รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลของภาวะความเป็นกรดต่อการสลายตัวและเมตาบอลิซึมของเซลไขมัน

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กรกฎาคม 2552

สัญญาเลขที่ RMU4980018

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและสำนักงานคณะกรรมการการอุดมศึกษา
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.และ สกอ.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัยและสำนักงานคณะกรรมการการอุดมศึกษาที่ให้การสนับสนุนทุนวิจัยมาโดยตลอด รศ.นพ.สุรเดช หงส์อิงค์ สำหนับการสนับสนุนเซลต้นกำเนิด ศ.นพ.บุญส่ง องค์พิพัฒนกุล ที่ช่วยเหลือด้านเงินทุนวิจัย คุณปิยะนุช ระเด่นอาหมัด และคุณวาสนา สถิตย์จันทรากุล ที่ช่วยทำงานวิจัยอย่างไม่เห็นแก่ความเหน็ดเหนื่อย และ สำนักงานวิจัยของคณะแพทยศาสตร์รพ.รามาธิบดี พร้อมด้วยเจ้าหน้าที่ทุกคนที่อำนวยความสะดวกในการทำวิจัย

สารบัญ

Abstract 1

Project Code: RMU4980018

Project Title: Role of Metabolic Acidosis on Adipocyte Lipolysis and Metabolism

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Project Period: July 2006 – July 2009

Keywords: acidosis, adipocyte, lipolysis, adiponectin, mesenchymal stem cell, stromal cell

Metabolic acidosis adversely affects musculoskeletal and endocrine systems as well as protein and lipid metabolism. Excess energy is stored as triglyceride in adipose tissue. During period of increased metabolic demand, free-fatty acid (FFA) and glycerol are released in response to catecholamine, a process called lipolysis. Catecholamine stimulates lipolysis through cAMP/protein kinase A pathway resulting in phosphorylation of hormone sensitive lipase (HSL) and perilipin (PLIN), which then cleaves triglyceride into FFA and glycerol. In vivo studies in humans and animals demonstrated decreased FFA availability in acute metabolic acidosis. The present study investigates the mechanism underlying the anti-lipolytic effect of metabolic acidosis in vitro. Mature adipocytes in culture were differentiated from human mesenchymal stem cells isolated from bone marrow. Concentrated HCl was added to lower the medium pH. Basal and isoproterenol-induced lipolysis was determined based on the amount of FFA and glycerol release into the culture medium corrected by protein content. Lowering pH from 7.4 to 6.9 suppressed basal lipolysis by upto 30% and isoproterenol-induced lipolysis by upto 70%. Manipulating cAMP/PKA system was unable to restore lipolysis. The role of serine/threonine protein phosphatase (PP) that dephosphorylates HSL and PLIN was next examined. Tautomycetin and okadaic acid, PP1 and PP2A inhibitors were able to overcome the anti-lipolysis suggesting the involvement of HSL and/or PLIN phosphorylation. In conclusion, metabolic acidosis inhibits adipocyte lipolysis through attenuation of phosphorylated HSL and/or PLIN.

Abstract 2

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Keywords: acidosis, adipocyte, lipolysis, adiponectin, mesenchymal stem cell, stromal cell

Metabolic acidosis adversely affects protein and lipid metabolism as well as endocrine function. Adipose tissue, a site where excess energy is stored as triglyceride, communicates with the rest of the body through synthesis and release adipokines such as leptin, adiponectin and TNF-alpha. Metabolic acidosis suppresses leptin in animals and cultured adipocytes. Adiponectin is known to enhance insulin sensitivity, improve endothelial function and possess anti-atherogenic and anti-inflammatory properties. Serum adiponectin is negatively correlated with cardiovascular outcome. The present study investigated the effect of metabolic acidosis on adiponectin mRNA expression and protein secretion. Adipocytes were differentiated from human mesenchymal stem cells (MSCs) isolated from bone marrow in the presence of PPAR-gamma agonist, dexamethasone and insulin. In order to reduce bicarbonate concentration, concentrated HCl was added to the culture medium lowering bicarbonate concentration from 24 to 12 mmol/L and pH from 7.4 to 6.9. Adiponectin mRNA and protein was determined at 48 and 96 hours by real time RT-PCR and ELISA respectively. Our MSCs were able to differentiate into mature adipocytes demonstrated by the morphological changes and the presence of GPDH enzyme activity. Metabolic acidosis significantly suppressed adiponectin mRNA by 40% ($p<0.005$) at 96 hours despite the slight increase in triglyceride accumulation. In conclusion, metabolic acidosis inhibits adiponectin gene transcription and protein secretion from adipocytes.

บทคัดย่อ 1

รหัสโครงการ: RMU4980018

ชื่อโครงการ: ผลของภาวะความเป็นกรดต่อการสลายตัวและเมตาบอลิซึมของเซลไขมัน

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คำหลัก: ภาวะกรด, เซลไขมัน, การสลายตัวของไขมัน, adiponectin, เซลต้นกำเนิด mesenchymal

ภาวะกรดในเลือดส่งผลร้ายต่อระบบกล้ามเนื้อ กระดูก ต่อมไร้ท่อ และเมตาบอลิซึมของโปรตีน และ ไขมัน พลังงานส่วนเกินที่ร่างกายได้รับจะถูกเก็บไว้ในรูปของไตรกลีเซอไรด์ในไขมัน เวลาที่ร่างกายจำเป็นต้องใช้พลังงาน catecholamine จะไปกระตุ้นให้เซลไขมันปลดปล่อย fee fatty acid และ glycerol ผ่านกระบวนการที่เรียกว่า lipolysis ซึ่งอาศัยการทำงานของ cAMP/PKA ในการกระตุ้นให้เกิด phosphorylation ของ hormone sensitive lipase (HSL) และ PLIN ทำให้มีการสลายตัวของไตรกลีเซอไรด์ไปเป็น free fatty acid จากการศึกษาในมนุษย์และสัตว์ทดลองพบว่าระดับของ free fatty acid ในเลือดลดลงในภาวะกรด ดังนั้นเราจึงทำการวิจัยเพื่อศึกษากลไกการยับยั้ง lipolysis in vitro โดยอาศัยเซลไขมันซึ่งถูกกระตุ้นให้เจริญเติบโตมาจากเซลต้นกำเนิด mesenchymal ที่ได้มาจากไขกระดูกในห้องทดลอง การสร้างภาวะกรดให้เกิดขึ้นในสารน้ำที่ใช้เลี้ยงเซลนั้นอาศัยการเติมกรด HCl เพื่อลดความเข้มข้นของ HCO_3 และ pH จาก 7.4 เป็น 6.9 จากนั้นวัด basal และ catecholamine-induced lipolysis จากปริมาณ glycerol ที่หลั่งออกมาในสารน้ำเลี้ยงเซล จากผลการศึกษาพบว่าภาวะกรดยับยั้ง basal lipolysis 30% และ catecholamine-induced lipolysis 70% การทดสอบ cAMP/PKA pathway พบว่าไม่เกี่ยวข้องกับการลดลงของ lipolysis สมมุติฐานอื่นที่เป็นไปได้ คือ การลดลงของ HSL และ PLIN phosphorylation ดังนั้นเราจึงศึกษาความผิดปกติของ serine/threonine protein phosphatase (PP) ซึ่งมีหน้าที่ในการ dephosphorylation ของ HSL และ PLIN ผลการทดลองพบว่า tautomycetin และ okadaic acid ซึ่งเป็นสารเคมีที่มีฤทธิ์ยับยั้ง PP1 และ PP2A สามารถเพิ่ม lipolysis ให้เท่ากับในสภาวะปกติได้ แสดงให้เห็นว่าการลดลงของ HSL และ/หรือ PLIN นั้นมีส่วนร่วมในการยับยั้ง lipolysis ในภาวะกรด สรุปได้ว่าภาวะกรดยับยั้ง lipolysis โดยทำให้มีการลดลงของปริมาณ phosphorylated HSL และ PLIN

บทคัดย่อ 2

รหัสโครงการ: RMU4980018

ชื่อโครงการ: ผลของภาวะความเป็นกรดต่อการสลายตัวและเมตาบอลิซึมของเซลไขมัน

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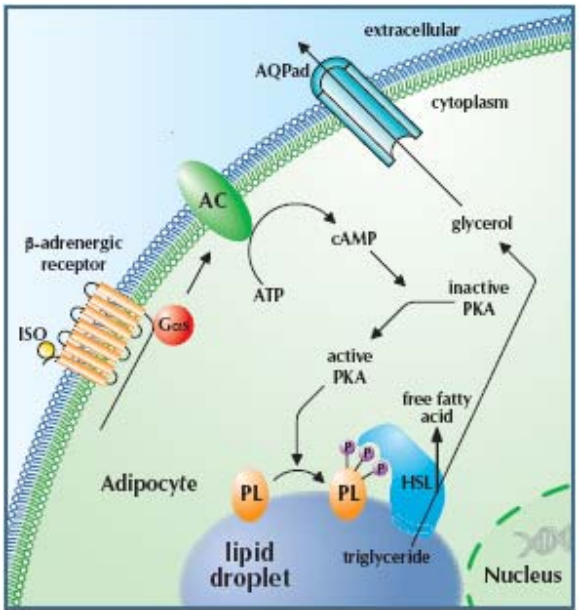
ระยะเวลาโครงการ: กรกฎาคม 2549 – กรกฎาคม 2552
คำหลัก: ภาวะกรด, เซลไขมัน, การสลายตัวของไขมัน, adiponectin, เซลต้นกำเนิด mesenchymal

ภาวะกรดในเลือดส่งผลกระทบต่อการทำงานของต่อมไร้ท่อ เมตาบอลิซึมของไขมัน และโปรตีน ไขมันนั้นนอกจากจะเป็นแหล่งสะสมพลังงานส่วนเกินแล้ว ยังผลิตโปรตีนชนิดต่างๆ เช่น leptin, adiponectin และ TNF-alpha เพื่อควบคุมการทำงานส่วนต่างๆ ของร่างกาย จากการศึกษาในอดีตพบว่าภาวะกรดสามารถยับยั้งการสร้างและหลั่ง leptin จากเซลไขมัน สำหรับ adiponectin นั้นยังไม่มีใครทำการศึกษา adiponectin มีคุณสมบัติในการเพิ่ม insulin sensitivity ช่วยให้การทำงานของเซล endothelium ดีขึ้น ป้องกันการเกิด atherosclerosis และ ลด inflammation ระดับของ serum adiponectin แปรผกผันกับ cardiovascular outcome การวิจัยนี้เป็นการศึกษาผลกระทบของภาวะกรดต่อการสร้างและหลั่ง adiponectin จากเซลไขมันที่เจริญเติบโตมาจากเซลต้นกำเนิด mesenchymal ของมนุษย์ (MSCs) ที่ได้มาจากไขกระดูกในห้องทดลอง การเลี้ยง MSCs ในสารน้ำเลี้ยวเซลที่ประกอบไปด้วย PPAR gamma agonist, dexamethasone และ insulin สามารถทำให้ MSCs เจริญเติบโตไปเป็นเซลไขมันได้ การสร้างภาวะกรดให้เกิดขึ้นในสารน้ำที่ใช้เลี้ยงเซลนั้นอาศัยการเติมกรด HCl เพื่อลดความเข้มข้นของ HCO_3 จาก 24 เป็น 12 และ pH จาก 7.4 เป็น 6.9 จากนั้นวัดระดับ adiponectin mRNA ที่ 48 และ 96 ชั่วโมงโดยวิธี real-time RT PCR และ วัดปริมาณ adiponectin protein โดยวิธี ELISA ผลการศึกษาพบว่า MSCs สามารถเจริญเติบโตไปเป็นเซลไขมันได้เป็นอย่างดี โดยจะเห็นได้จากรูปร่างและปริมาณของ GPDH enzyme ภาวะกรดนั้นทำให้มีการลดลงของ adiponectin mRNA ประมาณ 40% ที่ 96 ชั่วโมง ($P<0.005$) และ adiponectin protein ที่หลั่งออกมาในสารน้ำเลี้ยงเซลลดลงประมาณ 20% ในขณะที่มีปริมาณของ triglyceride เพิ่มมากขึ้น สรุปได้ว่าภาวะกรดยับยั้งการสร้าง adiponectin mRNA และ การหลั่ง adiponectin โปรตีนจากเซลไขมัน

Introduction

Metabolic acidosis is an inevitable consequence of chronic kidney disease secondary to diminished acid secretion from the diseased kidneys. Metabolic acidosis complicates several other conditions such as hypotension, sepsis, diabetic ketoacidosis, diarrhea, alcohol intoxication and poisoning. Metabolic acidosis is also a key manifestation of distal renal tubular acidosis, a condition prevalent among women in northeastern part of Thailand. Protein-energy malnutrition, inflammation, increased protein catabolism, reduced albumin synthesis, insulin resistance and diminished thyroid hormone secretion are associated with metabolic acidosis.[1-3] Excess energy is stored in adipose tissue in a form of triglyceride. During periods of increased metabolic demand such as physical exercise, trauma and stress, triglycerides are hydrolyzed into free fatty acid and glycerol (lipolysis) under the influence of catecholamine. Free fatty acid is further metabolized by various energy demanding tissues.[4, 5] The binding of catecholamine to ß-adrenergic receptor results in an increase in cAMP, which stimulates protein kinase A (PKA) that in turn phosphorylates hormone sensitive lipase (HSL) located in the cytoplasm of fat cells. The phosphorylation of HSL in response to PKA occurs at Ser563, 659 and Ser660.[6, 7] HSL is also negatively regulated through phosphorylation at Ser565 by AMP-activated protein kiase (AMPK) and calcium/calmodulin-dependent protein kinase II.[8] In addition to phosphorylation of HSL, PKA activation also phosphorylates perilipin (PLIN) located on the lipid droplet surface. The function of PLIN under basal condition is prevention of lipolysis. Phosphorylation of PLIN allows the translocation of phosphorylated-HSL (p-HSL) from cytosol to lipid droplet surface gaining access to triglycerides.



Adipolysis assay - Induction of adipolysis by β-adrenergic receptor agonists. Binding of isoproterenol (ISO) to the β-adrenergic receptor activates the G-protein, Gαs, which stimulates adenylate cyclase (AC) to produce cyclic AMP (cAMP). Protein kinase A (PKA) is activated by cAMP to phosphorylate the lipid droplet surface protein, perilipin (PL). Hormone-sensitive lipase (HSL) docks onto phosphorylated PL and breaks down triglyceride into glycerol and free fatty acid. Glycerol is released into the extracellular space through aquaporin adipose (AQPad).

Figure 1. Catecholamine induced Adipocyte lipolysis

Recently, adipocyte triglyceride lipase (ATGL) was identified as a second major lipase in adipose tissue. Unlike HSL that located mainly in the cytoplasm, ATGL is located both in the cytoplasm and on the surface of lipid droplet. ATGL does not appear to be regulated directly by PKA phosphorylation.[9] Data from both murine and human seem to suggest the importance of ATGL in basal lipolysis.[10, 11] ATGL activity is strongly stimulated by an activator protein annotated as comparative gene identification-58 (CGI-58). In unstimulated adipocytes, CGI-58 binds to PLIN preventing its access to ATGL. Phosphorylation of PLIN releases CGI-58 allowing the interaction with ATGL.[12]

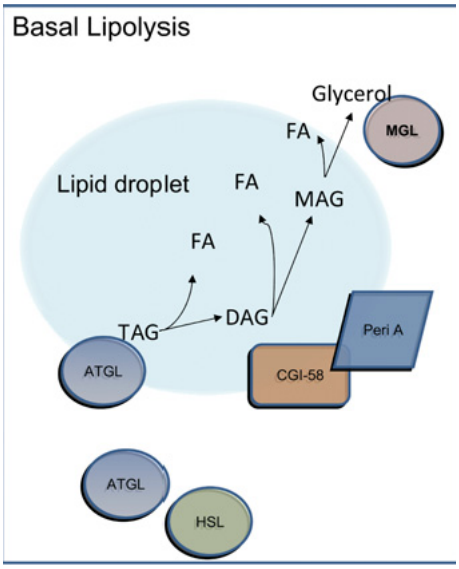


Figure 2. ATGL in basal lipolysis

Over three decades ago, the anti-lipolytic effect of metabolic acidosis was observed both in vivo and in vitro.[13-16] The decrease in cAMP was proposed to be responsible for the inhibition of lipolysis. However, repletion of cAMP and/or inhibition of phosphodiesterase (PDE), the cAMP metabolizing enzyme, was not able to restore adipocyte lipolysis. The mechanism underlying the anti-lipolytic effect of metabolic acidosis remains elusive. The anti-lipolytic effect of insulin is mediated by PI3-kinase/Akt pathway, whereas AMP-activated protein kinase has also been shown to exert the anti-lipolytic effect.[17, 18] The signaling mechanisms mediating the effect of extracellular acidosis varied from model to model. In bone, extracellular acidosis induced elevation of inositol-3-phosphate (IP3) and cytosolic free Ca2+ concentration from activation of phospholipase C/protein kinase C pathway.[19, 20] In aortic smooth muscle cells and heart, extracellular acidosis stimulated p38-MAPK, whereas, in immune cells, PI3-kinase/Akt and ERK-MAPK were activated.[21-23]

In addition to storage and release of metabolic fuel, adipocytes communicate with the rest of the body through production of hormones and cytokines including leptin, adiponectin, TNF-alpha and interleukin-6. Leptin, after binding to specific receptors in the hypothalamus regulates energy balance by reducing food intake and increase energy expenditure. A study in renal failure animals revealed lower serum leptin in animals with acidosis (Figure 3).[24] An in vitro study using mouse adipocytes demonstrated direct inhibition of leptin secretion by metabolic acidosis (Figure 4).[25] Similarly, an increase in serum leptin levels was found in patients with dRTA after alkaline supplement.[26] Adiponectin is another adipocyte-derived plasma protein with insulin sensitizing, anti-atherogenic and anti-inflammatory properties.[27] Plasma levels of adiponectin are negatively associated with obesity, insulin resistance, metabolic syndrome, cardiovascular disease and mortality.[28]. The effect of metabolic acidosis on adiponectin secretion from adipocytes is largely unexplored.

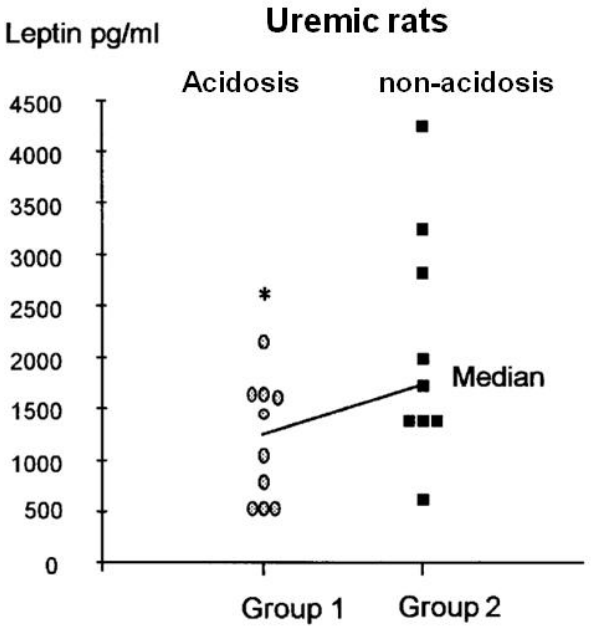


Figure 3. Leptin secretion in acidotic and non-acidotic uremic rats (Teta D, Cli Sci 1999, 97:363-368)

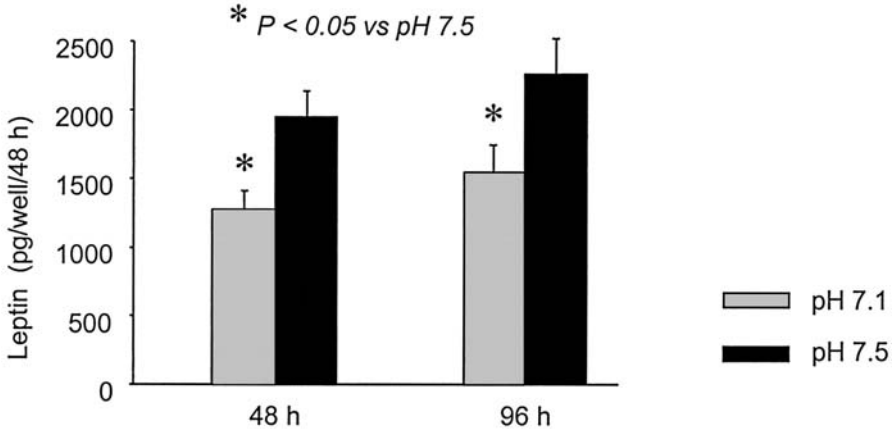


Figure 4. Leptin secretion in from mouse adipocytes in acidic medium (Teta D, J AM Soc Nephrol 2003, 9(14):2248-54)

Under appropriate conditions, multipotent mesenchymal stem cells (MSCs) can differentiate into chondrocytes, adipocytes and fibroblasts (Figure 5) Human MSCs (hMSCs) can be induced to differentiate into functional adipocytes with characteristics similar to those observed in primary cultures of human preadipocytes (which are lineage-committed progenitor cells) [29, 30]. To date several murine fat cell

lines have been utilized in experimental fat cell research. Since there are important species-specific differences in adipocyte function, fat cells differentiated from hMSCs may be of value in this respect. In a preliminary study, we have established the differentiation protocol for adipocytes from hMSCs using DMEM:DMEM/F12 1:1 containing PPAR-gamma agonist (Troglitazone), dexamethasone, isobutyl-1-methylxanthine (IBMX) and insulin. The differentiation process takes 3-4 weeks followed by 1 week of adipocyte maturation in the maintenance media containing dexamethasone and insulin. In our experiments, 30-70% of hMSCs upto the 5th passage were able to differentiate into mature adipocytes. After the 5th passage, the cells lost the differentiation ability.

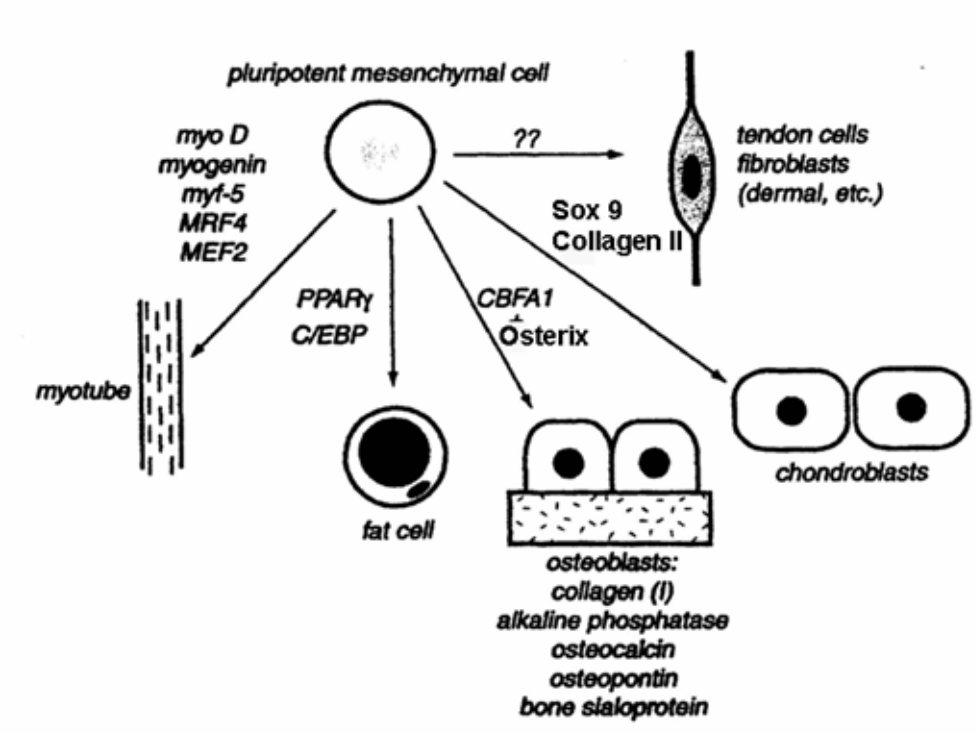


Figure 5. The differentiation pathways of multipotent MSCs

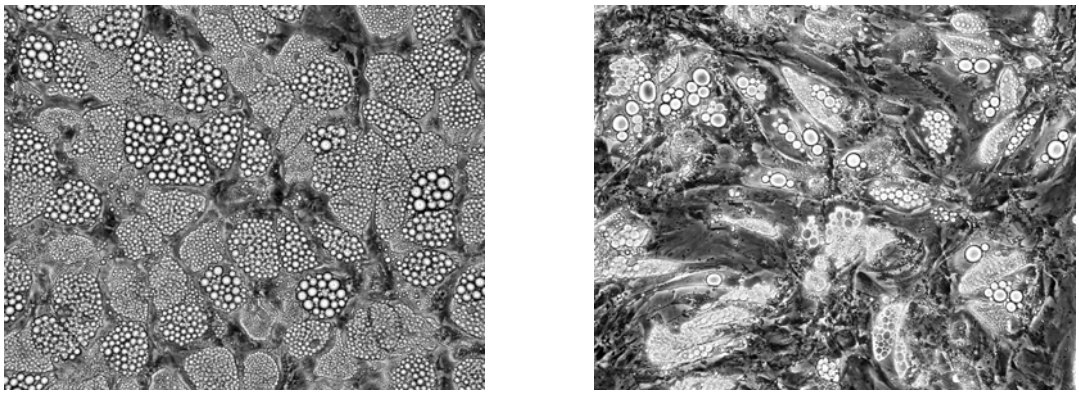


Figure 6. Adipocytes differentiated from hMSCs

The following study is an in vitro study that investigated the effect of metabolic acidosis on lipolysis of cultured hMSCs-derived adipocytes, the signaling mechanism of metabolic acidosis in adipocytes and the role of chronic metabolic acidosis on adiponectin gene expression and secretion.

Objectives

Part 1: The effect of metabolic acidosis on adipocyte lipolysis

1. To examine the effect of metabolic acidosis on adipocyte lipolysis
2. To investigate the signaling mechanisms underlying the alteration of adipocyte lipolysis

Part 2: The effect of chronic metabolic acidosis on adiponectin expression in cultured adipocytes

1. To examine the effect of metabolic acidosis on adiponectin mRNA and protein secreted from adipocytes

Summary of results

Part 1: The effect of metabolic acidosis on adipocyte lipolysis

1. Acute and chronic metabolic acidosis inhibits basal and catecholamine-induced lipolysis by 30-70%
2. Interfering with cAMP/PKA, PLC/PKC/IP3, p38 –MAPK, ERK-MAPK and AMP-activated protein kinase pathway was unable to restore adipocyte lipolysis
3. Inhibition of Ca2+/calmodulin-dependent kinase may be able to restore adipocyte lipolysis during metabolic acidosis suggesting its involvement in the signaling pathway.

4. Combination of protein phosphatase 1 and 2a inhibitor (Tautomycetin and Okadaic acid) was able to restore adipocyte lipolysis by upto 90% suggesting the impaired phosphorylation of HSL and/or PLIN was responsible for the attenuation of lipolysis

Part 2: The effect of chronic metabolic acidosis on adiponectin expression in cultured adipocytes

1. Chronic metabolic acidosis upto 96 hours inhibits adiponectin mRNA expression by real-time RT-PCR
2. Chronic metabolic acidosis upto 96 hours inhibits adiponectin protein secreted from cultured adipocytes measured by ELISA

Conclusion

Part 1: The effect of metabolic acidosis on adipocyte lipolysis

Metabolic acidosis inhibits adipocyte lipolysis possibly through activation of Ca2+/calmodulin-dependent kinase resulting in attenuated phosphorylation of HSL and/or PLIN

Part 2: **The effect of chronic metabolic acidosis on adiponectin expression in cultured adipocytes**

Chronic metabolic acidosis inhibits adiponectin mRNA expression and adiponectin protein secretion from cultured adipocytes suggesting adverse influence of metabolic acidosis on cardiovascular outcome

Materials and Methods

Isolation and culture of bone marrow derived MSCs

Bone marrow samples were obtained using a bone marrow biopsy needle inserted through posterior iliac crest of a healthy bone marrow donor after an informed consent. Bone marrow mononuclear cells (BMMCs) were separated by density gradient centrifugation with 1.073 g/ml Percoll solution (Sigma, MO, USA). Briefly, 10 ml of heparinized bone marrow cells were mixed in an equal volume of Dulbecco's Modified Eagle's Medium (DMEM) (BioWhittaker, MD, USA) and centrifuged at 900g for 10 min at room temperature. The washed cells were resuspended in DMEM at a density of 4 x 10^7 cells/ml, and 5 ml aliquot was layered over 1.073 g/ml Percoll solution and centrifuged at 1,000g for 30 min at room temperature. The interface mononuclear cells were collected and washed twice with DMEM. Total cell count and cell viability were evaluated by 0.2% Trypan blue exclusion. A total of 2x10^6 cells/ml of BMMCs were cultured in DMEM complete medium supplemented with 10% fetal bovine serum (FBS) (GibcoBRL, NY, USA) and 1% penicillin-streptomycin (GibcoBRL) at 37 C in 5% CO2 incubator. On day 3 of cultivation, non-adherent cells were discarded and this process was repeated every 4 days. Upon 90% confluency, MSCs were trypsinized by 0.05% trypsin (Gibco BRL) and passaged for the next expansion. This study has been approved by ethical committee on Research Involving Human Subjects at Ramathibodi hospital, Mahidol University.

Adipocyte differentiation from hMSCs

HMSCs passage 1-5 were grown in multi-well tissue culture plates (Nunc, Denmark) until 1-2 days post-confluency. Adipocyte differentiation was induced in DMEM:DMEM/F12 (Hyclone) 1:1+10% FBS containing Troglitazone (P-PARγ agonist) 5 ug/mL, dexamethasone 10^{-6} M , IBMX (phosphodiesterase inhibitor) 25 ug/mL and insulin 10 ug/mL. The media was changed twice per week. The differentiation was continued for 3 weeks followed by 1 week of adipocyte maturation in the DMEM:DMEM/F12 1:1 containing dexamethasone 10^{-6} M and insulin 10 ug/mL

Induction of metabolic acidosis in vitro

Adipocytes differentiate well in DMEM/F12, a HEPES based medium, that normally gives pH of 7.2-7.3 and HCO_3 concentration of 8 mmol/L in 5% CO2.[30-32] We adopt to add DMEM, a bicarbonate based media, to DMEM/F12 in order to obtain a

more physiological environment. To simulate metabolic acidosis in vivo, hydrochloric acid (HCl) was added to DMEM:DMEM/F12 2:1 for the entire treatment period. pH, pCO2 and HCO_3 concentration were as follow

Table 1. Media gas analysis

HCL (ul/mL of media)	pH	pCO_2 (mmHg)	HCO_3 (mmol/L)
0	7.4	39	23
2	6.9	38	10

Reagents

Isoproterenol, insulin, IBMX and dexamethasone, free glycerol reagent, glycerol standard solution, Bradford reagent and triglyceride reagent, protease inhibitor cocktails, phosphatase inhibitor solution 1 and 2 were purchased from Sigma (St Louis, USA). Forskolin, dBuCAMP, Okadaic acid, tautomycetin, SB203580, PD98059, wortmannin, dorsomorphin, trifluoperazine hydrochloride, calmidazolium, KN-62 and W-7 were purchased from Tocris (Bristol, UK).

Determination of adipocyte lipolysis

The night before the experiment, the media was changed to DMEM:DMEM/F12 2:1 with 1% FBS. On the day of the experiment, after washing with PBS, the cells were pre-incubated with various inhibitors or activators for 1-2 hours in 125 ul of incubation solution (phenol red free-DMEM:DMEM/F/12 2:1 + 2% albumin). After the pre-incubation was complete, the media was removed and subsequently changed to incubation solution containing isoproterenol +/- HCL +/- various inhibitors or activators as indicated. Incubation was continued for another 2 hours before the media was collected for glycerol and free fatty acid analysis. After washing with PBS, lysis of the cells was performed in the presence of cell lysis buffer containing 5% triton-X 100, 20 mM tris, 150 mM NaCl and 2 mM EDTA. After few freezing and thawing cycles, the cells were visualized under the microscope to confirm complete cell lysis. The lysis buffer was further diluted 1:10 before protein concentration was analyzed by Bradford method.

Glycerol analysis was performed in 96-well plate by adding 25 ul of the media to 175 ul of free glycerol reagent. The plate was incubated for 15 minutes at room temperature prior to reading the absorbance at 550 nM in ELISA reader. Free fatty

acid analysis was performed using Free Fatty acid detection kit (Zen-Bio, USA) following manufacturer instruction.

Determination of protein phosphatase (PP) activity

HMSCS were plated and differentiated in 6-well plates. On the day of the experiment, matured adipocytes were exposed to DMEM:DMEM/F-12 2:1 +/- HCL 2 ul/mL for 5, 15, 45 and 90 minutes at 37°C in 5% CO_2 incubator. The cells were washed in cold PBS, lysed in cell lysis buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 20 mM, Imidazole and 4% protease inhibitor cocktail, scraped off the plates and homogenized though 23 gauge needle several times. The amount of the protein was measured by BCA protein assay and stored at -80°C until assay.

Immunoprecipitation of PP1 and PP2A and determination of protein phosphatase activity were performed using Ser/Thr Phosphatase Assay Kit 1 (Millipore, USA) according to the manufacturer's instruction. Briefly, anti PP1 and PP2A antibodies were added to the protein samples and incubated with protein A agarose beads for 2 hours. After the incubation was complete, supernatant was removed and the beads were washed several times in TBS. Phosphatase activity was determined based on its ability to dephosphorylate the phosphopeptide (R-K-pTI-R-R. The amount of phosphate release was measured by malachite green method and read at absorbance 650 nM as described by manufacturer.

Adiponectin mRNA expression by real-time RT PCR

After adipocytes were fully matured, the media was changed to DMEM:DMEM/F-12 2:1 + 10% FBS +/- HCL 2 ul/mL in order to lower media pH. The media was changed every other day. After 48 and 96 hours, the cells were harvested for RNA extraction and the media was collected for measurement of adiponectin protein by ELISA (Zen-Bio, USA). Total RNA was isolated using Trizol (Life Technologies, NY, USA) method as described by manufacturer. cDNAs were reverse transcribed from 1 ug of total RNA using Reverse Transcription System (Promega, WI, USA) with random hexamer as primer as described by manufacturer. cDNAs obtained from the reaction were diluted 1:5 in DNAse free water. Quantitative real-time RT-PCR was performed using ABI Prism 7500 Sequence Detection System (Applied Biosystems, CA, USA). 3-6 uL of cDNAs was analyzed in 25 ul reaction of quantitative real-time PCR mastermix. Multiplexed PCR reaction was performed with

both target and reference genes (beta-actin) in the same reaction. Each sample was analyzed in triplicate. The probe for beta-actin was fluorescently labeled with CAL Flur Orange 560 and BHQ (Biosearch Tech, USA), whereas the probes for adiponectin were labeled with 6-carboxyfluorescein and BHQ. Primer concentrations for adiponectin and actin were 50 and 20 nM respectively. Probe concentrations of adiponectin and actin were 200 nM. The nucleotide sequences of primers and probes are shown in Table 2. Relative expression levels of the gene of interest, normalized by the amount of beta-actin, were calculated by Sequence Detection Software version 1.2 (Applied Biosystems) using Relative Quantification Study approach. The average values of ΔCts from each sample were used for statistical analysis.

Table 2. Primer and Probe Sequences

Gene	Forward and reverse Primers (5′-3′)	Probe (5′-3′)
Adiponectin	cccaaagaggagagaggaagct gccagagcaatgagatgcaa	ttcccagatgccccagcaagtgtaac
Beta-Actin	ttgccgacaggatgcagaa gccgatccacacggagtact	agcacaatgaagatcaagatcattgctcctcct

Determination of triglyceride and protein concentration

After removal the media and washing with PBS, the cells were lysed in cell lysis buffer containing 5% triton-X 100, 20 mM tris, 150 mM NaCl and 2 mM EDTA. After few freezing and thawing cycles, the cells were scraped of the plates, homogenized 20-25 strokes through 23 guage needle on ice. Supernatant was used for analysis of protein by Bradford method. The rest of the cell samples were heat at 80°C for 5 minutes to solubilize triglyceride into solution. After centrifugation, cell supernatant was transferred into a new tube and incubated with Triglyceride reagent (enzyme lipase) for 2 hours in 37°C water bath to convert all triglyceride into glycerol. Measurement of triglyceride was carried out using Free glycerol reagent as above.

Results

Establish a protocol for adipocyte differentiation from hMSCs

We have been able to establish a protocol for adipocyte differentiation from hMSCs as described in Materials and Methods section above. Maturation of adipocytes is confirmed by the presence of glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity using enzyme kinetic method. GPDH has ability to oxidize NADH resulting in the decrease in its activity. The slopes of the graph are used to calculate the enzyme activity (figure 2). Moreover, these adipocytes showed lipolytic activity in basal as well as in stimulated condition as determined by glycerol and FFAFFA release into the culture medium see below

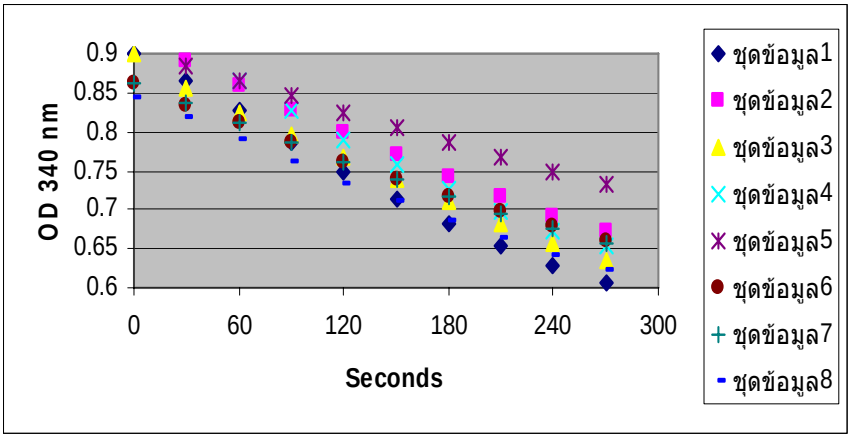


Figure 7. GPDH enzyme activity of matured adipocytes

Part 1: The effect of metabolic acidosis on adipocyte lipolysis

1. Role of metabolic acidosis on adipocyte lipolysis

The effect of acute and chronic metabolic acidosis on basal and isoproterenol-induced adipocyte lipolysis was next examined. Acute metabolic acidosis (2-12 hours) suppressed basal lipolysis by 20-70% as determine by glycerol release ($P<0.05$), while chronic metabolic acidosis (24-72 hours) variably suppressed lipolysis by 0-50 % (Figure 8). The optimum concentration of isoproterenol-induced lipolysis was next examined. Isoproterenol 1 uM has been shown to exert maximal stimulation on lipolysis and metabolic acidosis attenuated glycerol release at all concentrations studied. At pH 7.4, isoproterenol was able to enhance adipocyte lipolysis by 2-7 folds (Figure 9). Acute and chronic metabolic acidosis inhibited isoproterenol-induced lipolysis by 30-90% ($P<0.01$-0.0005) (Figure 10)

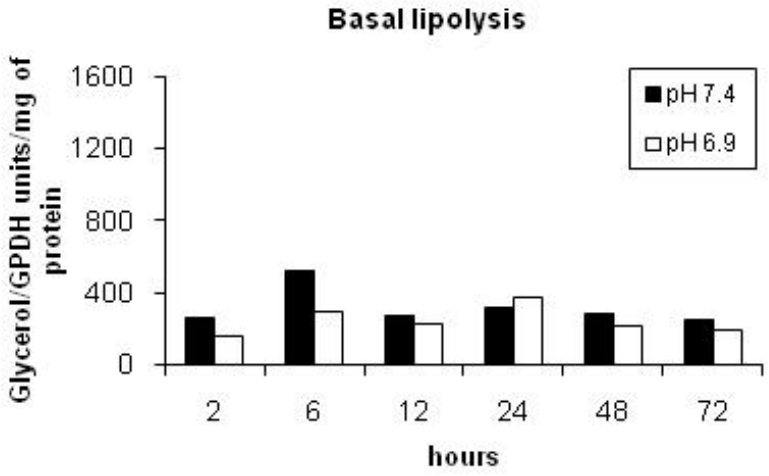


Figure 8. Acute and chronic basal lipolysis under pH 7.4 and 6.9

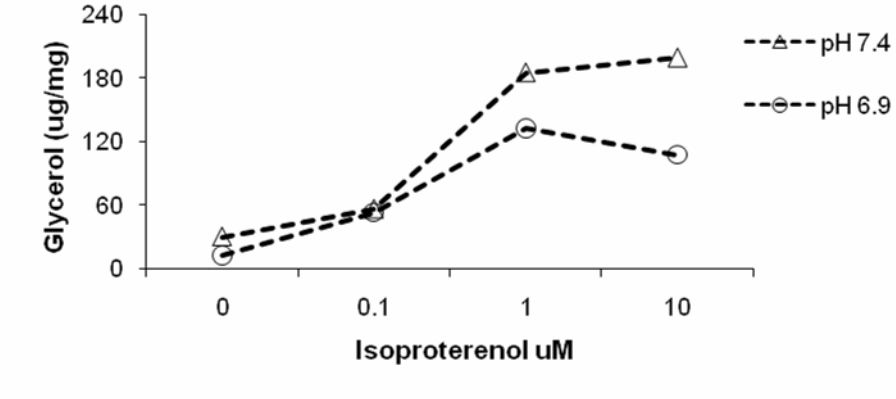


Figure 9. Isoproterenol induced adipocyte lipolysis

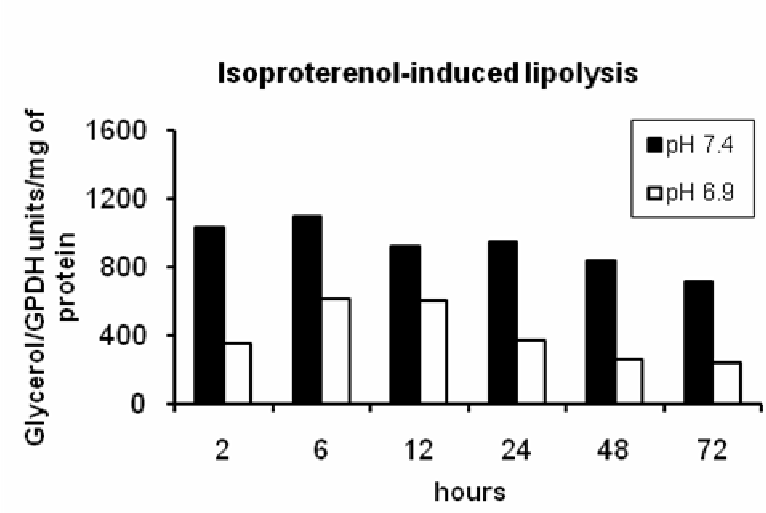


Figure 10. Acute and chronic isoproterenol-induced lipolysis under pH 7.4 and 6.9

The decrease in lipolysis was also confirmed by the amount of FFA release. Glycerol and FFA decreased in response to metabolic acidosis in a dose dose-dependent manner (Figure 11 and 12).

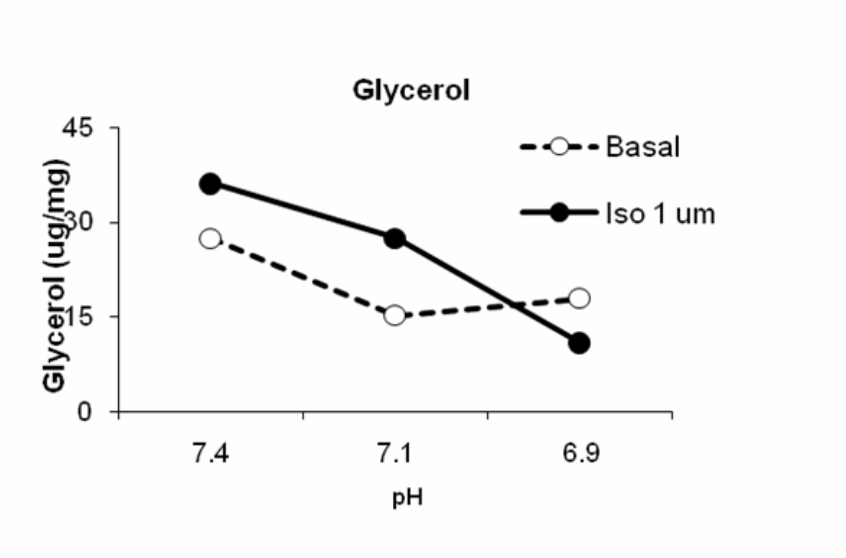


Figure 11. Dose dependent decrease in glycerol release

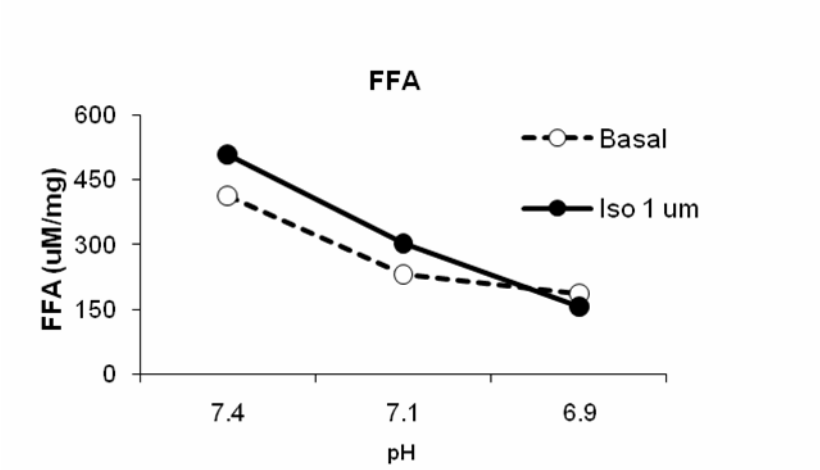


Figure 12. Dose dependent decrease in FFA release

2. The underlying mechanisms of anti-lipolytic effect of metabolic acidosis

3.1 Phospholipase C and inorsitol-3-phosphate

Extracellular H+ is sensed by proton sensors present on the cell surface. These G-protein-coupled receptors are signaled through activation of phospholipase C and/or cAMP pathways. First, we examined the involvement of phospholipase C (PCL) pathway in metabolic acidosis-mediated inhibition of adipocyte lipolysis. $CuCl_2$, an inhibitor of proton sensor resulted in a further inhibition of lipolysis (data not shown) suggesting that the effect of metabolic acidosis on inhibition of adipocyte lipolysis is mediated through other mechanism. Similarly, 2-APB, an IP3 receptor inhibitor, and U-73122, a PLC inhibitor, attenuated adipocyte lipolysis excluding the involvement of PLC/IP3 pathway in antilipolytic effect of metabolic acidosis (data not shown).

3.2 Cyclic AMP and protein kinase A pathway

Since catecholamine stimulates lipolysis through activation of cAMP/PKA pathway, it is possible that metabolic acidosis interferes with catecholamine action. The confluent adipocyte cell layers were preincubated with various activators and inhibitors of cAMP/PKA pathway including forskolin, a direct activator of adenylate cyclase, dibutyryl cAMP (dBucAMP), a non-hydrolyzable cAMP analog, 8Bromo-cAMP, a hydrolyzable cAMP analog and IBMX, a non-specific PDE inhibitor for upto 2 hours. Isoproterenol-induced lipolysis in the presence of these activators and inhibitors were determined. Forskolin, dBucAMP and IBMX were unable to restore adipocyte lipolysis (Figure 13, 14, 15 and 16)

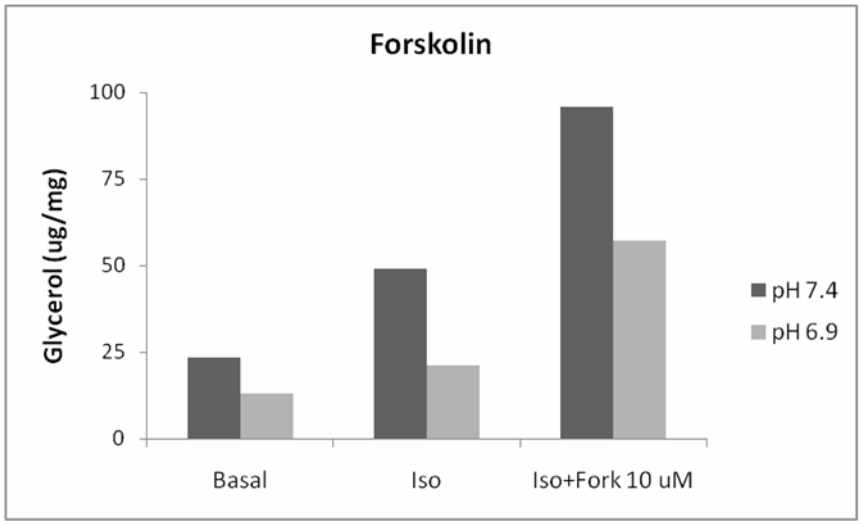


Figure 13. Forskolin and adipocyte lipolysis

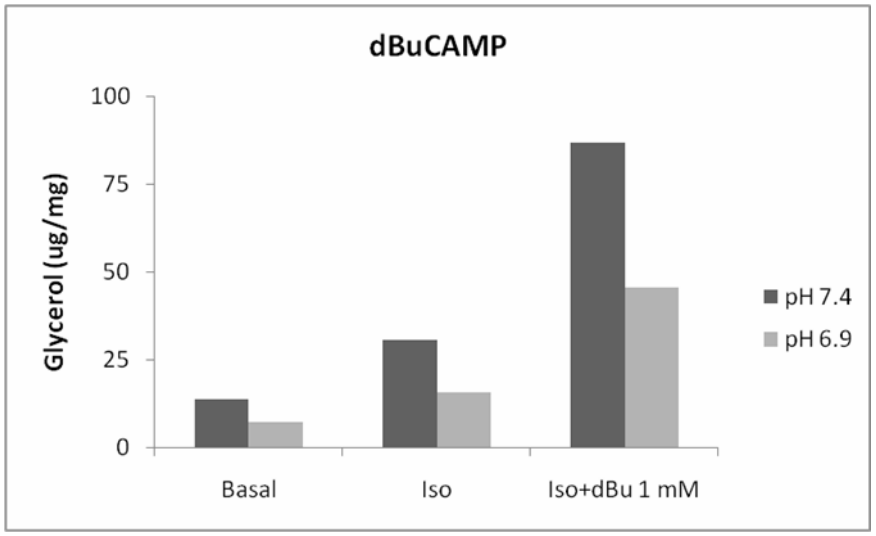


Figure 14. dBucAMP and adipocyte lipolysis

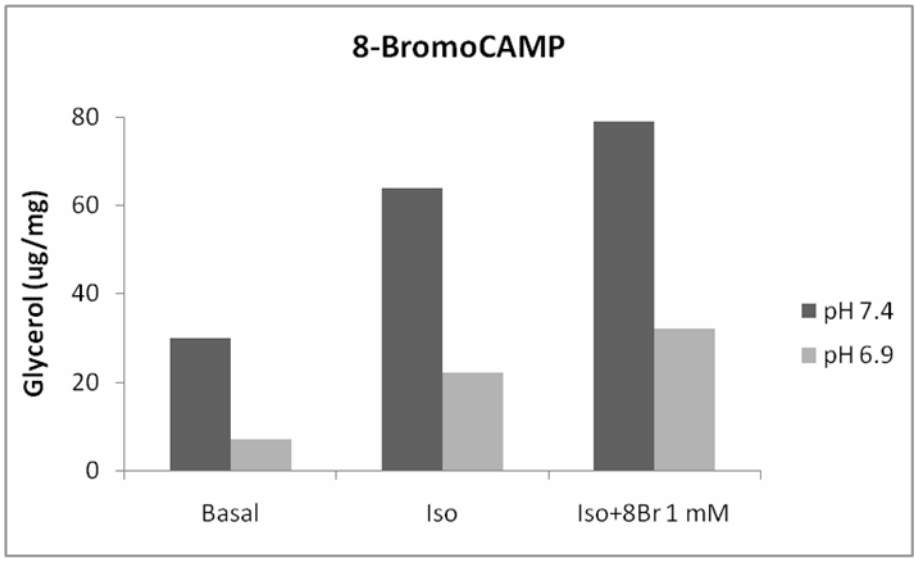


Figure 15. 8-Bromo-cAMP and adipocyte lipolysis

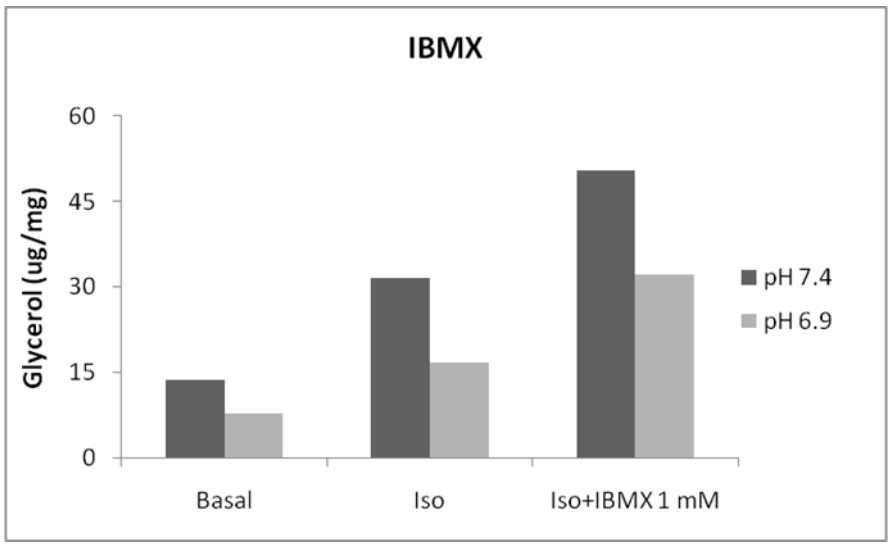


Figure 16. IBMX and adipocyte lipolysis

The effects of two specific PDE 3 and PDE 4 inhibitors, cilostamide and rolipram were next examined. Cilostamide 2 uM and rolipram 50 uM were unable to restore adipocyte lipolysis (Figure 17).

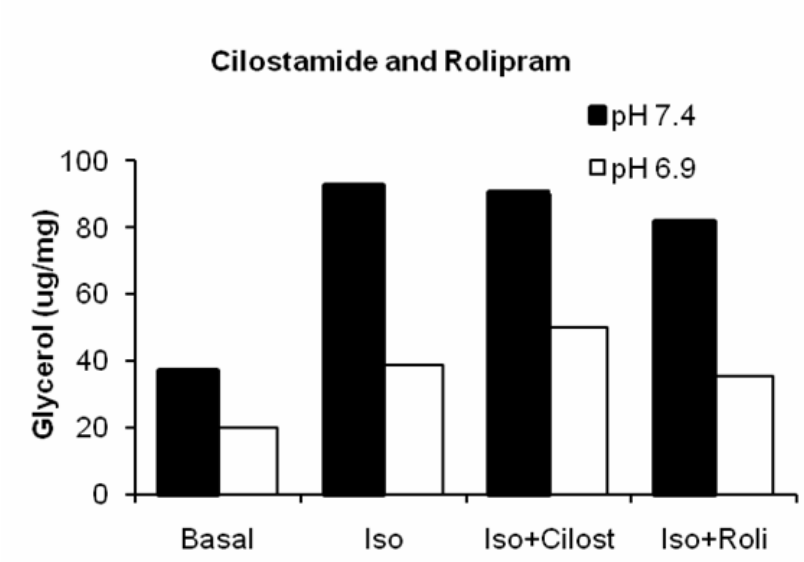


Figure 17. Cilostamide and rolipram and adipocyte lipolysis

3.3 *Protein phosphatases*

Normally, protein phosphatases dephosphorylate the target proteins at serine/threonine (PP1 and PP2A) and/or tyrosine (PP2B) residues in order to make the effect of the target protein transitory. The decreased phosphorylation of HSL and PLIN were likely responsible for the anti-lipolytic effect of metabolic acidosis. In order to confirm this, the confluent adipocyte cell layers were preincubated with tautomucetin 10 uM, a preferential PP1 inhibitor and okadaic acid 2 uM, a preferential PP2A inhibitor for upto 2 hours before basal and Isoproterenol-induced lipolysis in the presence of these inhibitors were determined. Tautomycetin and Okadaic acid partially restored basal and isoproterenol-induced lipolysis (Figure 18 and 19). The combination of the two nearly completely restored adipocyte lipolysis suggesting the decreased phosphorylation of HSL and/or PLIN was responsible for anti-lipolytic effect of metabolic acidosis. However, this did not seem to occur through the downregulation of cAMP/PKA pathway, the major regulatory pathway of adipocyte lipolysis. FK-506, the PP2B inhibitor had no effect on adipocyte lipolysis (data not shown).

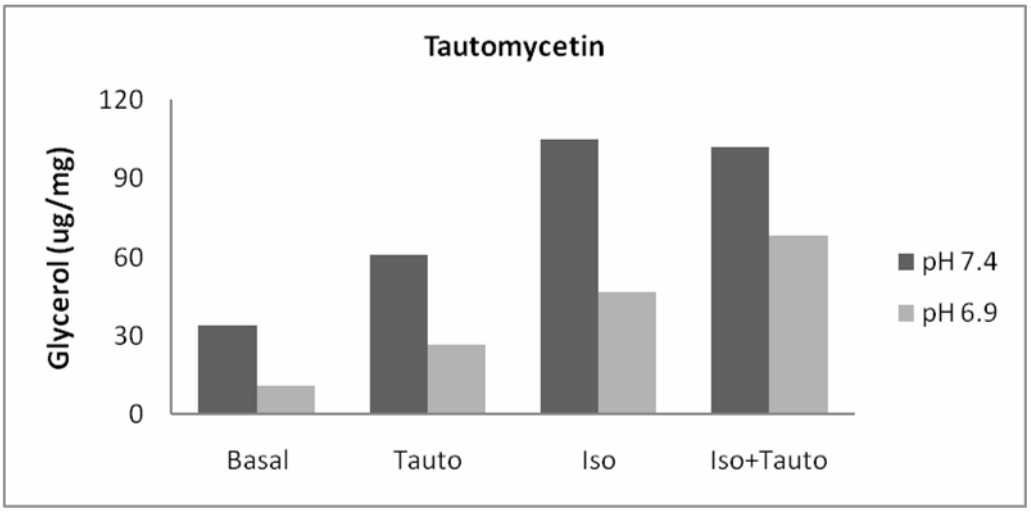


Figure 18. Tautomycetin and adiplocyte lipolysis

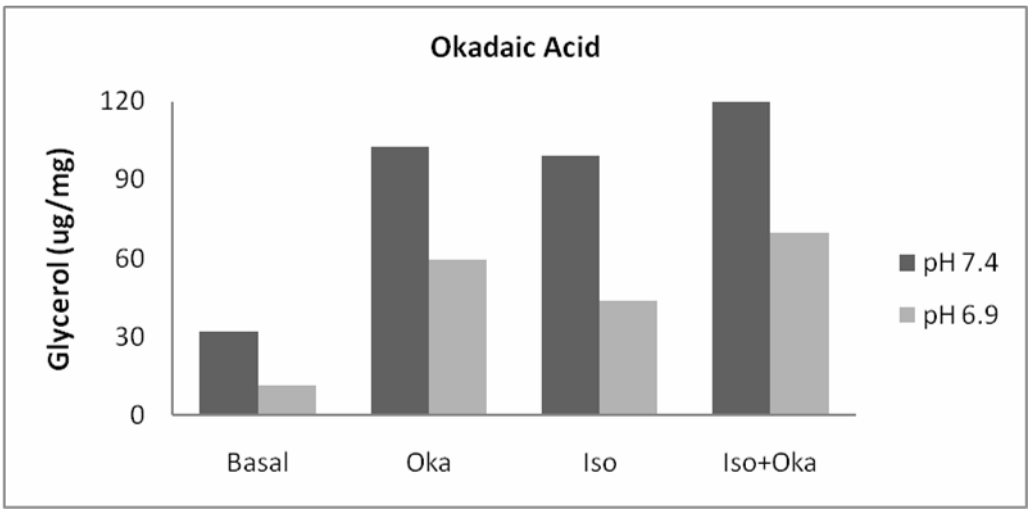


Figure 19. Okadaic acid and adipocyte lipolysis

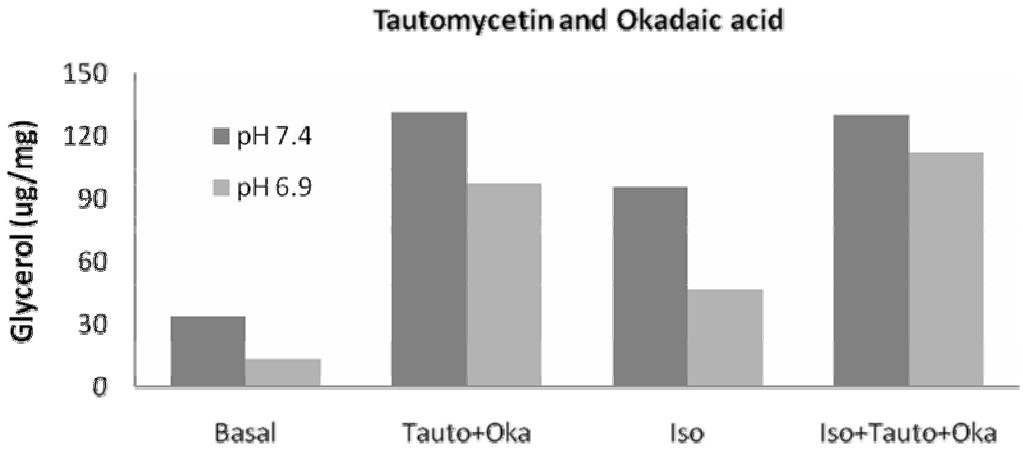


Figure 20. Tautomycetin and okadaic acid and adipocyte lipolysis

We next examined whether metabolic acidosis enhanced protein phosphatases activities. Using immunoprecipitation method with antibodies specific to PP1 and PP2A, protein phosphatases activities were determined by the ability to hydrolyze phosphopeptide substrate. The concentration of phosphate was measured by calorimetric method. We found that metabolic acidosis augmented PP2A only slightly but markedly suppressed PP1 (Figure 21 and 22). These results suggested that the decline in HSL and/or PLIN phosphorylation was not due to the activation of protein phosphatases and pathways other than cAMP/PKA were responsible for the suppressed phosphorylation.

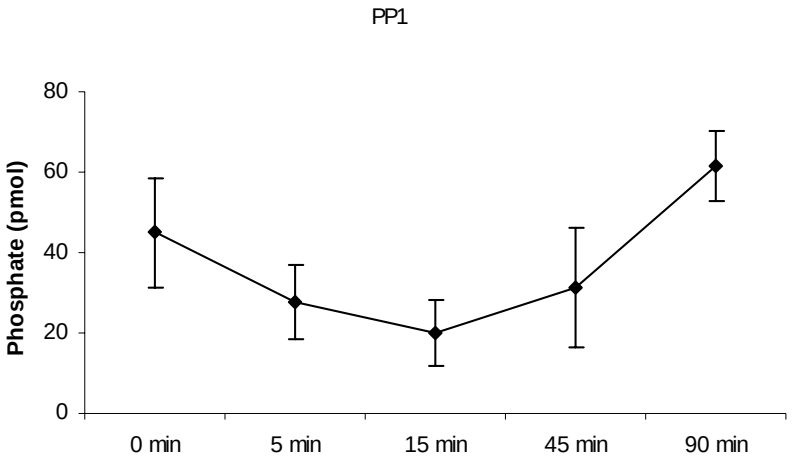


Figure 21. PP1 activity in cultured adipocytes during metabolic acidosis

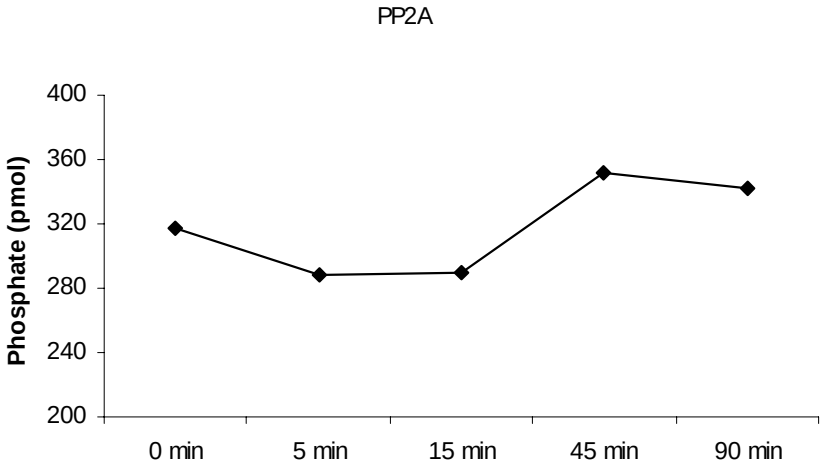


Figure 22. PP2A activity in cultured adipocytes during metabolic acidosis

3.4 *P38 –MAPK, ERK-MAPK, PI3-kinase/Akt, Protein kinase C and AMP-activated protein kinase*

By using the following inhibitors, we investigated the role of these signaling pathways in inhibition of lipolysis

Signaling pathway	Inhibitors	Reference
P38-MAPK	SB203580 1-10 uM	Stathopoulou K[22]
ERK-MAPK	PD98059 50-100 uM	Martinez D[23]
PI3-kinase/Akt	Wortmannin 0.1-1 uM	Xue B[33]
Protein kinase C	Chelerythrine 10-20 uM	Xue B[33]
AMP-activated protein kinase	Dorsomorphin 10-100 uM	Gao Y[34]

The confluent adipocyte cell layers were preincubated with the inhibitors for upto 2 hours before isoproterenol-induced lipolysis was determined. None of these inhibitors were able to restore adipocyte lipolysis (Figure 23-27). It is worth mentioning that PD98059, chelerythrine and dorsomorphin further inhibited lipolysis suggesting that ERK-MAPK, protein kinase C and AMP-activated protein kinase pathways are likely to promote adipocyte lipolysis

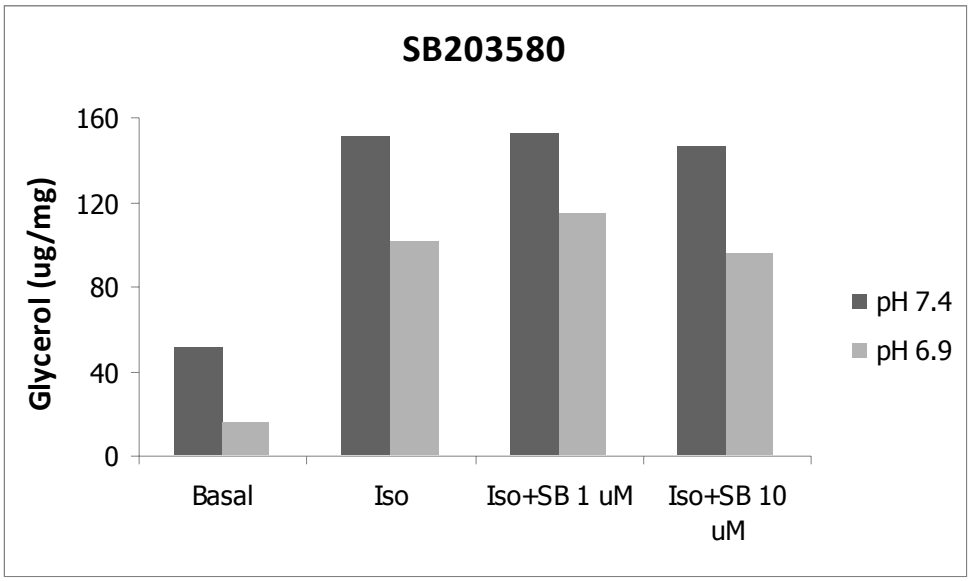


Figure 23. SB203580 and adipocyte lipolysis

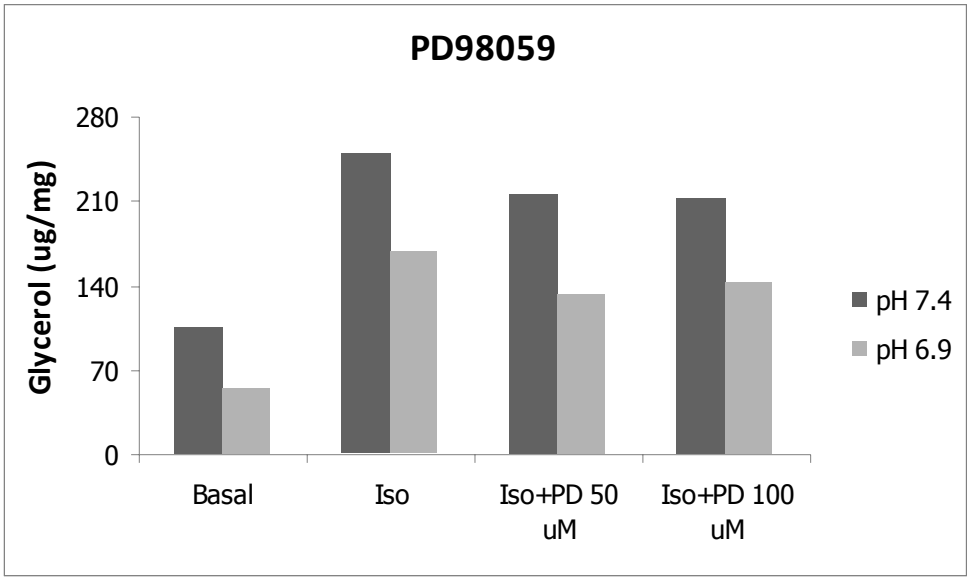


Figure 24. PD98059 and adipocyte lipolysis

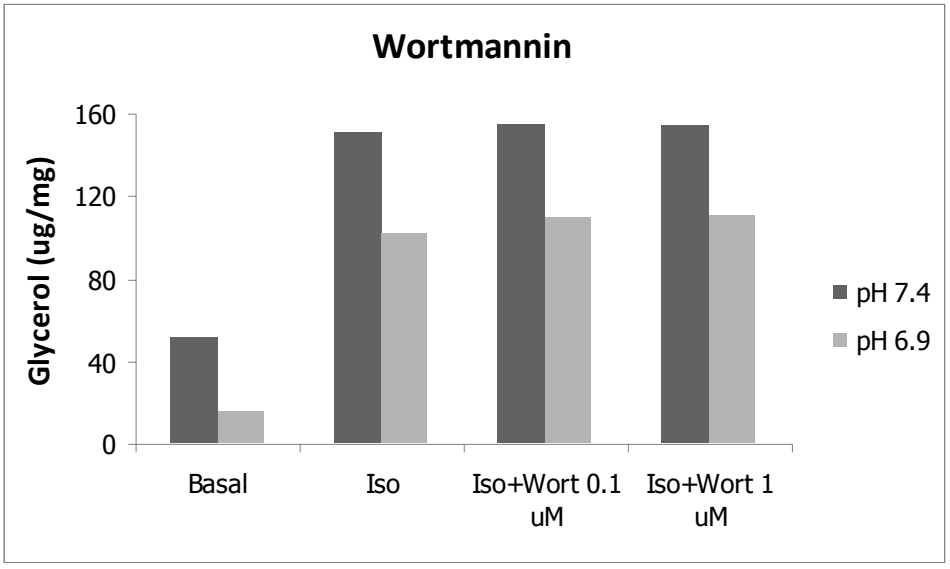


Figure 25. Wortmannin and adipocyte lipolysis

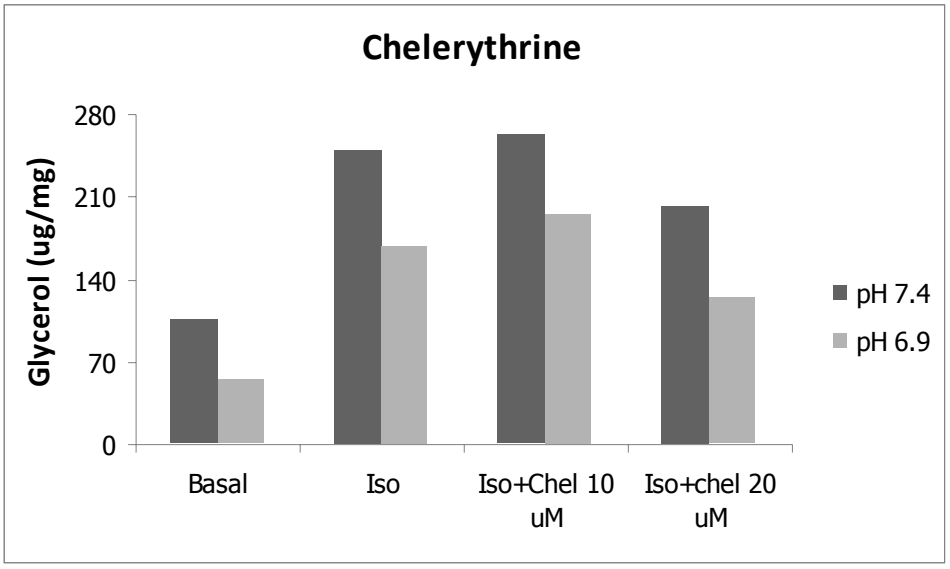


Figure 26. Chelerythrine and adipocyte lipolysis

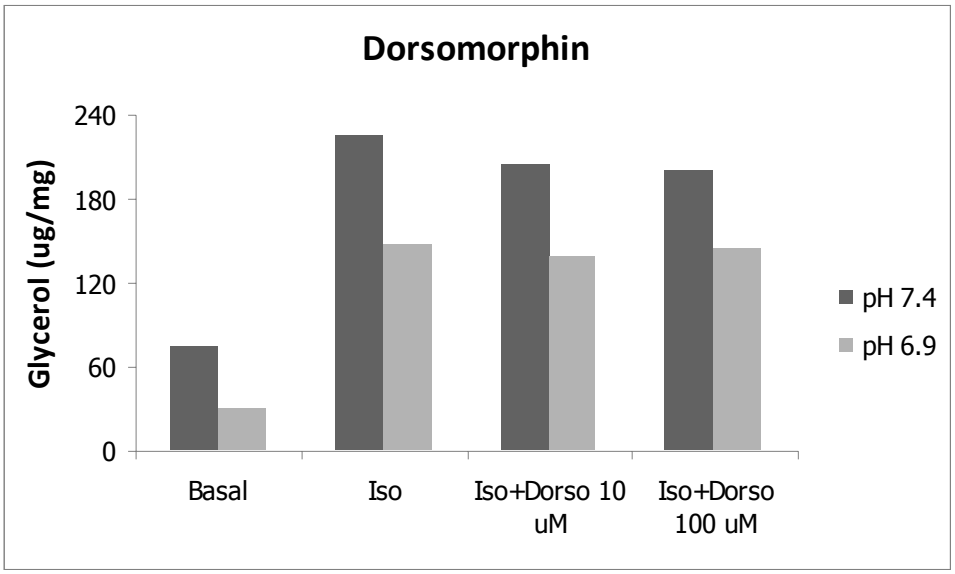


Figure 26. Dorsomorphin and adipocyte lipolysis

3.5 *Ca^{2+}/calmodulin-dependent kinase*

The following inhibitors including trifluoperazine hydrochloride 10-100 uM, W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) 10-100 uM, calmidazolium 10-100 nM and KN-62 1-10 uM[33] were used to test the involvement of Ca^{2+}/calmodulin-dependent kinase in metabolic acidosis mediated inhibition of lipolysis. The confluent adipocyte cell layers were preincubated with the inhibitors for upto 2 hours before isoproterenol-induced lipolysis was determined (Figure 28-

31). Trifluperazine and W-7 markedly inhibited adipocyte lipolysis, wheareas, calmidazodium partially restored adipocyte lipolysis suggesting the involvement of Ca^{2+}/calmodulin-dependent kinase in anti-lipolytic effect of metabolic acidosis. However, the effect of calmidazolium on adipocyte lipolysis will require further confirmation in subsequent experiments.

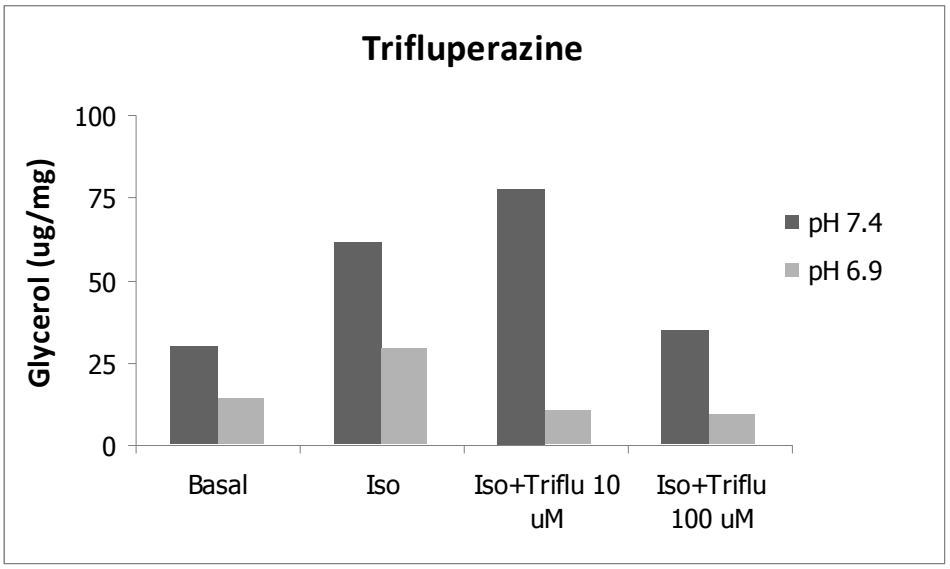


Figure 28. Trifluperazine and adipocyte lipolysis

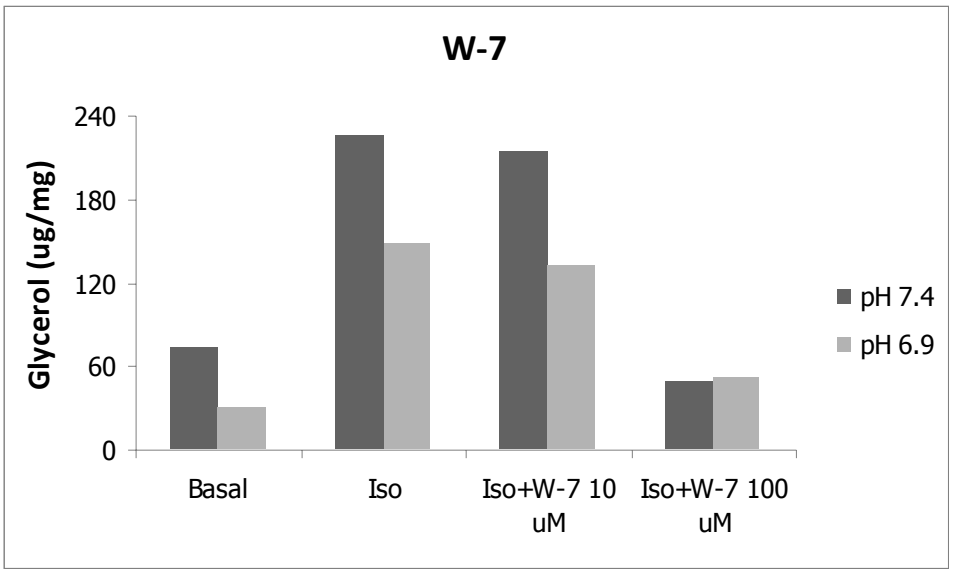


Figure 29. W-7 and adipocyte lipolysis

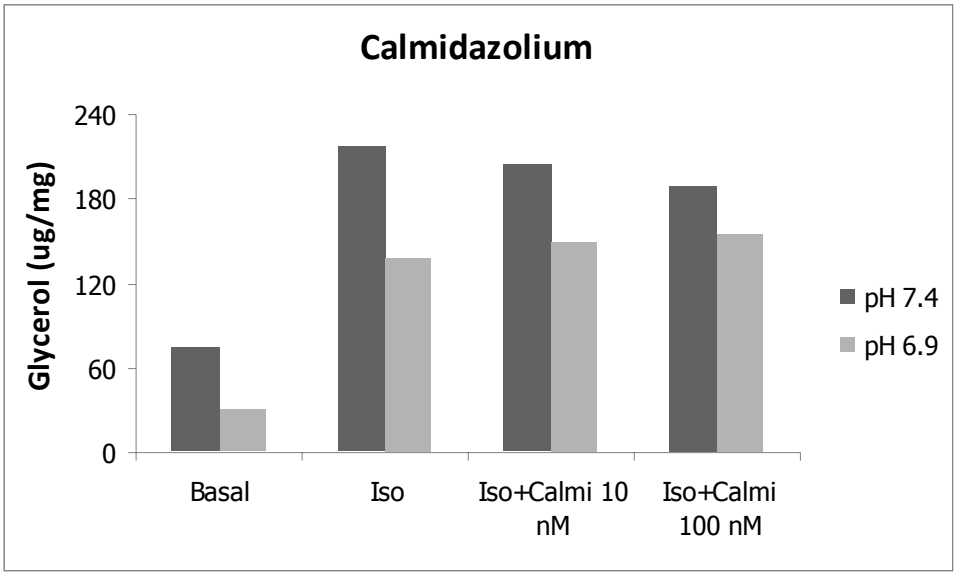


Figure 30. Calmidazolium and adipocyte lipolysis

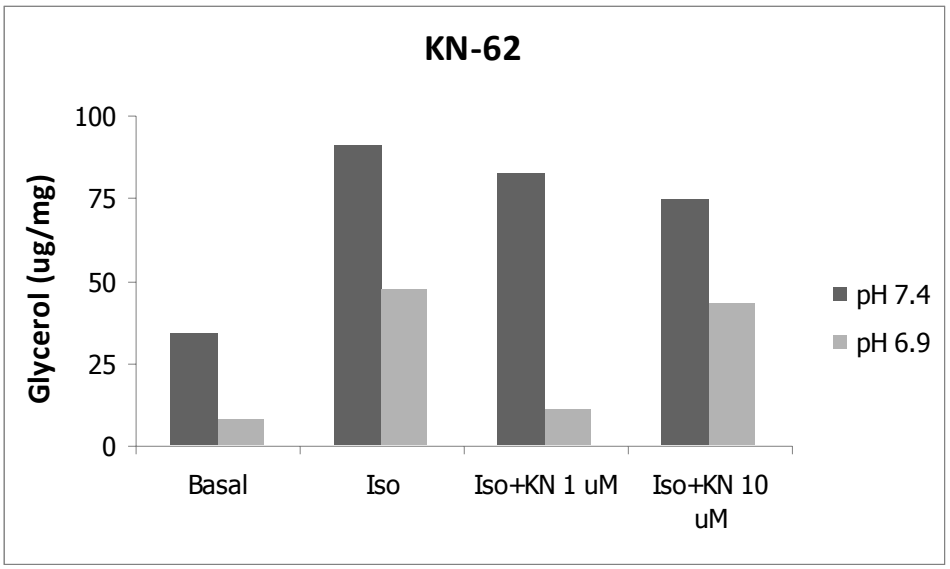


Figure 31. KN-62 and adipocyte lipolysis

4 The phosphorylation of HSL and PLIN

Based on the above experiments, we hypothesize that the phosphorylation of HSL and/or PLIN was diminished, therefore, western blot analysis of the phosphorylated HSL and PLIN will be confirmed next as well as whether the phosphorylation was restored by calmidazolium, one of the Ca^{2+}/calmodulin-dependent kinase inhibitor. At present time, the work of this part is in progress.

Part 2: The effect of chronic metabolic acidosis on adiponectin expression in cultured adipocytes

1. The effect of chronic metabolic acidosis on adiponectin mRNA expression

Confluent matured adipocytes were grown at pH 7.4 and 6.9 for 48 and 96 hours before RNA extraction and the expression of adiponectin mRNA was determined by realtime RT-PCR. The expression of adiponectin mRNA was corrected by beta-actin. Metabolic acidosis suppressed adiponectin mRNA at 96 hours (Figure 32).

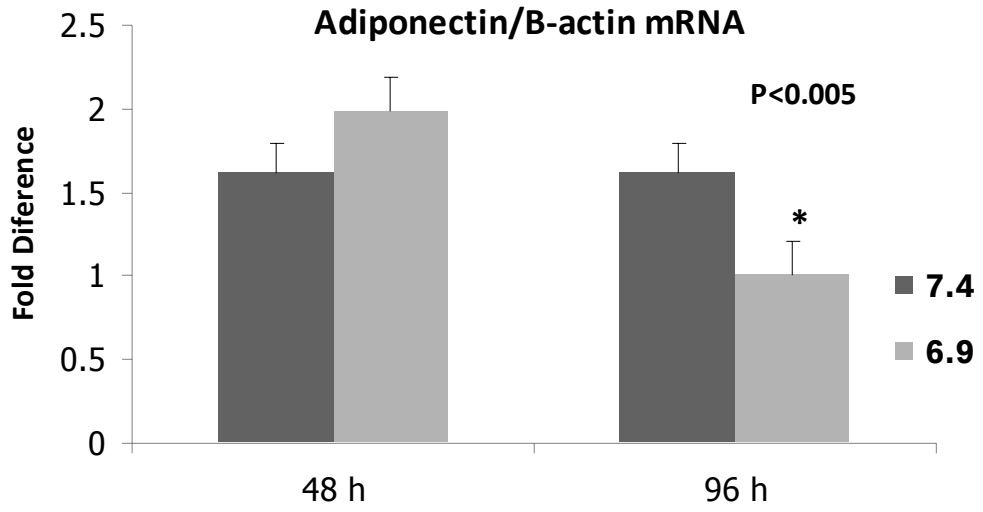


Figure 32. Adiponectin mRNA expression by real-time RT PCR

2. The effect of chronic metabolic acidosis on adiponectin protein

Confluent matured adipocytes were grown at pH 7.4 and 6.9 for 48 and 96 hours before the media was collected for measurement of adiponectin by ELISA. Metabolic acidosis suppressed adiponectin secretion from cultured adipocytes at 48 and 96 hours (Figure 33).

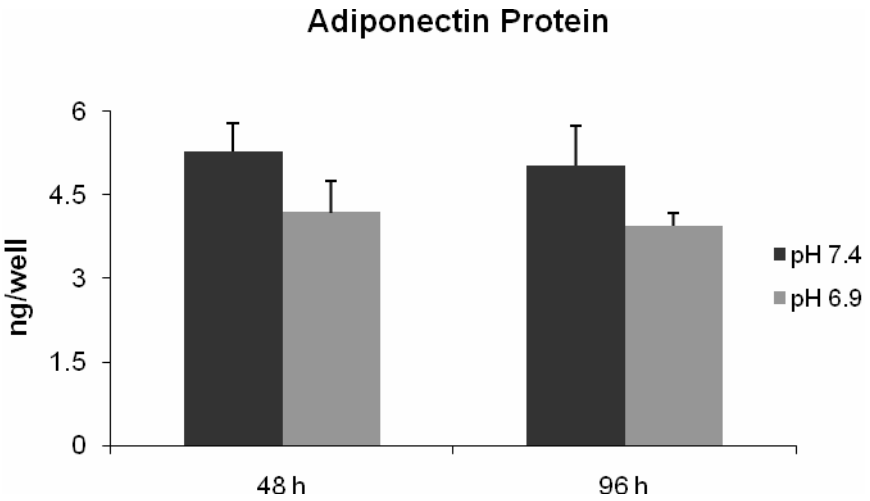


Figure 33. Adiponectin Protein secretion by ELISA

3. The effect of chronic metabolic acidosis on triglyceride accumulation and cell proliferation

Metabolic has been known to inhibit adipocyte lipolysis and cell proliferation.[35] Therefore, we determined whether metabolic acidosis alters triglyceride accumulation and cell proliferation which may influence adiponectin secretion. Confluent matured adipocytes were grown at pH 7.4 and 6.9 for 48 and 96 hours before the cells were scraped off the plate, lysed in cell lysis buffer and homogenized thoroughly. After centrifugation, the cell supernatant was used for triglyceride and protein measurement. As mentioned above, metabolic acidosis promotes triglyceride accumulation despite the decrease in cell proliferation suggested by the decrease in protein concentration (Figure 34 and 35). Cell proliferation will be confirm by MTT assay. At present time, this work is in progress.

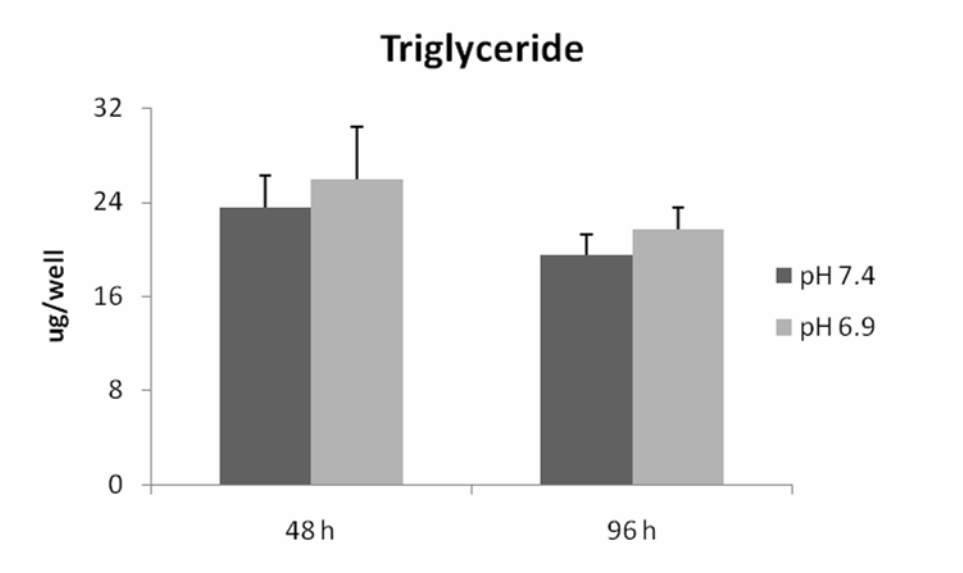


Figure 34. Triglyceride accumulation of adipocytes during metabolic acidosis

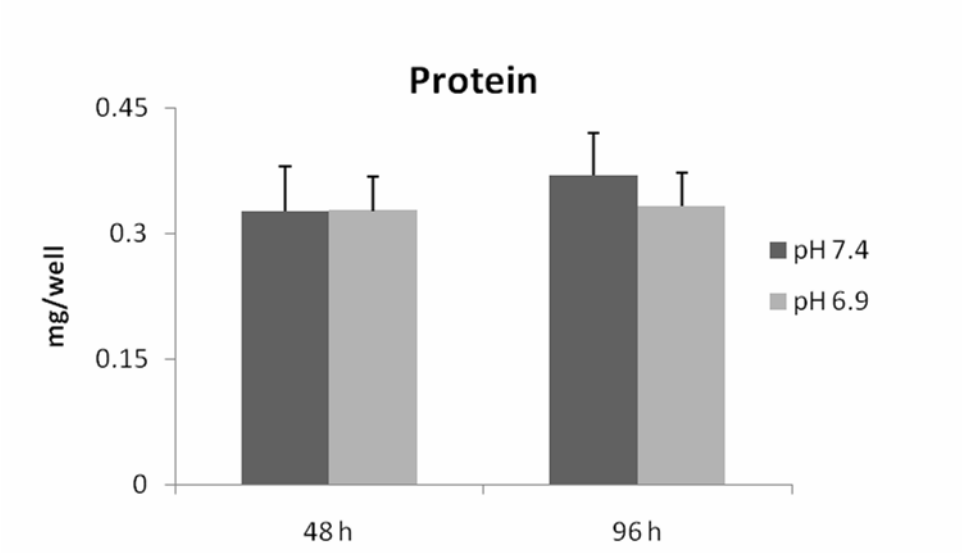


Figure 34. The amount of protein extracted from adipocytes during metabolic acidosis

Discussion

Part 1: The effect of metabolic acidosis on adipocyte lipolysis

Several years ago, Hood et al reported the first in vivo evidence of decreased free fatty acid availability in human subjected to NH_4Cl induced- metabolic acidosis.[13] Here we have confirmed that metabolic acidosis directly inhibited basal and catecholamine-induced adipocyte lipolyisis by upto 70%. The mechanisms underlying the anti-lipolytic effect of acute metabolic acidosis were further investigated. Alteration of catecholamine action through cAMP/PKA pathway was first examined. As reported few decades earlier, modifying this pathway with several activators and inhibitors including forskolin, a direct activator of adenylate cyclase, dBucAMP, a non-hydrolyzable analog of cAMP, 8-Bromo-cAMP, a hydrolyzable cAMP analog and IBMX, a phosphodiesterase inhibitor was unable to restore adipocyte lipolysis suggesting the involvement of other signaling mechanism.[14-16] In bone, metabolic acidosis stimulates osteoclast and enhances bone resorption through proton sensing receptor resulting in an increase in intracellular calcium through activation of PLC/IP3 pathway.[20] We tested whether this applies to the anti-lipolytic effect in adipocytes. In contrast to what we expected, $CuCl_2$, an inhibitor of proton sensor, 2-APB, an IP3 receptor inhibitor, and U-73122, a PLC inhibitor further inhibited lipolysis excluding the involvement of proton sensor in this case.

Lipolysis occurs in response to increased phosphorylation of HSL and PLIN. Protein phosphatases are responsible for the dephosphorylation process in order to make the effect of target protein transient. If the decrease in HSL and/or PLIN phosphorylation were responsible for the anti-lipolytic effect of metabolic acidosis, protein phosphatase inhibitors should be able to restore lipolysis. Tautomycetin, a PP1 inhibitor, and okadaic acid, PP2A inhibitor were able to partially restore lipolysis. The combination of the two almost completely restored adipocytes lipolysis which confirmed our hypothesis of attenuated HSL and/or PLIN phosphorylation. Next we tested whether metabolic acidosis directly stimulated protein phosphatases. Unfortunately, metabolic acidosis appeared to suppress PP1 activity suggesting the alternate pathway for decrease in HSL and/or PLIN phosphorylation.

Other signaling mechanisms that may be involved were then examined. Using various inhibitors for P38 –MAPK, ERK-MAPK, PI3-kinase/Akt, protein kinase C and AMP-activated protein kinase pathways were unable restore lipolysis. Some of these inhibitors suppressed lipolysis even further suggesting the roles of some of the signaling pathways in promoting lipolysis. A group of inhibitors for Ca^{2+}/calmodulin-

dependent kinase were next tested. Most of this the inhibitors in this group including trifluperazine and W-7 suppressed lipolysis even furthur except for calmidazolium that appeared restore lipolysis. We are currently in the process of confirming this finding.

In conclusion, acute and chronic metabolic acidosis inhibits basal and catecholamine-induced adipocytes lipolysis. This was not due to the alteration of catecholamine action via cAMP/PKA pathway but appeared to be working through Ca^{2+}/calmodulin-dependent kinase resulting in decreased phosphorylation of HSL and/or PLIN.

Part 2: The effect of chronic metabolic acidosis on adiponectin expression in cultured adipocytes

Previous reports demonstrated the effect of metabolic acidosis in inhibition of leptin secretion from adipocytes both in vivo and in vitro.[24, 25, 36, 37] Adiponectin is another protein produced exclusively from adipose tissue. Adiponectin has insulin sensitizing, anti-arthrogenic and anti-inflammatory properties.[27] Plasma levels of adiponectin are negatively associated with obesity, insulin resistance, metabolic syndrome, cardiovascular disease and mortality.[28]. Here we demonstrated that metabolic acidosis inhibits adiponectin gene expression and protein secretion from cultured adipocytes. This was unlikely to be due to the effect of metabolic acidosis on cell hypoproliferation since the triglyceride accumulation actually increased despite the suppressed adiponectin. Metabolic acidosis complicates several conditions such as hypotension, sepsis, diabetic ketoacidosis, diarrhea, alcohol intoxication and poisoning. Metabolic acidosis is also a key manifestation of distal renal tubular acidosis, a condition prevalent among women in northeastern part of Thailand. Protein-energy malnutrition, inflammation, increased protein catabolism, reduced albumin synthesis, insulin resistance and diminished thyroid hormone secretion are associated with metabolic acidosis. [1-3] The present study demonstrated the effect of metabolic acidosis on adipose tissue function from lipolysis to adipocyte hormone regulation. The suppression of adiponectin can adversely affect cardiovascular system and other metabolic function, therefore, emphasis should be made in metabolic acidosis correction in order to maintain normal physiological function.

In conclusion, metabolic acidosis suppressed adipocyte adiponectin mRNA and protein secretion. The consequence of such changes may translate into altered cardiovascular and other metabolic function.

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Suggestions for future research projects

The mechanisms underlying how chronic metabolic acidosis alters cell function in several systems are still somewhat unclear. Acidosis seems to work through different signaling mechanisms in different systems. If there is a pH set point for the body is still unresolved. There exists the difference in response of the body to respiratory and metabolic acidosis. It is at this point uncertain if proton sensors are responsible for the alternation of physiological condition of organs other than bone. The role of metabolic acidosis on adipose tissue has rarely been studied. The understanding of this issue is beneficial not only during physical illness but will be invaluable to metabolic syndrome and obesity research.

Output

1. **ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ**

   1.1. **Disthabanchong S**, Niticharoenpong K, Radinahamed P, Stitchantrakul W, Hongeng S, Ongphiphadhanakul, B. Metabolic Acidosis lowers serum adiponectin through inhibition of adiponectin mRNA expression and protein secretion from adipocytes. (Manuscript in preparation)

   1.2. **Disthabanchong S**, Radinahamed P, Stitchantrakul W, Hongeng S. Metabolic acidosis inhibits adipocyte lipolysis through suppression of HSL and/or PLIN phosphorylation. Work in progress

2. **การนำผลงานวิจัยไปใช้ประโยชน์**

   2.1. เชิงสาธารณะ

   ผลงานวิจัยนี้ได้ก่อให้เกิดเครือข่ายความร่วมมือในการทำวิจัยเซลต้นกำเนิดระหว่างภาควิชาอายุรศาสตร์ และภาควิชากุมารเวชศาสตร์ และเครือข่ายการวิจัยเซลไขมันกับหน่วยโภชนาการของภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี

   2.2. เชิงวิชาการ

   ผลงานวิจัยนี้สามารถใช้ในการพัฒนาการเรียนการสอน, เพิ่มศักยภาพของผู้ทำวิจัย และเผยแพร่ให้กับนักวิจัยรุ่นใหม่และนักศึกษาได้เรียนรู้และใช้เป็นแบบอย่าง เช่น ในการสร้างสมมุติฐาน และออกแบบระเบียบวิธีวิจัย

   2.3. อื่นๆ

   การเสนอผลงานในที่ประชุมวิชาการ และ หนังสือ

   1. **Disthabanchong S**, Radinahamed P, Stitchantrakul W, Hongeng S and Ongphiphadhanakul B. *Metabolic Acidosis Inhibits Adipocyte Lipolysis through Activation of Phosphoprotein Phosphatase.* Abstract TH-PO169. Accepted for a poster presentation at the American Society of Nephrology Annual Meeting on 6th November 2008, Philadelphia, Pennsylvania, USA

   2. **Disthabanchong S**, Radinahamed P, Stitchantrakul W, Hongeng S and Rajatanavin R. *Metabolic Acidosis Inhibits Adipocyte Lipolysis*. Abstract S-PO-0018. Accepted for a poster presentation at World Congress of Nephrology on 22nd April 2007, Rio de Janeiro, Brazil

   3. **Disthabanchong S**, Niticharoenpong K, Stitchantrakul W, Domrongkitchaiporn S, Ongphiphadhanakul B. *Suppressed Leptin and Adiponectin Secretion in Ammonium Chloride-induced Acidosis*. Abstract F-

FC003. Accepted for an oral presentation at the American Society of Nephrology Annual Meeting on 17th November 2006, San Diego, USA