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Estrogen and Exercise May Enhance β-Cell Function and Mass via Insulin Receptor Substrate 2 Induction in Ovariectomized Diabetic Rats

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The prevalence and progression of type 2 diabetes have increased remarkably in postmenopausal women. Although estrogen replacement and exercise have been studied for their effect in modulating insulin sensitivity in the case of insufficient estrogen states, their effects on β-cell function and mass have not been studied. Ovariectomized (OVX) female rats with 90% pancreatectomy were given a 30% fat diet for 8 wk with a corresponding administration of 17β-estradiol (30 μg/kg body weight) and/or regular exercise. Amelioration of insulin resistance by estrogen replacement or exercise was closely related to body weight reduction. Insulin secretion in first and second phases was lower in OVX during hyperglycemic clamp, which was improved by estrogen replacement and exercise but not by weight reduction induced by restricted diets. Both estrogen replacement and exercise overcame reduced pancreatic β-cell mass in OVX rats via increased proliferation and decreased apoptosis of β-cells, but they did not exhibit an additive effect. However, restricted diets did not stimulate β-cell proliferation. Increased β-cell proliferation was associated with the induction of insulin receptor substrate-2 and pancreatic homeodomain protein-1 via the activation of the cAMP response element binding protein. Estrogen replacement and exercise shared a common pathway, which led to the improvement of β-cell function and mass, via cAMP response element binding protein activation, explaining the lack of an additive effect with combined treatments. In conclusion, decreased β-cell mass leading to impaired insulin secretion triggers glucose dysregulation in estrogen insufficiency, regardless of body fat. Regular moderate exercise eliminates the risk factors of contracting diabetes in the postmenopausal state. (*Endocrinology* 146: 4786–4794, 2005)

INSULIN RESISTANCE IS a characteristic feature of type 2 diabetes mellitus (DM). The insulin-resistant state persists when islet hyperplasia and hyperinsulinemia compensate to maintain normoglycemia (1–3), but DM develops upon failure of the β-cells to secrete sufficient insulin to satisfy the rising insulin demand (2, 3). The reason for the eventual β-cell failure is likely decreased pancreatic β-cell mass. Indeed, postmortem study indicates a reduced β-cell mass in patients with DM (4).

The development of insulin resistance and DM is linked to both genetic and environmental factors, such as aging, obesity, diet, and exercise (5–8). Even though aging is an important factor for insulin resistance in both men and women (8, 9), the prevalence and progression of DM increases markedly in postmenopausal women. This phenomenon is closely related to estrogen insufficiency during which increased body weight (bw) and fat mass exacerbate insulin resistance. Exercise, estrogen replacement, or body fat loss appears to have potentially beneficial effects for the improvement of glucose tolerance or pancreatic β-cell function in the postmenopausal state (6, 9, 10). However, the underlying mechanisms and effects on pancreatic β-cell mass remain largely unknown.

Activation of cAMP response element binding protein (CREB) increases insulin receptor substrate (IRS) 2 expression in the Min6 insulinoma cell line and in mouse islets (11–13), leading to potentiation of the IGF-I/insulin signaling cascade and subsequently enhanced β-cell function and mass (13). Estrogen has been shown to activate CREB in neurons (14, 15), whereas exercise enhances the expression and phosphorylation of CREB in rat hippocampus (16). Moreover, recent studies have shown that estrogen induces the expression of IRS2 in breast carcinoma cells (5, 17, 18). Thus, it is conceivable that estrogen replacement and/or exercise decreases the progression of DM in estrogen-deficient states by modulating pancreatic β-cell function and mass via induction of IRS2 expression. To test this hypothesis, we challenged 90% pancreatectomized (Px) and ovariectomized (OVX) rats with estrogen, exercise, or restricted diets to investigate the effects on insulin resistance and pancreatic β-cell function and mass. Furthermore, the molecular mechanism, which modulates β-cell function and mass via estrogen replacement, exercise, and weight loss, was determined. We observed that exercise, regardless of estrogen replacement, was beneficial for preventing DM progression by im-

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Abbreviations: AGE, Advanced glycated end product; BrdU, 5-bromo-2-deoxyuridine; bw, body weight; CREB, cAMP response element binding protein; DM, type 2 diabetes mellitus; GLUT, glucose transporter; HGO, hepatic glucose output; IRS, insulin receptor substrate; OVX, ovariectomy; OVX-ER, OVX rats administered with estrogen replacement; OVX-ER-EX, OVX rats administered with estrogen replacement and exercise; OVX-EX, OVX rats that did regular and moderate exercise; OVX-P, OVX rats pair-fed to Sham rats; OVX-R, OVX rats fed restricted diets to maintain bw at the level of Sham rats; PDX, pancreatic homeodomain protein; Px, pancreatectomy; Sham, Sham-operated rat; Sham-EX, Sham rats that did exercise.

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proving not only insulin sensitivity but also β-cell function and mass, associated with enhanced insulin/IGF-I signaling cascade in islets via IRS2 induction. Weight reduction improved insulin sensitivity but did not prevent DM development and progression in estrogen insufficient states due to a lack of effect on pancreatic β-cell mass.

Materials and Methods

Experimental animals

All female Sprague Dawley rats weighing 277 ± 22 g had 90% of their pancreas removed using the Hosokawa technique (19). After a 90% Px, the pancreas that remained was only within 2 mm of the common bile duct and extended from the duct to the first part of the duodenum. Px rats with random-fed serum glucose levels less than 9.4 mM were excluded for the experiment after 2 wk of surgery. The Px rats included in the experiment showed characteristics of mild DM. All experimental animals were housed individually in stainless steel cages in a controlled environment (23 C and a 12-h light, 12-h dark cycle). All surgical and experimental procedures were performed according to the guidelines of the Animal Care and Use Review Committee (Hoseo University, Korea). Serum glucose levels, food intake, and bw were measured weekly every Tuesday at 1000 h.

Experiment 1

Px rats were randomly divided into six groups. Four groups were assigned into OVX, whereas two groups were formed for the sham operation (Sham). The OVX rats were assigned into the following four groups: 1) estrogen replacement + exercise (OVX-ER-EX), 2) estrogen replacement (OVX-ER), 3) exercise (OVX-EX), and 4) placebo (saline; OVX). The Sham rats were assigned into the following two groups: 1) exercise (Sham-EX) and 2) placebo (Sham). All rats freely consumed a 30% fat diet for 8 wk. Rats in the estrogen replacement groups were ip administered daily with 30 μg 17β-estradiol/kg bw. Rats in the exercise groups ran on an uphill treadmill at 20 m/min for 30 min four times a week.

Experiment 2

Px rats were divided into four groups: 1) OVX rats were pair-fed with Sham rats (OVX-P), 2) OVX rats consumed restricted diets to match bw to Sham rats (OVX-R), 3) OVX rats fed *ad libitum* (OVX), and 4) Sham rats. The rats consumed 30 En% fat diets in the designated experimental design. All assays were performed identically in experiments 1 and 2.

Insulin secretion and insulin resistance

After 7 wk of treatment, catheters were surgically implanted into the right carotid artery and left jugular vein of rats anesthetized with ip injections of ketamine and xylazine (100 and 10 mg/kg bw, respectively). After 5–6 d of implantation, a hyperglycemic clamp was performed in conscious and fasted rats to measure insulin secretion capacity (20). Bolus glucose (375 mg glucose/kg bw) was infused through the cannula for the first 5 min of the clamp, and 25% glucose was administered through the cannula to maintain the blood glucose levels at 6 mM above the fasting level. Blood was collected from the carotid artery at 0, 2, 5, 10, 60, 90, and 120 min, and glucose and insulin levels were also subsequently measured.

Two days after use of the hyperglycemic clamp, a euglycemic hyperinsulinemic clamp (21) was used under the same conditions as the hyperglycemic clamp to determine insulin resistance. Insulin-stimulated whole-body glucose flux was estimated using a prime continuous infusion of [3-^{3}H]glucose (10 μCi bolus, 0.1 μCi/min; NEN Life Science Products Life Science, Boston, MA) throughout the clamps. Regular human insulin (Humulin, Eli Lilly and Co., Indianapolis, IN) was continuously infused at a rate of 20 pmol/kg/min to raise plasma insulin concentration to approximately 1100 pM. Blood samples were collected from arteries at 10-min intervals for glucose estimation, and 25% glucose was infused at variable rates as needed to clamp glucose levels at approximately 6 mM. For the determination of plasma [3-^{3}H]glucose concentrations, plasma was deproteinized with $ZnSO_4$ and $Ba(OH)_2$, dried to remove ^3H_2O, resuspended in water, and disintegrations per minute of ^{3}H were recorded. The plasma concentration of ^3H_2O was determined by the difference between ^{3}H counts without and with drying. Rates of whole-body glucose uptake and basal glucose turnover were determined as the ratio of the [^{3}H] glucose infusion rate to the specific activity of plasma glucose (disintegrations per minute per micromole) during the final 30 min of the respective experiments. Hepatic glucose production during clamps was determined by subtracting the glucose infusion rate from the whole-body glucose uptake. Serum glucose levels were analyzed with Glucose Analyzer II (Beckman, Palo Alto, CA). Serum insulin, leptin, and 17β-estradiol levels were measured by RIA (Linco Research, St. Charles, MO). The advanced glycated end products (AGEs) of sc tissue were measured using fluorescence methods as previously described (22).

The rats were anesthetized with sodium pentobarbital (35 mg/kg bw) (Nembutal, Abbott Laboratories, North Chicago, IL) and were killed by decapitation at the end of the clamp. Tissues were rapidly dissected, weighed, and frozen in liquid nitrogen. The tissue was stored at −70 C until further analysis could be performed.

Islet isolation

Pancreatic islets from nine to 11 rats of each group were isolated by collagenase digestion (11). Through the pancreatic duct, 3 ml of 1.0 mg/ml collagenase (Sigma, St. Louis, MO) in DMEM-high glucose was injected into the pancreas of the rats anesthetized with sodium pentobarbital. The pancreas was immediately removed and incubated at 37 C for 15 min. The digested pancreas was washed with DMEM-high glucose four times on ice, and islets were isolated with a separation medium consisting of Ficoll reagent (Sigma). The islets washed with cold DMEM-high glucose were pooled for two to three rats from each group.

Immunohistochemistry and islet morphometry

Five to six rats from each group were treated with 5-bromo-2-deoxyuridine (BrdU; Roche Molecular Biochemicals, Indianapolis, IN; 100 μg/kg bw) at the end of 8-wk experimental periods. Six hours after injection, the pancreas samples were prepared and analyzed as previously described (11). The pancreas was dissected, fixed in a 4% paraformaldehyde solution (pH 7.2) overnight at room temperature, and embedded in paraffin blocks. Serial 5-μm paraffin-embedded tissue sections were mounted on slides. After rehydration, sections were immunostained as previously described (11) to determine β-cell area, BrdU incorporation, and apoptosis.

β-cell proliferation was examined by the incorporation of BrdU in β-cells from the rats injected with BrdU. The incorporation was determined by performing a double-label immunohistochemistry with anti-insulin (Zymed Laboratories, South San Francisco, CA) and BrdU antibodies (Roche Molecular Biochemicals) on rehydrated paraffin-embedded sections. Apoptosis of β-cells was measured by terminal deoxynucleotidyl transferase dUTP nick-end labeling kit (Roche, Mannheim, Germany) in paraffin sections of pancreas and counterstained with hematoxylin and eosin to visualize islets.

β-Cell areas were measured by acquiring images from two sets of eight to 10 distal, random, nonoverlapping images at ×20 of insulin-stained pancreatic sections. Results of β-cell quantification are expressed as the percentage of the total surveyed pancreas area containing insulin-positive cells. β-Cell proliferation was expressed in the number of BrdU-positive β-cells per millimeter squared of pancreas and was calculated as the BrdU-positive β-cells in total β-cell nuclei per pancreas section, two sections per animal, and five to six animals per group. Apoptosis of β-cells was determined by counting the total apoptotic bodies in β-cell nuclei, and the calculation was the same as that of β-cell proliferation.

Immunoblot analysis

Pooled islets from each group were lysed in a lysis buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 137 mM NaCl, 1% NP40, 10% glycerol, 12 mM β-glycerol phosphate, and supplemented protease inhibitors (11). Insoluble materials were removed by centrifugation. After measuring the protein concentration of the supernatants by BCA protein assay kit (Pierce Chemical, Rockford, IL) based on the Lowry method (23), they

were used for immunoblotting analysis. Lysate samples with equivalent protein levels (600 μg for immunoprecipitation and 30 μg for immunoblotting) were immunoprecipitated with anti-IRS2 antibodies before or were directly resolved by SDS-PAGE. Immunoblotting with specific antibodies against IRS2, pancreatic homeodomain protein (PDX)-1, glucokinase, and glucose transporter (GLUT) 2 was used as previously described (11, 24). Protein expression was measured by quantifying the intensity of each band using Imagequant TL (Amersham Biosciences, Piscataway, NJ), and an equal amount of total protein was loaded onto sodium dodecyl sulfate gels for analysis by polyacrylamide gel electrophoresis. The concentration of each target protein was normalized to levels of β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) and p85 protein (Cell Signaling Technology, Beverly, MA), which remained constant throughout the experimental period (24, 25), and immunostained using anti-β-actin and anti-p85 antibodies. These experiments were performed four times.

Statistical analysis

All results are expressed as a mean ± SE. Statistical analysis was performed using the SAS statistical analysis program (26). One-way ANOVA were carried out in OVX rats. Multiple comparisons of OVX groups were undertaken by Tukey tests. Exercise effects in Sham rats were determined by Student's *t* test. Differences with $P < 0.05$ were considered statistically significant.

Results

Body weight, food intake, and serum 17β-estradiol levels

OVX rats exhibited higher bw than Sham rats throughout the experimental period, paralleled by increased calorie consumption and increased epididymal (visceral) fat mass (Table 1A). In addition, OVX rats exhibited decreased energy expenditure because pair feeding with Sham rats did not lower bw to Sham levels (Table 1B). Body weight and epididymal fat mass was lowered in the OVX-ER-EX, OVX-ER, OVX-EX, and OVX-R groups (Table 1, A and B). Exercise (OVX-EX) decreased epididymal fat mass to a greater degree than estrogen replacement (OVX-ER) ($P < 0.05$, Student's *t* test), but the combination of estrogen and exercise treatment (OVX-ER-EX) did not show additive effects.

Serum leptin levels were not changed by OVX, estrogen replacement, or exercise. Serum 17β-estradiol levels of the OVX rats decreased to one third of those of the Sham rats (Table 1A). Exercise was not able to increase 17β-estradiol levels in the absence of estrogen replacement.

Serum glucose and insulin levels

To ascertain the effects of estrogen deficiency/replacement and exercise on metabolic function, we measured fasted serum glucose and insulin and levels of AGE as an indicator of effects on long-term glucose homeostasis. Levels of AGE reflected changes in serum glucose and insulin levels throughout the experimental period (Table 2, A and B). OVX rats exhibited hyperglycemia, hyperinsulinemia, and increased AGE levels during an overnight fast. Exercise improved glucose homeostasis in OVX rats to exhibit near normoglycemia (Table 2A). Estrogen replacement lowered serum glucose, insulin, and AGE contents, although to a lesser degree than exercise (Table 2A). Combined treatment of exercise and estrogen replacement did not have additive effects. This improvement was involved in weight reduction with exercise and estrogen replacement. Weight reduction with restricted diets showed the same results of improving glucose homeostasis in experiment 2. Although OVX-R rats on restricted diets also displayed near-normal fasted glucose and insulin, OVX-P rats pair-fed to the Sham group remained hyperglycemic and hyperinsulinemic, suggesting a detrimental effect of increased bw (Table 2B).

Overall, exercise had the greatest effect on improving glucose homeostasis. In Sham rats, exercise lowered AGE levels in sc tissues ($P < 0.05$), serum insulin ($P < 0.05$), and glucose ($P = 0.08$). In OVX rats, exercise decreased the high AGE levels ($P < 0.05$ compared with Sham, Student's *t* test) to those found in Sham animals (Table 2, A and B). The effect of exercise on insulin levels was even more dramatic, lowering serum insulin below that reported in the Sham group.

Insulin resistance during euglycemic hyperinsulinemic clamp

OVX rats displayed decreased whole-body glucose disposal rates compared with Sham during the euglycemic hyperinsulinemic clamp, reflecting a high degree of peripheral insulin resistance ($P < 0.05$, Fig. 1A). Improved glucose disposal rate was observed in OVX-EX and OXV-ER groups, but again, no additive effects of combining these treatments were observed. Thus, exercise alone was sufficient to normalize glucose disposal rates in OVX rats. Restricted diets and pair

TABLE 1. Body weight, energy intake, and serum leptin and 17β-estradiol levels after 8-wk experimental period

A	OVX-ER-EX (n = 9)	OVX-ER (n = 8)	OVX-EX (n = 9)	OVX (n = 9)	Sham-EX (n = 9)	Sham (n = 9)
bw (g)	249.6 ± 8.5^{b}	235.7 ± 7.5^{b}	241.6 ± 6.6^{b}	301.7 ± 7.6^{a}*	261.7 ± 5.9	268.4 ± 8.0
Epididymal fat (g)	1.5 ± 0.2^{c}	2.9 ± 0.3^{b}	1.9 ± 0.2^{c}	5.5 ± 0.6^{a}**	1.7 ± 0.2	2.3 ± 0.2†
Energy intake (kJ/d)	6.5 ± 0.2^{b}	6.4 ± 0.3^{b}	6.7 ± 0.2^{b}	7.6 ± 0.3^{a}*	6.6 ± 0.2	6.5 ± 0.3
Serum leptin (ng/ml)	3.3 ± 0.2	3.0 ± 0.3	3.4 ± 0.2	3.2 ± 0.2	3.2 ± 0.2	3.5 ± 0.3
Serum 17β-estradiol (pg/ml)	12.8 ± 0.5^{a}	12.3 ± 0.4^{a}	1.8 ± 0.6^{b}	1.6 ± 0.7^{b}***	5.7 ± 1.9	5.3 ± 1.8

B	OVX-P (n = 9)	OVX-R (n = 9)	OVX (n = 8)	Sham (n = 8)
bw (g)	269.3 ± 7.8^{a}	238.7 ± 6.8^{b}	286.3 ± 8.0^{a}*	245.4 ± 7.1
Epididymal fat (g)	2.6 ± 0.2^{b}	1.2 ± 0.1^{c}	5.1 ± 0.4^{a}**	1.9 ± 0.2
Energy intake (kJ/d)	6.1 ± 0.2^{b}	3.8 ± 0.2^{c}	7.4 ± 0.3^{a}*	6.2 ± 0.2
Serum leptin (ng/ml)	3.1 ± 0.2^{a}	2.4 ± 0.2^{b}	3.3 ± 0.2^{a}*	3.4 ± 0.2

Values are mean ± SE. ER, Estrogen replacement; EX, exercise; P, pair-fed with Sham; R, restricted diet to match with bw of Sham rats.
*, Significantly different among all groups of OVX rats at $P < 0.05$; **, at $P < 0.01$; ***, at $P < 0.001$.
Values on the same column with different superscripts (^{a}, ^{b}, ^{c}) were significantly different at $P < 0.05$ by Tukey test.
†, Significantly different from the Sham-EX group at $P < 0.05$ by Student's *t* test.

TABLE 2. Serum glucose and insulin levels and AGEs in SC tissues after 8-wk experimental period

A	OVX-ER-EX (n = 9)	OVX-ER (n = 8)	OVX-EX (n = 9)	OVX (n = 9)	Sham-EX (n = 9)	Sham (n = 9)
Serum glucose (mM)	6.2 ± 0.2^{b}	6.7 ± 0.3^{b}	6.3 ± 0.2^{b}	7.7 ± 0.2^{a}*	6.1 ± 0.2	6.6 ± 0.2
AGE (arbitrary unit/mg collagen)	5.0 ± 0.2^{c}	5.9 ± 0.3^{b}	5.1 ± 0.2^{c}	8.1 ± 0.4^{a}	4.9 ± 0.2	5.6 ± 0.2†
Serum insulin (pM)	346 ± 21^{c}	422 ± 22^{b}	331 ± 28^{c}	566 ± 29^{a}**	324 ± 26	458 ± 20†

B	OVX-P (n = 9)	OVX-R (n = 9)	OVX (n = 8)	Sham (n = 8)
Serum glucose (mM)	7.2 ± 0.3^{ab}	6.8 ± 0.3^{b}	7.8 ± 0.4^{a}*	6.7 ± 0.3
AGE (arbitrary unit/mg collagen)	7.8 ± 0.2^{a}	6.5 ± 0.4^{b}	8.3 ± 0.4^{a}*	5.9 ± 0.3
Serum insulin (pM)	539 ± 28^{a}	438 ± 31^{b}	582 ± 32^{a}*	479 ± 28

Values are mean ± SE. ER, Estrogen replacement; EX, exercise; P, pair-fed with Sham; R, restricted diet to match with bw of Sham rats.
*, Significantly different among all groups of OVX rats at $P < 0.05$; **, at $P < 0.01$.
Values in the same column with different superscripts (^{a}, ^{b}, ^{c}) were significantly different at $P < 0.05$ by Tukey test.
†, Significantly different from the Sham-EX group at $P < 0.05$ by Student's *t* test.

feeding increased the rates by 155 ± 21% ($P < 0.05$) and 119 ± 18%, respectively, compared with OVX rats. The increase of glucose disposal rates in restricted diets was similar to those of Sham rats. Thus, exercise, estrogen replacement, or restricted diets were sufficient to normalize glucose disposal rates in OVX rats.

Consistent with the hyperglycemia observed in OVX rats, hepatic glucose output (HGO) in the basal (fasted) state was higher in OVX rats than in the Sham group (Fig. 1B). Under clamped conditions of 1100 pM serum insulin levels, OVX rats exhibited the highest HGO (Fig. 1C) with a simultaneous decrease in glycogen storage in the liver (Fig. 1D). Estrogen replacement and exercise reversed the increased basal and clamped HGO in OVX rats, but again, no additive effect was observed upon combining the treatments (Fig. 1, B and C). Exercise significantly ($P < 0.05$) suppressed basal and clamped HGO in both OVX and Sham rats (Fig. 1, B and C). Basal HGO was decreased to the level of Sham rats in the rats of the OVX-R group but not in the OVX-P group (data not shown). Clamped HGO in OVX-R rats was decreased in 25% of OVX rats ($P < 0.05$), resulting in attaining levels similar to those of Sham rats. Thus, insulin resistance induced by OVX was reversed by estrogen replacement, exercise, and weight reduction.

Insulin secretion during hyperglycemic clamp

To characterize the effects of OVX on β-cell function, glucose-stimulated insulin secretion was measured by hyperglycemic clamp. Glucose infusion rates required to elevate basal serum glucose levels by 6 mM were lower in OVX, suggesting decreased insulin secretion capacity (Fig. 2A). Estrogen replacement, exercise, or a combination of these treatments returned them to levels similar to those in Sham rats (Fig. 2A). Glucose infusion rates in OVX-R (12.5 ± 0.6 mg/kg bw·min) and OVX-P (11.7 ± 0.5 mg/kg bw·min) groups were comparable with those observed in OVX (11.9 ± 0.6 mg/kg bw·d), suggesting that weight reduction with restricted diets had no beneficial effect on insulin secretion capacity. We then analyzed the time course of insulin secretion to determine the point at which β-cell failure occurred. Acute phase insulin secretion in the OVX group was impaired and delayed compared with Sham, whereas second phase insulin secretion was similar in both groups until 120 min, when the OVX group exhibited decreased insulin secretion (Fig. 2, B and C). The impaired insulin secretion in OVX rats after prolonged hyperglycemia may be explained by decreased pancreatic β-cell mass. The impaired acute phase insulin secretion in OVX rats, which is a primary cause of the insulin resistance, was recovered to Sham levels in the OVX-ER, OVX-EX and OVX-ER-EX groups (Fig. 2B). First phase insulin secretion in the OVX-R group improved but not to the level of Sham rats (Fig. 2D). Furthermore, second phase insulin secretion in OVX rats was not modulated by restricted diets and pair feeding (Fig. 2D).

Glucokinase and GLUT2 expression

The glucose sensing mechanism in islets is regulated, in part, by glucokinase and GLUT2 to modulate insulin secretion (27). The expression of these proteins was analyzed under our different treatment paradigms to assess their role in the impaired insulin secretion observed in Fig. 2. GLUT2 expression remained unchanged under all conditions (Fig. 3, A and B). Glucokinase expression was lower in the OVX group compared with Sham but increased in the OVX-ER-EX, OVX-ER, and OVX-EX groups ($P < 0.05$) (Fig. 3A). Restricted diets in OVX rats also elevated glucokinase expression to the level of Sham rats (Fig. 3B). These changes in glucokinase expression occurred during first phase insulin secretion, suggesting that the impaired secretion observed in OVX rats during this phase was mediated via reduced glucokinase expression.

The proliferation and apoptosis of pancreatic β-cells

Decreased insulin secretion at 120 min in OVX rats may be associated with a reduction in β-cell mass. β-Cell area, visualized by antiinsulin immunostaining, was quantified for each of our experimental groups (Table 3). As expected, β-cell area was reduced in OVX rats. OVX-ER rats displayed β-cell size comparable with Sham, whereas β-cell area was elevated even further in the Sham-EX, OVX-ER-EX, and OVX-EX groups (Table 3A). Restricted and diets pair feeding in OVX rats did not expand the β-cell area, however (Table 3B). β-Cell mass was determined by the pancreas weight multiplied by the β-cell area, and because the pancreas weight was not significantly different among all groups, the β-cell mass paralleled the β-cell area.

To investigate the mechanisms controlling β-cell mass, the proliferation and apoptosis of β-cells were measured. BrdU incorporation into β-cells, representing the degree of prolif-

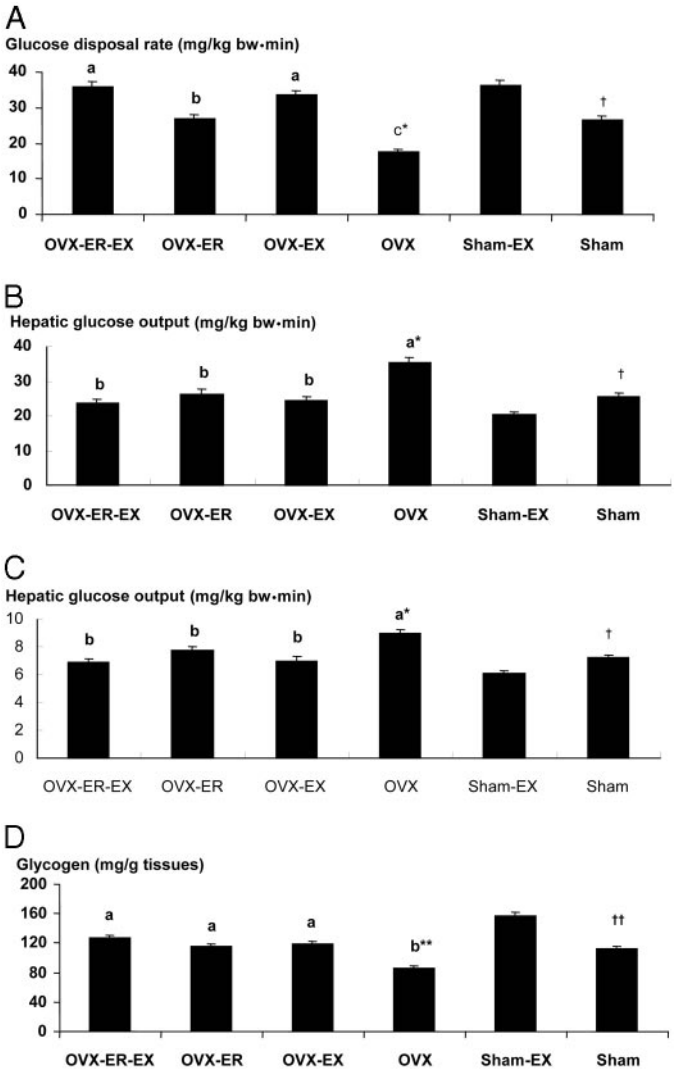

FIG. 1. Insulin resistance, glucose disposal rates, hepatic glucose output (HGO) at basal state, HGO at steady state, and liver glycogen contents. At the end of experimental periods of estrogen replacement (ER) and exercise (EX), a euglycemic hyperinsulinemic clamp was performed in overnight-fasted OVX and Sham diabetic rats to determine whole-body glucose disposal rates (A) and HGO in basal (B) and steady states at 1100 pM serum insulin (C). Liver glycogen contents (D) were also measured after decapitation. The sample size in each group was the same as Table 1A. *, Significantly different among four different groups of OVX rats at $P < 0.05$. **, $P < 0.01$. Values on the same column with *different superscripts* (a–c) were significantly different at $P < 0.05$ by Tukey test. †, Significantly different from the Sham-EX group at $P < 0.05$ by Student's t test.

eration, was decreased in OVX rats, whereas the number of apoptotic bodies was increased (Table 3A). OVX-ER, OVX-EX, and OVX-ER-EX groups displayed increased proliferation and decreased apoptosis, with no additive effect of combining treatments. As a result of proliferation and apoptosis, estrogen replacement alone was not enough to recover β-cell mass in OVX rats. However, exercise by itself was sufficient to bring about a full recovery to the level exhibited in Sham rats. Restricted diets and pair feeding did not affect β-cell mass or growth in OVX rats, although OVX-R decreased β-cell apoptosis (Table 3B).

To investigate the molecular pathways associated with the increase of β-cell mass, IRS2 and PDX-1 expression was measured. Estrogen replacement or exercise increased IRS2 and PDX-1 expression in islets from OVX rats, and the combination of treatments was not additive (Fig. 4). Increased expression of IRS2 and PDX-1 indirectly implied an enhanced IGF-I/insulin signaling cascade resulting in an enlargement of pancreatic β-cell mass. Pair feeding and restricted diets in OVX rats did not elevate IRS2 and PDX-1 expression (data not shown).

Discussion

We observed marked increases in insulin resistance, bw, and total body fat content in Px and OVX rats, reflecting the increased prevalence of DM and obesity in postmenopausal women. Glucose disposal rates, insulin secretion capacity, and β-cell mass were similarly lowered in these animals. Estrogen replacement lowered bw and reversed the hyperglycemia/hyperinsulinemia to levels displayed in Sham rats. The use of restricted diets to lower bw improved insulin sensitivity but failed to fully recover the metabolic phenotype because pancreatic β-cell function and mass were not improved. Regular exercise had the most beneficial effects, improved insulin sensitivity, glucose metabolism and β-cell function, and mass in both Sham and OVX rats. These data indicate that in estrogen-deficient states, weight loss or estrogen replacement is beneficial treatments for diabetes prevention, but regular moderate exercise is the most effective treatment to restore metabolic homeostasis.

It is well documented that estrogen removal causes a marked increase in body energy stores in OVX rats (6, 9, 10, 28, 29). The loss of estrogen may therefore contribute to weight gain in humans, as has been reported in women during and after menopause (29, 30). The increase in body fat mass is a crucial trigger in elevating insulin resistance in estrogen-insufficient states (28, 29). Several reports have noted that restricted diets increase insulin sensitivity through the loss of body fat (31, 32) and enhance insulin secretion in obese humans (33). Weight loss itself reverses insulin resistance and insulin hypersecretion by enhancing first phase insulin secretion (31, 33). However, until now, it has not been clear whether the weight loss itself modulates insulin secretion capacity and pancreatic β-cell mass. We addressed this issue by comparing the effects of weight loss by restricted diets to Sham rats. Restricted diets increased first phase insulin secretion and insulin sensitivity as effectively as estrogen replacement and exercise in OVX diabetic rats. However, restricted diets did not enhance second phase insulin secretion during hyperglycemic clamp and failed to restore pancreatic β-cell mass or proliferation, suggesting that although this treatment initially delayed the prevalence and progression of DM, the effect could not be sustained. β-Cell mass and growth was partially rescued by restricted diets but not to the extent seen by estrogen replacement or exercise. These data suggest that enhancement of second phase insulin secretion and β-cell mass expansion may be crucial factors required to fully reverse insulin resistance and that only exercise was able to rescue of both these conditions in OVX rats.

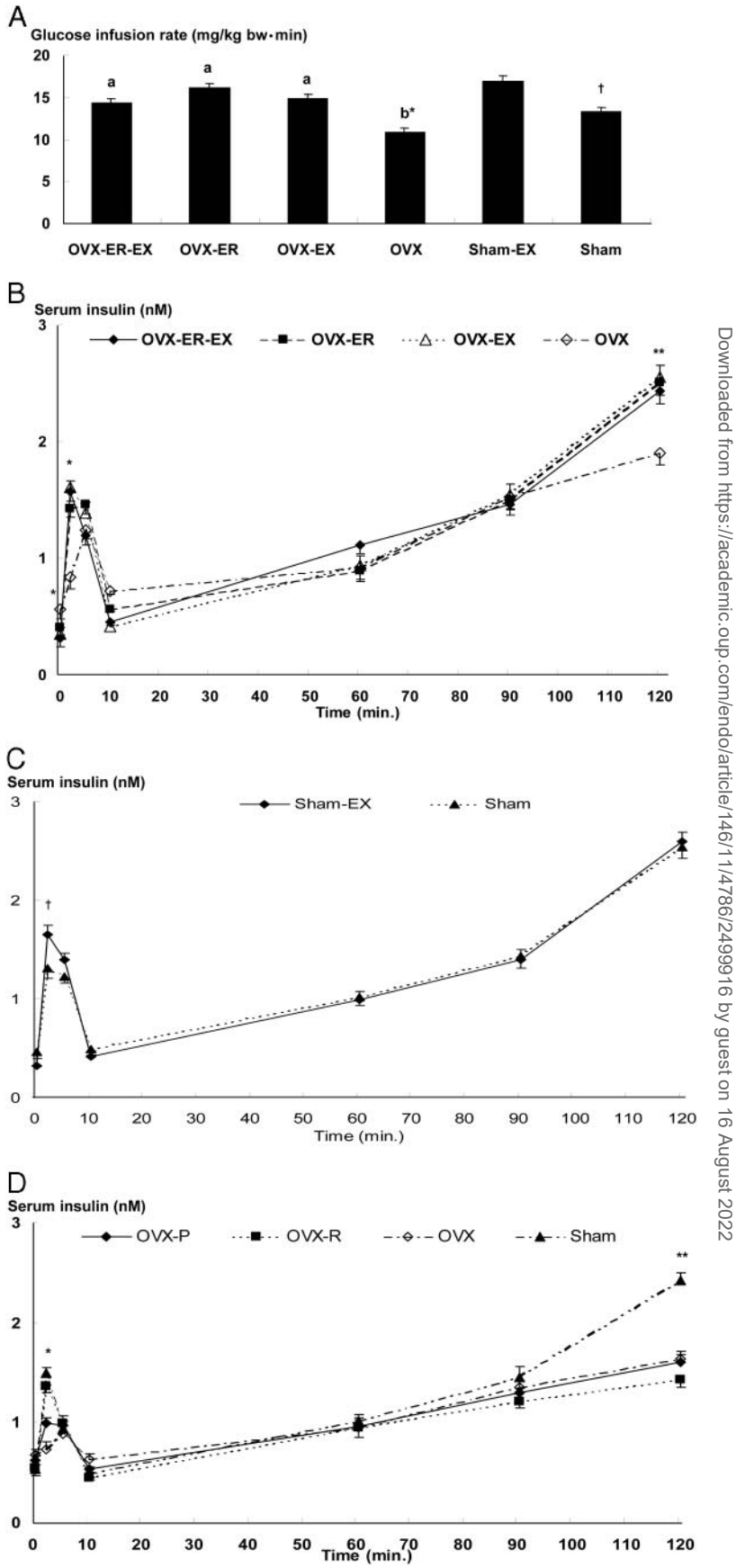

FIG. 2. Insulin secretion during hyperglycemic clamp. A hyperglycemic clamp was performed in overnight-fasted mice to determine insulin secretion patterns and capacity at the end of experimental periods. Experiment 1 determined the effects of estrogen replacement (ER) and exercise (EX) on glucose infusion rates to maintain blood glucose levels at 6 mM above the fasting levels during hyperglycemic clamp (A) and serum insulin levels (B and C) in OVX and Sham diabetic rats. The sample size in each group was the same as Table 1A. Experiment 2 determined the changes of serum insulin levels (D) during hyperglycemic clamp at the end of restricted diets (R) to maintain bw to Sham rats and pair feeding (P) to Sham rats. The sample size in each group was the same as Table 1B. *, Significantly different among all groups of OVX rats at $P < 0.05$. **, $P < 0.01$. Values in the same column with *different superscripts* (a and b) were significantly different at $P < 0.05$ by Tukey test. †, Significantly different from the Sham-EX group at $P < 0.05$ by Student's t test.

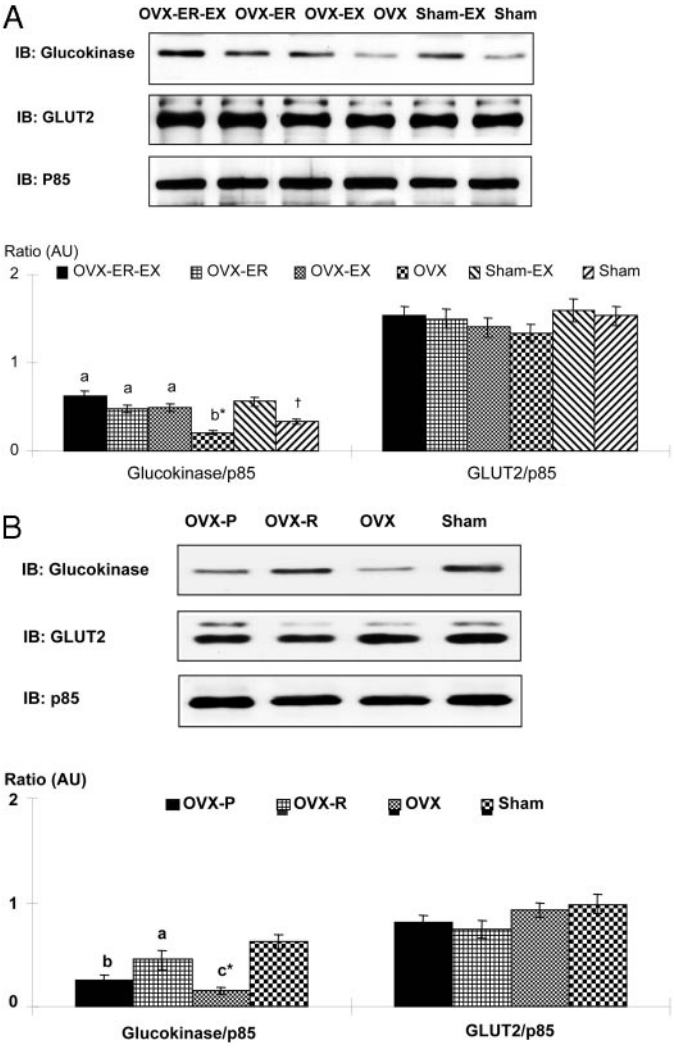

FIG. 3. Expression of glucokinase and GLUT2 associated with β-cell function in islets. Islets isolated from the rats in each group at the end of experimental periods were lysed with lysis buffer, and an equal amount of protein was used for immunoblotting analysis. Glucokinase and GLUT2 expression was determined with specific antibodies by immunoblotting analysis. These experiments were repeated four times, and the results were expressed as mean ± SE. Experiment 1 determined the effect of estrogen replacement (ER) and exercise (EX) on the expression of glucokinase and GLUT2 in OVX and Sham rats (A). In experiment 2, expression levels of glucokinase in islets from rats with restricted diets (R) to maintain bw at levels comparable to Sham rats or pair feeding (P) with Sham rats were analyzed (B). *, Significantly different among all groups of OVX rats at $P < 0.05$. **, $P < 0.01$. Values on the same column with *different superscripts* (a–c) were significantly different at $P < 0.05$ by Tukey test. †, Significantly different from the Sham-EX group at $P < 0.05$ by Student's t test.

In our study and others (34, 35), OVX animals are not hyperleptinemic, despite increased bw. This may be explained by decreased leptin synthesis in the absence of estrogen (36) or impaired leptin signaling in the brain via decreased receptor expression (37), although the exact role of estrogen in these processes is not known. Leptin treatment is partially effective in modulating appetite and limiting weight gain in OVX rats (38). Thus, an increase in bw was possibly related to an altered leptin signaling cascade in OVX rats without a corresponding alteration of serum leptin levels. The low leptin in OVX animals may explain their decreased energy expenditure, as displayed in our study by the failure of pair feeding to reduce OVX bw to Sham bw.

Several studies have reported the effects of estrogen on insulin secretion, but a link between estrogen and the regulation of pancreatic β-cell mass has yet to be demonstrated (5, 17, 39, 40). Estrogen enhanced glucose-induced insulin secretion within 3–5 min of perfusion into whole rat pancreas (5, 39) and in mouse islets (17, 40). The mechanism is not fully understood, but estrogen appears to act synergistically with glucose to close K_{ATP} channels through a cGMP-dependent phosphorylation process, consequently resulting in a calcium-mediated stimulation of insulin secretion (41). This may therefore explain the apparent suppression of glucose-induced insulin secretion in the absence of estrogen.

IRS2 is a crucial mediator of β-cell growth and survival (1, 11), demonstrated clearly in mouse models in which IRS proteins were genetically deleted. Although IRS1 knockout mice develop insulin resistance due to impaired insulin signaling in skeletal muscle and adipose tissues, they do not develop DM due to islet hyperplasia and hyperinsulinemia (1, 2). In contrast, IRS2 knockout mice are unable to increase their β-cell mass sufficiently to fulfill the increased insulin requirement, and they develop severe DM (2, 11). These studies demonstrate the crucial role of IRS2 in β-cell growth. In the present study, the effects of estrogen deficiency on IRS2 expression was investigated and found to be reduced in OVX rat islets. Estrogen or exercise reversed this effect to normal levels of IRS2 expression; no additive effects were seen upon combining the treatments, however. This suggests that these treatments employ a common mechanism (such as induction of IRS2 expression through the activation of CREB) (11, 12) that improves β-cell function and subsequently reverse insulin resistance.

Induction of IRS2 expression via the activation of CREB in Min6 cells and islets (11, 12) accounted for the fact that the upstream region of IRS2 gene coding contains a cAMP response element. When elevated levels of IRS2 potentiated an IGF-I/insulin signaling cascade, it increased β-cell proliferation and decreased apoptosis. It has also been shown that estrogen activates CREB. In the present study, IRS2 expression increased in islets due to exercise and/or estrogen treatment. We did not measure the phosphorylation of proteins involved in an IGF-I/insulin signaling cascade because the phosphorylation can be altered during isolation of islets from the rats. However, increased expression levels of IRS2 and PDX-1 levels as a result of estrogen replacement and/or exercise led us to the conclusion that an IGF-I/insulin signaling cascade would be potentiated in the islets. Many studies showed that the expression levels of PDX-1 in islets were consistent with the proliferation of β-cells, resulting in increased mass (11, 12, 42). Even though the phosphorylation of IRS2, protein kinase B, and forkhead was not measured in the islets, phosphorylation was expected to be enhanced by exercise and/or estrogen treatment.

In summary, the prevalence of DM can increase remarkably in parallel with an increase in bw and fat after menopause. However, bw and fat reduction with restricted diets can only partially reverse diabetic prevalence and progression in an estrogen insufficient state because the decrement

TABLE 3. Area, mass, proliferation, and apoptosis of β-cells

A	OVX-ER-EX (n = 5)	OVX-ER (n = 6)	OVX-EX (n = 5)	OVX (n = 6)	Sham-EX (n = 5)	Sham (n = 6)
β-Cell area (%)	34.5 ± 1.4^a	28.7 ± 0.9^b	33.1 ± 1.3^a	23.5 ± 0.9^c*	33.9 ± 1.4	27.9 ± 1.0†
β-Cell mass (mg)	83.8 ± 3.3^a	67.9 ± 3.4^b	77.2 ± 2.6^a	53.5 ± 2.5^c**	91.3 ± 4.1	66.8 ± 2.7†
β-Cell proliferation (no. of BrdU$^+$ cells/mm^2 pancreas)	2.1 ± 0.1^a	1.9 ± 0.1^a	2.2 ± 0.2^a	1.3 ± 0.1^b*	2.4 ± 0.2	1.8 ± 0.1†
Apoptotic bodies (no. of apoptotic bodies/mm^2 pancreas)	0.42 ± 0.02^c	0.54 ± 0.02^b	0.41 ± 0.02^c	0.68 ± 0.02^a*	0.36 ± 0.02	0.48 ± 0.02†

B	OVX-P (n = 5)	OVX-R (n = 6)	OVX (n = 6)	Sham (n = 5)
β-Cell area (%)	25.1 ± 1.1	26.3 ± 0.9	24.7 ± 1.0	29.5 ± 1.2
β-Cell mass (mg)	56.4 ± 2.2	59.3 ± 2.2	55.2 ± 2.5	67.2 ± 2.8
β-Cell proliferation (no. of BrdU$^+$ cells/mm^2 pancreas)	1.4 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	2.0 ± 0.1
Apoptotic bodies (no. of brown dots/mm^2 pancreas)	0.63 ± 0.03^a	0.51 ± 0.02^b	0.68 ± 0.02^a*	0.46 ± 0.2

Values are mean ± SE. ER, Estrogen replacement; EX, exercise; P, Pair-fed with Sham; R, restricted diet to match with bw of Sham rats.
*, Significantly different among all groups of OVX rats at $P < 0.05$; **, at $P < 0.01$.
Values in the same column with *different superscripts* (^{a}, ^{b}, ^{c}) were significantly different at $P < 0.05$ by Tukey test.
†, Significantly different from the Sham-EX group at $P < 0.05$ by Student's t test.

cannot improve pancreatic β-cell function and mass. Regular exercise, regardless of estrogen replacement, had the most beneficial effects on insulin sensitivity and β-cell function and mass, even though estrogen replacement alone also improved them. These results suggested that estrogen and exercise have a direct effect on improving insulin secretion capacity and β-cell proliferation, leading to preventing the prevalence of DM and delaying its progression. Therefore, menopausal women, to improve insulin sensitivity and β-cell function and mass, should be recommended to do regular, moderate exercise.

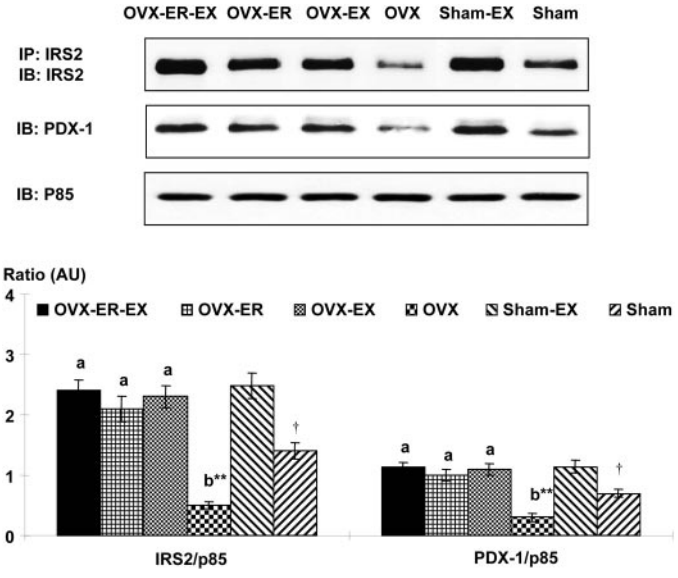

FIG. 4. Expression of IRS-2 and PDX-1 associated to β-cell mass in islets. In experiment 1, islets were isolated from the OVX and Sham diabetic rats treated with estrogen (ER) or exercise (EX) at the end of 8 wk of treatment. Islets were lysed with lysis buffer, and an equal amount of protein was used for immunoblotting analysis. IRS2 expression was measured by immunoprecipitation followed by immunoblotting analysis. The expression of other proteins was determined with specific antibodies by immunoblotting analysis only. These experiments were repeated four times, and the results were expressed as mean ± SE. **, Significantly different among four different groups of OVX rats at $P < 0.01$. Values in the same column with different superscripts (a and b) were significantly different at $P < 0.05$ by Tukey test. †, Significantly different from the Sham-EX group at $P < 0.05$ by Student's t test.

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