รายงานวิจัยฉบับสมบูรณ์

ผลของการออกกำลังกายอย่างสม่ำเสมอต่อสัญญาณอินซูลินและ MAPK ในกล้ามเนื้อลายของหนูที่เกิดภาวะดื้อต่ออินซูลินจากการตัดรังไข่

โดย ผู้ช่วยศาสตราจารย์ ดร. วิฑูร แสงศิริสุวรรณ

มิถุนายน 2556

สัญญาเลขที่ RMU5380010

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและสำนักงานคณะกรรมการการอุดมศึกษา

ABSTRACT

Project Code: RMU5380010

Project Title: Insulin signaling and mitogen-activated protein kinases in skeletal muscle of exercise-trained ovariectomized rats

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Insulin resistance of skeletal muscle glucose transport due to prolonged loss of ovarian function in ovariectomized (OVX) rats is accompanied by other features of the metabolic syndrome and may be confounded by increased calorie consumption. No study has reported the cellular mechanisms underlying the insulin-resistant state in skeletal muscle following prolonged ovariectomy with or without dietary manipulation. To address these issues, a number of metabolic characteristics, including glucose tolerance, insulin-stimulated skeletal muscle glucose transport, serum lipid profile, and visceral fat accumulation were determined in OVX rats that either had free access to food, pair feeding (PF) with SHAM or received a 35% reduction in food intake (calorie restriction; CR) for 12 weeks. In addition, the protein expression and functionality of specific elements of the PI3-kinase and MAPK pathways including insulin receptor (IR), insulin receptor substrate-1 (IRS-1), the p85 subunit of PI3-kinase, Akt, c-Jun NH_2-terminal kinase (SAPK/JNK), and p38 MAPK were evaluated. Skeletal muscle insulin resistance in OVX rats was associated with impaired tyrosine phosphorylation of IR and IRS-1, IRS-1 associated p85 subunit of PI3-kinase, and Akt Ser^{473} phosphorylation, whereas insulin-stimulated phosphorylation of IRS-1 Ser^{307}, SAPK/JNK Thr^{183}/Tyr^{185}, and p38 mitogen-activated protein kinase (MAPK) Thr^{180}/Tyr^{182} was enhanced. PF did not restore insulin-stimulated glucose transport, indicating that insulin resistance in OVX rats is a consequence of ovarian hormone deprivation. In contrast, impaired insulin sensitivity and defective insulin signaling were not observed in the skeletal muscle of OVX+CR rats.

Additionally, the cellular mechanisms by which exercise training improved insulin action in OVX rats were assessed. The protein expression and functionality of signaling elements in skeletal muscle were evaluated in OVX rats that remained sedentary, performed exercise training (ET), received 2.5 μg/kg of 17-β estradiol (E_2), or underwent both treatments. Individual treatment with ET or E_2 increased GLUT-4 protein abundance in OVX muscles. ET improved IRS-1/pY but not the downstream signaling elements whereas E_2 restored the defects in the insulin signaling cascade. The combined treatment brought about an additive effect on insulin-stimulated glucose transport. However, no interactive effects of ET and E_2 on the signaling proteins involved in the regulation of glucose transport were observed. Conclusively, the present investigation provided evidence for the first time that 1) CR effectively prevented the development of insulin resistance and impaired insulin signaling in the skeletal muscle of OVX rats; and 2) the beneficial effect of ET on insulin action on skeletal muscle glucose transport in OVX rats was not due to adaptations of proteins in the insulin signaling cascade.

Keywords: insulin resistance, insulin signaling, MAPK, calorie restriction, exercise, estrogen, ovariectomy

บทคัดย่อ

รหัสโครงการ: RMU5380010

ชื่อโครงการ: ผลของการออกกำลังกายอย่างสม่ำเสมอต่อสัญญาณอินซูลินและ MAPK ในกล้ามเนื้อลายของหนูที่เกิดภาวะดื้อต่ออินซูลินจากการตัดรังไข่

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ภาวะดื้อต่ออินซูลินที่พบในกล้ามเนื้อของหนูแรทที่ถูกตัดรังไข่ อาจเกิดขึ้นได้จากการขาดฮอร์โมนจากรังไข่หรือจากการกินที่เพิ่มขึ้นซึ่งเป็นผลจากการตัดรังไข่ออก การศึกษาที่หนึ่งทำการเปรียบเทียบตัวแปรทางเมแทบอลิสมในสัตว์ทดลองสี่กลุ่มได้แก่ หนูแรทที่ไม่ถูกตัดรังไข่ หนูแรทตัดรังไข่ออก หนูแรทตัดรังไข่ออกที่กินอาหารในปริมาณเท่ากับหนูแรทที่ไม่ถูกตัดรังไข่ และหนูแรทตัดรังไข่ออกที่กินอาหารปริมาณน้อยกว่าหนูแรทตัดรังไข่ร้อยละ 35 เป็นเวลา 12 สัปดาห์ ตัวแปรที่ศึกษา ได้แก่ ความสามารถของร่างกายในการรักษาระดับกลูโคสในเลือด ผลของอินซูลินในการกระตุ้นอัตราการขนถ่ายกลูโคสของกล้ามเนื้อลาย ระดับไขมันในเลือด และปริมาณในไขมันที่สะสมในช่องท้อง ปริมาณและการทำงานของโปรตีนสัญญาณอินซูลินและ MAPK ในกล้ามเนื้อลาย ผลการทดลองพบว่าหนูตัดรังไข่มีความผิดปกติในการทำงานของโปรตีนสัญญาณอินซูลินและ MAPK ภาวะดื้อต่ออินซูลินยังคงมีในหนูตัดรังไข่ที่กินอาหารปริมาณเท่ากับหนูแรทที่ไม่ถูกรังไข่ ดังนั้นภาวะดื้อต่ออินซูลินในหนูตัดรังไข่เกิดจากการขาดฮอร์โมนจากรังไข่และไม่เกี่ยวข้องกับการกินที่เพิ่มขึ้น อย่างไรก็ตาม ภาวะดื้อต่ออินซูลินและความบกพร่องของสัญญาณอินซูลินและ MAPK ในหนูตัดรังไข่ที่ถูกจำกัดอาหารลงร้อยละ 35 มีค่าใกล้เคียงกับหนูแรทที่ไม่ถูกตัดรังไข่

การศึกษาที่สองทำการประเมินผลของการออกกำลังกายอย่างสม่ำเสมอต่อปริมาณและการทำงานของโปรตีนสัญญาณอินซูลินและ MAPK ในกล้ามเนื้อลายในหนูตัดรังไข่ พบว่าการออกกำลังกายอย่างสม่ำเสมอลดภาวะดื้อต่ออินซูลิน ไม่มีผลต่อความบกพร่องของโปรตีนสัญญาณในกล้ามเนื้อของหนูตัดรังไข่ แต่เพิ่มการแสดงออกของโปรตีนขนถ่ายกลูโคสในกล้ามเนื้อ อัตราการขนถ่ายกลูโคสเข้ากล้ามเนื้อของหนูตัดรังไข่มีค่าสูงขึ้นในกลุ่มที่ออกกำลังกายอย่างสม่ำเสมอร่วมกับได้รับฮอร์โมนเอสโตรเจนทดแทน อย่างไรก็ตาม การออกกำลังกายและฮอร์โมนทดแทนไม่มีผลเสริมกันต่อการเปลี่ยนแปลงของโปรตีนสัญญาณหรือตัวแปรอื่นๆที่เกี่ยวข้อง การศึกษาทั้งสองส่วนสรุปได้ว่า 1) การจำกัดอาหารลงร้อยละ 35 สามารถป้องกันการเกิดภาวะดื้อต่ออินซูลินและความบกพร่องของโปรตีนสัญญาณในกล้ามเนื้อลายของหนูตัดรังไข่ และ 2) กลไกที่การออกกำลังกายอย่างสม่ำเสมอในการเพิ่มฤทธิ์ของอินซูลินในการกระตุ้นการขนถ่ายกลูโคส โดยไม่เกี่ยวข้องกับการเปลี่ยนแปลงของโปรตีนสัญญาณในกล้ามเนื้อลาย

คำหลัก: insulin resistance, insulin signaling, MAPK, calorie restriction, exercise, estrogen, ovariectomy

เนื้อหางานวิจัย

Introduction

The metabolic syndrome is a clustering of multifaceted condition characterized by a number of atherogenic risk factors, including central obesity, glucose intolerance, insulin resistance of skeletal muscle glucose metabolism, compensatory hyperinsulinemia, dyslipidemia, and essential hypertension. Individuals with this condition are at markedly elevated risk of diabetes and cardiovascular disease. Pathophysiology of the metabolic syndrome and diabetes is complex and involves both genetic and acquired factors, such as excessive caloric intake, physical inactivity, and aging. Although ageing is a considerable factor in both men and women, the prevalence and progression of the metabolic syndrome, diabetes and cardiovascular disease strikingly increases in women after menopause. This phenomenon is associated with estrogen deprivation during which increased fat mass and body weight aggravate the insulin-resistant condition. Nevertheless, the roles of ovarian sex hormone in regulation of substrate metabolism are not well identified. Furthermore, the mechanisms underlying defects of insulin action on glucose homeostasis at the whole-body and skeletal muscle tissue levels brought about by withdrawal of female sex hormone remain to be elucidated. We have previously shown that exercise training could prevent insulin resistance condition caused by deprivation of ovarian sex hormone, which was partly due to enhanced glucose transporter-4 protein expression. However, it remains unknown either in clinical, integrative, cellular, or molecular terms how exercise training would affect insulin action on insulin-responsive metabolic processes in an animal model of insulin resistance caused by estrogen deprivation mimicking menopausal condition. Therefore, the present study aims to examine the effects of prolonged estrogen deprivation and the role of calorie management and exercise training on signaling molecules known to be involved in insulin-stimulated skeletal muscle glucose transport.

Observations from the first study of the present investigation indicated that the resistance to insulin-stimulated skeletal muscle glucose transport activity in OVX rats is characterized by the impaired insulin stimulation of IR-β, IRS-1/pY, IRS-1/p85 of PI3-kinase, and Akt with concomitant enhanced insulin-stimulated SAPK/JNK and p38 MAPK activity. Pair-feeding paradigm does not restore insulin action at the whole-body or skeletal muscle level, indicating that insulin resistance in OVX rats is a consequence of ovarian hormone deprivation rather than a result of OVX-

induced hyperphagia. Importantly, a further reduction in calorie intake with moderate degree of calorie restriction effectively prevents the development of skeletal muscle insulin resistance and the defective signaling in the skeletal muscle of OVX rats.

The second study of this investigation determined the cellular mechanisms that underlie the improvement in insulin action of exercise-trained muscle of OVX rats were assessed. Endurance exercise training in OVX rats enhances skeletal muscle protein expression of estrogen receptor-alpha and GLUT-4, decreases phosphorylation levels of Akt and p38 MAPK, without significant alteration of expression or functionality of IR-β, PI3-kinase, AS160, AMPK-α, and SAPK/JNK. Moreover, the improved insulin-stimulated glucose transport activity in skeletal muscle of exercise-trained OVX rat is accompanied by reduced IRS-1 serine phosphorylation with a corresponding decrease in SAPK/JNK and p38 MAPK activation, without the effects on IRS-1/p85 and serine phosphorylation of Akt in skeletal muscle of OVX rat, indicating that the insulin sensitizing effects of exercise training on the insulin signaling pathway in skeletal muscle of OVX rat is limited to the enhanced tyrosine phosphorylation of IRS-1 and does not bring about adaptations of the downstream insulin signaling elements. Estrogen replacement restored the insulin signaling cascade and GLUT-4 protein abundance. The combination of estrogen replacement and exercise training leads to greater increases in insulin action on skeletal muscle glucose transport activity than either intervention individually, suggesting the additive effect of these two interventions to enhance insulin action in skeletal muscle of OVX rats. However, the interactive effects of endurance exercise training and estrogen replacement on skeletal muscle insulin action in OVX rats are not responsible for by adaptations of signaling proteins determined in the present study.

Objectives

1. To determine whether the effects of prolonged ovariectomy on the phenotypic characteristics of the metabolic syndrome is the results of estrogen deprivation or a secondary consequence of ovariectomy.
2. To determine the effects of prolonged ovariectomy with or without calorie restriction on the expression and function of the proteins in the insulin signaling and MAPK pathways.

3. To investigate how exercise training modulates the expression and function of the proteins in the insulin signaling and MAPK pathways in sham-operated rats, ovariectomized rats and ovariectomized rats receiving estrogen replacement.

Methods

Study 1

Female Sprague-Dawley strain rats (The National Laboratory Animal Care of Thailand, Salaya, Nakhon Pratom) were received at 8-week of age, weighing approximately 180-200 g. The animals were housed at the Center of Animal Facilities of the Faculty Science, Mahidol University. At 11-week-old, the animals were randomly assigned to either sham operation (SHAM) or bilateral ovariectomy (OVX). Surgical procedure was performed under anesthesia through bilateral skin incision at the lower back. Amount of food intake and body weight was determined daily throughout the experimental period. Following a 7-day period of recovery from the surgery, the animals were assigned to one of the following groups: sham-operated (SHAM), OVX (OVX), OVX + Pair-Feeding (OVX + PF), or OVX + Calorie Restriction (OVX + CR)

The rats in the SHAM and OVX groups were fed *ad libitum*. The amount of food given to animals in the OVX+PF group was matched to that consumed by the SHAM group, whereas animals in the OVX+CR group were given 65% of the amount of chow consumed by the OVX-control group. After a 12-week experimental period, whole-body insulin sensitivity of each animal was assessed by oral glucose tolerance tests (OGTTs). One week after the OGTTs, rats were subjected to evaluate insulin action on glucose transport activity in soleus muscle. Muscle strips were incubated in the absence or in the presence of a maximally effective concentration of insulin. After the first incubation period, four of the soleus strips (two incubated without insulin and two incubated with insulin) were removed, trimmed of fat and connective tissue, and quickly frozen in liquid nitrogen. These strips were subsequently used for the determination of signaling elements in response to insulin activation by immunoblotting and immunoprecipitation. The remaining muscle strips were incubated for 20 min at 37 C in incubation medium containing 1 mM 2-[1,2-^{3}H]deoxyglucose (2-DG, 300 μCi/mmol), 39 mM [U-^{14}C]mannitol (0.8 μCi/mmol), and insulin, if previously present. At the end of the incubation period, the muscle strips were removed, trimmed of excess fat and connective tissue, and immediately frozen with liquid nitrogen and weighed. The frozen muscles were solubilized in 0.5 N NaOH, and scintillation cocktail was added. The specific

intracellular accumulation of 2-DG was then determined. The plantaris muscle and the liver were collected for the determination of tissue triglyceride content. After the removal of muscle tissues, blood sample was collected from the abdominal vein and prepared for serum sample to determine the levels of total cholesterol, HDL-cholesterol and LDL-cholesterol. Immediately after completion of blood collection, the heart was detached, frozen in liquid nitrogen and weighed. Intra-abdominal fat and the uterine were removed and weighed.

Study 2

The effects of endurance exercise training on the signaling pathways and lipid profile were assessed in the SHAM rats. Eight-week-old female Sprague-Dawley strain rats weighing between 180-200 g were housed at the Center of Animal Facilities of the Faculty Science, Mahidol University. The room temperature was controlled at 22°C, and a 12:12-h reversed light-dark cycle (lights on at 18:00) was maintained so that exercise training occurs during the dark cycle when the animals are most active. The animals had free access to water and pellet rat chow. Animals underwent sham-operation at the 11-week-old. Following a 7-day period of recovery after operation, the animals were randomly divided into either sham-operated (SHAM) or sham-operated receiving exercise training (SHAM + ET).

In a separate experiment, the effects of endurance exercise training on the signaling pathways and lipid profile were assessed in the OVX rats. Eight-week-old female Sprague-Dawley strain rats weighing between 180-200 g were housed at the Center of Animal Facilities of the Faculty Science, Mahidol University. The room temperature was controlled at 22°C, and a 12:12-h reversed light-dark cycle (lights on at 18:00) was maintained so that exercise training occurs during the dark cycle when the animals are most active. The rats were randomly assigned to either sham operation (SHAM) or bilateral ovariectomy (OVX). Following a 7-day period of recovery from the operation, the OVX animals were divided into 4 subgroups that were given one of the following treatments: OVX + vehicle (OVX), OVX + 2.5 μg/kg body weight of 17β-estradiol (OVX + E_2), OVX + exercise training + vehicle (OVX + ET), or OVX + 2.5 μg/kg body weight of 17β-estradiol + exercise training (OVX + E_2 + ET)

The animal in the estradiol-treated group was administered subcutaneously with 0.1 ml of 17β-estradiol (2.5 μg/kg body weight) diluted in corn oil for 3 times per week. The animals receiving vehicle treatment were treated with 0.1 ml of corn oil subcutaneously 3 times per week. Following the 12-week experimental period, blood and tissues collection were performed as

described in the Study 1. Muscle tissue was used for examining the effects of insulin stimulation on the cellular signaling machineries. Results on the signaling pathways from the OVX animals were compared with those obtained from the SHAM animals to examine whether there is a disparity between the responses to exercise training in the insulin-resistant OVX rats versus the insulin-sensitive SHAM rats.

Results & Discussion: Study 1

Body and tissue weight. A significant difference in average body weight between SHAM and OVX rats was observed starting at week 1 after surgery (Fig. 1A). In weeks 2-13, the body weight of the OVX group continued to elevate and was significantly higher than all other groups. At week 2, the body weights of both OVX+PF and OVX+CR groups were higher ($P < 0.05$) than that of the SHAM rats (Fig. 1A). During weeks 3-13, the body weight of the OVX+PF rats was higher ($P < 0.05$) than that of the SHAM or OVX+CR group (Fig. 1A). The final average body weight and body weight gain per day of the OVX rats were higher ($P < 0.05$) than those of the SHAM rats (Table 1). In contrast, the weight gains in the OVX+PF and OVX+CR rats were lower by 40% and 59%, respectively, compared with the OVX group (Table 1). Uterine weights in the three OVX groups were 4-5 fold lower than that of the SHAM rats (Table 1), indicating the effectiveness of ovariectomy. OVX rats displayed a 69% higher visceral fat content than SHAM rats (Table 1). However, increased visceral fat accumulation in OVX rats was suppressed ($P < 0.05$) in the OVX+PF and OVX+CR groups by 32% and 44%, respectively.

Table 1. Initial and final body weight, total energy intake, uterine and visceral fat weight, fasting plasma insulin and glucose concentration in sham-operated (SHAM), ovariectomized (OVX) and OVX rats that undergone pair feeding (OVX + PF) and calorie restriction (OVX + CR).

	SHAM	OVX	OVX + PF	OVX + CR
Body weight (g)				
Initial weight	231.1 ± 2.8	239.6 ± 2.4	238.1 ± 4.6	238.1 ± 4.8
Final weight	295.3 ± 3.9	390.2 ± 8.4 *	330.0 ± 2.4 *†	296.0 ± 1.6 †§
Body weight gain/day (g)	0.77 ± 0.06	1.64 ± 0.09 *	1.00 ± 0.04 *†	0.68 ± 0.05 †§
Total energy intake (kcal)	4,522 ± 72	5,771 ± 142 *	4,369 ± 20 †	3,795 ± 50 †§
Uterine weight (mg)	522.0 ± 43.9	111.6 ± 8.2 *	134.0 ± 20.6 *	111.4 ± 10.6 *
Visceral fat weight (g)	15.7 ± 1.1	26.5 ± 3.5 *	17.9 ± 1.7 †	14.9 ± 1.2 †
Insulin (μU/ml)	8.7 ± 0.6	10.3 ± 0.6	12.4 ± 0.9	11.2 ± 1.2
Glucose (mg/dl)	111.4 ± 2.8	122.9 ± 2.7	124.5 ± 1.9	122.4 ± 3.1

Values are means ± SE for 10 animals/group. * $P < 0.05$ vs. SHAM group. † $P < 0.05$ vs. OVX group. § $P < 0.05$ vs. OVX + PF.

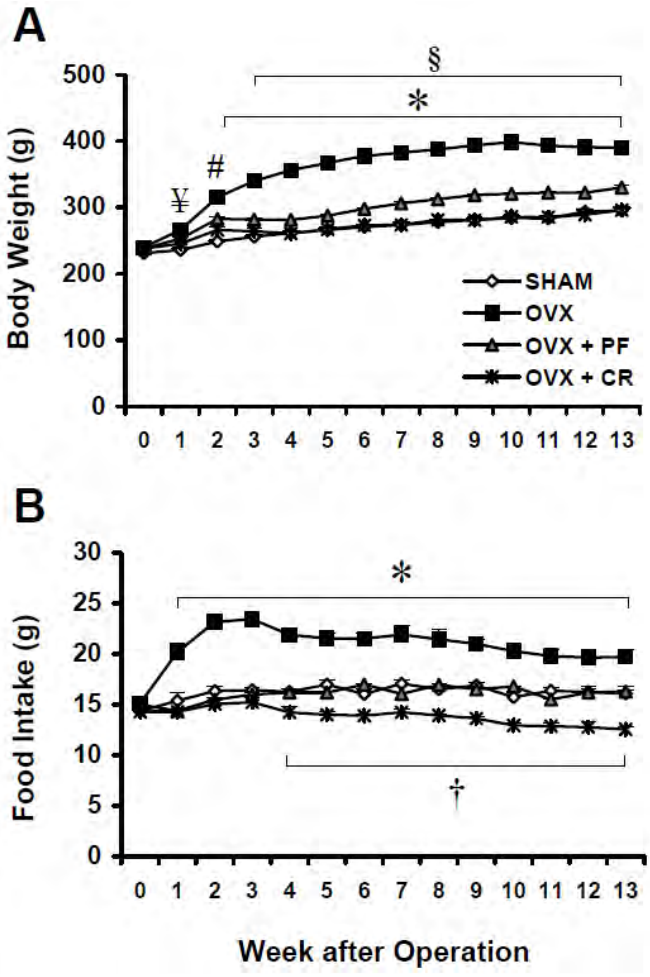


Fig. 1. Body weight (A) and food intake (B) of sham-operated control (SHAM) and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Measurements of body weight and food intake were performed at least 3 times per week during the experiment, and the weekly average values are presented. Values are means ± SE for 10 animals/group. ¥P < 0.05 SHAM vs. OVX; #P < 0.05 SHAM vs. OVX+PF; *P < 0.05 OVX vs. all other groups; §P < 0.05 OVX+PF vs. all other groups; †P < 0.05 OVX+CR vs. all other groups.

Food intake. OVX rats had a higher (P < 0.05) average food intake than the other three groups in weeks 1-13 (Fig. 1B), and the average food intake in the OVX+CR rats was significantly lower than that of the other three groups during weeks 5-13 (Fig. 1B). The total energy intake was calculated by multiplying the energy in each gram of rat chow by the average daily food intake, which was calculated from at least 3 collections in one week during the 12-week experimental period. The total energy intake of the OVX rats was significantly higher than that of the SHAM rats and was reduced to 75% and 66% of the total intake of the OVX rats by pair feeding and calorie restriction, respectively (Table 1).

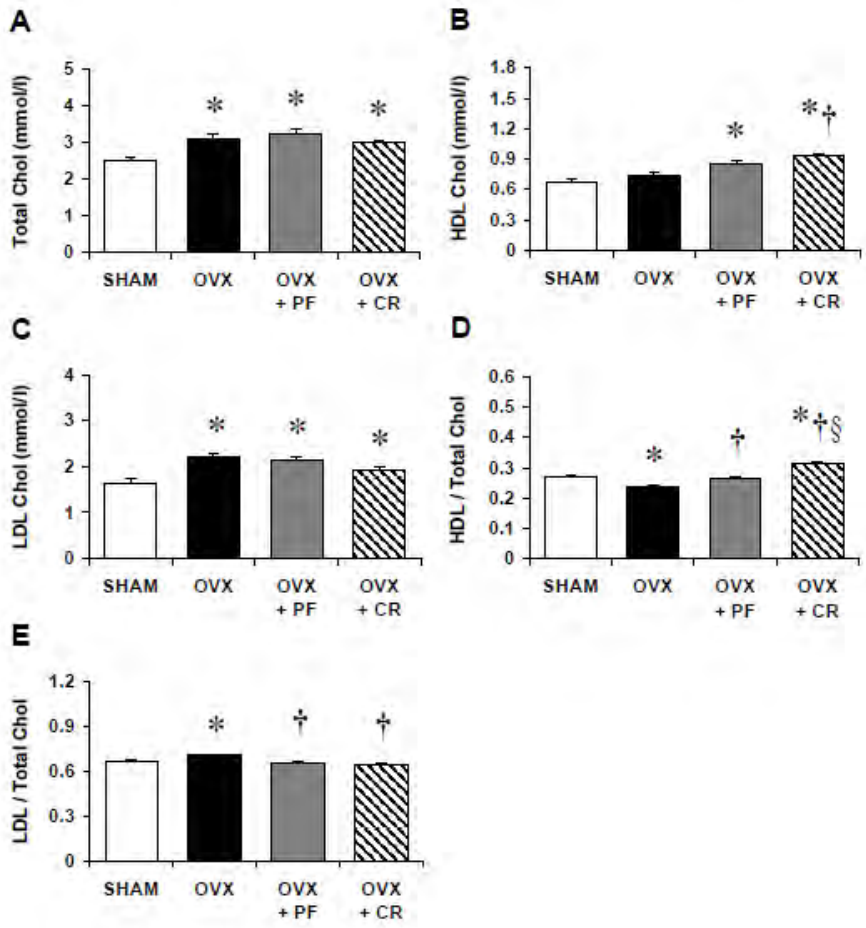


Fig. 2. Serum lipid levels of sham-operated control (SHAM) and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). (A) total; (B) high-density lipoprotein (HDL) cholesterol; (C) low-density lipoprotein (LDL) cholesterol; (D) ratio of HDL to total cholesterol (HDL/total); and (E) ratio of LDL to total cholesterol (LDL/total). Values are means ± SE for 10 animals/group. *P < 0.05 vs. SHAM; †P < 0.05 vs. OVX; §P < 0.05 vs. OVX+PF.

Serum lipid profile. The serum levels of total cholesterol (TC), HDL-cholesterol (HDL), LDL-cholesterol (LDL), and the HDL and LDL to TC ratios are demonstrated in Figure 2. Compared with SHAM rats, ovariectomy resulted in significant increases in TC (26%) and LDL (34%) but not HDL (Fig. 2). Compared to OVX, pair feeding and calorie restriction did not affect TC or LDL but led to a significant reduction (8-10%) in the LDL/TC ratio. However, pair feeding increased HDL by 15% and the HDL/TC ratio by 10%, while the highest improvements (P < 0.05) in HDL (26%) and the HDL/TC ratio (31%) were observed in the OVX rats with calorie restriction (Fig. 2).

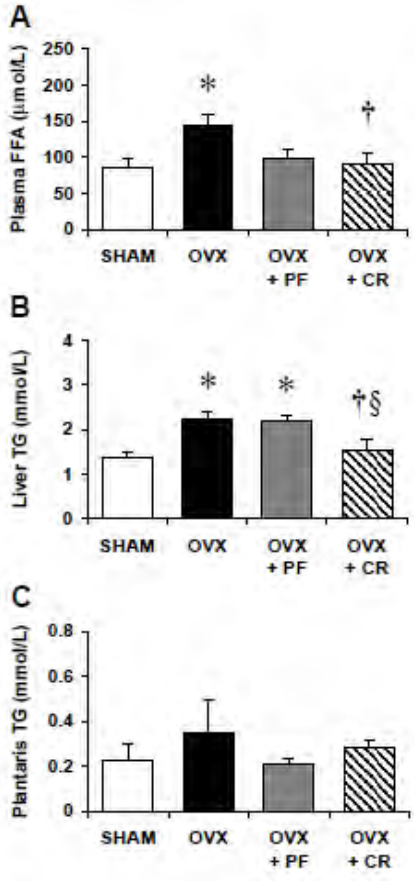


Fig. 3. Effects of sham operation (SHAM) and ovariectomy (OVX) without or with pair feeding (PF) or moderate calorie restriction (CR) on (A) plasma free fatty acid (FFA) levels, (B) liver triglyceride (TG) content, and (C) TG content in plantaris muscle. Values are means ± SE for 8 animals/group. *$P < 0.05$ vs. SHAM; †$P < 0.05$ vs. OVX; §$P < 0.05$ vs. OVX+PF.

Plasma free fatty acids (FFAs) and tissue triglycerides (TG). Elevated FFAs and increased ectopic fat accumulation have been implicated in the etiology of the insulin-resistant state. To determine the potential role of FFAs and ectopic fat in the insulin-resistant condition in OVX rats, the plasma level of FFAs and the TG content in the liver and the plantaris muscle were assessed. The muscular TG content was measured in the plantaris, due to limited availability of soleus muscle. Ovariectomy resulted in a 67% increase ($P < 0.05$) in plasma FFAs (Fig. 3A) and a 59% increase ($P < 0.05$) in the liver TG content (Fig. 3B). The elevation of FFAs in the OVX rats was reduced 32-37% by pair feeding and calorie restriction. On the other hand, the liver TG content was lowered ($P < 0.05$) only by calorie restriction (Fig. 3B). The TG content in the plantaris was, however, not significantly different among the treatments (Fig. 3C).

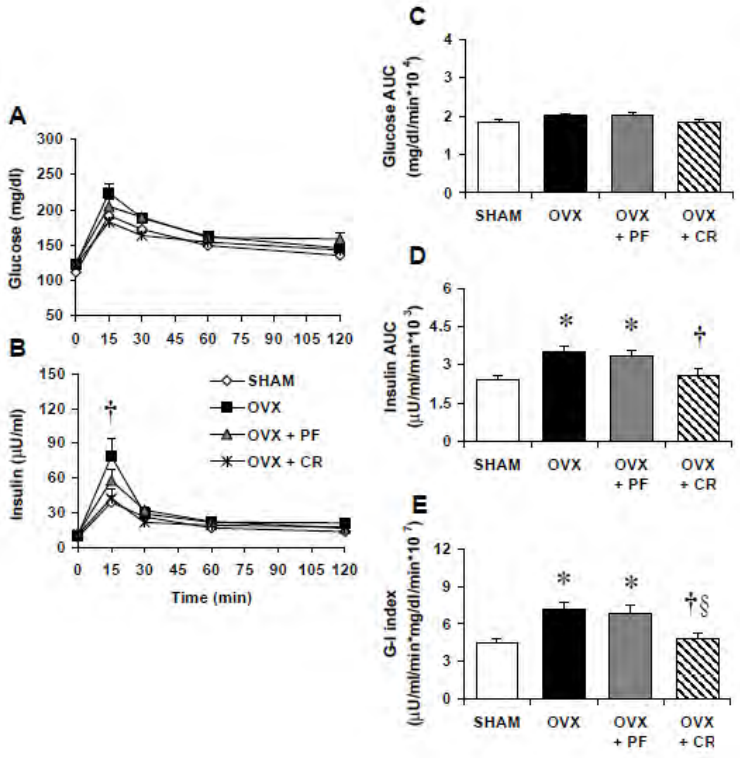


Fig. 4. Glucose (A) and insulin (B) responses during an oral glucose tolerance test and the area under the curve (AUC) for glucose (C) and insulin (D) as well as the glucose-insulin index (G-I index) (E) in sham-operated (SHAM) and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Data for the AUC were calculated from glucose (A) and insulin (B) responses. The G-I index was the product of glucose AUC and insulin AUC for each individual animal. Values are means ± SE for 10 animals/group. *$P < 0.05$ vs. SHAM; †$P < 0.05$ vs. OVX; §$P < 0.05$ vs. OVX+PF.

Whole body insulin action. Data from the OGTT, including plasma levels of glucose and insulin, the area under the curve for glucose (glucose AUC) and insulin (insulin AUC) and the glucose-insulin (G-I) index, are presented in Figure 4. The G-I index was calculated as the product of the respective glucose and insulin AUCs, and it is inversely related to whole-body insulin sensitivity. Plasma glucose (Fig. 4A) and insulin concentrations (Fig. 4B) were not significantly different between the groups throughout the test, except the insulin level at the 15-min time point in the OVX rats was higher ($P < 0.05$) than that in the other groups. There were no significant differences in glucose AUCs among all experimental groups (Fig. 4C). However, ovariectomy resulted in a significant increase in the insulin AUC compared with the SHAM group (Fig. 4D). Pair feeding failed to reduce the insulin AUC, whereas calorie restriction produced a significant reduction (27%). Similarly, the G-I index in the OVX group was 61% higher than that of the SHAM group (Fig. 4E), and calorie restriction significantly reduced the G-I index by 33% (Fig. 4E).

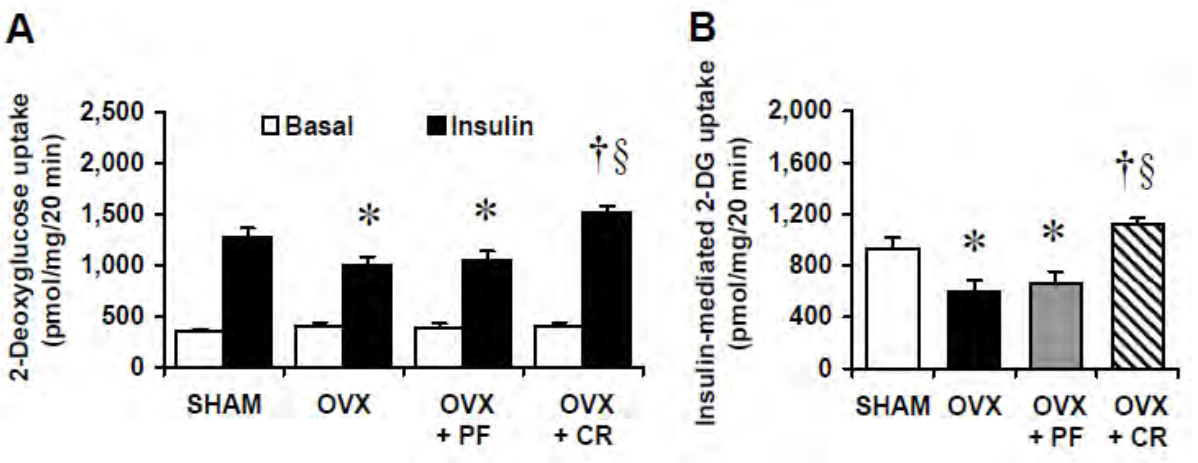


Fig. 5. *In vitro* rates of 2-deoxyglucose (2-DG) uptake in the absence (blank bars) and presence (filled bars) of insulin (2 mU/ml) (A) and net increases above basal rates of 2-deoxyglucose uptake due to insulin (B) in soleus muscle of sham-operated (SHAM) and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Values are means ± SE for 10 animals/group. **P* < 0.05 vs. SHAM; †*P* < 0.05 vs. OVX; §*P* < 0.05 vs. OVX+PF.

Insulin action on skeletal muscle glucose transport. Basal and insulin-stimulated glucose transport activities in isolated soleus are shown in Figure 5. No significant differences in basal 2-DG uptake were observed among the experimental groups. Compared to the SHAM group, the insulin-stimulated 2-DG uptake and the insulin-induced 2-DG transport above basal levels in muscle from OVX rats were reduced ($P < 0.05$) by 21% and 35%, respectively. These data clearly indicate the insulin-resistant state of the skeletal muscle of the OVX rats. Pair feeding in OVX animals did not produce a significant change in the insulin-stimulated 2-DG transport rate. In contrast, calorie restriction enhanced the insulin-mediated 2-DG uptake in OVX rats by 86% (Fig. 5B).

Expression and functionality of signaling elements in skeletal muscle. To assess the adaptive changes in GLUT-4 protein and signaling elements in response to prolonged estrogen deprivation without and with dietary manipulation, protein expression and phosphorylation levels were determined in non-incubated soleus. The protein expression levels of IR-β, IRS-1, the p85 subunit of PI3-kinase, Akt, SAPK/JNK, and p38 MAPK as well as the phosphorylation levels of IR-β (Tyr^{1158}/Tyr^{1162}/Tyr^{1163}), IRS-1 (Ser^{307}), Akt (Ser^{473}), SAPK/JNK (Thr^{183}/Tyr^{185}), and p38 MAPK (Thr^{180}/Tyr^{182}) were not different among groups (Fig. 6). However, a 12-week period of ovariectomy resulted in a reduction ($P < 0.05$) in the total GLUT-4 protein level in the soleus by

39% (Fig. 6). There was no significant effect of the pair feeding paradigm on total GLUT-4 protein content, whereas the reduced GLUT-4 protein level in OVX animals was partially attenuated by calorie restriction (P = 0.062 between SHAM vs. OVX+CR).

Insulin-stimulated activity of insulin signaling and MAPK in skeletal muscle. The ability of insulin to activate signaling elements in the PI3-K and MAPK pathways in skeletal muscle was examined in the incubated muscle. There were no differences in the protein expression of IR-β, IRS-1, Akt (Fig. 7), SAPK/JNK 1, SAPK/JNK 2/3, and p38 MAPK (Fig. 8) in the soleus muscle among the groups. However, insulin stimulation of tyrosine phosphorylation of IR-β and IRS-1, of IRS-1 associated with the p85 subunit of PI3-kinase, and of serine phosphorylation of Akt in the soleus muscle of OVX rats was significantly reduced when compared with the SHAM group (Fig. 7). Interestingly, ovariectomy led to the enhanced activation (P < 0.05) of insulin-stimulated serine^{307} phosphorylation of IRS-1 (Fig. 7), Thr^{183}/Tyr^{185} phosphorylation of SAPK/JNK, and Thr^{180}/Tyr^{182} phosphorylation of p38 MAPK in skeletal muscle (Fig. 8). Pair feeding and calorie restriction blunted the decreases in phosphorylated IR-β and IRS-1/pY, the IRS-1 associated with the p85 subunit of PI3-kinase and the serine phosphorylation of Akt (Fig. 7). Such dietary manipulation also abolished the increases in the level of phosphorylation of IRS-1 serine^{307} (Fig. 7), SAPK/JNK 1, SAPK/JNK 2/3, and p38 MAPK (Fig. 8), thereby suggesting significant improvement of the defective insulin signaling induced by estrogen deprivation.

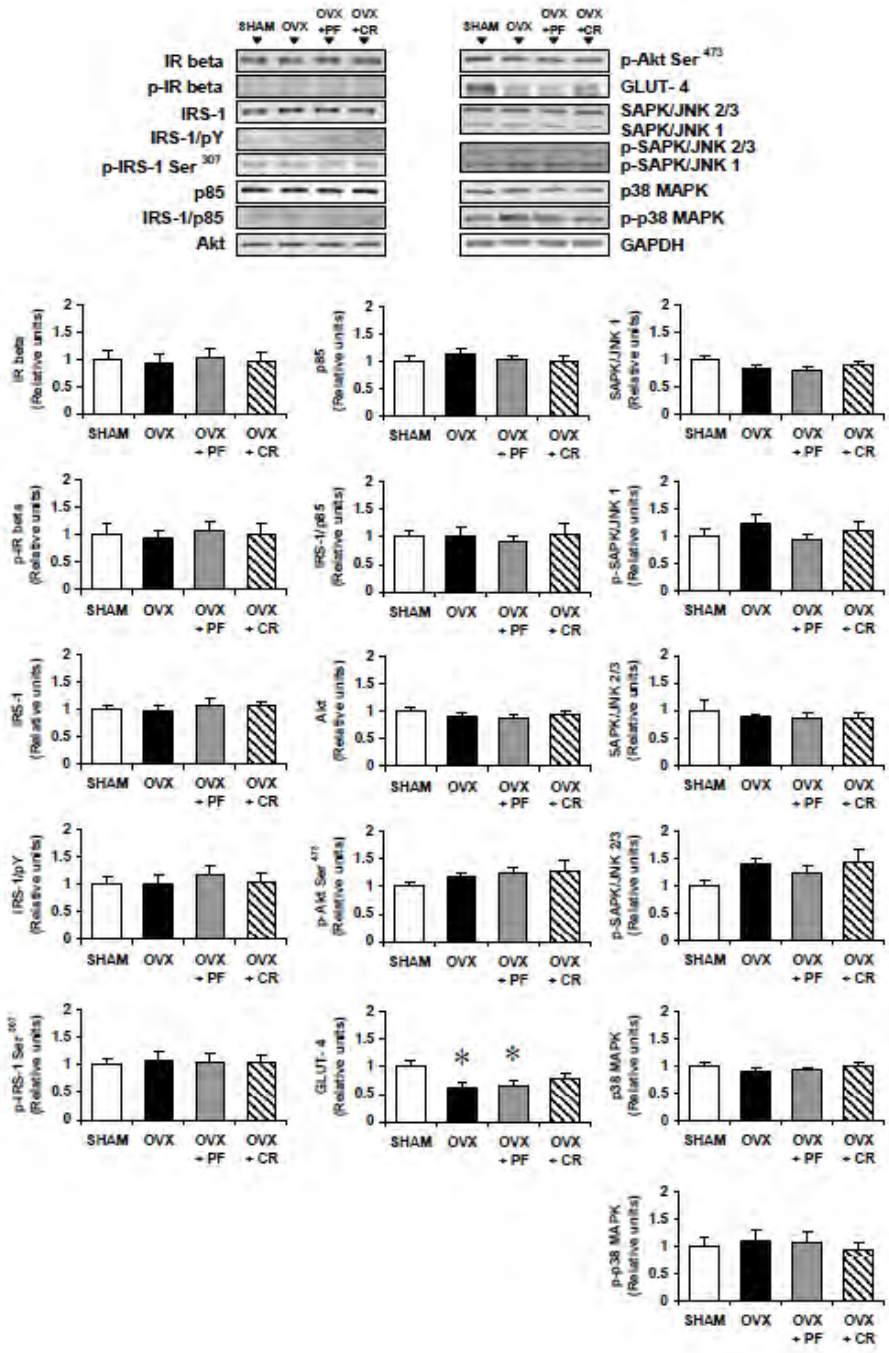


Fig. 6. Effects of pair feeding (PF) or moderate calorie restriction (CR) on the expressions of signaling proteins IR-β (IR beta), IR-β (Tyr[1158]/Tyr[1162]/Tyr[1163]) phosphorylation (p-IR beta), IRS-1, tyrosine phosphorylation of IRS-1 (IRS-1/pY), IRS-1 (Ser[307]) phosphorylation (p-IRS-1 Ser[307]), the p85 subunit of PI3-K (p85), IRS-1 associated with the p85 subunit of PI3-K (IRS-1/p85), Akt, Akt (Ser[473]) phosphorylation (p-Akt Ser[473]), GLUT-4, SAPK/JNK 1, SAPK/JNK 1 (Thr[183]/Tyr[185]) phosphorylation (p-SAPK/JNK 1), SAPK/JNK 2/3, SAPK/JNK 2/3 (Thr[183]/Tyr[185]) phosphorylation (p-SAPK/JNK 2/3), p38 MAPK, and p38 MAPK (Thr[180]/Tyr[182]) phosphorylation (p-p38 MAPK) in non-incubated soleus muscles from OVX rats. For IRS-1/pY and IRS-1/p85, muscle lysate was immunoprecipitated with agarose-conjugated anti-IRS-1 antibody prior to immunoblotting against tyrosine and p85, respectively. IRS-1/pY and IRS-1/p85 were normalized to IRS-1 protein expression. The rest of the signaling proteins were determined by immunoblot analysis and were normalized to GAPDH. Data are presented as fold change over the SHAM group. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 8-10 animals/group. *$P < 0.05$ vs. SHAM.

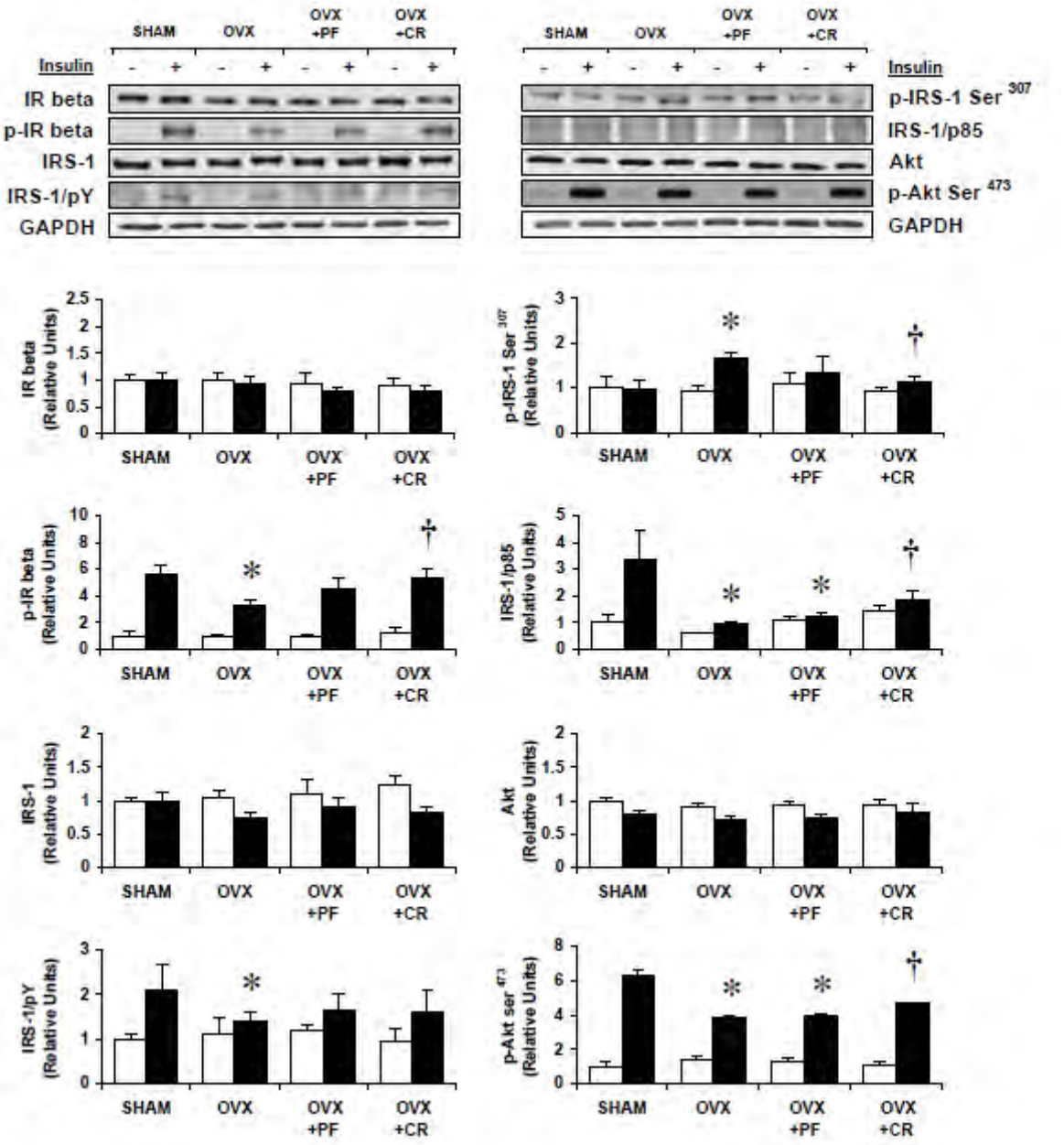


Fig. 7. Insulin-induced expression of IR-β (IR beta), IR-β (Tyr^{1158}/Tyr^{1162}/Tyr^{1163}) phosphorylation (p-IR beta), IRS-1, tyrosine phosphorylation of IRS-1 (IRS-1/pY), IRS-1 (Ser^{307}) phosphorylation (p-IRS-1 Ser^{307}), IRS-1 associated with the p85 subunit of PI3-K (IRS-1/p85), Akt, and Akt (Ser^{473}) phosphorylation (p-Akt Ser^{473}) in soleus muscles from SHAM and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Muscles were incubated in the absence (blank bars) or the presence (filled bars) of insulin (2 mU/ml). For IRS-1/pY and IRS-1/p85, muscle lysate was immunoprecipitated with agarose-conjugated anti-IRS-1 antibody prior to immunoblotting against tyrosine and p85, respectively. IRS-1/pY and IRS-1/p85 were normalized to IRS-1 protein expression. The rest of the signaling proteins were determined by immunoblot analysis and were normalized to GAPDH. Data are presented as fold change over the SHAM group.Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 8 animals/group. *P < 0.05 vs. SHAM; †P < 0.05 vs. OVX.

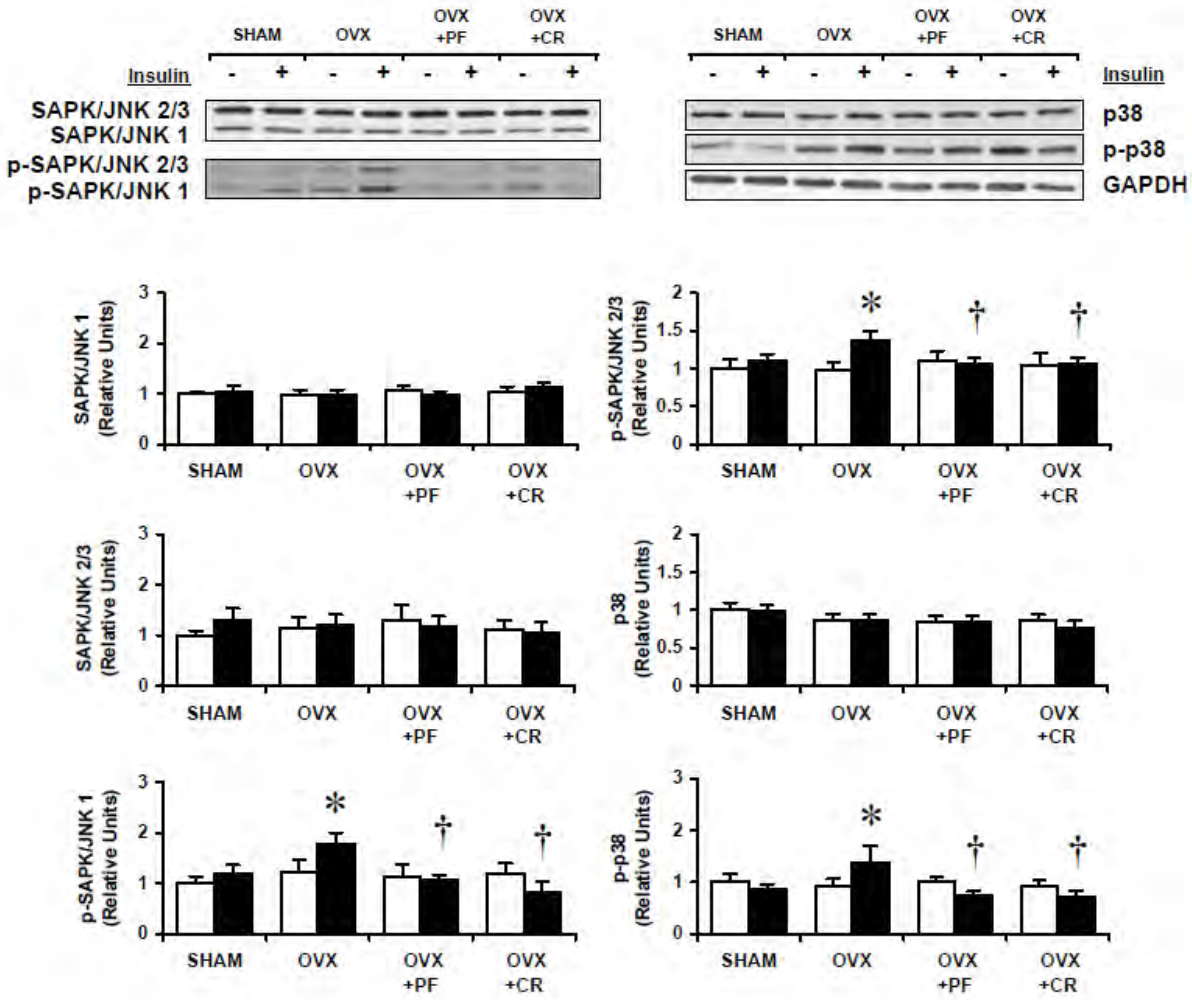


Fig. 8. Insulin-induced expression of SAPK/JNK 1, SAPK/JNK 2/3, SAPK/JNK 1 and SAPK/JNK 2/3 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 1), p38 MAPK, and p38 MAPK (Thr^{180}/Tyr^{182}) phosphorylation (p-p38 MAPK) in soleus muscles from SHAM and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Muscles were incubated in the absence (blank bars) or the presence (filled bars) of insulin (2 mU/ml). Data are presented as fold change over the SHAM group. Results were normalized to GAPDH. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 8 animals/group. *P < 0.05 vs. SHAM; †P < 0.05 vs. OVX.

The present study provides evidence that the insulin-resistant condition observed in OVX rats is primarily a consequence of loss of ovarian function and is independent of the increased food intake that accompanies the condition. Novel information presented in this study include our findings that the skeletal muscle of OVX rats is characterized by defects in insulin signaling, including reduced insulin action on the tyrosine phosphorylation of IR and IRS-1, the IRS-1 associated p85 subunit of PI3-kinase and the serine phosphorylation of Akt, with concomitant increases being observed in insulin-stimulated SAPK/JNK and p38 MAPK activity. In addition, the

present study is the first to show that the development of insulin resistance and the defective signaling elements in skeletal muscle observed in OVX rats can be prevented by moderate degree of calorie restriction (~35% less calorie intake than the OVX group).

The metabolic alterations in OVX rats on a pair feeding (PF) diet were assessed. The amount of total energy intake in the OVX+PF group is equivalent to a 25% reduction in the total energy intake by OVX rats. The PF dietary regime reduced visceral fat content and improved the lipid profile, as indicated by higher HDL/TC and lower LDL/TC ratios. Nevertheless, the OVX+PF rats exhibited a positive energy state because their body weight gain was significantly higher than that of the SHAM rats. In addition, the increased lipid content in the liver, increased plasma free fatty acid, impaired glucose tolerance and defective insulin action on skeletal muscle glucose transport in OVX rats were not attenuated by pair feeding. Based on these findings, it may be concluded that the insulin-resistant condition observed in OVX rats is primarily a consequence of ovarian hormone deprivation.

A critical role of estrogens in glucose homeostasis has been supported by clinical and experimental studies. In this study, we demonstrated that insulin-stimulated glucose transport activity was reduced in the skeletal muscle of OVX rats with a corresponding reduction in the level of total GLUT-4 protein expression. As normal activation of the PI3-kinase pathway is essential for the recruitment of GLUT-4 to facilitate glucose transport, our findings regarding the defective insulin stimulation of the tyrosine phosphorylation of IR-β and IRS-1, IRS-1 associated with p85 of PI3-kinase and the serine phosphorylation of Akt probably explain the insulin resistance of glucose transport activity observed in the skeletal muscle of OVX rats. These results are consistent with the notion that a defect in the proximal step of the insulin signaling pathway results in reduced functionality of the downstream signaling elements. Therefore, the defective signaling response to insulin stimulation in the muscle of OVX rats where GLUT-4 expression is already impaired would aggravate insulin resistance.

In addition to its metabolic functions through the PI3-kinase pathway, insulin exerts mitogenic effects via the mitogen-activated protein kinase (MAPK) pathway. The present study demonstrated that the insulin resistance of the PI3-kinase pathway in OVX rats was accompanied by increased IRS-1 serine phosphorylation with a corresponding increase in SAPK/JNK and p38 MAPK activation. This observation seems to support the concept that intact stimulation of the MAPK pathway by insulin plays an important role in the development of insulin resistance in the

skeletal muscle of OVX rats. Nevertheless, our observation that impaired glucose tolerance and skeletal muscle insulin resistance do exist in OVX+PF rats without an increase in insulin-activated SAPK/JNK and p38 MAPK is more consistent with the interpretation that intact stimulation of the MAPK pathway by insulin is not required for the insulin-resistant condition in OVX rats.

Insulin resistance is attributed to numerous factors such as increased visceral fat accumulation, plasma free fatty acids, and fat deposition in non-adipose tissues. We did observe a significant increase in plasma free fatty acids, visceral fat content, and triglyceride content in the liver but not the plantaris muscle of OVX rats. Accordingly, these metabolic changes may account, in part, for the insulin-resistant state observed in OVX rats. In this study, we found that pair feeding reduced visceral fat content and tended to decrease the level of plasma free fatty acid in OVX rats without improving whole body or skeletal muscle glucose metabolism. Thus, it appears that visceral fat accumulation and plasma free fatty acids may not be the primary event leading to the development of the insulin-resistant condition in OVX rats. Our findings that the enhanced hepatic triglyceride content in OVX rats is not attenuated by pair feeding support the idea that this factor may be important in the development of insulin resistance in OVX rats.

Although PF did not significantly increase insulin sensitivity in OVX rats, a further reduction in total energy intake (~35% less than the OVX group) effectively prevented the progression of metabolic defects, including the insulin-resistant condition at whole body and skeletal muscle levels of OVX rats. We found that these metabolic adaptations in the OVX+CR rats were accompanied by significant diminution of visceral fat accumulation, the plasma level of free fatty acid, and the triglyceride content in the liver. One of the important findings in this study is the favorable effect of CR on insulin-stimulated glucose transport and the signaling elements in skeletal muscle of OVX rats. Without a significant improvement in the expression of the GLUT-4 protein, calorie restriction fully recuperated the impaired insulin action on skeletal muscle glucose transport activity in OVX rats. Intriguingly, the improved insulin action on the functionality of the insulin signaling elements and glucose transport in skeletal muscle was associated with a significant reduction in insulin-stimulated IRS-1 serine phosphorylation. Because the effect of CR on the insulin signaling pathway includes the proximal step such as insulin-stimulated IR-β and IRS-1, we speculate that the favorable effect of CR on skeletal muscle insulin action is associated with the suppression of hepatic fat accumulation and the plasma free fatty acid level. Therefore, the systemic improvements as a result of CR could be substantial for the beneficial

adaptation from the proximal step of the PI3-kinase pathway and the enhanced activity of the glucose transport system in skeletal muscle.

Taken together, our observation from the Study 1 indicated that the resistance to insulin-stimulated skeletal muscle glucose transport activity in OVX rats is characterized by the impaired insulin stimulation of IR-β, IRS-1/pY, IRS-1/p85 of PI3-kinase, and Akt with concomitant enhanced insulin-stimulated SAPK/JNK and p38 MAPK activity. Pair feeding improved serum lipid profiles in OVX rats without a significant improvement of insulin action at the whole-body or skeletal muscle level, suggesting a causal role of ovarian hormone deprivation in the progression of the insulin-resistant condition. A further reduction in calorie intake, however, prevents the development of skeletal muscle insulin resistance and the defective signaling in the skeletal muscle of rats undergone prolonged ovariectomy.

Results & Discussion: Study 2

Body & tissue weight and food intake. Body weight gain observed in OVX rats were prevented in estrogen-treated groups, whereas the average body weight of OVX rats that received exercise training alone did not present any significantly different compared with OVX control rats (Table 2). Uterine weights were 4-5 folds decreased in OVX and OVX + ET groups compared with SHAM group, indicating the effectiveness of ovariectomy. Estrogen replacement reversed the uterine weight to the level of SHAM rats (Table 2). Exercise training resulted in significantly enhanced heart weight to body weight ratio in OVX rats, and an additional enhancement of the heart weight to body weight ratio was found in OVX + E2 + ET group. Accumulation of visceral fat in OVX rats was suppressed in OVX + ET group. Suppression of visceral fat accumulation was found in OVX + E2 ($P < 0.05$) and it further reduced ($P < 0.05$) in combined treatment group (Table 2). Total energy intake was significantly higher in OVX rats compared with SHAM rats ($P < 0.05$). Exercise training did not present any change of the total energy intake in OVX rats. In contrast with the exercise training groups, estrogen replacement caused significantly decreased in total energy intake by 12% in OVX + E2 group and 18% in OVX + E2 + ET group (Table 2). Compared with SHAM group, ovariectomy led to a significant decrease in serum estrogen by 37% ($P < 0.05$). Exercise training in OVX rats displayed no change in estrogen levels.

Table 1. Initial and final body weight, total energy intake, uterine, heart, and fat weight, serum estrogen, fasting plasma glucose and insulin concentration in sham-operated (SHAM) and ovariectomized (OVX) rats that received estrogen replacement (E2) and exercise training (ET).

	SHAM	OVX	OVX + E2	OVX + ET	OVX + E2 + ET
Body weight (g)					
Initial weight	231.53 ± 3.37	223.21 ± 4.05	226.98 ± 4.42	228.71 ± 3.18	230.14 ± 3.35
Final weight (BW)	304.72 ± 7.03	362.95 ± 7.62 *	300.84 ± 4.74 †§	342.43 ± 5.22 *	291.71 ± 7.41 †§
Body weight gain/day (g)	0.68 ± 0.06	1.47 ± 0.07 *	0.70 ± 0.06 †§	1.21 ± 0.04 *†	0.64 ± 0.05 †§
Total energy intake (kcalx10^3)	3.74 ± 0.14	4.51 ± 0.10 *	3.95 ± 0.10 †	4.13 ± 0.09	3.70 ± 0.09 †§
Uterine weight (UW; mg)	509.83 ± 34.96	116.63 ± 4.21 *	496.16 ± 36.66 †§	109.66 ± 3.78 *	543.74 ± 30.93 †§
UW/kg BW	1.68 ± 0.13	0.32 ± 0.01 *	1.66 ± 0.13 †§	0.32 ± 0.01 *	1.86 ± 0.09 †§
Serum E2 (pg/ml)	39.62 ± 8.60	14.73 ± 3.95 *	30.99 ± 4.15 †	16.71 ± 3.14 *	30.11 ± 4.09 †
Heart weight (HW; mg)	932.95 ± 34.02	1045.85 ± 24.11	950.88 ± 14.06	1032.49 ± 37.27	1034.65 ± 33.85
HW/kg BW	3.06 ± 0.08	2.88 ± 0.03	3.16 ± 0.05 †	3.10 ± 0.07 †	3.55 ± 0.06 *†#§
Fat weight (g)	11.83 ± 0.83	15.70 ± 1.13 *	9.59 ± 0.53 †	10.75 ± 0.98 †	7.10 ± 0.59 *†§
FW/kg BW	39.24 ± 3.12	42.78 ± 2.56	31.85 ± 1.64 †	31.38 ± 2.67 †	23.16 ± 2.23 *†#
Fasting plasma glucose (mg/dl)	117.55 ± 2.71	139.42 ± 13.65	116.23 ± 4.79	137.04 ± 11.67	136.86 ± 5.80
Fasting plasma insulin (µU/ml)	11.88 ± 1.00	13.11 ± 1.56	11.97 ± 1.29	14.71 ± 1.44	15.15 ± 0.87

Values are means ± SE for 6-8 animals/group. * $P < 0.05$ vs. SHAM group; † $P < 0.05$ vs. OVX group; # $P < 0.05$ vs. E2 group; § $P < 0.05$ vs. OVX+ET group.

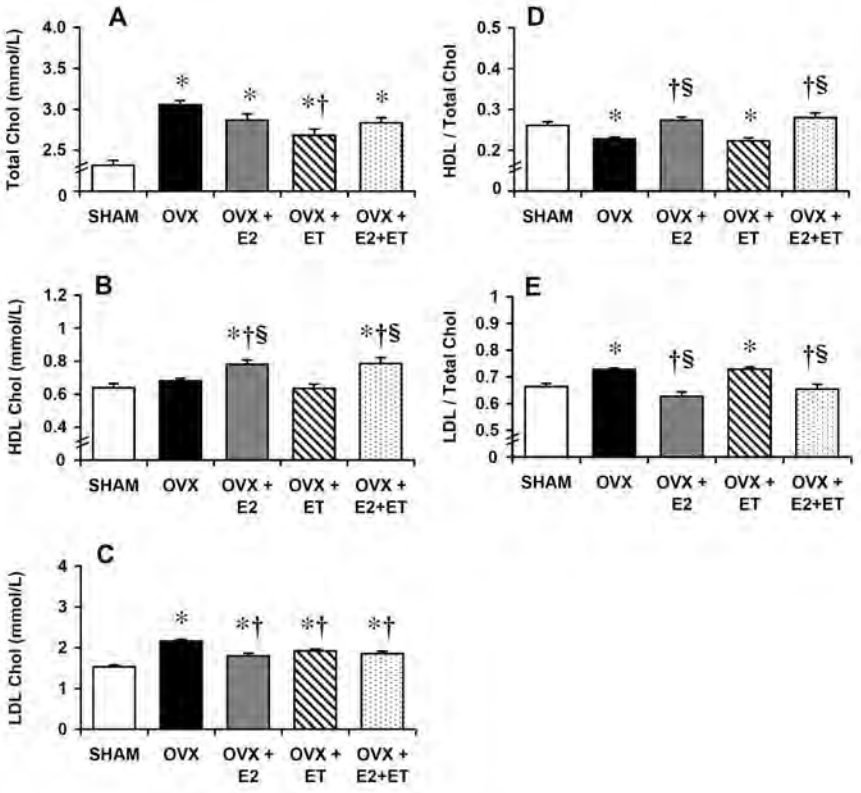


Fig. 9. Effects of estrogen (E2) and/or exercise training (ET) treatments on serum lipid levels of ovariectomized (OVX) rats. A: total cholesterol; B: high-density lipoprotein (HDL) cholesterol; C: low-density lipoprotein (LDL) cholesterol: D: ratio of HDL to total cholesterol (HDL/Total); E: ratio of LDL to total cholesterol (LDL/Total). Values are means ± SE for 8 animals/group. *P < 0.05 vs. SHAM; †P < 0.05 vs. OVX; §P < 0.05 vs. OVX + ET.

Serum lipid profiles. The serum levels of total cholesterol, HDL cholesterol (HDL), LDL cholesterol (LDL), and the HDL and LDL to total cholesterol ratios are demonstrated in Figure 9. Compared with SHAM rats, ovariectomy resulted in significant increases in total cholesterol (32%) and LDL (40%) but not HDL. Estrogen replacement alone increased HDL cholesterol and the HDL to total cholesterol ratio by 15% and 20%, respectively ($P < 0.05$). Furthermore, estrogen reduced LDL and the LDL to total cholesterols ratio by 17% and 14%, respectively ($P < 0.05$). Exercise training alone causes significantly decreased of total cholesterol and LDL cholesterol but it did not increase HDL cholesterol. Combined treatment of exercise training and estrogen presented the same trend of changes as the estrogen treated group, that are increased HDL cholesterol by 16% and the HDL to total cholesterol ratio by 23%, whereas it decreased LDL cholesterol by 14% and the LDL to total cholesterol ratio by 10% ($P < 0.05$).

Plasma free fatty acids (FFAs) and tissue triglycerides (TG). Assessments of plasma level of FFAs and the TG content in the liver and the plantaris muscle are for determining the potential role of FFAs and ectopic fat in the insulin resistant condition due to ovariectomy. The muscular TG content was measured in the plantaris, due to limited availability of soleus muscle. Triglyceride levels in serum, liver, and plantaris, and FFAs levels in plasma are presented in Figure 10. OVX rats had higher ($P < 0.05$) plasma FFAs (56%) and liver TG (53%) but not serum TG or plantaris TG compared with that of the SHAM rats. Interestingly, estrogen replacement can reduce ($P < 0.05$) liver TG (39%) and plasma FFAs (38%), whereas it increased serum TG (39%). Exercise training decreased serum and liver TG by 51% and 21%, respectively ($P < 0.05$). Combined treatment between estrogen replacement and exercise training expressed decrements ($P < 0.05$) of plasma FFAs (45%) and liver TG (54%).

Whole body insulin action. Plasma glucose and insulin concentrations were not significantly different between the groups throughout the test, except the insulin level at the 15-min time point in the OVX rats was higher ($P < 0.05$) than that of the other groups. Significantly higher of the insulin AUCs and G-I index were observed in OVX group. Estrogen treatment alone, exercise training alone, and combined treatments decreased insulin AUCs by 43-48% and improved G-I index by 49-52% ($P < 0.05$) but did not affected the glucose AUCs (Figures 11-12).

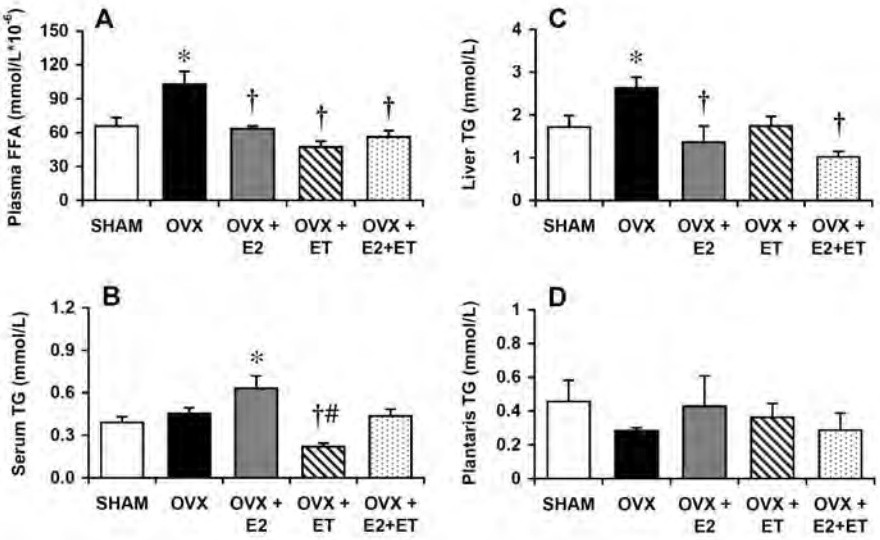


Fig. 10. Plasma free fatty acid (FFA; A), serum triglyceride (TG; B) content, TG content in liver (C) and plantaris muscle (D) of sham-operated control rats (SHAM) and ovariectomized (OVX) rats with or without estrogen replacement (E2) and/or exercise training (ET). Values are means ± SE for 8-10 animals/group. *P < 0.05 vs. SHAM; †P < 0.05 vs. OVX; §P < 0.05 vs. OVX + ET; #P < 0.05 vs. OVX + E2.

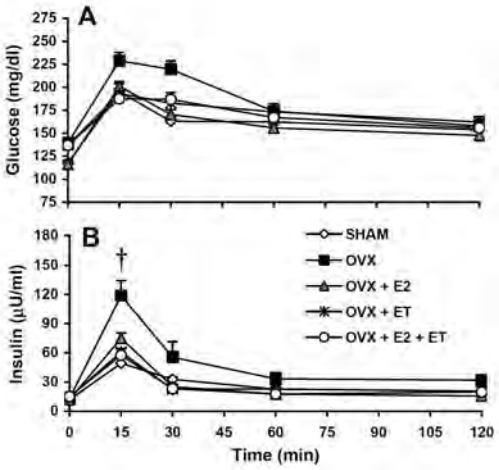


Fig. 11. Glucose (A) and insulin (B) responses during and OGTT in sham-operated rats (SHAM) and ovariectomized (OVX) rats treated with or without estrogen (E2) and/or exercise training (ET). Values are means ± SE for 10 animals/group. †P < 0.05 vs. OVX group.

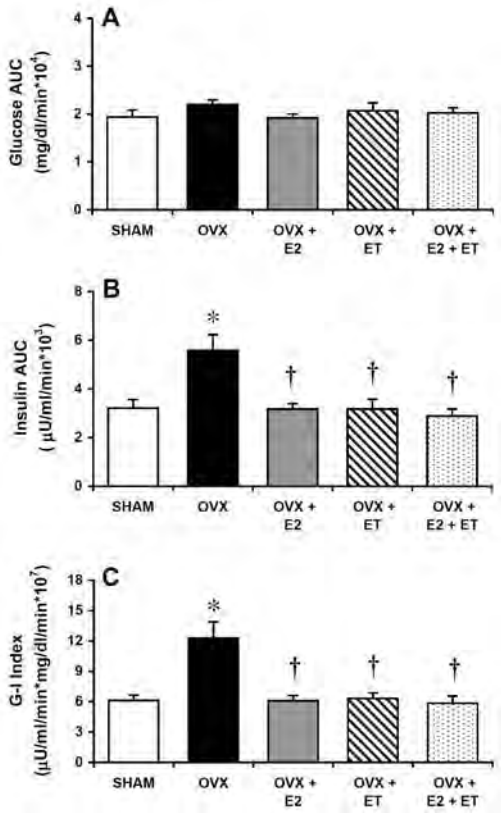


Fig. 12. Area under the curve (AUC) for glucose (A) and insulin (B), and the glucose-insulin index (G-I index; C) in sham-operated rats (SHAM) and ovariectomized (OVX) rats treated with or without estrogen (E2) and/or exercise training (ET). Data for AUC were calculated from glucose and insulin responses from the OGTTs. The G-I index was the product of glucose and insulin AUC for individual rat. Values are means ± SE for 10 animals/group. *P < 0.05 vs. SHAM; †P < 0.05 vs. OVX.

Insulin action on skeletal muscle glucose transport. Basal and insulin-stimulated glucose transport activities in isolated soleus are shown in Figure 13. Basal 2-DG transport activities did not different among the experimental groups. Insulin-stimulated and insulin-mediated 2-DG transport activities decreased due to ovariectomy by 38% and 49%, respectively (P < 0.05). OVX rats treated with estrogen or exercise training alone improved both insulin-stimulated and insulin-mediated 2-DG transport activities by 36-65% (P < 0.05). The combined treatment group further increased insulin-stimulated and insulin-mediated 2-DG transport activities by 72% and 112%, respectively (P < 0.05).

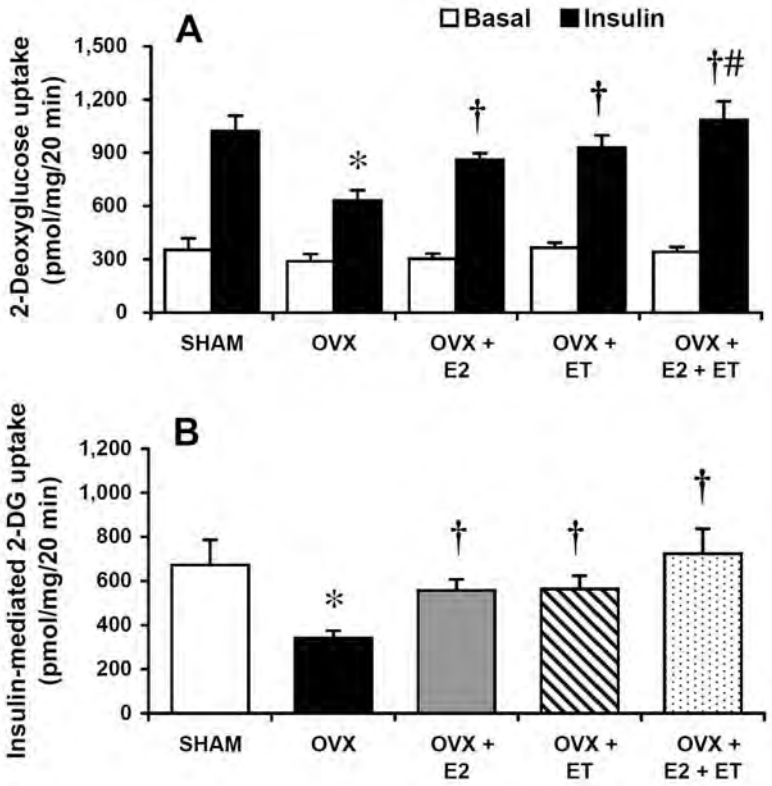


Fig. 13. *In vitro* rates of 2-deoxyglucose (2-DG) transport activities in the absence (blank bars) of insulin (2 mU/ml) (A) and net increases above basal rates of 2-DG transport activities due to insulin (B) in soleus muscles of sham-operated control (SHAM) and ovariectomized (OVX) rats without or with estrogen replacement (E2) and/or exercise training (ET). Values are means ± SE for 8-10 animals/group. *P < 0.05 vs. SHAM; †P < 0.05 vs. OVX; #P < 0.05 vs. OVX + E2.

Expression and functionality of signaling elements in skeletal muscle. The total protein expression levels of IR β (IR beta), IRS-1, the p85 subunit of PI3-kinase, Akt, AS160, AMPK α, SAPK/JNK, p38 MAPK, and estrogen receptor β (ER beta) as well as the phosphorylation levels of IR β (Tyr^{1158}/Tyr^{1162}/Tyr^{1163}) (p-IR beta), IRS-1 (Ser^{307}), AMPK α (Thr^{172}), and SAPK/JNK (Thr^{183}/Tyr^{185}) were not different among groups (Figure 14). Compared with SHAM group, GLUT-4 protein expression decreases by 38% in OVX group (P < 0.05) (Figure 14). Estrogen, exercise training alone, and combined treatments improved GLUT-4 by 61-80% when compared with OVX group (P < 0.05). Interestingly, phosphorylation levels of Akt (Ser^{473}) presented significantly decreased in exercise treatment alone (34%) and combined treatment (57%) groups. Phosphorylation levels of p38 MAPK (Thr^{180}/Tyr^{182}) decreased by 43-61% in exercise-trained groups (P < 0.05). Moreover, estrogen receptor α total protein expression

levels in exercise-trained group were higher when compared with all other groups ($P < 0.05$) (Figure 14). It is very interesting that exercise training in insulin-sensitive (SHAM) rats demonstrated its effects on total protein expression and functionality of AMPK α in non-incubated soleus (Figure 15), that are exercise training increased 37% of AMPK α and 67% of phosphorylation of AMPK α (Thr^{172}) ($P < 0.05$).

Insulin-stimulated activity of insulin signaling and MAPK in skeletal muscle. The PI3-K and MAPK pathways were examined in the incubated muscle to reflex the ability of insulin to activate signaling elements in skeletal muscle of sham-operated-rats (SHAM) and ovariectomized (OVX) rats treated with or without estrogen replacement and/or exercise training. Insulin stimulation of tyrosine phosphorylation of IR β and IRS-1, IRS-1 associated with the p85 subunit of PI3-kinase, and serine phosphorylation of Akt (Figure 16) in the soleus muscle of OVX rats was significantly reduced when compared with that of the SHAM rats. Whereas ovariectomy enhanced insulin-stimulated $serine^{307}$ phosphorylation of IRS-1 (Figure 16), Thr^{183}/Tyr^{185} phosphorylation of SAPK/JNK (Figure 17), and Thr^{180}/Tyr^{182} phosphorylation of p38 MAPK (Figure 17) in skeletal muscle ($P < 0.05$). Compared with the OVX rats, all treatments led to enhance tyrosine phosphorylated IR β by 12-23% while and IRS-1 (IRS-1/pY) by 2-3 folds ($P < 0.05$) (Figure 16). For IRS-1 associated with the p85 subunit of PI3-kinase, and serine phosphorylation of Akt, only estrogen treatment alone and combined treatment with exercise training attenuated the ovariectomy-induced impairment. Estrogen alone and estrogen combined with exercise training can increase insulin stimulation of IRS-1 associated with the p85 subunit of PI3-kinase by 81 and 67% (Figure 16), and serine phosphorylation of Akt by 51 and 70% (Figure 16) ($P < 0.05$). Phosphorylation levels of SAPK/JNK (31-51%) and p38 (21-45%) (Figure 17) ($P < 0.05$). In addition, effects of exercise training on insulin stimulating protein expression or functionality of insulin signaling and MAPK pathways in SHAM rats were also evaluated. Results showed no significantly change in insulin signaling and MAPK elements in exercise-trained SHAM rats (Figure 19). Except the phosphorylation of AMPK α (Thr^{172}), significantly changes in both basal and insulin stimulated phosphorylation were found in exercise-trained SHAM rats compared with the sedentary SHAM rats (Figure 18).

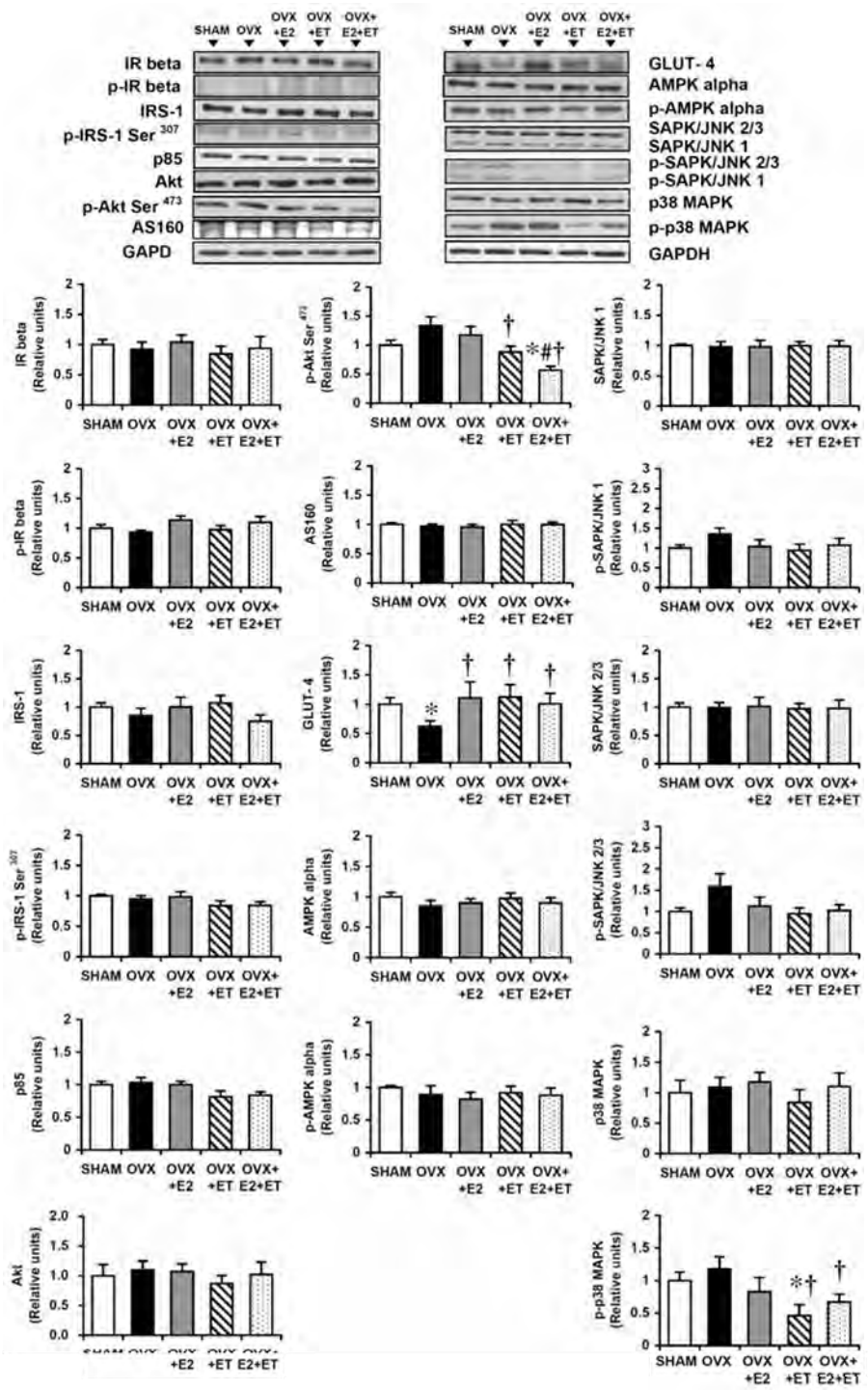


Fig. 14. Immunoblot analysis of signaling proteins IR-β (IR beta), IR-β (Tyr^{1158}/Tyr^{1162}/Tyr^{1163}) phosphorylation (p-IR beta), IRS-1, IRS-1 (Ser^{307}) phosphorylation (p-IRS-1 Ser^{307}), the p85 subunit of PI3-K (p85), Akt, Akt (Ser^{473}) phosphorylation (p-Akt Ser^{473}), GLUT-4, SAPK/JNK 1, SAPK/JNK 1 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 1), SAPK/JNK 2/3, SAPK/JNK 2/3 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 2/3), p38 MAPK, and p38 MAPK (Thr^{180}/Tyr^{182}) phosphorylation (p-p38 MAPK) in non-incubated soleus muscles of sham-operated control (SHAM) and ovariectomized (OVX) rats without or with estrogen replacement (E2) and/or

exercise training (ET). Proteins were normalized to GAPDH. Data are presented as fold change over the sham-operated (SHAM) group. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 8-10 animals/group. *P < 0.05 vs. SHAM.

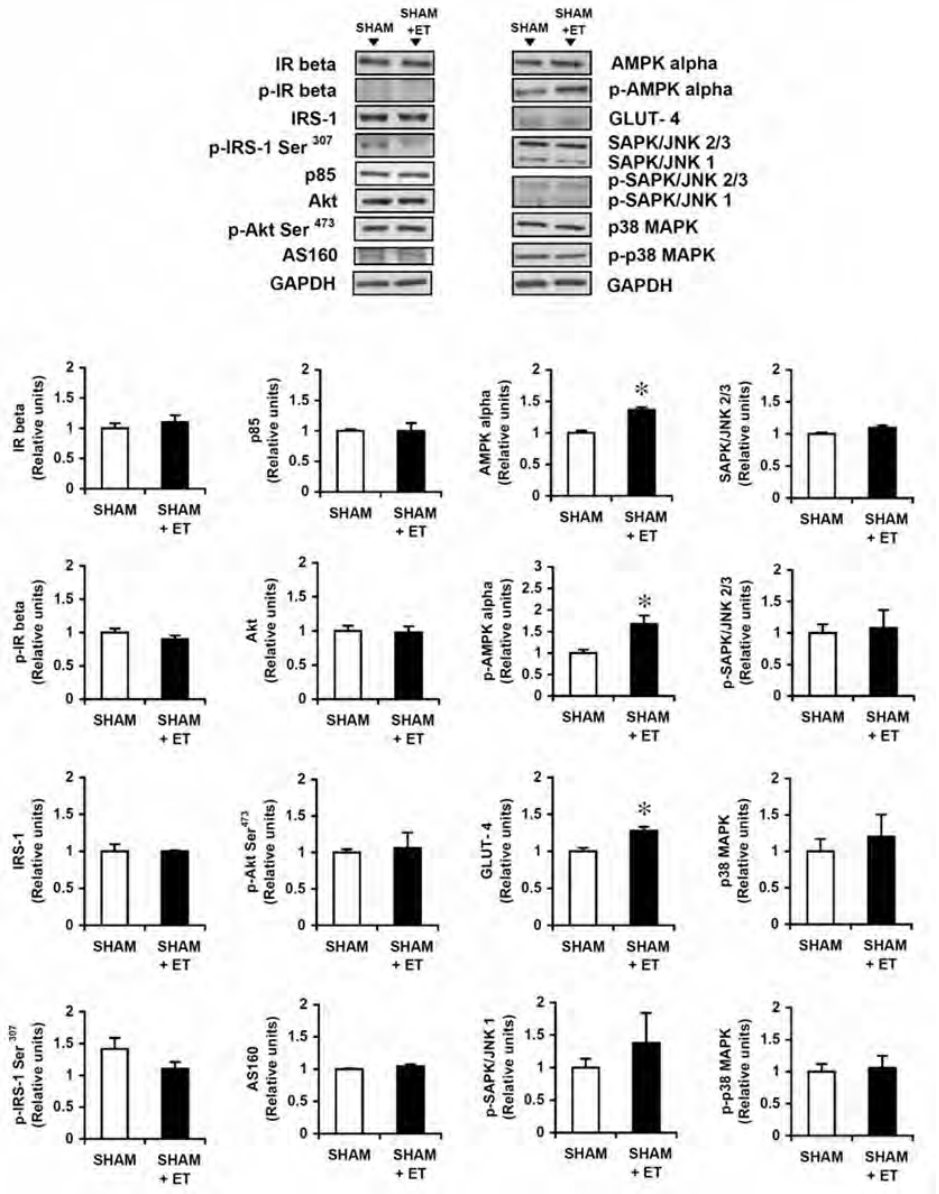


Fig. 15. Immunoblot analysis of signaling proteins IR-β (IR beta), IR-β (Tyr^{1158}/Tyr^{1162}/Tyr^{1163}) phosphorylation (p-IR beta), IRS-1, IRS-1 (Ser^{307}) phosphorylation (p-IRS-1 Ser^{307}), the p85 subunit of PI3-K (p85), Akt, Akt (Ser^{473}) phosphorylation (p-Akt Ser^{473}), GLUT-4, SAPK/JNK 1, SAPK/JNK 1 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 1), SAPK/JNK 2/3, SAPK/JNK 2/3 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 2/3), p38 MAPK, and p38 MAPK (Thr^{180}/Tyr^{182}) phosphorylation (p-p38 MAPK) in non-incubated soleus muscles of sham-operated control without (SHAM) or with exercise training (SHAM+ET). Proteins were normalized to GAPDH. Data are presented as fold change over the SHAM group. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 6-8 animals/group. *P < 0.05 vs. SHAM.

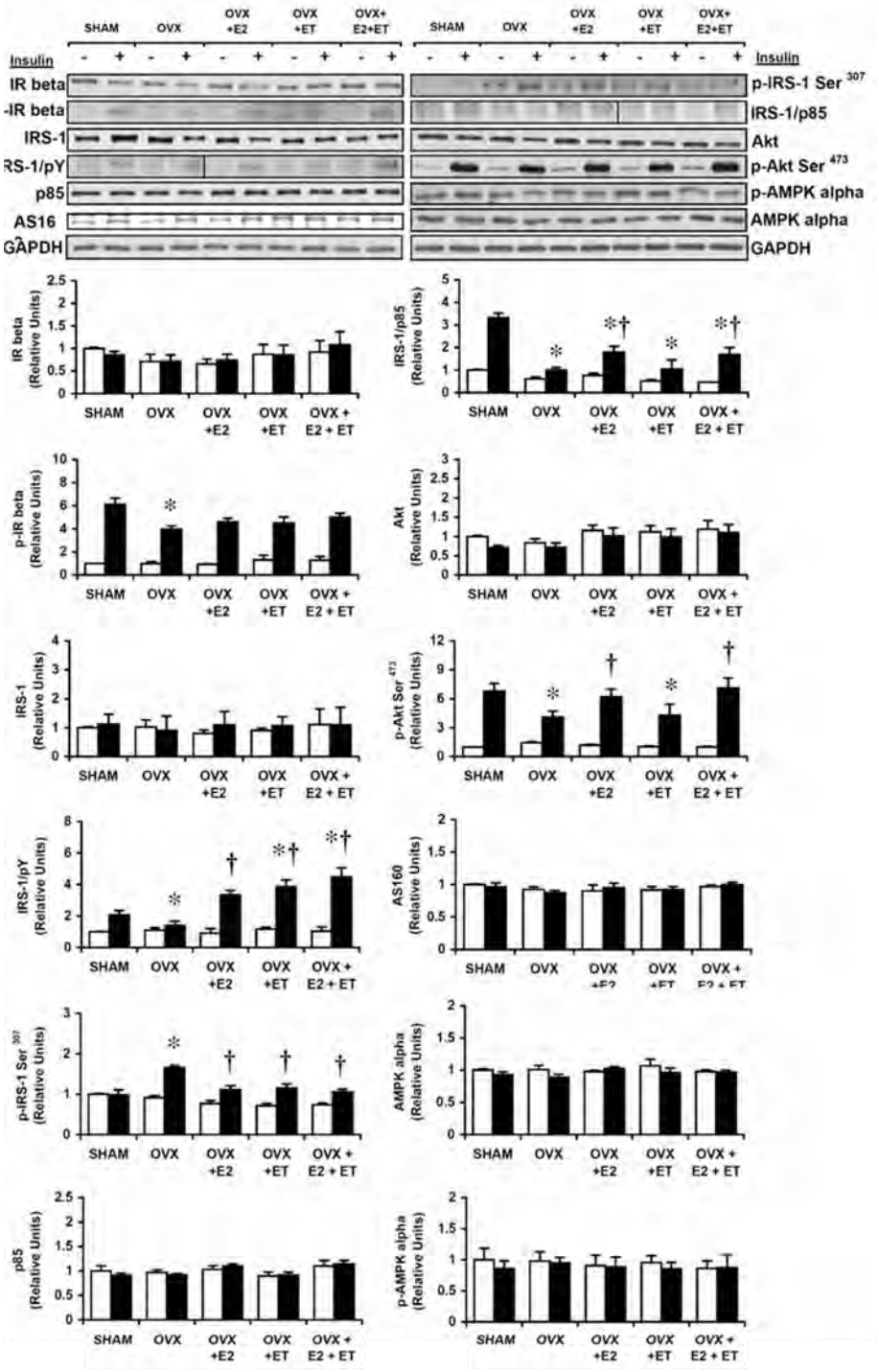


Fig. 16. Insulin-induced expression of IR-β (IR beta), IR-β phosphorylation (p-IR beta), IRS-1, tyrosine phosphorylation of IRS-1 (IRS-1/pY), IRS-1 (Ser^{307}) phosphorylation (p-IRS-1 Ser^{307}), IRS-1 associated with the p85 subunit of PI3-K (IRS-1/p85), Akt, and Akt phosphorylation (p-Akt Ser^{473}) in soleus muscles from SHAM and ovariectomized (OVX) rats without or with estrogen replacement (E2) and/or exercise training (ET). Muscles were incubated in the absence (blank bars) or the presence (filled bars) of insulin (2 mU/ml). For IRS-1/pY and IRS-1/p85, muscle lysate was immunoprecipitated with agarose-conjugated anti-IRS-1 antibody prior to immunoblotting against tyrosine and p85, respectively. IRS-1/pY and IRS-1/p85 were normalized to IRS-1 protein expression. Data are presented as fold change over the SHAM group. Values are means ± SE for 8 animals/group. *$P < 0.05$ vs. SHAM; †$P < 0.05$ vs. OVX.

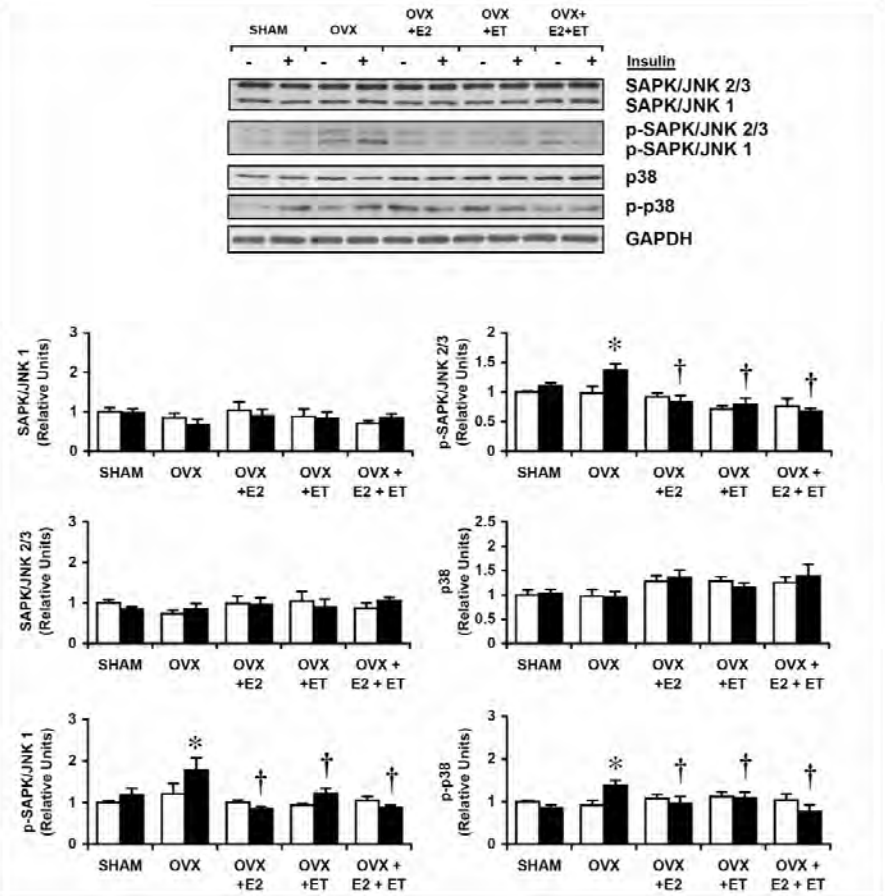


Fig. 17. Insulin-induced expression of SAPK/JNK 1, SAPK/JNK 2/3, SAPK/JNK 1 and SAPK/JNK 2/3 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 1), p38 MAPK, and p38 MAPK (Thr^{180}/Tyr^{182}) phosphorylation (p-p38 MAPK) in soleus muscles from SHAM and ovariectomized (OVX) rats without or with estrogen replacement (E2) and/or exercise training (ET). Muscles were incubated in the absence (blank bars) or the presence (filled bars) of insulin (2 mU/ml). Data are presented as fold change over the SHAM group. Results were normalized to GAPDH. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 8 animals/group. *$P < 0.05$ vs. SHAM; †$P < 0.05$ vs. OVX.

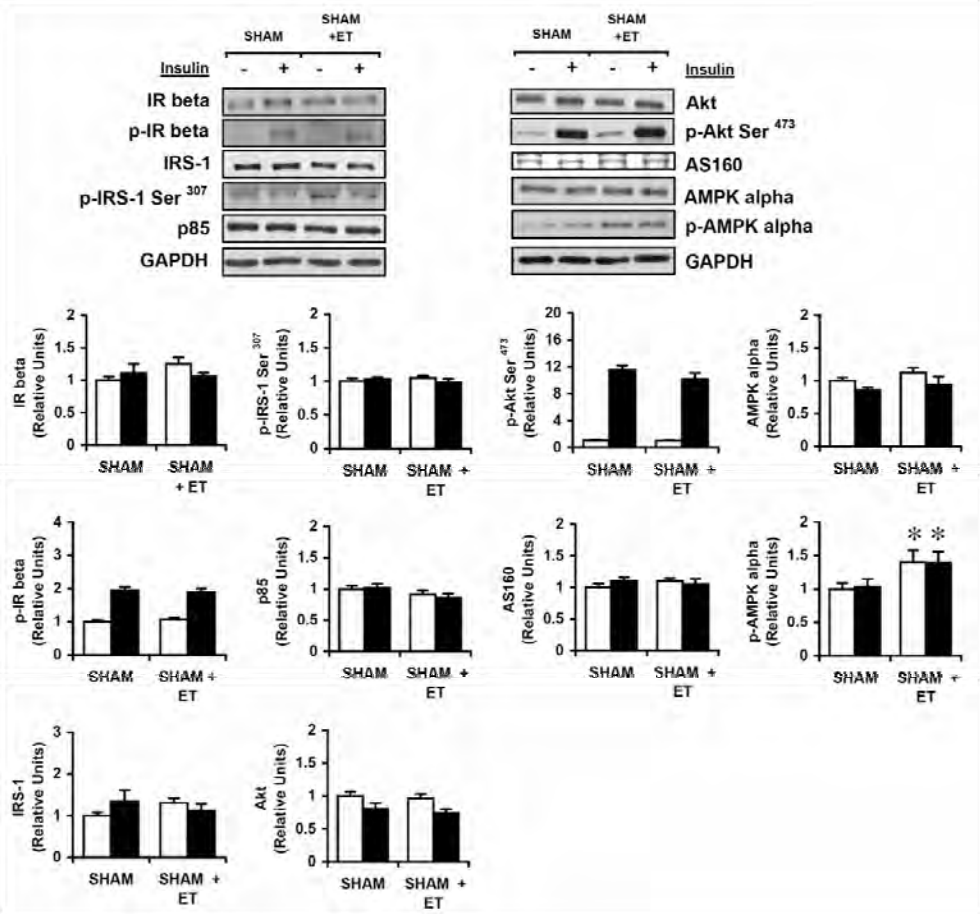


Fig. 18. Insulin-induced expression of IR-β (IR beta), IR-β (Tyr^{1158}/Tyr^{1162}/Tyr^{1163}) phosphorylation (p-IR beta), IRS-1, tyrosine phosphorylation of IRS-1 (IRS-1/pY), IRS-1 (Ser^{307}) phosphorylation (p-IRS-1 Ser^{307}), IRS-1 associated with the p85 subunit of PI3-K (IRS-1/p85), Akt, and Akt (Ser^{473}) phosphorylation (p-Akt Ser^{473}) in soleus muscles from sham-operated control without (SHAM) or with exercise training (SHAM+ET). Muscles were incubated in the absence (blank bars) or the presence (filled bars) of insulin (2 mU/ml). Data are presented as fold change over the SHAM group. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 6-8 animals/group. *P < 0.05 vs. SHAM.

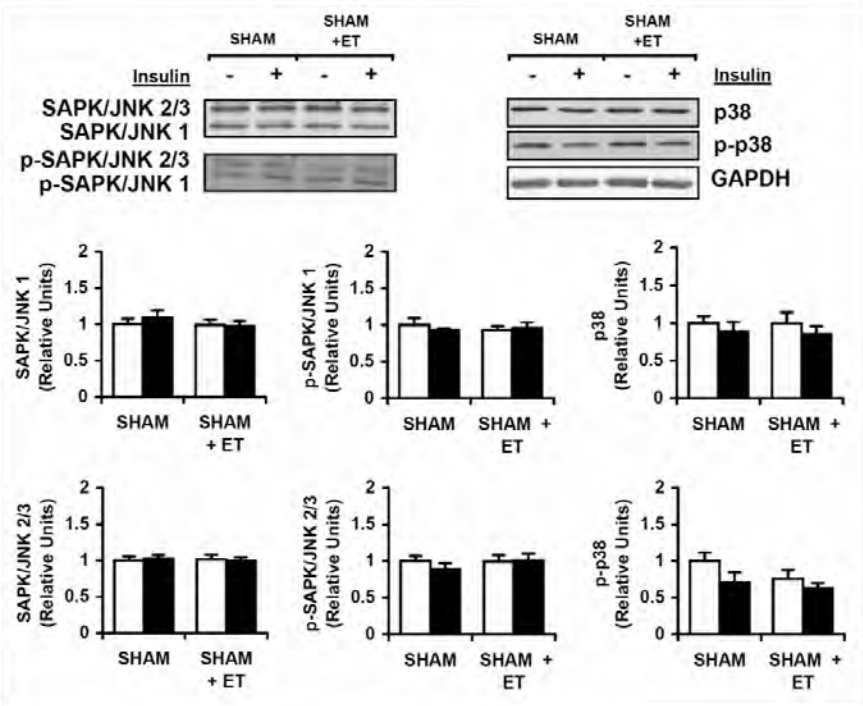


Fig. 19. Insulin-induced expression of SAPK/JNK 1, SAPK/JNK 2/3, SAPK/JNK 1 and SAPK/JNK 2/3 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 1), p38 MAPK, and p38 MAPK (Thr^{180}/Tyr^{182}) phosphorylation (p-p38 MAPK) in soleus muscles from sham-operated control without (SHAM) or with exercise training (SHAM+ET). Muscles were incubated in the absence (blank bars) or the presence (filled bars) of insulin (2 mU/ml). Data are presented as fold change over the SHAM group. Results were normalized to GAPDH. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 6-8 animals/group. *P < 0.05 vs. SHAM.

Endurance exercise training is an effective intervention that improves insulin action on whole body glucose disposal or on skeletal muscle glucose transport activity. The molecular mechanisms for increased glucose transport with exercise training may be related to changes in expression and/or functionality of key signaling proteins involved in the regulation of glucose transport. Insulin resistance at the whole body and skeletal muscle levels in the OVX rat can be overcome with endurance exercise training, and a critical adaptation underlying the enhanced insulin-stimulated glucose transport activity after exercise training in OVX rat is an upregulation of GLUT-4 protein expression. However, the potential cellular mechanisms that underlie the beneficial adaptations associated with exercise training leading to enhanced insulin action on the insulin-resistant skeletal muscle in OVX rat were not completely elucidated.

A primary finding of the second part of this investigation is that the adaptive responses to endurance exercise training include increased protein expression of ERα and GLUT-4, and decreased phosphorylation levels of Akt and p38 MAPK, without significant alteration of expression or functionality of IR β, PI3-kinase, AS160, AMPK α, and SAPK/JNK. In addition, enhanced insulin action on glucose transport in skeletal muscle of exercise-trained OVX rat occurs with increased insulin-stimulated tyrosine phosphorylation of IRS-1, decreased insulin-stimulated serine phosphorylation of IRS-1, and reduced SAPK/JNK and p38 MAPK activity. Interestingly, several adaptive changes in the protein expression and functionality of the insulin signaling and MAPK pathways observed in skeletal muscle of exercise-trained OVX rat are not apparent in exercise-trained SHAM rat while a single similar adaptation in skeletal muscle of OVX and SHAM animals to endurance exercise training is an upregulation of GLUT-4 protein expression. The distinctive adaptations in SHAM animal to endurance exercise training are increases in protein expression and functionality of AMPK α and insulin-stimulated AMPK α activity.

Evidence that provided a comprehensive evaluation on the effects of exercise training on the insulin signaling elements is limited and has reported inconsistent observations. In the present study, AMPK α activity in skeletal muscle of exercise-trained SHAM rat was augmented while AS160 was unaltered. Based on the findings in the present study, it appears that an improvement in insulin signaling at the level of IRS-1/Akt in skeletal muscle is not required for the enhanced glucose transport process observed in exercise-trained SHAM rat. Our findings are consistent with the notion that elevated protein expression and activity of AMPK α in response to exercise training where GLUT-4 expression is also increased contribute to an enhancement in insulin-stimulated skeletal muscle glucose transport activity in exercise-trained SHAM rat.

A significant reduction in serine phosphorylation of Akt was observed in skeletal muscle of exercise-trained OVX rat when compared with OVX control. This finding indicated that Akt activation is not involved in the insulin sensitizing effect of endurance exercise training in OVX rat. Furthermore, an increase in insulin-stimulated glucose transport activity and IRS-1/pY in skeletal muscle of exercise-trained OVX rat is accompanied by reduced IRS-1 serine phosphorylation with a corresponding decrease in SAPK/JNK and p38 MAPK activation. Nevertheless, endurance exercise training did not restore the impaired insulin stimulation on IRS-1/p85 and serine phosphorylation of Akt in skeletal muscle of OVX rat. Thus, the effects of

exercise training on the insulin signaling pathway in skeletal muscle of OVX rat is limited to the enhanced tyrosine phosphorylation of IRS-1 and does not bring about adaptation of the downstream insulin signaling elements including IRS-1/p85 and serine phosphorylation of Akt.

Our findings that visceral fat accumulation and circulating free fatty acids were substantially decreased suggest that systemic inflammatory state would be attenuated in exercise-trained OVX rats. In fact, we have demonstrated in this study that endurance exercise training led to a significant reduction in phosphorylation level of p38 MAPK. Furthermore, we did observe that insulin-stimulated phosphorylation of SAPK/JNK and p38 MAPK were decreased in skeletal muscle of exercise-trained OVX rats. Therefore, the reduced activity of SAPK/JNK and p38 MAPK and the enhanced tyrosine and reduced serine phosphorylation of IRS-1 in skeletal muscle of exercise-trained OVX rat appear to be important for the improved insulin action. However, our observation that the downstream insulin signaling elements were not activated along with IRS-1 has weakened the functional implication of the reduced MAPK activation in exercise-trained OVX muscles.

One critical finding from this study is that the enhanced protein expression and activity of AMPK α in exercise-trained SHAM rat is not apparent in exercise-trained OVX rat as endurance exercise training in OVX rat did not bring about adaptation in the levels of protein expression and phosphorylation of AMPK α or insulin-stimulated AMPK α activity. Our finding supported the idea proposed by previous investigations that the AMPK pathway may function abnormally in phenotypes of obesity and insulin resistance.

The physiological actions of estrogens are mediated by estrogen receptor alpha (ER α) and estrogen receptor beta (ER β). In this study, we have demonstrated that ovariectomy brought about significant reduction in circulating levels of estrogen without affecting the protein expression of ER α and ER β. However, we did observe a significant increase in protein expression of ER α in exercise-trained OVX muscles. Accordingly, we speculate that the reduced inflammatory mediators and/or the improved insulin-stimulated glucose transport activity in exercise-trained OVX muscle could be related to the training effect on ERα expression. Collectively, our results support the notion that endurance exercise training does not amend the defect in insulin signaling through the PI3-kinase/Akt pathway in OVX rat. Nevertheless, an improvement in insulin-stimulated skeletal muscle glucose transport in OVX rat after endurance exercise training is associated with the increase in GLUT-4 protein expression and a corresponding reduction in the activity of SAPK/JNK and p38 MAPK.

Therefore, it appears that the key adaptation of OVX skeletal muscle to exercise training is an increase in expression of GLUT-4 protein content.

We have previously (Saengsirisuwan et al., 2009) demonstrated that estrogen replacement effectively corrected the phenotypic characteristics of obesity and insulin resistance in OVX rat. In addition, the potential interaction of endurance exercise training in combination with estrogen replacement was evaluated. Even though these two interventions did not work in an additive fashion, we proposed that the potential interactive effects may be overruled by the hyperestrogenic condition as the uterine weights of OVX+E_2 group were twice as much of the SHAM group. In this study, the dose of estrogen given to each rat was reduced to 2.5 μg. The values of the uterine weights and serum estrogen in OVX rats receiving estrogen replacement and SHAM animals were comparable. In agreement with the previous study, estrogen replacement restored the phenotypic characteristics of obesity and insulin resistance and improved insulin action at the whole-body and skeletal muscle levels. Additionally, several changes at the cellular level such as the defective insulin signaling and MAPK pathways observed in OVX skeletal muscle were attenuated by estrogen replacement. Importantly, the combination of estrogen replacement and exercise training resulted in greater increases in insulin action on skeletal muscle glucose transport activity than either intervention individually, indicating the additive effect of these two interventions to enhance insulin action in skeletal muscle of OVX rats. Nevertheless, this interactive effect on skeletal muscle insulin action did not bring about a greater improvement in whole-body insulin sensitivity as determined by oral glucose tolerance test.

What are the underlying mechanisms for the interactive effect of estrogen replacement and exercise training on skeletal muscle insulin action? Our findings that estrogen replacement corrected the defective insulin signaling factors and GLUT-4 protein expression while the key adaption to endurance exercise training was an upregulation of GLUT-4 protein were insufficient to explain the additive effect. However, it is rational to propose that endurance exercise training did improve the capacity for glucose transport by increasing the metabolic machineries in glucose metabolism such as the non-oxidative and oxidative pathways.

Conclusively, this study provided an extensive evaluation of the adaptive response of skeletal muscle in OVX rats to 12 weeks of endurance exercise training by tracking alterations in protein expression and the phosphorylation level of the insulin signaling and MAPK cascades. At the systemic level, training improved the ectopic fat accumulation and lipid profile.

The effects of exercise training on insulin signaling were observed including increased tyrosine phosphorylation of IRS-1 but unaltered the downstream signaling molecules. This dissociation does not support generally improved insulin signaling to glucose transport activity. In turn, it appears that the observed training effects are at least partly a result of increased cellular protein content of GLUT-4. On the other hand, estrogen replacement did restore the insulin signaling cascade and GLUT-4 protein abundance. Endurance exercise training in concert with estrogen replacement brought about the greatest improvement in skeletal muscle insulin action. As the interactive effect of these two interventions was not attributed to additive effects on GLUT-4 protein abundance, it remains to be verified whether the mechanism for this interaction will be due to the adaptive changes in other unidentified metabolic factors.

Output ที่ได้จากโครงการ

1. ผลงานวิจัยในวารสารวิชาการนานาชาติ

1.1 ผลงานเรื่องที่ 1

Prasannarong M, Vichaiwong K, Saengsirisuwan V*. Calorie restriction prevents the development of insulin resistance and impaired insulin signaling in skeletal muscle of ovariectomized rats. *Biochimica et Biophysica Acta – Molecular Basis of Disease.* 1822 (6): 1051-1061, 2012. (IF = 5.211) (ภาคผนวก)

1.2 อยู่ระหว่างการดำเนินการเพื่อเผยแพร่ผลงานเรื่องที่ 2 ในวารสารวิชาการ โดยคาดว่าชื่อเรื่องและชื่อวารสารของผลงานวิจัยจะเป็นดังนี้

ชื่อเรื่อง Endurance exercise training improves insulin-stimulated glucose transport and tyrosine phosphorylation of IRS-1 but not PI3-kinase and Akt in skeletal muscle of ovariectomized rats.

ชื่อวารสาร Journal of Applied Physiology (IF = 3.753)

2. การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

ทุนวิจัยที่ได้รับส่วนหนึ่งเป็นทุนสนับสนุนการทำวิจัยของนักศึกษาระดับปริญญาเอก จำนวนหนึ่งราย คือ นางสาวมุจลินทร์ ประสานณรงค์ ปัจจุบันทำงานเป็นอาจารย์ สังกัดคณะเทคนิคการแพทย์ มหาวิทยาลัยเชียงใหม่

3. Abstract in international and national meetings

3.1 Abstract การนำเสนอผลงานแบบโปสเตอร์ในการประชุมวิชาการระดับนานาชาติ

73rd Scientific Sessions, American Diabetes Association, Chicago, Illinois, June 21-25, 2013.

- Saengsirisuwan V, Prasannarong M, Vichaiwong K. Endurance exercise training improves insulin-stimulated glucose transport and tyrosine phosphorylation of IRS-1 but not PI3-kinase and Akt in skeletal muscle of ovariectomized rats (Abstract). *Diabetes.* 2013 (ยังไม่ทราบเลขที่หน้า ณ วันที่ส่งรายงาน)

3.2 Abstract การนำเสนอผลงานแบบโปสเตอร์ในการประชุมวิชาการระดับนานาชาติ

71st Scientific Sessions, American Diabetes Association, San Diego, California, June 24-28, 2011.

- Prasannarong M, Vichaiwong K, Saengsirisuwan V. Weight control diminishes progression of insulin resistance due to estrogen-deprived condition (Abstract). *Diabetes.* 2011; 60 (supp 1): 1854.

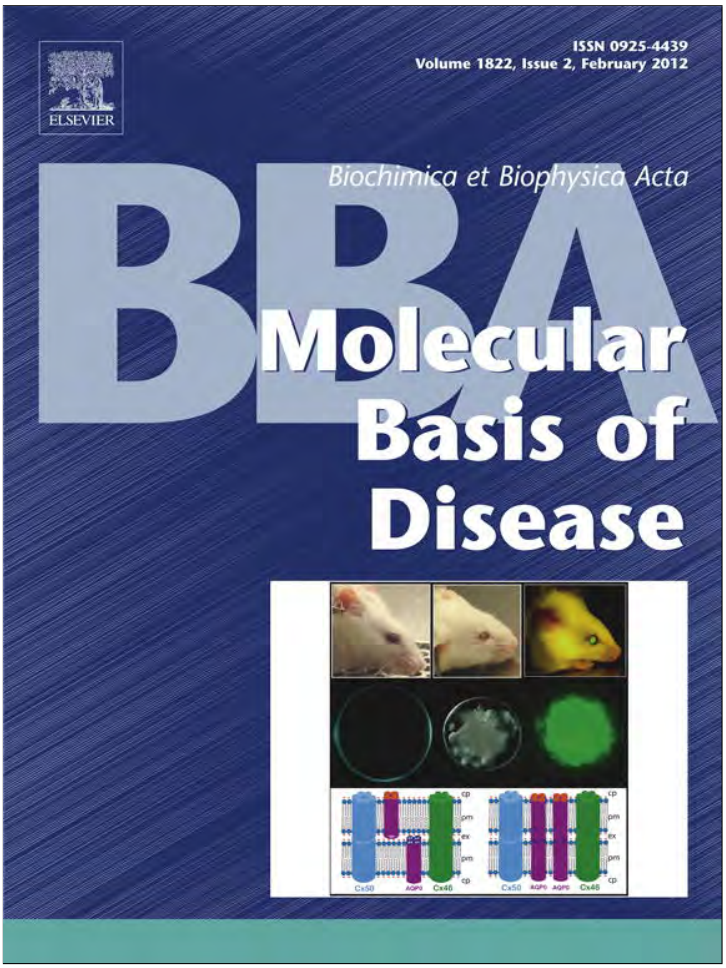
3.3 Abstract การนำเสนอผลงานแบบปากเปล่าในการประชุมวิชาการระดับชาติ

งานวิจัยที่เกี่ยวกับ obesity ในประเทศไทย ณ การประชุมวิชาการสรีรวิทยาสมาคม ครั้งที่ 41 ประจำปี 2555 ที่คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล ระหว่างวันที่ 2-4 พฤษภาคม 2555

- Prasannarong M, Vichaiwong K, Saengsirisuwan V. Calorie restriction prevents the development of insulin resistance and impaired insulin signaling in skeletal muscle of ovariectomized rats (Abstract). *Journal of Physiological and Biomedical Sciences.* 2012; 25 (1): p. 40.

3.4 ผู้รับทุนเป็นวิทยากรนำเสนอผลงานวิจัยในการอภิปรายกลุ่ม (symposium) หัวข้อ งานวิจัยที่เกี่ยวกับ obesity ในประเทศไทย เรื่อง Relief of insulin resistance and obesity: Role of exercise training ณ การประชุมวิชาการสรีรวิทยาสมาคม ครั้งที่ 41 ประจำปี 2555 ที่คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล ระหว่างวันที่ 2-4 พฤษภาคม 2555

ภาคผนวก



(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

Biochimica et Biophysica Acta 1822 (2012) 1051–1061

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Calorie restriction prevents the development of insulin resistance and impaired insulin signaling in skeletal muscle of ovariectomized rats

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ABSTRACT

Insulin resistance of skeletal muscle glucose transport due to prolonged loss of ovarian function in ovariectomized (OVX) rats is accompanied by other features of the metabolic syndrome and may be confounded by increased calorie consumption. In this study, we investigated the role of calorie consumption in the development of insulin resistance in OVX rats. In addition, we examined the cellular mechanisms underlying skeletal muscle insulin resistance in OVX rats. Female Sprague-Dawley rats were ovariectomized (OVX) or sham-operated (SHAM). OVX rats either had free access to food, pair feeding (PF) with SHAM or received a 35% reduction in food intake (calorie restriction; CR) for 12 weeks. Compared with SHAM, ovariectomy induced skeletal muscle insulin resistance, which was associated with decreases (32–70%) in tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1), IRS-1 associated p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase), and Akt Ser^{473} phosphorylation whereas insulin-stimulated phosphorylation of IRS-1 Ser^{307}, SAPK/JNK Thr^{183}/Tyr^{185}, and p38 mitogen-activated protein kinase (MAPK) Thr^{180}/Tyr^{182} was increased (24–62%). PF improved the serum lipid profile but did not restore insulin-stimulated glucose transport, indicating that insulin resistance in OVX rats is a consequence of ovarian hormone deprivation. In contrast, impaired insulin sensitivity and defective insulin signaling were not observed in the skeletal muscle of OVX + CR rats. Therefore, we provide evidence for the first time that CR effectively prevents the development of insulin resistance and impaired insulin signaling in the skeletal muscle of OVX rats.

1. Introduction

The insulin resistance syndrome is a condition characterized by insulin resistance of skeletal muscle glucose metabolism and is accompanied by several metabolic and cardiovascular abnormalities, including central obesity, dyslipidemia, essential hypertension, hyperinsulinemia, and glucose intolerance [1]. The pathophysiology of the insulin resistance syndrome is complex and involves several factors. Aging is a major factor in both men and women. In women, the systemic loss of ovarian function at menopause is associated with an increase in the amount of visceral fat [2,3], a higher prevalence of atherogenic lipid profiles, insulin resistance and type 2 diabetes [4,5]. As women often spend nearly one third of their lives in the postmenopausal state, understanding how ovarian hormone depletion contributes to the regulation of glucose metabolism could provide essential information toward reducing the incidence of insulin resistance and type 2 diabetes. Ovariectomized (OVX) animals have been used as a model for human menopause and for studying the metabolic consequences due to loss of ovarian function. We have previously reported that prolonged (12 weeks) ovariectomy leads to the development of a systemic metabolic condition, including increased visceral fat accumulation, an abnormal serum lipid profile, impaired glucose tolerance, and defective insulin action on skeletal muscle glucose transport [6]. As ovariectomy results in increased daily food intake and promotes weight gain [6–8], the consequences of ovarian hormone deprivation in obesity and insulin resistance are complicated by the effects of increased calorie intake. Thus, it remains unknown whether the metabolic defects that result from ovariectomy are confounded by ovariectomy-induced hyperphagia.

It is well documented that a 25–40% caloric restriction (CR) diet (below *ad libitum* intake) enhances insulin sensitivity in both insulin-sensitive and insulin-resistant skeletal muscle [9–13]. The underlying molecular process responsible for the enhanced insulin sensitivity appears to be associated with increased activation of certain insulin signaling elements [9–13]. However, the benefits of calorie restriction on insulin action in rat skeletal muscle under prolonged ovarian hormone depletion have not been determined.

Insulin enhances cellular metabolic functions mainly through the phosphatidylinositol 3-kinase (PI3-kinase) pathway and stimulates mitogenic effects via the mitogen-activated protein kinase (MAPK) signaling pathway [14,15]. Accumulating evidence indicates a connection between the MAPK pathway and insulin resistance in skeletal muscle [16,17]. To date, no study has reported the cellular mechanisms underlying the insulin-resistant state in skeletal muscle

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following prolonged ovariectomy with or without dietary manipulation. It is, therefore, unknown whether the insulin-dependent signaling elements in skeletal muscle are affected. In this context, the aims of this study were: 1) to investigate whether the insulin resistance in OVX rats is directly attributed to ovarian hormone depletion or is secondary to increased caloric intake; 2) to examine the effects of calorie restriction on the insulin-resistant condition in OVX rats; and 3) to examine whether the insulin resistance of glucose transport in skeletal muscle of OVX rats is associated with alterations in signaling molecules involved in the skeletal muscle glucose transport system. To address these issues, a number of metabolic characteristics, including glucose tolerance, insulin-stimulated skeletal muscle glucose transport, serum lipid profile, and visceral fat accumulation were determined in OVX rats that received a pair feeding diet (PF) diet or calorie restriction (CR). In addition, we evaluated the protein expression and functionality of specific elements of the PI3-kinase and MAPK pathways including insulin receptor (IR), insulin receptor substrate-1 (IRS-1), the p85 subunit of PI3-kinase, Akt, c-Jun NH_2-terminal kinase (SAPK/JNK), and p38 MAPK.

2. Materials and methods

2.1. Animal treatments

Animal procedures were approved by the Animal Care and Use Committee, Faculty of Science, Mahidol University, in accordance with the International Guiding Principles for Biomedical Research Involving Animals of CIOMS. Eight-week-old female Sprague-Dawley rats, weighing between 180 and 200 g, supplied by the National Laboratory Animal Center, Thailand, were individually housed in an 8×10 in. hygienic hanging cage at the Center of Animal Facilities, Faculty of Science, Mahidol University. The housing unit was maintained at 22 C with a 12/12-hour light/dark cycle (light on from 0600 to 1800 h). The rats were randomly assigned to either sham operation (SHAM, n = 10) or bilateral ovariectomy (n = 30). Surgery was performed under anesthesia through bilateral skin incisions at the lower back. Animals were allowed to recover for 7 days after surgery. Body weight was monitored every other day. The amount of food intake of each animal was measured over a 24 h period using a metabolic cage (Model 3701 M081, Tecniplast, Italy), and the measurements were conducted at least 3 times per week. Ovariectomized animals were further divided into 3 groups: OVX-control (OVX, n = 10), pair-fed OVX (OVX + PF, n = 10), or calorie-restricted OVX (OVX + CR, n = 10). Rats in the SHAM and OVX-control groups were given regular rat chow (Perfect Companion, Samutprakarn, Thailand) *ad libitum*. The average amount of food consumed by SHAM or OVX groups was recorded to determine the amount of food given to the OVX + PF or OVX + CR groups in the following day. The amount of food given to animals in the OVX + PF group was matched to that consumed by the SHAM group, whereas animals in the OVX + CR group were given 65% of the amount of chow consumed by the OVX-control group. Rats in the OVX + PF and OVX + CR groups were given a portion of their feed allotment in two periods (50% between 0800 and 0900 h and 50% between 1700 and 1800 h).

2.2. Oral glucose tolerance test

Following a 12-week treatment, an oral glucose tolerance test (OGTT) was performed on each animal as follows. In the evening (1800 h) of the day before the test, all rats were food-restricted to 4 g of chow. On the day of the test (0900 h), tail blood was collected before glucose feeding (1 g/kg body wt) by gavage and at 15, 30, 60, and 120 min after glucose challenge. Blood sample was mixed with anticoagulant (18 mM final concentration of EDTA) and centrifuged at 13,000 *g* at 4 C for 1 min. Plasma was kept at −80 C and used for the determination of glucose (Gesellschaft fur Biochemica und Diagnostica, Wiesbaden, Germany) and insulin (Linco Research, St. Charles, MO). Aliquots of plasma at the 0-min time point were kept frozen and used for subsequent analyses of free fatty acid levels using an enzymatic kit (BioVision, Inc., Mountain View, CA). Immediately after the OGTT, each animal was subcutaneously given 2.5 ml of sterile 0.9% saline to replace fluid loss.

2.3. Measurement of muscle glucose transport activity

Five days after the OGTT, all animals were food-restricted as described above. At 0800 h, animals were weighed and anesthetized with an intraperitoneal administration of a mixture of ketamine (50 mg/kg body wt) and xylazine (10 mg/kg body wt). Soleus muscles were isolated and prepared for *in vitro* incubation. The two soleus muscles were divided into four strips each. To investigate the adaptive changes in skeletal muscle signaling and GLUT-4 proteins due to prolonged ovariectomy and calorie restriction, two non-incubated soleus strips were quickly frozen in liquid nitrogen for subsequent analyses. Six fresh soleus strips (~25 mg) were incubated for 60 min at 37 C in 3 ml of oxygenated Krebs-Henseleit buffer (KHB) supplemented with 8 mM D-glucose, 32 mM D-mannitol, and 0.1% radioimmunoassay-grade bovine serum albumin (Sigma Chemical, St. Louis, MO). Of these six, three muscle strips were incubated in the absence of insulin, and the other three strips were incubated in the presence of a maximally effective concentration of insulin (2 mU/ml; Human R, Eli Lilly, Indianapolis, IN). The flasks were continuously gassed with a mixture of 95% O_2 and 5% CO_2 throughout the incubation and glucose transport assay. After the first incubation period, four of the soleus strips (two incubated without insulin and two incubated with insulin) were removed, trimmed of fat and connective tissue, and quickly frozen in liquid nitrogen. These strips were subsequently used for the determination of signaling elements in response to insulin activation by immunoblotting and immunoprecipitation. The remaining muscle strips were rinsed for 10 min at 37 C in 3 ml of oxygenated KHB containing 40 mM D-mannitol, 0.1% BSA, and insulin, if previously present. Muscle strips were incubated for 20 min at 37 C in 2 ml of KHB containing 1 mM 2-[1,2-^{3}H]deoxyglucose (2-DG, 300 μCi/mmol; PerkinElmer Life Sciences, Boston, MA), 39 mM [U-^{14} C]mannitol (0.8 μCi/mmol; PerkinElmer Life Sciences), 0.1% BSA, and insulin, if previously present. At the end of the incubation period, the muscle strips were removed, trimmed of excess fat and connective tissue, and immediately frozen with liquid nitrogen and weighed. The frozen muscles were solubilized in 0.5 ml of 0.5 N NaOH, and 10 ml of scintillation cocktail (Ultima Gold™; PerkinElmer Life Sciences) was added. The specific intracellular accumulation of 2-DG was determined as described previously [18] using mannitol to correct for 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).

2.4. Tissue and blood collection

The plantaris muscle and the liver were collected for the determination of tissue triglyceride content. Blood was collected from the abdominal vein using a 5 ml syringe. Whole blood was allowed to clot and then centrifuged at 3,000 *g* at 4 C for 20 min to obtain serum, which was kept at -80 C until the determination of serum lipids. Immediately after blood collection, visceral fat was collected from the superficial area covering the alimentary tract, and the uterus was removed and weighed. The heart was removed for the animal euthanasia.

2.5. Biochemical assays

Serum levels of total cholesterol, high-density lipoprotein (HDL)-cholesterol, and low-density lipoprotein (LDL)-cholesterol were determined using Dimension RxL Max (DADE Behring, Marburg, Germany).

The triglyceride contents in the liver and plantaris muscle were assessed using enzymatic kits (BioVision).

2.6. Analyses of signaling elements in skeletal muscle

Muscles were homogenized in ice-cold lysis buffer: 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM EDTA, 10 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 2 mM Na_3VO_4, 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. Aliquots of supernatant were frozen at −80 C, and a portion of these homogenates was used for the determination of total protein (BCA method, Sigma Chemical). Proteins in the homogenates were separated on 8% or 12% polyacrylamide gels and transferred electrophoretically onto nitrocellulose paper. Protein blots of samples from incubated and non-incubated muscles were incubated with the appropriate dilution of commercially available antibodies against phospho-insulin receptor (IR)/IGF1R (Tyr^{1158}/Tyr^{1162}/Tyr^{1163}) (Millipore, Billerica, MA), insulin receptor beta (4B8), phospho-IRS-1 (Ser^{307}), IRS-1, the p85 subunit of PI3-kinase (Millipore), phospho-Akt (Ser^{473}), Akt, phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), SAPK/JNK, phospho-p38 MAPK (Thr^{180}/Tyr^{182}), and p38 MAPK. Protein blots of samples from non-incubated muscles were also incubated with commercially available antibodies against GLUT-4. Subsequently, all blots were incubated with goat anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase-conjugated (IgG-HRP) secondary antibody. All antibodies, if not specified above, were purchased from Cell Signaling Technology (Beverly, MA). Protein bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences) on hyper film (Amersham Biosciences, Buckinghamshire, England). Images were digitized, and band intensities were quantified using an imaging densitometer (GS-800, Bio-Rad) and Quantity One software.

For the measurements of tyrosine-phosphorylated IRS-1 (IRS-1/pY) and IRS-1 associated p85 (IRS-1/p85), immunoprecipitation and subsequent immunoblotting were performed. The muscle homogenates were diluted to 2 mg/ml (IRS-1/pY) or 3 mg/ml (IRS-1/p85). Subsequently, 0.5 ml of diluted homogenate was immunoprecipitated with 25 μl of agarose-conjugated anti-IRS-1 antibody (Millipore). After an overnight incubation at 4 C, the samples were centrifuged, and the supernatant was removed. The beads were washed three times with ice-cold PBS, mixed with SDS sample buffer, and boiled for 5 min. The proteins were separated by SDS-PAGE on 8% polyacrylamide gels and were transferred to nitrocellulose membranes. Immunoblotting for detection of IRS-1/p85 was completed as described above for the detection of the protein expression of p85. For analysis of IRS-1/pY, the nitrocellulose membrane was incubated in anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology). Thereafter, the membranes were incubated with secondary goat anti-mouse antibody conjugated with HRP (Santa Cruz Biotechnology). Protein bands of interest were exposed, visualized, and quantified as described above.

2.7. Statistical analysis

All values are expressed as means ± SE. Differences among groups were determined by one-way ANOVA with Tukey's post hoc test. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Body and tissue weight

A significant difference in average body weight between SHAM and OVX rats was observed starting at week 1 after surgery (Fig. 1A). In weeks 2–13, the body weight of the OVX group continued to elevate and was significantly higher than all other groups. At week 2, the body weights of both OVX + PF and OVX + CR groups were higher ($P<0.05$) than that of the SHAM rats (Fig. 1A). During weeks 3–13, the body weight of the OVX + PF rats was higher ($P<0.05$) than that of the SHAM or OVX + CR group (Fig. 1A). The final average body weight and body weight gain per day of the OVX rats were higher ($P<0.05$) than those of the SHAM rats (Table 1). In contrast, the weight gains in the OVX + PF and OVX + CR rats were lower by 40% and 59%, respectively, compared with the OVX group (Table 1). Uterine weights in the three OVX groups were 4–5 fold lower than that of the SHAM rats (Table 1), indicating the effectiveness of ovariectomy. OVX rats displayed a 69% higher visceral fat content than SHAM rats (Table 1). However, increased visceral fat accumulation in OVX rats was suppressed ($P<0.05$) in the OVX + PF and OVX + CR groups by 32% and 44%, respectively.

3.2. Food intake

OVX rats had a higher ($P<0.05$) average food intake than the other three groups in weeks 1–13 (Fig. 1B), and the average food intake in the OVX + CR rats was significantly lower than that of the other three groups during weeks 5–13 (Fig. 1B). The total energy intake was calculated by multiplying the energy in each gram of rat chow by the average daily food intake, which was calculated from at least 3 collections in one week during the 12-week experimental period.

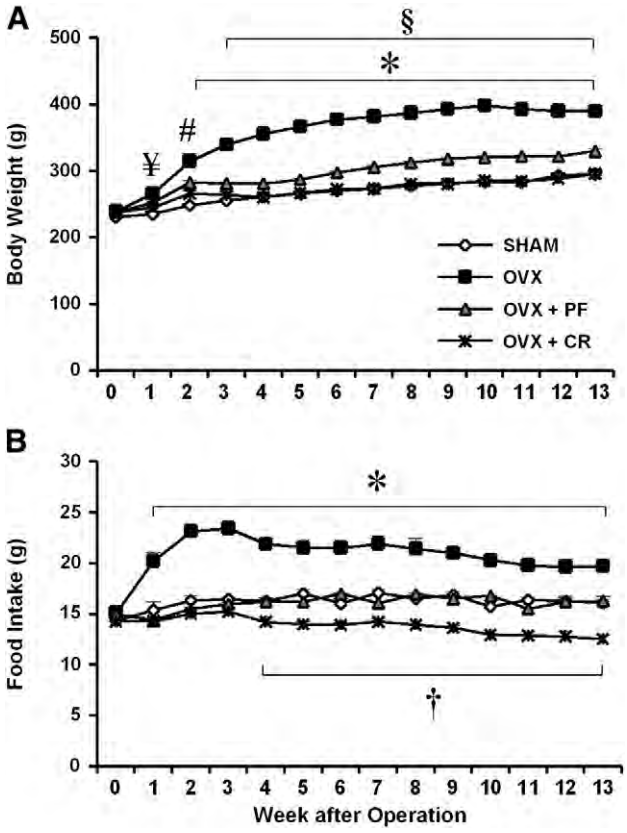

Fig. 1. Body weight (A) and food intake (B) of sham-operated control (SHAM) and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Measurements of body weight and food intake were performed at least 3 times per week during the experiment, and the weekly average values are presented. Values are means ± SE for 10 animals/group. ¥$P<0.05$ SHAM vs. OVX; #$P<0.05$ SHAM vs. OVX + PF; *$P<0.05$ OVX vs. all other groups; §$P<0.05$ OVX + PF vs. all other groups; †$P<0.05$ OVX + CR vs. all other groups.

Table 1
Initial and final body weight, total energy intake, uterine and visceral fat weight, fasting plasma insulin and glucose concentration in sham-operated (SHAM), ovariectomized (OVX), and OVX rats that undergone pair feeding (OVX + PF) and calorie restriction (OVX + CR).

	SHAM	OVX	OVX + PF	OVX + CR
Body weight (g)				
Initial weight	231.1 ± 2.8	239.6 ± 2.4	238.1 ± 4.6	238.1 ± 4.8
Final weight	295.3 ± 3.9	390.2 ± 8.4*	330.0 ± 2.4*†	296.0 ± 1.6†§
Body weight gain/day (g)	0.77 ± 0.06	1.64 ± 0.09*	1.00 ± 0.04*†	0.68 ± 0.05†§
Total energy intake (kcal)	4522 ± 72	5771 ± 142*	4369 ± 20†	3795 ± 50†§
Uterine weight (mg)	522.0 ± 43.9	111.6 ± 8.2*	134.0 ± 20.6*	111.4 ± 10.6*
Visceral fat weight (g)	15.7 ± 1.1	26.5 ± 3.5*	17.9 ± 1.7†	14.9 ± 1.2†
Insulin (μU/ml)	8.7 ± 0.6	10.3 ± 0.6	12.4 ± 0.9	11.2 ± 1.2
Glucose (mg/dl)	111.4 ± 2.8	122.9 ± 2.7	124.5 ± 1.9	122.4 ± 3.1

Values are means ± SE for 10 animals/group.
* $P<0.05$ vs. SHAM group.
† $P<0.05$ vs. OVX group.
§ $P<0.05$ vs. OVX + PF.

The total energy intake of the OVX rats was significantly higher than that of the SHAM rats and was reduced to 75% and 66% of the total intake of the OVX rats by pair feeding and calorie restriction, respectively (Table 1).

3.3. Serum lipid profile

The serum levels of total cholesterol (TC), HDL-cholesterol (HDL), LDL-cholesterol (LDL), and the HDL and LDL to TC ratios are

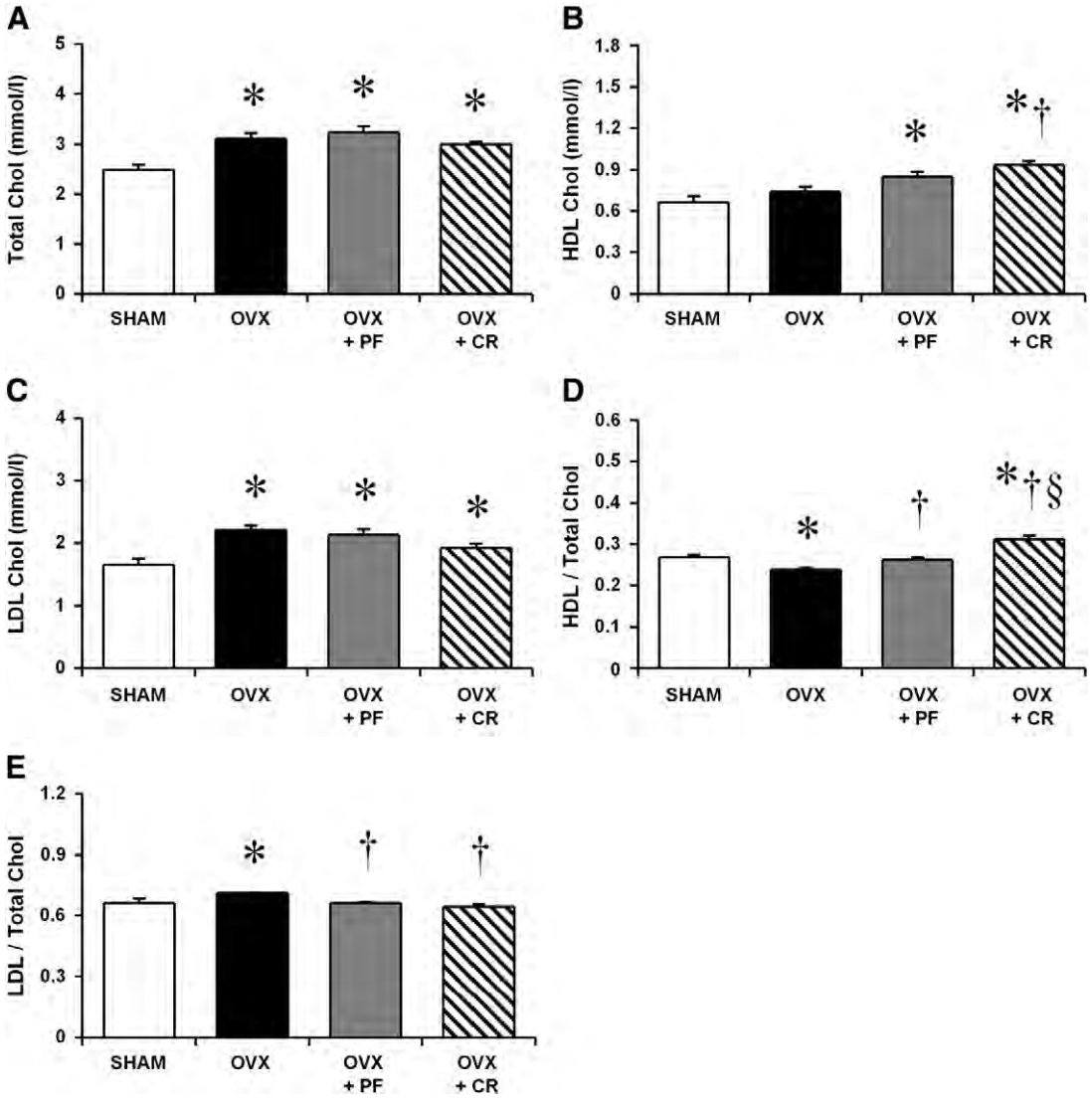

Fig. 2. Serum lipid levels of sham-operated control (SHAM) and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). (A) total; (B) high-density lipoprotein (HDL) cholesterol; (C) low-density lipoprotein (LDL) cholesterol; (D) ratio of HDL to total cholesterol (HDL/total); and (E) ratio of LDL to total cholesterol (LDL/total). Values are means ± SE for 10 animals/group. *$P<0.05$ vs. SHAM; †$P<0.05$ vs. OVX; §$P<0.05$ vs. OVX + PF.

demonstrated in Fig. 2. Compared with SHAM rats, ovariectomy resulted in significant increases in TC (26%) and LDL (34%) but not HDL (Fig. 2). Compared to OVX, pair feeding and calorie restriction did not affect TC or LDL but led to a significant reduction (8–10%) in the LDL/TC ratio. However, pair feeding increased HDL by 15% and the HDL/TC ratio by 10%, while the highest improvements ($P<0.05$) in HDL (26%) and the HDL/TC ratio (31%) were observed in the OVX rats with calorie restriction (Fig. 2).

3.4. Plasma free fatty acids (FFAs) and tissue triglycerides (TG)

Elevated FFAs and increased ectopic fat accumulation have been implicated in the etiology of the insulin-resistant state [19,20]. To determine the potential role of FFAs and ectopic fat in the insulin-resistant condition in OVX rats, we assessed the plasma level of FFAs and the TG content in the liver and the plantaris muscle. The muscular TG content was measured in the plantaris, due to limited availability of soleus muscle. Ovariectomy resulted in a 67% increase ($P<0.05$) in plasma FFAs (Fig. 3A) and a 59% increase ($P<0.05$) in the liver TG content (Fig. 3B). The elevation of FFAs in the OVX rats was reduced 32–37% by pair feeding and calorie restriction. On the other hand, the liver TG content was lowered ($P<0.05$) only by calorie restriction (Fig. 3B). The TG content in the plantaris was, however, not significantly different among the treatments (Fig. 3C).

3.5. Whole body insulin action

Data from the OGTT, including plasma levels of glucose and insulin, the area under the curve for glucose (glucose AUC) and insulin (insulin AUC) and the glucose-insulin (G-I) index, are presented in Fig. 4. The G-I index was calculated as the product of the respective glucose and insulin AUCs, and it is inversely related to whole-body insulin sensitivity [21]. Plasma glucose (Fig. 4A) and insulin concentrations (Fig. 4B) were not significantly different between the groups throughout the test, except the insulin level at the 15-min time point in the OVX rats was higher ($P<0.05$) than that in the other groups. There were no significant differences in glucose AUCs among all experimental groups (Fig. 4C). However, ovariectomy resulted in a significant increase in the insulin AUC compared with the SHAM group (Fig. 4D). Pair feeding failed to reduce the insulin AUC, whereas calorie restriction produced a significant reduction (27%). Similarly, the G-I index in the OVX group was 61% higher than that of the SHAM group (Fig. 4E), and calorie restriction significantly reduced the G-I index by 33% (Fig. 4E).

3.6. Insulin action on skeletal muscle glucose transport

Basal and insulin-stimulated glucose transport activities in isolated soleus are shown in Fig. 5. No significant differences in basal 2-DG uptake were observed among the experimental groups. Compared to the SHAM group, the insulin-stimulated 2-DG uptake and the insulin-induced 2-DG transport above basal levels in muscle from OVX rats were reduced ($P<0.05$) by 21% and 35%, respectively. These data clearly indicate the insulin-resistant state of the skeletal muscle of the OVX rats. Pair feeding in OVX animals did not produce a significant change in the insulin-stimulated 2-DG transport rate. In contrast, calorie restriction enhanced the insulin-mediated 2-DG uptake in OVX rats by 86% (Fig. 5B).

3.7. Expression and functionality of signaling elements in skeletal muscle

To assess the adaptive changes in GLUT-4 protein and signaling elements in response to prolonged estrogen deprivation without and with dietary manipulation, protein expression and phosphorylation levels were determined in non-incubated soleus. The protein

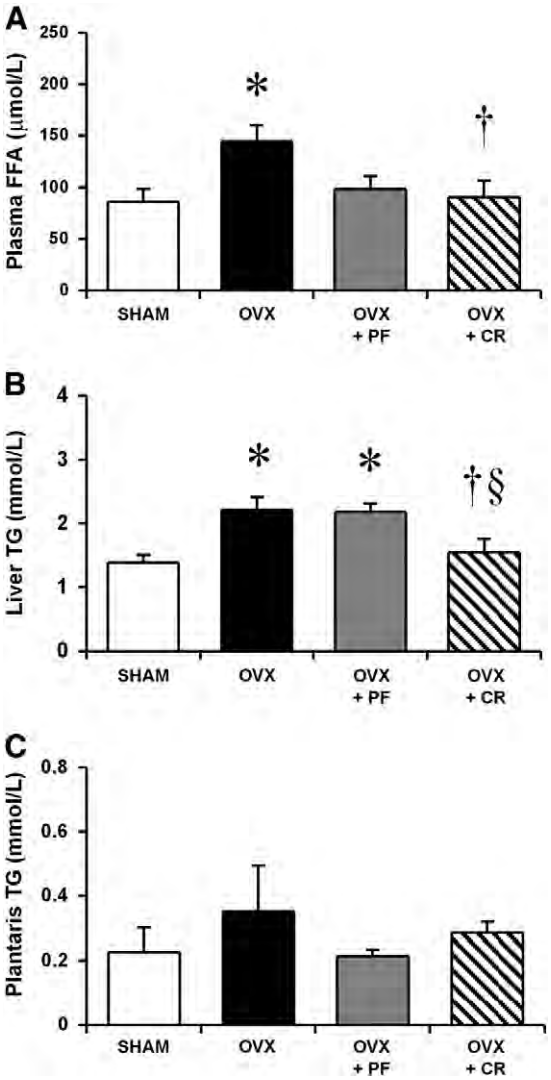

Fig. 3. Effects of sham operation (SHAM) and ovariectomy (OVX) without or with pair feeding (PF) or moderate calorie restriction (CR) on (A) plasma free fatty acid (FFA) levels, (B) liver triglyceride (TG) content, and (C) TG content in plantaris muscle. Values are means ± SE for 8 animals/group. *$P<0.05$ vs. SHAM; †$P<0.05$ vs. OVX; §$P<0.05$ vs. OVX + PF.

expression levels of IR-β, IRS-1, the p85 subunit of PI3-kinase, Akt, SAPK/JNK, and p38 MAPK as well as the phosphorylation levels of IR-β ($Tyr^{1158}/Tyr^{1162}/Tyr^{1163}$), IRS-1 ($Ser^{307}$), Akt ($Ser^{473}$), SAPK/JNK ($Thr^{183}/Tyr^{185}$), and p38 MAPK ($Thr^{180}/Tyr^{182}$) were not different among groups (Fig. 6). However, a 12-week period of ovariectomy resulted in a reduction ($P<0.05$) in the total GLUT-4 protein level in the soleus by 39% (Fig. 6). There was no significant effect of the pair feeding paradigm on total GLUT-4 protein content, whereas the reduced GLUT-4 protein level in OVX animals was partially attenuated by calorie restriction ($P=0.062$ between SHAM vs. OVX + CR).

3.8. Insulin-stimulated activity of insulin signaling and MAPK in skeletal muscle

The ability of insulin to activate signaling elements in the PI3-K and MAPK pathways in skeletal muscle was examined in the incubated muscle. There were no differences in the protein expression of IR-β, IRS-1, Akt (Fig. 7), SAPK/JNK 1, SAPK/JNK 2/3, and p38 MAPK

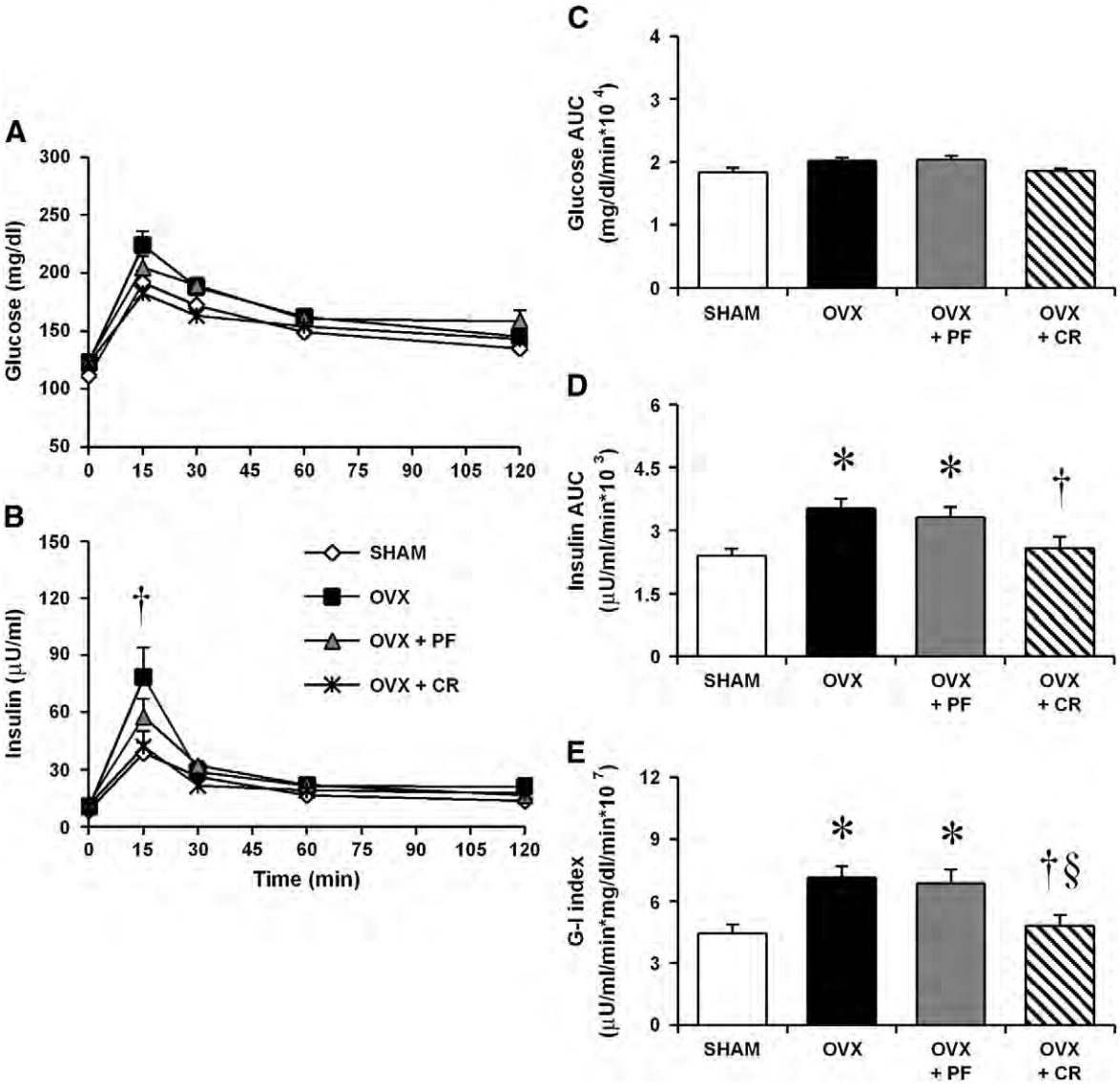

Fig. 4. Glucose (A) and insulin (B) responses during an oral glucose tolerance test and the area under the curve (AUC) for glucose (C) and insulin (D) as well as the glucose-insulin index (G–I index) (E) in sham-operated (SHAM) and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Data for the AUC were calculated from glucose (A) and insulin (B) responses. The G–I index was the product of glucose AUC and insulin AUC for each individual animal. Values are means ± SE for 10 animals/group. *$P<0.05$ vs. SHAM; †$P<0.05$ vs. OVX; §$P<0.05$ vs. OVX + PF.

(Fig. 8) in the soleus muscle among the groups. However, insulin stimulation of tyrosine phosphorylation of IR-β and IRS-1, of IRS-1 associated with the p85 subunit of PI3-kinase, and of serine phosphorylation of Akt in the soleus muscle of OVX rats was significantly reduced when compared with the SHAM group (Fig. 7). Interestingly, ovariectomy led to the enhanced activation ($P<0.05$) of insulin-stimulated serine^{307} phosphorylation of IRS-1 (Fig. 7), Thr^{183}/Tyr^{185} phosphorylation of SAPK/JNK, and Thr^{180}/Tyr^{182} phosphorylation of

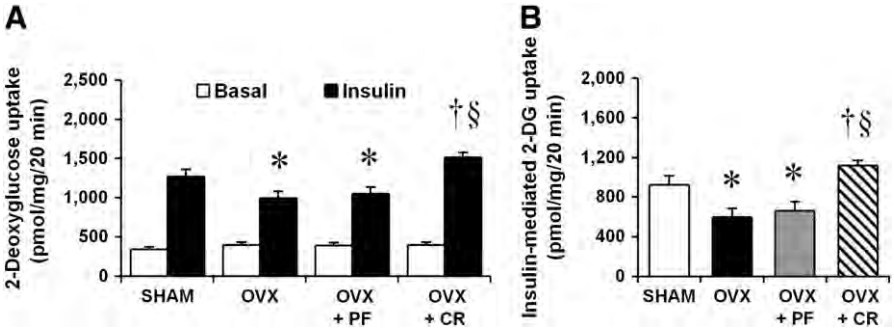

Fig. 5. *In vitro* rates of 2-deoxyglucose (2-DG) uptake in the absence (blank bars) and presence (filled bars) of insulin (2 mU/ml) (A) and net increases above basal rates of 2-deoxyglucose uptake due to insulin (B) in soleus muscle of sham-operated (SHAM) and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Values are means ± SE for 10 animals/group. *$P<0.05$ vs. SHAM; †$P<0.05$ vs. OVX; §$P<0.05$ vs. OVX + PF.

p38 MAPK in skeletal muscle (Fig. 8). Pair feeding and calorie restriction blunted the decreases in phosphorylated IR-β and IRS-1/pY, the IRS-1 associated with the p85 subunit of PI3-kinase and the serine phosphorylation of Akt (Fig. 7). Such dietary manipulation also abolished the increases in the level of phosphorylation of IRS-1 serine^{307} (Fig. 7), SAPK/JNK 1, SAPK/JNK 2/3, and p38 MAPK (Fig. 8), thereby suggesting significant improvement of the defective insulin signaling induced by estrogen deprivation.

4. Discussion

The present study provides evidence that the insulin-resistant condition observed in OVX rats is primarily a consequence of loss of ovarian function and is independent of the increased food intake that accompanies the condition. We have also presented novel information that the skeletal muscle of OVX rats is characterized by defects in insulin signaling, including reduced insulin action on the tyrosine phosphorylation of IR and IRS-1, the IRS-1 associated p85 subunit of PI3-kinase and the serine phosphorylation of Akt, with concomitant increases being observed in insulin-stimulated SAPK/JNK and p38 MAPK activity. In addition, the present study is the first to show that the development of insulin resistance and the defective signaling elements in skeletal muscle observed in OVX rats can be prevented by moderate degree of calorie restriction (~35% less calorie intake than the OVX group).

Excessive calorie intake is one of the causal factors leading to the insulin-resistant state and diabetes [22]. With ovarian hormone depletion, OVX rat becomes hyperphagic and gains weight [6–8,23]. Consequently, the metabolic defects occurring after ovariectomy could be confounded by ovariectomy-induced hyperphagia. In this study, we assessed the metabolic alterations in OVX rats on a pair feeding (PF) diet. The amount of total energy intake in the OVX + PF group is equivalent to a 25% reduction in the total energy intake by OVX rats. The PF dietary regime reduced visceral fat content and improved the lipid profile, as indicated by higher HDL/TC and lower LDL/TC ratios. Nevertheless, the OVX + PF rats exhibited a positive energy state because their body weight gain was significantly higher than that of the SHAM rats. In addition, the increased lipid content in the liver, increased plasma free fatty acid, impaired glucose tolerance and defective insulin action on skeletal muscle glucose transport in OVX rats were not attenuated by pair feeding. Based on these findings, it may be concluded that the insulin-resistant condition observed in OVX rats is primarily a consequence of ovarian hormone deprivation.

A critical role of estrogens in glucose homeostasis has been supported by clinical and experimental studies. For example, postmenopausal therapy with estrogen alone reduces the incidence of insulin resistance and type 2 diabetes risks [24–26]. Moreover, insulin resistance develops when there is no estrogen in aromatase-knockout mice [27] or when estrogen receptor alpha (ERα) is deficient [28,29]. The impaired glucose tolerance and reduced insulin sensitivity in ERα knockout mice was attributed to hepatic insulin resistance [28], impaired insulin action [29] and GLUT-4 expression [30] in skeletal muscle. However, insulin action on glucose transport in rat skeletal muscle was not affected by acute incubations (10 min) with estradiol [31] but was increased in skeletal muscle from normal rats after a 3-day treatment with an ERα agonist [32]. In this study, we demonstrated that insulin-stimulated glucose transport activity was reduced in the skeletal muscle of OVX rats with a corresponding reduction in the level of total GLUT-4 protein expression. As normal activation of the PI3-kinase pathway is essential for the recruitment of GLUT-4 to facilitate glucose transport, our findings regarding the defective insulin stimulation of the tyrosine phosphorylation of IR-β and IRS-1, IRS-1 associated with p85 of PI3-kinase and the serine phosphorylation of Akt probably explain the insulin resistance of glucose transport activity observed in the skeletal muscle of OVX rats. These results are consistent with the notion that a defect in the proximal step of the insulin signaling pathway results in reduced functionality of the downstream signaling elements. Therefore, the defective signaling response to insulin stimulation in the muscle of OVX rats where GLUT-4 expression is already impaired would aggravate insulin resistance.

In addition to its metabolic functions through the PI3-kinase pathway, insulin exerts mitogenic effects via the mitogen-activated protein kinase (MAPK) pathway [14,15]. Several lines of evidence indicate that MAPKs such as SAPK/JNK and p38 MAPK can negatively modulate the PI3-kinase pathway [16,17,19,33]. These kinases mainly phosphorylate serine residues on IRS-1, resulting in reduced activities of the downstream insulin signaling factors [16,17,19,33]. The differential responses of the insulin signaling elements and these MAPKs have been previously reported [33,34]. For example, increased phosphorylation of SAPK/JNK with impaired activation of IRS-1 and Akt in skeletal muscle has been revealed in individuals with insulin resistance [34]. Moreover, de Alvaro et al. [33] found that decreased insulin stimulation of the PI3-kinase pathway in skeletal muscle cells induced by TNF-α did occur in a p38 MAPK-dependent manner. The present study demonstrated that the insulin resistance of the PI3-kinase pathway in OVX rats was accompanied by increased IRS-1 serine phosphorylation with a corresponding increase in SAPK/JNK and p38 MAPK activation. This observation seems to support the concept that intact stimulation of the MAPK pathway by insulin plays an important role in the development of insulin resistance in the skeletal muscle of OVX rats. Nevertheless, our observation that impaired glucose tolerance and skeletal muscle insulin resistance do exist in OVX + PF rats without an increase in insulin-activated SAPK/JNK and p38 MAPK is more consistent with the interpretation that intact stimulation of the MAPK pathway by insulin is not required for the insulin-resistant condition in OVX rats.

It is well documented that insulin resistance is attributed to numerous factors such as increased visceral fat accumulation, plasma free fatty acids, and fat deposition in non-adipose tissues [35]. For instance, it has been reported that increased long-chain fatty acyl-CoAs induce defects in IR-β and IRS-1 functionality [36,37] by activating serine phosphorylation of these signaling elements [37,38]. Indeed, we did observe a significant increase in plasma free fatty acids, visceral fat content, and triglyceride content in the liver but not the plantaris muscle of OVX rats. Accordingly, these metabolic changes may account, in part, for the insulin-resistant state observed in OVX rats. In this study, we found that pair feeding reduced visceral fat content and tended to decrease the level of plasma free fatty acid in OVX rats without improving whole body or skeletal muscle glucose metabolism. Thus, it appears that visceral fat accumulation and plasma free fatty acids may not be the primary event leading to the development of the insulin-resistant condition in OVX rats.

Hepatic triglyceride content is directly correlated with the severity of insulin resistance in both the liver and skeletal muscle [39–41], independent of intra-abdominal fat and overall obesity [41]. Increased lipid content has been reported in the liver of postmenopausal women [42,43] and OVX rodents [20,44–47] and is associated with an upregulation of the expression of lipogenic markers [44,48] and a decrease in fatty acid oxidation [20,44]. Estrogen replacement can prevent hepatic lipid accumulation [20,44,45] and normalize the altered lipogenesis [44,48] and lipid oxidation [44,45] in the liver of OVX rodents. Our findings that the enhanced hepatic triglyceride content in OVX rats is not attenuated by pair feeding support the idea that this factor may be important in the development of insulin resistance in OVX rats. This notion is supported by the evidence that hepatic steatosis is an early event in the development of ovariectomy-induced insulin resistance and obesity in mice [49].

Although PF did not significantly increase insulin sensitivity in OVX rats, a further reduction in total energy intake (~35% less than the OVX group) effectively prevented the progression of metabolic defects, including the insulin-resistant condition at whole body and skeletal muscle levels of OVX rats. We found that these metabolic

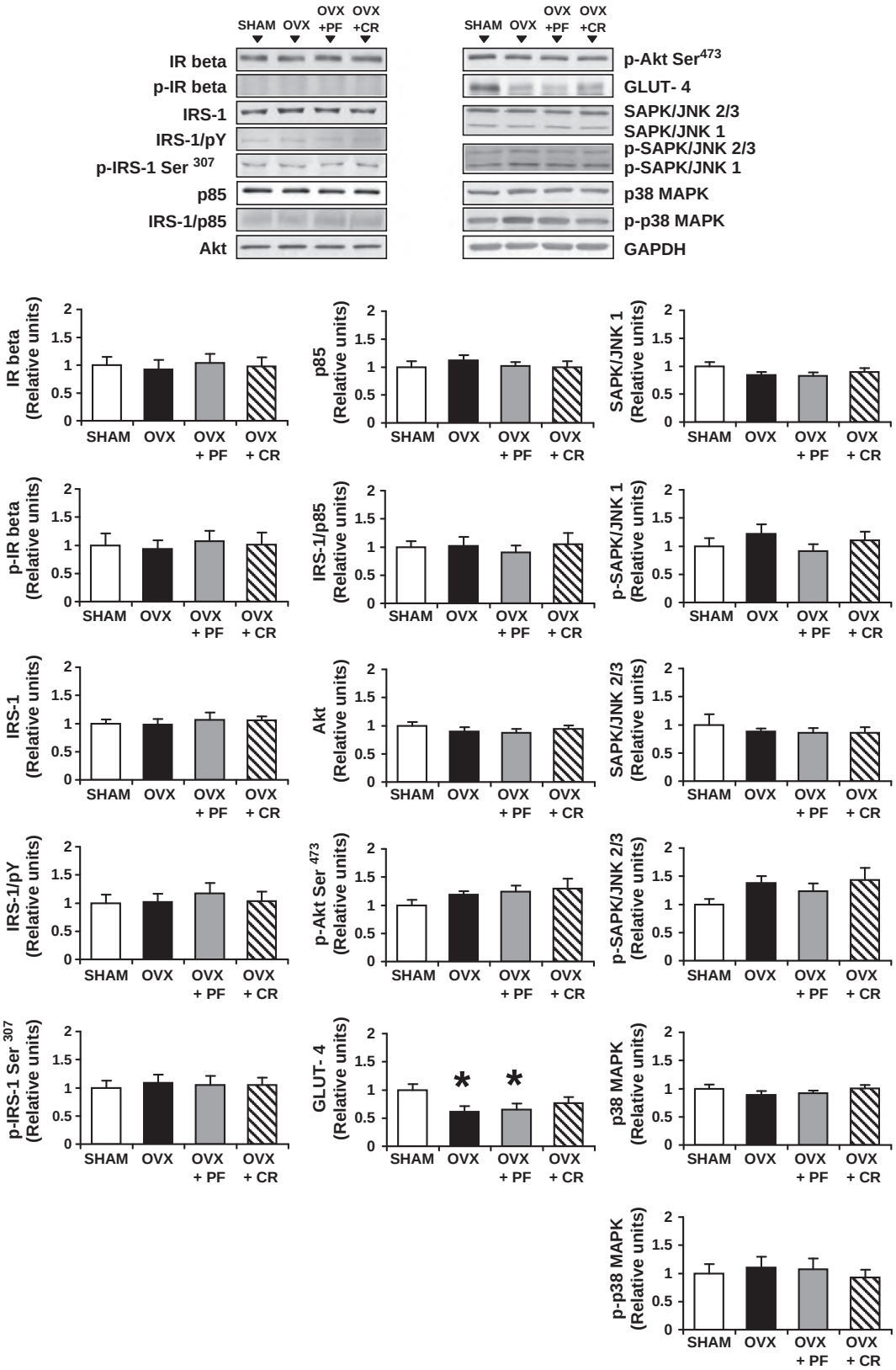

Fig. 6. Effects of pair feeding (PF) or moderate calorie restriction (CR) on the expressions of signaling proteins IR-β (IR beta), IR-β ($Tyr^{1158}/Tyr^{1162}/Tyr^{1163}$) phosphorylation (p-IR beta), IRS-1, tyrosine phosphorylation of IRS-1 (IRS-1/pY), IRS-1 (Ser^{307}) phosphorylation (p-IRS-1 Ser^{307}), the p85 subunit of PI3-K (p85), IRS-1 associated with the p85 subunit of PI3-K (IRS-1/p85), Akt, Akt (Ser^{473}) phosphorylation (p-Akt Ser^{473}), GLUT-4, SAPK/JNK 1, SAPK/JNK 1 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 1), SAPK/JNK 2/3, SAPK/JNK 2/3 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 2/3), p38 MAPK, and p38 MAPK (Thr^{180}/Tyr^{182}) phosphorylation (p-p38 MAPK) in non-incubated soleus muscles from ovariectomized (OVX) rats. For IRS-1/pY and IRS-1/p85, muscle lysate was immunoprecipitated with agarose-conjugated anti-IRS-1 antibody prior to immunoblotting against tyrosine and p85, respectively. IRS-1/pY and IRS-1/p85 were normalized to IRS-1 protein expression. The rest of the signaling proteins were determined by immunoblot analysis and were normalized to GAPDH. Data are presented as fold change over the sham-operated (SHAM) group. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 8–10 animals/group. $^{*}P<0.05$ vs. SHAM.

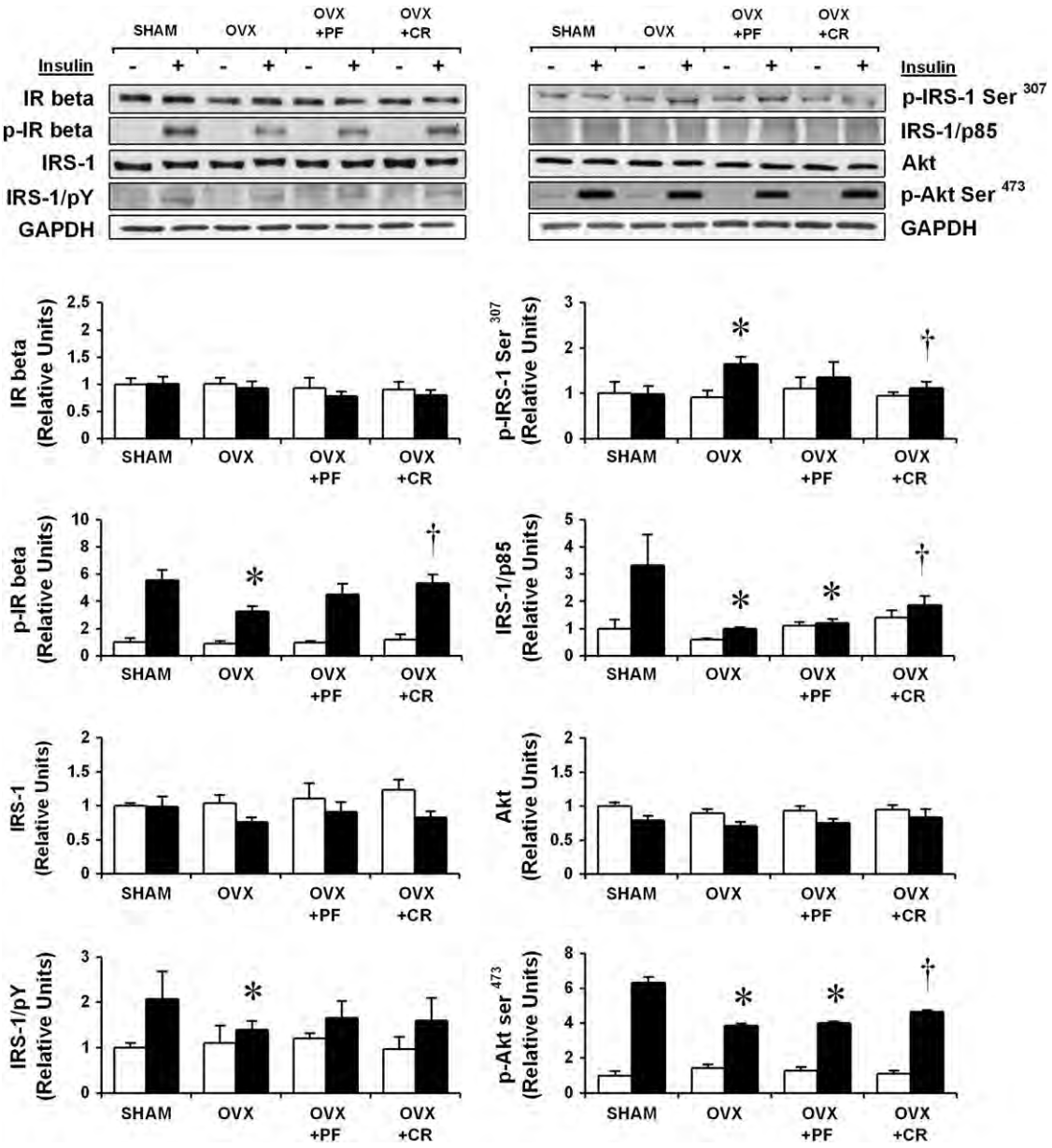

Fig. 7. Insulin-induced expression of IR-β (IR beta), IR-β ($Tyr^{1158}/Tyr^{1162}/Tyr^{1163}$) phosphorylation (p-IR beta), IRS-1, tyrosine phosphorylation of IRS-1 (IRS-1/pY), IRS-1 (Ser^{307}) phosphorylation (p-IRS-1 Ser^{307}), IRS-1 associated with the p85 subunit of PI3-K (IRS-1/p85), Akt, and Akt (Ser^{473}) phosphorylation (p-Akt Ser^{473}) in soleus muscles from SHAM and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Muscles were incubated in the absence (blank bars) or the presence (filled bars) of insulin (2 mU/ml). For IRS-1/pY and IRS-1/p85, muscle lysate was immunoprecipitated with agarose-conjugated anti-IRS-1 antibody prior to immunoblotting against tyrosine and p85, respectively. IRS-1/pY and IRS-1/p85 were normalized to IRS-1 protein expression. The rest of the signaling proteins were determined by immunoblot analysis and were normalized to GAPDH. Data are presented as fold change over the SHAM group. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 8 animals/group. *$P<0.05$ vs. SHAM; †$P<0.05$ vs. OVX.

adaptations in the OVX + CR rats were accompanied by significant diminution of visceral fat accumulation, the plasma level of free fatty acid, and the triglyceride content in the liver. One of the important findings in this study is the favorable effect of CR on insulin-stimulated glucose transport and the signaling elements in skeletal muscle of OVX rats. Without a significant improvement in the expression of the GLUT-4 protein, calorie restriction fully recuperated the impaired insulin action on skeletal muscle glucose transport activity in OVX rats. Intriguingly, the improved insulin action on the functionality of the insulin signaling elements and glucose transport in skeletal muscle was associated with a significant reduction in insulin-stimulated IRS-1 serine phosphorylation. Because the effect of CR on the insulin signaling pathway includes the proximal step such as insulin-stimulated IR-β and IRS-1, we speculate that the favorable effect of CR on skeletal muscle insulin action is associated with the suppression of hepatic fat accumulation and the plasma free fatty acid level. Therefore, the systemic improvements as a result of CR could be substantial for the beneficial adaptation from the proximal step of the PI3-kinase pathway and the enhanced activity of the glucose transport system in skeletal muscle.

There are some limitations to this study. The soleus muscle (mainly slow twitch fibers) was used in this study to demonstrate the effects of OVX and CR on insulin action and related signaling elements. However, it remains to be verified whether the same responses will be observed in muscles with different fiber type composition such as the fast twitch

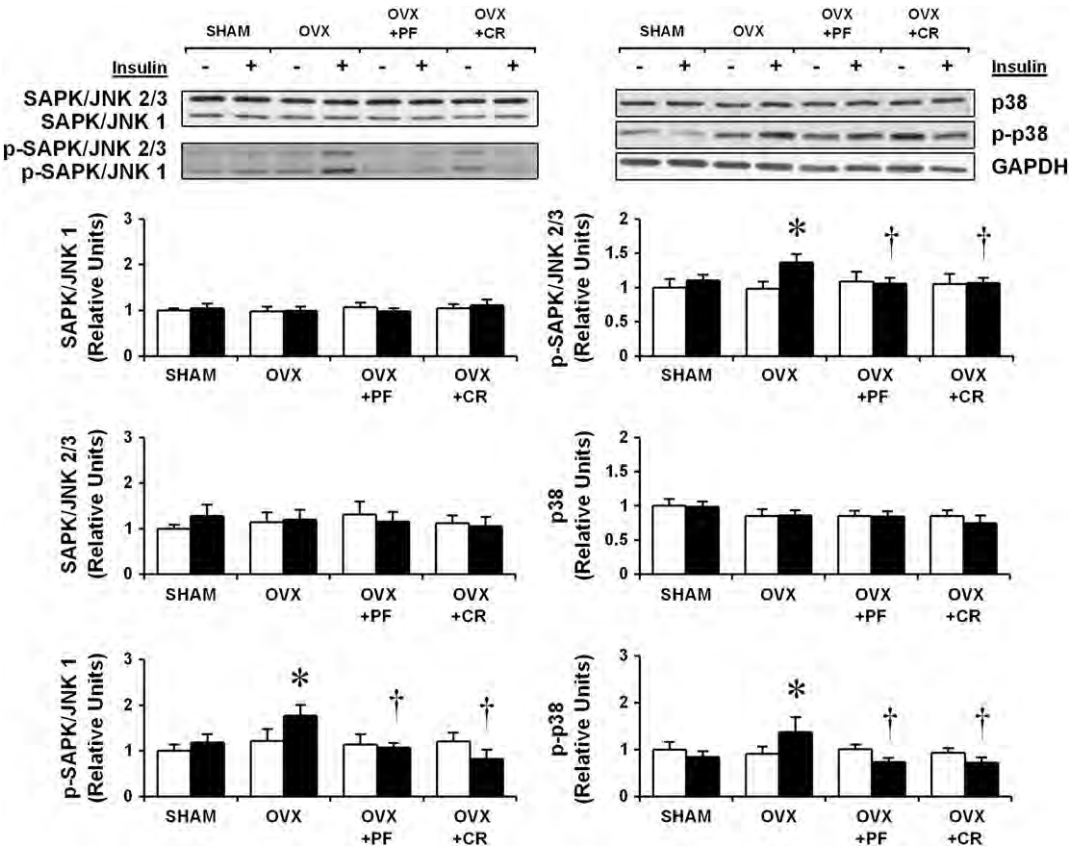

Fig. 8. Insulin-induced expression of SAPK/JNK 1, SAPK/JNK 2/3, SAPK/JNK 1 and SAPK/JNK 2/3 (Thr^{183}/Tyr^{185}) phosphorylation (p-SAPK/JNK 1), p38 MAPK, and p38 MAPK (Thr^{180}/Tyr^{182}) phosphorylation (p-p38 MAPK) in soleus muscles from SHAM and ovariectomized (OVX) rats without or with pair feeding (PF) or moderate calorie restriction (CR). Muscles were incubated in the absence (blank bars) or the presence (filled bars) of insulin (2 mU/ml). Data are presented as fold change over the SHAM group. Results were normalized to GAPDH. Representative bands from the autoradiograph are displayed at the top of the figure. Values are means ± SE for 8 animals/group. *$P<0.05$ vs. SHAM; †$P<0.05$ vs. OVX.

or the mixed fibers. Also, as we use a maximally effective concentration of insulin to assess insulin action on skeletal muscle glucose transport, the determination of insulin sensitivity with lower doses of insulin in the physiological range is important and should be addressed in future investigations.

5. Conclusions

In summary, the present investigation demonstrates for the first time that the resistance to insulin-stimulated skeletal muscle glucose transport activity in OVX rats is characterized by the impaired insulin stimulation of IR-β, IRS-1/pY, IRS-1/p85 of PI3-kinase, and Akt with concomitant enhanced insulin-stimulated SAPK/JNK and p38 MAPK activity. Pair feeding improved serum lipid profiles in OVX rats without a significant improvement of insulin action at the whole-body or skeletal muscle level, suggesting a causal role of ovarian hormone deprivation in the progression of the insulin-resistant condition. A further reduction in calorie intake, however, prevents the development of skeletal muscle insulin resistance and the defective signaling in the skeletal muscle of rats undergone prolonged ovariectomy.

Conflict of interest

The authors declare that they have no conflict of interest.

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