รายงานวิจัยฉบับสมบูรณ์

โครงการ: ผลของกรดโรสมารินิคต่อภาวะดื้อต่อฮอร์โมนอินซูลินที่ถูกเหนี่ยวนำโดยแองจิโอเทนซิน II

(Potential protection of angiotensin II-induced insulin resistance by rosmarinic acid)

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ดร. มุจลินทร์ ประสานณรงค์ มหาวิทยาลัยเชียงใหม่

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โครงการเสร็จสิ้นเมื่อ พฤศจิกายน 2558

สัญญาเลขที่ TRG5680065

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
มหาวิทยาลัยเชียงใหม่

บทคัดย่อ

รหัสโครงการ: TRG5680065

ชื่อโครงการ: ผลของกรดโรสมารินิคต่อภาวะดื้อต่อฮอร์โมนอินซูลินที่ถูกเหนี่ยวนำโดยแองจิโอเทนซิน II

ชื่อนักวิจัย: ดร. มุจลินทร์ ประสานณรงค์ (มหาวิทยาลัยเชียงใหม่)

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ฮอร์โมนแองจิโอเทนซิน II (angiotensin II) ก่อให้เกิดภาวะความดันโลหิตสูง และภาวะดื้อต่อฮอร์โมนอินซูลินทั้งในระดับร่างกายและกล้ามเนื้อลายจากการเพิ่มขึ้นของความเครียดออกซิเดชัน (oxidative stress) อาจกระตุ้นการทำงานของไมโทเจน-แอคทิเวเตดโปรตีนไคเนส (MAPKs) และยับยั้งการทำงานของโปรตีนในสัญญาณอินซูลิน (Akt-dependent insulin signaling) ส่งผลให้เกิดภาวะดื้อต่อฮอร์โมนอินซูลินต่อไป จากผลการศึกษาก่อนหน้าพบว่ากรดโรสมารินิค (rosmarinic acid) สามารถลดความเครียดออกซิเดชันได้ การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของกรดโรสมารินิคต่อความผิดปกติที่เกิดจากการได้รับฮอร์โมนแองจิโอเทนซิน II ได้แก่ ภาวะความดันโลหิตสูง และภาวะที่ร่างกายหรือกล้ามเนื้อลายดื้อต่อฮอร์โมนอินซูลิน โดยการป้อนกรดโรสมารินิคแก่หนูตะเภาที่ได้รับฮอร์โมนแองจิโอเทนซินนาน 2 สัปดาห์ เพียงหนึ่งครั้ง (10 20 และ 40 มิลลิกรัมต่อกิโลกรัม) หรือวันละครั้งนาน 2 สัปดาห์ (10 20 และ 40 มิลลิกรัมต่อกิโลกรัม) ผลการศึกษาพบว่า การป้อนเพียงหนึ่งครั้งสามารถลดความดันโลหิตได้ โดยเฉพาะการป้อนกรดโรสมารินิคที่มีความเข้มข้นสูงเพียงหนึ่งครั้ง สามารถเพิ่มอัตราการขนถ่ายกลูโคสเข้าเซลล์กล้ามเนื้อลายภายใต้การกระตุ้นของฮอร์โมนอินซูลิน และเพิ่มการทำงานของ ERK1/2 ในกล้ามเนื้อลายได้อีกด้วย นอกจากนี้ การป้อนกรดโรสมารินิคนาน 2 สัปดาห์ช่วยป้องกันผลของฮอร์โมนแองจิโอเทนซินที่มีผลลดปริมาณการกิน น้ำหนักตัว และปริมาณไขมันในช่องท้องได้ โดยสรุป การศึกษานี้อาจกล่าวได้ว่า การใช้กรดโรสมารินิคทั้งแบบการใช้เพียงหนึ่งครั้งหรือใช้ติดต่อกันเป็นระยะเวลาหนึ่ง อาจใช้เป็นทางเลือกสำหรับการช่วยลดผลเสียที่เกิดจากฮอร์โมนแองจิโอเทนซิน II ได้ อย่างไรก็ตามผลเพิ่มอัตราการขนถ่ายกลูโคสเข้าเซลล์กล้ามเนื้อลายที่พบในการศึกษานี้อาจไม่เกี่ยวข้องกับการเพิ่มขึ้นของการทำงานของ ERK1/2 ที่พบในกล้ามเนื้อลาย ดังนั้นการศึกษาวิจัยในอนาคตควรศึกษาเพิ่มเติมเกี่ยวกับการเปลี่ยนแปลงการทำงานของโปรตีนในเส้นทางอื่นเพื่อนำมาซึ่งการอธิบายผลของกรดโรสมารินิคที่สามารถเพิ่มอัตราการนำเข้ากลูโคสเข้าสู่เซลล์กล้ามเนื้อลายได้

คำหลัก : กรดโรสมารินิค, แองจิโอเทนซิน II, กล้ามเนื้อลาย, ภาวะดื้อต่ออินซูลิน, ไมโทเจน-แอคทิเวเตดโปรตีนไคเนส

Abstract

Project Code: TRG5680065

Project Title: Potential protection of angiotensin II-induced insulin resistance by rosmarinic acid

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Project Period: 1 June 2013 to 31 May 2015

Angiotensin II (ANG II) causes high blood pressure and insulin resistance via inducing oxidative stress may bring about the activation of mitogen-activated protein kinases (MAPKs) and inhibition of Akt-dependent insulin signaling. Antioxidative activity of rosmarinic acid (RA) has been revealed. This study was designed to explore whether rosmarinic acid has acute or chronic therapeutic potential for lowering blood pressure, insulin stimulating the whole-body insulin sensitivity and skeletal muscle glucose transport activity in ANG II-treated rats. Acute RA rats received RA 10, 20, and 40 mg/kg by single gavage, whereas chronic RA rats received one time per day for 2 weeks. The results show that both acute and chronic treatments of RA have blood pressure lowering effects. Only a high dose of acute treatment increased insulin-mediated glucose transport activity and ERK1/2 activity. Chronic treatment of RA reduces effects of ANG II on anorexia, body weight and visceral adiposity reducing. These results imply that RA may possess ANG II effects on cardiometabolic abnormalities, including, high blood pressure, anorexia, and decreases of body weight and visceral adiposity. However, the induction of glucose transport activity by RA may not involve with increasing ERK1/2 activity. To clarify the increasing effect of RA, future study should explore protein activity in other signaling pathways that involve with skeletal muscle glucose transport activity.

Keywords : rosmarinic acid, angiotensin II, skeletal muscle, insulin resistance, mitogen-activated protein kinase

Executive Summary

The metabolic syndrome is a cluster of cardio-metabolic abnormalities, which include insulin resistance, impaired glucose tolerance, hypertension, obesity, and dyslipidemia. This major public-health problem put individuals at high risk for type 2 diabetes and cardiovascular disease. Skeletal muscle insulin resistance is commonly found in insulin-resistant and hypertensive conditions. The proposed mechanisms that linked between insulin resistance and hypertension include the renin-angiotensin system (RAS).

Angiotensin II (ANG II), a hypertensive element of RAS, is often elevated in hypertension. Several lines of investigation have shown critical roles of ANG II in skeletal muscle insulin resistance. Direct administration of ANG II to non-hypertensive skeletal muscle can induce skeletal muscle insulin resistance of the glucose transport process. The mechanisms behind these effects are related to Akt-dependent insulin signaling and reactive oxygen species (ROS) production. It has been shown that ANG II-induced impaired Akt-dependent insulin signaling by reducing pAkt Ser473 and pGSK-3. The effect on ANG II on insulin signaling proteins was rescued by a superoxide radical scavenger. An activation of ROS subsequently activates mitogen-activated protein kinases (MAPKs), including p38 MAPK, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). Connection between MAPKs and insulin resistance has been indicated in skeletal muscle.

RA is a natural pure compound isolated from many herbs that belong to Lamiaceae family such as rosemary, sage, basil, mint. These plants can be found in many parts of Thailand and are largely and routinely used in Thai cooking recipe. RA is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. Many interesting biological activities of RA have been revealed including its anti-oxidant, anti-inflammatory, lipid-lowering effects. Blood pressure-lowering and whole-body insulin sensitizing effects of RA have been reported in fructose-induced hypertensive and insulin-resistant rats. In addition, an inhibitory effect of RA on ROS and MAPKs activities in cardiac muscle cell line by adriamycin, an anti-cancer drug that causes development of severe cardiomyopathy failure after long-term use, has been reported. To date, although several therapeutic potentials of RA have been revealed, it remains unknown how RA affects the glucose transport system in both insulin-sensitive and insulin-resistant skeletal muscle.

วัตถุประสงค์

Objectives

1. To assess the acute effects of RA treatment in rats with insulin resistance-induced by ANG II at the whole-body and skeletal muscle levels.

2. To examine the molecular mechanisms (i.e. MAPKs and Akt-dependent insulin signaling) underlying the acute effects of RA in insulin-resistant skeletal muscle induced by ANG II infusion.

3. To evaluate the effects of chronic administration of RA in insulin-resistant rat induced by ANG II at the whole-body and skeletal muscle levels.

4. To examine the adaptive changes at the molecular level (i.e. MAPKs and Akt-dependent insulin signaling) in skeletal muscle of ANG II-induced insulin-resistant rat following the chronic administration of RA.

วิธีทดลอง

Material and methods

Animals and experimental design

Eight-week-old male Sprague-Dawley rats supplied by the National Laboratory Animal Center, Thailand, were given regular rat chow and water ad libitum. The housing unit was maintained at 22°C with a 12/12-hr light/dark cycle. Animal procedures were approved by the Institution of Animal Care and Use Committee, Faculty of Science, Mahidol University, in accordance with those of the National Laboratory Animal Center, Thailand.

Experiment 1: To determine the acute effects of RA on insulin sensitivity in ANG II-infused rats, rats were implanted with subcutaneous osmotic mini pumps (model 2006, Alzet, CA) that delivered ANG II (250 ng/kg/min). After 2 weeks of ANG II infusion, rats were fed with RA by single gavage at different concentration, including 10, 20, and 40 mg/kg. Thirty minutes after the RA treatment, oral glucose tolerance tests (OGTTs) were performed. Insulin and glucose concentrations were measured and used for calculating the whole-body insulin sensitivity.

Experiment 2: To examine acute effects of RA on ANG II-treated rats at the infusion rate of 250 ng/kg/min, ANG II-infused rats were fed with RA at 10, 20, and 40 mg/kg by single gavage. Thirty minutes after the RA treatment, rats were anesthetized and skeletal muscle tissues were collected for determining insulin action on glucose transport activities and evaluating signaling proteins, for instance, MAPKs

(phosphorylation and protein abundance of ERK, JNK, and p38 MAPK) and Akt-dependent insulin signaling pathways.

Experiment 3: The objective of this experiment is to evaluate the chronic effects of RA administrations (10, 20, and 40 mg/kg) in ANG II-treated rats at the infusion rate of 250 ng/kg/min for 2 weeks. Following a daily administration with RA at different doses by gavage for 2 weeks, OGTTs will be performed to determine the whole-body insulin resistance.

Experiment 4: To the chronic effects of RA administrations (10, 20, and 40 mg/kg) in ANG II-treated rats at the infusion rate of 250 ng/kg/min for 2 weeks. After 2 weeks of RA administration, rats will be anesthetized and skeletal muscles will be collected for determining insulin action on glucose transport activities and evaluating signaling proteins, for example MAPKs and Akt-dependent insulin signaling pathways.

Measurement of whole-body insulin sensitivity

To test the whole-body insulin sensitivity, rats underwent oral glucose tolerance tests (OGTTs). In the evening (1800 hr) of the day before the test, rats were food-restricted to 4 g of chow. In the next morning (0800–0900 hr), tail blood was collected into microfuge tubes containing anticoagulant (EDTA) at 0, 15, 30, 60, and 120 min after glucose feeding (1 g/kg) by gavage. The blood samples were centrifuged before collecting the plasma samples, which were used subsequently for determining glucose and insulin concentrations. Each animal was given of sterile 0.9% saline subcutaneously right after the test for body fluid replacement.

Assessment of skeletal muscle glucose transport activity

After food restriction, rats were weighed and deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (75 mg/kg). Soleus muscles were dissected and subsequently divided into two portions. Muscle strips were incubated for 30 min at 37°C in oxygenated Krebs-Henseleit buffer (KHB) supplemented with 8 mM D-glucose, 32 mM D-mannitol, 0.1% radioimmunoassay-grade bovine serum albumin, and the present or absent of different 5 mU/ml insulin. All flasks were gassed continuously with 95% O2-5% CO2 throughout incubation procedure or glucose transport measurements. Then each strip was rinsed for 10 min at 37°C in 3 ml of oxygenated KHB containing 40 mM D-mannitol, 0.1% BSA, and/or insulin, if previously present. Thereafter, muscle strips were incubated for 20 min at 37°C in 2 ml of KHB containing 1 mM 2-[1,2-

3H]deoxyglucose (2-DG; 300 mCi/mmol), 39 mM [U-14C]mannitol (0.8 mCi/mmol), 0.1% BSA, and/or insulin if previously present. At the end of the incubation, muscle was removed, trimmed of excess fat and connective tissue, and frozen with liquid nitrogen immediately and weighed. The frozen muscles were solubilized in 0.5 ml of 0.5 N NaOH, and then 10 ml of scintillation cocktail was added. The specific intracellular accumulation of 2-DG was determined by using mannitol to correct for the extracellular accumulation of 2-DG. Glucose transport activity was measured as the intracellular accumulation of 2-DG (in pmol/mg muscle wet weight/20 min).

Data analysis and statistical method

Values are reported as the means ± SE. One-way analyses of variance (ANOVA) with Tukey's HSD post hoc test were used to determine the significant difference from among groups. Statistical analyses will be performed using SPSS 16.0 (SPSS Inc., Chicago, IL). A value of $p < 0.05$ is considered to be statistically significant.

ผลการทดลอง

Results

Effects of ANG II and an acute administration of RA on body weight, total calorie intake, and organ weights

Body weight of ANG II treated rat was significantly lower than SHAM rats from day 10 to 14 after starting ANG II infusion (Figure 1). Total calorie intake was significantly lower in ANG II-treated rats compared with the SHAM rats (Table 1). Liver weight, intra abdominal fat weight, and intra abdominal fat to body weight ratio in ANG II group were lower than those in SHAM whereas ANG II increased heart weight to body weight ratio. Acute RA administration did not affect liver weight, heart weight to body weight ratio, total intra abdominal fat weight, and total intra abdominal fat weight to body weight ratio in ANG II-treated animals, as shown in Table 1.

Table 1. Initial and final body weight, total calorie intake, and organ weights of SHAM and ANG II treated rats with or without acute administrations of RA at 10, 20, and 40 mg/kg. Numbers of rats for each group was 6-10. *p < 0.05 vs SHAM group.

	SHAM	ANG II	RA-10a	RA-20a	RA-40a
Body weight (g)					
Initial weight	373.55 ± 5.58	372.25 ± 5.83	364.41 ± 9.07	365.47 ± 7.23	368.03 ± 5.48
Final weight (BW)	408.53 ± 6.34	366.40 ± 13.23 *	358.02 ± 9.77 *	362.86 ± 9.28 *	358.94 ± 9.18 *
Total energy intake (kcalx10^3)	859.96 ± 33.26	720.06 ± 41.04 *	718.95 ± 38.93 *	727.59 ± 30.38 *	721.98 ± 10.06 *
Liver weight (LW; mg)	10.80 ± 0.31	9.30 ± 0.20 *	9.25 ± 0.25 *	9.66 ± 0.50 *	9.93 ± 0.50 *
LW/kg BW	26.96 ± 0.85	27.04 ± 0.86	26.67 ± 0.58	27.86 ± 0.82	28.01 ± 0.85
Heart weight (HW; mg)	1.12 ± 0.04	1.26 ± 0.02	1.27 ± 0.04	1.29 ± 0.04	1.31 ± 0.03
HW/kg BW	2.81 ± 0.07	3.67 ± 0.17 *	3.66 ± 0.11 *	3.75 ± 0.13 *	3.71 ± 0.12 *
Intra abdominal fat weight (FW; g)	18.61 ± 1.25	13.13 ± 2.34 *	12.67 ± 0.79 *	12.63 ± 0.77 *	13.69 ± 0.77 *
FW/kg BW	46.51 ± 3.32	37.34 ± 5.06 *	35.46 ± 1.89 *	36.29 ± 0.94 *	38.05 ± 1.66 *

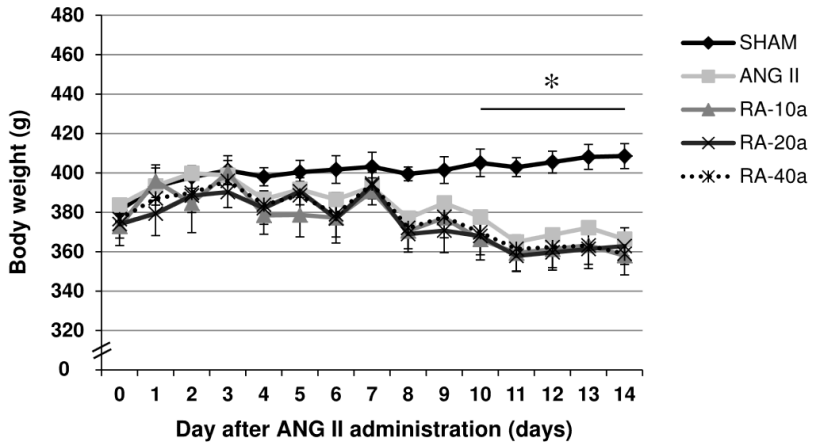

Figure 1. Time course of body weight of SHAM and ANG II treated rats before acute administrations of RA (10, 20, and 40 mg/kg). Numbers of rats for each group was 6-10. *p < 0.05 vs SHAM group.

Acute administration of RA attenuated ANG II-induced high blood pressure

ANG II infusion (250 ng/kg/min) for 2 weeks increased systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MAP) in rats (Figure 2). After two weeks of inducing hypertension, acute administrations of RA (10, 20, and 40 mg/kg) was performed. Arterial blood pressures (SBP, DBP, and MAP) were measured before and 30 minutes after RA administration. The results showed that all doses of RA had antihypertensive effects, which reduced SBP, DBP, and MAP to the level of SHAM rats (Figure 2).

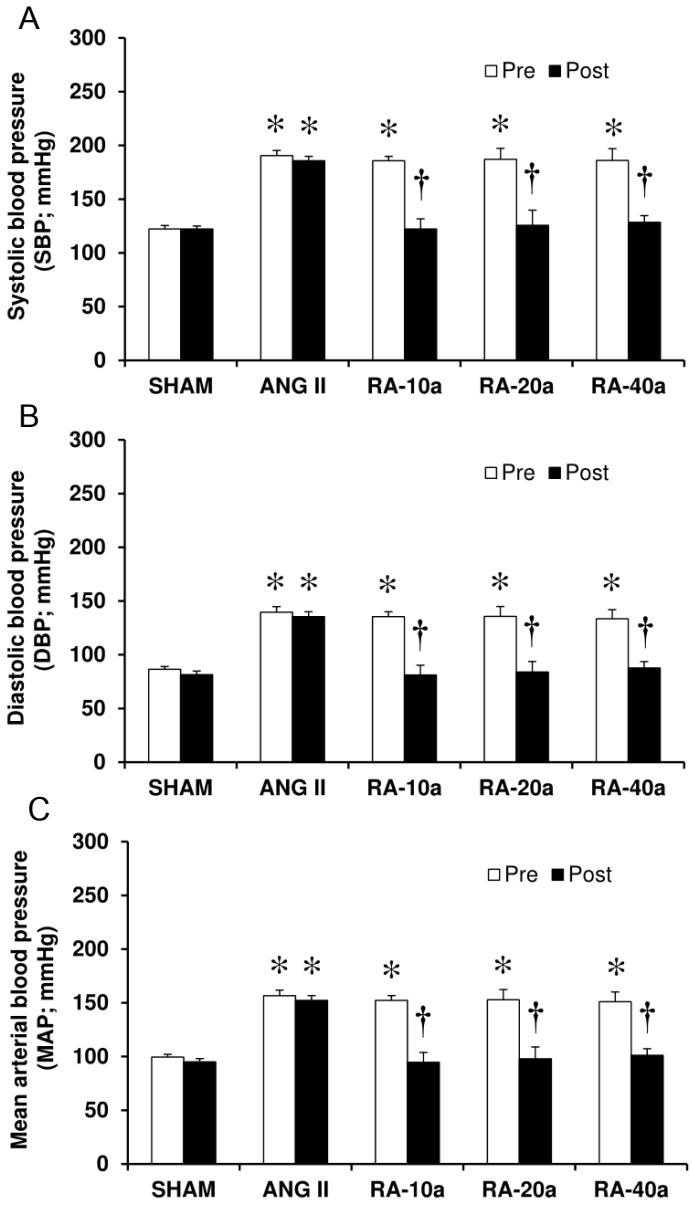

Figure 2. SBP (A), DBP (B), and MAP (C) of SHAM, ANG II, and RA treatment groups (10, 20, and 40 mg/kg) at 30 minutes after acute administrations of water (SHAM and ANG II) or RA (RA groups). Values are mean ± SE of 6-10 rats per group. *p < 0.05 vs SHAM group; †p < 0.05 vs ANG II group.

Effects of ANG II and an acute administration of RA on the whole-body insulin sensitivity and skeletal muscles

Glucose-insulin index (G-I index) has been used to represent the whole-body insulin sensitivity. Although the results showed no significant difference of G-I index among groups (Figure 3E), ANG II infusion caused a reduction in insulin AUC (Figure 3D).

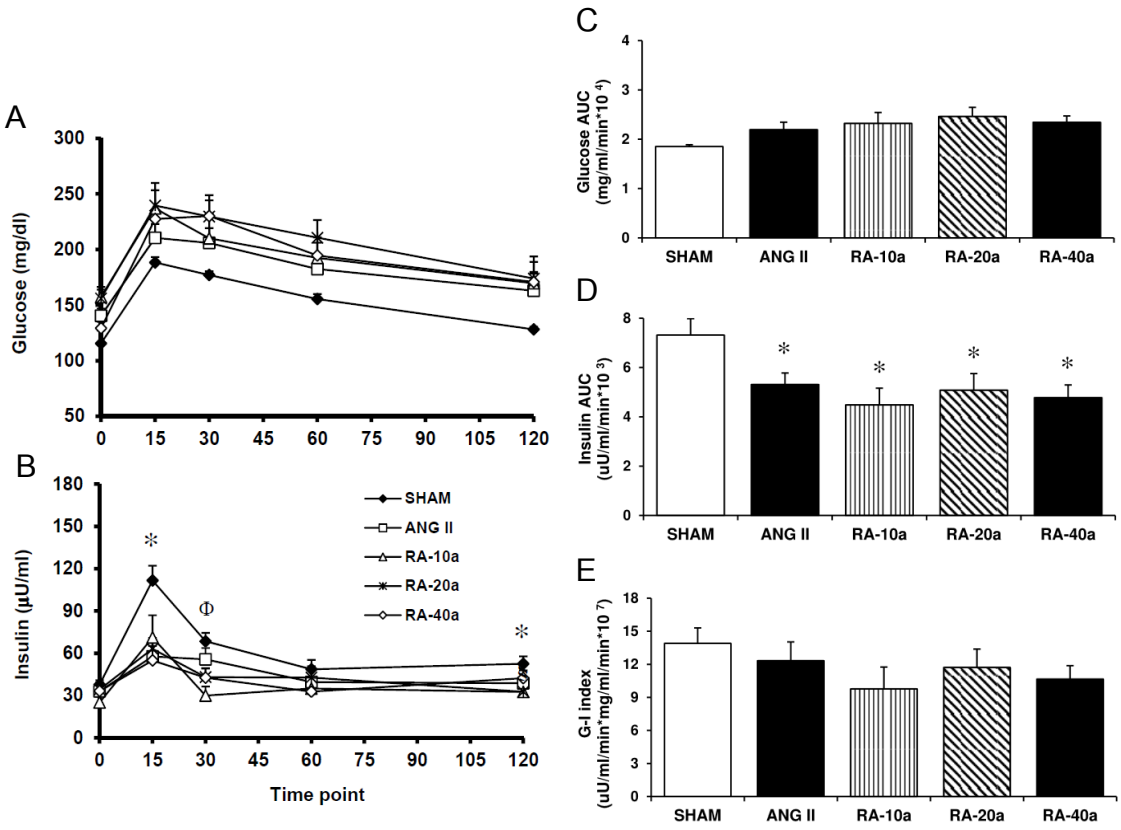

Figure 3. Glucose (A) and insulin (B) concentrations in plasma during oral glucose tolerance tests (OGTTs), glucose (C) and insulin (D) area under the curve (AUC), and glucose-insulin index (G-I index) (E) of SHAM, ANG II, and RA treatment groups (10, 20, and 40 mg/kg). Values are mean ± SE of 5-8 rats per group. *$p < 0.05$ vs SHAM group; $^{\Phi}p < 0.05$ RA-10a, RA-20a, or RA-40a vs SHAM group.

After 30 minutes of acute administration of RA by gavage, soleus muscles were dissected, separated into small muscle strips, and incubated in two conditions, which were condition with insulin stimulation (insulin; Figure 4A) or without insulin stimulation (basal; Figure 4A) . The differences between basal and insulin stimulating of glucose transport activities were presented as insulin-mediated glucose transport activities (Figure 4B). The results showed significantly higher of insulin-stimulated glucose transport activity after acute administrations of RA at 20 and 40 mg/kg (R-20a and R-40a, respectively) compared with SHAM group (Figure 4A). However, only an administration of RA at 40 mg/kg presented significantly higher than that of SHAM group (Figure 4B).

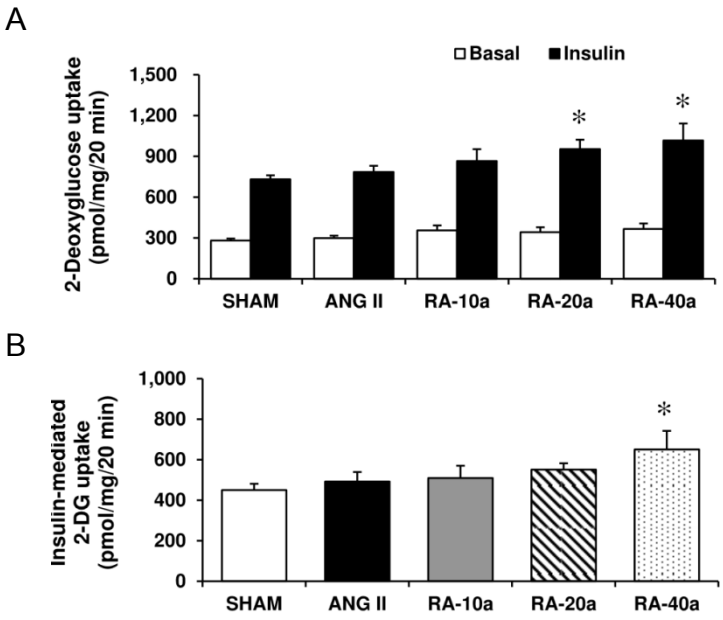

Figure 4. Glucose transport activity at basal, insulin stimulation (A), and different changes between basal and insulin stimulation (B) of SHAM, ANG II, and acute administration of RA groups (10, 20, and 40 mg/kg). *p < 0.05 vs SHAM group.

Effects of ANG II and a chronic administration of RA on body weight, total calorie intake, and organ weights

Body weight and total calorie intake of ANG II and RA 10 mg/kg treated rats were significantly lower than SHAM rats from day 10 to 14 after starting ANG II infusion (Table 2 and Figure 5). Compared with the SHAM rats, only RA 20 mg/kg treated rats did not show significantly reduced intra abdominal fat weight and total intra abdominal fat weight to body weight ratio, as shown in Table 2.

Table 2. Initial and final body weight, total calorie intake, and organ weights of SHAM and ANG II treated rats with or without chronic administrations of RA at 10, 20, and 40 mg/kg. Numbers of rats for each group was 6-10. *p < 0.05 vs SHAM group

	SHAM	ANG II	RA-10c	RA-20c	RA-40c
Body weight (g)					
Initial weight	374.10 ± 6.08	372.92 ± 7.30	383.71 ± 5.87	373.28 ± 5.89	379.00 ± 4.71
Final weight (BW)	400.80 ± 4.79	369.16 ± 9.57 *	363.99 ± 11.71 *	383.77 ± 11.85	373.24 ± 9.82
Total energy intake (kcalx10^3)	858.65 ± 37.16	725.75 ± 64.41 *	715.44 ± 30.77 *	819.78 ± 35.36	749.74 ± 42.88
Liver weight (LW; mg)	11.64 ± 0.36	10.02 ± 0.27 *	10.44 ± 0.45	10.99 ± 0.43	10.68 ± 0.38
LW/kg BW	28.80 ± 0.75	28.53 ± 0.64	28.63 ± 0.60	28.64 ± 0.69	28.58 ± 0.55
Heart weight (HW; mg)	1.21 ± 0.04	1.34 ± 0.04	1.32 ± 0.04	1.30 ± 0.04	1.30 ± 0.04
HW/kg BW	2.98 ± 0.10	3.75 ± 0.14 *	3.65 ± 0.14 *	3.42 ± 0.13	3.41 ± 0.10
Intra abdominal fat weight (FW; g)	18.32 ± 1.21	13.33 ± 1.01 *	13.43 ± 1.24 *	14.29 ± 1.08	14.82 ± 0.54
FW/kg BW	45.21 ± 2.64	36.73 ± 2.18 *	36.46 ± 2.75 *	37.92 ± 1.53	37.30 ± 2.15

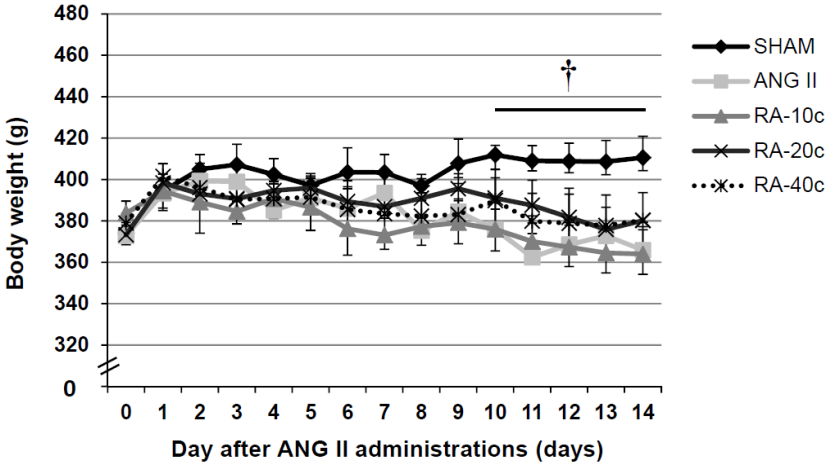

Figure 5. Time course of body weight of SHAM and ANG II with or without chronic administrations of RA (10, 20, and 40 mg/kg). Numbers of rats for each group was 6-10. †$p < 0.05$ is ANG II or RA 10 mg/kg vs SHAM group.

Chronic administration of RA attenuated ANG II-induced high blood pressure

Arterial blood pressures (SBP, DBP, and MAP) were measured every week. Rats treated with ANG II infusion (250 ng/kg/min) for 2 weeks presented increased SBP, DBP, and MAP (Figure 6). All doses of RA (10, 20, and 40 mg/kg) had antihypertensive effects. All arterial blood pressures reduced since the first week after administrations (Figure 6).

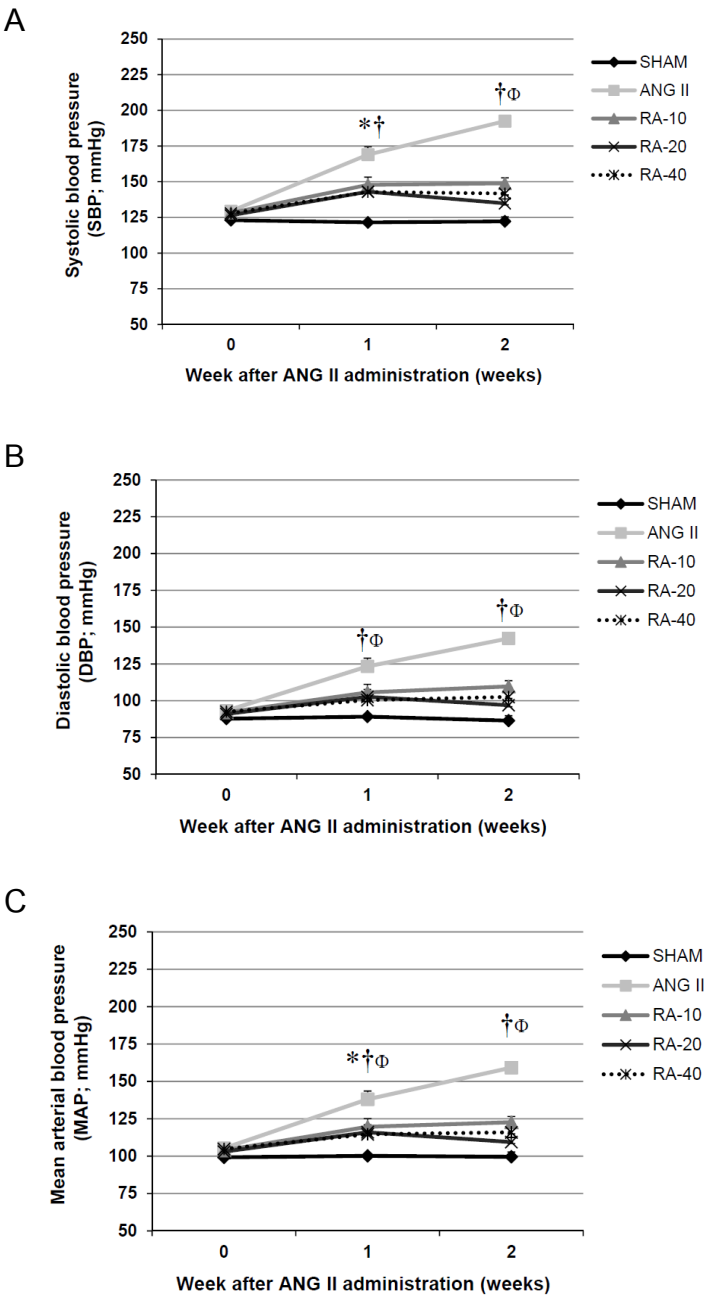

Figure 6. SBP (A), DBP (B), and MAP (C) of SHAM, ANG II, and RA treatment groups (10, 20, and 40 mg/kg). Values are mean ± SE of 6-10 rats per group. *p < 0.05 vs SHAM group; †p < 0.05 vs ANG II group; $^{\Phi}$p< 0.05 RA 10 or 40 mg/kg vs SHAM group.

Effects of ANG II and a chronic administration of RA on the whole-body insulin sensitivity and skeletal muscles

Glucose-insulin index (G-I index) has been used to represent the whole-body insulin sensitivity. Although the results showed no significant difference of G-I index among groups (Figure 7E), ANG II infusion caused a reduction in insulin AUC (Figure 7D).

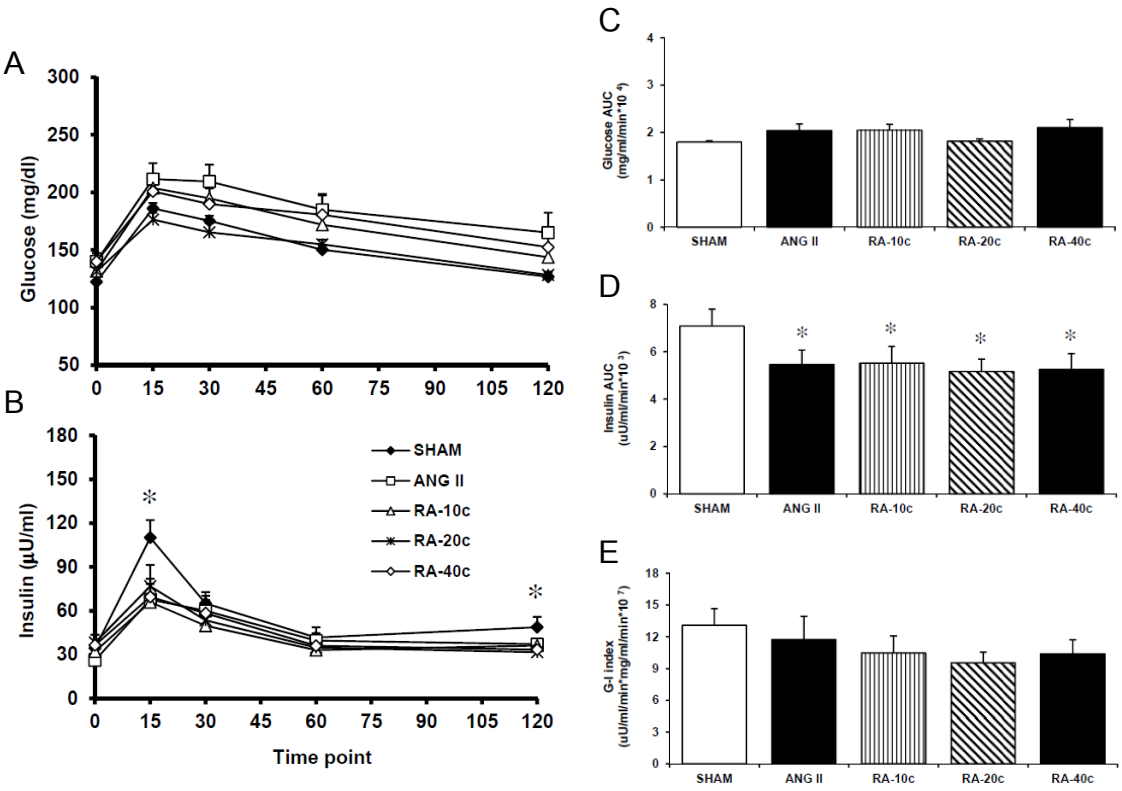

Figure 7. Glucose (A) and insulin (B) concentrations in plasma during oral glucose tolerance tests (OGTTs), glucose (C) and insulin (D) area under the curve (AUC), and glucose-insulin index (G-I index) (E) of SHAM, ANG II, and RA treatment groups (10, 20, and 40 mg/kg). Values are mean ± SE of 5-9 rats per group. *p < 0.05 vs SHAM group.

To determine whether chronic treatment of RA affects glucose transport activity, soleus muscle strips were incubated in the condition of absent (basal; Figure 8A) or present (insulin; Figure 8A) of insulin. The differences between basal and insulin stimulating of glucose transport activities were presented as insulin-mediated glucose transport activities (Figure 8B). Although RA treatment showed no significantly different among group, RA 20 mg/kg treated rats had trend of increasing insulin-meditated glucose transport activity ($p = 0.056$) when compared with SHAM rats (Figure 8B).

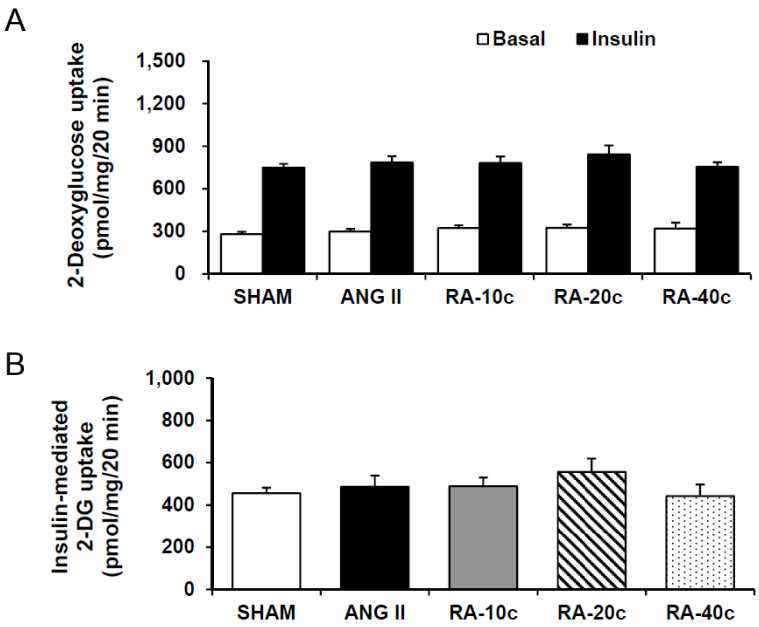

Figure 8. Glucose transport activity at basal, insulin stimulation (A), and different changes between basal and insulin stimulation (B) of SHAM, ANG II, and chronic administration of RA groups (10, 20, and 40 mg/kg).

Effects of RA treatments on the whole-body and skeletal muscle insulin sensitivity

Angiotensin II infusion for 14 days caused significantly lower plasma insulin concentration during OGTT. Neither acute nor chronic administrations of RA can gain the response back to the level of SHAM rats (Figure 3 and Figure 7). Skeletal muscle glucose transport activities did not change after ANG II infusion. It is interesting that acute RA administration of 20 and 40 mg/kg caused significantly increase in insulin-stimulated glucose transport activities by 31% and 39% when compared with SHAM rats, whereas only 40 mg/kg of RA caused increase in insulin-mediated glucose transport activities (the difference between basal and insulin-stimulated condition) by 45% (Figure 4, $p < 0.05$). No effect of ANG II was observed on skeletal muscle signaling elements in both basal and insulin-stimulated condition in this study (Figure 9). Thirty minutes after acute treatment of RA at 40 mg/kg increased insulin-stimulated ERK1/2 activity was in agreement with increased insulin-mediated glucose transport activities in slow-twitch skeletal muscle (Figure 10, $p < 0.05$).

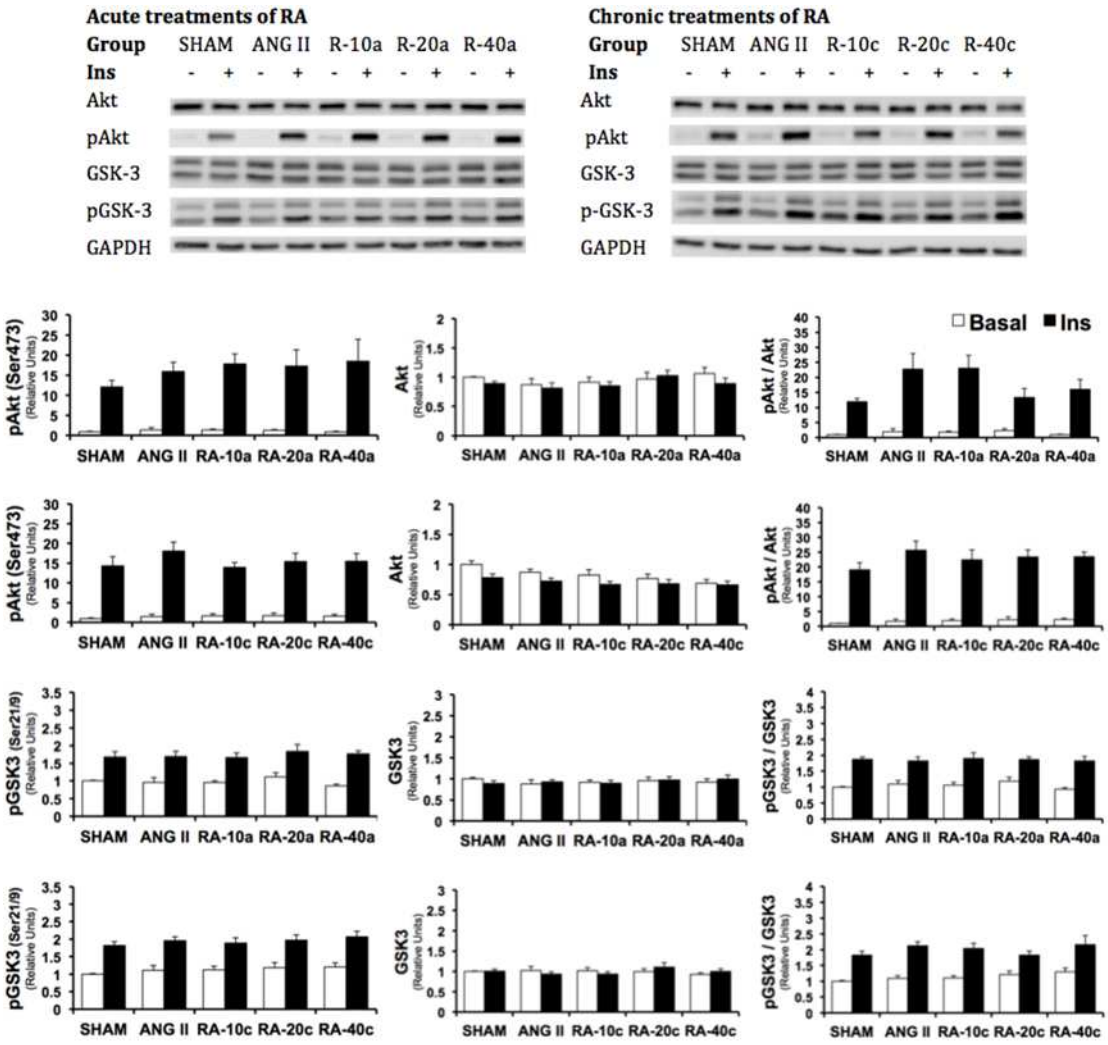

Figure 9. Insulin signaling of SHAM, ANG II, acute (10a, 20a, and 40a mg/kg BW), and chronic administration of RA groups (10c, 20c, and 40c mg/kg BW); *p < 0.05 vs SHAM group.

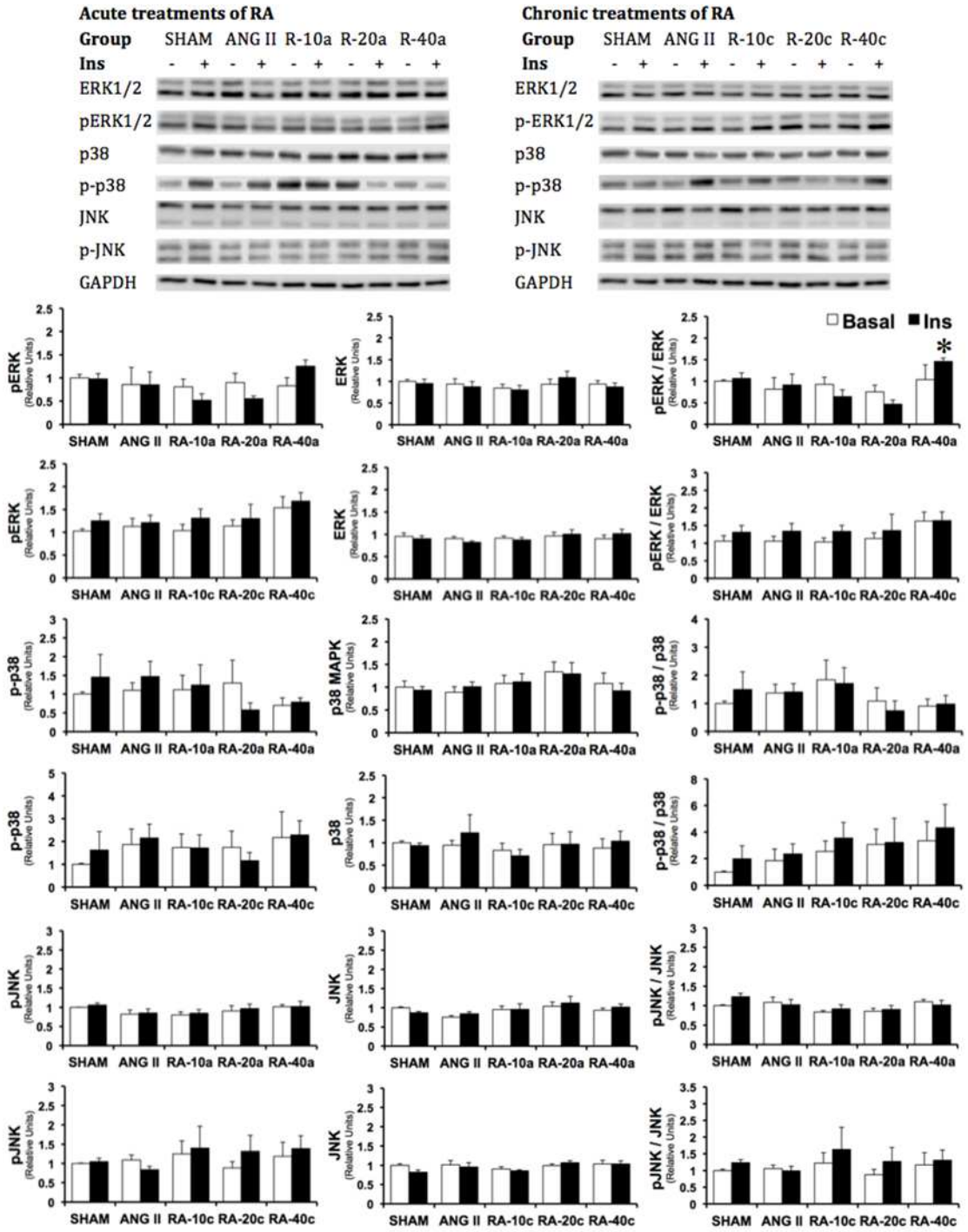

Figure 10. MAPK signaling of SHAM, ANG II, acute (10a, 20a, and 40a mg/kg BW), and chronic administration of RA groups (10c, 20c, and 40c mg/kg BW); *p < 0.05 vs SHAM group.

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Discussion and conclusion

The novel findings of this study are (1) RA has acute effect on reducing high blood pressure caused by ANG II; (2) the acute treatment of high dose RA (40 mg/kg) raises glucose transport activity in slow–twitch skeletal muscle that may not involve with the enhancement of ERK1/2 activity.

In vivo studies about the effects of ANG II inducing blood pressure in mammalian has been revealed. Cardiac hypertrophy is an adaptive change in response to ANG II-induced high blood pressure or by the effects of ANG II itself (Dostal and Baker, 1992; Rockman et al., 1994; Brink et al., 1999; Wang et al., 2001). In agreement with the previous studies, ANG II infusion (250 ng/kg/min) for 14 days used in this study shows an increase of heart weight to body weight ratio that may be prevented by chronic treatment of RA 20 and 40 mg/kg (Table 2). High blood pressure induced by ANG II observed in this study was blunted by all doses of RA treatments without showing any dose–dependent effect. Possible mechanisms of chronic RA treatment lowering blood pressure have been proposed. These involve with a decrease of ROS production that subsequently increases NO production, decreases of ET-1 and ACE activities (Karthik et al., 2011). Moreover, ACE inhibition (Li et al., 2008) and antioxidant (Kim et al., 2005) effects of RA have also been proposed. Although a chronic effect RA on lowering blood pressure has been recognized, an acute effect remains unrevealed. Therefore, our findings provide a new finding that an acute RA treatment also has blood pressure-lowering effect on ANG II-induced hypertensive rats (Figure 2). One possible explanation for this difference may relate to an acute systemic vasodilation due to RA treatments that decrease the total peripheral resistance, and subsequently decrease blood pressures. It is interesting that acute treatment of RA reduces blood pressure (33–42%) slightly more potent than the chronic treatment (23–32%) that may involve with a peak action of RA after an acute administration.

The present study found that RA might prevent changes of visceral fat weight, calorie intake, and final body weight that were induced by ANG II. Effects of ANG II on energy balance have been observed. Brink et al. (2001) stated that a reduction of body weight after infuse ANG II 500 ng/kg/min for 7 days did not due to only ANG II-induced anorexia itself, but it consistent with a catabolic response to ANG II. In our study, visceral fat weight decreased due to an increase in lipolysis that stimulated by chronic ANG II infusion. The mechanisms of lipolysis are involved with increase in local and

general sympathetic activity by ANG II. Beta-blockers can prevent lipolysis that induced by ANG II infusion (Cabassi et al., 2005). Considering ACE inhibitory effect, RA reduces ANG II production via inhibiting ACE activity; therefore, lipolysis could be prevented. Together, RA preventive effects on negative energy balance were observed in this study, and may involve with decrease in ACE activity that may decrease ANG II production.

Although the whole body insulin sensitivity did not show significantly decrease due to ANG II during the glucose tolerance tests, the significant reduction of plasma insulin concentration at the 15– and 120–minute time points, and the area under the curve of insulin were observed. One mechanism of the absolute effect of ANG II on reducing plasma insulin concentration during the glucose tolerance tests might be involved with an ANG II-induced beta cells dysfunction (Skipworth et al., 2011). The treatments of RA in this study did not show a positive effect on plasma insulin concentration during the glucose tolerance tests. In contrast to our study, Govindaraj and Sorimuthu Pillai (2015) studied the effects of oral administration of RA-100 mg/kg in diabetic rats for 30 days. They reported that RA can improve the whole-body insulin sensitivity, protects beta cell mass of pancreas, increased insulin level, and decreased glucose level. Moreover, Karthick et al. (2011) reported improvement of systemic insulin sensitivity, blood pressure, lipid profile, myocardial damage markers, and oxidative stress markers in high fructose-fed rats treated with RA 10 mg/kg for 45 days. Therefore, the possibility that our results fail to report an improving effect of RA on the whole-body insulin sensitivity should be a lower dose of RA used and a shorter duration of the RA treatments than the previous study. However, the used doses in the present study were low, it was suggested that RA was effective at least for high blood pressure induced by ANG II. Therefore, further studies are needed to define the higher dose or longer duration effect of RA treatment on improving insulin sensitivity in ANG II-treated rats.

The present study firstly reports that the pretreatments of RA (40 mg/kg) for 30 minutes increased insulin-stimulated glucose transport activities in slow-twitch skeletal muscles of ANG II-treated rats, whereas there were no changes in the chronic RA treatments. The major intracellular signaling of insulin-dependent skeletal muscle glucose transport that is the PI3-kinase/Akt pathway was not affected by acute or chronic RA administrations, whereas increased ERK1/2 activity was observed in the present study. Previous study indicated that chronic treatment of ANG II 200 ng/kg for 8

weeks abolished phosphorylation of Akt Ser473, Akt Thr308, AS160Thr642, and AMPKα Thr172 compared to control rats (Lastra et al., 2013). Although, the present study used higher dose of ANG II, we treated rats only 14 days, thus, we did not found significant changes in Akt Ser473 and GSK-3α/β phosphorylation. Similar to the present study, Lee et al. (2007) presented no significant effect on p38 MAPK and Akt phosphorylation in B16 melanoma mouse cells after treated with RA. In addition, effect of RA on increasing ERK activity has been indicated, for example, chronic treatment of RA 10 mg/kg for 14 days improved depressive-like behaviors or stress-related disorders via increases of ERK1/2 phosphorylation in hippocampus of rats that underwent chronic unpredictable stress (Jin et al., 2013) or an enhanced single prolonged stress paradigm (Nie et al., 2014). They suggest that RA might be used as an anti-depressant drugs or anti-stress-related disorders. Although there are positive effects of ERK on neuronal tissue, the significantly increased ERK1/2 activity in our study seem not to be a benefit for skeletal muscle glucose insulin sensitivity. Wojtaszewski et al. (1999) reported that ERK signaling pathway did not play a potent role in both insulin- and contraction-stimulated glucose transport and glycogen synthase activity in rats' skeletal muscles. Csibi et al., (2010) stated that ANG II decreases insulin sensitivity in skeletal muscle is the inhibition of Akt through at least 2 RNS-dependent pathways: 1) through the transient activation of the MEK-ERK1/2 pathway, which inhibits IRS1/2-dependent signaling; and 2) through direct nitration of Akt. Therefore, significant increased glucose transport activity due to a high dose of chronic RA treatment should be explained by other aspects. Since an ACE inhibitor activity of RA (Li et al., 2008), it can induce vasodilation through an antagonist of AT1 receptor and increase NO production. It is possible that the beneficial effects of RA on glucose transport activities were due to a simple increase availability of insulin and glucose due to vasodilation of capillaries inside the muscle strip during glucose transport measurements. In addition, NO production can improve GLUT-4 translocation in mammalian skeletal muscle (review in Henriksen and Jacob, 2003).

In conclusion, our data suggest that ANG II exhibit significant induced hypertension, cardiac hypertrophy, impaired metabolic manifestations, and energy balance, including decreased body weight, energy intake, intra abdominal fat, and plasma insulin concentration in response to glucose challenges. Both acute and chronic RA treatments in vivo could reduce hypertension. High dose of chronic RA may prevent cardiac hypertrophy, metabolic and energy balance impairments that caused by ANG II

administration. It is interesting that high dose of acute RA increased insulin-mediated skeletal muscle glucose transport activity that may not involve with enhancement of ERK1/2 activity. Taken together, these findings suggest benefits of RA on reducing of hypertension, cardiac hypertrophy, and anorexia; improving of body weight and visceral adiposity; and increasing slow-twitch muscle glucose transport in ANG II-treated rats.

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ผู้วิจัยอยู่ในระหว่างการจัดทำ manuscript เพื่อเผยแพร่ในวารสารระดับนานาชาติ ดังเอกสารแนบในภาคผนวก

ภาคผนวก

Rosmarinic acid improves insulin resistance and cardiovascular hypertension effects of angiotensin II

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Keyword: rosmarinic acid, angiotensin II, skeletal muscle, insulin resistance, mitogen-activated protein kinase

Abstract

Angiotensin II (ANG II) causes high blood pressure and insulin resistance via inducing oxidative stress may bring about the activation of mitogen-activated protein kinases (MAPKs) and inhibition of Akt-dependent insulin signaling. Antioxidative activity of rosmarinic acid (RA) has been revealed. This study was designed to explore whether rosmarinic acid has acute or chronic therapeutic potential for lowering blood pressure, insulin stimulating the whole-body insulin sensitivity and skeletal muscle glucose transport activity in ANG II-treated rats. The results show that both acute and chronic treatments of RA have blood pressure lowering effects. Only a high dose of acute treatment increased insulin-mediated glucose transport activity and ERK1/2 activity. Chronic treatment of RA reduces effects of ANG II on anorexia, body weight and visceral adiposity reducing. These results imply that RA may posses ANG II effects on cardiometabolic abnormalities, including, high blood pressure, anorexia, and decreases of body weight and visceral adiposity. However, an induction of glucose transport activity by RA may not involve with increasing ERK1/2 activity.

Introduction

Skeletal muscle insulin resistance is accompanied by cardio-metabolic abnormalities, including insulin resistance, impaired glucose tolerance, hypertension, obesity, and dyslipidemia (DeFronzo and Ferrannini, 1991; Reaven, 1993). Fail to control normal glucose transport of skeletal muscle is a major defect of maintaining normal circulating glucose level (Zeirath, 2000). Insulin is responsible for stimulating glucose transport activity in skeletal muscle. Insulin binds to the α-subunit, an extracellular portion of the insulin receptor and consequently enhances activities of tyrosine kinase in the β–subunit, an intracellular portion of the receptor. Auto-phosphorylation of the the β-subunits by the tyrosine kinase activity, stimulates the downstream signaling molecules comprising of insulin receptor substrate-1, p85 regulatory subunit and p110 catalytic subunit of phosphoinositide-3-kinase (PI3-kinase), the downstream targets of PI3-kinase, which are phosphoinositide-dependent kinase (PDK), Akt or protein kinase B, and the Akt-substrate protein AS160. The activation of these steps ultimately brings about the translocation of GLUT-4 containing vesicles to the plasma membrane allowing more glucose to diffuse into the cell (Shepherd & Kahn, 1999; Zierath et al., 2000).

Defective insulin signaling is a hallmark of skeletal muscle insulin resistance. The defects ultimately affect the normal GLUT-4 translocation to plasma membrane leading to a decrease rate of insulin-stimulated glucose transport into the skeletal muscle. Early studies have reported an attenuation of insulin signaling in insulin-resistant animals and humans. Long term high-fat fed lean Zucker rats presented a significant decrease in insulin-stimulated glucose uptake and impairments of insulin receptor β, tyrosine phosphorylated IRS-1, serine phosphorylated Akt, and glycogen synthase kinase-3β (GSK-3β) in skeletal muscle (Henriksen et al., 2007). In human subjects, non-obese insulin-resistant skeletal muscles showed an increase in serine phosphorylation of IRS-1 (negatively regulates IRS function), decrease in tyrosine phosphorytion of IRS-1 and threonine phosphorylation of Akt (Masharani et al., 2011).

The renin-angiotensin system (RAS) is a physiological system that regulates blood pressure. The precursor of RAS, angiotensinogen, is converted to angiotensin I (ANG I) by renin and ANG I consequently converted to angiotensin II (ANG II) by

angiotensin converting enzyme (ACE) (Santos et al., 2008; Xu et al., 2011). ANG II is a major hypertensive element of RAS that induces hypertensive effects of the cardiovascular system. Biological responses of ANG II are activated through the ANG II type 1 receptor (AT1R). Previous studies have reported that elevated ANG II involved with hypertension and insulin resistance (Gress et al., 2000; Reviewed in Henriksen et al., 2011; Togashi et al., 2012). Treatment of RAS blockage drugs in hypertensive patients, e.g. ACE inhibitors and AT1R blockers, effectively reduced blood pressure and oxidant production, enhanced insulin-stimulated skeletal muscle glucose transport activity, improved fasting glucose concentration, and reduced type 2 diabetes incidence (Blendea, 2005; Gress et al., 2000; The NAVIGATOR Study Group, 2010). In addition to the systemic roles of ANG II, direct negative roles of ANG II on skeletal muscle insulin sensitivity have been evaluated. Previous studies reported an induction of skeletal muscle insulin resistance by ANG II in normotensive rats by decreased of insulin-stimulated glucose transport activities. The underlying mechanisms may relate to overproduction of reactive oxygen species (ROS) that induce overactivities of MAPKs and inhibition of Akt-dependent insulin signaling cascade. These effects have been confirmed by several investigations about a superoxide radical scavenger that attenuates insulin resistance of ANG II-treated muscle (Diamond-Stanic and Henriksen, 2010; Wei et al., 2006; reviewed in Henriksen and Prasannarong, 2013), supporting a connection between ANG II action and insulin resistance in muscle. NADPH oxidase is activated after ANG II binds to AT1R. The activation of the NADPH oxidase is subsequently generating ROS that in turn leads to activations of MAPKs such as JNK, ERK, and p38 MAPK. Activation of MAPKs leads to the phosphorylation at serine residue of IRS-1 and followed by decreased interaction with PI3-K and the phosphorylation of serine residue of Akt (Aguirre et al., 2000; Hotamisligil et al., 2006; Henriksen et al., 2011; Prasannarong et al., 2012). Thus, the decreased GLUT-4 translocation and decreased glucose transport activities are observed in the ANG II-treated skeletal muscle. Moreover, skeletal muscle glycogen synthesis process decreased due to a decrease of the phosphorylation at the serine residue of GSK-3 due to ANG II (Diamond-Stanic and Henriksen, 2010). Recently, ANG II has been addressed as an important molecular mechanism that links the etiology of hypertension and insulin resistance. Therefore, strategies that modify RAS activities and resulting in decreased ANG II may be effective treatments for attenuating or treating individuals who have hypertension and insulin resistance.

RA is a natural pure compound isolated from many herbs that belong to Lamiaceae family such as rosemary, sage, basil, mint. These plants can be found in many parts of Thailand and are largely and routinely used in Thai cooking recipe. RA is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. The structure of RA is shown in Figure 1. A number of biological activities of RA have been revealed, including lipid lowering effects, anti-inflammation, antioxidant, decrease blood pressure and insulin sensitive activities (Sahu et al., 1999; Gao et al., 2005; Qiao et al., 2005; Sa´ et al., 2009; Kim et al., 2015). Long-term treatment of RA in fructose-induced hypertension and insulin resistance rats brought about improvements of the whole-body insulin sensitivity and circulating lipid levels as well as decreases in oxidative stress and cardiac damage markers (Karthik et al., 2011). Anti-inflammatory properties of RA were performed in animal model. In response to RA, carrageenan-induced pleurisy model was used for evaluating anti-inflammatory and nociceptive response of cells in the pleural exudates. Anti-inflammatory effects including reductions of polymorphonuclear and mononuclear cells migration into the pleural cavity were observed in the animals treated with RA (Gamaro et al., 2011). Sanbongi et al. (2003) studied in diesel exhaust particle (DEP) induced lung injury. The results suggested that anti-inflammatory effects of RA in this study model were associated with the changes in pro-inflammatory cytokine and chemokine expression include IL-1β, KC, MIP-1α, and MCP-1 that play a crucial role in initiating and progressing inflammatory response and involving pathology of metabolic abnormalities and insulin resistance. This study further revealed antioxidative activities of RA by reducing DEP generate ROS production. These evidences showed that RA has anti-inflammatory and antioxidative effects by inhibiting DEP-induced proinflammatory cytokine and ROS productions. Kim et al. (2005) proposed molecular mechanism explaining an antioxidative effect of RA treatment in adriamycin-induced injury of cardiac muscle cell line study. They reported that RA plays an antioxidative role by inhibiting ROS and MAPKs activities.

Although, the findings on chronic administrations of RA indicate its cardio-metabolic preventive role in lipid lowering, anti-inflammation, antioxidant, and blood pressure lowering effects have been revealed, the effect of RA on insulin action on skeletal muscle has yet to be investigated. Therefore, this study evaluated the potential effects of acute and chronic treatments of RA on ANG II-treated rats, in the aspects of

the whole-body insulin sensitivity, slow-twitch skeletal muscle insulin sensitivity and signaling proteins that involve glucose transport activities.

Materials and Methods

Materials

Rosmarinic acid was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Angiotensin II was purchased from AnaSpec Inc. (Fremont, CA, USA). Osmotic mini pump was purchased from DURECT Co. (Cupertino, CA, USA), Rat insulin RIA kits were purchased from Millipore (St. Charles, MO, USA). Glucose enzymatic colorimetric tests were purchased from HUMAN Gesellschaft für Biochemica und Diagnostica mbH (Wiesbaden, Germany). Antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Animals

Experiments were carried out using 8-week-old male Sprague Dawley rats (National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand). One week after arrival, rats were randomly divided to SHAM and ANG II-treated groups. ANG II (250 ng/kg/min) was delivered by implanting osmotic mini pump (model 2006, Alzet, CA) subcutaneously for 14 days. During the experiment, all SHAM and ANG II rats were gavaged with water, whereas the acute RA-treated rats received RA 10, 20, or 40 mg/kg by single gavage at 30 min before blood or tissue collection, and the chronic RA-treated rats received RA by gavage one time per day for 14 consecutive days. The chronic RA-treated rats received the last gavage at 1600-1700 hr before blood and tissue collections in the next day. Pharmacokinetic study reported that $t_{1/2}$ of RA was 56.9 min (Konishi et al., 2005) and its distribution in skeletal muscle tissue was found after single gavage for 30 min (Ritschel et al., 1989).

Oral glucose tolerance tests (OGTT)

OGTT were performed to determine the whole-body insulin sensitivity. In the evening (1800 hr) of the day before the test, rats were restricted to 4 g of chow. In the next morning (0800–0900 hr), tail blood were collected into microfuge tubes containing anticoagulant (18 mM final concentration of EDTA) at 0, 15, 30, 60, and 120 min after glucose feeding (1 g/kg) by gavage. The blood samples were centrifuged at 13,000×g

at 4°C for 1 min and plasma samples were collected for determining glucose and insulin concentrations (Saengsirisuwan et al., 2009). Then, each rat was given sterile 0.9% saline subcutaneously as soon as the test was completed for replacing body fluid loss. Plasma insulin and glucose concentrations were measured by RIA and enzymatic colorimetric test, respectively. Glucose–insulin index were calculated from an area under the curve (AUC) of glucose multiplied by insulin AUC.

Glucose transport activities (GT)

After food restriction, rats were weighed and deeply anesthetized with and intraperitoneal injection of pentobarbital sodium (75 mg/kg). After deeply anesthetized, soleus muscles were dissected and subsequently divided into two portions. Each muscle strip (weighing approximately 25 mg) was incubated at 37°C in 3 ml of oxygenated Krebs–Henseleit buffer (KHB) supplemented with 8 mM D-glucose, 32 mM D-mannitol, 0.1% radioimmunoassay-grade bovine serum albumin, and the present or absent of 5 mU/ml insulin. Each flask was gassed with 95% O_2–5% CO_2 throughout incubation period of glucose transport activity measurements. At the end of the incubation, muscles were removed from the flask, trimmed off excess fat and connective tissue, frozen with liquid nitrogen, and immediately weighed. Then, the muscle strips were solubilized in 0.5 ml of 0.5 N NaOH for 1 hour and mixed with 10 ml of scintillation cocktail. The specific intracellular accumulation of 2–DG was determined by using mannitol to correct for the extracellular accumulation of 2–DG. Glucose transport activities were measured as the intracellular accumulation of 2–DG (in pmol/mg muscle wet weight/20 min) (Henriksen and Halseth, 1994).

Skeletal muscle protein abundance and phosphorylation using immunoblotting

Muscle strips were collected after incubations in the same solution type that was used for measuring GT in the condition of present or absent of 5 mU/ml insulin. After an incubation, each muscle strip was trimmed off excess fat and connective tissue, quickly frozen in liquid nitrogen and kept at -80°C until performing immunoblots. Muscle strips were homogenized in ice-cold lysis buffer: 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM CaCl2, 1mM MgCl2, 2 mM EDTA, 10 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 2 mM Na3VO4, 10 μg/ml aprotinin and leupeptin, and 2 mM PMSF. After a 20-min incubation on ice, the homogenates were centrifuged at 13,000 g for 20 min at 4°C. Proteins in the

homogenates were separated on polyacrylamide gels and transferred electrophoretically onto nitrocellulose paper. Blots were incubated with an appropriate dilution of commercially available antibodies against phospho-Akt (Ser473), Akt 1/2, phospho-GSK-3α/β (Ser21/Ser9), GSK-3α/β, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, GAPDH. Subsequently, all blots were incubated with anti-rabbit IgG, HRP–linked antibody. Protein bands were visualized by enhanced chemiluminescence. Images were digitized, and band intensities were quantified using Image Studio software (LI-COR Inc., Lincoln, NE, USA)

Statistical analysis

Values are reported as the means ± SE. One-way analyses of variance (ANOVA) with Tukey's HSD post hoc test were used to determine the significant difference from among groups. Statistical analyses will be performed using SPSS 16.0 (SPSS Inc., Chicago, IL). A value of $P < 0.05$ is considered to be statistically significant.

Results

In the present study, the potential therapeutic effects of acute and chronic RA on insulin resistance induced by ANG II were performed in rats. Whole-body insulin sensitivity, slow-twitch skeletal muscle glucose transport activities, and changes of the involved signaling proteins, including MAPKs and insulin signaling cascade) at the presence or absence of insulin were measured.

Impact of ANG II on blood pressure, organ weights, and energy balance

Administrations of ANG II for 14 days caused increases in systolic, diastolic, and mean arterial blood pressure since the first week (38–39%) and after two weeks of the infusions (58–65%) (Figure 2, $P < 0.05$). At the end of experiment ANG II rats presented increases in heart weight to body weight ratio (26–34%), decrease in liver weights (Table 1, $P < 0.05$; Table 2, $P < 0.05$), but no change of liver weight to body weight ratio. Considering the energy balance, reductions of total energy intake (16%), body weight (8–12%), and intra-abdominal fat weight (27–32%) were observed (Table 1, $P < 0.05$; Table 2, $P < 0.05$).

Effects of ANG II on the whole-body and skeletal muscle insulin sensitivity

Chronic treatments of angiotensin II decrease a rising of plasma insulin concentration during the OGTT. Although the insulin AUC of ANG II-treated rats was significantly reduced for 22% and 27% when compared to SHAM rats, there was no significant change in glucose AUC or G-I index (Figure 3). There were no change in slow-twitch muscle glucose transport activities (Figure 4) and its protein elements due to ANG II (Figure 5).

Impact of acute and chronic of RA on blood pressure, organ weights, and energy balance

All doses of acute or chronic RA treatment attenuated the effects of ANG II on increasing blood pressure. A reduction of blood pressure was found in acute treatment of RA by 33–42% and chronic treatment of RA by 23–32%, as shown in Figure 2 ($P < 0.05$). Both types of RA administration did not significantly alter liver weight that decreased by the effect of ANG II (Table 2). In contrast, chronic RA treatments (20 and 40 mg/kg) may prevent the effects of ANG II that caused increased heart weight to body weight ratio, reduced intra abdominal fat weight, fat weight to the body weight ratio, body weight, and energy intake (Table 2).

Effects of RA treatments on the whole-body and skeletal muscle insulin sensitivity

Angiotensin II infusion for 14 days caused significantly lower plasma insulin concentration during OGTT. Neither acute nor chronic administrations of RA can gain the response back to the level of SHAM rats (Figure 3). Skeletal muscle glucose transport activities did not change after ANG II infusion. It is interesting that acute RA administration of 20 and 40 mg/kg caused significantly increase in insulin-stimulated glucose transport activities by 31% and 39% when compared with SHAM rats, whereas only 40 mg/kg of RA caused increase in insulin-mediated glucose transport activities (the difference between basal and insulin-stimulated condition) by 45% (Figure 4, $P < 0.05$). No effect of ANG II was observed on skeletal muscle signaling elements in both basal and insulin-stimulated condition in this study (Figure 5). Thirty minutes after acute treatment of RA at 40 mg/kg increased insulin-stimulated ERK1/2 activity was in agreement with increased insulin-mediated glucose transport activities in slow-twitch skeletal muscle (Figure 6, $P < 0.05$).

Discussion

The novel findings of this study are (1) RA has acute effect on reducing high blood pressure caused by ANG II; (2) the acute treatment of high dose RA (40 mg/kg) raises glucose transport activity in slow–twitch skeletal muscle that may not involve with the enhancement of ERK1/2 activity.

In vivo studies about the effects of ANG II inducing blood pressure in mammalian has been revealed. Cardiac hypertrophy is an adaptive change in response to ANG II-induced high blood pressure or by the effects of ANG II itself (Dostal and Baker, 1992; Rockman et al., 1994; Brink et al., 1999; Wang et al., 2002). In agreement with the previous studies, ANG II infusion (250 ng/kg/min) for 14 days used in this study shows an increase of heart weight to body weight ratio that may be prevented by chronic treatment of RA 20 and 40 mg/kg (Table 2). High blood pressure induced by ANG II observed in this study was blunted by all doses of RA treatments without showing any dose–dependent effect. Possible mechanisms of chronic RA treatment lowering blood pressure have been proposed. These involve with a decrease of ROS production that subsequently increases NO production, decreases of ET-1 and ACE activities (Karthik et al., 2011). Moreover, ACE inhibition (Li et al., 2008) and antioxidant (Kim et al., 2005) effects of RA have also been proposed. Although a chronic effect RA on lowering blood pressure has been recognized, an acute effect remains unrevealed. Therefore, our findings provide a new finding that an acute RA treatment also has blood pressure-lowering effect on ANG II-induced hypertensive rats (Figure 2). One possible explanation for this difference may relate to an acute systemic vasodilation due to RA treatments that decrease the total peripheral resistance, and subsequently decrease blood pressures. It is interesting that acute treatment of RA reduces blood pressure (33–42%) slightly more potent than the chronic treatment (23–32%) that may involve with a peak action of RA after an acute administration.

The present study found that RA might prevent changes of visceral fat weight, calorie intake, and final body weight that were induced by ANG II. Effects of ANG II on energy balance have been observed. Brink et al. (2001) stated that a reduction of body weight after infuse ANG II 500 ng/kg/min for 7 days did not due to only ANG II-induced anorexia itself, but it consistent with a catabolic response to ANG II. In our study,

visceral fat weight decreased due to an increase in lipolysis that stimulated by chronic ANG II infusion. The mechanisms of lipolysis are involved with increase in local and general sympathetic activity by ANG II. Beta-blockers can prevent lipolysis that induced by ANG II infusion (Cabassi et al., 2005). Considering ACE inhibitory effect, RA reduces ANG II production via inhibiting ACE activity; therefore, lipolysis could be prevented. Together, RA preventive effects on negative energy balance were observed in this study, and may involve with decrease in ACE activity that may decrease ANG II production.

Although the whole body insulin sensitivity did not show significantly decrease due to ANG II during the glucose tolerance tests, the significant reduction of plasma insulin concentration at the 15– and 120–minute time points, and the area under the curve of insulin were observed. One mechanism of the absolute effect of ANG II on reducing plasma insulin concentration during the glucose tolerance tests might be involved with an ANG II-induced beta cells dysfunction (Skipworth et al., 2011). The treatments of RA in this study did not show a positive effect on plasma insulin concentration during the glucose tolerance tests. In contrast to our study, Govindaraj and Sorimuthu Pillai (2015) studied the effects of oral administration of RA-100 mg/kg in diabetic rats for 30 days. They reported that RA can improve the whole-body insulin sensitivity, protects beta cell mass of pancreas, increased insulin level, and decreased glucose level. Moreover, Karthick et al. (2011) reported improvement of systemic insulin sensitivity, blood pressure, lipid profile, myocardial damage markers, and oxidative stress markers in high fructose-fed rats treated with RA 10 mg/kg for 45 days. Therefore, the possibility that our results fail to report an improving effect of RA on the whole-body insulin sensitivity should be a lower dose of RA used and a shorter duration of the RA treatments than the previous study. However, the used doses in the present study were low, it was suggested that RA was effective at least for high blood pressure induced by ANG II. Therefore, further studies are needed to define the higher dose or longer duration effect of RA treatment on improving insulin sensitivity in ANG II-treated rats.

The present study firstly reports that the pretreatments of RA (40 mg/kg) for 30 minutes increased insulin-stimulated glucose transport activities in slow-twitch skeletal muscles of ANG II-treated rats, whereas there were no changes in the chronic RA

treatments. The major intracellular signaling of insulin-dependent skeletal muscle glucose transport that is the PI3-kinase/Akt pathway was not affected by acute or chronic RA administrations, whereas increased ERK1/2 activity was observed in the present study. Previous study indicated that chronic treatment of ANG II 200 ng/kg for 8 weeks abolished phosphorylation of Akt Ser473, Akt Thr308, AS160Thr642, and AMPKα Thr172 compared to control rats (Lastra et al., 2013). Although, the present study used higher dose of ANG II, we treated rats only 14 days, thus, we did not found significant changes in Akt Ser473 and GSK-3α/β phosphorylation. Similar to the present study, Lee et al. (2007) presented no significant effect on p38 MAPK and Akt phosphorylation in B16 melanoma mouse cells after treated with RA. In addition, effect of RA on increasing ERK activity has been indicated, for example, chronic treatment of RA 10 mg/kg for 14 days improved depressive-like behaviors or stress-related disorders via increases of ERK1/2 phosphorylation in hippocampus of rats that underwent chronic unpredictable stress (Jin et al., 2013) or an enhanced single prolonged stress paradigm (Nie et al., 2014). They suggest that RA might be used as an anti-depressant drugs or anti-stress-related disorders. Although there are positive effects of ERK on neuronal tissue, the significantly increased ERK1/2 activity in our study seem not to be a benefit for skeletal muscle glucose insulin sensitivity. Wojtaszewski et al. (1999) reported that ERK signaling pathway did not play a potent role in both insulin- and contraction-stimulated glucose transport and glycogen synthase activity in rats' skeletal muscles. Csibi et al., (2010) stated that ANG II decreases insulin sensitivity in skeletal muscle is the inhibition of Akt through at least 2 RNS-dependent pathways: 1) through the transient activation of the MEK-ERK1/2 pathway, which inhibits IRS1/2-dependent signaling; and 2) through direct nitration of Akt. Therefore, significant increased glucose transport activity due to a high dose of chronic RA treatment should be explained by other aspects. Since an ACE inhibitor activity of RA (Li et al., 2008), it can induce vasodilation through an antagonist of AT1 receptor and increase NO production. It is possible that the beneficial effects of RA on glucose transport activities were due to a simple increase availability of insulin and glucose due to vasodilation of capillaries inside the muscle strip during glucose transport measurements. In addition, NO production can improve GLUT-4 translocation in mammalian skeletal muscle (review in Henriksen and Jacob, 2003).

In conclusion, our data suggest that ANG II exhibit significant induced hypertension, cardiac hypertrophy, impaired metabolic manifestations, and energy balance, including decreased body weight, energy intake, intra abdominal fat, and plasma insulin concentration in response to glucose challenges. Both acute and chronic RA treatments *in vivo* could reduce hypertension. High dose of chronic RA may prevent cardiac hypertrophy, metabolic and energy balance impairments that caused by ANG II administration. It is interesting that high dose of acute RA increased insulin-mediated skeletal muscle glucose transport activity that may not involve with enhancement of ERK1/2 activity. Taken together, these findings suggest benefits of RA on reducing of hypertension, cardiac hypertrophy, and anorexia; improving of body weight and visceral adiposity; and increasing slow-twitch muscle glucose transport in ANG II-treated rats. Therefore, RA may be an alternative strategy for protecting against ANG II-induce cardiometabolic abnormalities.

Abbreviations and nomenclature

ACE	Angiotensin converting enzyme
ANG II	Angiotensin II
GSK	Glycogen synthase kinase
ERK	Extracellular regulated kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
RA	Rosmarinic acid
RAS	Renin-angiotensin system
ROS	Reactive oxygen species

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Table 1. Initial and final body weight, total calorie intake, and organ weights of SHAM and ANG II treated rats with or without acute administrations of RA at 10, 20, and 40 mg/kg BW. Numbers of rats for each group was 6-10. *p < 0.05 vs SHAM group.

Table 2. Initial and final body weight, total calorie intake, and organ weights of SHAM and ANG II treated rats with or without chronic administrations of RA at 10, 20, and 40 mg/kg BW. Numbers of rats for each group was 6-10. *p < 0.05 vs SHAM group.

Figure 1. The structure of rosmarinic acid (RA).

Figure 2. Systolic, diastolic, and mean arterial blood pressure of SHAM, ANG II, acute RA treatment (10a, 20a, and 40a mg/kg BW), and chronic RA treatment groups (10c, 20c, and 40c mg/kg BW) ; values are mean ± SE of 6-10 rats per group. *p < 0.05 vs SHAM group; †p < 0.05 vs ANG II group; Φp < 0.05 R-10c vs SHAM group.

Figure 3. Plasma glucose and insulin concentrations during oral glucose tolerance tests (OGTTs), glucose and insulin area under the curve (AUC), and glucose-insulin index (G-I index) of SHAM, ANG II, acute RA (10a, 20a, and 40a mg/kg) and chronic RA (10c, 20c, and 40c mg/kg) groups; values are mean ± SE of 5-8 rats per group. *p < 0.05 vs SHAM group; Φp < 0.05 RA-10a, RA-20a, or RA-40a vs SHAM group.

Figure 4. Glucose transport activity at basal, insulin stimulation, and different changes between basal and insulin stimulation of SHAM, ANG II, acute (10a, 20a, and 40a mg/kg BW), and chronic administration of RA groups (10c, 20c, and 40c mg/kg BW). Numbers of rats for each group was 6-10. *p < 0.05 vs SHAM group.

Figure 5. Insulin signaling of SHAM, ANG II, acute (10a, 20a, and 40a mg/kg BW), and chronic administration of RA groups (10c, 20c, and 40c mg/kg BW); *p < 0.05 vs SHAM group.

Figure 6. MAPK signaling of SHAM, ANG II, acute (10a, 20a, and 40a mg/kg BW), and chronic administration of RA groups (10c, 20c, and 40c mg/kg BW); *p < 0.05 vs SHAM group.

Table 1.

	SHAM	ANG II	RA-10a	RA-20a	RA-40a
Body weight (g)					
Initial weight	373.55 ± 5.58	372.25 ± 5.83	364.41 ± 9.07	365.47 ± 7.23	368.03 ± 5.48
Final weight (BW)	408.53 ± 6.34	366.40 ± 13.23 *	358.02 ± 9.77 *	362.86 ± 9.28 *	358.94 ± 9.18 *
Total energy intake (kcalx10^3)	859.96 ± 33.26	720.06 ± 41.04 *	718.95 ± 38.93 *	727.59 ± 30.38 *	721.98 ± 10.06 *
Liver weight (LW; mg)	10.80 ± 0.31	9.30 ± 0.20 *	9.25 ± 0.25 *	9.66 ± 0.50 *	9.93 ± 0.50 *
LW/kg BW	26.96 ± 0.85	27.04 ± 0.86	26.67 ± 0.58	27.86 ± 0.82	28.01 ± 0.85
Heart weight (HW; mg)	1.12 ± 0.04	1.26 ± 0.02	1.27 ± 0.04	1.29 ± 0.04	1.31 ± 0.03
HW/kg BW	2.81 ± 0.07	3.67 ± 0.17 *	3.66 ± 0.11 *	3.75 ± 0.13 *	3.71 ± 0.12 *
Intra abdominal fat weight (FW; g)	18.61 ± 1.25	13.13 ± 2.34 *	12.67 ± 0.79 *	12.63 ± 0.77 *	13.69 ± 0.77 *
FW/kg BW	46.51 ± 3.32	37.34 ± 5.06 *	35.46 ± 1.89 *	36.29 ± 0.94 *	38.05 ± 1.66 *

Table 2.

	SHAM	ANG II	RA-10c	RA-20c	RA-40c
Body weight (g)					
Initial weight	374.10 ± 6.08	372.92 ± 7.30	383.71 ± 5.87	373.28 ± 5.89	379.00 ± 4.71
Final weight (BW)	400.80 ± 4.79	369.16 ± 9.57 *	363.99 ± 11.71 *	383.77 ± 11.85	373.24 ± 9.82
Total energy intake (kcalx10^3)	858.65 ± 37.16	725.75 ± 64.41 *	715.44 ± 30.77 *	819.78 ± 35.36	749.74 ± 42.88
Liver weight (LW; mg)	11.64 ± 0.36	10.02 ± 0.27 *	10.44 ± 0.45	10.99 ± 0.43	10.68 ± 0.38
LW/kg BW	28.80 ± 0.75	28.53 ± 0.64	28.63 ± 0.60	28.64 ± 0.69	28.58 ± 0.55
Heart weight (HW; mg)	1.21 ± 0.04	1.34 ± 0.04	1.32 ± 0.04	1.30 ± 0.04	1.30 ± 0.04
HW/kg BW	2.98 ± 0.10	3.75 ± 0.14 *	3.65 ± 0.14 *	3.42 ± 0.13	3.41 ± 0.10
Intra abdominal fat weight (FW; g)	18.32 ± 1.21	13.33 ± 1.01 *	13.43 ± 1.24 *	14.29 ± 1.08	14.82 ± 0.54
FW/kg BW	45.21 ± 2.64	36.73 ± 2.18 *	36.46 ± 2.75 *	37.92 ± 1.53	37.30 ± 2.15

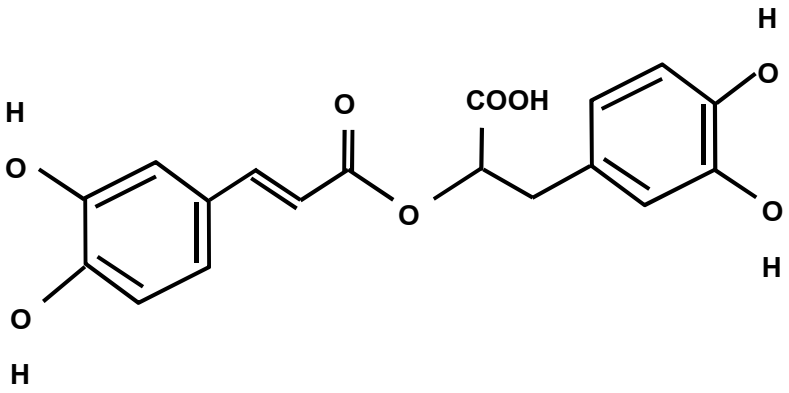

Figure 1.

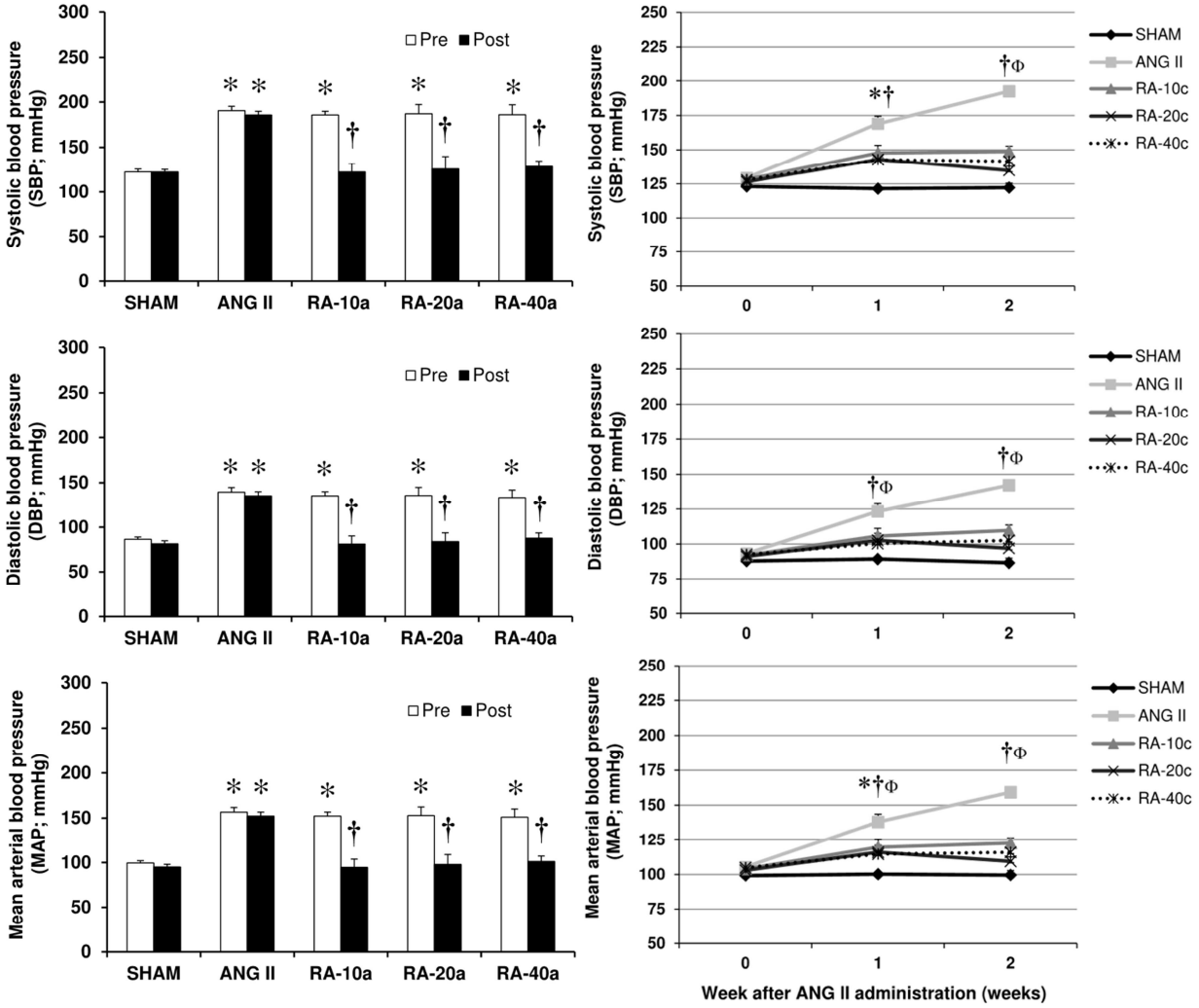
Systolic blood pressure (SBP; mmHg)
Diastolic blood pressure (DBP; mmHg)
Mean arterial blood pressure (MAP; mmHg)
Pre
Post
SHAM
ANG II
RA-10a
RA-20a
RA-40a
SHAM
ANG II
RA-10c
RA-20c
RA-40c
Week after ANG II administration (weeks)

Figure 2.

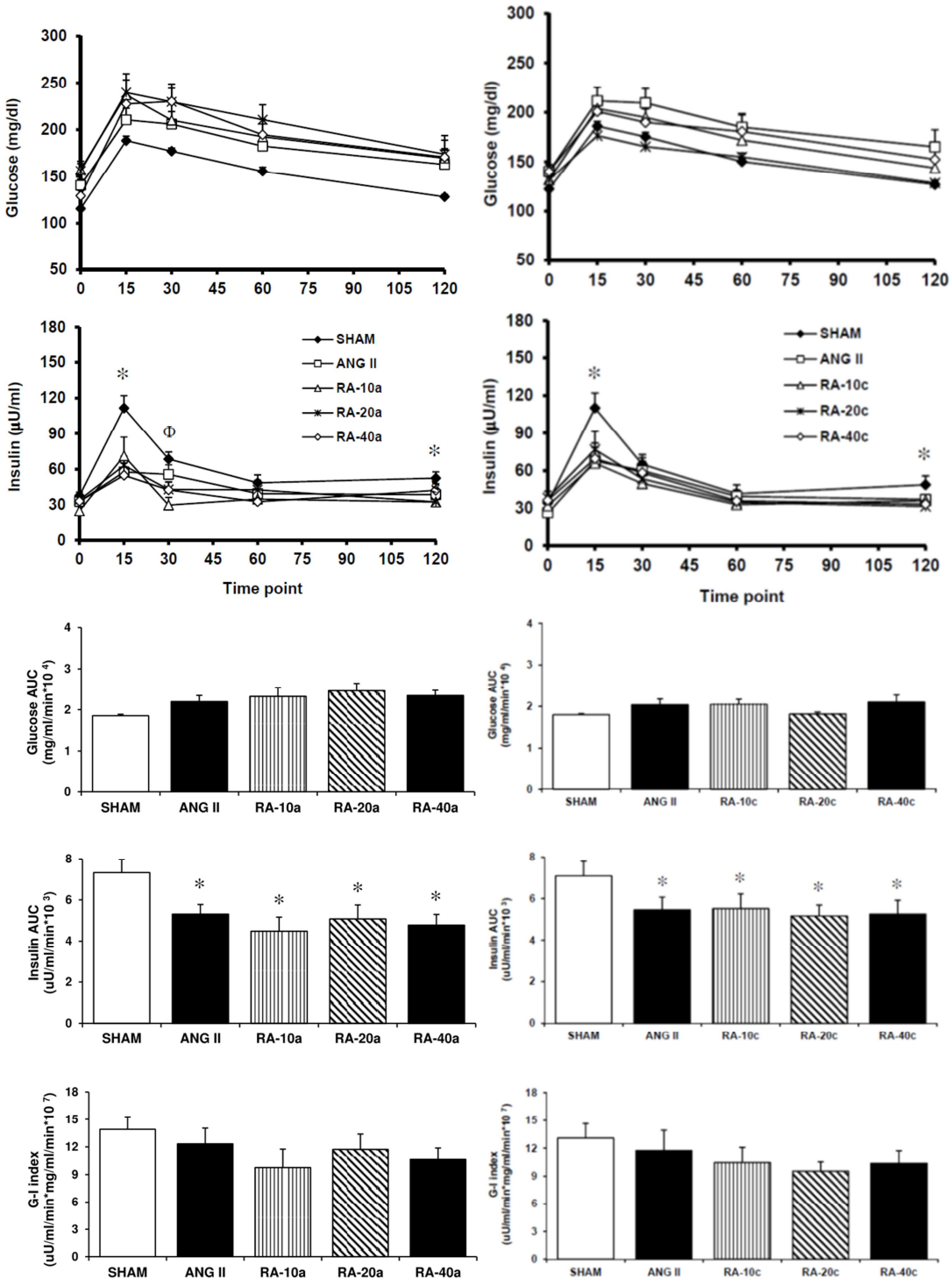
Glucose (mg/dl)
Insulin (μU/ml)
SHAM
ANG II
RA-10a
RA-20a
RA-40a
RA-10c
RA-20c
RA-40c
Time point
Glucose AUC (mg/ml/min*10 4)
Insulin AUC (uU/ml/min*10 3)
G-I index (uU/ml/min*mg/ml/min*10 7)

Figure 3.

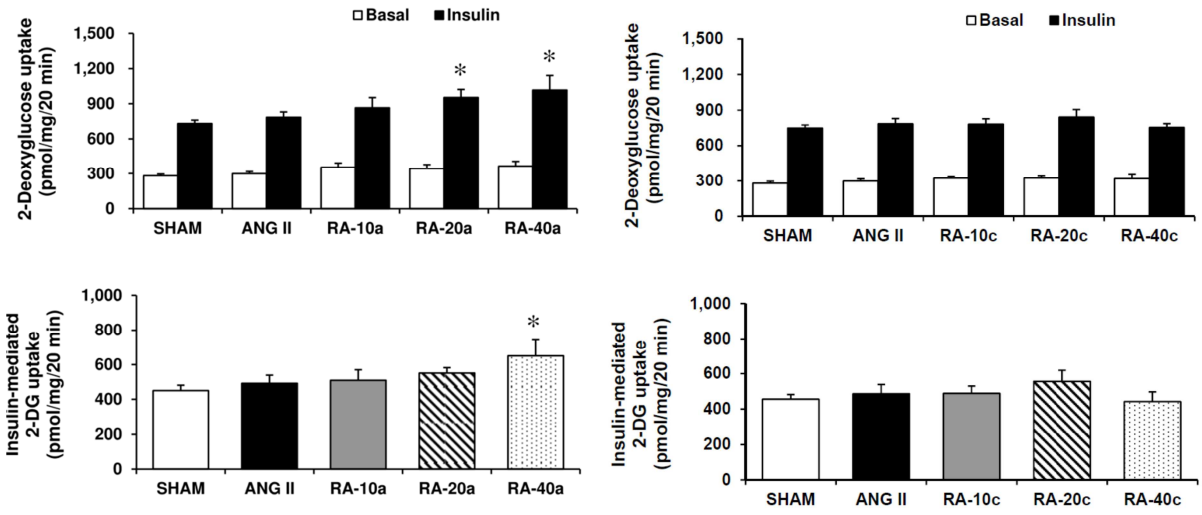
Basal
Insulin
2-Deoxyglucose uptake (pmol/mg/20 min)
1,500
1,200
900
600
300
0
SHAM
ANG II
RA-10a
RA-20a
RA-40a
RA-10c
RA-20c
RA-40c
Insulin-mediated 2-DG uptake (pmol/mg/20 min)
1,000
800
600
400
200
0
*

Figure 4.

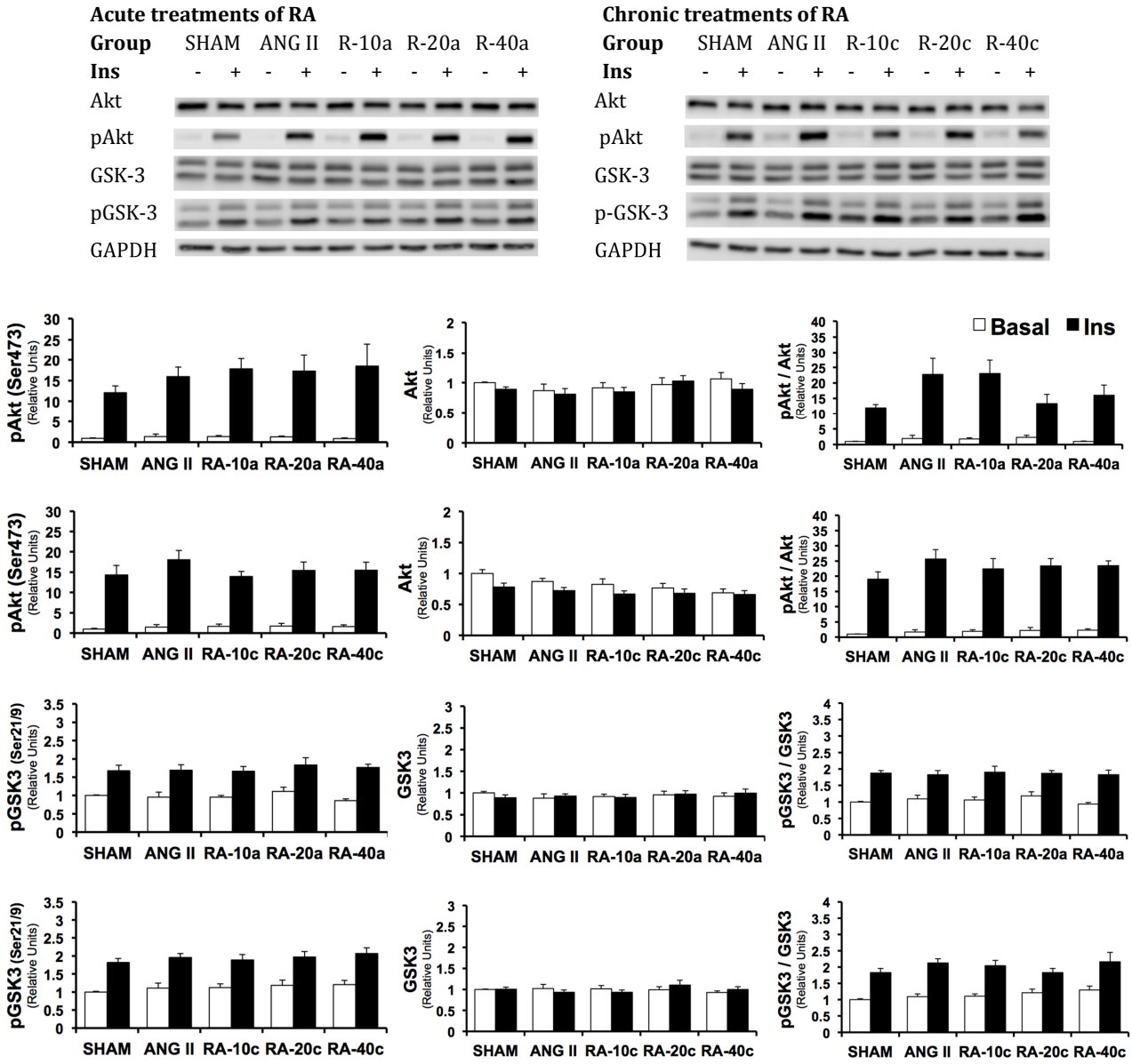
Acute treatments of RA
Group SHAM ANG II R-10a R-20a R-40a
Ins - + - + - + - + - +
Akt
pAkt
GSK-3
pGSK-3
GAPDH
Chronic treatments of RA
Group SHAM ANG II R-10c R-20c R-40c
Ins - + - + - + - + - +
Akt
pAkt
GSK-3
p-GSK-3
GAPDH
pAkt (Ser473) (Relative Units)
Akt (Relative Units)
pAkt / Akt (Relative Units)
Basal
Ins
SHAM ANG II RA-10a RA-20a RA-40a
SHAM ANG II RA-10c RA-20c RA-40c
pGSK3 (Ser21/9) (Relative Units)
GSK3 (Relative Units)
pGSK3 / GSK3 (Relative Units)

Figure 5.

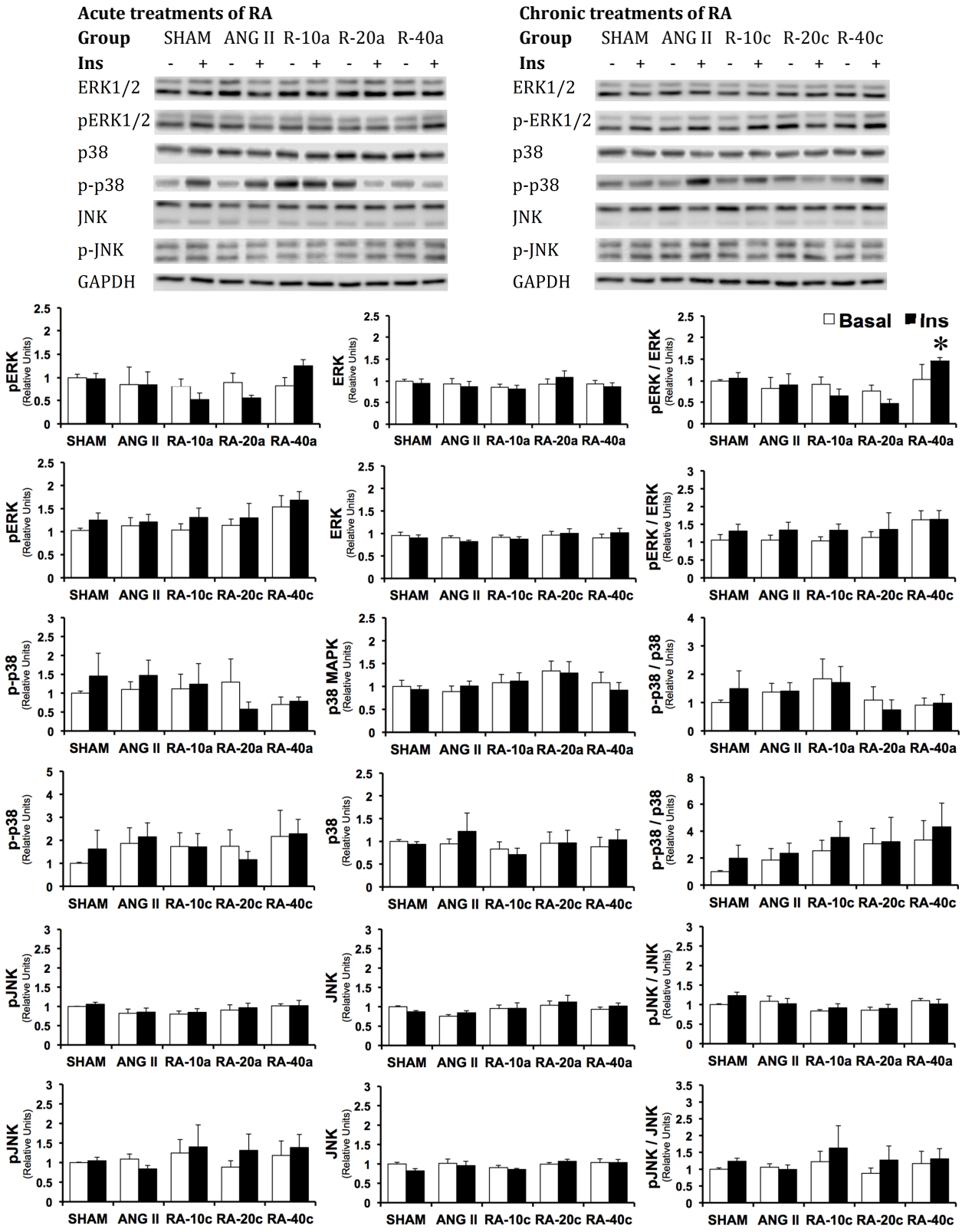
Acute treatments of RA
Group SHAM ANG II R-10a R-20a R-40a
Ins - + - + - + - + - +
ERK1/2
pERK1/2
p38
p-p38
JNK
p-JNK
GAPDH
Chronic treatments of RA
Group SHAM ANG II R-10c R-20c R-40c
Ins - + - + - + - + - +
ERK1/2
p-ERK1/2
p38
p-p38
JNK
p-JNK
GAPDH
Basal
Ins
*
pERK (Relative Units)
ERK (Relative Units)
pERK / ERK (Relative Units)
p-p38 (Relative Units)
p38 MAPK (Relative Units)
p-p38 / p38 (Relative Units)
p38 (Relative Units)
pJNK (Relative Units)
JNK (Relative Units)
pJNK / JNK (Relative Units)
SHAM ANG II RA-10a RA-20a RA-40a
SHAM ANG II RA-10c RA-20c RA-40c

Figure 6.