation in the intestinal absorptive cells to elevate the 'baseline calcium flux' by ~2-fold (Step-1), that is further increased (Step-2) by the transient PRL surge (400–800 ng/mL) ~15–90 min after suckling. Only Step-1 calcium absorption is dependent on *de novo* transcription of genes required for calcium absorption, for example those encoding TRPV6 and calbindin-D_{9k} (Box 1). However, the Step-2 calcium flux is well correlated with milk volume, and it is possible that the PRL surge during suckling serves as a signal to coordinate calcium mobilization from various sources to supply calcium for milk production [10]. Because inhibition of

pituitary PRL secretion completely abolishes Step-1 and -2 calcium absorption [10], PRL might serve as a single salient factor for this maternal adaptation.

The duodenum and cecum are highly responsive to PRL [10,20]. Both transcellular and paracellular calcium transport in the duodenum are markedly stimulated by PRL during the reproductive periods [10]. Although the major driving force for paracellular intestinal calcium transport is the calcium concentration gradient, the tight junction acts as a barrier that restricts paracellular ion movement in a charge- and size-selective manner [21]. Paracellular permeability is thus regulated by tight junction proteins including occludin, ZO-1 and claudins [21]. PRL overcomes this barrier by lowering the transepithelial resistance and increasing calcium permeability, in part, by downregulating

Box 1. Redundancy in intestinal calcium transport

Calcium traverses the intestinal epithelium via paracellular and transcellular pathways, with the former being present in most intestinal segments and predominant in the presence of ~2–6 mM luminal calcium as found with a normal diet [50]. In the presence of 10 mM luminal calcium, for example after oral calcium supplementation, the paracellular calcium flux can be ~5 times as high as the transcellular flux [10,14]. However, transcellular calcium transport becomes more significant during low calcium intake or increased calcium demand, for example during pregnancy and lactation [10,24,50]. Nevertheless, the cellular and molecular mechanisms are still controversial and require further investigation.

Transcellular calcium transport in the intestine is a three-step active process, consisting of apical calcium uptake, cytoplasmic calcium translocation and basolateral calcium extrusion. The calcium channel TRPV6 and calbindin-D_{9k} were believed to be responsible for the apical uptake and facilitated diffusion across the cytoplasm, respectively. Thereafter, plasma membrane Ca²⁺-ATPase-1b (PMCA_{1b}) and Na⁺/Ca²⁺ exchanger-1 (NCX1) are responsible for ~85% and ~15% of calcium efflux, respectively [51]. Bianco and co-workers reported a 60% decrease in intestinal calcium absorption, growth retardation and low femoral bone mineral density in the TRPV6-null mice [52]. However, more recent investigations showed that TRPV6 knockout, calbindin-D_{9k}

knockout, and TRPV6/calbindin-D_{9k} double knockout mice manifested normal serum calcium and 1,25(OH)₂D₃-dependent active calcium transport [53]. Several alternative or compensatory mechanisms can partially explain these discrepancies. For example, calcium might enter intestinal epithelial cells via other channels, including voltage-dependent calcium channels (Ca_v), and especially when cells are slightly depolarized by apical sodium entry in the presence of high luminal glucose [sodium and glucose enter the cell together via the sodium-dependent glucose transporter (SGLT)-1] [48]. Cytoplasmic calcium translocation might also be accomplished by other calcium-binding proteins such as calmodulin and parvalbumin [25,51], or by vesicular transport or 'tunneling' through the endoplasmic reticulum [51].

It is reasonable to ask the question – why does the gut use so many transport mechanisms and transporters for calcium absorption? Because calcium is essential for many processes in the body and is obtained solely from the diet, such redundancy guarantees that calcium absorption is always sufficient and matches calcium demand. It is also possible that these various mechanisms of calcium absorption are differentially regulated by different hormones or conditions. For example, estrogen, endurance exercise and chronic metabolic acidosis can upregulate TRPV6 expression [7,51,54,55], whereas Ca_v1.3 is essential for glucose-stimulated calcium absorption [48].

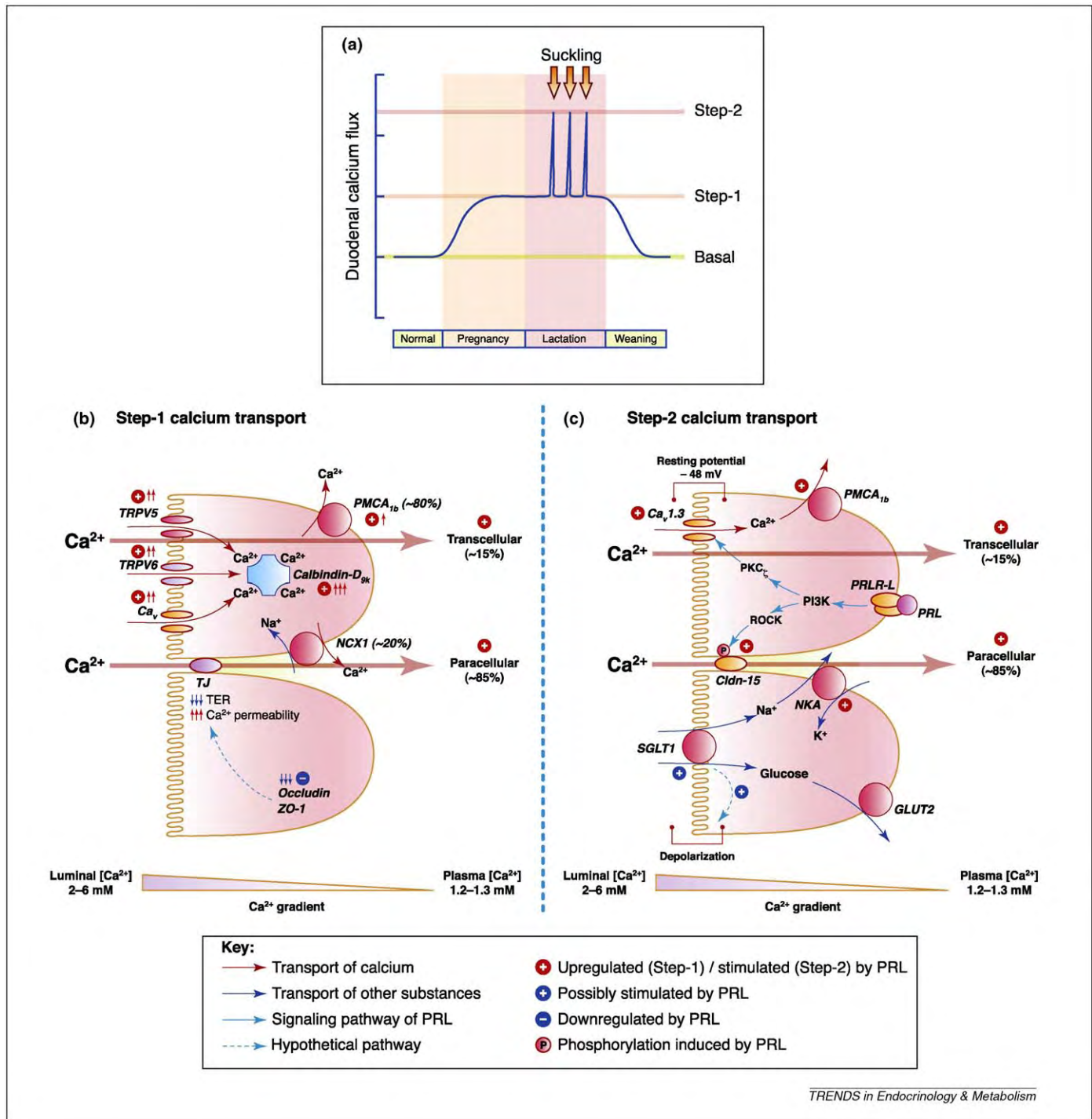


Figure 2. Hypothetical diagram of two-step stimulation of duodenal calcium absorption by PRL and its possible cellular mechanisms [10]. **(a)** Hyperprolactinemia during pregnancy and lactation elevates the 'basal calcium flux' to a new baseline (Step-1), whereas the suckling-induced PRL surge exerts a transient stimulatory effect on the calcium flux, that is further increased to the Step-2 level. This adaptation disappears after weaning. In the presence of a calcium gradient (i.e. luminal calcium > plasma free ionized calcium) and/or solvent drag, the PRL-enhanced paracellular calcium transport is predominant [14,23]. **(b)** The Step-1 calcium flux is achieved by PRL-induced upregulation of genes encoding proteins related to transcellular calcium transport (i.e. TRPV5, TRPV6, Ca_v , calbindin-D_{9k} and PMCA_{1b}), and downregulation of genes encoding tight junction (TJ) proteins (i.e. occludin and ZO-1) that are generally essential for the paracellular barrier functions as indicated by a high transepithelial resistance (TER) and restriction of paracellular ion permeability [10]. The degree of up- or downregulation is indicated by the number of small arrows. **(c)** Step-2 calcium transport through the transcellular pathway requires PRL-stimulated apical calcium entry via Ca_v 1.3, and calcium extrusion via PMCA_{1b}, whereas transport through paracellular pathway involves PRL-induced phosphorylation of claudin (Cldn)-15 [10]. PRL signaling is mediated by several proteins, such as long isoform (-L) of PRLR, phosphoinositide 3-kinase (PI3K), Rho-associated kinase (ROCK) and protein kinase C (PKC)-ζ [14,20,22]. Glucose absorption via the sodium-dependent glucose transporter (SGLT)-1 and the glucose transporter (GLUT)-2 is also important for PRL action because sodium entry via SGLT1 can induce depolarization of the apical membrane, thereby activating Ca_v 1.3 for transcellular calcium transport [48], whereas sodium extrusion via Na^+/K^+ -ATPase (NKA) can increase the paracellular hyperosmotic gradient for solvent drag [5,23]. In addition, an increase in paracellular calcium transport induced by solvent drag also results from PRL-stimulated NKA activity [5].

occludin and ZO-1 (Figure 2), thereby leading to Step-1 paracellular calcium transport [10]. Step-2 paracellular calcium transport, on the other hand, is related to the PRL-induced serine phosphorylation of claudin-15 [10]. The downstream PRL signal transduction pathway is not completely understood, but appears to involve phosphoinositide 3-kinase (PI3K)- and Rho-associated kinase (ROCK)-related pathways, and not the classical Janus kinase-2 pathway [10,20,22].

Moreover, calcium also traverses the paracellular space of the proximal small intestine along with osmotic water flow – a process known as solvent drag-induced calcium transport [23]. Solvent drag is induced after sodium entering the cells via sodium-dependent glucose transporter (SGLT)-1, and exiting the cells via Na^+/K^+ -ATPase, accumulates in the paracellular space as a standing hyperosmotic gradient (Figure 2) [5,23]. PRL stimulates this solvent drag-induced calcium transport by stimulating Na^+/K^+ -ATPase activity [5].

Regarding transcellular calcium transport, PRL-induced upregulation of crucial transporter genes including those encoding TRPV5, TRPV6 and calbindin- D_{9k} , accomplishes Step-1 transcellular calcium transport [7,10,24]. A recent microarray study in duodenal epithelial cells suggested that PRL also upregulated other potential calcium transporters, for example the voltage-dependent L-type calcium channel (Ca_v) and parvalbumin [25], perhaps to ensure adequate intestinal calcium absorption during pregnancy and lactation. However, it remains to be investigated whether PRL directly upregulates these calcium transporter genes, or acts indirectly by stimulating renal and/or local $1,25(\text{OH})_2\text{D}_3$ production [24].

In addition, acute PRL exposure directly activates the long isoform of PRLR and its downstream mediators, PI3K and protein kinase C- ζ , thereby leading to Step-2 transcellular calcium transport [10,22]. It appears likely that short isoforms of PRLR do not mediate PRL signaling in intestinal absorptive cells (for a review on PRLRs, see Ref. [9]). PRL-enhanced apical calcium uptake occurs very rapidly, within ~8–10 min after PRL exposure, and requires $\text{Ca}_v1.3$ [5,10,22] (Figure 2). We recently demonstrated that TRPV5 and TRPV6 were not involved because double knock-down of TRPV5 and TRPV6 did not abolish PRL-enhanced transcellular calcium transport [26]. Moreover, PRL also stimulates the basolateral calcium extrusion by increasing plasma membrane Ca^{2+} -ATPase-1b (PMCA $_{1b}$) activity [5]. It is noteworthy that the increased numbers of calcium transporter proteins (e.g. TRPV6 and calbindin- D_{9k}) induced by long-term PRL exposure (Step-1) might help to enhance Step-2 transcellular calcium absorption and/or increase the responsiveness of cells to acute PRL exposure during suckling [10].

Enhanced bone turnover and bone calcium release by PRL: friend or foe?

Pregnant mammals, including humans, sheep and rats, exhibit bone calcium accumulation in early and mid-pregnancy [1]. However, there is a slight decrease in maternal bone mineral density due to rapid fetal bone calcium accretion in the third trimester in humans, whereas maternal bone calcium accumulation in rats appears to

continue until early lactation [1,27]. Activated osteoclast activity at both trabecular and cortical sites is already evident throughout pregnancy, presumably to prepare the maternal calcium pool for fetal transfer [1,28]. Regulation of maternal bone calcium accumulation is partially PRL-dependent [27,29] and might also be associated with increased plasma levels of progesterone, insulin-like growth factor-I and osteoprotegerin (OPG), the last of which counterbalances osteoclast-mediated bone resorption [1,30]. After delivery, bone resorption is markedly enhanced to release calcium for lactogenesis [3,31]. A ~10–30% reduction in bone mineral density (i.e. osteopenia) and a dramatic decline in bone strength that often accompany lactation sometimes lead to pregnancy and lactation-associated osteoporosis (PLO) featuring back pain, vertebral fracture, and height loss [3,32,33]. Decreased ovarian estrogen and increased mammary production of PTH-related peptide (PTHrP) – a hormone that induces osteoclast formation – can be responsible for maternal osteopenia [33], and PRL might act in concert to fine-tune maternal bone turnover or might even cause PLO.

PRL was previously thought to indirectly enhance bone resorption by suppressing ovarian production of estrogen that normally induces net bone gain [16,18]. However, osteoblasts strongly express PRLR, indicating a direct action of PRL on bone [16,34,35]. At the cellular level, PRL downregulates OPG while concurrently upregulating the receptor activator of nuclear factor- κB ligand (RANKL), a factor generally synthesized and/or released from osteoblasts to increase the number and activity of osteoclasts [16] (Figure 3). Although PRL can also increase osteoblast number *in vivo* [16], fully differentiated or functional osteoblasts could be decreased because PRL downregulates Runx2 (a transcription factor required for osteoblast differentiation), and two important differentiation markers, alkaline phosphatase and osteocalcin [16,34,36]. After weaning, bone mass is restored in both humans and rodents [1,33], coincidentally with decreases in osteoblast-derived RANKL [31] and plasma levels of PRL and PTHrP [33]. The long-term effects of lactation-induced osteopenia on subsequent bone health is controversial, ranging from full recovery or minor deficits to a permanent decrease in bone mass [3,31–33,37]. Preexisting low bone mass prior to pregnancy might be a cause of incomplete recovery [38]. However, for the sake of maternal bone health, massive and/or permanent bone loss, if any, should be prevented [39].

Possible regulatory action of PRL on renal calcium handling

Renal calcium excretion is increased during pregnancy, but is decreased during lactation in both humans and rodents [29,33]. Pregnancy-induced hypercalciuria is caused by excess calcium absorption from the intestine (i.e. absorptive hypercalciuria; Figure 1) [33]. Moreover, elevated plasma calcitonin levels in pregnancy could contribute to maternal calciuresis [33].

PRLR expression was reported in renal tubular cells [15], suggesting the kidney as a probable target organ for PRL. As demonstrated by the calcium balance technique,

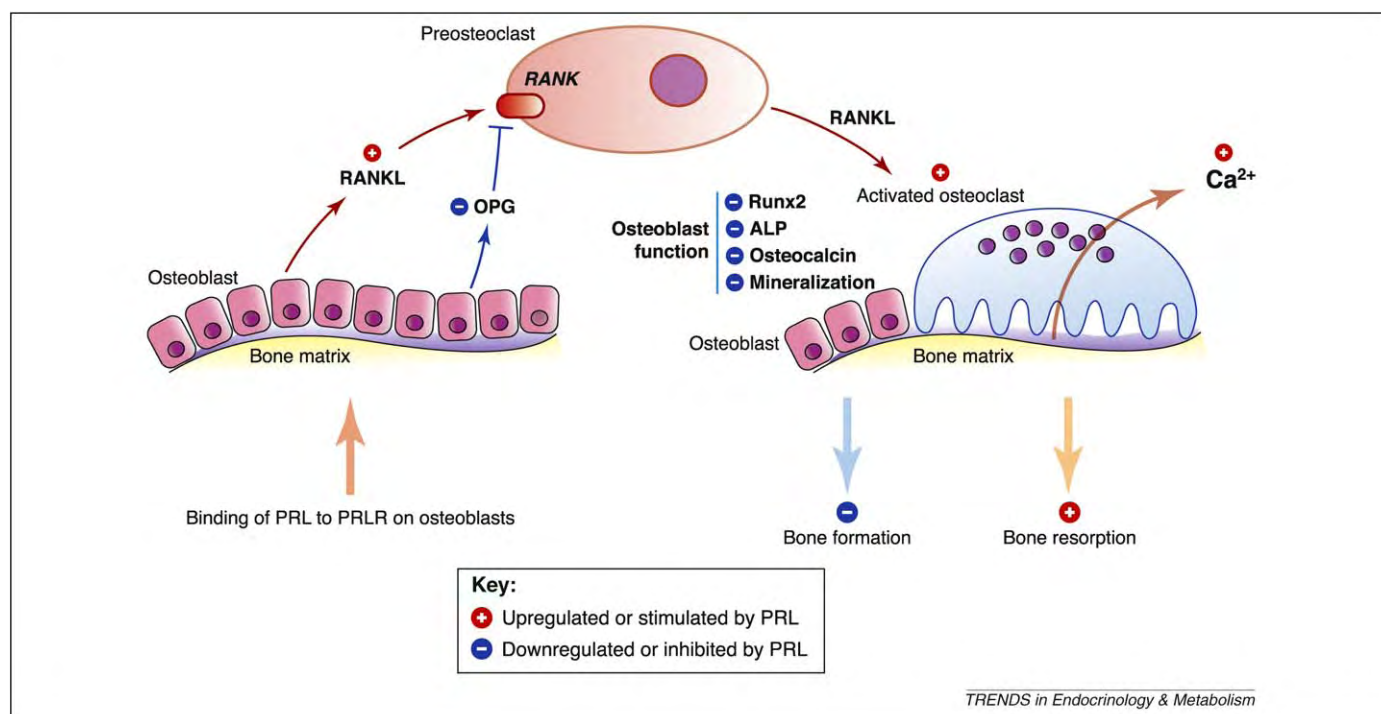


Figure 3. Possible mechanism of PRL-enhanced bone resorption in late lactation. PRL exerts a direct action on bone through osteoblasts because osteoclasts do not express PRLR [34]. Binding of PRL to PRLR on osteoblasts upregulates the expression of the receptor activator of nuclear factor- κ B (RANK) ligand (RANKL), and downregulates osteoprotegerin (OPG), a decoy factor that blocks the RANKL–RANK interaction. After binding to its receptor RANK, RANKL induces osteoclastogenesis, activates osteoclasts, and maintains the survival of activated osteoclasts, thereby inducing bone demineralization to release more calcium into the plasma [16]. PRL also suppresses osteoblast functions by decreasing the expression of *Runx*-related transcription factor (*Runx*)-2, alkaline phosphatase (ALP) and osteocalcin, as well as the osteoblast-induced matrix mineralization [16,34,36]. The net result of PRL action on bone is therefore to stimulate bone resorption and inhibit bone formation.

PRL injection decreased renal calcium excretion in both non-mated and pregnant rats [29,40]. Although it was suggested that exogenous PRL enhanced renal calcium reabsorption [29,40], the role of endogenous PRL released during pregnancy and lactation remains uncertain because inhibition of pituitary PRL release by the dopaminergic agonist bromocriptine did not show a significant increase in renal calcium excretion [29]. Recent investigation showed that combined exposure to PRL and glucocorticoid directly modulates the barrier function of tight junctions in cultured renal epithelial monolayer, possibly by inducing redistribution of tight junction proteins occludin and ZO-1 [41]. Further *in vivo* investigations will be required to confirm a direct action of endogenous PRL on the renal tubular epithelium and on renal calcium handling in lactating animals.

Possible role of PRL in calcium transfer for fetal development and lactogenesis

Placental calcium transfer to the fetus and fetal bone mineralization largely take place in the third trimester in humans and the last five days of pregnancy in rats [42]. Several hormones including PTH and PTHrP are essential for placental calcium transfer in rodents [43]. Although the placenta is responsive to PRL and PRL-like factors [9], the physiological role of PRL in regulating placental calcium transport is currently not known. Nevertheless, it is possible that PRL contributes to calcium transfer across the placenta because it can increase calcium supply to the mother by stimulating intestinal calcium absorption [10].

After parturition, the mammary gland secretes up to 1000 mg/day of milk calcium, the concentration of which

varies according to the stage of lactation (~5–8 mM) [1–3,44]. Because milk calcium is the sole calcium source for neonatal bone development and cellular metabolism, milk calcium secretion in mothers on a low calcium diet is maintained by the release of calcium from bone [2,44]. However, the hormone(s) responsible for controlling milk calcium secretion remains elusive.

Generally, transcellular calcium transport from plasma to the acinar lumen of the mammary gland consists of (i) calcium entry at the blood-facing plasma membrane, presumably via canonical transient receptor potential (TRPC) channels, (ii) uptake of cytoplasmic calcium into the Golgi apparatus by secretory pathway Ca^{2+} -ATPase system, and (iii) calcium extrusion via PMCA_2 and exocytosis [44,45]. In the Golgi apparatus and exocytotic vesicles, calcium binds to citrate, phosphate, and milk protein casein, the expression of which is enhanced by PRL [44,46]. Administration of exogenous PRL to lactating rats increases milk calcium concentration by ~10% [29]. Recently, PRL was reported to increase the expression of secretory pathway Ca^{2+} -ATPase-2 (SPCA2), and knockdown of SPCA2 abolished PRL-induced calcium uptake into the Golgi apparatus [47]. However, PRL did not alter TRPC channel mRNA expression levels [45]. Although the signaling pathway remains unknown, it is possible that the lactogenic hormone PRL directly regulates milk calcium secretion.

Possible directions for nutraceutical research and maternal health care

Because milk calcium is derived from both diet and bone in as yet unknown proportions, oral calcium supplementation should shift the proportion of milk calcium toward the

Box 2. Outstanding questions

- Does PRL enhance calcium uptake from the plasma into mammary epithelial cells? If so, what are the responsible calcium channels or transporters?
- Is PRL responsible for the maintenance of milk calcium concentration? What is the proportion of milk calcium content derived from diet versus bone?
- What is the exact role of PRL in renal calcium handling? Does PRL directly stimulate calcium reabsorption in the kidney?
- Does PRL directly enhance placental calcium transfer? How does maternal PRL contribute to skeletal development in the fetus?
- Can oral calcium supplementation prior to breastfeeding or PRL surge efficiently prevent maternal bone resorption, or even PLO and postmenopausal osteoporosis later in life, by supplying a larger proportion of calcium from the diet, thus reducing calcium mobilization from bone for milk production?

dietary source [10,39]. However, bone loss in breastfeeding mothers with relatively high calcium intake suggests that calcium supplementation alone is probably not enough to achieve this shift [1,37]. Because Step-2 calcium absorption is triggered by the suckling-induced PRL surge, to maximize the benefit of calcium supplementation we postulate that calcium should be administered orally at an appropriate time (e.g. 30–60 min) prior to breastfeeding in 3–4 divided doses/day (e.g. ~300–400 mg/dose; estimated from the recommended dietary intake of ~1200 mg/day). However, the effectiveness and potential long-term benefits of this regimen in humans remain to be validated.

Furthermore, certain nutrients such as glucose (and perhaps galactose, another substrate of SGLT1) can induce plasma membrane depolarization and increase Na^+/K^+ -ATPase activity, thereby leading to $\text{Ca}_v1.3$ activation and solvent drag (Figure 2), respectively, both of which are important for PRL-stimulated duodenal calcium absorption [22,23,48]. Moreover, microflora can ferment prebiotics (non-digestible food ingredients) into acidic compounds, that then solubilize insoluble calcium complexes and promote calcium absorption [49], and especially in the cecum where PRL also markedly stimulates both transcellular and paracellular calcium transport [14,20]. It is therefore suggested that nutraceutical scientists might develop a calcium-rich product containing such additional ingredients as a supplement for breastfeeding mothers.

Concluding remarks

Several lines of evidence corroborate that PRL, with elevated plasma levels during mammalian reproductive periods, can be considered to be the cardinal calciotropic hormone that regulates maternal calcium homeostasis. PRL appears not only to provide more 'calcium input' during pregnancy and lactation, but also controls 'calcium output' via milk secretion (Figure 1). In other words, PRL acts as a coordinator to regulate and synchronize the calcium-regulating system to mobilize a sufficient amount of calcium for fetal development and lactogenesis. Calcium adequacy is achieved by PRL-enhanced intestinal calcium absorption, renal calcium reabsorption and bone resorption [6,10,14,27,29]. Although PRL is clearly a calcium regulating hormone, there are several questions that await further investigation (Box 2).

On the basis of findings that PRL stimulates intestinal calcium absorption in a two-step manner, it is suggested that oral calcium supplement for breastfeeding mothers should be given at appropriate times and doses to optimize calcium absorption. The development of a new calcium supplement that is well-absorbed in PRL-responsive intestinal segments including duodenum or cecum [14,20] should provide maximum benefit. Future holistic and integrative approaches in understanding physiological functions of PRL and maternal calcium homeostasis are important for preventing PLO and promoting fetomaternal bone health.

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Bone modeling in bromocriptine-treated pregnant and lactating rats: possible osteoregulatory role of prolactin in lactation

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Suntornsaratoon P, Wongdee K, Goswami S, Krishnamra N, Charoenphandhu N. Bone modeling in bromocriptine-treated pregnant and lactating rats: possible osteoregulatory role of prolactin in lactation. *Am J Physiol Endocrinol Metab* 299: E426–E436, 2010. First published June 15, 2010; doi:10.1152/ajpendo.00134.2010.—The lactogenic hormone prolactin (PRL) directly regulates osteoblast functions in vitro and modulates bone remodeling in nulliparous rats, but its osteoregulatory roles in pregnant and lactating rats with physiological hyperprolactinemia remained unclear. Herein, bone changes were investigated in rats treated with bromocriptine (Bromo), an inhibitor of pituitary PRL release, or Bromo+PRL at different reproductive phases, from mid-pregnancy to late lactation. PRL receptors were strongly expressed in osteoblasts lining bone trabeculae, indicating bone as a target of PRL actions. By using dual energy X-ray absorptiometry, we found a significant increase in bone mineral density in the femora and vertebrae of pregnant rats. Such pregnancy-induced bone gain was, however, PRL independent and may have resulted from the increased cortical thickness. Bone trabeculae were modestly changed during pregnancy as evaluated by bone histomorphometry. On the other hand, lactating rats, especially in late lactation, showed massive bone loss in bone trabeculae but not in cortical shells. Further study in Bromo- and Bromo+PRL-treated rats suggested that PRL contributed to decreases in trabecular bone volume and number and increases in trabecular separation and eroded surface, as well as a paradoxical increase in bone formation rate in late lactation. Uncoupling of trabecular bone formation and resorption was evident in lactating rats, with the latter being predominant. In conclusion, pregnancy mainly induced cortical bone gain, whereas lactation led to trabecular bone loss in both long bones and vertebrae. Although PRL was not responsible for the pregnancy-induced bone gain, it was an important regulator of bone modeling during lactation.

bone histomorphometry; hyperprolactinemia; ion chromatography; osteopenia; uncoupling

IN PREGNANT AND BREASTFEEDING WOMEN, massive calcium loss occurs for fetal development (~200–300 mg/day) and lactogenesis (~300–1,000 mg/day), respectively (4, 23, 36). A huge amount of calcium demand is accomplished, in part, by enhanced intestinal calcium absorption during these reproductive periods (9). Our recent studies in rats demonstrated that the lactogenic hormone prolactin (PRL), released from the anterior pituitary gland during pregnancy (~100–200 ng/ml) and lactation (~200–300 ng/ml), was the principal calciotropic maternal hormone, which was capable of stimulating calcium absorption in the small intestine and proximal large intestine (7, 21). Moreover, lactation-induced bone resorption provides additional calcium to match the increased calcium demand of

the offspring, which in turn induces reversible osteopenia in mothers (20, 36).

In both humans and rodents, hormonal regulation of bone changes during pregnancy and lactation is not completely understood, but it is not directly regulated by the major calciotropic hormones, namely parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (9, 30, 36). Other hormones with elevated plasma levels, such as PRL, PTH-related peptide (PTHrP), calcitonin, and insulin-like growth factor (IGF)-I, might contribute to the maternal bone changes, perhaps with each exerting action at different times and in a bone site-specific manner (4, 20). Among these hormones, PRL is of special interest, since its plasma levels are increased ~10-fold and ~20-fold in pregnant and lactating rats, respectively (6). In addition, primary osteoblasts cultured from rat bones were found to strongly express mRNAs and proteins of PRL receptors (PRLR), suggesting that bone could be another target tissue of PRL actions (40).

A recent investigation in nulliparous rats with hyperprolactinemia induced by pituitary transplantation showed that PRL could induce both bone formation and bone resorption, with the latter being predominant, thereby leading to net bone loss and osteopenia (40). PRLR^{-/-} mice also manifested an impairment of bone growth and mineralization of bone matrix (11). Moreover, pathological hyperprolactinemia in nonpregnant patients with prolactinoma or chronic uses of antipsychotic drugs (i.e., dopaminergic antagonists) may lead to progressive osteopenia and osteoporosis (27, 42). Such bone loss in rodents was apparent mainly in the primarily trabecular site (e.g., vertebrae) or the trabecular parts of the long bone (e.g., metaphysis or secondary spongiosa) but was rarely observed in the cortex (8, 44). The PRL-related bone loss may result from direct actions of PRL on osteoblasts as well as indirectly from the PRL-induced hypoeostrogenemia (8, 12, 27, 40).

Although pregnancy and lactation are considered a physiological hyperprolactinemic state (9), little is currently known regarding the effects of PRL on cortical and trabecular sites at different phases of the reproductive periods (e.g., early vs. late lactation). Generally, longitudinal studies of PRL actions on maternal bone metabolism are carried out in animals treated with bromocriptine (Bromo), an agonist of the PRL-inhibiting factor dopamine, which can inhibit pituitary PRL release by ~80–90% (3, 7, 35), in the presence or absence of exogenous PRL administration (43). The uses of PRL^{-/-} or PRLR^{-/-} mice are not possible due to infertility (2). Furthermore, disruption of PRL synthesis in the first week of pregnancy in rodents could lead to abortion (2). Thus, by using Bromo-treated lactating rats, the effect of PRL on the long bone could be demonstrated by densitometric analysis (43), but whether

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microstructural changes in the cortices and trabeculae were different remained to be investigated. Nevertheless, the aforementioned findings in nulliparous rats (40, 44) suggested that PRL could have action on maternal bone, particularly in the trabeculae.

Therefore, the objectives of the present longitudinal study were 1) to investigate macroscopic and microscopic bone changes by using dual energy X-ray absorptiometry (DEXA) and bone histomorphometry, respectively, in pregnant and lactating rats, from mid-pregnancy to day 15 postweaning, 2) to reveal the possible roles of PRL in regulating maternal bone changes, and 3) to demonstrate differential responses of maternal cortical and trabecular bones to PRL in different reproductive periods.

MATERIALS AND METHODS

Animals. Pregnant and age-matched nulliparous Sprague-Dawley rats (8 wk old) were obtained from the National Laboratory Animal Centre in Thailand. They were housed in the husbandry unit under a 12:12-h light-dark cycle (lights on at 0600) for at least 5 days prior to the experiments and were fed standard chow and distilled water ad libitum. The room had a temperature of $25 \pm 2^\circ\text{C}$ with average illuminance of 200 lux. This study was approved by the Animal Care and Use Committee of the Faculty of Science, Mahidol University. All animals were cared for in accordance with the principles and guidelines of the American Physiological Society's "Guiding Principles in the Care and Use of Animals."

Experimental design. Bone changes were investigated in different reproductive phases, i.e., mid-pregnancy (day 14, P14), late pregnancy (day 21, P21), early lactation (day 8, L8), mid-lactation (day 14, L14), late lactation (day 21, L21), and day 15 postweaning (PW). Since bone growth and calcium accretion are normally age dependent, we used age-matched nulliparous rats as a control group. Ages of P14, P21, L8, L14, L21, and PW rats were 10, 11, 12, 13, 14, and 16 wk old, respectively. Body weights of all rats were recorded weekly (Supplemental Fig. S1; supplemental materials are found in the online version of this paper at the Journal website). After delivery, litter size was adjusted to eight pups per dam for lactating groups, whereas in nonsuckling groups all pups were permanently separated from dams from birth. Such nonsuckling mothers were reported to have no hyperprolactinemia because the suckling-induced PRL surge was absent, and plasma PRL levels rapidly declined within 12 h after cessation of suckling (26, 34). In some experiments, dams were injected daily subcutaneously for 7 days with 4 mg/kg Bromo sc (Sigma, St. Louis, MO) or Bromo+PRL (purified from ovine pituitary gland; catalog no. L6520, Sigma) before being killed. PRL doses for pregnant and lactating rats were 0.4 and 0.6 mg/kg, respectively (7). These PRL regimens have been reported to completely restore the Bromo-induced decrease in the intestinal calcium absorption in pregnant and lactating rats (7). Finally, all rats in each pregnant (P14 and P21) or lactating (L8, L14, and L21) group were killed on the same day, between 0800 and 1000.

Preparation of bone samples. As previously described by Charoenphandhu et al. (8), femora (primarily cortical sites) and L5 vertebrae (primarily trabecular sites) were cleaned and subjected to densitometric analysis. In some experiments, femoral length and dry and ash weights were recorded. Femora were dried in an oven at 80°C for 3 days and then ashed at 800°C overnight in a muffle furnace (model 48000; Thermolyne, Dubuque, IA). After bone ash was dissolved with 3.0 N HCl, the samples were diluted with a solution containing 0.38% wt/vol SrCl_2 and 0.9% vol/vol HClO_4 and were analyzed for total calcium and magnesium contents by atomic absorption spectrophotometry (for calcium only; model SpectrAA-300, Varian Techtron, Springvale, Australia) and ion chromatography (for calcium and magnesium).

Tibiae (primarily cortical sites) were also removed, cleaned, and fixed for PRLR expression study and histomorphometric analysis of their cortical and trabecular portions.

Immunohistochemical analysis of PRLR expression. Tibiae were dissected from P21 and L8 rats. After cleaning, they were fixed overnight in 0.1 M phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Decalcification was later performed by immersing bone specimens in 15% wt/vol ethylenediaminetetraacetic acid (EDTA; Sigma) at 25°C for 3 wk. Decalcifying solution was replaced every 3 days. After being embedded in paraffin, bone specimens were cut longitudinally into 7- μm sections, which were later incubated at 37°C for 30 min in antigen retrieval solution (0.01 mg/ml proteinase K, 50 mM Tris·HCl, pH 8.0, and 5 mM EDTA). To inhibit background endogenous peroxidase activity, sections were incubated for 1 h with 10% H_2O_2 . Nonspecific binding was blocked for 2 h by 4% bovine serum albumin, 10% normal goat serum, and 0.7% Tween-20 in PBS. Thereafter, sections were incubated at 4°C overnight with 1:50 rabbit polyclonal primary antibody against PRLR (catalog no. sc-30225; Santa Cruz Biotechnology, Santa Cruz, CA). After being washed with 0.7% Tween-20 in PBS, sections were incubated for 1 h at room temperature with 1:500 biotinylated goat anti-rabbit IgG (catalog no. 656140; Zymed, South San Francisco, CA) followed by incubation for 1 h with streptavidin-conjugated horseradish peroxidase solution (Zymed) and 3,3'-diaminobenzidine chromogen (Pierce, Rockford, IL). As for the negative control, sections were incubated with 0.7% Tween-20 in PBS in the absence of PRLR primary antibody. Sections were counterstained with hematoxylin and visualized under a light microscope (model BX51TRF; Olympus, Tokyo, Japan).

Measurement of bone calcium and magnesium contents by ion chromatography. A modular ion chromatographic system (Waters, Milford, MA) comprised a model-600 controller, model-600 pump, model-432 conductivity detector, and a cation column containing silica gel with sulfonic acid group (125×4 mm inner diameter, 5 μm particle size, model Nucleosil 5SA; Metrohm, Herisau, Switzerland). All components were connected through the Waters SAT/IN module to a Millennium 32 workstation, which performed system control, acquisition, and data analysis. The eluent for simultaneous separation of the divalent cations consisted of 4 mM tartaric acid, 0.5 mM citric acid, and 3 mM ethylenediamine in 5% vol/vol acetone and were degassed for 5 min before use. Eluent flow rates were adjusted at 1.5 ml/min, and the column was equilibrated in eluent for 45 min prior to sample charging. The analytic condition was optimized for isocratic elution using a 100- μl injection loop and total run time of 10 min. Calcium and magnesium were eluted at 5.9 and 7.0 min, respectively. Standard stock solutions were prepared by dissolving appropriate amounts of CaCl_2 or MgCl_2 in ultrapure Milli-Q water to obtain a final concentration of 1 M and kept at -20°C . Working standard solutions were prepared fresh by diluting the stock solutions to 10, 20, 50, 100, 150, and 200 μM with Milli-Q water (pH ~ 2.5 – 3.5 adjusted with 2 M HNO_3) for six-point calibration. The detection limit of both calcium and magnesium at a signal-to-noise ratio of 3:1 appeared to be ~ 0.02 and ~ 0.04 μM , respectively. The six-point calibrations for calcium and magnesium showed percent residual standard deviations of 0.9 and 0.5 and correlation coefficients of 0.9989 and 0.9993, respectively. A reproducibility test was performed by injecting standard solutions 10 times on the same day as well as on different days. The relative standard deviations of peak area of calcium and magnesium were varied by less than 0.5% and 3–5%, respectively, during intraday vs. interday tests.

For the analyses of bone calcium and magnesium contents, each bone sample was dissolved in 3 N HCl and diluted 100-fold with distilled water. Thereafter, all samples were filtered through 0.22- μm membranes prior to injection into the column. All samples were analyzed in duplicate and reported as millimoles per gram of dry weight of bone.

DEXA. Bone mineral density (BMD) and bone mineral content (BMC) were determined in the ex vivo whole femora and L5 vertebrae by DEXA (model Lunar PIXImus2; GE Medical Systems, Madison, WI), operated with software version 2.10, as previously described (8). The system was calibrated daily with a standard material of known BMD and BMC of 0.0690 g/cm² and 0.697 g, respectively.

Bone histomorphometry. A tibia was cleaned of adhering tissues and bone marrow. Bone was then dehydrated in 70, 95, and 100% vol/vol ethanol for 3, 3, and 2 days, respectively. Dehydrated bone was embedded in methyl methacrylate resin at 42°C for 48 h. The resin-embedded tibia was cut longitudinally with a microtome equipped with a tungsten carbide blade (model RM2255; Leica, Nussloch, Germany) to obtain 7- and 12- μ m-thick sections for the staining and unstaining histomorphometric techniques, respectively (16, 18). For the staining technique, longitudinal sections were mounted on standard microscope slides, deplastinated, dehydrated, and processed for Goldner's trichrome staining (45). The unstained slides were examined for the double lines of calcein labeling (10 mg/kg sc injected at 6-day interval, Sigma). Imaging analysis was performed under a fluorescent/light microscope using the computer-assisted Osteomeasure system (Osteometric, Atlanta, GA), operated with software version 4.1. The region of interest covered secondary spongiosa (the trabecular part of proximal tibia at 1–2 mm distal to the epiphyseal plate), which was analyzed to obtain static and dynamic parameters from stained and unstained sections, respectively. Static parameters included trabecular bone volume normalized by tissue volume (BV/TV, %), trabecular number (Tb.N, mm⁻¹), trabecular separation (Tb.Sp, μ m), trabecular thickness (Tb.Th, μ m), osteoblast surface normalized by bone surface (Ob.S/BS, %), osteoclast surface (Oc.S/BS, %), and eroded surface (ES/BS, %). Dynamic parameters were double labeled surface (dLS/BS, %), mineralizing surface (MS/BS, %), mineral apposition rate (MAR, μ m/day), and bone formation rate (BFR/BS, μ m³/ μ m²/day). The nomenclature, symbols, and units complied with the report of the American Society for Bone and Mineral Research Nomenclature Committee (31).

For the cortical measurements, midfrontal tibial sections and mid-sagittal vertebral sections were used. Cortical thicknesses of tibiae were averaged from three points, i.e., at 2, 3, and 4 mm distal to the epiphyseal plate, whereas those of L5 vertebrae were averaged from the thickness at one-third and two-thirds of the length of the L5 vertebral body. Cortical thicknesses were measured with Motic Images Plus 2.0 (Motic Instruments, Richmond, Canada).

Statistical analysis. Results are expressed as means $\pm$ SE. Two sets of data were compared with an unpaired Student's *t*-test. One-way analysis of variance with a Newman-Keuls multiple comparison test was used for multiple sets of data. The level of significance for all statistical tests was *P* < 0.05. Data were analyzed with GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA).

RESULTS

Expression of PRLR proteins in bone. Prior to the investigation of the PRL effects on bone, the expression of PRLR proteins in bone was determined by immunohistochemical technique. As shown in Fig. 1, brownish signals of PRLR proteins were observed in flat osteoblasts lining bone trabeculae in the tibiae of nulliparous, pregnant (P21), and lactating (L8) rats. Several hematopoietic cells in bone marrow also showed positive PRLR signals, whereas no signal was observed in osteocytes embedded in bone trabeculae and cortices.

Densitometric analyses of femora and L5 vertebrae of pregnant and lactating rats. At a macroscopic level, bone densitometric analysis using DEXA in femora (a primarily cortical site) and L5 vertebrae (a primarily trabecular site) demonstrated that BMD was markedly increased in mid- (P14) and late pregnancy (P21) but was decreased in late lactation (L21)

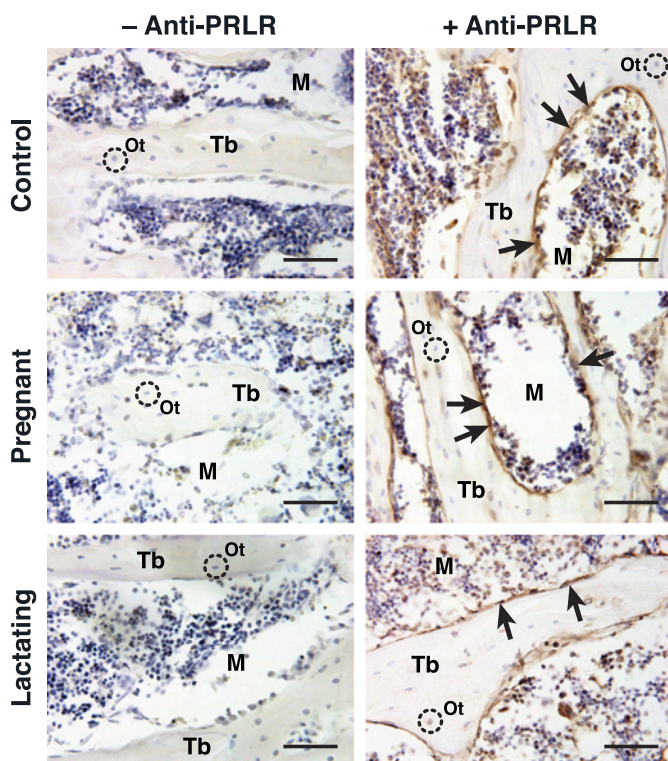


Fig. 1. Immunolocalization of prolactin receptor (PRLR) proteins in the tibiae of control (nulliparous), pregnant (P21), and lactating (L8) rats (*n* = 3–4 rats per group). Immunostained (+Anti-PRLR) and negative control (–Anti-PRLR) sections were incubated in the presence and absence of anti-PRLR antibody, respectively. Immunoreactive signals were observed in flat osteoblasts (arrows) lining bone trabeculae (Tb) and some leukocytes in bone marrow (M), but not in osteocytes (Ot). Bars, 50 μ m.

compared with the age-matched controls (Fig. 2, A and B). In femora, an increase in BMD was maintained until early lactation (L8; Fig. 2A). Fifteen days after weaning, femoral BMD was restored, whereas vertebral BMD remained lower than the control level. Although whole bone BMC was also increased during pregnancy, BMC of L21 lactating rats was comparable to that of age-matched control rats (Fig. 2, C and D).

Neither exogenous PRL nor Bromo affected femoral and vertebral BMD during pregnancy (Fig. 2, A and B). On the other hand, Bromo restored femoral BMD but not vertebral BMD in L21 rats (Fig. 2, A and B). However, PRL administration did not decrease femoral BMD in Bromo-treated L21 rats (Fig. 2A). Interestingly, femoral BMDs in L8 and L21 nonsuckling rats were greater than that in age-matched control rats (Fig. 2A).

Femoral length, ash weight, and total contents of calcium and magnesium. Femoral length was significantly increased during pregnancy and lactation (Fig. 3A). Thereafter, the femoral elongation slowed down, as the femoral length of post-weaning rats was comparable to that of control rats (Fig. 3A). Femoral ash weight was increased only in pregnancy, but not in lactation, compared with age-matched control rats (Fig. 3B). Neither Bromo nor PRL altered femoral length and ash weight in pregnant rats. However, Bromo completely abolished the lactation-induced increase in femoral length. Exogenous PRL administration partially reversed the Bromo effect on femoral length in L21 rats (Fig. 3A).

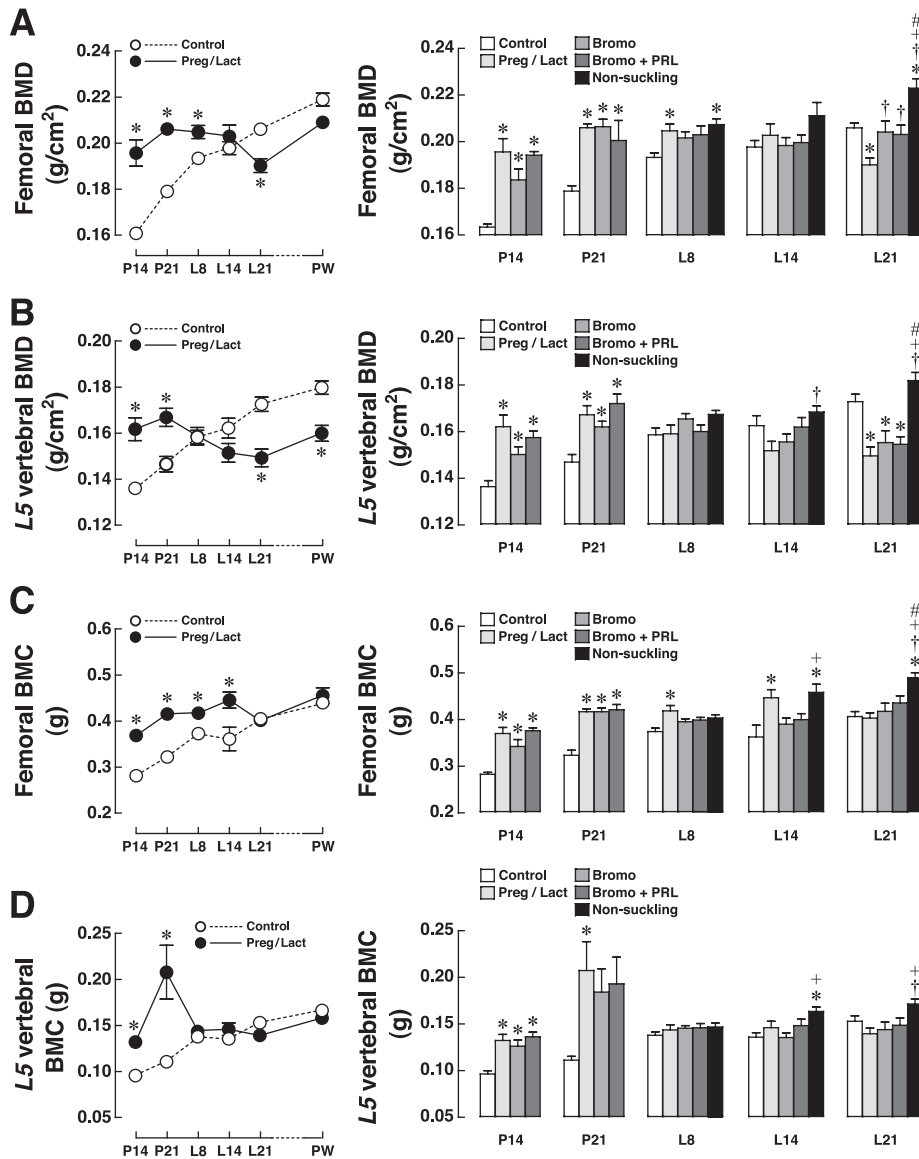


Fig. 2. Bone mineral density (BMD; A and B) and bone mineral content (BMC; C and D) of ex vivo femora (primarily cortical sites) and L5 vertebrae (primarily trabecular sites) of age-matched control, pregnant (P14 and P21), lactating (L8, L14 and L21), and 15-day postweaning (PW) rats, as determined by DEXA. Some pregnant and lactating rats were administered bromocriptine (Bromo) for 7 days ($4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ sc) or Bromo plus PRL sc (Bromo+PRL). PRL doses for pregnant and lactating rats were 0.4 and $0.6 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, respectively. Some rats were permanently separated from their pups after parturition (nonsuckling). All values in line graphs and bar graphs are presented as means $\pm$ SE ($n = 7-8$ rats per each condition). * $P < 0.05$ vs. age-matched control group; † $P < 0.05$ vs. corresponding lactating group; + $P < 0.05$ vs. Bromo group; # $P < 0.05$ vs. Bromo+PRL group.

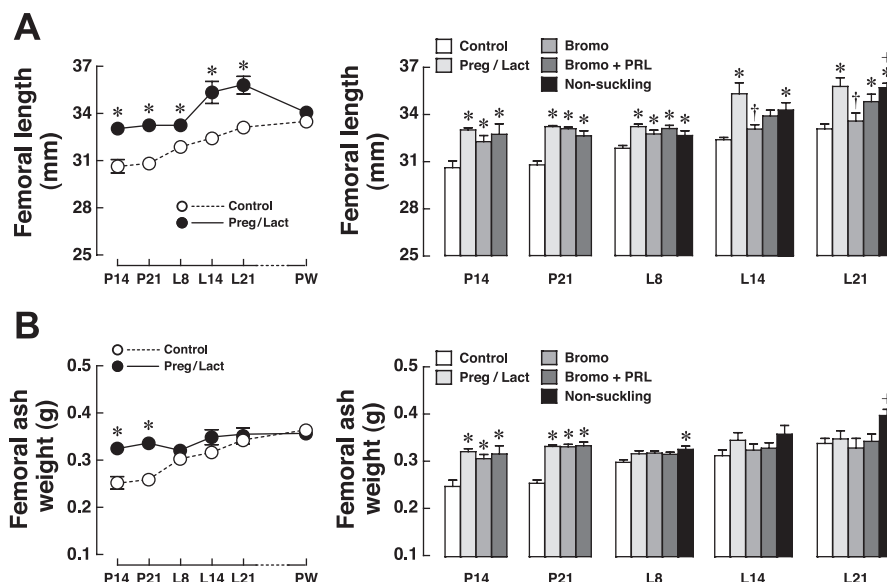
Since PRL was reported to inhibit *in vitro* matrix mineralization (39), we further investigated the total calcium and mineral contents normalized by the dry weight of the femora. Normally, bone dry weight includes dry weight of protein matrix and weight of minerals (e.g., calcium, magnesium, and phosphate). Therefore, if the studied conditions do not change the stoichiometry of mineralized bone matrix (i.e., relative quantities of minerals per weight of protein matrix), the total calcium and magnesium contents normalized by the dry weight should be constant. As demonstrated by ion chromatography, these parameters were not altered in pregnant and lactating rats and remained constant after Bromo or Bromo+PRL administration (Fig. 4). In addition, calcium content in bone ash was greater than magnesium content ~ 40 -fold. A constant total calcium content normalized by dry femoral weight was also confirmed by atomic absorption spectrophotometry (Supplemental Fig. S2).

Cortical thicknesses of tibiae and L5 vertebrae. Goldner's trichrome staining of tibial and vertebral sections revealed that the thicknesses of tibial and vertebral cortical shells as

well as of trabeculae were markedly increased during pregnancy (Figs. 5A and 6, A and B). The pregnancy-induced increase in cortical thickness was not dependent on PRL (data not shown). In L14 lactating rats, the thickness of tibial cortical shell, but not vertebral cortical shell, remained greater than that in the control groups (Figs. 5B and 6, C and D). In contrast, bone trabeculae in both tibiae and L5 vertebrae became thinner during lactation (Fig. 5B).

Histomorphometry of trabecular microstructure in pregnant and lactating rats. Further investigation by bone histomorphometry in the trabecular portion of tibiae demonstrated that trabecular bone volume and trabecular number were not changed during pregnancy compared with the age-matched controls but were markedly decreased after mid-lactation, which lasted until day 15 postweaning (Fig. 7, A and B). Trabecular thickness was modestly altered during pregnancy and lactation with a significant increase only at day 15 postweaning (Fig. 7C). Trabecular separation was significantly increased after mid-lactation (Fig. 7D), consistent with a decrease in trabecular bone volume during the same period.

Fig. 3. Length (A) and ash weight (B) of femora dissected from age-matched control, pregnant (P14 and P21), lactating (L8, L14 and L21), and 15-day postweaning (PW) rats. Ash weight was determined after placing femora in a muffle furnace at 800°C overnight. Some pregnant and lactating rats were administered Bromo for 7 days ($4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ sc) or Bromo+PRL. PRL doses for pregnant and lactating rats were 0.4 and 0.6 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, respectively. Some rats were permanently separated from their pups after parturition (nonsuckling). All values in line graphs and bar graphs are presented as means $\pm$ SE ($n = 4-6$ rats per condition). * $P < 0.05$ vs. age-matched control group; † $P < 0.05$ vs. the corresponding lactating group; + $P < 0.05$ vs. Bromo group.



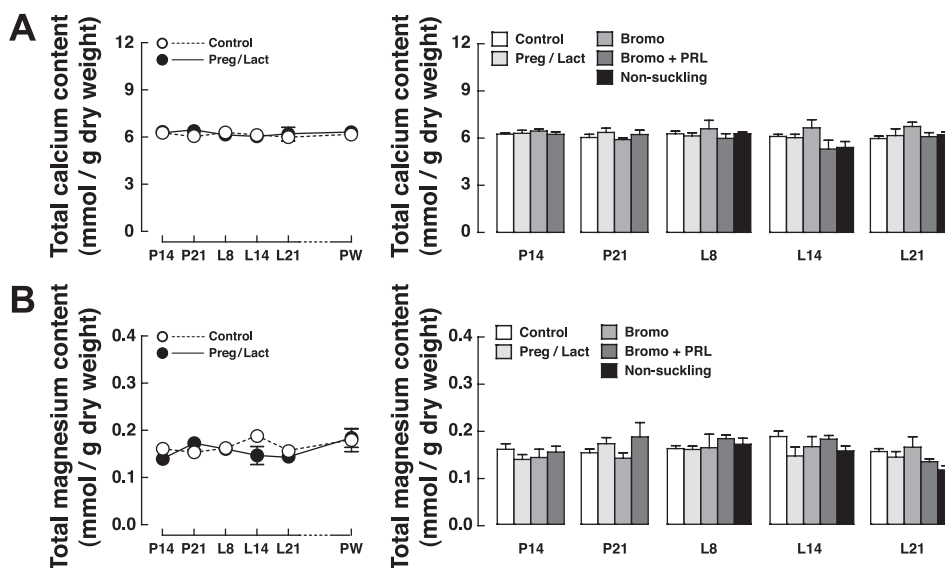
Regarding bone formation-related parameters, although osteoblast surface was decreased during mid-pregnancy (Fig. 8A), double labeled surface, mineral apposition rate, and bone formation rate were significantly increased (Fig. 8, B–D) compared with the corresponding age-matched control groups. However, these parameters were relatively constant or modestly changed between late pregnancy and mid-lactation but were later drastically increased in late lactation and postweaning (Fig. 8, A–D). Mineralizing surface (Supplemental Fig. S3) was consistent with bone formation rate (Fig. 8D).

Interestingly, despite having net bone gain during pregnancy, as demonstrated by DEXA, osteoclast surface and eroded surface in tibial trabeculae had already increased from mid-pregnancy and lasted until late lactation (Fig. 9, A and B). These results suggested that trabecular bone formation and resorption were uncoupled during these reproductive periods, thereby leading to bone gain and bone loss in pregnant and lactating rats, respectively. In other words, “bone modeling” may be present, since bone formation was not proportionally

coupled to bone resorption (13). Both osteoclast surface and eroded surface returned to the control values postweaning (Fig. 9, A and B).

Most histomorphometric parameters, with the exception of osteoblast surface, double labeled surface, and bone formation rate, were not altered in Bromo-treated or Bromo+PRL-treated pregnant rats (Figs. 7–9), suggesting that PRL did not have much influence on trabecular bone changes during pregnancy. On the other hand, during the lactation period, especially in late lactation, changes in several parameters related to both bone formation and resorption, e.g., bone volume, trabecular number, trabecular separation, osteoblast surface, double labeled surface, mineral apposition rate, bone formation rate, osteoclast surface, and eroded surface, were regulated by PRL (Figs. 7–9). Multinucleated or activated osteoclasts were more abundant in Bromo+PRL-treated L21 rats than in Bromo-treated L21 rats (data not shown). However, PRL-induced changes in these parameters showed different times of responses as reflected by nonuniform responses to exogenous

Fig. 4. Total calcium content (A) and total magnesium content (B) normalized by dry weight of femora obtained from age-matched control, pregnant (P14 and P21), lactating (L8, L14 and L21), and 15-day postweaning (PW) rats, as determined by ion chromatography. Some pregnant and lactating rats were administered Bromo 7 days ($4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ sc) for or Bromo+PRL. PRL doses for pregnant and lactating rats were 0.4 and 0.6 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, respectively. Some rats were permanently separated from their pups after parturition (nonsuckling). All values in line graphs and bar graphs are presented as means $\pm$ SE ($n = 4-6$ rats per condition).



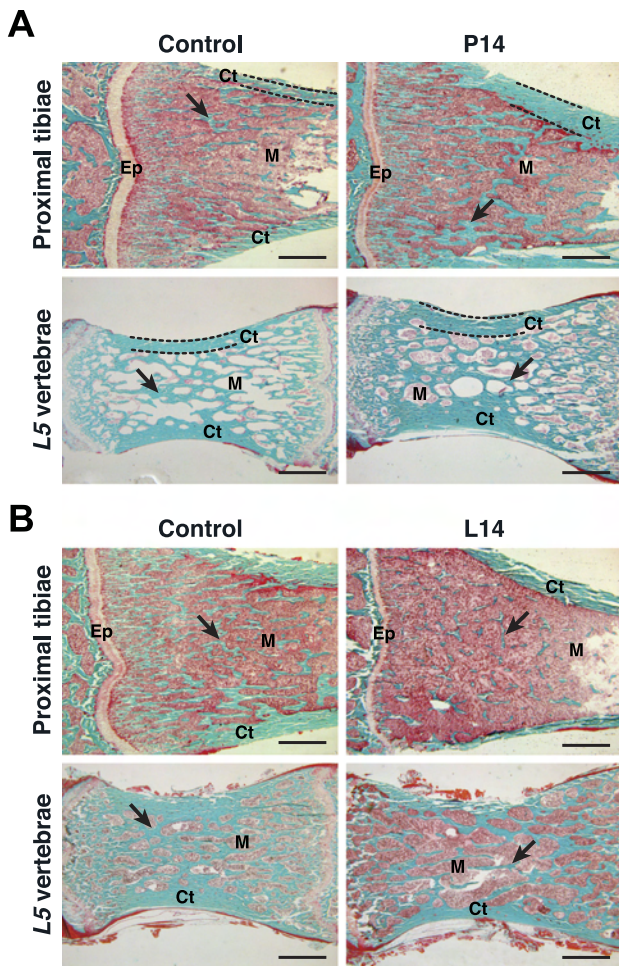


Fig. 5. Goldner's trichrome staining of tibiae (primarily cortical sites) and L5 vertebrae (primarily trabecular sites) dissected from P14 (A) and L14 rats (B) and their corresponding age-matched controls ($n = 3-4$ rats per group). Cortical shell (Ct; bone tissue between dashed lines in A), epiphyseal plate (Ep), bone trabeculae (arrows), and bone marrow (M) were identified. Mineralized bone matrix, erythrocytes, and cytoplasm were stained green, orange, and red, respectively. Bars, 1,000 μm .

PRL administration (Figs. 7–9). It is noteworthy that trabecular thickness in the rat tibiae was relatively constant from late pregnancy until late lactation, and also not responsive to both Bromo and PRL (Fig. 7C).

In nonsuckling rats, which had no hyperprolactinemia and the suckling-induced PRL surge (1, 34), most parameters with the exception of trabecular thickness were not much different from those of the age-matched control rats (Figs. 7–9).

DISCUSSION

How maternal bone adapts during pregnancy and lactation is crucial for growth, development, and calcium metabolism of the offspring. Herein, we provided evidence that bone changes during these periods were complex and time-dependent (pregnancy vs. lactation), and varied with sites of bone (femora/tibiae vs. vertebrae) as well as types of microstructure (cortical shells vs. trabeculae). Specifically, pregnancy predominantly induced femoral and vertebral bone gain in the cortical shells, whereas long-term lactation eventually led to trabecular bone loss. Lactation-induced trabecular bone loss, but not pregnancy-

induced cortical bone gain, was found to be regulated by PRL. However, other hormones with elevated plasma levels, such as IGF-I and PTHrP, might also be responsible for bone changes during pregnancy and lactation, respectively (4, 28, 47).

In mid- and late pregnancy, the observed increases in femoral and vertebral BMD were due to an increase in cortical thickness, presumably by enhancing periosteal bone formation (4). Increased femoral bone length (or size), which probably results from pregnancy-enhanced endochondral bone growth (4), may also contribute to the increased femoral BMD in pregnant rats. Since bone size of pregnant rats was larger than that of their age-matched control rats, their whole bone BMC and ash weight were markedly increased during this period. However, the stoichiometry of calcium and magnesium in bone matrix remained unchanged. In contrast to the cortical parts, despite an increase in trabecular thickness in mid-pregnancy, changes in the trabecular microstructure in P14 and P21 rats were modest. Although P14 pregnant rats manifested the enhanced trabecular bone turnover, as indicated by the augmented bone formation and resorption, both processes were still coupled, and no change in trabecular bone volume was observed. These coupled processes in P14 rats could not be explained simply by osteoblast-induced bone formation, since osteoblast surface was indeed decreased in a PRL-dependent manner (Fig. 8A). It was hypothesized that surplus minerals supplied by the intestine might accelerate bone calcium acquisition (7, 9), as indicated by the twofold increase in the double labeled surface and mineral apposition rate in P14 rats (Fig. 8, B and C). On the other hand, uncoupling of trabecular bone formation and resorption, the latter of which being predominant, was evident in late pregnancy and continued throughout lactation. Therefore, the differential changes in cortical and trabecular structures (i.e., net bone gain and loss occurred at

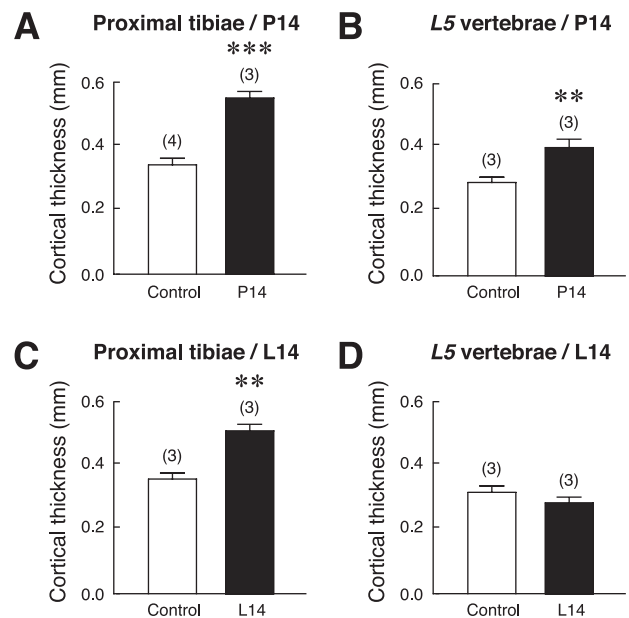


Fig. 6. Average thicknesses of cortical shells in proximal tibiae and L5 vertebrae of P14 (A–B) and L14 (C–D) rats. ** $P < 0.01$, *** $P < 0.001$ vs. its respective control group. Nos. in parentheses represent nos. of experimental animals.

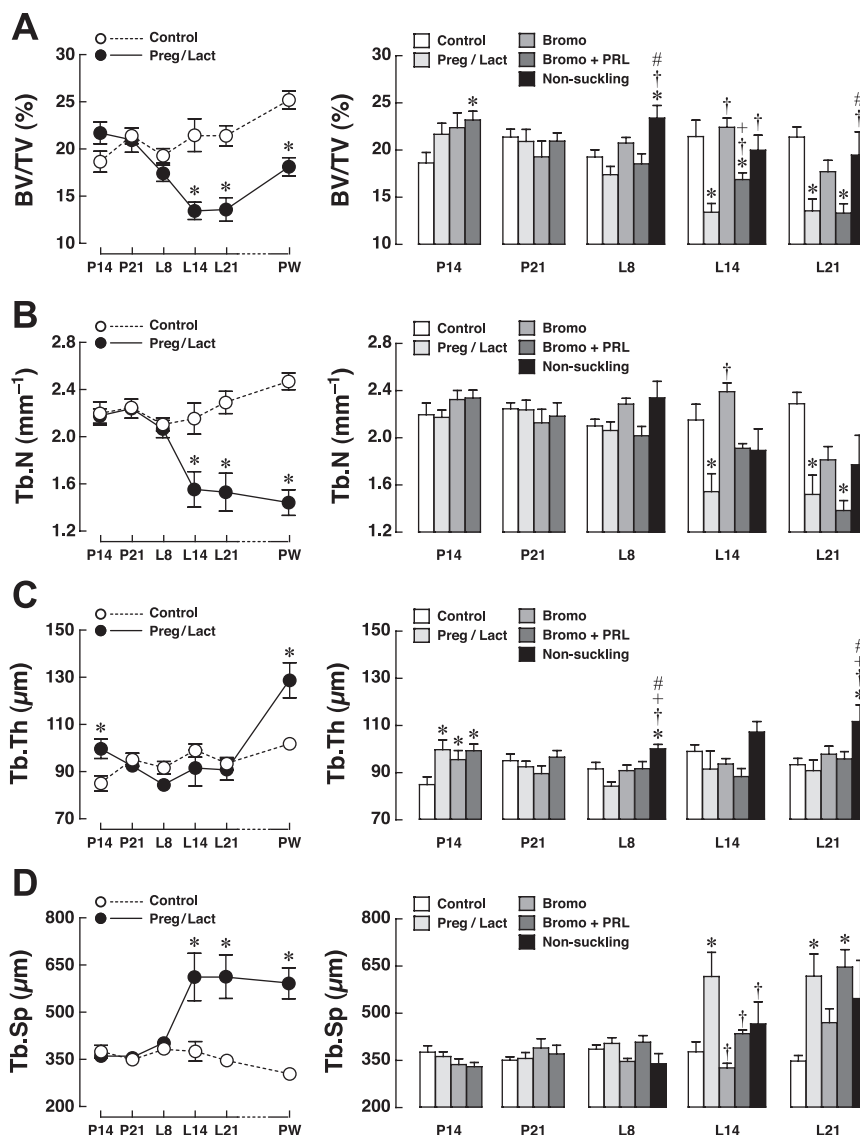


Fig. 7. A, trabecular bone volume normalized by tissue volume (BV/TV); B, trabecular number (Tb.N); C, trabecular thickness (Tb.Th); D, trabecular separation (Tb.Sp) of the trabecular portion (secondary spongiosa) of tibiae obtained from age-matched control, pregnant (P14 and P21), lactating (L8, L14 and L21), and 15-day postweaning (PW) rats, as determined by bone histomorphometry. Some pregnant and lactating rats were administered Bromo for 7 days ($4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \text{ sc}$) or Bromo+PRL. PRL doses for pregnant and lactating rats were 0.4 and $0.6 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, respectively. Some rats were permanently separated from their pups after parturition (nonsuckling). All values in line graphs and bar graphs are presented as means $\pm$ SE ($n = 7-8$ rats per condition). * $P < 0.05$ vs. age-matched control group; † $P < 0.05$ vs. corresponding lactating group; + $P < 0.05$ vs. Bromo group; # $P < 0.05$ vs. Bromo+PRL group.

different parts of the same bone) indicated a process of bone modeling in this phase of the reproductive cycle (13).

It was postulated that pregnancy-induced bone gain was essential for the preparation of the maternal calcium pool during the last 5 days of pregnancy in rodents and the third trimester in humans for fetal skeletal mineralization and the upcoming lactogenesis (4). In addition, thickening of the cortical shells was important for maintaining bone shape and strength to compensate for the gradual increase in the trabecular separation during lactation (16). Such pregnancy-induced cortical adaptation was certainly under the regulation of hormone(s) other than PRL, such as progesterone and IGF-I (4, 17, 28). PRL might indirectly promote maternal bone gain through stimulation of intestinal calcium absorption to provide more calcium for mineralization (7). However, $1,25(\text{OH})_2\text{D}_3$ and PTHrP might not participate directly in this bone gain, because bone mass was found to be increased in the vitamin D receptor knockout mice (17) and the plasma PTHrP levels were significantly increased in late pregnancy (20), after BMD had already been increased.

During lactation, progressive bone loss was evident, especially in the trabecular portions of both long bones and vertebrae. Since the trabecular surface was much greater than the exposed cortical surface (13), the lactation-induced bone loss occurred mainly in bone trabeculae rather than in cortical shells, thereby leading to a significant decrease in BMD in late lactation. Nevertheless, the endocortical surface of the rat tibiae showed greater erosion during lactation compared with the end of pregnancy (25). Previous investigations in humans also demonstrated that calcium lost during breastfeeding may cause a 10% loss of the total body skeletal mass (22, 37, 41). In rodents nursing around six to eight pups, bone mass could be decreased by $\sim 30\%$ in late lactation (5, 32, 47). Nevertheless, BMC and ash weight were not decreased, because bone size, as indicated by the femoral bone length, in lactating rat was actually larger than that of their age-matched controls. Similar to the pregnant rats, the stoichiometry of calcium and magnesium in bone matrix of lactating rats remained constant (Fig. 4), suggesting that the newly formed osteoid might be rapidly mineralized. It was possible that there was a sufficient amount

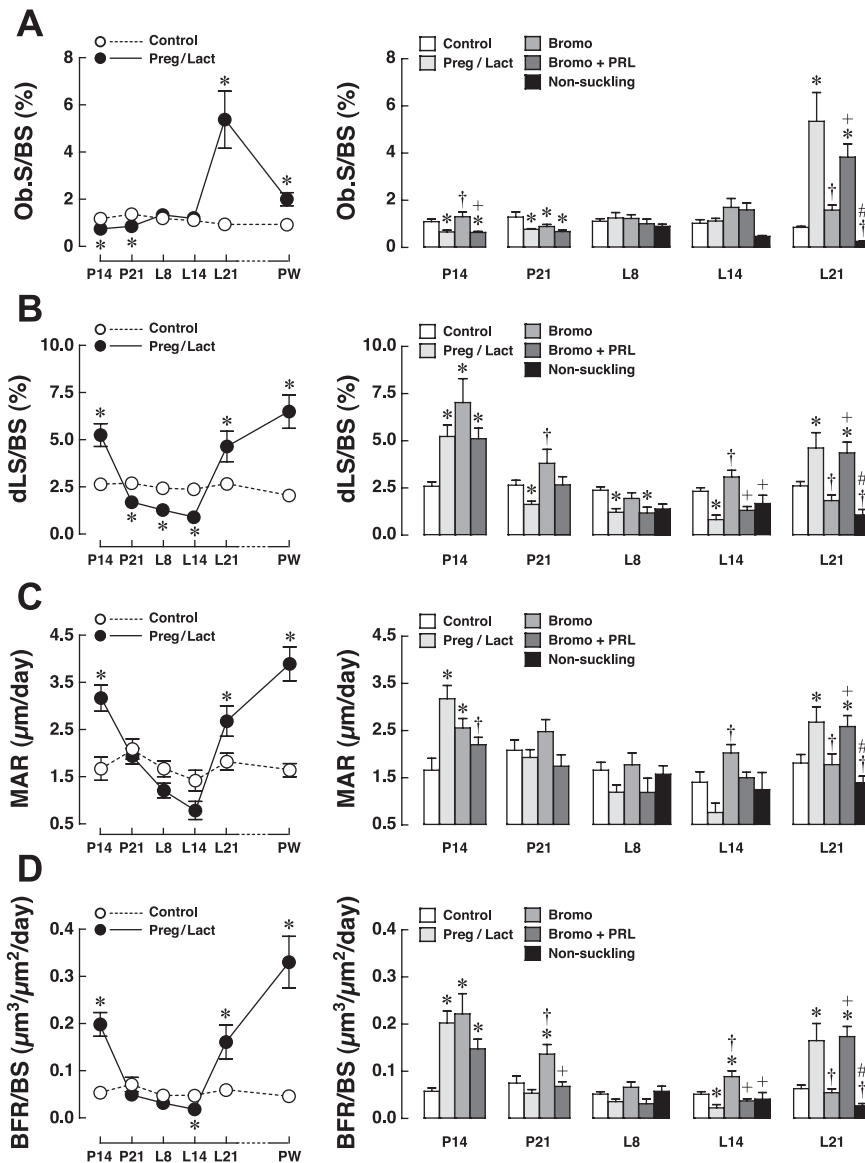


Fig. 8. Trabecular bone formation-related parameters: A, osteoblast surface normalized by trabecular bone surface (Ob.S/BS); B, double labeled surface (dLS/BS); C, mineral apposition rate (MAR); D, bone formation rate (BFR/BS) of the secondary spongiosa of tibiae obtained from age-matched control, pregnant (P14 and P21), lactating (L8, L14 and L21), and 15-day postweaning (PW) rats. Some pregnant and lactating rats were administered Bromo for 7 days ($4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ sc) or Bromo + PRL. PRL doses for pregnant and lactating rats were 0.4 and 0.6 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, respectively. Some rats were permanently separated from their pups after parturition (non-suckling). Ob.S/BS is a static histomorphometric parameter from the Goldner's trichrome-stained sections; dLS/BS, MAR, and BFR/BS are dynamic histomorphometric parameters from unstained sections. All values in line graphs and bar graphs are presented as means $\pm$ SE ($n = 6-8$ rats per condition). * $P < 0.05$ vs. age-matched control group; † $P < 0.05$ vs. corresponding pregnant or lactating groups; + $P < 0.05$ vs. Bromo group; # $P < 0.05$ vs. Bromo + PRL group.

of mineral supply for mineralization, perhaps from the intestine and kidney (9); otherwise, a decrease in normalized bone calcium content would be apparent as in dietary calcium deficiency with osteomalacia (15, 24, 33, 38).

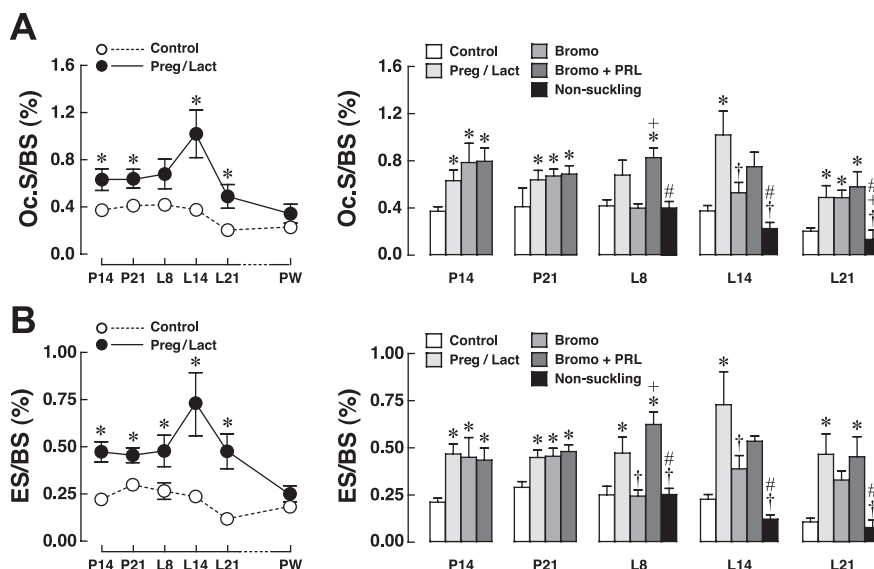
It was evident that trabecular bone loss in lactating rats was induced by PRL, the plasma levels of which were $\sim 200-300$ ng/ml (normal levels $\sim 7-10$ ng/ml) and could further elevate up to $\sim 600-800$ ng/ml during suckling (1, 6, 7). The presence of PRLR in osteoblasts corroborated a direct effect of PRL on bone metabolism (12, 14, 40). Besides its direct action, PRL may indirectly induce bone resorption by suppressing ovarian estrogen synthesis (48). Furthermore, PRL may be responsible for calcium availability for bone mineralization, as it was the principal stimulator of intestinal calcium absorption in lactating rats (7, 9, 10).

Evidence that supported the PRL actions on bone in this study were based on the use of Bromo, which suppressed PRL release through stimulation of the pituitary D_2 dopaminergic receptors and in turn reduced circulating PRL levels by $\sim 80-90\%$ (3, 7, 35). Since osteoblasts did not express D_2 receptors

(43), Bromo should not exert a direct action on this cell type. Although all histomorphometric parameters related to osteoblast functions and bone formation in L21 rats (e.g., osteoblast surface and bone formation rate) were completely inhibited by Bromo and restored by exogenous PRL supplement, changes in some other parameters seemed to be partially PRL dependent (e.g., osteoclast surface in L21 rats). It was possible that other hormones, such as PTHrP from the mammary glands, also contributed to the lactation-induced trabecular bone loss as reported previously (46). On the other hand, the PRL-induced bone changes were not clearly observed at the macroscopic level with DEXA, presumably because the gross changes in femoral BMD required prolonged duration of 2–7 wk of PRL exposure (40, 44).

Furthermore, the results in nonsuckling rats confirmed that the factor(s), either PRL or PTHrP, that induced trabecular bone loss during lactation was related to suckling. Normally, the plasma PRL levels decreased rapidly within 1–2 days after cessation of breastfeeding (26, 34). However, the significant increases in BMD and BMC in nonsuckling rats suggested that

Fig. 9. Trabecular bone resorption-related parameters: osteoclast surface normalized by trabecular bone surface (Oc.S/BS; A), and eroded surface (ES/BS; B) of the secondary spongiosa of tibiae obtained from age-matched control, pregnant (P14 and P21), lactating (L8, L14 and L21), and 15-day postweaning (PW) rats. Some pregnant and lactating rats were administered Bromo for 7 days (4 mg·kg⁻¹·day⁻¹ sc) or Bromo+PRL. PRL doses for pregnant and lactating rats were 0.4 and 0.6 mg·kg⁻¹·day⁻¹, respectively. Some rats were permanently separated from their pups after parturition (non-suckling). Both Oc.S/BS and ES/BS are static histomorphometric parameters from Goldner's trichrome-stained sections. All values in line graphs and bar graphs are presented as means ± SE (n = 6–8 rats per condition). *P < 0.05 vs. age-matched control group; †P < 0.05 vs. corresponding lactating group; +P < 0.05 vs. Bromo group; #P < 0.05 vs. Bromo+PRL group.



the absence of suckling pups and normal weaning might differentially affect bone metabolism, because BMD and BMC continued to increase in nonsuckling rats (Fig. 2), although their trabecular histomorphometric parameters showed similar modest changes (Figs. 7–9). It was, therefore, speculated that an endocrine factor that increased cortical bone growth during pregnancy remained active after parturition, perhaps due to the lack of antagonistic signals triggered by suckling.

Previous investigations concerning the direct effect of PRL on osteoblast-induced bone formation at the cellular and molecular levels agreed with the present histomorphometric findings. Specifically, PRL was found to downregulate *Runt*-related transcription factor-2, alkaline phosphatase, and osteocalcin (12, 40). Such direct PRL actions explained the PRL-induced decreases in osteoblast surface in tibial trabeculae of P14 rats and double labeled surface and bone formation rate of L14 rats. Interestingly, in L21 rats there were paradoxical increases in all bone formation-related parameters, which could be prevented by Bromo and restored by exogenous PRL administration (Fig. 8). The exact explanation of this paradox is not known, but it might be related to the PRL-enhanced intestinal calcium absorption (7, 19) and/or the calcitonin-induced renal calcium reabsorption (20), both of which could provide extra calcium for mineralization. Alternatively, extensive bone resorption in late lactation might lead to substantial release of the embedded growth factors, thus in turn stimulating bone formation (13).

Regarding PRL effects on bone resorption, a previous study using human osteoblasts showed that PRL upregulated the expression of receptor activator of nuclear factor- κ B ligand (RANKL), while concurrently downregulating the osteoprotegerin (OPG) expression (40), thereby increasing the number of active osteoclasts and their activities. The PRL-induced increase in the RANKL/OPG ratio explained the present findings that osteoclast surface and eroded surface were markedly increased in mid- and late lactation. Besides PRL, mammary gland-derived PTHrP, which could directly stimulate osteoclasts, may also participate in maternal bone resorption, especially when its plasma levels were elevated from mid-pregnancy to late lactation (20, 46).

After weaning, BMD was restored by increasing trabecular bone formation (Fig. 8) and decreasing bone resorption (Fig. 9). BMD of the primarily cortical sites (e.g., femora) recovered faster than that of the trabecula-rich sites (e.g., L5 vertebrae). It was apparent that the repair of trabecular microstructure in rats could not be accomplished within 15 days postweaning, but might require up to 20 wk of recovery (4). Similarly, in humans, bone mass could be restored after cessation of breastfeeding, although some mothers might develop the so-called pregnancy- and lactation-induced osteoporosis featuring bone pain, height loss, and fragility fracture (29).

In conclusion, we demonstrated herein that pregnancy increased bone mass predominantly in the cortical shells of both long bones and vertebrae, whereas lactation decreased bone mass predominantly in bone trabeculae. Thus, trabecular structures were an important source of calcium for lactogenesis. Interestingly, bone modeling was discernible during these reproductive periods as a result of increased bone length and uncoupling of bone formation and bone resorption. Although the pregnancy-induced cortical bone gain appeared to be PRL independent, PRL still contributed to maternal bone changes during pregnancy since it could decrease osteoblast surface in P14 rats. On the other hand, suppression of bone formation in mid-lactation and stimulation of bone resorption in mid- and late lactation were clearly PRL dependent and could be explained by the direct PRL actions on osteoblasts (39, 40). The present *in vivo* data, therefore, provided further information on a dynamic, time-dependent, site-specific adaptation of maternal bone, as well as differential osteoregulatory roles of PRL during pregnancy and lactation.

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DISCLOSURES

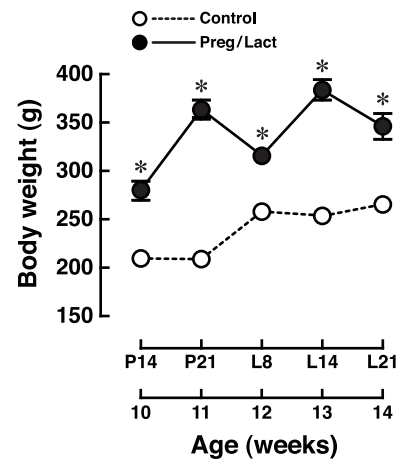
No conflicts of interest are reported by the authors.

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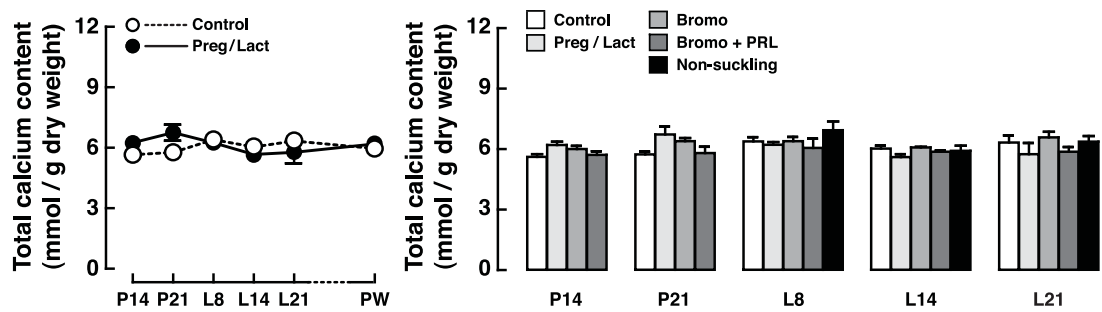
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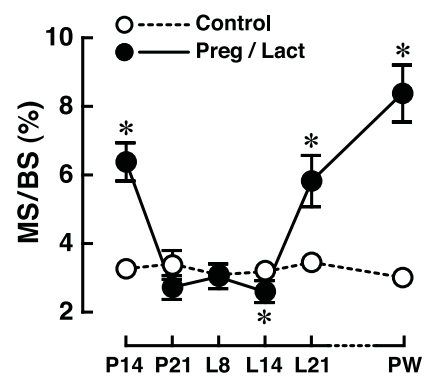




Supplemental Figure S1: Suntornsaratoon et al.



Supplemental Figure S2: Suntornsaratoon et al.



Supplemental Figure S3: Suntornsaratoon et al.

Legends to supplemental figures

Supplemental Figure S1. Body weight of pregnant (P14 and P21), lactating (L8, L14 and L21) and age-matched control rats ($n = 8-10$ rats per group). * $P < 0.05$ compared with the corresponding age-matched control group.

Supplemental Figure S2. Total calcium content normalized by dry weight of femora obtained from age-matched control, pregnant (P14 and P21), lactating (L8, L14 and L21) and 15-day postweaning (PW) rats, as determined by atomic absorption spectrophotometer. Some pregnant and lactating rats were administered for 7 days with 4 mg/kg/day bromocriptine s.c. (Bromo), or Bromo plus PRL s.c. (Bromo+PRL). The PRL doses for pregnant and lactating rats were 0.4 and 0.6 mg/kg/day, respectively. Some rats were permanently separated from their pups after parturition (non-suckling). All values in line graphs and bar graphs are presented as means $\pm$ SE ($n = 4-6$ rats per condition).

Supplemental Figure S3. Mineralizing surface normalized by trabecular bone surface (MS/BS) of the secondary spongiosa of tibiae obtained from age-matched control, pregnant (P14 and P21), lactating (L8, L14 and L21) and 15-day postweaning (PW) rats. All values are presented as means $\pm$ SE ($n = 7-8$ rats per each condition). * $P < 0.05$ compared with age-matched control group.

Possible chondroregulatory role of prolactin on the tibial growth plate of lactating rats

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Abstract Besides calcium accretion in the cortical envelope, a marked increase in the length of long bone was observed in pregnant and lactating rats, and thus the growth plate change was anticipated. Since several bone changes, such as massive trabecular bone resorption in late lactation, were found to be prolactin (PRL)-dependent, PRL may also be responsible for the maternal bone elongation. Herein, we investigated the growth plate change and possible chondroregulatory roles of PRL in the tibiae of rats at mid-pregnancy until 15 days postweaning. We found that the tibial length of lactating rats was increased and was inversely correlated with the total growth plate height, as well as the heights of proliferating zone (PZ) and hypertrophic zone (HZ), but not the resting zone (RZ). Chondrocytes in all zones expressed PRL receptors as visualized by immunohistochemistry, suggesting that the growth plate cartilage was a target of PRL action. Further investigations in lactating rats treated with an inhibitor of pituitary PRL release, bromocriptine, with or without PRL supplement, revealed the PRL-induced decreases in total growth plate

height and HZ height from early to late lactation. However, decreases in RZ and PZ heights were observed only in late and mid-lactation, respectively. Thus, this was the first report on the chondroregulatory action of PRL on the growth plate of long bone in lactating rats. The results provided better understanding of the maternal bone adaptation during lactation.

Keywords Cartilage · Chondrocytes · Goldner's trichrome · Hyperprolactinemia · Immunohistochemistry · Prolactin receptor

Introduction

During pregnancy and lactation, the microstructure of maternal bone is markedly changed to meet mineral demand for fetal skeletal development and milk production (Bowman and Miller 2001; Charoenphandhu et al. 2010). Specifically, pregnancy induces bone calcium accretion in the cortical and trabecular parts of the long bone as a reserved pool for later use, whereas lactation induces progressive trabecular bone loss (Charoenphandhu et al. 2010; Suntornsaratoon et al. 2010a, b). Although maternal osteopenia in humans is usually asymptomatic and reversible, some individuals may experience severe pathological bone loss, known as pregnancy/lactation-induced osteoporosis with low-back pain, loss of height, fragility fracture, and non-traumatic vertebral compression (Ofiuoglu and Ofiuoglu 2008). Besides cortical and trabecular bone changes, femoral bone length in rats was also increased during pregnancy and appeared to continue through late lactation, presumably to further expand the calcium reserve pool, and to maintain bone strength (Suntornsaratoon et al. 2010a, b). Such increased bone

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length probably results from the pregnancy- and/or lactation-induced endochondral bone growth, which is the principal mechanism for elongation of long bones, especially femora, tibiae, and humeri (Bowman and Miller 2001; Rauch 2005). This process might also help replenish the metaphyseal spongiosa that is markedly resorbed during lactation (Rauch 2005; Suntornsaratoon et al. 2010a).

Generally, endochondral bone growth initiates at the growth plate cartilage with a sequence of chondrocyte proliferation, differentiation, hypertrophy, and apoptosis, thereby permitting vascular invasion and entrance of osteoprogenitor cells to replace degenerated chondrocytes with calcified bone matrix (Gartner and Hiatt 2001; Olsen 2006; Rauch 2005). In tibiae and femora, the growth plate cartilage is organized into three zones, namely resting zone (RZ), proliferating zone (PZ) and hypertrophic zone (HZ) (Rauch 2005). Van Buul-Offers and colleagues (1984) reported that longitudinal bone growth in rodents was closely associated with the heights of the growth plate zones, and an increase in tibial length showed negative correlations with the heights of RZ and PZ. Therefore, changes in the growth plate zones were anticipated in pregnant and lactating rats with the increased bone length.

Complex maternal bone changes are tightly regulated by a number of humoral factors, such as osteoprotegerin, insulin-like growth factor (IGF)-1, and parathyroid hormone-related peptide (PTHrP) (Hong et al. 2005; Kovacs 2005; O'Brien et al. 2006). Moreover, a recent investigation in rats revealed that prolactin (PRL) released from the pituitary gland during pregnancy (plasma levels ~ 100 – 200 ng/mL) and lactation (up to ~ 650 ng/mL; normal levels ~ 7 – 10 ng/mL) was also an important regulator of bone metabolism during these reproductive periods (Seriwatanachai et al. 2008; Suntornsaratoon et al. 2010a). After binding to PRL receptors (PRLR) on osteoblasts, PRL induced trabecular bone resorption and calcium release through the synthesis of an osteoblast-derived osteoclastogenic factor, namely receptor activator of nuclear factor- κ B ligand (Seriwatanachai et al. 2008). Besides the osteoblasts, chondrocytes isolated from the rat articular cartilage were also found to express functional PRLR, which could prevent apoptosis of this cell type in vitro (Zermeño et al. 2006). Nevertheless, whether the growth plate changes during pregnancy and lactation were also PRL-dependent remained unknown.

Therefore, the objectives of the present study were (1) to demonstrate that growth plate chondrocytes expressed PRLR proteins, (2) to investigate changes in the growth plate zones during pregnancy and lactation, and (3) to provide evidence that growth plate changes in these reproductive periods were dependent on PRL.

Materials and methods

Animals

Non-mated (nulliparous) and pregnant Sprague-Dawley rats were obtained from the National Laboratory Animal Centre, Salaya, Nakhon Pathom, Thailand. They were housed in standard stainless steel cages under 12-h light/dark cycle for at least 7 days prior to the experiments and were fed standard chow and distilled water ad libitum. The room temperature was $25 \pm 2^\circ\text{C}$ with average illuminance of 200 lux and relative humidity of ~ 50 – 60% . This study has been approved by the Animal Care and Use Committee of the Faculty of Science, Mahidol University, Thailand.

Experimental design

The present experimental design was based on the previous experiment by Suntornsaratoon et al. (2010a, b) and used a new collection of bone specimens. In brief, the tibial growth plate changes were investigated in different reproductive phases, i.e., mid-pregnancy (day 14 of pregnancy; P14), late pregnancy (day 21; P21), early lactation (day 8 of lactation; L8), mid-lactation (day 14; L14), late lactation (day 21; L21) and day 15 postweaning (PW), as well as in the age-matched nulliparous controls. The ages of P14, P21, L8, L14, L21, and PW rats were 10, 11, 12, 13, 14, and 16 weeks, respectively. After delivery, the litter size was adjusted to 8 pups/dam for all lactating groups. In some experiments, dams were daily injected for 7 days prior to the experiment with 4 mg/kg bromocriptine s.c. (Bromo) (Sigma, St. Louis, MO, USA), or Bromo + PRL s.c. (purified from ovine pituitary gland; catalog no. L6520; Sigma). PRL doses for pregnant and lactating rats were 0.4 and 0.6 mg/kg/day, respectively, as reported previously (Charoenphandhu et al. 2009). This regimen of Bromo administration was found to successfully abolish hyperprolactinemia in maternal rats (Charoenphandhu et al. 2009). Finally, maternal rats were killed on the aforementioned designated time. Their tibiae and femora were removed and measured for total length.

Histomorphometric measurement of growth plate height

After being removed from maternal rats, tibiae were cleaned off adhering connective tissue and bone marrow. Tibial length was measured by a vernier caliper. Each specimen was then dehydrated serially in 70, 95, and 100% v/v ethanol for 3, 3, and 2 days, respectively, as described previously (Assapun et al. 2009). Dehydrated bone was embedded in methyl methacrylate resin at 42°C for 48 h. The resin-embedded bone was cut longitudinally into 7- μm sections by

a microtome equipped with a tungsten carbide blade (model RM2255; Leica, Nussloch, Germany). Bone sections were then mounted on microscope slides, deplastinated, dehydrated, and processed for the Goldner's trichrome staining (van't Hof et al. 2003). Growth plate height was measured under a light microscope (model BX51TRF; Olympus, Tokyo, Japan) with the computer-assisted Motic Images Plus 2.0 (Motic Instruments Inc., Richmond, Canada).

Histomorphometric measurement of growth plate zones (i.e., RZ, PZ and HZ) was previously described by Yakar and colleagues (2002). In brief, all measurements were performed within the central two-thirds of growth plate section. The height of each zone was determined by using the following criteria: (1) PZ contains three or more cells aligned with the long axis of the bone and each cell was not greater than 10 μm in height, (2) HZ is measured from the end of PZ to the border of primary spongiosa (i.e., at the site of vascular invasion), and (3) RZ is the subtraction of PZ and HZ heights from the total growth plate height.

Immunohistochemical localization of PRLR

Tibiae were dissected from lactating (L8) rats. After removal of adhering tissue and bone marrow, they were fixed overnight at 4°C in 0.1 M phosphate-buffered saline (PBS) containing 4% w/v paraformaldehyde. Decalcification was performed by immersing bone specimens in 15% w/v ethylenediaminetetraacetic acid (EDTA; Sigma) for 3 weeks (Wongdee et al. 2010). Decalcifying solution was changed every 3 days. After being embedded in paraffin, the specimens were cut longitudinally into 7- μm sections which were later incubated at 37°C for 30 min in antigen retrieval solution (0.01 mg/mL proteinase K, 50 mM Tris-HCl pH 8.0 and 5 mM EDTA). Thereafter, the sections were incubated for 1 h with 3% H_2O_2 to inhibit endogenous peroxidase activity. Non-specific bindings were blocked by 2-h incubation with 4% bovine serum albumin, 10% normal goat serum, and 0.7% Tween-20 in PBS. The sections were then incubated at 4°C overnight with 1:50 rabbit polyclonal primary antibody against PRLR (catalog no. sc-30225; Santa Cruz Biotechnology, CA, USA). After being washed with 0.7% Tween-20 in PBS, the sections were incubated for 1 h at room temperature with 1:500 biotinylated goat anti-rabbit IgG (catalog no. 656140; Zymed, South San Francisco, CA, USA), followed by 1-h incubation with streptavidin-conjugated horseradish peroxidase solution (Zymed) and 3,3'-diaminobenzidine chromogen (Pierce, Rockford, IL, USA). As for the negative control, the sections were incubated with 0.7% Tween-20 in PBS in the absence of PRLR primary antibody. Finally, the sections were counterstained with hematoxylin (Sigma), and examined under a light microscope and Image Pro Plus 5 (Media Cybernetics, Bethesda, MD, USA).

Statistical analysis

Unless otherwise specified, the results are expressed as means $\pm$ SE. Two sets of data were compared by the unpaired Student's *t*-test. One-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison test was used for multiple sets of data. The level of significance for all statistical tests was $P < 0.05$. Data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software Inc., San Diego, CA, USA).

Results

Correlations between the heights of growth plate zones and tibial length

Consistent with the previous report (Suntornsaratoon et al. 2010a), the tibial and femoral lengths of maternal rats were significantly increased by ~ 2 and $\sim 3\%$, respectively, at late lactation (L21) when compared with the age-matched controls (Fig. 1). Correlation plots indicated that the tibial length of L21 rats was negatively correlated with the total growth plate height, as well as PZ and HZ heights (Fig. 2). However, there was no correlation between the tibial length and RZ height (Fig. 2b).

Expression of PRLR in growth plate

Prior to investigation of possible PRL effect on growth plate, PRLR protein expression in the growth plate was first demonstrated by immunohistochemistry in decalcified tibial sections. As shown in Fig. 3, positive brownish signals of PRLR were localized in chondrocytes in all three zones of tibial growth plate cartilage from L21 lactating rats.

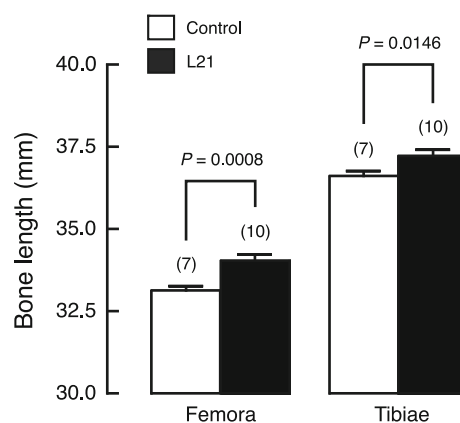


Fig. 1 Femoral and tibial lengths of L21 and age-matched control rats. Numbers in parentheses represent the numbers of experimental animals. *P*-values (unpaired Student's *t*-test) are presented above the corresponding bars

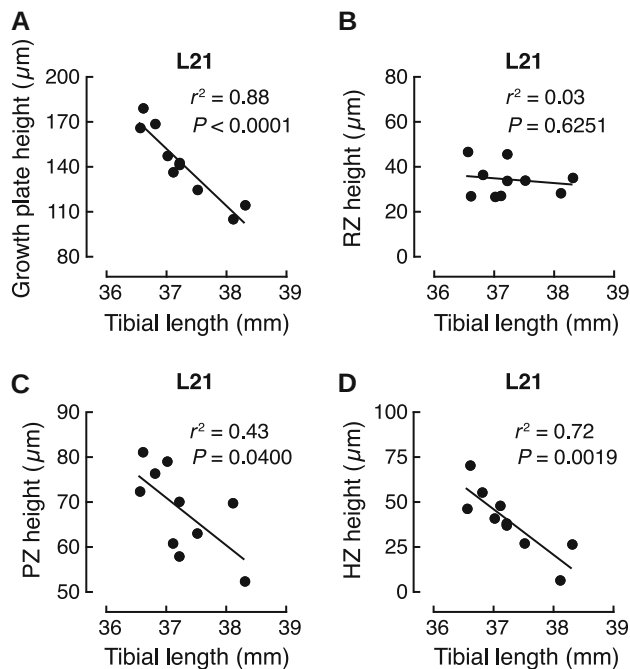


Fig. 2 Graphs of the lengths versus the heights of **a** growth plate, **b** resting zone (RZ), **c** proliferating zone (PZ), and **d** hypertrophic zone (HZ) in tibiae obtained from L21 rats. *P*-values, coefficients of determination (r^2), and regression lines are presented. The tibial length of L21 rats showed negative correlations with the heights of growth plate, PZ and HZ ($P < 0.05$), but showed no correlation with RZ height ($P > 0.05$)

However, in RZ, PZ and upper 2/3 of HZ, PRLR proteins were abundant in the cytoplasm, whereas they were localized at the cell periphery, presumably on the plasma membrane, in hypertrophic chondrocytes near primary spongiosa (lower 1/3 of HZ; Fig. 3f). PRLR proteins were also expressed in osteoblasts covering bone trabeculae (visualized at 1,000 $\times$; data not shown), but not in the embedded osteocytes. Similar pattern of PRLR protein expression was observed in the tibial growth plate cartilage of non-pregnant rats (Fig. 4).

Changes in growth plate height during pregnancy and lactation

Microscopic examination of tibial growth plate in different reproductive phases was performed in Goldner's trichrome-stained undecalcified sections. The total growth plate height of proximal tibia was initially decreased at late pregnancy (P21) by $\sim 22\%$, when compared with the corresponding age-matched controls (Fig. 5). Thereafter, it was progressively decreased to the lowest point ($\sim 70\%$ of the control value) in late lactation (L21), and was partially restored postweaning. Representative photomicrographs of the growth plate in L21 rats are also presented (Fig. 6). Inhibition of pituitary PRL secretion by Bromo completely

prevented the reduction in growth plate heights in L14 and L21 rats (Figs. 5, 6). Bromo also partially restored the decreased growth plate height in L8 rats, but did not alter that in P21 rats (Fig. 5). Moreover, exogenous PRL supplement to Bromo-treated lactating rats reduced the growth plate heights to the values observed in lactation (Figs. 5, 6). The results suggested that the growth plate change during lactation was dependent on PRL.

Changes in the heights of growth plate zones during pregnancy and lactation

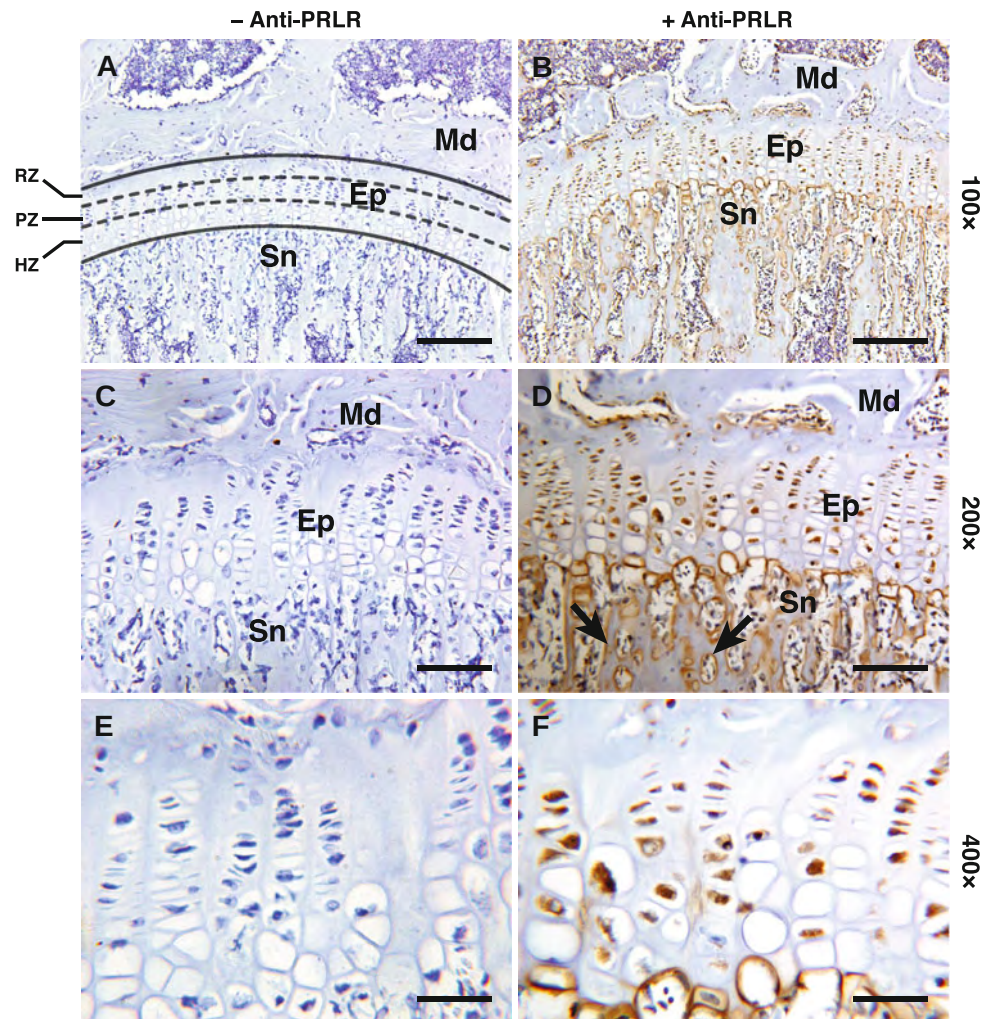
Histomorphometric analysis of Goldner's trichrome-stained tibial sections further revealed that the heights of all growth plate zones were markedly changed during pregnancy and lactation but within different timeframe. Although the RZ height (Fig. 7a) was significantly decreased only at late lactation (L21), the heights of PZ (Fig. 7b) and HZ (Fig. 7c) were dramatically reduced, especially from late pregnancy (P21) to mid-lactation (L14). A decrease in HZ height persisted until late lactation (L21). Fifteen days after weaning, PZ and HZ heights were still significantly lower than those of the controls.

Further investigations in Bromo- and Bromo + PRL-treated rats revealed that the pregnancy-induced changes in growth plate zones were independent of pituitary PRL (Fig. 7). On the other hand, the lactation-induced decreases in RZ and PZ heights in late and mid-lactation, respectively, were found to be PRL-dependent (Fig. 7a–b). Moreover, PRL was responsible for the reduction in HZ height throughout the lactating period (Fig. 7c). Our results therefore suggested the chondroregulatory action of PRL in maternal long bone during lactation.

Discussion

Whether PRL plays a role in the regulation of growth plate cartilage during pregnancy and lactation has not been known. Here, we provided, for the first time, suggestive evidence on the chondroregulatory action of PRL in the tibial growth plate, especially in lactating rats. Normally, PRL is one of the maternal calcitropic hormones, which helps supply sufficient calcium for the developing fetuses and milk production, in part, by increasing intestinal calcium absorption and enhancing bone resorption (Ajibade et al. 2010; Charoenphandhu et al. 2010). In order to supply adequate calcium from maternal bone for milk production, calcium accumulation in cortical envelope and continuing trabecular formation are requisite throughout the reproductive periods, particularly in pregnancy and early lactation (Bowman and Miller 2001; Miller et al. 1986; Suntornsarattoon et al. 2010a, b). Thereafter, in late

Fig. 3 a–f Immuno-histochemical localization of PRLR proteins in paraffin-embedded decalcified tibial sections from lactating rats (L8; $n = 4$) at 100 $\times$ (bars, 200 μm), 200 $\times$ (bars, 100 μm), and 400 $\times$ (bars, 50 μm) magnifications. The immuno-stained (+Anti-PRLR) and corresponding negative control (–Anti-PRLR) sections were incubated in the presence and absence of anti-PRLR antibody, respectively. Images of negative control do not show immunoreactive signal. The positive *brownish* signals are observed in chondrocytes in all three zones of the epiphyseal plate (Ep), i.e., resting zone (RZ), proliferating zone (PZ) and hypertrophic zone (HZ). Positive signals are also localized in osteoblasts in the primary spongiosa (Sn), but not in osteocytes embedded in mineralized matrix (Md) of bone trabeculae (arrows)



lactation, both cortical envelope and metaphyseal trabeculae are resorbed to release the stockpiled calcium under a concerted regulation of several hormones, such as PRL and PTHrP (Charoenphandhu et al. 2010; Kovacs 2005). Since bone resorption usually exceeds bone formation during lactation, osteopenia does occur in nursing mothers; however, bone loss is gradually reversed postweaning (Charoenphandhu et al. 2010; Kovacs 2005).

Elongation of the maternal bone during the reproductive periods is not uncommon in rodents (Dengler-Criss and Catania 2009). In primiparous mole rats, the lumbar vertebral length was markedly increased during pregnancy, and this lengthening was dampened after delivery (Dengler-Criss and Catania 2009). Our previous study in rats also demonstrated that, in addition to the increased bone length, the cortical envelope of long bone was also thickened during pregnancy, presumably to help maintain bone strength and/or prevent fracture during the lactation-induced massive trabecular bone loss (Suntornsaratoon et al. 2010a). This increase in bone length in lactating rats was previously shown to be dependent on PRL

(Suntornsaratoon et al. 2010a). Moreover, as shown in the present study, the tibial length was negatively correlated with the total growth plate height, PZ height, and HZ heights, similar to that reported previously in mice (van Buul-Offers et al. 1984), thereby supporting the hypothesis that the increased length of maternal long bone resulted from the adaptive changes in the growth plate cartilage, and particularly from the accelerated endochondral ossification.

Data from the Bromo-treated P21 rats (Fig. 7b–c) indicated that the pregnancy-induced changes in growth plate zones were independent of pituitary PRL because PRL supplement was unable to restore the zone heights. It is currently not known why Bromo, a dopaminergic agonist, slightly but significantly diminished the change in HZ height in P21 rats (Fig. 7c). However, the direct Bromo effect on growth plate cartilage could not be excluded despite lack of reports on dopaminergic D2 receptor expression in growth plate cartilage of pregnant rats. A previous report using radioligand binding assays in human articular cartilage suggested that, under certain conditions

Fig. 4 a–d Immuno-histochemical localization of PRLR proteins in paraffin-embedded decalcified tibial sections from non-pregnant rats (normal rats; $n = 5$) at 200 $\times$ (bars, 100 μm) and 400 $\times$ (bars, 50 μm) magnifications. The immuno-stained (+Anti-PRLR) and corresponding negative control (–Anti-PRLR) sections were incubated in the presence and absence of anti-PRLR antibody, respectively. Images of negative control sections do not show immunoreactive signal. The positive *brownish* signals are observed in chondrocytes in all three zones of the epiphyseal plate (*Ep*)

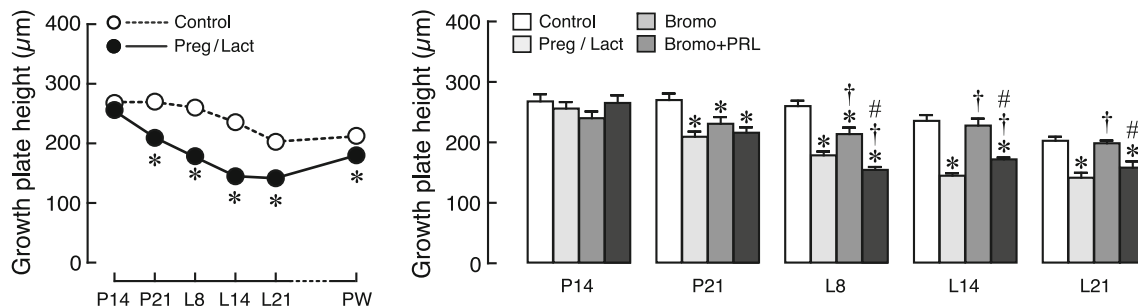
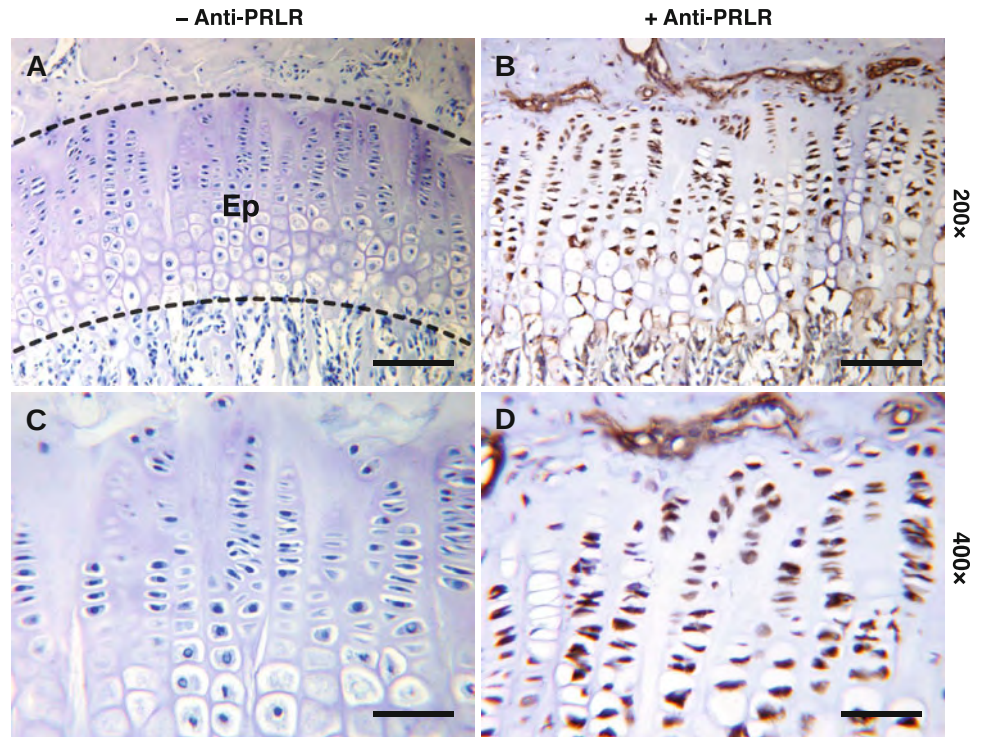


Fig. 5 Growth plate height of tibiae obtained from pregnant (P14 and P21), lactating (L8, L14 and L21), 15-day postweaning (PW), and age-matched control rats. Some pregnant and lactating rats were administered for 7 days with 4 mg/kg/day bromocriptine s.c. (Bromo), or Bromo and PRL s.c. (Bromo + PRL). The PRL doses

for pregnant and lactating rats were 0.4 and 0.6 mg/kg/day, respectively. All values in line graphs and bar graphs are presented as means $\pm$ SE ($n = 6$ –10 rats per condition). * $P < 0.05$ compared with age-matched control group. † $P < 0.05$ compared with the corresponding lactating group. # $P < 0.05$ compared with Bromo group

(e.g., osteoarthritis), chondrocytes might be responsive to dopamine agonist (Vignon et al. 1990).

On the other hand, the lactation-induced decreases in the total growth plate height as well as RZ, PZ, and HZ heights in different phases of lactation were found to be PRL-dependent (Fig. 7). Although decreases in the total height and HZ height observed throughout the entire lactating period were induced by PRL, the responses of the chondrocytes in RZ and PZ to PRL were less consistent (Fig. 7). Localization of PRLR proteins on the plasma membrane of hypertrophic chondrocytes (Fig. 3f) could partially explain why the PRL effects were clearly observed in HZ. It was also possible

that PRL acted specifically on fully differentiated chondrocytes in HZ rather than on young proliferating and progenitor cells in PZ and RZ, respectively. The differential responsiveness to PRL was previously reported in human undifferentiated mesenchymal stem cells and their chondrocyte-like progeny expressing intermediate and long isoforms of PRLR, respectively (Ogueta et al. 2002), the latter of which was the functional PRLR isoform in several cell types, including mammary and intestinal epithelial cells (Binart et al. 2010; Thongon et al. 2008).

Although the underlying mechanism of PRL action on the growth plate is not known, the strong expression of

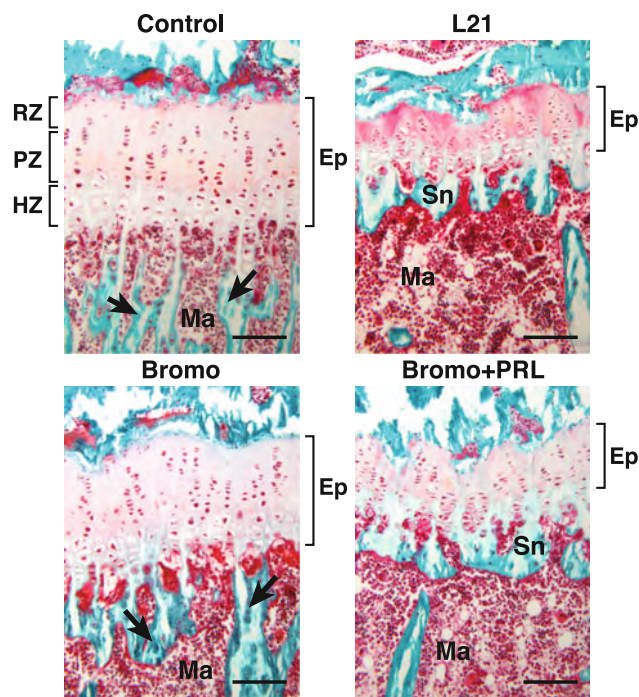


Fig. 6 Representative photomicrographs of Goldner's trichrome-stained tibial growth plates dissected from L21, Bromo-treated L21, Bromo + PRL-treated L21, and age-matched control rats. Primary spongiosa (Sn) and all three zones of epiphyseal plate (Ep), i.e., resting zone (RZ), proliferating zone (PZ) and hypertrophic zone (HZ) were identified. Mineralized trabeculae (arrows) and marrow cells (Ma) were stained green and red, respectively. Bars, 100 μ m

PRLR in the growth plate chondrocytes of lactating rats suggested a direct action of PRL. Coss and co-workers (2000) also showed that PRLR was widely expressed in the growth plate chondrocytes in the digits of neonatal rats. Besides the growth plate chondrocytes, PRLR expression has been reported in articular chondrocytes from both humans and rats (Ogueta et al. 2002; Zermeño et al. 2006). In the articular chondrocytes in vitro, PRL has been found to inhibit apoptosis induced by serum starvation (Zermeño et al. 2006) and to increase type II collagen expression (Ogueta et al. 2002). Moreover, PRL could directly induce proliferation and chondrogenic differentiation of the human marrow-derived mesenchymal stem cells to mature chondrocytes (Ogueta et al. 2002). Although the chondrocytes in articular cartilages may have different functions from those in the growth plate since the articular chondrocytes do not proceed to hypertrophic differentiation, the aforementioned findings corroborated that PRL is a regulator of the growth of cartilage.

In addition to possible stimulatory effects of PRL on chondrogenesis (Ogueta et al. 2002; Zermeño et al. 2006), a previous study that showed the delay of calvarial

ossification and a decrease in femoral mineral apposition rate in PRLR knockout mice also suggested a stimulatory action of PRL on osteogenesis (Clément-Lacroix et al. 1999). Since the growth plate height is normally maintained by keeping a precise balance between chondrogenesis and osteogenesis, the PRL-induced growth plate narrowing might be due to the enhanced turnover of chondrocyte replication and growth plate ossification, the latter of which probably occurred at the faster rate. Nevertheless, once bone trabeculae which serve as calcium storage pools are formed, later on, by as yet unknown mechanism, PRL appears to enhance bone resorption at the rate that matches the maternal requirement (Charoenphandhu et al. 2010).

In addition to PRL, other hormones, such as IGF-1, placental lactogen (PL) and PTHrP, released during pregnancy and lactation might participate in the present growth plate changes. Plasma levels of IGF-1 and PL were elevated in pregnant humans and rats (Freeman et al. 2000; O'Brien et al. 2006). Both systemic and local IGF-1 productions were known to be essential for chondrogenesis and endochondral ossification (Wang et al. 2006; Yakar et al. 2002). PL can bind to PRLR (Cohick et al. 1996), and thus can mimic the PRL effect on cartilage. In addition, growth plate changes during certain phases of lactation might be due to the circulating PTHrP from the mammary glands and its downstream Indian hedgehog signaling pathways (Goltzman 2010; Maeda et al. 2007; Olsen 2006). Nevertheless, further experiments are required to elucidate the interplay between these maternal hormones and their influence on the growth plate function.

However, the chondroregulatory actions of PRL should be apparent only in mammals with persistent growth plate cartilage. In adolescent humans, the accelerated endochondral ossification might be a compensatory mechanism which helped to maintain bone growth during lactation, despite having lactation-induced bone loss. We also further speculated that, in lactating mammals without growth plates, the PRL-induced bone loss became more pronounced, especially when the intestinal calcium absorption was restricted by inadequate calcium intake.

In conclusion, there was a decrease in the tibial growth plate height from late pregnancy to 15 days postweaning. Such a decrease in the total height in lactation showed a negative correlation with the tibial length, and was mainly due to a decrease in HZ height in a PRL-dependent manner. The presence of PRLR protein expression in growth plate chondrocytes further suggested that PRL exerted a direct action on the tibial growth plate cartilage. Although additional investigations are needed to demonstrate the underlying mechanism, the present results are helpful in understanding better the maternal bone adaptation during

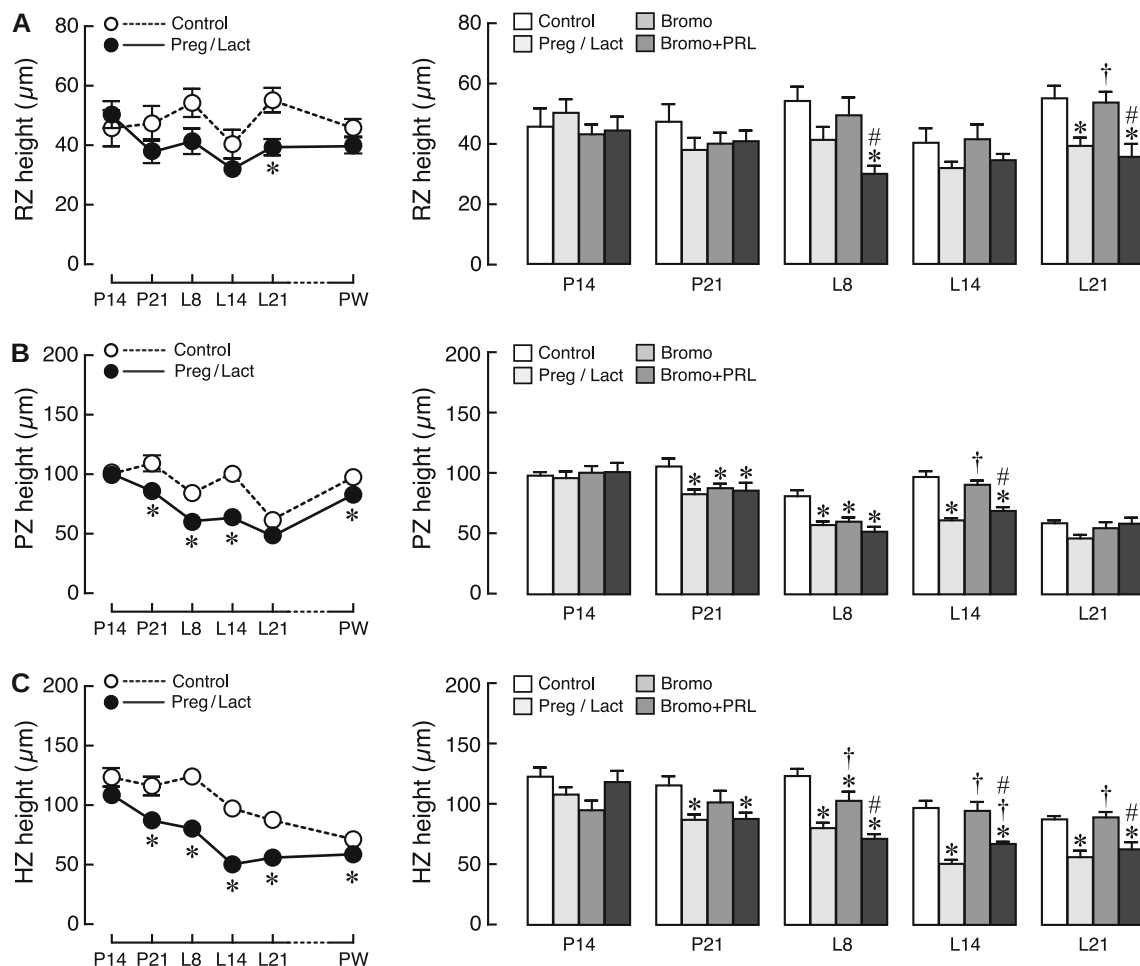


Fig. 7 The heights of **a** resting zone (RZ), **b** proliferating zone (PZ), and **c** hypertrophic zone (HZ) of tibial growth plate obtained from pregnant (P14 and P21), lactating (L8, L14 and L21), 15-day postweaning (PW), and age-matched control rats. Some pregnant and lactating rats were administered for 7 days with 4 mg/kg/day bromocriptine s.c. (Bromo), or Bromo and PRL s.c. (Bromo + PRL).

lactation, and substantiate the chondroregulatory role of PRL in lactating rats.

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Conflict of interest The authors declare no conflicts of interest.

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Prolactin alters the mRNA expression of osteoblast-derived osteoclastogenic factors in osteoblast-like UMR106 cells

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Abstract Prolactin (PRL) is known to participate in the lactation-induced maternal bone loss, presumably by inducing the release of receptor activator of nuclear factor- κ B ligand (RANKL), a potent osteoclastogenic factor from osteoblasts. Since maternal bone resorption was too massive to be solely explained by RANKL and osteoclasts did not express PRL receptors (PRLR), the involvement of some other osteoblast-derived osteoclastogenic modulators was anticipated. Herein, the authors used quantitative real-time PCR to investigate the mRNA expressions of various osteoclastogenic factors in osteoblast-like UMR106 cells directly exposed to PRL for 48 h. These cells were found to express PRLR and respond to 300 ng/ml PRL by increasing RANKL mRNA expression. This PRL concentration (comparable to plasma PRL levels in lactation) also induced the upregulation of monocyte chemoattractant protein (MCP)-1, cyclooxygenase (Cox)-2, and ephrin-B1, whereas a higher concentration (500 ng/ml) was required to upregulate tumor necrosis factor (TNF)- α and interleukin (IL)-1. However, 100–500 ng/ml PRL affected

neither the cell proliferation, the cell viability nor the mRNA expressions of macrophage colony-stimulating factor, IL-6, ephrin type-B receptor 4 and ephrin-B2. In conclusion, besides RANKL overexpression, PRL upregulated the expressions of other osteoclastogenic modulators, i.e., MCP-1, Cox-2, TNF- α , IL-1, and ephrin-B1, thus, further explaining how PRL induced bone loss in lactating mothers.

Keywords Hyperprolactinemia · Osteoblast-derived factors · Osteoclastogenesis · Prolactin receptor · RANKL · Real-time PCR

Introduction

With the clearly observed link between hyperprolactinemia and the lactation-induced maternal bone loss [1], prolactin (PRL) has been postulated to be an important regulator of maternal bone metabolism during lactation [2], in which the average plasma PRL levels are markedly elevated by ~30-fold (i.e., ~7–10 ng/ml in non-mated rats vs. ~200–300 ng/ml in lactating rats). The authors' recent investigation in lactating rats showed that PRL markedly stimulated osteoclastic bone resorption, particularly in the trabecular areas [1]. The resorptive effects of PRL on bone were accomplished by its direct actions on osteoblasts, but not osteoclasts, since only osteoblasts were found to express functional PRL receptors (PRLR) [3, 4]. At the molecular level, the PRL-induced bone resorption was partially explained by an overexpression of the receptor activator of nuclear factor- κ B (RANK) ligand (RANKL) [5], an osteoblast-derived factor that increased osteoclast differentiation and survival [6].

Since progressive bone loss in lactating rats was too massive to be explained solely by the RANKL

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overproduction [1, 5, 7], PRL was likely to modulate the expression of other genes essential for osteoclastogenesis. In general, besides RANKL which interacts with its receptors RANK on the plasma membrane of pre-osteoclasts [6], a number of osteoblast-derived paracrine factors, such as macrophage colony-stimulating factors (MCSF), interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)- α , are essential for proliferation and activation of pre-osteoclasts [6, 8, 9]. In addition, an upregulation of cyclooxygenase (Cox)-2 in osteoblasts may lead to an overproduction of prostaglandin E2, which in turn enhanced the osteoclastic bone resorption [10], whereas inhibition of Cox-2 activity by celecoxib and indomethacin restricted the osteoclast formation in a dose-dependent manner [11].

Besides using cytokines as signals between osteoblasts and osteoclasts, in bone remodeling, these bone cells can communicate with each other through a direct cell–cell contact using the ephrin receptor (Eph)/ephrin system [12]. The tyrosine kinase receptor Eph and its ligand ephrin are transmembrane proteins, both of which can initiate the signaling cascades [12]. An interaction between ephrin type-B receptor 4 (EphB4) expressed in osteoblasts and ephrin-B2 in osteoclasts was reported to inhibit osteoclastogenesis, while stimulating osteoblastogenesis through a bidirectional signaling, thereby inducing the transition from bone resorption to formation [13]. Thus, EphB4 and ephrin-B2 were parts of a negative feedback loop that counterbalanced the osteoblast-derived osteoclastogenic factors. In addition, ephrin-B1 was also essential for normal skeletal development in both humans and rodents [14, 15]. Ephrin-B1 knockout mice exhibited several bone anomalies, such as rib mispairing and fusion of sternebrae [14].

Therefore, the objectives of this study were (i) to determine the mRNA expressions of genes related to the osteoblast-derived osteoclastogenic paracrine factors in rat osteoblast-like UMR106 cells directly exposed to PRL; and (ii) to investigate the direct effect of PRL on the mRNA expression of Eph/ephrin in osteoblast-like cells. Unlike the previous investigations that used the pathological PRL concentration of 1000 ng/ml (as in prolactinoma) [5], in this study, the authors used high physiological concentrations of PRL, i.e., 100, 200–300, and 400–500 ng/ml which were comparable to the average plasma levels in pregnancy, lactation, and suckling-induced PRL surge, respectively [2, 16]. Thus, the results were more closely related to what happened in lactating mothers.

Materials and methods

Cell culture

Rat osteoblast-like UMR106 cells (American Type Culture Collection [ATCC] no. CRL-1661) were propagated in

six-well culture plates with Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria), and 100 U/ml penicillin–streptomycin (Gibco, Grand Island, NY, USA). Cells were incubated with 5% CO₂ at 37°C, and subcultured according to the ATCC's protocol. Despite being a rat osteosarcoma cell line, Partridge et al. [17] provided evidence that UMR106 cells were more similar to the differentiated rat osteoblasts than their osteosarcoma parent because they exhibited high alkaline phosphatase activity and were well responsive to parathyroid hormone. This cell type has been reported to express several markers of normal differentiated osteoblasts, e.g., type I collagen, osteocalcin, and osteopontin [18, 19]. Therefore, UMR106 cell line is an appropriate model for studying the PRL regulation of osteoblast gene expression.

Experimental design

Total RNA of confluent UMR106 cells were collected at 96 h after seeding in six-well plates to demonstrate the mRNA expression profile of short (-S) and long (-L) isoforms of PRLR, as well as the anti-osteoclastogenic factor, osteoprotegerin (OPG), and various osteoclastogenic factors, namely, RANKL, MCSF, monocyte chemoattractant protein (MCP)-1, Cox-2, TNF- α , IL-1, IL-6, EphB4, ephrin-B1, and ephrin-B2, by using conventional reverse transcription (RT)-PCR. To investigate the time-dependent effects of PRL on osteoblast mRNA expression, confluent UMR106 cells were incubated in 500 ng/ml PRL (purified from ovine pituitary gland; catalog no. L6520; Sigma) for 0, 12, 24, 48, and 72 h before determination of RANKL expression by quantitative real-time PCR (qRT-PCR). The optimal incubating time (i.e., 48 h) from the time-dependent study was used in the subsequent experiments. For the dose-dependent study, confluent UMR106 cells were incubated with normal medium (0 ng/ml PRL; control) or medium containing various concentrations of PRL, i.e., 100, 200, 300, 400, and 500 ng/ml, for 48 h before determining the expression levels of PRLR and osteoclastogenic modulators by qRT-PCR. The effects of PRL on osteoblast proliferation and viability were determined by 5-bromo-2'-deoxyuridine (BrdU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively.

Cell proliferation assay

UMR106 cells were seeded in 96-well culture plates (3,000 cells/well). Culture media also contained 10 μ M BrdU to allow BrdU molecules to be incorporated into the newly synthesized DNA of proliferating cells. After a 48-h incubation with various concentrations of PRL, cell

proliferation was determined by BrdU-based cell proliferation ELISA kit (catalog no. 11647229001; Roche, Mannheim, Germany) according to the manufacturer's instruction. The absorbance of each well was determined at 370 nm with a reference wavelength of 490 nm by a microplate reader (model 1420; Wallac, Turku, Finland). Experiments with seven independent samples per each PRL concentration ($n = 7$) were performed in triplicate.

Cell viability assay

UMR-106 cells were seeded in 96-well culture plates (3,000 cells/well). After a 48-h incubation with various concentrations of PRL, MTT (catalog no. M2128; Sigma) was pipetted into each well to obtain a final concentration of 1 mg/ml. Cells were subsequently incubated at 37°C for 3.5 h, after which the culture medium was discarded. Each well was then filled with 150 μ l of MTT solvent (isopropanol containing 4 mM HCl and 0.1% v/v Nonidet P-40), and the plate was finally incubated in the dark for 15 min. In living cells, yellow MTT was enzymatically reduced to purple formazan dye in the mitochondria. The absorbance of each well was determined at 590 nm with a reference wavelength of 620 nm by a microplate reader (model 1420; Wallac). Experiments with seven independent samples per each PRL concentration ($n = 7$) were performed in triplicate.

Total RNA preparation

The total RNA samples were prepared from UMR106 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Purity of the total RNA was evaluated by NanoDrop-2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) reading at 260 and 280 nm, the ratio of which was in the range of 1.8–2.0. One microgram of total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-rad, Hercules, CA, USA) to cDNA by a conventional thermal cycler (model MyCycler; Bio-rad). Rat β -actin served as a control gene to check the consistency of the reverse transcription (percent coefficient of variation <5%).

Quantitative real-time PCR (qRT-PCR)

Primers used in this study are shown in Table 1. All the studied genes were first determined by conventional RT-PCR, as previously described [18, 20]. Conventional RT-PCR was performed using GoTaq Green Master Mix (Promega, Madison, WI, USA) and a thermal cycler (model MyCycler; Bio-rad). qRT-PCR and melting curve analyses were operated by Bio-rad MiniOpticon with iQ

SYBR Green SuperMix (Bio-rad), as previously described [18, 21]. PCR was performed for 40 cycles at 95°C for 60 s, 51–57°C annealing temperature (Table 1) for 30 s, and 72°C for 30 s. PCR products were also visualized on 2% agarose gel stained with 1 μ g/ml ethidium bromide (Sigma) under UV transilluminator (Alpha Innotech, San Leandro, USA). PCR experiments were performed in triplicate with 12 independent samples for each experimental condition ($n = 12$).

Statistical analysis

Unless otherwise specified, the results are expressed as means $\pm$ SE, whereas the qRT-PCR data are presented as log₂ means $\pm$ SE. Two-group comparisons were performed by Student's *t*-test. Multiple comparisons were performed by one-way analysis of variance (ANOVA), followed by Dunnett's post-test. The level of significance was $P < 0.05$. Data were analyzed by GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Before the investigation of PRL effects on mRNA expressions of osteoblast-derived osteoclastogenic factors, the background expression profile of all the studied genes and PRLRs was determined in osteoblast-like UMR106 cells. As shown in Fig. 1a, untreated UMR106 cells strongly expressed OPG, RANKL, MCSF, MCP-1, Cox-2, TNF- α , IL-1, IL-6, EphB4, ephrin-B1 and ephrin-B2. They were also found to constitutively express both PRLR-S and PRLR-L (Fig. 1b), the former of which was of greater abundance by 207% (Fig. 1c), suggesting that UMR106 cells could natively respond to PRL. This was in contrast to the human osteoblast-like MG-63 and Saos-2 cells in which 1,25-dihydroxyvitamin D₃ was necessary to induce PRLR expression [22].

Since it was reported that PRL was a key mediator of maternal bone resorption in lactating rodents [1], and that in the calvaria, RANKL mRNA levels and the RANKL/OPG ratio were significantly increased before returning rapidly to the baseline after weaning [7], the PRL effect on RANKL/OPG expression in the present in vitro UMR106 model was first confirmed. After being exposed to 300 ng/ml PRL for 48 h, UMR106 cells markedly increased RANKL mRNA expression by $\sim$ 2-fold (Fig. 1d), while OPG mRNA expression was not altered (Fig. 1e). The RANKL/OPG ratio was, therefore, significantly elevated by $\sim$ 45% (Fig. 1f), consistent with that reported by Ardeshirpour et al. [7]. Thus, the present in vitro model could be used to represent the osteoblastic response to PRL in lactating animals.

Table 1 Primers used in the PCR experiments

Gene	Accession no.	Primer (forward/reverse)	Product length (bp)	Annealing temperature (°C)
Prolactin receptors				
PRLR-S	NM_012630	5'-TTCTACCACCATCGCAAC-3' 5'-CTGATCTCGTTTGTCATTGAG-3'	120	53
PRLR-L	NM_001034111	5'-TCAAGCAACCGCAGACTC-3' 5'-CAGTTTAGCCAATCGTTCCA-3'	107	53
Anti-osteoclastogenic factor				
OPG	NM_012870	5'-ATTGGCTGAGTGTCTGGT-3' 5'-CTGGTCTCTGTTTGTATGC-3'	140	53
Osteoclastogenic factors				
RANKL	NM_057149	5'-TCGCTCTGTTCTGTACT-3' 5'-AGTGCTTCTGTGTCTTCG-3'	145	53
MCSF	NM_023981	5'-ATCCAGGCAGAGACTGACAGA-3' 5'-CGCAGTGTAGATGAACCATCC-3'	182	55
MCP-1	NM_031530	5'-TGAGTCGGCTGGAGAACTA-3' 5'-ATTGGGGTCAGCACAGAT-3'	104	51
Cox-2	NM_017232	5'-TATCAGGTCATCGGTGGAGAG-3' 5'-CGAAGCCAGATGGTAGCATAC-3'	161	56
TNF- α	NM_012675	5'-GCGTGTTTCATCCGTCTCTA-3' 5'-ACTACTTCAGCGTCTCGTGTGT-3'	198	55
IL-1	NM_031512	5'-TCAAGCAGAGCACAGACCTGT-3' 5'-TGAGAGACCTGACTTGGCAGA-3'	197	56
IL-6	NM_012589	5'-GCAAGAGACTTCCAGCCAGT-3' 5'-AGCCTCCGACTTGTGAAGTG-3'	145	54
EphB4	NM_010144	5'-GTGTATGCCACGATACGCTT-3' 5'-ACTGTGTCCACCTTGATGTAGG-3'	167	56
Ephrin-B1	NM_017089	5'-GTCTCTCTCCTTACCTTCGGCA-3' 5'-CCAAGCAGCCTCTTCTCTT-3'	200	57
Ephrin-B2	NM_001107328	5'-AACACTCTCCACAGCACACG-3' 5'-TGGGCAGAAGACACTGTCTG-3'	134	56
Housekeeping gene				
β -actin	NM_031144	5'-CAGAGCAAGAGAGGCATCCT-3' 5'-GTCATCTTTTCACGGTTGGC-3'	185	56

PRLR-S short isoform of prolactin receptor, *PRLR-L* long isoform of prolactin receptor, *OPG* osteoprotegerin, *RANKL* receptor activator of nuclear factor- κ B ligand, *MCSF* macrophage colony-stimulating factor, *MCP-1* monocyte chemoattractant protein-1, *Cox-2* cyclooxygenase-2, *TNF- α* , tumor necrosis factor- α , *IL* interleukin, *EphB4* ephrin type-B receptor 4

To determine the optimal incubating time with PRL, confluent UMR106 cells were incubated with 500 ng/ml PRL for 0, 12, 24, 48, and 72 h. After 48 and 72 h of continuous PRL exposure, RANKL mRNA expression was upregulated by ~ 2 -fold (Fig. 2a). Therefore, the 48-h incubating period was used in the subsequent experiments. Moreover, PRL concentrations used in this study (100–500 ng/ml) did not affect the mRNA expressions of the housekeeping gene β -actin (Fig. 2b) or PRLR-S and -L (Fig. 2c). A 48-h exposure to 100–500 ng/ml PRL affected neither osteoblast proliferation (Fig. 3a) nor viability (Fig. 3b).

The mRNA expressions of six osteoblast-derived osteoclastogenic factors, namely, MCSF, MCP-1, Cox-2, TNF- α , IL-1, and IL-6 were thereafter demonstrated in UMR106 cells exposed for 48 h to various PRL concentrations. As shown in Fig. 4, the mRNA levels of MCP-1, which served to recruit osteoclast progenitors [12, 23], were increased by ~ 2 -fold after exposure to 200–500 ng/ml PRL. Cox-2 mRNA expression was also upregulated by ~ 3 -fold in the presence of 300 ng/ml PRL. Only the higher physiological PRL concentration of 500 ng/ml upregulated TNF- α and IL-1 by ~ 3 -fold and ~ 2 -fold,

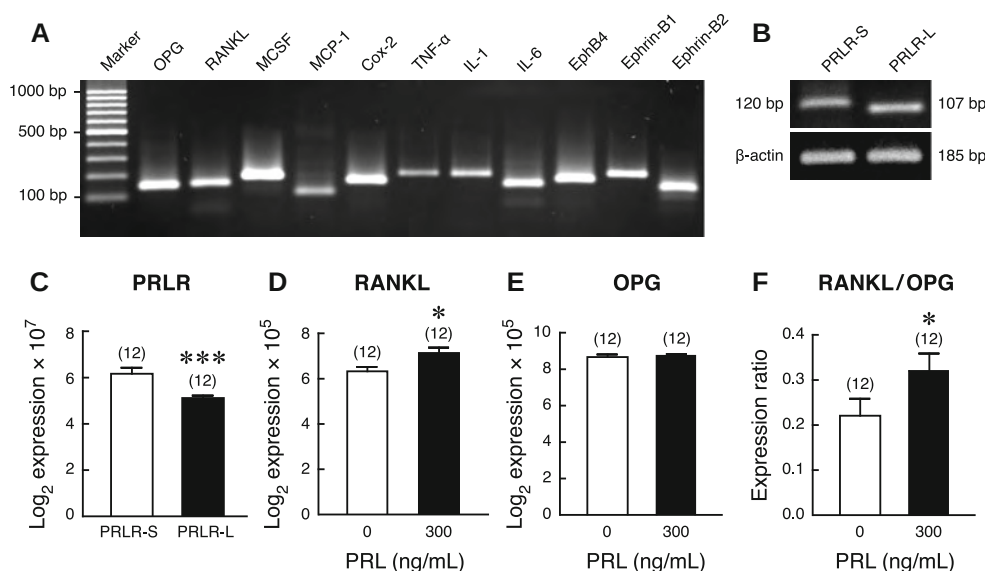


Fig. 1 **a** Representative electrophoretic bands of the osteoblast-derived osteoclast-regulating factors, namely *OPG* osteoprotegerin, *RANKL* receptor activator of nuclear factor- κ B ligand, *MCSF* macrophage colony-stimulating factor, *MCP-1* monocyte chemoattractant protein-1, *Cox-2* cyclooxygenase-2, *TNF- α* tumor necrosis factor- α , *IL-1*, *IL-6*, *EphB4*, *ephrin-B1*, and *ephrin-B2* in UMR106 cells at 96 h after seeding. The culture medium was without PRL. Conventional PCR was performed for 40 cycles. **b** Representative electrophoretic bands of short (-S) and long (-L) isoforms of PRLR in UMR106 cells. Expression of β -actin, a housekeeping gene, was also

presented along with the studied genes. **c** Expression of PRLR-S and PRLR-L in UMR106 cells at 96 h after seeding, as determined by qRT-PCR. *** P < 0.001 compared with the PRLR-S expression level. **d–f** Expression of RANKL, OPG, and their expression ratio (RANKL/OPG) in UMR106 cells exposed for 48 h to 300 ng/ml PRL, a physiological concentration comparable to that observed in lactation. The mRNA expression of each gene was quantified in triplicate by qRT-PCR and normalized by β -actin expression. * P < 0.05 compared with the control group (0 ng/ml PRL). Numbers in parentheses represent the numbers of independent samples

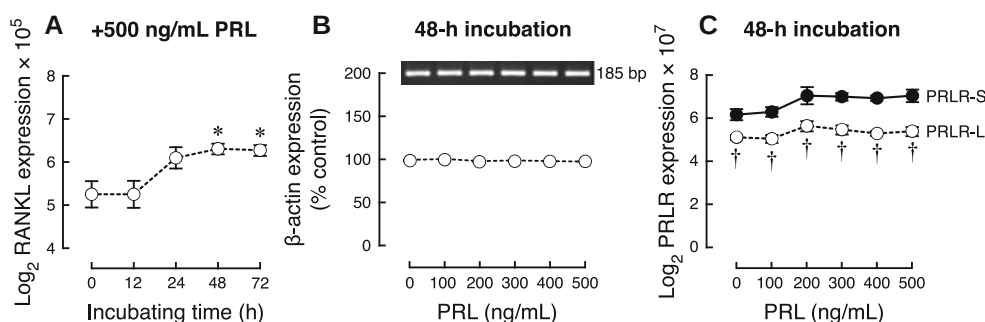


Fig. 2 **a** Expression of RANKL in UMR106 cells after direct exposure to 500 ng/ml PRL for 0 (control), 12, 24, 48, or 72 h. * P < 0.05 compared with the control group (one-way ANOVA). **b** Expression of β -actin, a housekeeping gene for normalization, in UMR106 cells directly exposed for 48 h to 0 (normal medium), 100, 200, 300, 400, or 500 ng/ml PRL. A representative electrophoretic

image is also depicted. **c** Expression of short (-S) and long (-L) isoforms of PRLR in UMR106 cells exposed for 48 h to 0 (normal medium), 100, 200, 300, 400 or 500 ng/ml PRL. † P < 0.001 compared with the corresponding PRLR-S expression level (Student's t -test). The mRNA expression of each gene in 12 independent samples (n = 12 per group) was determined by qRT-PCR

respectively. In contrast, neither the MCSF nor the IL-6 mRNA expressions were affected by 100–500 ng/ml PRL.

Besides activating osteoclasts by the cytokine-mediated mechanism, osteoblasts also activate osteoclasts by a direct contact through Eph/ephrin interaction [12, 13]. As depicted in Fig. 5, only ephrin-B1 mRNA expression was significantly increased by ~2-fold after a 48-h exposure to

300 ng/ml PRL. The upregulation of ephrin-B1 and Cox-2 by 300 ng/ml, but not by lower or higher concentrations, could be considered as biphasic PRL responses as previously reported in other tissues, such as small intestine and Leydig cells [24, 25]. None of the PRL concentrations had any effect on the mRNA expression of EphB4 and ephrin-B2.

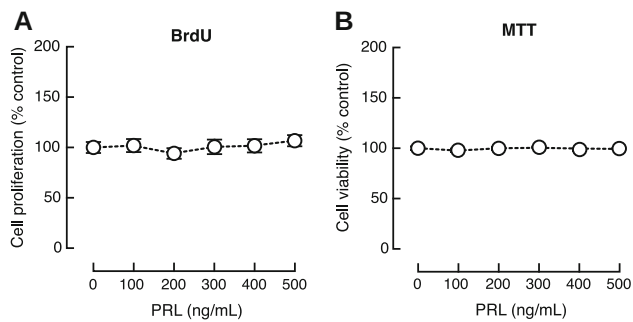


Fig. 3 **a** Proliferation and **b** viability of UMR106 cells after direct exposure for 48 h to 0 (normal medium; control), 100, 200, 300, 400, or 500 ng/ml PRL. Cell proliferation and viability in seven independent samples ($n = 7$ per group) were evaluated in triplicate by BrdU and MTT assays, respectively. The values of the control group (0 ng/ml PRL) were normalized to 100%

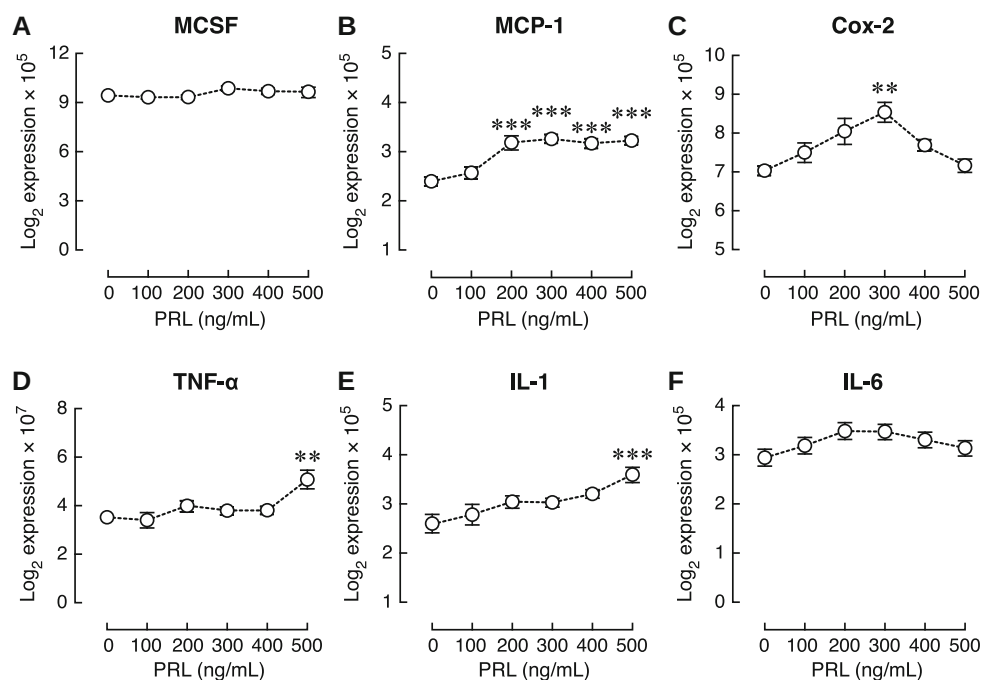
Discussion

The lactation-related skeletal changes, including rapid trabecular bone resorption and osteopenia [26, 27], were induced by the interplay between a number of hormones, such as PRL and parathyroid hormone-related peptide [1, 2, 28]. After binding to its receptors on osteoblasts, PRL was proposed to participate in the lactation-induced suppression of osteoblast functions, for example, by inhibiting alkaline phosphatase activity [4]. Moreover, PRL activated the osteoclast functions, as indicated by the increased active osteoclast surface and eroded surface in bone trabeculae [1]. Although little is known regarding the molecular mechanism of PRL action on bone resorption, its

actions on the osteoclasts could only occur through the osteoblasts because osteoclasts did not express functional PRLR [4]. In General, osteoblasts recruit and activate osteoclasts by (i) upregulating RANKL and/or downregulating OPG, a decoy receptor of RANKL [6]; (ii) enhancing the productions of osteoclast-regulating factors, such as MCSF, IL-1, IL-6, TNF- α , MCP-1, and prostaglandin E2 (via Cox-2); and (iii) mediating osteoclast maturation through Eph/ephrin interaction [6, 8–10]. Herein, the evidence, for the first time, was provided in this study that osteoblasts could respond to high physiological levels of PRL by enhancing the mRNA expression of RANKL, MCP-1, Cox-2, TNF- α , IL-1, and ephrin-B1.

Previous investigations suggested that an increase in RANKL/OPG ratio, presumably under the regulation of PRL, was responsible for the enhanced osteoclastic bone resorption in lactating rodents [5, 7]. Consistent with that reported in the calvaria of lactating mice [7], the RANKL/OPG ratio in UMR106 cells was mainly increased by an upregulation of RANKL, but not by a downregulation of OPG. However, a higher pathological PRL concentration (1,000 ng/ml) could also downregulate OPG mRNA levels in osteoblasts, and chronic hyperprolactinemia in pituitary-grafted rats led to a reduction in serum OPG levels [5], thereby further enhancing the RANKL action. These findings also agreed with a report in humans that serum OPG levels in prolonged lactation were lower than that in near-term pregnancy [29]. It was apparent that the responses of osteoblasts to PRL in term of RANKL/OPG expressions were dependent on the age of the animals from which bone cells were derived. This was partly due to the

Fig. 4 Expression of **a** macrophage colony-stimulating factor (MCSF), **b** monocyte chemoattractant protein (MCP)-1, **c** cyclooxygenase (Cox)-2, **d** tumor necrosis factor (TNF)- α , **e** interleukin (IL)-1, **f** IL-6 in UMR106 cells directly exposed for 48 h to 0 (normal medium; control), 100, 200, 300, 400, or 500 ng/ml PRL. The mRNA expression of each gene in 12 independent samples ($n = 12$ per group) was determined by qRT-PCR, and was normalized to β -actin mRNA expression. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group (0 ng/ml PRL)



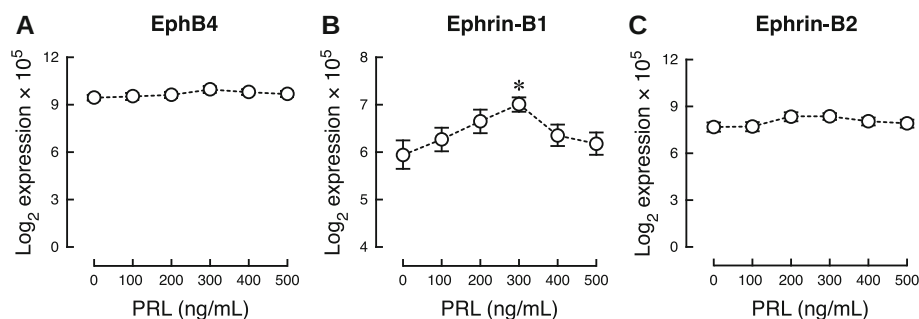


Fig. 5 Expression of **a** ephrin type-B receptor 4 (EphB4), **b** ephrin-B1, **c** ephrin-B2 in UMR106 cells directly exposed for 48 h to 0 (normal medium; control), 100, 200, 300, 400, or 500 ng/ml PRL. The mRNA expression of each gene in 12 independent samples

($n = 12$ per group) was determined by qRT-PCR, and was normalized to β -actin mRNA expression. * $P < 0.05$ compared with the control group (0 ng/ml PRL)

age-dependent effect of PRL, which induced net bone gain in young growing individuals [30], but net bone loss in adults [31]. It was previously shown that human fetal osteoblast cells responded to PRL by decreasing the RANKL/OPG ratio [32], whereas primary osteoblasts derived from adolescent rats showed no change in the RANKL/OPG ratio [33]. Since the present PRL-induced increase in RANKL/OPG ratio agreed well with the high ratio observed in lactating mice [7], the rat osteoblast-like UMR106 cell line was an appropriate model for studying PRL effects.

Although the PRL-induced increase in RANKL/OPG ratio could explain osteoclast hyperactivity and trabecular erosion in lactating animals [5], this ratio alone could not fully explain the increase in osteoclast “number” during lactation because RANKL predominantly promoted the differentiation, but not proliferation, of osteoclast precursors [34]. It was, therefore, possible that other cytokines from osteoblasts and stromal cells, such as MCSF and MCP-1, contributed to the recruitment and proliferation as well as differentiation of osteoclast precursors [6, 12, 23]. In this study, despite having no effect on MCSF mRNA expression, 200–500 ng/ml PRL markedly upregulated the mRNA expression of the chemotactic cytokine MCP-1, which might in turn increase the number of osteoclast precursors in the vicinity of the resorptive area.

Other osteoblast-derived humoral factors, including TNF- α , IL-1, and prostaglandin E2 (produced by Cox-2), have been known to potentiate osteoclastogenesis and osteoclast activity [8, 10]. The release of prostaglandin E2 from osteoblasts as a result of upregulated Cox-2 was capable of enhancing the osteoclastic bone resorption [10, 11]. TNF- α also directly stimulated pre-osteoclast differentiation in vitro [9, 35–37]. In addition, TNF- α together with IL-1 helped in maintaining MCP-1 expression, which in turn facilitated the osteoclast recruitment, differentiation, and fusion [12, 23, 38]. Incidentally, the interactions among these factors could be complicated by

the dose-specific responses of osteoblasts to PRL. For example, the upregulation of particular genes, such as TNF- α and IL-1, was observed only at a high PRL concentration of 500 ng/ml, implicating that the overexpression of TNF- α and IL-1 occurred only during suckling (when plasma PRL surge reached 500 ng/ml) to further stimulate the osteoclasts that had already been exposed to high RANKL levels induced by the baseline PRL concentration (~ 300 ng/ml) in lactating rats. Previous studies have provided corroborative evidence that the TNF- α -induced osteoclast differentiation in vitro was augmented in the presence of RANKL [37, 39], and the RANKL-primed osteoclasts did exist in ample number in maternal bone [7]. Indeed, the increased activity of osteoclasts might contribute considerably to the provision of a large quantity of calcium for milk production during suckling. It was apparent that intestinal calcium absorption and bone calcium release correlated with milk production [16, 27].

Besides inducing the mRNA expression of several osteoclastogenic factors, PRL also upregulated the expression of ephrin-B1, suggesting that PRL facilitated osteoblast-osteoclast communication through a direct cell–cell contact. Under normal conditions, a direct binding of EphB4 localized on the plasma membrane of osteoblasts to its ligand type-B ephrin (particularly ephrin-B2) on osteoclasts is known to stimulate osteoblastic bone formation, while inhibiting osteoclastic bone resorption [12, 40]. On the other hand, a direct interaction between ephrin-B1 on osteoblasts and EphB1 on monocytes or osteoclast precursors might have a role in the regulation of osteoclastogenesis [41, 42]. It was, therefore, possible that PRL enhanced bone resorption, in part, through the potentiation of EphB1/ephrin-B1 signaling, rather than the suppression of EphB4/ephrin-B2 signaling. However, the physiological significance of EphB1/ephrin-B1 interaction in bone resorption remains elusive, and further experiment is required to demonstrate an in vivo osteoblast-osteoclast contact in maternal bone.

Similar to Cox-2 mRNA expression, the PRL effect on ephrin-B1 expression was observed only in the presence of

300 ng/ml PRL, with lower or higher concentrations having no significant effects. Such biphasic and/or dose-specific responses to PRL were previously reported in several tissues and cells, e.g., small intestine, Leydig cells, mammary cells, and adrenocortical carcinoma cells [24, 25, 43, 44]. The exact explanation for this phenomenon is currently unknown but is generally explained by the increased amount of non-functional PRL–PRLR complex at high PRL concentrations. Normally, binding of one PRL molecule with two PRLRs induces dimerization of PRLRs, which in turn activates the downstream signaling cascade. At very high PRL concentrations, PRL binds to PRLR as a non-functional 1:1 PRL–PRLR complex rather than the functional 1:2 complex [45, 46], resulting in reduced downstream PRL signal transduction. The presence of different PRLR isoforms in the same cell type further added to the complexity to PRL signaling. Similar to the endothelial cells and hepatocytes [47–49], primary rat osteoblasts [5] and UMR106 cells (Fig. 1c) predominantly expressed more PRLR-S than PRLR-L, although PRL actions in several other tissues, such as intestine and mammary glands, were mainly mediated by PRLR-L [2, 45]. Little is known regarding the physiological significance of PRLR-S function, but the presence of PRLR-S was able to rescue mammapoiesis in PRLR^{+/-} mice, and induced a specific response to PRL [47, 50, 51]. It was also possible that the observed PRL actions in UMR106 cells were mediated by PRLR-S.

In addition to the PRL-induced activation of osteoclasts through osteoblasts, PRL also suppressed the osteoblastic functions, e.g., osteocalcin expression and alkaline phosphatase activity [4, 52]. Although a 48-h exposure to 100–500 ng/ml PRL had no effect on the osteoblast proliferation (Fig. 3a) or cell viability (Fig. 3b), prolonged exposure to 100 ng/ml PRL for 5–21 days did inhibit osteoblast proliferation in vitro, but did not induce apoptosis of osteoblasts [52]. However, the long-term effects of PRL on osteoblast proliferation and functions might not be of physiological significance since bone surface covered by differentiated osteoblasts was not increased in 8–14 days of lactation [1].

In conclusion, besides RANKL, we provided corroborative evidence that high physiological PRL concentrations were capable of upregulating several other osteoblast-derived osteoclastogenic mediators, namely MCP-1, Cox-2, TNF- α , IL-1, and ephrin-B1, in UMR106 cells without affecting cell proliferation and viability. These mediators have been known to recruit osteoclast precursors to the resorptive area, and/or to induce proliferation, differentiation, and activation of osteoclasts [6, 8–10, 12]. Although further investigation is required to demonstrate the PRL-induced changes in protein expression levels as well as the related physiological significance in vivo and

underlying molecular mechanisms, the findings of this study introduced, for the first time, the possible contributions of other osteoclastogenic mediators besides RANKL, which might help us explain the PRL-induced osteoclastic bone resorption in lactating mothers. Moreover, it was shown that UMR106 cell line that constitutively expressed PRLR was probably the most suitable in vitro model for studying PRL effects on osteoblasts since a high physiological PRL concentration (300 ng/ml), comparable to the plasma levels in lactation, increased the RANKL/OPG ratio by principally upregulating RANKL mRNA expression, agreeing with the pattern observed in lactating mice [7].

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Conflict of interest None.

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REVISED MANUSCRIPT

Parathyroid hormone (PTH) rapidly enhances CFTR-mediated HCO_3^-
secretion in intestinal epithelium-like Caco-2 monolayer:
A novel ion regulatory action of PTH

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Keywords: impedance analysis; phosphoinositide 3-kinase (PI3K); protein kinase A (PKA);
small interfering RNA; Ussing chamber

Abstract

Besides being a Ca^{2+} -regulating hormone, parathyroid hormone (PTH) has also been shown to regulate epithelial transport of certain ions, such as Cl^- , HCO_3^- and Na^+ , particularly in the kidney. Although the intestinal epithelium also expressed PTH receptors, little was known regarding its mechanism in the regulation of intestinal ion transport. We investigated the ion regulatory role of PTH in intestinal epithelium-like Caco-2 monolayer by Ussing chamber technique and alternating current impedance spectroscopy. It was found that Caco-2 cells rapidly responded to PTH within 1 min by increasing apical HCO_3^- secretion. CFTR served as the principal route for PTH-stimulated apical HCO_3^- efflux, which was abolished by various CFTR inhibitors, namely NPPB, GlyH-101 and CFTRinh-172, as well as by siRNA against CFTR. Concurrently, the plasma membrane resistance was decreased with no changes in the plasma membrane capacitance or paracellular permeability. HCO_3^- was probably supplied by basolateral uptake via the electrogenic $\text{Na}^+/\text{HCO}_3^-$ co-transporter and by methazolamide-sensitive carbonic anhydrase, while the resulting intracellular H^+ might be extruded by both apical and basolateral Na^+/H^+ exchangers. Furthermore, the PTH-stimulated HCO_3^- secretion was markedly reduced by protein kinase A (PKA) inhibitor (PKI 14–22 amide) and phosphoinositide 3-kinase (PI3K) inhibitors (wortmannin and LY-294002), but not by intracellular Ca^{2+} chelator (BAPTA-AM) or protein kinase C inhibitor (GF-109203X). In conclusion, the present study provided evidence that PTH directly and rapidly stimulated apical HCO_3^- secretion through CFTR in PKA- and PI3K-dependent manner, which was a novel non-calcitropic, ion regulatory action of PTH in the intestinal epithelium.

Keywords: impedance analysis; phosphoinositide 3-kinase (PI3K); protein kinase A (PKA); small interfering RNA; Ussing chamber

Introduction

Besides being widely known for its hypercalcemic and hypophosphatemic actions, parathyroid hormone (PTH) has been demonstrated to regulate transepithelial transport of various ions, such as Na^+ , Cl^- and HCO_3^- , especially in the gastric mucosa and renal proximal tubule (7, 16, 18, 33). Specifically, in the amphibian gastric epithelium, PTH was found to inhibit HCO_3^- secretion, thereby leading to acidification of the gastric lumen and, in turn, dissolution of Ca^{2+} from insoluble complexes, perhaps for better Ca^{2+} absorption in the small intestine (18). Moreover, PTH inhibited the tubular Na^+ and HCO_3^- reabsorption in the renal proximal tubule (6, 7, 16), which functioned as part of body Na^+ and acid-base balance, respectively. Despite a number of studies pertaining to ion regulatory action of PTH on the kidney, virtually nothing is currently known regarding this PTH effect on intestinal epithelium, which is another important regulatory site for ion homeostasis.

PTH usually accomplished its ion regulatory action through a direct modulation of relevant ion transporters. For example, evidence from renal epithelial cells suggested that PTH regulated Na^+ and acid-base balance, in part, by inhibiting Na^+/H^+ exchanger (NHE)-3 via cAMP-dependent protein kinase A (PKA) and/or protein kinase C (PKC) signaling pathways (3). PTH might also be capable of controlling cellular volume as it could directly stimulate Cl^- efflux through the outward rectifying apical Cl^- channels in cultured rabbit renal proximal tubular cells (53). Since intestinal epithelial cells are equipped with a number of cAMP-dependent transporters, such as cystic fibrosis transmembrane conductance regulator (CFTR) and NHE3 in the apical membrane, and NHE1 and electrogenic $\text{Na}^+/\text{HCO}_3^-$ co-transporter-1 [NBCe1; also known as solute carrier (SLC) 4A4] (4, 62) in the basolateral membrane, as well as PTH

84 signaling machinery, including PTH receptor (PTHR) isoform-1 (PTHR1) (37), the direct effect
85 of PTH on intestinal ion transport was anticipated.

86 CFTR in the apical membrane of intestinal epithelial cells provided an important route
87 for Cl^- secretion into the intestinal lumen along the entire length of intestine in a cAMP-, cGMP-
88 and/or intracellular Ca^{2+} -dependent manner (21, 55). In addition to Cl^- , the effluxes of other
89 anions, including HCO_3^- , could also be carried out by CFTR, as observed in the pancreatic duct
90 (52) and mouse duodenum (57). Under normal conditions, the apical CFTR activity was minimal
91 since most channels were closed and some CFTR proteins remained in subapical vesicles (2, 21).
92 Several cAMP-producing mediators, such as vasoactive intestinal peptide (VIP) and forskolin,
93 not only induced CFTR opening but also stimulated recruitment of vesicular CFTR to the apical
94 membrane (2). Since PTH has been reported to elevate intracellular cAMP levels (35), it was
95 possible that PTH induced cAMP-dependent CFTR activation, thereby stimulating anion
96 secretion in intestinal epithelial cells. By using alternating current (AC) impedance spectroscopy,
97 one could demonstrate whether PTH induced CFTR opening and/or fusion of CFTR-rich vesicles
98 to the apical membrane, as these processes usually affect the resistive and capacitive properties
99 of the plasma membrane, respectively (12, 40, 44, 50).

100 In the intestinal epithelium and Caco-2 monolayer, there are a number of other
101 transporters involved in the CFTR-mediated Cl^- and HCO_3^- secretion. The basolateral influx of
102 Cl^- occurs through $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC)-1, whereas that of HCO_3^- may be
103 achieved either by the cytoplasmic carbonic anhydrase-mediated production from CO_2 (36) or
104 the basolateral uptake via NBCe1 (32). NHE was also essential for the extrusion of H^+ produced
105 by carbonic anhydrase (5, 52). The effects of PTH on these intestinal proteins were elusive, but if

it did have action on intestinal anion transport, these transporters were plausibly used in the process.

Therefore, the present study aimed to (i) investigate whether PTH directly regulate anion secretion in intestinal epithelial cells by using Ussing chamber system equipped with direct current (DC) circuitry; (ii) to determine ion species (e.g., Cl^- or HCO_3^-) and relevant ion transporters regulated by PTH; (iii) to monitor changes in the resistive and capacitive properties of the plasma membrane after PTH stimulation by using AC impedance spectroscopy; and (iv) to investigate the responsible signaling pathways of PTH for anion transport. In this study, the colorectal adenocarcinoma-derived Caco-2 cell monolayer was used because it possessed the apical brush border, polarization and anion transport functions resembling the native epithelium from the small intestine (25, 34). Moreover, the monolayer was devoid of the underlying connective tissue and smooth muscle layers, both of which might hinder measurements of transepithelial ion current and plasma membrane impedance (61).

Materials and methods

Cell culture

Caco-2 cells [American Type Culture Collection (ATCC) no. HTB-37] were propagated in 75 cm^2 T-flask (Corning, NY, USA) containing Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 15% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% nonessential amino acid (Sigma), 1% L-glutamine (Gibco), and 100 U/mL penicillin-streptomycin (Gibco) under humidified 5% CO_2 atmosphere at 37°C. Cells were subcultured as recommended by ATCC. In electrophysiological studies and impedance analysis, Caco-2

monolayers were prepared by plating cells at 5×10^5 cells/well on polyester Snapwells (12-mm diameter and 0.4- μ m pore size; Corning). In real-time PCR study, Caco-2 cells were cultured at 5×10^5 cells/well in 6-well polystyrene plates (Corning). Culture medium was changed daily, and Snapwells or 6-well plates were maintained at 37°C for 14 days in an incubator with humidified 5% CO₂.

Experimental design

Caco-2 cells were first harvested for PTHR mRNA expression study by quantitative real-time PCR. To investigate whether PTH directly regulated anion transport, Caco-2 monolayers were exposed on the apical and/or basolateral sides to various concentrations of PTH 1–34 [active form of PTH; purity >95% as assayed by high-performance liquid chromatography (HPLC); catalog no. P3796; Sigma], 10 nmol/L PTH 13–34 (inactive PTH; purity >95% as assayed by HPLC; catalog no. P2780; Sigma), and 10 μ mol/L forskolin (catalog no. F6886; Sigma). Forskolin is an adenylyl cyclase activator which served as a positive control for anion secretion through CFTR (57). In some experiments, Caco-2 monolayers were treated on the basolateral side with 10 nmol/L heat-inactivated PTH 1–34 (heated at 121°C for 40 min), or 10 nmol/L PTH 1–34 plus various concentrations of polyclonal antibody against PTH 1–34 (catalog no. ab14164; Abcam, Cambridge, UK). The PTH-stimulated anion transport was represented by the magnitudes of changes in the transepithelial potential difference (V_t) and equivalent short-circuit current (I_{sc}). AC impedance spectroscopy was used to determine ohmic resistance and capacitance of the plasma membrane during PTH 1–34 and forskolin stimulation, whereas paracellular permeability was determined by unidirectional ³H-mannitol flux study. Furthermore, Caco-2 monolayers were bathed on both sides with Cl[−]-depleted and/or HCO₃[−]-depleted solution

in order to identify the responsible anion species. pH of bathing solution was monitored by pH-sensing electrode connected to PowerLab/4SP (ADInstruments, Colorado Springs, CO, USA). In the subsequent series of experiments, the monolayers were independently exposed to a number of transporter inhibitors (Table 1) prior to PTH 1–34 treatments to identify the PTH-regulated transporters. Involvement of CFTR was also confirmed by using CFTR knockdown Caco-2 monolayers. Finally, the responsible signaling pathways were identified by pre-treating Caco-2 monolayers on both apical and basolateral sides with inhibitors of known PTH signaling pathways, i.e., cAMP-dependent PKA (10 μ mol/L myristoylated PKI 14–22 amide; Calbiochem, San Diego, CA, USA), intracellular Ca^{2+} [50 μ mol/L 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl ester); BAPTA-AM; Calbiochem], Ca^{2+} -dependent PKC (1 μ mol/L GF-109203X; A.G. Scientific, San Diego, CA, USA), and PI3K (200 nmol/L wortmannin and 75 μ mol/L LY-294002; Tocris Bioscience, Bristol, UK). The signaling pathway inhibitors were of the optimal concentrations as reported previously (10, 28, 31, 65).

Small interfering RNA (siRNA) transfection

Three pairs of siRNA oligonucleotides targeted for CFTR (siCFTR1, 5'–GAAAGCUGUGUCUGUAAAC–3' and 5'–AAGUUUACAGACACAGCUUUC–3'; siCFTR2, 5'–GACUUUAGCCAUGAAUAUC–3' and 5'–AAGAUUAUCAUGGCUAAAGUC–3'; and siCFTR3, 5'–GUGAAAGACUUGUGAUUAC–3' and 5'–AAGUAAUCACAAGUCUUUCAC–3') were designed by siRNA Target Designer 1.51 and synthesized by T7 RiboMax Express RNAi System (Promega, Madison, WI, USA). Caco-2 cells were first seeded on a Snapwell at 5×10^5 cells/cm². Regarding in vitro transfection, 10 μ g/mL polyethyleneimine (PEI) and siCFTR1–3 (1 μ mol/mL each) were simultaneously added into culture medium on day 12 after

seeding, as described previously (10). On day 14 (i.e., 48 h after in vitro transfection), siRNA-treated monolayers were used for the experiments. Efficiency of siRNA was evaluated by quantitative real-time PCR.

Quantitative real-time PCR

The PCR technique was modified from the method of Charoenphandhu et al. (10). In brief, total RNA was extracted from Caco-2 cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. The RNA samples were treated with RQ1 DNase (Promega) and later purified with RNeasy Mini kit (Qiagen, Valencia, CA, USA). One microgram of total RNA was reverse-transcribed with oligo-dT₂₀ primer and iScript kit (Bio-rad, Hercules, CA, USA). Primers used in the present study are listed in Table 2. Real-time PCR was performed by Bio-rad MiniOpticon PCR system with iQ SYBR Green SuperMix (Bio-rad) to quantify the mRNA expression of PTHR1, PTHR2 and CFTR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a housekeeping gene for normalization. Relative mRNA expression was calculated from the threshold cycle (C_T) by $2^{-\Delta C_T}$ method.

Bathing solution

Normal bathing solution contained (in mmol/L) 118 NaCl, 4.7 KCl, 1.1 MgSO₄, 1.25 CaCl₂, 23 NaHCO₃, 12 D-glucose, 2.5 L-glutamine, and 2 mannitol (all purchased from Sigma). The solution (37°C) was continuously aerated with humidified 5% CO₂ in 95% O₂ to maintain a constant partial pressure of CO₂ (pCO₂) and pH 7.4. Osmolality was 290–295 mmol/kg H₂O as measured by an osmometer (model 3320; Advanced Instruments, Norwood, MA, USA). Distilled water used in this work had electrical resistance higher than 18.3 MΩ cm and free-

ionized Ca^{2+} less than 2.5 nmol/L. In Cl^- -depleted experiments, Cl^- in the solution was replaced by gluconate, and Ca^{2+} concentration was increased to 2.5 mmol/L to compensate for the Ca^{2+} -buffering capacity of gluconate (15). Under HCO_3^- -depleted conditions, HCO_3^- in the solution was replaced by N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonate (HEPES; Sigma), and Na^+ concentration was adjusted by sodium gluconate. HEPES-containing solution was aerated by humidified 100% O_2 .

Measurement of electrical parameters in direct current (DC) mode

The DC electrical parameters were mainly for representing the epithelial electrogenic ion transport during PTH exposure. I_{sc} was used as a fair measure of the active ion flux across the monolayer. A Snapwell with Caco-2 monolayer was mounted in Ussing chamber as described previously (56). Two pairs of salt bridges made of 2 mol/L KCl and 2% wt/vol agar connected Ag/AgCl half-cells (World Precision Instrument, Sarasota, FL, USA) with the chamber, and served as electrodes for measuring V_t or passing electrical current. Each V_t -sensing electrode was placed near the Snapwell, whereas the current-passing electrode was placed at the end of each hemi-chamber. Each Ag/AgCl half-cell was connected to a preamplifier/DC current-generating unit (model ECV-4000; World Precision Instrument), and finally to PowerLab/4SP (operated with Chart version 5.4.1; ADInstruments) for digital recording. During the experiment, square pulse current (I ; 3 μA magnitude, 800 ms current duration, and 6 pulses/min) was applied across the monolayer and the corresponding voltage change (ΔV) was recorded (low-pass filter 1 Hz; sampling frequency 200 s^{-1}). Transepithelial resistance (R_t) and I_{sc} were calculated by Equations 1 and 2, respectively.

$$R_t = \Delta V / I \quad (\text{Eq. 1})$$

$$I_{sc} = V_t / R_t \quad (\text{Eq. 2})$$

AC impedance spectroscopy and analysis

AC impedance analysis was to determine a decrease in plasma membrane resistance due to activated ion channels and transporters, and/or changes in membrane capacitance after fusion of CFTR-rich vesicles with the apical membrane. A Snapwell with 14-day-old Caco-2 monolayer was mounted in Ussing chamber. Two pairs of Ag/AgCl half-cells were connected to the chamber as described in the previous section. Impedance spectroscopy was performed by using 1255B frequency response analyzer and 1287A electrochemical interface (Solartron, Farnborough, Hampshire, UK). Sine-wave alternating current ($35 \mu\text{A}/\text{cm}^2$ root mean square amplitude, 0.5 Hz – 10 kHz) was applied across Caco-2 monolayer at different time points, i.e., before adding 10 nmol/L PTH 1–34 or 10 $\mu\text{mol}/\text{L}$ forskolin, and at 1, 5, 10, 15, 20, 25, 30, 35 and 40 min after adding PTH 1–34 or forskolin. The Nyquist plot [real part of impedance (Z_{re}) vs. imaginary part of impedance (Z_{im})], Bode magnitude plot [frequency vs. impedance magnitude ($|Z|$)] and Bode phase plot [frequency vs. phase angle (θ)] were graphed and fitted with nonlinear least squares fitting algorithm by Zplot/Zview software package 3.0 (Scribner Associates, Southern Pines, NC, USA). The equivalent circuit of Caco-2 monolayer (Figure 1A) was automatically analyzed by Zplot/Zview. This circuit consisted of 4 ohmic resistors and 2 capacitors, i.e., 2 parallel RC components which represented the resistance (R_a and R_b) and capacitance (C_a and C_b) of the apical and basolateral plasma membranes, paracellular resistor (R_p), and series resistor (R_s) which represented the resistance of bathing solution and Snapwell. R_a , R_b , C_a and C_b were important parameters obtained from Zplot/Zview.

Determination of R_p or paracellular conductance (G_p ; $G_p = 1/R_p$) was required prior to analyses of R_a , R_b , C_a and C_b since R_p was an initial parameter essential for curve fitting by Zplot/Zview (40). According to the method of Wills et al. (60), G_p was the y-intercept in a linear plot between transepithelial conductance (G_t ; $G_t = 1/R_t$) and I_{sc} , as followed.

$$G_t = I_{sc}/E_b + G_p \quad (\text{Eq. 3})$$

where E_b is electromotive force of the basolateral membrane. G_t and I_{sc} values were obtained during PTH 1–34 or forskolin stimulation (50). Since R_a , R_b , C_a and C_b from impedance analysis were considered valid only when R_p was not changed by the PTH- or forskolin-enhanced transcellular ion transport, the constant R_p was verified by the nystatin-induced membrane permeabilization method (60). In brief, the monolayer was first incubated with or without 10 nmol/L PTH 1–34 for 10 min. Once I_{sc} appeared steady, 10 $\mu\text{g/mL}$ nystatin (Calbiochem) was added into the apical solution every 5 min for 6 times, while I_{sc} and G_t were determined every 2.5 min. Generally, nystatin gradually increased the permeability of the apical membrane to small monovalent ions, thereby eliminating the electrical resistance and electromotive force of the apical membrane. The comparable R_p values of control and PTH-treated monolayers [269.04 ± 15.48 ($n = 4$) vs. 268.66 ± 11.43 ($n = 4$) $\Omega \text{ cm}^2$; $P = 0.492$] suggested that an increase in the transcellular ion movement and a decrease in the apical membrane resistance had no effect on R_p . Therefore, R_p could be used as a constant for impedance analysis.

Unidirectional mannitol flux measurement

Mannitol transport across Caco-2 monolayer was determined as described previously (56). After 15-min incubation, the apical side of Ussing chamber was filled with fresh bathing solution containing ^3H -mannitol (final specific activity of ~ 70 mCi/mol; Amersham, Buckinghamshire, UK). Radioactivity of ^3H -mannitol was measured by liquid scintillation spectrophotometer (model Tri-Carb 3100 TR; Perkin-Elmer, CT, USA). Unidirectional flux ($J_{\text{H} \rightarrow \text{C}}$; $\text{nmol h}^{-1} \text{ cm}^{-2}$) of mannitol from the hot side (H) to the cold side (C) was calculated by Equations 4 and 5.

$$J_{\text{H} \rightarrow \text{C}} = R_{\text{H} \rightarrow \text{C}} / (S_{\text{H}} \times A) \quad (\text{Eq. 4})$$

$$S_{\text{H}} = C_{\text{H}} / C_{\text{M}} \quad (\text{Eq. 5})$$

where $R_{\text{H} \rightarrow \text{C}}$ is the rate of tracer appearance in the cold side (cpm/h); S_{H} is the specific activity in the hot side (cpm/nmol); A is the surface area of Snapwell (cm^2); C_{H} is the mean radioactivity in the hot side (cpm); and C_{M} is the total mannitol content in the hot side (nmol).

Statistical analysis

Results are expressed as means $\pm$ SE. Unless otherwise specified, two sets of data were analyzed by Student's t -tests, whereas multiple group comparisons were performed by one-way analysis of variance (ANOVA) followed by Dunnett's post-test. Levels of mRNA expression were compared by nonparametric Mann-Whitney test. The dose-response relationship of PTH was fitted by nonlinear least squares method with four-parameter logistic equation (41). The differences were considered significant when P -values were less than 0.05. All statistical tests were performed by GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Results

PTH 1–34 directly enhanced electrogenic ion transport in a dose-dependent manner.

Prior to investigation of PTH 1–34 effects on intestinal epithelium-like Caco-2 monolayer, expression of PTHR was first carried out in Caco-2 cells. As shown in Figure 1B, Caco-2 cells expressed mRNAs of both PTHR1 and PTHR2, the latter of which was less abundant by ~3,000-fold. Direct exposure to PTH 1–34 on the basolateral side transiently increased the negative potential on the apical side (peak response), followed by a sustained negative-potential plateau until PTH 1–34 was washed out (Figure 2A). The V_t peak response occurred very rapidly within 67.50 ± 2.07 s ($n = 20$), and underwent depolarization with a time constant of 111.70 s (95% confidence interval 98.38–129.30 s; $n = 20$). The peak interval thus lasted for 347.00 ± 4.18 s ($n = 20$). The peak I_{sc} changes (i.e., ΔI_{sc}) were calculated at the V_t peak responses, and were later used to represent electrogenic ion transport across the monolayer. Since PTH 1–34 is known to exert its actions through cAMP-dependent PKA signaling pathway (35), an adenylyl cyclase activator forskolin was applied to the basolateral side. Similar to PTH, forskolin rapidly induced the V_t peak response within 80.00 ± 6.71 s ($n = 6$), followed by a sustained negative-potential plateau (Figure 2B).

Further investigations revealed that PTH 1–34 regulated ion transport when it was applied on the basolateral side, but not the apical side (Figure 2C). A dose-response study demonstrated a dose-dependent stimulatory effect of PTH 1–34 on ΔI_{sc} with a half maximal effective concentration (EC_{50}) of ~3.2 nmol/L (Figure 2D; $\text{Log } EC_{50} = -8.50 \pm 0.05$ and Hill coefficient = 3.76). Therefore, in the present study, we used PTH 1–34 concentration of 10 nmol/L (~3 times the EC_{50}), which appeared to have comparable potency with 10 $\mu\text{mol/L}$

forskolin (Figure 2E). On the other hand, Caco-2 monolayers which were directly exposed to truncated PTH 13–34, heat-inactivated PTH 1–34, or PTH 1–34 plus 1:1,000–1:10 PTH antibody showed a decrease in ΔI_{sc} (Figure 2E), suggesting that the observed Caco-2 cell responses were specific to the actions of PTH 1–34. Taken together, our results indicated that PTH 1–34 directly and rapidly stimulated electrogenic ion transport in Caco-2 monolayer in a dose-dependent manner.

The PTH 1–34 decreased the apical and basolateral membrane resistance.

The AC impedance analysis further revealed that PTH 1–34 and forskolin markedly altered the transcellular impedance, which was the sum of impedance of the apical and basolateral plasma membranes of Caco-2 cells. Representative Nyquist plots, Bode magnitude plots and Bode phase plots at the peak response in PTH 1–34- or forskolin-treated Caco-2 monolayer (i.e., 1 min after adding PTH 1–34 or forskolin) were depicted in Figure 3. The obtained parameters (i.e., Z_{re} , Z_{im} , $|Z|$ and θ) were automatically used by Zplot/Zview software for the calculations of R_a , R_b , C_a and C_b , based on the epithelial equivalent circuit in Figure 1A. Under control conditions (i.e., without PTH 1–34), R_a of Caco-2 cells was $2,141.30 \pm 222.50 \Omega \text{ cm}^2$ ($n = 17$), and the fractional electrical resistance of the apical membrane [R_f ; $R_f = R_a/(R_a+R_b)$] was 0.67 ± 0.01 ($n = 17$), consistent with the previous report in Caco-2 cells using microelectrode recording (26). The basal C_a and C_b in our Caco-2 cells were 28.98 ± 0.69 and $12.36 \pm 0.23 \mu\text{F}/\text{cm}^2$ ($n = 17$) respectively, similar to that reported in mammalian colonic cells using impedance analysis (59). The higher C_a value compared to C_b value may result from the presence of apical brush border in the polarized Caco-2 cells.

As shown in Figure 4, PTH 1–34 transiently decreased the apical membrane resistance during the first 15 min, whereas forskolin exposure resulted in a sustained decrease in the apical resistance until the end of the 40-min experiment. Both PTH 1–34 and forskolin rapidly decreased the basolateral membrane resistance throughout the 40-min exposure. On the other hand, neither PTH 1–34 nor forskolin affected the apical and basolateral membrane capacitance of Caco-2 cells. The impedance results, therefore, suggested that PTH 1–34 enhanced ion movement across both apical and basolateral membranes (i.e., transcellular ion transport), presumably via ion channels and/or transporters, thereby leading to a decrease in plasma membrane resistance.

In addition, an electrophysiological study in DC mode at 10 min after PTH 1–34 exposure demonstrated no change in R_t (Figure 5A), which is predominantly determined by the tight junction resistance when the cellular resistance is relatively high (27). The paracellular resistance R_p calculated from the method of Wills et al. (60) was similar to the R_t results (Figure 5B). No change in the transepithelial mannitol transport (using ^3H -mannitol as a paracellular marker; Figure 5C) also confirmed that PTH 1–34 had no effect on the epithelial paracellular pathway.

Apical CFTR mediated HCO_3^- efflux in response to PTH 1–34.

Furthermore, Caco-2 monolayers were directly exposed to various inhibitors to demonstrate the ion channels/transporters responsible for the PTH 1–34-enhanced anion transport. It was apparent that apical exposure to non-selective Cl^- channel inhibitor NPPB or selective CFTR inhibitor CFTRinh-172 manifested a decrease in ΔIsc in a dose-dependent manner (Figure 6A). Another CFTR inhibitor GlyH-101 and CFTR siRNA, the latter of which diminished CFTR

mRNA expression by ~80-fold (Figure 6B), showed similar inhibition (Figures 6A and 6C–D). However, DIDS which is capable of blocking apical Ca^{2+} -activated Cl^- channels and $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers did not affect ΔIsc induced by PTH 1–34 (Figure 6A). A subsequent series of experiments using Cl^- - and/or HCO_3^- -depleted bathing solution revealed that it was HCO_3^- , and not Cl^- , which was responsible for ΔIsc during PTH 1–34 stimulation since ΔIsc was diminished by >80% only under HCO_3^- -depleted conditions (Figure 6E). Furthermore, decreases in Cl^- concentrations in both sides by gluconate replacement did not affect the transepithelial potential difference V_t (Supplemental Figure S1). No change in ΔIsc after basolateral exposure to NKCC1 inhibitor, bumetanide, confirmed that Cl^- was not involved in the PTH 1–34-enhanced anion transport (Figure 7A). K^+ also did not contribute to the observed ΔIsc because the PTH 1–34 responses were not abolished by the inhibitors of Ca^{2+} - and cAMP-activated K^+ channels (i.e., chromanol 293B and charybdotoxin, respectively) (Figure 7B). Although apical HCO_3^- efflux induced by PTH 1–34 was substantial, it was still unable to alter the pH of the well buffered apical or basolateral solution (Supplemental Figure S2).

The basolateral NBCe1 and cytoplasmic carbonic anhydrase contributed to the PTH 1–34-enhanced HCO_3^- transport.

In most epithelia, the sources of HCO_3^- for apical efflux were derived from the basolateral NBCe1-mediated HCO_3^- uptake and carbonic anhydrase-mediated intracellular HCO_3^- production (32, 36). As shown in Figure 7A, the PTH 1–34-enhanced HCO_3^- transport was abolished by DIDS and a cardiac glycoside ouabain known to inhibit the basolateral NBCe1 and Na^+/K^+ -ATPase, respectively. Interestingly, ΔIsc was also decreased by a carbonic anhydrase

inhibitor, methazolamide (Figure 7A), suggesting that intracellularly produced HCO_3^- partly contributed to the PTH 1–34-enhanced HCO_3^- transport.

Since the carbonic anhydrase activity is known to be dependent on NHE function, which helps cells to extrude intracellular H^+ produced by carbonic anhydrase (5, 52), NHE inhibitors, high-dose amiloride (100 and 1,000 $\mu\text{mol/L}$) and EIPA, were applied to the solution to inhibit apical NHE3 or basolateral NHE1 activities. We found that both high-dose amiloride and EIPA added either to the apical or basolateral solution significantly decreased ΔIsc induced by PTH 1–34 (Figure 7C). However, low-dose amiloride (10 $\mu\text{mol/L}$), which inhibited only epithelial sodium channel (ENaC), and not NHE, had no effect on the PTH 1–34 responses (Figure 7C).

The PTH 1–34-enhanced HCO_3^- transport was mediated by PKA and PI3K signaling pathways.

Although PTH is known to exert its actions mainly through the cAMP-dependent PKA pathway, other signaling pathways, namely Ca^{2+} -dependent PKC and PI3K, have been reported to mediate PTH actions in the renal proximal tubular epithelium (1, 16, 38) and intestinal epithelium (23), respectively. In the present study, Caco-2 monolayers were exposed on both sides to inhibitors of cAMP/PKA, Ca^{2+} /PKC or PI3K pathways prior to PTH 1–34 stimulation. It was found that only PKA inhibitor (myristoylated PKI 14–22 amide) and PI3K inhibitors (wortmannin and LY-294002), but not intracellular Ca^{2+} chelator (BAPTA-AM) or panspecific PKC inhibitor (GF-109203X), significantly diminished ΔIsc induced by PTH 1–34 (Figure 8). Thus, PTH 1–34 probably enhanced the electrogenic HCO_3^- transport in Caco-2 monolayer via PKA and PI3K signaling pathways.

Discussion

As an ancient peptide hormone first evolved in arthropods and mollusks (29), PTH has a plethora of regulatory actions pertaining to neural modulation, ion and water transport, extracellular and intracellular pH balance, cellular volume regulation, control of skeletal development, and Ca^{2+} /phosphate homeostasis (16–18, 29, 39, 53, 54). In mammals, the direct PTH actions, prominently confined to the kidney and bone, include inhibition of proximal tubular HCO_3^- and phosphate reabsorption, stimulation of distal tubular Ca^{2+} reabsorption, and enhancement of bone resorption (6, 54). However, direct effect of PTH on the intestine remained elusive, and whether PTH could exert an ion regulatory action in the intestinal epithelium was not known. Herein, we provided corroborative evidence, for the first time, that PTH did exert a direct ion regulatory effect on the intestinal epithelium-like Caco-2 monolayer by enhancing apical HCO_3^- efflux through CFTR via PKA and PI3K signaling pathways. The PTH-enhanced transcellular HCO_3^- transport peaked within 1 min concurrently with a decrease in the plasma membrane resistance.

The direct effect of PTH on the intestinal epithelial cells has long been anticipated since the reports of PTHR expression in rat small intestine and chick duodenum (37, 42); however, its exact ion regulatory action in mammalian intestine had never been demonstrated experimentally. Nemere and Norman previously showed that vascular perfusion of isolated chick duodenum with PTH 1–34 led to an enhancement of ^{45}Ca transport within 12 min (43). Although the finding in chick duodenum was consistent with the calciotropic role of PTH, the PTH-enhanced rapid Ca^{2+} absorption was not observed in Caco-2 monolayer (Laohapitakworn et al., unpublished observation). Generally, in mammals including humans and rodents, PTH enhances the intestinal Ca^{2+} absorption indirectly by stimulating renal synthesis of $1,25(\text{OH})_2\text{D}_3$ (54). Since PTH increased the intracellular cAMP levels in most target cells (35), and the cAMP-regulated ion

transporters (e.g., CFTR, NHE1, NHE3, NBCe1 and Na^+/K^+ -ATPase) were abundant in mammalian intestinal cells (30, 51, 64), it was possible that PTH could directly activate these transporters, leading to ion transport across mammalian intestinal epithelium.

In the present study, direct exposure to PTH on the basolateral side resulted in a rapid hyperpolarization of the epithelial monolayer (i.e., the luminal side became more negative), similar to that observed with forskolin which was known to induce apical anion efflux (Figure 2). The increase in ΔI_{sc} induced by PTH 1–34 was highly correlated with decreases in the apical and basolateral membrane resistance (Figure 4). Various modulators, such as forskolin and prostaglandin E_2 , that increased intracellular cAMP levels were previously shown to similarly reduce the resistance of both apical and basolateral membranes (40, 44, 50). This phenomenon was usually explained by the increased opening of ion channels or increased activities of transcellular ion transporters (40). Although some cAMP-dependent mediators, such as prostaglandin E_2 , were capable of altering the paracellular permeability (47), the absence of changes in R_p and transepithelial mannitol transport suggested that paracellular pathway was not involved. If paracellular ion transport was increased by PTH, the charge- and/or size-selective properties of the tight junction would have been changed (58), thereby leading to a decrease in R_p and/or an increase in mannitol flux. The present electrophysiological study and impedance analysis thus indicated that PTH directly stimulated anion transport only via the transcellular pathway.

By using a Cl^- -depleted solution, we further showed that the PTH-induced electrical responses in Caco-2 monolayer were due to electrogenic transport of HCO_3^- , but not Cl^- . Since ion flux (J ; $\text{pmol s}^{-1} \text{ cm}^{-2}$) caused by the electrogenic monovalent ion transport was roughly estimated from the magnitude of an electrical current change (i ; $\mu\text{A}/\text{cm}^2$) and Faraday constant

(F ; $F = 96485.34 \text{ A}\cdot\text{s mol}^{-1}$) as $J = i \times 10^6/F$, and ΔIsc was decreased by $\sim 13 \text{ }\mu\text{A}/\text{cm}^2$ under the HCO_3^- -depleted condition (Figure 6E), the calculated HCO_3^- flux at the peak PTH response should be as high as $\sim 13 \times 10^6/96485.34 \approx 135 \text{ pmol s}^{-1} \text{ cm}^{-2}$. Indeed, the cAMP-activated HCO_3^- transport was not uncommon. In the rat colon, forskolin was found to stimulate HCO_3^- secretion in a bumetanide-insensitive fashion (48). Although an increase in the luminal negative potential could also be explained by an activation of ENaC-mediated apical Na^+ uptake or basolateral K^+ efflux, the findings that ENaC inhibitor (low-dose amiloride), and Ca^{2+} - and cAMP-activated K^+ channel inhibitors (chromanol 293B and charybdotoxin, respectively) had no effect on ΔIsc suggested no significant contribution from the electrogenic Na^+ and K^+ transport through these channels. However, the presence of residual ΔIsc after exposure to high-dose NPPB (200 $\mu\text{mol/L}$) or high-dose CFTRinh-172 (20 $\mu\text{mol/L}$) indicated a minor contribution of unidentified electrogenic transporters in the PTH-treated Caco-2 cells.

Further investigation using various inhibitors of anion channels or transporters strongly indicated that CFTR was the principal anion channel for the apical HCO_3^- efflux. CFTR contribution was also confirmed by the diminished ΔIsc in PTH-treated CFTR knockdown Caco-2 monolayer. Generally, CFTR—as a non-rectifying anion channel—transports a wide variety of anion species, including Cl^- , HCO_3^- , Br^- and I^- (49). Since CFTR opening usually increases the apical membrane permeability to Cl^- , PTH might first alter the dynamic CFTR selectivity toward HCO_3^- , perhaps by the mechanism previously reported by Reddy and Quinton (46), to obtain such a high HCO_3^- conductance. Contributions of other apical anion channels or transporters, such as Ca^{2+} -activated Cl^- channels and $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers were unlikely as the PTH-induced electrical response was insensitive to DIDS in the apical solution. Lavery and co-workers reported a stimulatory effect of PTH on CFTR activity in chick renal proximal tubule

(33). Nevertheless, an increase in HCO_3^- transport could be due to increases in channel opening and/or CFTR number in the apical membrane. Some cAMP-producing secretagogues, e.g., VIP, cholera toxin and forskolin, are known to promote recruitment of vesicular CFTR to the apical membrane (2). Fusion of the intracellular vesicles to the apical membrane usually leads to an increase in the apical membrane area, thus culminating in the proportional change in membrane capacitance (12). Therefore, the unaltered apical membrane capacitance C_a in this study suggested no recruitment of CFTR to the apical membrane, and the augmented CFTR function was probably due to the PTH-induced increase in channel opening.

The present results further suggested that HCO_3^- came from intracellular production from CO_2 by methazolamide-sensitive carbonic anhydrase and basolateral uptake by DIDS-sensitive NBCe1 (Figure 7). Besides providing the driving force for NBCe1 activity, Na^+/K^+ -ATPase might also help maintain the intracellular hyperpolarized potential for apical HCO_3^- efflux, as well as cell volume during a rapid HCO_3^- production (63). During the process of HCO_3^- production, excess H^+ was extruded from the cells by the basolateral NHE1 and apical NHE3 (52). A marked inhibition of the PTH-stimulated HCO_3^- secretion after high-dose amiloride and EIPA treatment implicated that NHE was imperative for the carbonic anhydrase-mediated HCO_3^- production. In fact, both HCO_3^- -producing mechanisms demonstrated in this study are ubiquitously present in other cAMP-dependent HCO_3^- -secreting epithelial cells, such as pancreatic ductal cells and cholangiocytes (5, 52).

Regarding the signal transduction, PTH appeared to exert its ion regulatory action in Caco-2 cells via PTHR1 rather than PTHR2 since the former was much more abundant. PTHR1 was previously reported to be the sole functional receptor for renal distal tubular Ca^{2+} reuptake and bone resorption (35), whereas the ion regulatory function of PTHR2 remained elusive. The

downstream signaling pathways of PTH were herein demonstrated to be cAMP/PKA- and PI3K-dependent. In general, the cAMP/PKA pathway is of utmost importance for the classical PTH actions, including the production of osteoblast-derived osteoclastogenic factors (35), activation of transient receptor potential vanilloid 5 Ca^{2+} channel for distal tubular Ca^{2+} reabsorption (14), and upregulation of 1α -hydroxylase for renal $1,25(\text{OH})_2\text{D}_3$ synthesis (8). Since CFTR, NBCe1, NHE1, NHE3, carbonic anhydrase and Na^+/K^+ -ATPase are known to be under cAMP/PKA regulation (3, 4, 9, 57, 62), PKA and/or its downstream molecules might direct PTH signals to activate all of these transporters and related proteins, thereby leading to the enhanced HCO_3^- secretion. In addition to cAMP/PKA, Gentili and co-workers showed that PTH 1–34 could also directly activate PI3K in rat duodenal enterocytes (23). Since the HCO_3^- efflux through CFTR in mouse duodenum was also previously reported to be mediated by PI3K (57), PTH in the present study may trigger CFTR opening via both PKA- and PI3K-dependent pathways.

The exact physiological significance of the PTH-stimulated HCO_3^- secretion in vivo remains elusive, but it might contribute to intestinal mucous and fluid secretion, optimization of extracellular pH in the brush border microclimate, and/or maintenance of the intracellular pH and volume (13, 22). Our preliminary study in the in situ ligated loops of various rat intestinal segments revealed a significant dilution of sodium ferrocyanide, a non-absorbable marker of water transport, only in the ileum within 20 min after a single intravenous PTH administration (Laohapitakworn et al., unpublished observation), suggesting that PTH induced fluid secretion in rat ileum. Unlike the well-buffered apical solution in Ussing chamber (i.e., the estimated PTH-induced HCO_3^- flux of $\sim 135 \text{ pmol s}^{-1} \text{ cm}^{-2}$ was not large enough to shift the $[\text{HCO}_3^-]/\text{pCO}_2$ ratio of the buffer solution), the unbuffered ileal fluid would have been alkalinized by the robust HCO_3^- secretion. Since the ileum is the principal site for phosphate absorption (45), this PTH

action could be of physiological significance as phosphate should precipitate as non-absorbable complexes in the ileal alkaline lumen (11, 24), and thus fulfilling the classical hypophosphatemic action of PTH (54). On the other hand, in lower vertebrates, the PTH function with regard to HCO_3^- secretion and the responsive gastrointestinal segment may differ from that in mammals. PTH appeared to inhibit antral and fundic HCO_3^- secretion in amphibian stomach, presumably to help acidifying the gastric lumen, thereby increasing free-ionized Ca^{2+} concentrations available for absorption in the small intestine (18). In marine fish, PTH-related peptide, which was capable of binding to PTHR1 (37), inhibited HCO_3^- secretion in the duodenum to prevent intraluminal Ca^{2+} precipitation, and thus indirectly enhanced duodenal Ca^{2+} absorption (20).

In conclusion, our electrophysiological study and impedance analysis provided corroborative evidence for the novel ion regulatory role of PTH in the intestinal-like Caco-2 monolayer, as depicted in Figure 9. After binding to its receptors in the basolateral plasma membrane of intestinal epithelial cells, PTH rapidly activated apical HCO_3^- secretion within 1 min via the cAMP/PKA and PI3K signaling pathways. The apical HCO_3^- efflux was mediated mainly by the PTH-induced opening of CFTR rather than insertion of new channels into the apical membrane. Meanwhile, NBCe1 and carbonic anhydrase served to supply HCO_3^- by inducing basolateral HCO_3^- uptake and intracellular HCO_3^- production, respectively. Other transporters, such as NHE1, NHE3 and Na^+/K^+ -ATPase, were also crucial for the PTH-stimulated HCO_3^- secretion. Although more experiments are required to systematically elucidate the physiological significance of PTH in vivo, the present work is the first report that shows a direct, non-calcitropic, ion regulatory effect of this classical hormone in mammalian intestinal epithelium.

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Disclosures

No conflicts of interest, financial or otherwise are declared by the authors.

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Legends to figures

Figure 1. (A) Electrical equivalent circuit of intestinal epithelium-like Caco-2 monolayer. Each plasma membrane is represented by two electrical parameters, i.e., capacitance (C) and resistance (R). The subscripts *a* and *b* designate the apical and basolateral membranes, respectively. Paracellular resistance (R_p) is mainly determined by the tight junction, which restricts paracellular ion movement in size- and charge-selective manner. The element R_s represents the sum of series resistance in bathing solution and Snapwell. C_a , C_b , R_a and R_b are evaluated by alternating current impedance spectroscopy. On the other hand, transepithelial potential difference (V_t), short-circuit current (I_{sc}) and transepithelial resistance (R_t) are determined in a direct current mode by using Ohm's equation. Under normal conditions and during apical anion efflux, the apical side of Caco-2 monolayer is negative with respect to the basolateral side. (B) mRNA expression of PTH receptor (PTHR) isoforms 1 and 2 in normal Caco-2 cells as quantified by real-time PCR. GAPDH is the housekeeping gene for normalization. Numbers in parentheses represent the numbers of independent specimens. ** $P < 0.01$ compared with PTHR1 expression level (Mann-Whitney test).

Figure 2. (A–B) Representative transepithelial potential difference (V_t) tracings of Caco-2 monolayers after exposure on the basolateral side to 10 nmol/L PTH 1–34 or adenylyl cyclase activator (10 μ mol/L forskolin). Repetitive deflections in V_t resulted from current injection for determination of short-circuit current (I_{sc}). The present study mainly focused on changes in I_{sc} from the baseline levels to peak response levels (i.e., peak I_{sc} change; ΔI_{sc}). (C) ΔI_{sc} in Caco-2 monolayers after exposure to apical or basolateral 10 nmol/L PTH 1–34. ** $P < 0.01$ compared

with the basolateral value. (D) A sigmoidal dose-response curve of Caco-2 monolayers directly exposed on the basolateral side to various concentrations of PTH 1–34. (E) ΔI_{sc} in Caco-2 monolayers after exposure on the basolateral side to 10 nmol/L PTH 1–34, 10 nmol/L PTH 13–34 (inactive form), 10 nmol/L heat-inactivated PTH 1–34, various concentrations of polyclonal antibody (Ab) against PTH plus 10 nmol/L PTH 1–34, or 10 μ mol/L forskolin. *** $P < 0.001$ compared with ΔI_{sc} in PTH 1–34 group. Numbers in parentheses represent the numbers of independent monolayers.

Figure 3. Representative Nyquist plots, Bode magnitude plots and Bode phase plots at the peak response in Caco-2 monolayer ($n = 7-10$) after exposure on the basolateral side to (A–C) 10 nmol/L PTH 1–34 or (D–F) 10 μ mol/L forskolin, as determined by alternating current impedance spectroscopy. Abscissa and ordinate of Nyquist plot graphed in the plane of complex numbers show real part (Z_{re}) and imaginary part (Z_{im}) of the impedance (Z), respectively. All parameters were obtained prior to (control) and at 1 min after adding PTH 1–34. Relationships between frequency (0.5 Hz – 10 kHz) vs. impedance magnitude ($|Z|$) or phase angle (θ) are shown in Bode magnitude plot and Bode phase plot, respectively.

Figure 4. Time-dependent changes in the apical and basolateral plasma membrane resistance and capacitance in Caco-2 monolayer after exposure on the basolateral side to (A–B) 10 nmol/L PTH 1–34 or (C–D) 10 μ mol/L forskolin, as determined by alternating current impedance spectroscopy. All electrical elements were determined for the baseline (0 min) and after exposure to PTH 1–34 or forskolin for 1, 5, 10, 15, 20, 25, 30, 35 and 40 min. * $P < 0.05$ compared with

the baseline value of the apical membrane. [†] $P < 0.05$ compared with the baseline value of the basolateral membrane.

Figure 5. (A) Transepithelial resistance (R_t), (B) paracellular resistance (R_p), and (C) apical-to-basolateral transport of mannitol (a paracellular marker) in control and 10 nmol/L PTH 1–34-treated Caco-2 monolayers. R_t was determined at 10 min after PTH 1–34 exposure, and appeared time-invariant since the plateau response was attained. Numbers in parentheses represent the numbers of independent monolayers.

Figure 6. (A) Peak I_{sc} change (ΔI_{sc}) in Caco-2 monolayers treated with 10 nmol/L PTH 1–34 in the presence or absence of vehicle for inhibitor preparation (0.3% vol/vol dimethyl sulfoxide; DMSO), non-selective CFTR/ Cl^- channel inhibitor (20, 50, 100 or 200 μ mol/L NPPB), selective CFTR inhibitors (2, 5, 10 or 20 μ mol/L CFTRinh-172, or 50 μ mol/L GlyH-101) or non-selective Cl^- channel/anion exchanger inhibitor (500 μ mol/L DIDS). PTH 1–34 was added to the basolateral solution, whereas DMSO and inhibitors were added into the apical solution (ap). Full names of inhibitors are presented in Table 1. *** $P < 0.001$ compared with ΔI_{sc} of PTH 1–34-treated group. (B) mRNA expression of CFTR in normal (control) Caco-2 monolayers and that transfected with siRNA against CFTR (siCFTR), as determined by quantitative real-time PCR. The fold difference value is also presented. * $P < 0.05$ compared with the control group (Mann-Whitney test). (C–D) Representative transepithelial potential difference (V_t) tracings and ΔI_{sc} of 10 nmol/L PTH 1–34-treated CFTR knockdown Caco-2 monolayers. Polyethyleneimine (PEI; 10 μ g/mL) was used as a transfectant. The control group exposed to neither PEI nor siCFTR. *** $P < 0.001$ compared with the control group. (E) ΔI_{sc} in 10 nmol/L PTH 1–34-treated Caco-2

monolayers exposed on both sides to Cl^- -depleted, HCO_3^- -depleted or $\text{Cl}^-/\text{HCO}_3^-$ -depleted solution. *** $P < 0.001$ compared with ΔIsc of $\text{Cl}^-/\text{HCO}_3^-$ -rich group. Numbers in parentheses represent the numbers of independent monolayers.

Figure 7. (A) Peak Isc change (ΔIsc) in Caco-2 monolayers exposed on the basolateral side (bl) to 10 nmol/L PTH 1–34 with and without DMSO, NKCC1 inhibitor (100 $\mu\text{mol/L}$ bumetanide), non-selective Cl^- channel/anion exchanger inhibitor (500 $\mu\text{mol/L}$ DIDS) or Na^+/K^+ -ATPase inhibitor (1000 $\mu\text{mol/L}$ ouabain), or on both sides to carbonic anhydrase inhibitor (1000 $\mu\text{mol/L}$ methazolamide). Na^+/K^+ -ATPase is essential for the activity of the basolateral DIDS-sensitive NBCe1. (B) ΔIsc in Caco-2 monolayers exposed on the basolateral side to 10 nmol/L PTH 1–34 with and without DMSO, Ca^{2+} -activated K^+ channel inhibitor (100 $\mu\text{mol/L}$ chromanol 293B) or cAMP-activated K^+ channel inhibitor (0.1 $\mu\text{mol/L}$ charybdotoxin). (C) ΔIsc in Caco-2 monolayers exposed on the basolateral side to 10 nmol/L PTH 1–34 with and without DMSO or NHE inhibitors (amiloride and EIPA). Amiloride (10, 100 or 1000 $\mu\text{mol/L}$) or EIPA (10, 20 or 50 $\mu\text{mol/L}$) were added to the apical solution to inhibit the apical NHE3, whereas 1000 $\mu\text{mol/L}$ amiloride or 50 $\mu\text{mol/L}$ EIPA in the basolateral solution were to inhibit the basolateral NHE1. Full names of inhibitors are presented in Table 1. *** $P < 0.001$ compared with ΔIsc of PTH 1–34-treated group (Dunnett's post-test). $^{\dagger\dagger} P < 0.01$ compared with ΔIsc of Caco-2 monolayers treated with DIDS plus PTH 1–34 (Newman-Keuls' post-test). Numbers in parentheses represent the numbers of independent monolayers.

Figure 8. Peak Isc change (ΔIsc) in Caco-2 monolayers exposed on the basolateral side to 10 nmol/L PTH 1–34 with and without DMSO or various inhibitors of known PTH signaling

mediators, i.e., PKA (10 $\mu\text{mol/L}$ PKI 14–22), Ca^{2+} /PKC (50 $\mu\text{mol/L}$ BAPTA-AM and 1 $\mu\text{mol/L}$ GF-109203X for Ca^{2+} and PKC, respectively), and PI3K (200 nmol/L wortmannin and 75 $\mu\text{mol/L}$ LY-294002). DMSO and/or inhibitors were present on both sides of the monolayer. *** $P < 0.001$ compared with ΔIsc of PTH 1–34-treated group. Numbers in parentheses represent the numbers of independent monolayers.

Figure 9. Hypothetical diagram of the PTH-stimulated apical HCO_3^- efflux in intestinal epithelium-like Caco-2 monolayer. Human PTH 1–34 which binds to PTH receptor (PTHR) stimulates cystic fibrosis transmembrane conductance regulator (CFTR)-mediated HCO_3^- transport, presumably via cAMP/protein kinase A (PKA) and phosphoinositide 3-kinase (PI3K) signaling pathways. HCO_3^- is supplied by carbonic anhydrase (CA) and electrogenic $\text{Na}^+/\text{HCO}_3^-$ co-transporter-1 (NBCe1), both of which are known to be PKA-dependent. Both apical Na^+/H^+ exchanger-3 (NHE3) and basolateral NHE1 might be of importance in the maintenance of intracellular pH, particularly in the presence of robust CA activity. Intracellular Na^+ concentration and hyperpolarized potential might be maintained by the basolateral Na^+/K^+ -ATPase (NKA). This PTH-stimulated HCO_3^- transport increases transepithelial potential difference (V_t) at the peak response from ~ 0.4 mV to ~ 3 mV. Some inhibitors used in this study are also presented, and their full names are mentioned in Table 1 or Materials and Methods.

Legends to supplemental figures

Supplemental Figure S1. Transepithelial potential difference (V_t) across 10 nmol/L PTH 1–34-treated Caco-2 monolayers exposed on both sides to 7, 25, 50, 75, 100 or 125 mmol/L Cl^- -containing solution. Cl^- in the bathing solution was replaced by gluconate. V_t was determined at the peak response. Numbers in parentheses represent the numbers of independent monolayers.

Supplemental Figure S2. (A) Apical and (B) basolateral pH difference (ΔpH) after Caco-2 monolayers were continuously exposed to 10 nmol/L PTH 1–34 for 3, 6, 9, 12 and 15 min. The initial apical and basolateral pH were 7.426 ± 0.002 ($n = 5$) and 7.422 ± 0.005 ($n = 5$), respectively, as measured by pH-sensing electrode. Numbers in parentheses represent the numbers of independent monolayers.

Table 1. Inhibitors of ion transporters and proteins related to ion transport.

Target transporter	Common name of inhibitor	Full name of inhibitor	Catalog no. / manufacturer	Concentration used ($\mu\text{mol/L}$)
CFTR	NPPB	5-nitro-2-(3-phenylpropylamino)benzoic acid	N4779/Sigma	20, 50, 100, 200 ^a
CFTR	GlyH-101	N-(2-naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene)glycine hydrazide	219671/Calbiochem	50 ^a
CFTR	CFTRinh-172	5-[(4-carboxyphenyl)methylene]-2-thioxo-3-[(3-trifluoromethyl)phenyl]-4-thiazolidinone	219670/Calbiochem	2, 5, 10, 20 ^a
Basolateral NBCe1	DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid	D3514/Sigma	500 ^b
Apical Cl ⁻ channels and anion exchangers	DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid	D3514/Sigma	500 ^a
NKCC1	Bumetanide	3-(aminosulfonyl)-5-(butylamino)-4-phenoxybenzoic acid	B3023/Sigma	100 ^b
Na ⁺ /K ⁺ -ATPase	Ouabain	Acocantherine, G-strophanthin, 1 β ,3 β ,5 β ,11 α ,14,19-hexahydroxycard-20(22)-enolide 3-(6-deoxy- α -L-mannopyranoside)	O3125/Sigma	1000 ^b
Carbonic anhydrase	Methazolamide	N-(4-methyl-2-sulfamoyl- Δ^2 -1,3,4-thiadiazolin-5-ylidene) acetamide	M4156/Sigma	1000 ^{a+b}
Apical NHE3	Amiloride	N-amidino-3,5-diamino-6-chloropyrazinecarboxamide	A7410/Sigma	10, 100, 1000 ^a
Apical NHE3	EIPA	5-(N-ethyl-N-isopropyl) amiloride	A3085/Sigma	10, 20, 50 ^a
Basolateral NHE1	Amiloride	N-amidino-3,5-diamino-6-chloropyrazinecarboxamide	A7410/Sigma	1000 ^b
Basolateral NHE1	EIPA	5-(N-ethyl-N-isopropyl) amiloride	A3085/Sigma	50 ^b
Ca ²⁺ -activated K ⁺ channel	Chromanil 293B	Trans-N-[6-cyano-3,4-dihydro-3-hydroxy-2,2'-dimethyl-2H-1-benzopyran-4-yl]-N-methylethanesulfonamide	C2615/Sigma	100 ^b
cAMP-activated K ⁺ channel	Charybdotoxin	Charybdotoxin	C7802/Sigma	0.1 ^b

CFTR, cystic fibrosis transmembrane conductance regulator; NBCe1, electrogenic Na⁺/HCO₃⁻ co-transporter-1; NKCC1, Na⁺/K⁺/Cl⁻ co-transporter-1; NHE, Na⁺/H⁺ exchanger; *a* and *b*, applied in the apical and basolateral solution, respectively.

Table 2. *Homo sapiens* oligonucleotide sequences used in PCR study.

Gene	Accession no.	Primer (forward/reverse)	Product length (bp)
PTH1	NM_000316	5'-ACCTGCACAGCCTCATCTTCA-3' 5'-CACACAGCCACGAAGACAGC-3'	103
PTH2	NM_005048	5'-TCTGGGAGACCAATGCAGTTG-3' 5'-GGCAGGCATACGAACACGAT-3'	118
CFTR	NM_000492	5'-CACTCCTCATGGGGCTAATCT-3' 5'-CTGATCTTCCCAGCTCTCTGA-3'	142
GAPDH	NM_002046	5'-CTGGTAAAGTGGATATTGTTG-3' 5'-GAGGCTGTTGTCATACTTCTC-3'	359

PTH1, parathyroid hormone receptor; CFTR, cystic fibrosis transmembrane conductance regulator; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

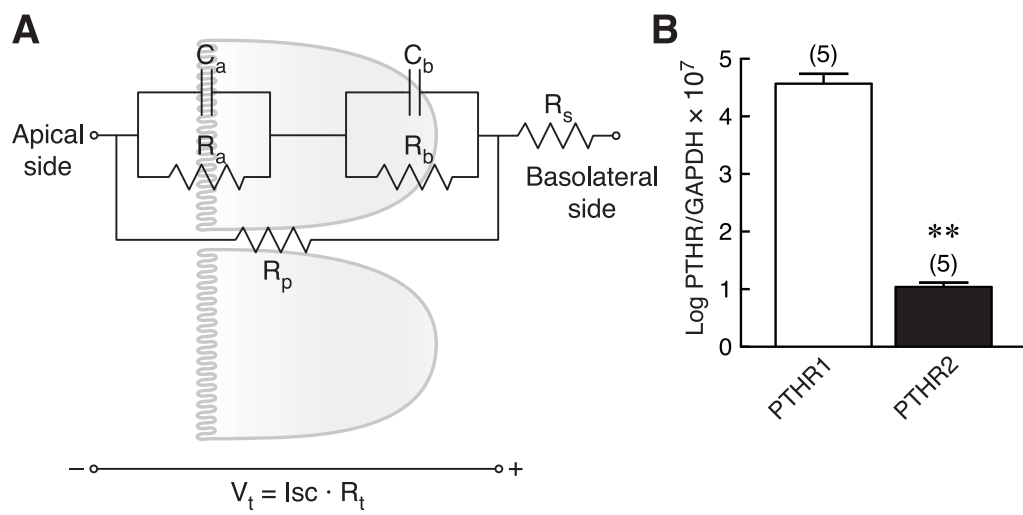


Figure 1: Laohapitakworn et al.

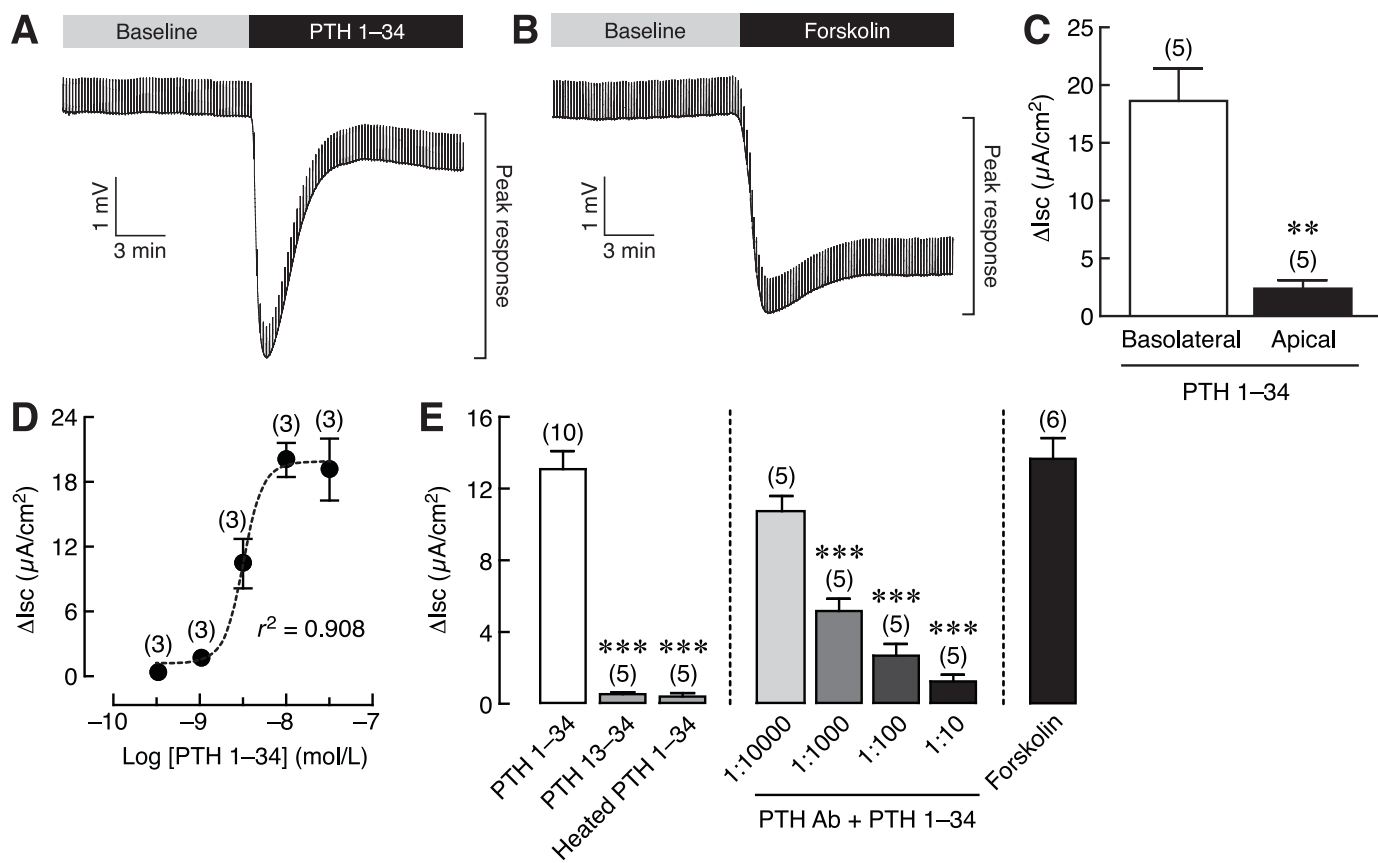


Figure 2: Laohapitakworn et al.

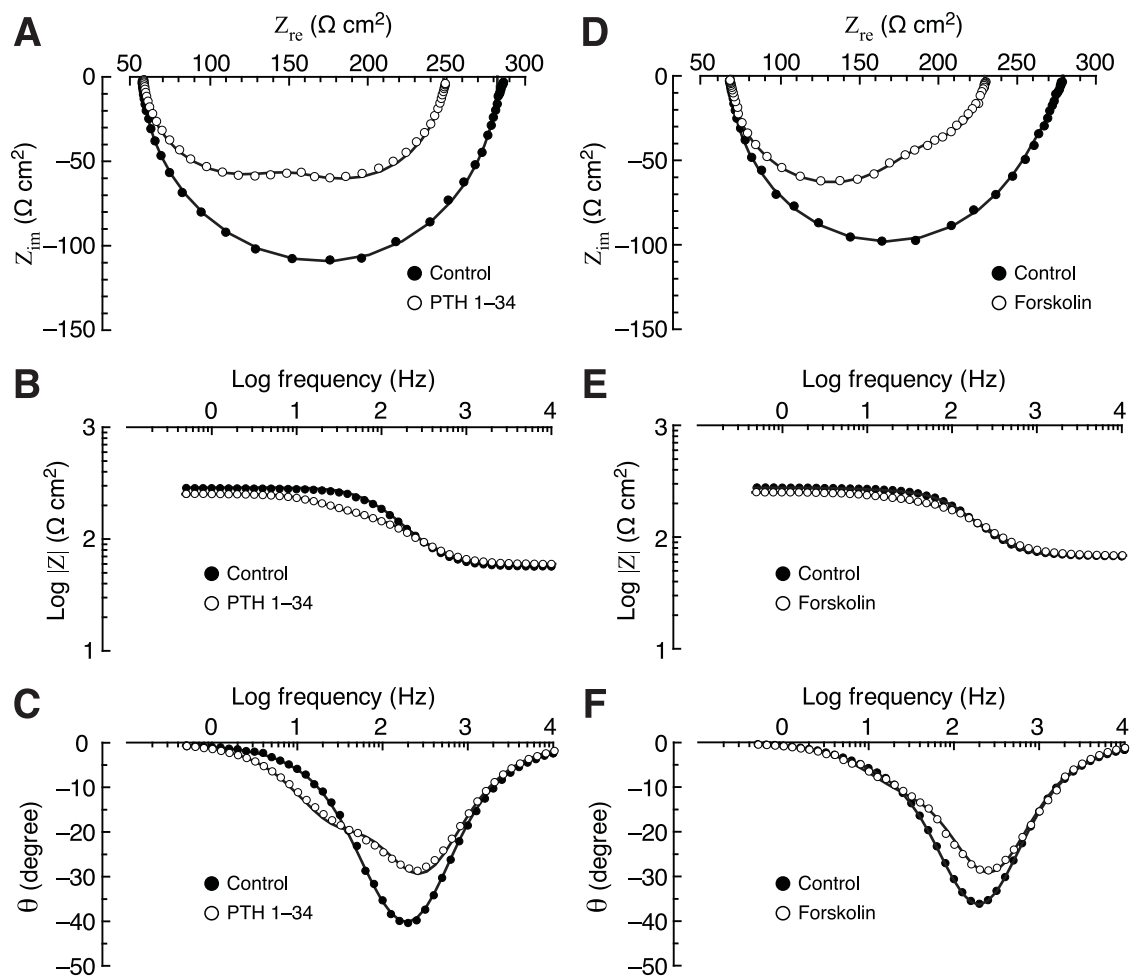


Figure 3: Laohapitakworn et al.

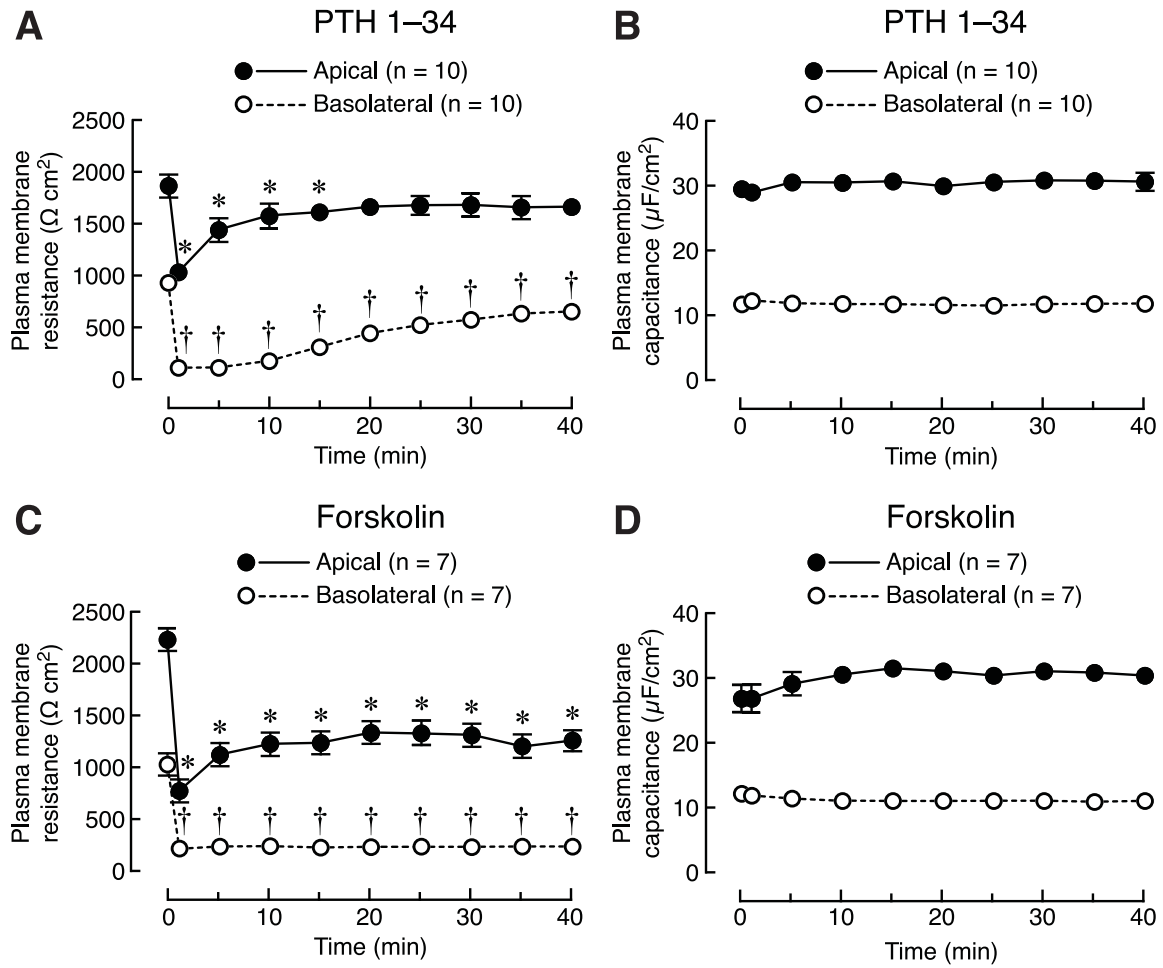


Figure 4: Laohapitakworn et al.

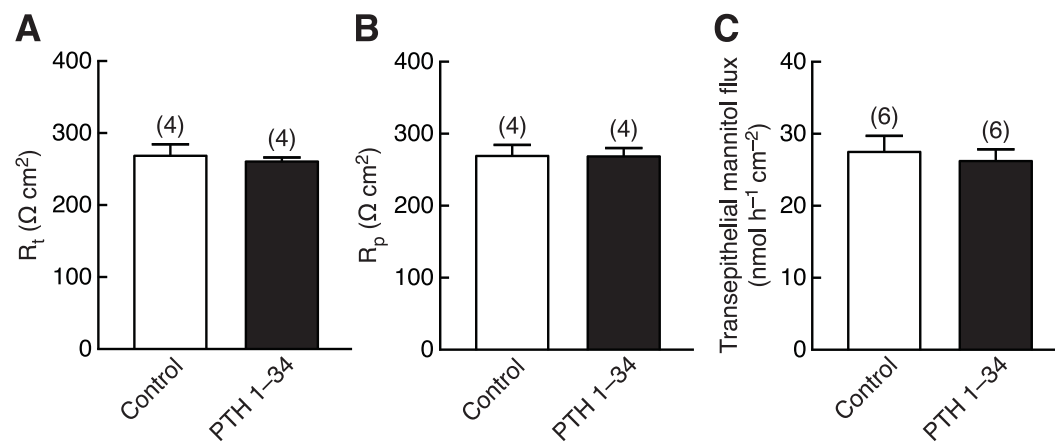


Figure 5: Laohapitakworn et al.

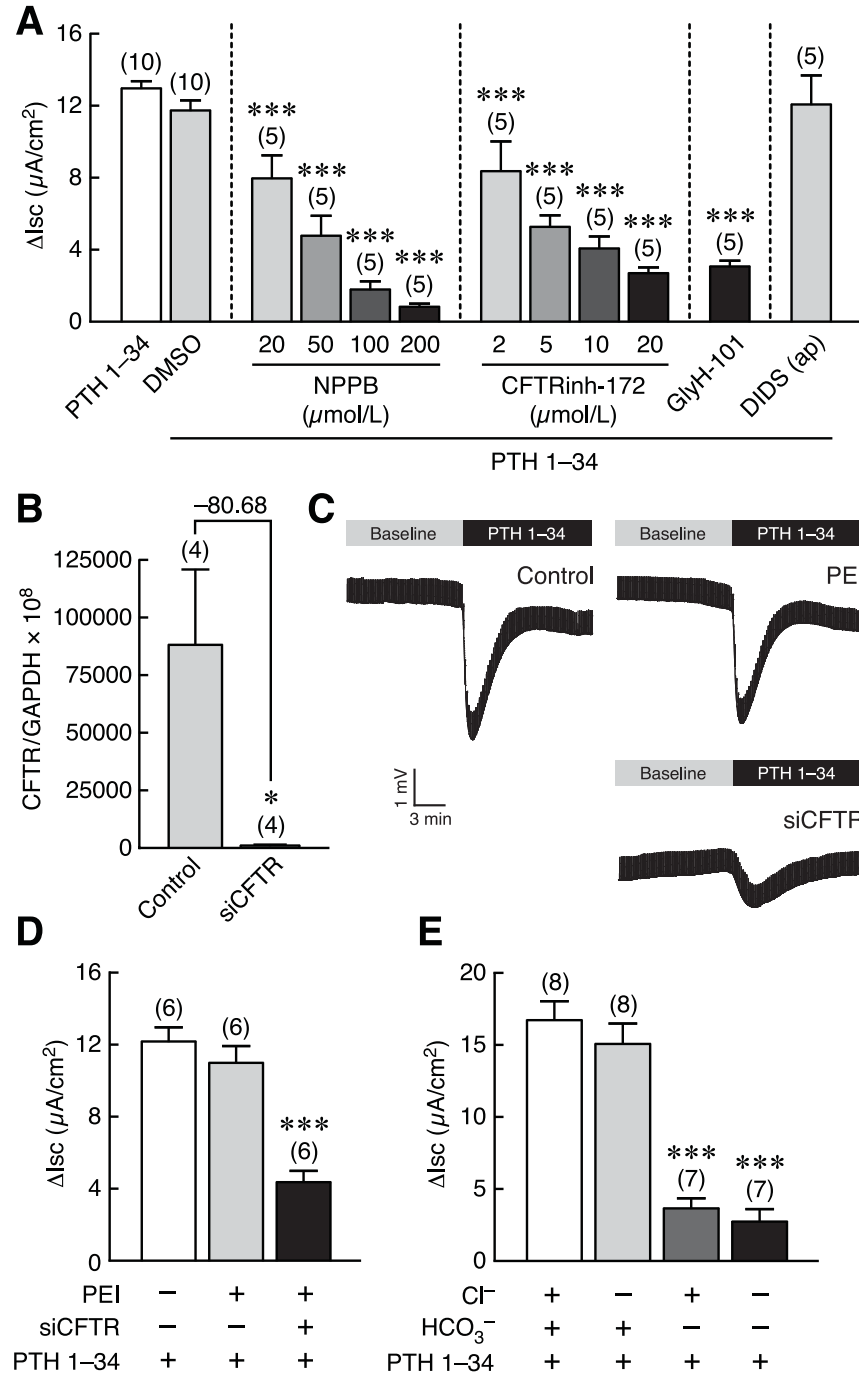


Figure 6: Laohapitakworn et al.

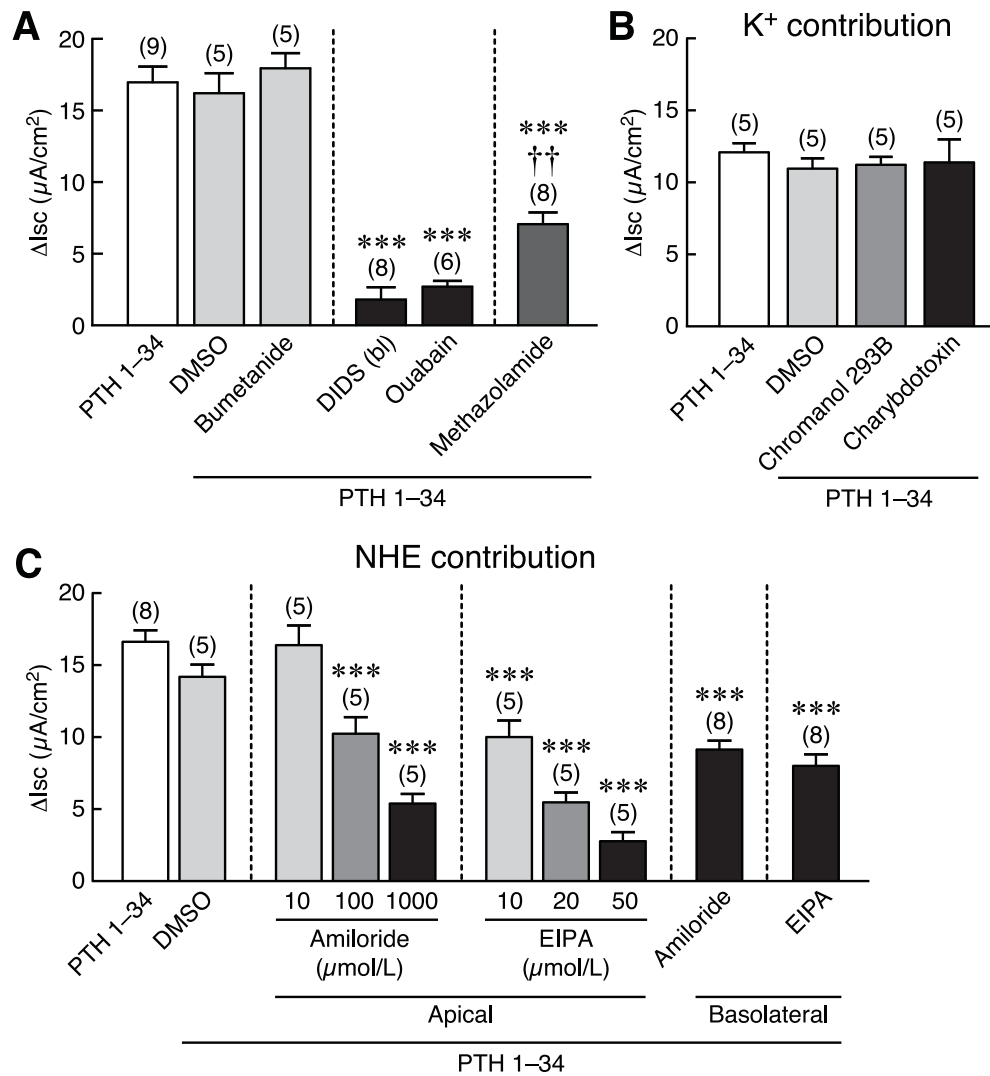


Figure 7: Laohapitakworn et al.

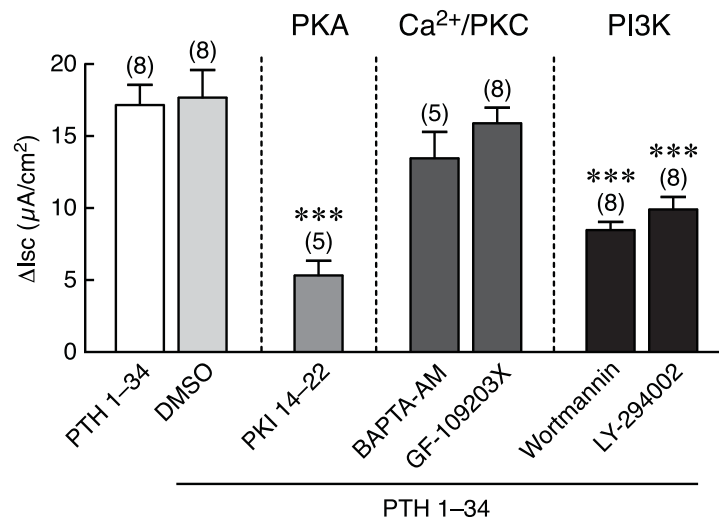
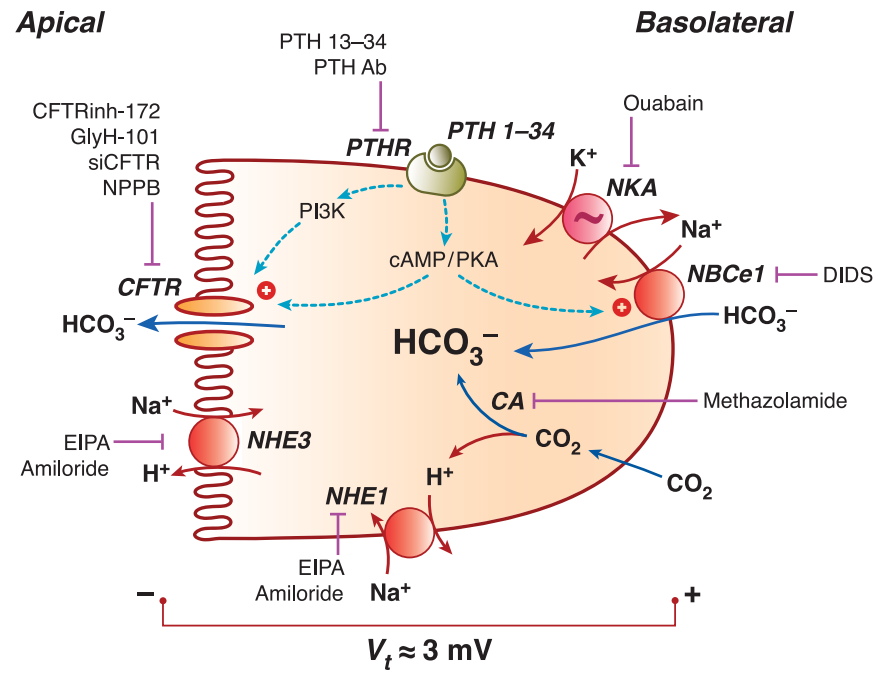


Figure 8: Laohapitakworn et al.



- Transport of HCO₃⁻ and CO₂
- Transport of other substances
- - - - - Hypothetical signaling pathways
- | Inhibitors
- ⊕ Possibly stimulated by PKA and/or PI3K

Figure 9: Laohapitakworn et al.



วันที่ข่าว 07 สิงหาคม 2552 เลขที่ข่าว 255208070088

บทความเรื่อง "ตอบแทนพระคุณแม่...ห่างไกลโรคระดูกพรุน"

ตอบแทนพระคุณแม่...ห่างไกลโรคระดูกพรุน

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หากจะกล่าวว่าแคลเซียมเป็นธาตุอาหารสารพัดประโยชน์ก็คงจะไม่มีเสียงค้าน เพราะแคลเซียมมีความจำเป็นต่อการทำงานของร่างกายทุกระบบ ไม่ว่าจะเป็นระบบไหลเวียนโลหิต การทำงานของสมอง และสร้างความแข็งแรงให้กับกระดูก ในแต่ละวันเราได้รับแคลเซียมจากอาหาร เช่น ผลิตภัณฑ์จากนม กุ้งแห้งตัวเล็ก ปลาเล็กปลาน้อย ถั่วแดง และงาดำ เป็นต้น เพื่อชดเชยกับการสูญเสียจากร่างกายทางปัสสาวะ แต่ “แม่” ของเรานั้นต้องเสียแคลเซียมในปริมาณมากเพื่อการเจริญเติบโตของลูกในครรภ์ก่อนที่ลูกจะเกิดเสียอีก นอกจากนี้ “แม่” ยังต้องเสียแคลเซียมอีกมากมายทางน้ำนมเพื่อเสริมสร้างให้ลูกเติบโตขึ้นอย่างสมบูรณ์และแข็งแรง เมื่อเวลาผ่านไป เราเติบโตขึ้นเป็นกำลังของสังคม ขณะที่ “แม่” สูงวัยขึ้นและกำลังก้าวเข้าสู่ช่วงชีวิตในวัยผู้สูงอายุ “แม่” ณ เวลานี้อาจต้องเผชิญกับการสูญเสียแคลเซียมจำนวนมากจากกระดูก ซึ่งอาจนำไปสู่โรคระดูกพรุน ซึ่งมีสาเหตุจากความเสื่อมที่เกิดขึ้นเองตามธรรมชาติ จากปัจจัยทางพันธุกรรมและการขาดฮอร์โมนเพศหญิงหลังหมดประจำเดือน อย่างไรก็ตาม ยังมีปัจจัยอีกมากมายที่เป็นตัวเสริมเร่งให้เกิดภาวะกระดูกพรุนได้รวดเร็วมากขึ้น เช่น การขาดการออกกำลังกาย การรับประทานอาหารที่มีแคลเซียมต่ำ ตลอดจนพยาธิสภาพบางประการ ได้แก่ ภาวะเลือดเป็นกรดเรื้อรัง และโรคทางไต เป็นต้น ภาวะกระดูกพรุนนี้เป็นเหตุให้คุณภาพชีวิตของ “แม่” ลดลง แต่ก็เป็นภาวะที่สามารถชะลอหรือบรรเทาได้หากได้รับกำลังใจและความเอาใจใส่จากลูก ๆ ทุกคน โดยทั่วไปการรักษาโรคระดูกพรุนสามารถทำได้ด้วยการใช้ยาหรือฮอร์โมน แต่การชะลอการเกิดหรือการป้องกันจะเป็นวิธีที่มีประสิทธิภาพมากกว่าการรักษาหลังเกิดโรคแล้ว ปัจจุบันมีอยู่หลายวิธีที่มีรายงานทางวิชาการว่าสามารถชะลอหรือลดความรุนแรงของโรคนี้ได้ อาทิเช่น การออกกำลังกายที่มีความเหมาะสม และการให้แคลเซียมเพื่อรับประทานเสริม การตอบแทนพระคุณแม่อย่างการชักชวนให้แม่ออกกำลังกายสม่ำเสมอและรับประทานอาหารแคลเซียมสูงจะเป็นประโยชน์ต่อสุขภาพกระดูกของแม่ในระยะยาว อย่างไรก็ตาม ในทางวิชาการยังมีข้อจำกัดสำหรับการช่วยให้กระดูกของ “แม่” แข็งแรงอยู่ 2 ประการดังนี้คือ

1. การออกกำลังกายที่มีแรงกระทำต่อกระดูกและข้อสูง อาจทำให้เกิดการบาดเจ็บของข้อต่อ หรือทำให้ข้อเสื่อมได้ ฉะนั้นจึงมีการตั้งสมมติฐานว่าการออกกำลังกายอาจต้องเป็นแบบแรงกระแทกต่ำ (Non-impact exercise) เช่น การว่ายน้ำ ที่ลดการบาดเจ็บของข้อต่อได้ แต่ต้องมีความรู้เรื่องผลของการออกกำลังกายแบบแรงกระแทกต่ำต่อการสร้างความแข็งแรงของกระดูกมีอยู่น้อยมาก งานวิจัยส่วนใหญ่เน้นที่การเพิ่มความแข็งแรงของกระดูกในคนอายุน้อยหรือนักกีฬา ซึ่งส่วนใหญ่เป็นการออกกำลังกายแบบมีแรงกระแทกสูง เช่น การวิ่ง หรือกระโดด
2. ประสิทธิภาพของกระบวนการดูดซึมแคลเซียมจากอาหารเข้าสู่ร่างกายจะลดลงตามอายุของ “แม่” ที่เพิ่มขึ้น ดังนั้นแคลเซียมจากอาหารแต่เพียงอย่างเดียวอาจไม่เพียงพอต่อความต้องการของร่างกาย จึงมีความจำเป็นต้องรับประทานแคลเซียมเสริม ซึ่งแม้จะทำให้ร่างกายได้รับแคลเซียมมากขึ้น แต่ร่างกายก็ไม่สามารถดูดซึมแคลเซียมเข้าสู่ร่างกายได้ทั้งหมด การเพิ่มประสิทธิภาพการดูดซึมแคลเซียมของร่างกายตลอดจนการพัฒนา รูปแบบของแคลเซียมที่จะเข้าสู่ร่างกายได้ดีจำเป็นต้องมีองค์ความรู้ด้านกลไกการดูดซึมแคลเซียมเข้าสู่ร่างกาย องค์ความรู้ดังกล่าวนี้เป็นที่ทราบและยอมรับมากกว่าหนึ่งทศวรรษ อย่างไรก็ตาม ผลการวิจัยในปัจจุบันที่ใช้เทคนิคอันทันสมัยมากขึ้นบ่งชี้ว่า องค์ความรู้ที่มีความคลาดเคลื่อนค่อนข้างมาก ดังนั้นนักวิจัยด้านแคลเซียมและกระดูกจำเป็นต้องศึกษาค้นคว้าเพิ่มเติมเพื่อพัฒนาองค์ความรู้ที่ถูกต้องขึ้นมาใหม่ สำหรับการต่อยอดความรู้เพื่อเพิ่มประสิทธิภาพการดูดซึมแคลเซียม

คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ได้เล็งเห็นประโยชน์ที่จะได้รับจากงานวิจัยด้านแคลเซียมและกระดูก ตลอดจนผลกระทบเชิงบวกขององค์ความรู้ในด้านนี้ต่อสังคมไทย จึงได้จัดตั้ง “เครือข่ายวิจัยด้านแคลเซียมและกระดูก” ขึ้น ภายใต้การนำของ ศาสตราจารย์ ดร. นทีทิพย์ กฤษณามระ โดยมีนักวิจัยที่มีความเชี่ยวชาญในสาขานี้เป็นสมาชิกเครือข่ายฯ กว่า 20 คน เช่น ผู้ช่วยศาสตราจารย์ ดร. นพ. นรุตพล เจริญพันธุ์ ซึ่งเป็นนักวิจัยรุ่นใหม่ของคณะฯ ภารกิจหลักของเครือข่ายฯ คือ ดำเนินการวิจัยเพื่อให้ได้ข้อมูลเกี่ยวกับการเพิ่มความแข็งแรงของกระดูกโดยการออกกำลังกายประเภทต่าง ๆ และการเพิ่มประสิทธิภาพการดูดซึมแคลเซียมเพื่อชะลอหรือลดความรุนแรงของโรคระดูกพรุน โดยเฉพาะใน “แม่” ที่เข้าสู่วัยหมดประจำเดือนและผู้สูงอายุ งานวิจัยอีกส่วนหนึ่งยังแสดงให้เห็นว่า มีความเป็นไปได้ที่จะป้องกันการสูญเสียมวลกระดูกของแม่ในระหว่างเลี้ยงบุตรด้วยน้ำนมแม่โดยให้แม่รับประทานแคลเซียมเสริมในปริมาณและช่วงเวลาที่เหมาะสม ทั้งนี้เพราะในระหว่างให้นมบุตรจะมีฮอร์โมนที่อยู่ในกระแสเลือดหลายชนิดที่สามารถเพิ่มประสิทธิภาพการดูดซึมแคลเซียมจากอาหารที่รับประทานเข้าสู่ร่างกายได้ในอัตราที่สูงขึ้นเป็นเท่าตัว ปัจจุบันงานวิจัยส่วนนี้ได้รับการสนับสนุนทุนวิจัยจากทางคณะฯ และสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) เมื่องานวิจัยสัมฤทธิ์ผลจะนำไปสู่ความเชื่อมั่นว่า “แม่” ผู้เป็นที่รักของลูกทุก ๆ คน มีกระดูกที่สมบูรณ์แข็งแรงได้ไปจนสูงวัย

วิจัยเด่นแนะแม่ดื่มนมก่อนให้นมลูก

กรุงเทพฯ ● นักวิจัยเจ้าของรางวัลนักวิจัยรุ่นใหม่ดีเด่น สกว.-สกอ. แนะนำแม่ดื่มนมก่อนให้นมลูก จะเสริมสร้างกระดูกของแม่ให้แข็งแรง ไม่ประสบปัญหากระดูกพรุนหรือกระดูกเปราะในช่วงสูงวัย

ผศ.นพ.ดร.นรุตพล เจริญพันธุ์ อาจารย์ประจำภาควิชาสรีรวิทยา และเครือข่ายวิจัยด้านแคลเซียมและกระดูก คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล เปิดเผยว่า จากการศึกษาเกี่ยวกับบทบาททางสรีรวิทยาของฮอร์โมนโปรแลคติน เพื่อนำแคลเซียมจากอาหารสู่กระดูกในระหว่างการตั้งครรภ์และให้นมลูก โดยได้รับการสนับสนุนทุนวิจัยจากสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) นั้นพบว่าสามารถเพิ่มความแข็งแรงของกระดูกในระหว่างที่แม่เลี้ยงลูกด้วยน้ำนม และทำให้น้ำนมแม่มีแคลเซียมอย่างเพียงพอ

นักวิจัยเจ้าของ “รางวัลนักวิทยาศาสตร์รุ่นใหม่” ประจำปี 2551 จากมูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยีในพระบรมราชูปถัมภ์ และ “รางวัลนักวิจัยรุ่นใหม่ดีเด่น สกว.-สกอ.” กล่าวว่า โดยทั่วไปร่างกายของแม่จะหลั่งฮอร์โมนโปรแลคตินออกมาจากต่อมใต้สมองอยู่แล้วในช่วงระหว่างการตั้งครรภ์และให้นมลูก เพื่อกระตุ้นการสร้างน้ำนมสำหรับเลี้ยงทารก ปรากฏการณ์นี้เป็นสิ่งที่ต้องเกิดขึ้นอยู่แล้วตามธรรมชาติ แต่ที่ทางคณะผู้วิจัยได้ค้นพบคือ ฮอร์โมนนี้ยังมีฤทธิ์กระตุ้นการดูดซึมแคลเซียมจากอาหารที่รับประทาน

ทานด้วย จึงคาดว่าจะมีความเป็นไปได้ที่จะสร้างสุขภาพกระดูกที่ดีให้กับแม่ โดยอาศัยการทำงานของฮอร์โมนดังกล่าว เมื่อแม่ได้รับแคลเซียมเสริมในเวลาและปริมาณที่เหมาะสม จะทำให้ได้รับแคลเซียมเข้าสู่ร่างกายในปริมาณสูงเพื่อเสริมความแข็งแรงของกระดูกโดยไม่ต้องพึ่งยาหรือฮอร์โมนอื่นๆ เสริม ซึ่งในเบื้องต้นคาดว่าจะช่วงเวลาที่เหมาะสมสำหรับการรับประทานนมหรือแคลเซียมเสริมคือ ประมาณ 15-30 นาทีก่อนเริ่มให้นมลูก โดยแคลเซียมที่เข้าสู่ร่างกายเพิ่มขึ้นนี้จะช่วยเสริมให้กระดูกของแม่แข็งแรง และมีปริมาณแคลเซียมในน้ำนมเพียงพอต่อความต้องการของร่างกายลูก

นอกจากนี้คณะผู้วิจัยยังพบอีกว่ามีปริมาณมากที่สามารถเพิ่มประสิทธิภาพการดูดซึมแคลเซียมจากทางเดินอาหารได้ และภาวะใดๆ ที่สามารถเพิ่มอัตราการดูดซึมแคลเซียม เช่น ภาวะเลือดเป็นกรดเรื้อรังก็มักจะกระตุ้นยีนชุดนี้ ผู้วิจัยยังแสดงให้เห็นว่าปัจจัยภายนอกร่างกาย เช่น การออกกำลังกาย และสารอาหารหลายชนิด ก็มีผลช่วยเพิ่มอัตราการดูดซึมแคลเซียมเข้าสู่ร่างกายเป็นอย่างมาก องค์ความรู้เรื่องปัจจัยภายนอกนี้ได้จากการประยุกต์ใช้เทคนิคทางชีวฟิสิกส์ที่พัฒนาขึ้นในประเทศไทย และยังมีความเป็นไปได้สูงว่าปัจจัยภายนอกทั้งการออกกำลังกายและสารอาหารต่างๆ จะไปช่วยเสริมฤทธิ์ของโปรแลคตินเพื่อช่วยให้แม่มีสุขภาพของกระดูกที่สมบูรณ์และแข็งแรงตั้งแต่เริ่มเป็นแม่ไปจนสูงวัย.



น้านมแม่ มีคุณค่าดีแท้แก่ทารก ช่วยเพิ่มพูนสติปัญญาและพัฒนาความผูกพันระหว่างแม่ลูก แต่ในระหว่างให้นมนั้น แม่ก็ต้องได้รับสารอาหารที่เป็นประโยชน์ด้วย เพื่อส่งผ่านสิ่งที่ดีไปให้ลูกอีกต่อหนึ่ง

ผศ. นพ. ดร.นฤตพล เจริญพันธุ์ อาจารย์ภาควิชาสรีรวิทยา และเครือข่ายวิจัยด้านแคลเซียมและกระดูก คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล เปิดเผยว่า จากการศึกษาเกี่ยวกับบทบาททางสรีรวิทยาของฮอร์โมนโพรแลคตินเพื่อนำแคลเซียมจากอาหารสู่กระดูกในระหว่างการตั้งครรภ์และให้นมลูก โดยได้รับการสนับสนุนทุนวิจัยจากสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) นั้น พบว่า สามารถเพิ่มความแข็งแรงของกระดูกในระหว่างที่แม่เลี้ยงลูกด้วยน้านม และทำให้น้านมแม่มีแคลเซียมอย่างเพียงพอ

อาจารย์ประจำภาควิชาสรีรวิทยา บอกว่า โดยทั่วไปร่างกายของแม่จะหลั่ง ฮอร์โมนโพรแลคติน ออกมาจากต่อมใต้สมองอยู่แล้ว ในช่วงระหว่างการตั้งครรภ์และให้นมลูกเพื่อกระตุ้นการสร้างน้านมสำหรับเลี้ยงทารก ปรากฏการณ์นี้เป็นสิ่งที่ต้องเกิดขึ้นอยู่แล้วตามธรรมชาติ

แต่ที่คณะผู้วิจัยได้ค้นพบคือ ฮอร์โมนนี้ยังมีฤทธิ์กระตุ้นการดูดซึมแคลเซียมจากอาหารที่รับประทานด้วยจึงคาดว่าจะมีความเป็นไปได้ที่จะสร้างสุขภาพกระดูกที่ดีให้กับแม่โดยอาศัยการทำงาน

เสริมแคลเซียมแม่ ก่อนให้ลูกดูดนม



ของฮอร์โมนดังกล่าว เมื่อแม่ได้รับแคลเซียมเสริมในเวลาและปริมาณที่เหมาะสมจะทำให้ได้รับแคลเซียมเข้าสู่ร่างกายในปริมาณสูงเพื่อเสริมความแข็งแรงของกระดูกโดยไม่ต้องพึ่งยาหรือฮอร์โมนอื่นๆ เสริม ในเบื้องต้นคาดว่าช่วงเวลาที่เหมาะสมสำหรับการรับประทานนมหรือแคลเซียมเสริม คือ ประมาณ 15-30 นาที ก่อนเริ่มให้นมลูก โดยแคลเซียมที่เข้าสู่ร่างกายเพิ่มขึ้นนี้จะช่วยเสริมให้กระดูกของแม่แข็งแรง และมีปริมาณแคลเซียมในน้านมเพียงพอต่อความต้องการของร่างกายลูก

คณะผู้วิจัยยังพบข้อมูลที่เป็นประโยชน์ เช่น มีความเป็นไปได้สูงว่าปัจจัยภายนอกทั้งการออกกำลังกายและสารอาหารต่างๆ จะไปช่วยเสริมฤทธิ์ของโพรแลคติน เพื่อช่วยให้แม่มีสุขภาพของกระดูกที่สมบูรณ์และแข็งแรงตั้งแต่เริ่มเป็นแม่ไปจนสูงวัย

สรุปว่า ตอนนี้นักแม่ที่อยู่ในช่วงกำลังให้นมลูก อยากให้ตัวเองแข็งแรงและลูกได้แคลเซียมเพียงพอ ก็ต้องเสริมแคลเซียมให้ตัวเองก่อนให้นมลูกสัก 15-30 นาที เพื่อจะได้เสริมกระดูกของแม่ให้แข็งแรงขึ้นและปริมาณแคลเซียมที่เพียงพอต่อความต้องการของลูกอีกด้วย.

เตลิทวิสต์ รับข่าวด่วนจาก **เตลิทวิสต์** ผ่าน SMS
 วันศุกร์ ที่ 21 สิงหาคม 2552
 เฉพาะลูกค้า **DTAC** และ **AIS**

วิสิมัคร	DTAC	ใช้บริการ	AIS
ข่าวทั่วไป	โทร. *75171		
ข่าวกีฬา	โทร. *73161		
ข่าวทั่วไป	โทร. *478900011		
ข่าวกีฬา	โทร. *478910011		

ค่าบริการ 29 บาท ต่อเดือน

หมายเหตุประชาชน

ดื่มนมเพิ่มแคลเซียมก่อนให้นมลูก

วันอังคาร ที่ 18 สิงหาคม 2552 เวลา 0:00 น

เนื้อหา ...

หนทางป้องกันโรคกระดูกพรุนช่วงสูงวัย

ท่ามกลางบรรยากาศวันแม่ที่ยังไม่จางหาย ความพยายามในการรณรงค์ให้ลูกดีมีนมแม่ตั้งแต่วันแรกเกิดจนถึง 6 เดือน ยังคงมีการประชาสัมพันธ์อย่างต่อเนื่อง เพราะคุณค่าทางอาหารอันเปี่ยมล้น

แต่ขณะที่ทารกได้รับสารอาหารที่มีคุณค่าประโยชน์มากมาย เช่น โปรตีน ไขมันที่จำเป็น และแคลเซียม โดยเฉพาะแคลเซียมซึ่งมีความสำคัญต่อการสร้างกระดูกของทารกแรกเกิด ตลอดจนการทำงานของหัวใจและสมอง ปริมาณแคลเซียมที่อยู่ในตัวของคนเป็นแม่ก็จะมีปริมาณลดลง ซึ่งเกิดขึ้นมาตั้งแต่ระยะตั้งครรภ์

ระหว่างตั้งครรภ์ผู้เป็นแม่จะใช้แคลเซียมไม่ต่ำกว่า 200 มิลลิกรัมต่อวัน เพื่อการเจริญเติบโตของทารกในครรภ์ ขณะที่ในระหว่างเลี้ยงลูกด้วยนมแม่ต้องใช้แคลเซียมราว 250-400 มิลลิกรัมต่อวัน

การสูญเสียแคลเซียมใน... ระหว่างตั้งครรภ์และเลี้ยงบุตรอาจยังไม่ทำให้คนเป็นแม่เห็นผลที่อาจเกิดขึ้นในทันที แต่เมื่อก้าวเข้าสู่ช่วงชีวิตในวัยผู้สูงอายุ จากปัจจัยทางพันธุกรรมและการขาดฮอร์โมนเพศหญิงหลังหมดประจำเดือนทำให้แม่ ณ เวลานั้นต้องเผชิญกับการสูญเสียแคลเซียมจำนวนมากจากกระดูก ซึ่งอาจนำไปสู่โรคกระดูกพรุนในที่สุด

แคลเซียมมีความจำเป็นต่อการทำงานของร่างกายทุกระบบ ไม่ว่าจะเป็นระบบไหลเวียนโลหิต การทำงานของสมอง และสร้างความแข็งแรงให้กับกระดูก ในแต่ละวันเราได้รับแคลเซียมจากอาหาร เช่น ผลิตภัณฑ์จากนม กุ้งแห้งตัวเล็ก ปลาเล็กปลาน้อย ถั่วแดง และงาดำ เป็นต้น เพื่อชดเชยกับที่สูญเสียจากร่างกายทางปัสสาวะ

สำหรับคนเป็นแม่ในช่วง 6 เดือนของการเลี้ยงลูกด้วยนมแม่ ร่างกายของแม่จะสร้างฮอร์โมนออกมาในกระแสเลือดหลายชนิด เช่น โพรแลคติน และวิตามินดี ที่สามารถเพิ่มประสิทธิภาพการดูดซึมแคลเซียมจากอาหารเข้าสู่ร่างกายได้เป็นเท่าตัว โดยไม่จำเป็นต้องให้ยาหรือฮอร์โมนเสริมจากภายนอก

"โดยทั่วไปร่างกายของแมงจะหลั่งฮอร์โมนโปรแลคตินออกมาจากต่อมใต้สมองอยู่แล้วในช่วงระหว่างการตั้งครรภ์และให้นมลูกเพื่อกระตุ้นการสร้างน้ำนมสำหรับเลี้ยงทารก ปรากฏการณ์นี้เป็นสิ่งที่ต้องเกิดขึ้นอยู่แล้วตามธรรมชาติ แต่ฮอร์โมนนี้ยังมีฤทธิ์กระตุ้นการดูดซึมแคลเซียมจากอาหารที่รับประทานด้วย จึงคาดว่าจะมีความเป็นไปได้ที่จะสร้างสุขภาพกระดูกที่ดีให้กับแม่โดยอาศัยการทำงานของฮอร์โมนดังกล่าว"

ผศ.นพ. ดนัย ตัณฑพล เจริญพันธุ์ อาจารย์ประจำภาควิชาสรีรวิทยา และเครือข่ายวิจัยด้านแคลเซียมและกระดูก คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ระบุ

เมื่อแม่ได้รับแคลเซียมเสริมในเวลาและปริมาณที่เหมาะสมจะทำให้ได้รับแคลเซียมเข้าสู่ร่างกายในปริมาณสูงเพื่อเสริมความแข็งแรงของ กระดูกโดยไม่ต้องพึ่งยาหรือฮอร์โมนอื่น ๆ เสริม

เบื้องต้นคาดว่าช่วงเวลาที่เหมาะสมสำหรับการรับประทานนมหรือแคลเซียมเสริม คือ ประมาณ 15-30 นาทีก่อนนมให้หมูลูก โดยแคลเซียมที่เข้าสู่ร่างกายเพิ่มขึ้นนี้จะช่วยเสริมให้กระดูกของแม่แข็งแรง และมีปริมาณแคลเซียมในน้ำนมเพียงพอต่อความต้องการของร่างกายลูก

นอกจากนี้ยังมีมีนจำนวนมากที่สามารถเพิ่มประสิทธิภาพการดูดซึมแคลเซียมจากทางเดินอาหารได้ และ
ภาวะใด ๆ ที่สามารถเพิ่มอัตราการดูดซึมแคลเซียม เช่น ภาวะเลือดเป็นกรดเรื้อรัง ซึ่งเป็นภาวะที่ร่างกาย
จะต้องปรับตัวเพื่อนำแคลเซียมเข้าสู่ร่างกายเพิ่มขึ้นนั้นก็มักจะกระตุ้นอินซูลินชนิดเดียวกันนี้ด้วย

ขณะที่ปัจจัยภายนอกร่างกาย เช่น การออกกำลังกาย ซึ่งต้องใช้แคลเซียมเพื่อสร้างกระดูก และสารอาหารหลายชนิด ก็มีส่วนช่วยเพิ่ม อัตราการดูดซึมแคลเซียมเข้าสู่ร่างกายเป็นอย่างมาก เช่นเดียวกับการที่สารอาหารต่าง ๆ จะไปช่วยเสริมฤทธิ์ของโพรแลคติน

แม้ว่าการออกกำลังกายจะเป็นวิธีหนึ่งที่จะช่วยเพิ่มแคลเซียมได้ แต่สำหรับผู้สูงอายุก็ยังมีความจำเป็นที่การออกกำลังกายที่มีแรงกระทำต่อกระดูกและข้อสูง อาจทำให้เกิดการบาดเจ็บของข้อต่อหรือทำให้ข้อเสื่อมได้ การออกกำลังกายจึงควรเป็นแบบแรงกระทำต่ำ เช่น การว่ายน้ำ ที่ลดการบาดเจ็บของข้อต่อได้

ส่วนการรับประทานแคลเซียมเสริม แม้ว่าจะทำให้ร่างกายได้รับแคลเซียมมากขึ้น แต่ร่างกายก็ไม่อาจดูดซึมแคลเซียมเข้าสู่ร่างกายได้ทั้งหมด

ดังนั้นก่อนที่จะเวลานั้นจะมาถึง แม่ทุก ๆ คนควรใช้ความสามารถของร่างกายที่มีประสิทธิภาพเพิ่มขึ้นตามธรรมชาติในระหว่างตั้งครรภ์และให้นมบุตรมาช่วยเสริมให้ร่างกายได้รับแคลเซียมมากยิ่งขึ้น ซึ่งเป็นอีกทางหนึ่งที่จะช่วยให้กระดูกของแม่แข็งแรงมากยิ่งขึ้น.

- หน้าขาวใส...ไม่ใช่ทุกคนจะมีได้

- การเปลี่ยนถ่ายไขกระดูก

- ตีมนมเพิ่มแคลเซียมก่อนให้นมลูก

- **ดิจิทัล คอนเทนต์ คลื่นลูกใหม่ สร้างโอกาสให้อุตสาหกรรมไทย**

- ราคาพุ่งน้ำมันเถื่อนอาละวาด

- 'ม.อ.วิชาการ52'เปิดโลกการเรียนรู้เพื่อชุมชน
- เปิดศูนย์บริการประชาชน (วังไซยา)

- **จุดบันทึกลดกินของดิบ**

- จดทะเบียนแรงงานข้ามชาติ ครึ่งสุดท้าย

- กะเพรา-ไทรพาปลดภัย

แนะแม่เต็มนมก่อนให้นมลูก เสริม-ชะลอ 'กระดูกพรุน'

กรุงเทพฯ : ผศ.นพ.ดร.นรุตพล จรรย์-พันธุ์ อาจารย์ประจำภาควิชาสรีรวิทยา และเครือข่ายวิจัยด้านแคลเซียมและกระดูก คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล เปิดเผยว่า จากการศึกษาบทบาททางสรีรวิทยาของฮอร์โมนโปรแล็กตินเพื่อนำแคลเซียมจากอาหารสู่กระดูกในระหว่างการตั้งครรภ์และให้นมลูก โดยได้รับทุนสนับสนุนจากฝ่ายวิชาการ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) พบว่าสามารถช่วยเพิ่มความแข็งแรงของกระดูกระหว่างที่แม่เลี้ยงลูกด้วยน้ำนม และทำให้น้ำนมแม่มีแคลเซียมเพียงพอ

ผศ.นพ.ดร.นรุตพลกล่าวเพิ่มเติมว่า โดยทั่วไปร่างกายของแม่จะหลั่งฮอร์โมนโปรแล็กตินออกมาจากต่อมใต้สมองอยู่แล้วในช่วงระหว่างการตั้งครรภ์

และให้นมลูก เพื่อกระตุ้นการสร้างน้ำนมสำหรับเลี้ยงทารกซึ่งเป็นกลไกตามธรรมชาติ แต่ที่ทางคณะผู้วิจัยได้ค้นพบคือฮอร์โมนนี้ยังมีฤทธิ์กระตุ้นการดูดซึมแคลเซียมจากอาหารที่รับประทานด้วย จึงคาดว่าเป็นไปได้ที่จะสร้างสุขภาพกระดูกที่ดีให้กับแม่โดยอาศัยการทำงานของฮอร์โมนดังกล่าว โดยเมื่อแม่ได้รับแคลเซียมเสริมในเวลาและปริมาณที่เหมาะสม จะทำให้ได้รับแคลเซียมเข้าสู่ร่างกายในปริมาณสูงเพื่อเสริมความแข็งแรงของกระดูกได้โดยไม่ต้องพึ่งยาหรือฮอร์โมนอื่นๆ ซึ่งในเบื้องต้นคาดว่าช่วงเวลาที่เหมาะสมสำหรับการรับประทานนมหรือแคลเซียมเสริมคือประมาณ 15-30 นาที ก่อนเริ่มให้นมลูก โดยแคลเซียมที่เข้าสู่ร่างกายเพิ่มขึ้นนี้จะช่วยเสริมให้กระดูกของแม่แข็งแรงและมีปริมาณแคลเซียมในน้ำนมเพียงพอ

แนะแม่ดีมีนม ก่อนให้นมลูก เพื่อกระดูกที่แข็งแรง

น้ามนแม่อุดมไปด้วยสารอาหารที่มีคุณประโยชน์มากมาย เช่น โปรตีน ไขมันที่จำเป็น และแคลเซียม โดยเฉพาะแคลเซียมนั้นมีความสำคัญต่อการสร้างกระดูกของทารกแรกเกิด ตลอดจนการทำงานของหัวใจและสมอง ในระหว่างตั้งครรภ์แม่จะใช้แคลเซียมไม่ต่ำกว่า 200 มิลลิกรัมต่อวันเพื่อการเจริญเติบโตของทารกในครรภ์ ส่วนระหว่างเลี้ยงลูกด้วยน้ามน แม่ต้องใช้แคลเซียมราว 250-400 มิลลิกรัมต่อวัน การให้แม่รับประทานอาหารที่มีแคลเซียมสูงในช่วงเวลาที่เหมาะสม โดยเฉพาะอย่างยิ่งระหว่างการให้นมบุตรจึงน่าจะได้ประโยชน์ทั้งต่อสุขภาพกระดูกของแม่และลูก

ในช่วง 6 เดือนของการเลี้ยงลูกด้วยน้ามนแม่ ร่างกายของแม่จะสร้างฮอร์โมนออกมาในกระแสเลือดหลายชนิด เช่น โพรแลคติน และวิตามินดี ที่สามารถเพิ่มประสิทธิภาพดูดซึมแคลเซียมจากอาหารเข้าสู่ร่างกายได้เป็นเท่าตัว โดยไม่จำเป็นต้องให้ยาหรือฮอร์โมนเสริมจากภายนอก กล่าวคือ แม่ทุกๆ คนควรใช้ความสามารถของร่างกายที่มีประสิทธิภาพเพิ่มขึ้นตามธรรมชาติในระหว่างตั้งครรภ์และให้นมบุตร มาช่วยเสริมให้ร่างกายได้รับแคลเซียมมากยิ่งขึ้น ซึ่งอาจเป็นทางหนึ่งที่ช่วยให้กระดูกของแม่แข็งแรงมากยิ่งขึ้น

การพัฒนางานวิจัยดังกล่าวมาโดย ผศ.ดร.นพ.นริศกมล เจริญพันธุ์ อาจารย์

ประจำภาควิชาสรีรวิทยา และเครือข่ายวิจัยด้านแคลเซียมและกระดูก คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ซึ่งได้รับการสนับสนุนจากฝ่ายวิชาการ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) โครงการปริญญาเอกกาญจนาภิเษก (คปก.) และฝ่ายวิจัย คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ในการศึกษาเกี่ยวกับบทบาททางสรีรวิทยาของฮอร์โมนโพรแลคตินเพื่อการนำแคลเซียมจากอาหารสู่กระดูกในระหว่างการตั้งครรภ์และให้นม ซึ่งมีศักยภาพสูงในการนำไปใช้ต่อยอดเพื่อเพิ่มความแข็งแรงของกระดูกในระหว่างที่แม่เลี้ยงลูกด้วยน้ามนและเพื่อให้ในน้ามนแม่มีแคลเซียมอย่างเพียงพอ ปัจจุบันทางคณะผู้วิจัยได้ตีพิมพ์ผลการวิจัยใน



วารสารวิชาการระดับนานาชาติกว่า 8 เรื่อง ภาย
ใต้การสนับสนุนของ สกว.

นักวิจัยเจ้าของ “รางวัลนักวิทยาศาสตร์
รุ่นใหม่” ประจำปี 2551 จากมูลนิธิส่งเสริม
วิทยาศาสตร์และเทคโนโลยีในพระบรมราชูปถัมภ์
และ “รางวัลนักวิจัยรุ่นใหม่ดีเด่น สกว.-สก.” กล่าว
ว่า จากภารกิจหลักของเครือข่ายวิจัยด้าน
แคลเซียมและกระดูก คณะวิทยาศาสตร์
มหาวิทยาลัยมหิดล ที่ดำเนินการวิจัยเพื่อให้ได้
ข้อมูลเกี่ยวกับการเพิ่มความแข็งแรงของกระดูก
โดยการออกกำลังกายประเภทต่างๆ และการ
เพิ่มประสิทธิภาพการดูดซึมแคลเซียมเพื่อเพิ่ม
ความแข็งแรงของกระดูกในผู้ที่เริ่มเป็น “แม่” และ
เพื่อชะลอหรือลดความรุนแรงของโรคกระดูกพรุน
ใน “แม่” ที่กำลังเข้าสู่วัยหมดประจำเดือนและ
วัยผู้สูงอายุ คณะผู้วิจัยจึงหวังว่างานวิจัยที่กำลัง
ดำเนินการอยู่นี้จะสามารถสร้างองค์ความรู้เพื่อ
นำไปใช้ต่อยอดในการวางนโยบายด้าน
สาธารณสุขและอนามัยแม่และเด็กได้ในอนาคต
สำหรับในผู้ที่เริ่มจะเป็น “แม่” นั้น

ผศ.ดร.นพ.นรุตพลกล่าวเพิ่มเติมว่า โดยทั่วไป
ร่างกายของแม่จะหลังฮอร์โมนโพเรแลคตินออก
มาจากต่อมใต้สมองอยู่แล้วในช่วงระหว่างการ
ตั้งครรภ์และให้นมลูก เพื่อกระตุ้นการสร้าง
น้ำนมสำหรับเลี้ยงทารก ปรากฏการณ์นี้เป็นสิ่ง
ที่ต้องเกิดขึ้นอยู่แล้วตามธรรมชาติ แต่ที่ทาง
คณะผู้วิจัยได้ค้นพบคือ ฮอร์โมนนี้ยังมีฤทธิ์
กระตุ้นการดูดซึมแคลเซียมจากอาหารที่
รับประทานด้วย จึงคาดว่าถ้ามีความเป็นไปได้ที่
จะสร้างสุขภาพกระดูกที่ดีให้กับแม่โดยอาศัย
การทำงานของฮอร์โมนดังกล่าว เมื่อแม่ได้รับ
แคลเซียมเสริมในเวลาและปริมาณที่เหมาะสม
จะทำให้ได้รับแคลเซียมเข้าสู่ร่างกายในปริมาณ
สูงเพื่อเสริมความแข็งแรงของกระดูกโดยไม่ต้อง
พึ่งยาหรือฮอร์โมนอื่นๆ เสริม ในเบื้องต้นคาด
ว่าช่วงเวลาที่เหมาะสมสำหรับการรับประทาน
นมหรือแคลเซียมเสริม คือ ประมาณ 15-30 นาที
ก่อนเริ่มให้นมลูก โดยแคลเซียมที่เข้าสู่ร่างกาย
เพิ่มขึ้นนี้จะช่วยเสริมให้กระดูกของแม่แข็งแรง
และมีปริมาณแคลเซียมในน้ำนมเพียงพอต่อ

ความต้องการของร่างกายลูก

นอกจากนี้ คณะผู้วิจัยยังพบอีกว่า มีเยื่อ
จำนวนมากที่สามารถเพิ่มประสิทธิภาพการ
ดูดซึมแคลเซียมจากทางเดินอาหารได้ และ
ภาวะใดๆ ที่สามารถเพิ่มอัตราการดูดซึม
แคลเซียม เช่น ภาวะเลือดเป็นกรดเรื้อรัง (ซึ่ง
ร่างกายจะต้องปรับตัวเพื่อนำแคลเซียมเข้าสู่
ร่างกายเพิ่มขึ้น) ก็มักจะกระตุ้นเยื่อชั้นนี้ ผู้วิจัย
ยังแสดงให้เห็นว่า ปัจจัยภายนอกร่างกาย เช่น
การออกกำลังกาย (ซึ่งต้องใช้แคลเซียมเพื่อ
สร้างกระดูก) และสารอาหารหลายชนิดก็มีส่วน
ช่วยเพิ่มอัตราการดูดซึมแคลเซียมเข้าสู่ร่างกาย
เป็นอย่างมาก องค์ความรู้เรื่องปัจจัยภายนอกนี้
ได้จากการประยุกต์ใช้เทคนิคทางชีวฟิสิกส์ที่
พัฒนาขึ้นในประเทศไทย และยังมีความเป็นไป
ได้สูงที่ว่าปัจจัยภายนอกทั้งการออกกำลังกาย
และสารอาหารต่างๆ จะไปช่วยเสริมฤทธิ์ของ
โพเรแลคตินเพื่อช่วยให้ “แม่” มีสุขภาพของ
กระดูกที่สมบูรณ์และแข็งแรงตั้งแต่เริ่มเป็นแม่
ไปจนสูงวัย ●



TRF Gallery

สื่อ Multimedia (252)

ประเภทคลิปวิดีโอ

- รายการวิจัยไทยคิด (57)
- รายการสารคดี (94)
- สัมภาษณ์นักวิจัย (18)
- สื่อบุคคล (53)
- อื่นๆ (30)
- ดูทั้งหมด

ฝ่ายงาน

- ฝ่ายนโยบายชาติและความสัมพันธ์ข้ามชาติ (ฝ่าย 1)
- ฝ่ายเกษตร (ฝ่าย 2)
- ฝ่ายสวัสดิภาพสาธารณะ (ฝ่าย 3)
- ฝ่ายชุมชนและสังคม (ฝ่าย 4)
- ฝ่ายอุตสาหกรรม (ฝ่าย 5)
- ฝ่ายวิชาการ
- ฝ่าย คปก.
- ฝ่ายประชาสัมพันธ์
- สกว.
- สำนักงาน สกว. ภาค
- หน่วยงานอื่นๆ

ผู้ดูแลระบบ

- ระบบจัดการ TRF Gallery

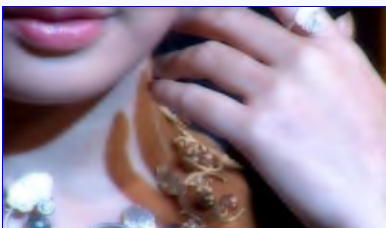


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	Title
	รหัส : 1000000263 ชื่อรายการ : รายการวิจัยไทยคิด ตอน ภาวะพร่องเอนไซม์ G6PD (จีซิกส์พีดี) รายละเอียด : รายการวิจัยไทยคิด ตอน ภาวะพร่องเอนไซม์ G6PD (จีซิกส์พีดี) ออกอากาศทาง ทวีไทย วันที่ 7 กุมภาพันธ์ 2553 เวลา : 26:52 นาที สถานที่เผยแพร่/ที่มา : ทวีไทย วันที่ 7 กุมภาพันธ์ 2553 วันที่อัปเดตข้อมูล : 7/2/2553 จำนวนผู้เข้าชม : 538 ครั้ง จำนวนผู้ดาวน์โหลด : 625 ชมคลิปวิดีโอออนไลน์ :  ดาวน์โหลดคลิปวิดีโอ :  ประเภทสื่อ : ไฟล์วิดีโอ
	รหัส : 1000000262 ชื่อรายการ : รายการวิจัยไทยคิด ตอน "แคลเซียม" มิตรแท้ กระดูกและฟัน รายละเอียด : รายการวิจัยไทยคิด ตอน "แคลเซียม" มิตรแท้ กระดูกและฟัน ออกอากาศทาง ทวีไทย วันที่ 31 มกราคม 2553 เวลา : 27:30 นาที สถานที่เผยแพร่/ที่มา : ทวีไทย วันที่ 31 มกราคม 2553 วันที่อัปเดตข้อมูล : 31/1/2553 จำนวนผู้เข้าชม : 634 ครั้ง จำนวนผู้ดาวน์โหลด : 719 ชมคลิปวิดีโอออนไลน์ :  ดาวน์โหลดคลิปวิดีโอ :  ประเภทสื่อ : ไฟล์วิดีโอ
	รหัส : 1000000261 ชื่อรายการ : รายการวิจัยไทยคิด ตอน เงิน เงิน เงิน ตอนที่ 3 รายละเอียด : รายการวิจัยไทยคิด ตอน เงิน เงิน เงิน ตอนที่ 3 ออกอากาศทาง ทวีไทย วันที่ 24 มกราคม 2553 เวลา : 28:16 นาที สถานที่เผยแพร่/ที่มา : ทวีไทย วันที่ 24 มกราคม 2553 วันที่อัปเดตข้อมูล : 24/1/2553 จำนวนผู้เข้าชม : 915 ครั้ง จำนวนผู้ดาวน์โหลด : 875 ชมคลิปวิดีโอออนไลน์ :  ดาวน์โหลดคลิปวิดีโอ :  ประเภทสื่อ : ไฟล์วิดีโอ
	รหัส : 1000000260 ชื่อรายการ : รายการวิจัยไทยคิด ตอน เงิน เงิน เงิน ตอนที่ 2 รายละเอียด : รายการวิจัยไทยคิด ตอน เงิน เงิน เงิน ตอนที่ 2 ออกอากาศทาง ทวีไทย วันที่ 17 มกราคม 2553 เวลา : 27:54 นาที สถานที่เผยแพร่/ที่มา : ทวีไทย วันที่ 17 มกราคม 2553 วันที่อัปเดตข้อมูล : 17/1/2553 จำนวนผู้เข้าชม : 1081 ครั้ง จำนวนผู้ดาวน์โหลด : 1086

Multidisciplinary research on bone and calcium metabolism

Asst. Prof. Narattaphol Charoenphandhu, M.D., Ph.D.

Department of Physiology, Faculty of Science, Mahidol University

Young Scientist Award 2008

Foundation for the Promotion of Science and Technology under the Patronage of His Majesty the King

The ultimate goal of the current research in our group is to answer the question of — how to help Thai people have strong and healthy bone? This question should bring awareness to the future risk that comes with aging population and should be included in the public health policy. Aging is accompanied by degenerative diseases, especially metabolic bone diseases which are expensive to treat and eventually lead to morbidity and mortality. In our perspective view, the solution can be achieved by prevention and early diagnosis of bone loss. To optimize efficiency of the prevention and treatment, one needs full understanding of the mechanisms involved in the body calcium-regulating system. To achieve this goal, our group has adopted vertical and horizontal approaches to multidisciplinary research on bone and calcium metabolism. Regarding the vertical approach, we use various available techniques, e.g., organ transplantation, electrophysiology, radionuclide tracer, bone histomorphometry, dual-energy X-ray absorptiometry (DEXA), confocal fluorescent microscopy, molecular cloning and various bioinformatic technology, to elucidate the calcium-regulating machinery from the whole-body level to molecular level. Recently, we have proposed a novel theory pertaining the transepithelial calcium transport in the intestine, as well as the optimal calcium concentration for effective calcium absorption. Our findings can be applied to develop better calcium-fortified tablets and calcium-enriched diet with higher fractional calcium absorption, especially for patients with osteopenia or osteoporosis secondary to menopause, aging, chronic metabolic acidosis and lactation. As for the horizontal approach, our group has applied knowledge in physics, chemistry, material science and computer science to develop working models, e.g., biophysical modeling, electrical circuit analyses and computational biology, to explain how calcium is transferred from diet to bone. We also collaborate with other research groups to apply nanotechnology, e.g., atomic force microscopy and nanoindentation, to evaluate bone strength at the nano scale. We expect that investigations of bone properties at the nano level will be available for use in the near future for early diagnosis of the bone diseases.

Multidisciplinary research on bone and calcium metabolism: From molecular to clinical study

Asst. Prof. Narattaphol Charoenphandhu, M.D., Ph.D.

Consortium for Calcium and Bone Research (COCAB), and Faculty of Science,
Mahidol University, Bangkok, Thailand

The ultimate goal of the current research in our group is to answer the question of – how to help Thai people have strong and healthy bone? This question should bring awareness to the future risk that comes with aging population and should be included in the public health policy. Aging is accompanied by bone degenerative disorders, especially metabolic bone diseases which are expensive to treat and eventually lead to morbidity and mortality. In our perspective view, the solution can be achieved by prevention and early diagnosis of bone loss. To optimize efficiency of the prevention and treatment, one needs full understanding of the mechanisms involved in the body calcium-regulating system. To achieve this goal, we adopt vertical and horizontal approaches to multidisciplinary research on bone and calcium metabolism. Regarding the vertical approach, we use various available techniques, e.g., organ transplantation, electrophysiology, radionuclide tracer, bone histomorphometry, dual-energy X-ray absorptiometry (DEXA), confocal fluorescent microscopy, molecular cloning and various bioinformatic technology, to elucidate the calcium-regulating machinery from the whole-body level to molecular level. Recently, we have proposed a novel theory pertaining the transepithelial calcium transport in the intestine, as well as the optimal calcium concentration for effective calcium absorption. Our findings can be applied to develop better calcium-fortified tablets and calcium-enriched diet with higher fractional calcium absorption, especially for patients with osteopenia or osteoporosis secondary to menopause, aging, chronic metabolic acidosis, pregnancy and lactation. As for the horizontal approach, we have applied knowledge in physics, chemistry, material science and computer science to develop working models, e.g., biophysical modeling, electrical circuit analyses and computational biology, to explain how calcium is transferred from diet to bone. We also collaborate with other research groups to apply nanotechnology, such as atomic force microscopy and nanoindentation, to evaluate bone strength at the nano scale. We expect that investigations of bone properties at the nano level will be available for use in the near future for early diagnosis of bone diseases.

Supported by Thailand Research Fund and Commission on Higher Education.

Keywords: acidosis, bone, calcium absorption, lactation, osteoporosis, pregnancy

Molecular Mechanism of the Prolactin-Enhanced Intestinal Ca²⁺ Absorption: Potential Application for the Prevention of Maternal Osteopenia

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Consortium for Calcium and Bone Research (COCAB) and Department of Physiology, Faculty of Science, Mahidol University, Thailand

*Corresponding author: naratt@narattsys.com

To supply a sufficient amount of calcium to the offspring, various hormones such as prolactin and parathyroid hormone-related peptide drastically accelerate maternal bone resorption, thus in turn liberating free ionized calcium for milk production. This adaptation often leads to osteopenia and, the lesser extent, overt osteoporosis in breastfeeding mothers. Besides being an activator of bone resorption, prolactin also helps to alleviate the stress on bone by stimulating intestinal calcium absorption. Our group has recently demonstrated that the prolactin-enhanced maternal calcium absorption occurs through both transcellular and paracellular pathways in a two-step manner. Specifically, chronic exposure to high plasma prolactin of ~ 200–300 ng/mL during lactation (Step 1) induces the upregulation of calcium transporters as determined by microarray and real-time PCR, whereas the suckling-induced prolactin surge of ~400–800 ng/mL rapidly increases the transcellular and paracellular calcium transport by ~2-fold (Step 2), presumably via voltage-dependent L-type calcium channel-1.3 and serine phosphorylation of the tight junction protein claudin-15, respectively. Therefore, we hypothesizes that oral calcium supplementation ~30 min prior to breastfeeding should shift the maternal calcium source from bone to diet, and would prevent the pregnancy/lactation-induced osteoporosis. In other words, the physicians can take advantage of this naturally occurring phenomenon and make use of the prolactin surge after breastfeeding for the benefit of both mother and newborn.

Acknowledgements: Supported by Mahidol University, Thailand Research Fund (TRF), and National Science and Technology Development Agency (NSTDA).

Keywords: bone, calcium, lactation, osteoporosis, prolactin

Bromocriptine can prevent lactation-induced bone loss in femoral metaphyses : Dual-energy X-ray absorptiometry study

Panan Suntornsaratoon¹, Nateetip Krishnamra^{1,2}, Narattaphol Charoenphandhu^{1,2,*}

*Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand
Consortium for calcium and Bone Research, Faculty of Science, Mahidol University, Bangkok,
Thailand*

During pregnancy and lactation, bone resorption is enhanced to supply calcium for fetal growth and milk production. Prolactin (PRL) has been hypothesized to be an important regulator of bone resorption during these two reproductive periods, since hyperprolactinemia in non-mated female rats could induce high bone turnover and trabecular bone loss. Pregnant and lactating Sprague-Dawley rats were treated for 7 days with bromocriptine 4 mg/kg s.c. (Bromo) to suppress pituitary PRL secretion. After the rats being sacrificed, the left femora and lumbar vertebra 5 (L5) were removed for bone mineral density (BMD) and content (BMC) analysis. During pregnancy, femoral and L5 BMD were higher when compared to age-matched controls and became lower in late lactation at the L5 (trabecular bone) and femoral metaphysis (trabecula-rich site), not diaphysis (cortex-rich site). Lactation-induced bone loss was inhibited in bromocriptine-treated lactating rats only at femoral metaphysis. Moreover, femoral diaphyseal BMC increased during pregnancy, early lactating and mid-lactating periods without change during late lactation. These lactation-induced increases in BMC were abolished during lactation. However, bromocriptine had no effect on femoral and L5 BMD/BMC during pregnancy. It could be concluded that the effect of PRL on bone was variable during the reproductive periods and site specific. While having no effect on femoral and L5 BMD/BMC during pregnancy, PRL was responsible for bone gain in early and mid-lactating periods, but loss during late lactating period.

Key words: Bone density, pregnancy, lactation

This work is supported in part by the King Prajadhipok and Queen Rambhai Barni Memorial Foundation and Faculty of Graduate studies, Mahidol University.

A longitudinal study of the prolactin-stimulated transepithelial calcium transport in the duodenum of pregnant and lactating rats

Kamonshanok Kraidith, Nateetip Krishnamra, Narattaphol Charoenphandhu

*Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand
Consortium for Calcium and Bone Research, Faculty of Science, Mahidol University, Bangkok, Thailand*

Abstract

Prolactin (PRL) is known to stimulate the duodenal calcium absorption in nonmated rats. However, the effects of PRL on intestinal calcium absorption in pregnant, lactating, and suckling rats as well as its mechanisms were not well understood. The present study, therefore, aimed to investigate the PRL-enhanced duodenal calcium transport during pregnancy and lactation, to determine the PRL-induced changes in the charge selectivity of the duodenal epithelium, and to investigate the physiological significance of the suckling-induced PRL surge in the regulation of duodenal calcium absorption in lactating rats. Rats were divided into day-14 and -21 pregnancy (P14 and P21), day-7, -14, and -21 lactation (L7, L14 and L21), and 15-day postweaning. Calcium flux and charge selectivity were determined by Ussing chamber techniques. The results showed that chronic PRL exposure was responsible for the elevated “baseline” of the duodenal calcium absorption during pregnancy and lactation, while the transient PRL surge during suckling rapidly enhanced the transepithelial calcium transport to the maximal capacity above the newly elevated baseline. The chronic effect of endogenous PRL on calcium transport was abolished by 7-day bromocriptine administration. Such an effect was restored by exogenous PRL administration. PRL altered the charge selectivity of the duodenal epithelium during pregnancy and lactation and made it more cation-selective by increasing cation selectivity. It could be concluded that PRL elevated the “baseline” of duodenal calcium absorption during pregnancy and lactation, and also altered the epithelial charge selectivity. A further increase in the duodenal calcium absorption resulted from the suckling-induced PRL surge.

Key words: calcium absorption, prolactin, transcellular transport, pregnancy, lactating rats

Publication:

Charoenphandhu N, Nakrasae L, Kraidith K, Teerapornpuntakit J, Thongchote K, Thongon N, Krishnamra K. Two step stimulation of intestinal Ca^{2+} absorption during lactation by long-term prolactin exposure and suckling-induced prolactin surge. *Am J Physiol Endocrinol Metab* 2009;297:E609–E619.

Corresponding author

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E-mail: naratt@narattsys.com

Kamonshanok K, Krishnamra N, Charoenphandhu N. “A longitudinal study of the prolactin-stimulated transepithelial calcium transport in the duodenum of pregnant and lactating rats” at the 39th Physiological Society of Thailand’s Annual Conference, Pattaya, Thailand, May 5-8, 2010.



6 ตุลาคม 2552

เรื่อง การประชุม "นักวิจัยรุ่นใหม่ ... พบ ... เมธีวิจัยอาวุโส สกว."

เรียน ผศ.นพ. นรุตพล เจริญพันธุ์ RSA5180001

สิ่งที่ส่งมาด้วย 1. "ร่าง" กำหนดการประชุม "นักวิจัยรุ่นใหม่ ... พบ ... เมธีวิจัยอาวุโส สกว."
2. รายละเอียดการนำเสนอผลงานแบบโปสเตอร์
3. รายละเอียดการพิจารณารางวัลนำเสนอผลงานแบบโปสเตอร์ดีเด่น
4. บทคัดย่อผู้นำเสนอผลงานแบบโปสเตอร์
5. แผนที่สถานที่จอดรถ จุฬาลงกรณ์มหาวิทยาลัย
6. แผนที่โรงแรม และป้ายติดกระเป๋

ตามที่ท่านตอบรับการเข้าร่วมประชุม "นักวิจัยรุ่นใหม่ ... พบ ... เมธีวิจัยอาวุโส สกว." ระหว่าง วันที่ 15 - 17 ตุลาคม 2552 ณ โรงแรมฮอลิเดย์อินน์ รีสอร์ท บีช ชะอำ จังหวัดเพชรบุรี มาแล้วนั้น ฝ่ายวิชาการ สกว. ไคร์ขอส่งร่าง กำหนดการประชุมและรายละเอียดเพิ่มเติม ดังนี้

1. วุฒิเมธีวิจัย เมธีวิจัย สกว. และผู้รับทุนวิจัยแบบมุ่งเป้าสาขาวิทยาศาสตร์นำเสนอผลงานวิจัยแบบโปสเตอร์ ณ ห้องประชุม Grand Sema Hall และ Park View Hall ในวันที่ 16 ตุลาคม 2552 เวลา 15.30-17.30 น. (ผู้เสนอผลงานประจำที่โปสเตอร์ของท่านเพื่อพบปะนักวิจัยที่สนใจและตอบข้อซักถามในวันที่ 16 ตุลาคม 2552 เวลา 15.30-16.30 น. และให้ข้อคิดเห็นแก่ผู้เสนอโปสเตอร์อื่นๆ ตามความสนใจในเวลา 16.30-17.30 น.)

ทั้งนี้ขอให้ท่านติดโปสเตอร์ในวันที่ 16 ตุลาคม 2552 ก่อนเวลา 15.00 น. และอยู่ประจำที่โปสเตอร์ของท่าน เพื่อพบปะนักวิจัยที่สนใจและตอบข้อซักถามตามเวลาที่กำหนด (และปลดโปสเตอร์ก่อนเวลา 19.00 น.)

2. เป็นกรรมการตัดสินรางวัลนำเสนอผลงานแบบโปสเตอร์ดีเด่น โดยมีรายชื่อคณะกรรมการพิจารณารางวัลเกณฑ์การพิจารณารางวัล ดังรายละเอียดในเอกสารที่แนบมาด้วย

ทั้งนี้ ขอความกรุณาส่งสรุปผู้สมควรได้รับรางวัล ที่ ศ.ดร. จำรัส ลิ้มตระกูล ภายในเวลา 15.00 น. ของวันที่ 16 ตุลาคม 2552

3. เพื่อให้การประชุมบรรลุวัตถุประสงค์โดยเฉพาะด้านการสร้างเครือข่ายวิจัย ฝ่ายวิชาการจึงได้คัดเลือกโครงการที่นำเสนอผลงานแบบโปสเตอร์ที่คาดว่าจะมีเนื้อหาที่ใกล้เคียงกับสาขาของท่าน เพื่อขอให้ท่านกรุณาประเมินโครงการที่นำเสนอด้วยโปสเตอร์ของนักวิจัยรุ่นใหม่ ในวันที่ 15 ตุลาคม 2552 เวลา 16.00-18.00 น. รหัส [REDACTED] จำนวน 7 เรื่อง

ทั้งนี้ได้ส่งบทคัดย่อของโครงการและแบบประเมินดังแนบ นอกจากนี้กรุณาซักถามและแลกเปลี่ยนความเห็นกับผู้เสนอโปสเตอร์อื่นๆ ที่ไม่ได้รับข้างต้นตามความสนใจของท่านด้วย

และขอให้เข้าร่วมรับฟัง ซักถามในการบรรยายของนักวิจัยอาวุโส ตลอดจนให้ข้อคิดเห็นในการนำเสนอผลงานวิจัยของผู้ได้รับทุนวิจัยท่านอื่นตลอดการประชุม

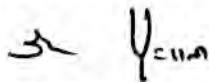
"ถ้าคุณต้องการทำวิจัย...biodata ให้โอกาส เภยสูงทุนุเบียมหรับทราบไทยวิจัย ที่ <http://biodata.trf.or.th>"

ชั้น 14 อาคาร เอส เอ็ม ทาวเวอร์ 979/17-21 ถนนพหลโยธิน แขวงสามเสนใน เขตพญาไท กรุงเทพฯ 10400
14th Floor, S M Tower, 979/17-21 Phaholyothin Road, Samsennai, Phayathai, Bangkok 10400, Thailand
Tel : +66 (0) 2278-8200 Fax : +66 (0) 2298-0476 [http:// www.trf.or.th](http://www.trf.or.th) E-mail : trf-info@trf.or.th

สร้างสรรค์ปัญญา เพื่อพัฒนาประเทศ

จึงเรียนมาเพื่อโปรดให้ความร่วมมือในกิจกรรมดังกล่าว สำหรับท่านที่ประสงค์จะเดินทางไปกับรถที่ สกว. จัดให้ รถจะ
ออกเวลา 06.00 น. (รถออกตรงเวลา) ในวันที่ 15 ตุลาคม 2552 ที่บริเวณลานจอดรถด้านหลังหอประชุมจุฬาลงกรณ์
มหาวิทยาลัย

ขอแสดงความนับถือ



(ศ.ดร. วิชัย บุญแสง)

ผู้อำนวยการฝ่ายวิชาการ
สำนักงานกองทุนสนับสนุนการวิจัย

Prolactin-Stimulated Calcium Absorption in the Duodenal Epithelium of Lactating Rats and Intestine-Like Caco-2 Monolayer

Charoenphandhu, N.^{1,2*}, Kraidith, K.¹, Nakkrasae, L.², Thongon, N.², Krishnamra, N.^{1,2}

¹Department of Physiology, Faculty of Science, Mahidol University, Bangkok 10400 Thailand

²Consortium for Calcium and Bone Research, Faculty of Science, Mahidol University, Bangkok 10400 Thailand

Abstract

During pregnancy and lactation, the intestinal calcium absorption is enhanced by a poorly understood mechanism to help alleviate negative calcium balance and osteopenia due to calcium loss for fetal growth and lactogenesis. Herein, we demonstrated that prolactin (PRL) was responsible for the lactation-enhanced duodenal calcium absorption by increasing the transepithelial calcium transport through both transcellular and paracellular pathways. Such PRL actions could also be mimicked in the intestine-like Caco-2 monolayer. Effects of PRL on the transcellular calcium transport appeared to be non-genomic, and required voltage-dependent L-type calcium channel (Ca_v) 1.3. Further calcium flux study showed that PRL increased the transcellular and paracellular calcium transport via the phosphoinositide 3-kinase (PI3K)/protein kinase C (PKC) and PI3K/RhoA-associated coiled-coil forming kinase (ROCK) pathways, respectively. The PRL-enhanced paracellular calcium permeability was dependent on the phosphorylation of cation-selective tight junction protein claudin-15 in a PI3K-dependent manner, and was absent in the claudin-15 knockdown Caco-2 monolayer. It could be concluded that PRL is the important hormone that stimulates the transcellular and paracellular calcium absorption in the duodenum of lactating rats and Caco-2 monolayer. This work, thus, provided corroborative evidence for better understanding of the cellular mechanism of PRL in intestinal epithelial cells.

Keywords: Caco-2 monolayer, calcium absorption, cation, claudin-15, lactation, paracellular transport, transcellular transport, Ussing chamber

Outputs

1. Thongon N, Nakkrasae L, Thongbunchoo J, Krishnamra N, Charoenphandhu N. *Enhancement of calcium transport in Caco-2 monolayer through PKC ζ -dependent Ca_v1.3-mediated transcellular and rectifying paracellular pathways by prolactin.* American Journal of Physiology – Cell Physiology **2009**; 296: C1373–C1382.
2. Charoenphandhu N, Wongdee K, Teerapornpuntakit J, Thongchote K, Krishnamra N. *Transcriptome responses of duodenal epithelial cells to prolactin in pituitary-grafted rats.* Molecular and Cellular Endocrinology **2008**; 296: 41–52.
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บทคัดย่อ

การเสนอผลงานแบบโปสเตอร์

ทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลาง

ในสถาบันอุดมศึกษา * เมธีวิจัย สกว. * วุฒิเมธีวิจัย สกว.

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สำนักงานคณะกรรมการการอุดมศึกษา (สกอ.)



Stimulation of Cecal Calcium Transport in Female Rats by Prolactin

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Abstract

During pregnancy and lactation, prolactin (PRL) in maternal circulation markedly enhances calcium absorption, especially in the small intestine to supply calcium for fetal growth and milk production. However, effects of PRL on the cecum, a part of the large intestine with the highest rate of calcium transport, were elusive. Here, we demonstrated by using quantitative real-time PCR that cecal epithelial cells of female rats expressed PRL receptors. In Ussing chamber experiments, 200–800 ng/mL PRL significantly stimulated the transcellular active cecal calcium absorption in a dose-dependent manner. PRL also altered the charge-selective property of the paracellular pathway in such a way that the movement of cations was increased, thereby enhancing the paracellular passive calcium transport. Such the PRL actions were abolished by phosphoinositide 3-kinase (PI3K) inhibitors, wortmannin and LY294002, but not by RNA polymerase-II inhibitor, 5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole. Thus, it could be concluded that PRL directly enhanced the transcellular active and paracellular passive calcium transport in the rat cecum through the non-genomic PI3K signaling pathway.

Keywords: calcium absorption, cecum, lactation, large intestine, paracellular transport, pregnancy, transcellular transport, using chamber

Outputs

1. Thongon N, Nakkrasae L, Thongbunchoo J, Krishnamra N, Charoenphandhu N. *Prolactin stimulates transepithelial calcium transport and modulates paracellular permselectivity in Caco-2 monolayer: Mediation by PKC and ROCK pathways*. American Journal of Physiology–Cell Physiology **2008**; 295: C1158–C1168.
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Possible osteoregulatory role of prolactin in lactation
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Osteopenia was commonly found in lactation because of a huge amount of maternal calcium loss for milk production. Prolactin (PRL), which becomes substantially high in plasma during pregnancy and lactation, has been hypothesized to be a crucial osteoregulator for maternal skeletal changes, since hyperprolactinemia was found to induce bone loss in the metaphysis of long bone in non-mated female rats. Here, bone changes were investigated in mated Sprague-Dawley rats treated for 7 days with bromocriptine (Bromo), an inhibitor of pituitary PRL release, or Bromo+PRL at various reproductive states, from mid-pregnancy to late lactation. After the rats were sacrificed, femora and tibiae were removed for bone length measurement, and immunohistochemistry and bone histomorphometry. The results showed that PRL receptors were strongly expressed in osteoblasts lining bone trabeculae, indicating that bone is a target tissue of PRL. During pregnancy, there were an increase in the cortical thickness and length of the long bone, but its trabecular structure was modestly changed. Such pregnancy-induced bone changes, however, were PRL independent. On the other hand, lactating rats showed massive bone loss in the trabeculae, but not in the cortical shells. Further study in Bromo- and Bromo+PRL-treated rats suggested that PRL was responsible for the decreased trabecular bone volume and the increased trabecular resorption. Lactating rats showed an uncoupling of trabecular bone formation and resorption with the latter being predominant. In conclusion, pregnancy mainly induced cortical bone gain, whereas lactation led to trabecular bone loss in long bones. PRL was also found to be an important regulator of bone modeling during lactation.

Key words: bone modeling, pregnancy, prolactin, lactation, trabecular structure

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Parathyroid hormone induces HCO_3^- secretion in intestinal cell line Caco-2

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Abstract

Introduction: Parathyroid hormone (PTH) has an important regulatory function on electrolyte transport especially in kidney and bone. However, its role in the intestine is still poorly understood. In this study, we investigated PTH receptor expression and a possibility of a direct effect of PTH on intestinal cells using the human epithelial colorectal adenocarcinoma cell line Caco-2.

Results: RT-PCR showed that Caco-2 expressed PTH receptor type 1 and 2 (PTH1R and PTH2R) mRNA 14 days after plating. Actions of PTH on electrolyte transport were examined in the *in vitro* Ussing chamber experiments. Results showed that PTH significantly increased the short-circuit currents (I_{sc}), which were indication of transepithelial ion transport, in a dose-dependent manner. While removal of Cl^- from the bathing solution had no effect, HCO_3^- depletion abolished PTH action. Bumetanide, an inhibitor of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC), did not inhibit response to PTH, whereas 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS), an inhibitor of $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC), diminished PTH action. Changes in I_{sc} were also partially blocked by carbonic anhydrase inhibitor methazolamide. Furthermore, a nonspecific Cl^- channel blocker 5-nitro-2-(3-phenyl propylamino)benzoic acid (NPPB), and cystic fibrotic transmembrane conductance regulator (CFTR) blockers, thiazolidinone CFTRinh-172 and glycine hydrazide GlyH-101 inhibited the PTH action. Effects of PTH were also abolished after cells were exposed to protein kinase A (PKA) inhibitor 14-22 amide (PKAI 14-22) and phosphoinositide 3-kinase (PI3K) inhibitors, LY294002 and wortmannin.

Conclusion: The present data suggested a novel action of PTH in stimulating HCO_3^- secretion in the intestine by enhancing HCO_3^- entry into the cell through NBC, and its secretion through CFTR. Carbonic anhydrase also helps generate HCO_3^- . PTH actions were mediated by the PKA and PI3K signaling pathways.

Keywords: anion secretion, carbonic anhydrase, cystic fibrosis transmembrane conductance regulator (CFTR), sodium bicarbonate cotransporter (NBC)

Acknowledgement: Medical Scholar Programme, Faculty of Science, Mahidol University, Bangkok, Thailand

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ABSTRACT NO. 00175

Prolactin-enhanced calcium absorption in the duodenum of lactating rats is mediated by the phosphoinositide 3-kinase (PI3K) pathway

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During lactation, the duodenal Ca^{2+} absorption is enhanced by unknown mechanism to help alleviate negative Ca^{2+} balance and osteopenia which result from Ca^{2+} loss for milk production. We demonstrated herein that the lactogenic hormone prolactin (PRL) was responsible for the lactation-enhanced Ca^{2+} absorption in rats by increasing the transepithelial Ca^{2+} transport in the duodenum via both transcellular and paracellular pathways. In Caco-2 monolayer, effects of PRL on Ca^{2+} absorption were observed within 60 min. Since PRL actions were not blocked by inhibitors of gene transcription and protein synthesis, they were likely to be non-genomic. Further Ca^{2+} flux study showed that PRL increased the transcellular active and paracellular passive Ca^{2+} transport via the PI3K/protein kinase C (PKC) and PI3K/RhoA-associated coiled-coil forming kinase (ROCK) pathways, respectively. The PRL-enhanced paracellular Ca^{2+} permeability required phosphorylation of the cation-selective tight junction protein claudin-15 in a PI3K-dependent manner, and was absent in the claudin-15-knockdown Caco-2 monolayer. It could be concluded that PRL is the important hormone that stimulates the transcellular and paracellular Ca^{2+} absorption in the duodenum of lactating rats via the phosphoinositide 3-kinase pathway.

Key words: calcium absorption; claudin-15; lactation; paracellular transport; transcellular transport; Ussing chamber

Prolactin stimulates transepithelial calcium transport in the cecum of female rats

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Prolactin (PRL) is known to stimulate calcium absorption in the small intestine in both lactating and unmated rats. However, effects of PRL on the cecum, a part of the large intestine with the highest rate of intestinal calcium transport, are controversial. We demonstrated herein by quantitative real-time PCR that cecal epithelial cells of female rats expressed PRL receptors. In Ussing chamber experiments, 200-800 ng/mL PRL enhanced the transcellular active calcium absorption in the cecum in a dose-dependent manner. PRL also altered the charge-selective property of the paracellular pathway in such a way that the movement of cations was increased, thereby enhancing the paracellular passive calcium transport. Such PRL actions in the cecum could be abolished by phosphoinositide 3-kinase (PI3K) inhibitors, wortmannin and LY294002, but not by RNA polymerase II inhibitor, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB). It could be concluded that PRL directly enhanced the transcellular and paracellular calcium transport in the cecum of female rats through the non-genomic PI3K signaling pathway.

Key words: calcium absorption; paracellular transport; transcellular transport; Ussing chamber

Subject: IUPS 2009 Congress Travel Award (official Letter)
Date: Wednesday, April 1, 2009 4:53 AM
From: Esther Samuel <ESamuel@The-APS.org>
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Narattaphol Charoenphandhu, MD, PhD
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Dear Dr. Charoenphandu,

I am pleased to inform you that International Physiology Committee and the Council of the American Physiological Society have approved your application for the IUPS 2009 Congress scheduled for July 27 – August 1 in Kyoto, Japan. The Society has allocated \$1,000 for you to attend the XXXVI International Congress of Physiological Sciences. We urge you to register for the Congress by April 17 in order to take advantage of early registration rates (<http://www.iups2009.com/>).

The award funds will be provided after the IUPS Congress. You will need to submit receipts providing evidence that you have attended the Congress. It is anticipated that you will need to send copies of your registration, airline, and hotel receipts. In addition, you will be asked to complete a survey designed to assess your opinions about the Congress. The survey link will be distributed to you in advance of the Congress.

Congratulations on your selection as a travel award recipient for the IUPS 2009 Congress. I hope to see you in Kyoto.

Sincerely yours,

A

Martin Frank, Ph.D.
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10 มีนาคม 2554

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ตามที่ สาขาชีวเคมีและชีววิทยาโมเลกุล สมาคมวิทยาศาสตร์แห่งประเทศไทยในพระบรมราชูปถัมภ์ ได้จัดให้มีการพิจารณาผู้สมควรได้รับรางวัลนักชีวเคมีและชีววิทยาโมเลกุล (BMB Award) ประจำปี พ.ศ. 2554 นั้น สาขาชีวเคมีและชีววิทยาโมเลกุล มีความยินดีที่จะเรียนให้ท่านทราบว่าท่านทั้งสองเป็นผู้ได้รับรางวัล นักชีวเคมีและชีววิทยาโมเลกุลดาวรุ่ง/รุ่นเยาว์ (Young BMB Award) ประจำปี พ.ศ. 2554 ร่วมกัน โดยได้รับรางวัลท่านละ 10000.- บาท และ โล่เกียติยศ ทั้งนี้ การมอบรางวัลจะดำเนินการในระหว่างการประชุมวิชาการ The Third BMB Conference ที่ จังหวัดเชียงใหม่ ในการนี้ สาขาฯ จัดให้ท่านให้สัมมนา ในเวลา ประมาณ 16:30 – 17:00 น. โดยบรรยายท่านละ 15 นาที ทั้งนี้สาขาฯ จะให้การสนับสนุนในด้านค่าเดินทางและที่พัก 1 คืน ที่โรงแรมดิเอ็มเพรส

จึงเรียนมาเพื่อทราบ และขอให้ท่านช่วยส่งหัวข้อสัมมนา และบทคัดย่อ ภายใน 15 มีนาคม 2554 หากมีข้อสงสัย โปรดติดต่อ ผศ.ดร. สุรพงษ์ พินิจกลาง (surapong_pin@utcc.ac.th, 081 3127007) หรือ รศ.ดร.ดวงพร สุทธิพงษ์ชัย (sctsc@mahidol.ac.th, โทร 02-2015609)

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(ผู้ช่วยศาสตราจารย์ ดร. สุรพงษ์ พินิจกลาง)

ประธานสาขาชีวเคมีและชีววิทยาโมเลกุล

สมาคมวิทยาศาสตร์แห่งประเทศไทยในพระบรมราชูปถัมภ์