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ORIGINAL PAPER

Endurance running exercise is an effective alternative to estradiol replacement for restoring hyperglycemia through TBC1D1/GLUT4 pathway in skeletal muscle of ovariectomized rats

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Abstract
Menopause is a risk factor for impaired glucose metabolism. Alternative treatment of estrogen for postmenopausal women is required. The present study was designed to investigate the effects of 5-week endurance running exercise (Ex) by treadmill on hyperglycemia and signal pathway components mediating glucose transport in ovariectomized (OVX) placebo-treated rats, compared with 4-week 17β-estradiol (E2) replacement or pair-feeding (PF) to the E2 group. Ex improved the hyperglycemia and insulin resistance index in OVX rats as much as E2 or PF did. However, Ex had no effect on body weight gain in the OVX rats. Moreover, Ex enhanced the levels of GLUT4 and phospho-TBC1D1 proteins in the gastrocnemius of the OVX rats, but E2 or PF did not. Instead, the E2 increased the Akt2/AS160 expression and activation in the OVX rats. This study suggests that endurance Ex training restored hyperglycemia through the TBC1D1/GLUT4 pathway in muscle by an alternative mechanism to E2 replacement.

Keywords Estradiol replacement · Hyperglycemia · Insulin resistance · TBC1D1/GLUT4 pathway · Running exercise training · Ovariectomized rat

Introduction

Postmenopausal women are at higher risk for metabolic disorders, such as metabolic syndrome and type 2 diabetes than premenopausal women [1, 2]. Because estrogens play an important role in the control of energy homeostasis in females, estrogen deficiency in menopausal status is associated with visceral fat accumulation [3, 4], impaired glucose tolerance, and insulin resistance. Similarly, ovariectomized (OVX) rats, an animal model widely used for studying the pathology of human menopause, develop body weight, visceral fat accumulation, and impairment of whole-body glucose homeostasis [5, 6]. Recently, we found that 17β-estradiol (E2) replacement restored the impairment of insulin sensitivity by increasing the activation of the insulin signaling pathway in the gastrocnemius muscle of OVX rats [7]. These findings suggest that E2 replacement restores glucose metabolism as its direct action in OVX rats. In addition, the inhibitory effect of estrogen against abdominal obesity may be partly associated with restoring the insulin sensitivity, since visceral fat accumulation contributes to glucose intolerance [2, 8].

Estrogen replacement in postmenopausal women is usually performed in combination with progesterone, a treatment known as hormone replacement therapy (HRT). The metabolic impact of HRT varies depending on the dose of the estrogen component, the type of progesterone, and the route of administration [10–12]. Previous studies have reported that HRT exerts a beneficial effect on the glucose metabolism [9]; however, it deteriorates insulin sensitivity, attributed to progesterone or high doses of estrogen [10–12]. Additionally, the general efficacy and safety of HRT is controversial due to the risks associated, including stroke and coronary heart disease, as well as an elevated risk of breast cancer, which were increased in the HRT trials performed by the Women's Health Initiative [13]. Therefore, it is essential to develop alternative treatments that restore the positive glucose metabolic effects of estrogen.

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Several human studies show that aerobic exercise (Ex) is insulin-sensitizing and that training is an effective substitute or adjunct for HRT [14, 15]. As evidenced in rodent studies, Ex training initiated at the onset of OVX maintained normal skeletal muscle glucose uptake, prevented visceral adipose accretion, and improved whole-body glucose tolerance in OVX rats [16, 17]. However, to our knowledge, mechanisms underlying the abilities of Ex training to improve glucose metabolism under reduced estrogen function are not fully understood.

Skeletal muscle is the major tissue responsible for uptake of glucose from the blood, accounting for 70–85% of whole-body glucose disposal [18]. Insulin and Ex/muscle contraction are two widely studied physiological stimuli that increase glucose uptake via the activation of intracellular signaling cascades [19–21]. The signaling mechanism by which insulin stimulates muscle glucose uptake is relatively well known, and involves phosphorylation of protein kinase B (Akt) and the Rab-GTPase activating protein (Rabs), an Akt substrate of 160 kDa (AS160) [22, 23]. In contrast, the signaling mechanism by which Ex acts is not fully understood, although studies have shown that activation of AMP-activated protein kinase (AMPK), an energy sensing kinase, is positively correlated with increases in muscle glucose uptake [24]. Furthermore, the downstream regulators of AMPK are still debated, while AS160 or TBC1 (Tre-2, BUB2, CDC16) domain family member 1 (TBC1D1), another Rabs of AS160 (also known as TBC1D4), is reported as a glucose uptake regulator in Ex/muscle contraction [19, 21, 25].

It is important to define the differences in molecular mechanism underlying beneficial effects of Ex training on glucose uptake in muscle of OVX rats compared with E2 replacement, whereby Ex is a critical alternative to estrogen replacement [14, 16]. Recently, several researchers have reported the effects of Ex training on glucose transporter 4 (GLUT4), Akt protein, or mRNA level in OVX rats [17, 26, 27], but those findings were inconsistent. In this study, we focused on the effects of Ex training on signal pathway components that mediate glucose uptake in skeletal muscle and adipose tissues of OVX rats, because our previous study showed that beneficial effects of E2 replacement on insulin sensitivity were mediated by enhancing activation of the Akt2/AS160 pathway in the gastrocnemius muscle, but not in liver [7].

In addition, whether estrogen reduction in the menopausal phase directly impairs the glucose uptake mechanism [28, 29] remains unclear, or whether estrogen deficiency-induced hyperphagia induces visceral fat accumulation, which promotes insulin resistance resulting in the impairment of glucose uptake as an indirect result of estrogen deficiency [30]. A previous study reported that pair-feeding (PF) with sham-operated female rats failed to improve insulin action at the whole-body or skeletal muscle level in OVX rats, suggesting ovarian hormone deprivation to be involved in the progression of insulin resistance as a direct cause [31]. Therefore, as per the second aim of this study, we also examined the effects of PF on plasma glucose levels and the signaling pathway components that mediate glucose transport in OVX rats fed with the same diet as the E2-replaced OVX rats. This experiment may give an answer to above-mentioned question whether estrogen directly restores glucose metabolism, or whether estrogen-induced anorexia and following leanness prevents deterioration of it. The present study may give first data simultaneously showing the effects of Ex training, E2 replacement, and PF on insulin-dependent or independent signaling pathways in muscle or adipose tissue of OVX rats.

This study was designed to test an initial hypothesis, that is whether Ex training in the form of endurance running improves hyperglycemia and the insulin resistance index in the basic condition without muscle contraction through the AMPK-TBC1D1/GLUT4 pathway, which is different from the pathway activated by E2 replacement in skeletal muscle of OVX rats. Furthermore, the second hypothesis is that is whether E2 directly restores glucose metabolism, or whether E2-induced anorexia and following leanness prevents deterioration of it in OVX rats.

Materials and methods

Animals

The Nara Women's University Committee on Animal Experiments approved the experimental protocol. In total, 24 female Wistar rats were used in this study. The rats were housed in standard rat cages (length: 40 cm, width: 25 cm, and depth: 25 cm) under controlled temperature and light conditions (26 ± 1 °C, a 12:12-h light–dark cycle, with lights on at 6:00 a.m.). Tap water and rodent chow (Oriental Yeast, Tokyo, Japan) were provided ad libitum.

Preparation for experiments

Ovariectomy and E2 (or placebo) replacement

Nine-week-old female rats were ovariectomized, followed by E2 or placebo (Pla) replacement as previously described [7, 32, 33]. In brief, after a 4-week-recovery period from OVX, the rats aged 13 weeks were assigned randomly to either the Pla ($n = 18$)- or the E2 ($n = 6$)-treated group, and were subcutaneously implanted with either E2 (1.5 mg/60-day release) or Pla pellets (Innovative Research of America, Sarasota, FL, USA). The Pla group rats were divided into control (Pla; $n = 6$), PF (Pla/PF; $n = 6$), and Ex (Pla/Ex; $n = 6$) groups.

Experimental protocols

PF study

Two days after Pla replacement, the Pla/PF group was pair-fed to the E2 group, i.e., given the average food intake of the E2 group in the previous day from 13 to 17 weeks of age. Food intake and body weight were monitored daily.

Endurance running Ex training

Before the Ex training protocol, the Pla/Ex group rats were familiarized with Ex by running at 10 m/min for 30 min/day on a custom-built, five-lane motorized rodent treadmill (KN-73, Natume, Tokyo, Japan) in the hours before dark for 2 weeks from 10 to 12 weeks of age, during which the rats had the intensity of Ex gradually increased. From 12 to 17 weeks of age, the rats ran 17 m/min of treadmill running for 60 min/day, 5 day/week for 5 weeks. The intensity of the running Ex may be moderate, as previous researchers have estimated that running at 28 m/min as high intensity or 8 m/min as low intensity elicited ~ 75% or ~ 45% of maximal O_2 uptake in female rats [34, 35].

Sampling for estimation of plasma glucose, insulin, and signaling pathway

All the rats fasted for 16 h before blood and tissue sampling, with free access to water. On the day of sampling, after the rats were deeply anesthetized by a pentobarbital sodium (45 mg/kg body weight) [36], blood samples were collected from cardiac puncture in the four groups. After euthanasia, the gastrocnemius muscles and mesenteric adipose tissues were excised and immediately frozen in liquid nitrogen, then stored at − 50 °C until further processing of Western blotting. Parts of these tissues were stored in RNA stabilization solution, until RT-qPCR analysis for AS160 and GLUT4 mRNAs was performed. The wet weights of the intra-abdominal (mesenteric, kidney-genital, and retroperitoneal) and subcutaneous (inguinal) adipose tissues were measured. The total visceral fat weight was calculated by the sum of the intra-abdominal fat weights.

Analytical methods for plasma glucose, insulin, and E2

The plasma glucose concentration was measured by a glucose oxidase method using a glucose assay kit (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin concentration was determined by the use of a rat insulin ELISA kit (FUJIFILM Wako Shibayagi, Gunma, Japan). Using these variables, insulin resistance was assessed by a homeostasis model assessment of the insulin resistance index (HOMA-IR), calculated using the following formula [37–39]:

$$\mathrm{HOMA-IR} = \text{fasting glucose concentration(mmol/l)} \times \text{fasting insulin concentration}(\mu\mathrm{IU/ml})/22.5$$

The E2 concentrations were measured commercially by an electro-chemiluminescence immunoassay (SRL Co, Nara, Japan).

Immunoblotting

Isolated muscle and mesenteric adipose tissue were immediately homogenized in homogenization buffer [320 mM sucrose; 10 mM Tris·HCl, pH 7.4; 1 mM EGTA; 10 mM β-mercaptoethanol; 50 mM NaF; 10 mM Na_3VO_4; 9 tablets of cOmplete EDTA-free protease inhibitor cocktail containing 0.2 mM PMSF, 20 μM leupeptin, and 0.15 μM pepstatin (Roche, Mannheim, Germany); 1% TritonX-100], as described previously [7]. The homogenates were centrifuged at 15,000*g* for 30 min at 4 °C. SDS samples containing equal amounts of protein were separated by SDS-PAGE on 10% polyacrylamide gels, and immunoblotted using a PVDF membrane (GE Healthcare, Buckinghamshire, UK) with the following antibodies: antibodies for Akt and phospho (p)-Akt Ser^{473}, p-Akt Thr^{308}, Akt2, p-Akt2 Ser^{474}, AMPKα, p-AMPKα Thr^{172}, and p-AS160 Thr^{642} were from Cell Signaling Technology (Danvers, MA, USA). The AS160 and p-TBC1D1 Ser^{237} antibody were from MILLIPORE (Temecula, CA, USA), and GLUT4, TBC1D1, and Tubulin antibody from Abcam (Cambridge, MA, USA). Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was obtained from Promega (Madison, WI, USA). The enhanced chemiluminescence (ECL, GE Healthcare Life Sciences, Buckinghamshire, UK) system was used for protein detection. Imaging and densitometry were performed using the imaging system Ez-Capture (ATTO, Tokyo, Japan) and image processing program CS Analyzer (ATTO, Tokyo, Japan).

RNA isolation and RT-qPCR

Total RNA was extracted using the TRI Reagent Solution (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The amount of total RNA extracted was determined, and its purity (absorption ratio of optical density 260 nm and 280 nm > 1.9) was verified spectrophotometrically using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Waltham, MA, USA). RT-qPCR was performed using a StepOne Software v2.1 system (Applied Biosystems). The commercially available TaqMan Gene Expression Assay (Applied Biosystems) for AS160 (Rn01468356_m1), GLUT4 (Rn00562597_m1), and β-2M (Rn00560856_m1)

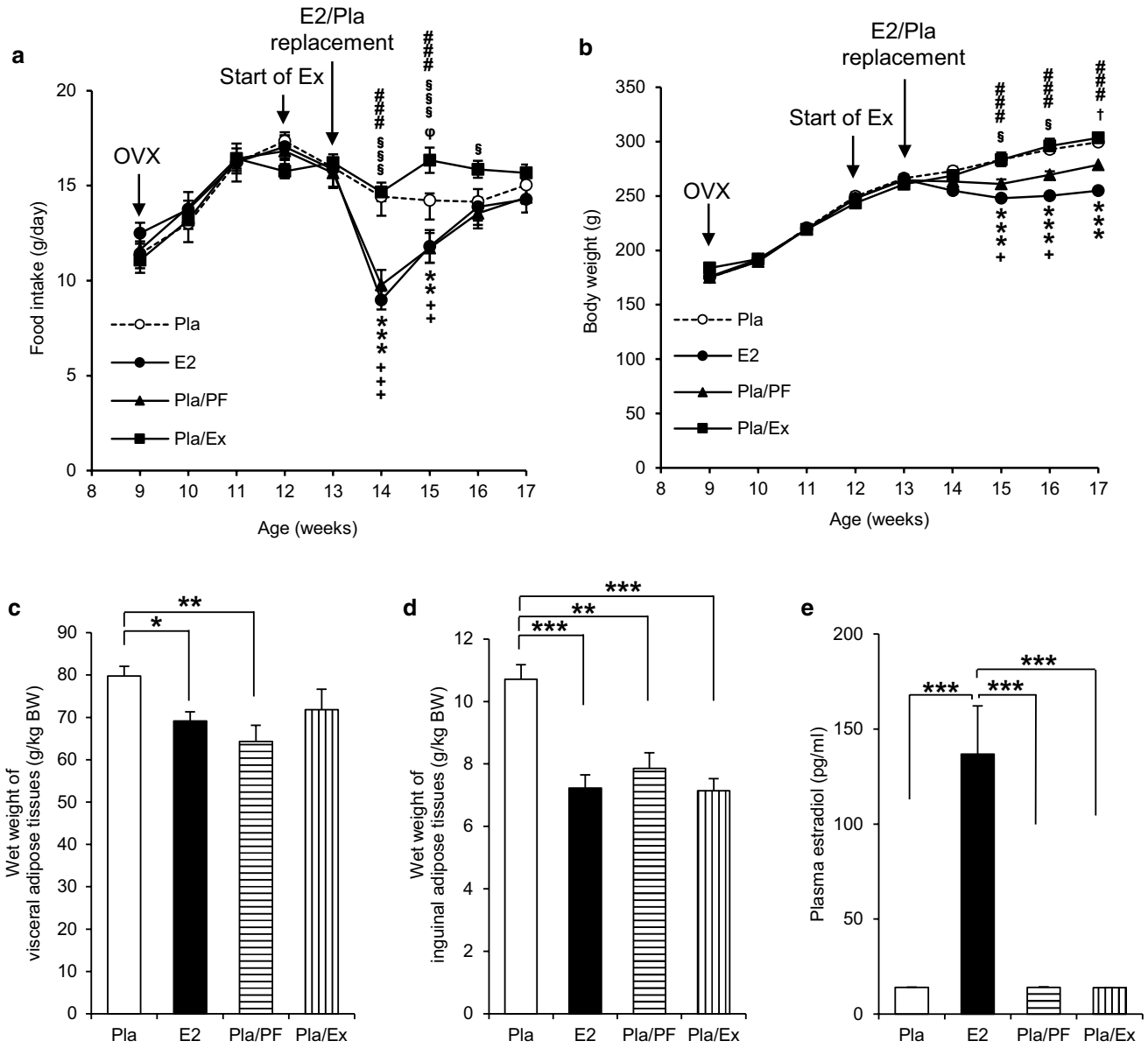


Fig. 1 Characterization of rats studied. Data are expressed as means ± SE. Line graphs represent course of change in mean food intake per day (**a**) and body weight (**b**) in the placebo (Pla, $n=6$)-, the 17β-estradiol (E2, $n=6$)-treated, the placebo/pair-feeding (Pla/PF, $n=6$), and the placebo/exercise (Pla/Ex, $n=6$) groups. Two-way repeated-measures ANOVA revealed significant differences in food intake and body weight between the four groups. $^{**}P<0.01$, $^{***}P<0.001$: E2 vs. Pla. $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$: Pla/PF vs. Pla. $^{\varphi}P<0.05$: Pla/Ex vs. Pla. $^{\dagger}P<0.05$: Pla/PF vs. E2. $^{\#\#\#}P<0.001$: Pla/Ex vs. E2. $^{\S}P<0.05$: Pla/Ex vs. Pla/PF. There was an interaction of time and group effects in food intake ($P_{\text{Time×Group}}<0.05$: E2 vs. Pla or Pla/Ex, Pla/Ex vs. Pla) and body weight ($P_{\text{Time×Group}}<0.05$: E2 vs. Pla/PF or Pla/Ex, $P_{\text{Time×Group}}<0.01$, E2 vs. Pla, Pla/PF vs. Pla or Pla/Ex). Bar graphs represent wet weights of visceral (the sum of weights of the mesenteric, kidney-genital, and retroperitoneal adipose tissues) (**c**), inguinal (**d**) adipose tissues per body weights, and plasma E2 concentration (**e**) in the Pla ($n=6$)-, the E2 ($n=6$)-treated, the Pla/PF ($n=6$), and the Pla/Ex ($n=6$) groups at 17 weeks of age. One-way ANOVA followed by a post hoc Tukey's HSD test revealed differences in wet weights of the visceral adipose tissues per body weights between the Pla and E2 or Pla/PF groups ($^{***}P<0.001$), and inguinal adipose tissues between the Pla and every other group ($^{***}P<0.001$). There is a difference in plasma E2 concentration between the E2 and every other group ($^{***}P<0.001$). OVX, ovariectomy. *BW* body weight

were used in this study. For the analysis, gene expression levels of AS160 were normalized using β-2M as a housekeeping gene, and expressed with respect to the average value for the Pla group. All reactions were performed in duplicate. The thermal cycling conditions were as follows: 95 °C for 20 s, followed by 40 cycles at 95 °C for 1 s and

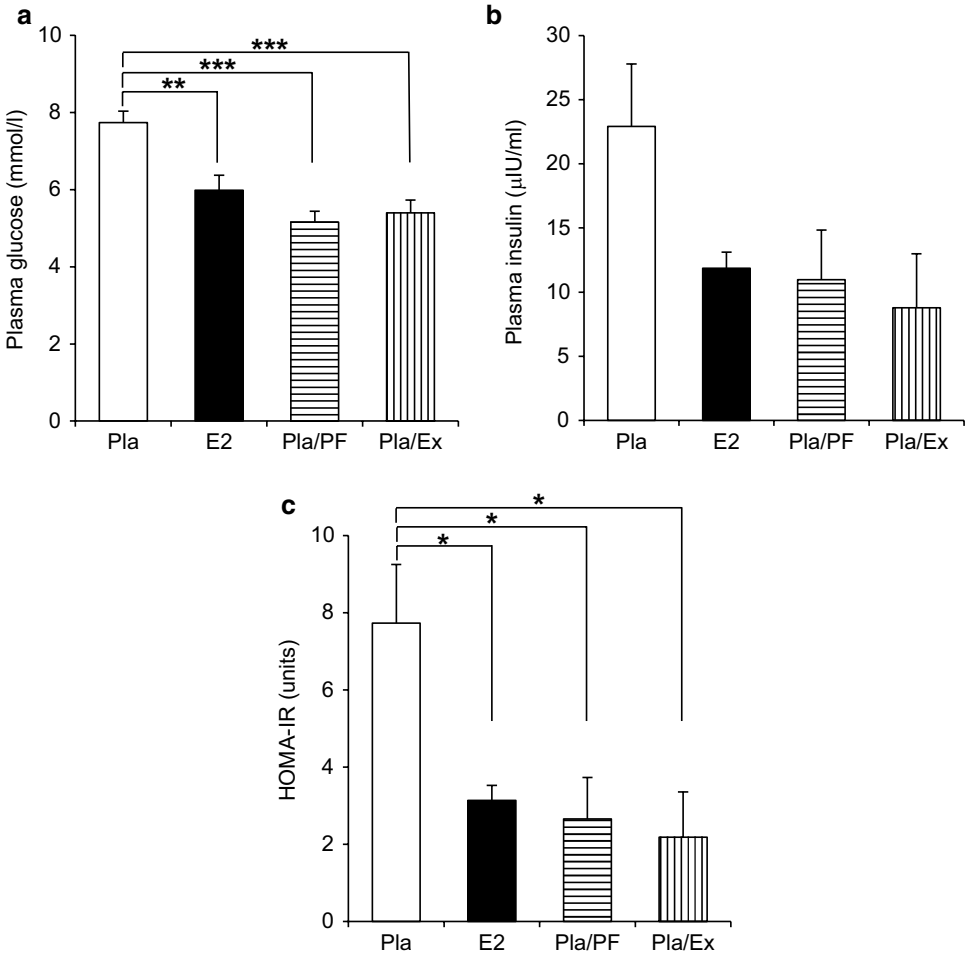


Fig. 2 Plasma concentrations of glucose (mmol/l) (**a**), insulin (μIU/ml) (**b**), and homeostasis model assessment of insulin resistance (HOMA-IR) index (**c**) in the placebo (Pla, $n=6$)-, the 17β-estradiol (E2, $n=6$)-treated, the placebo/pair-feeding (Pla/PF, $n=6$), and the placebo/exercise (Pla/Ex, $n=6$) groups. Data are expressed as means ± SE and were analyzed by one-way ANOVA. This was followed by a post hoc Tukey's HSD test. *$P<0.05$, **$P<0.01$, and ***$P<0.001$, differences between the Pla and every other group

60 °C for 20 s. No amplification of fragments occurred in the control samples without reverse transcriptase. The mRNA quantity was calculated using the $\Delta\Delta C_t$ (comparative C_t) method under the assumption that primer efficiencies were relatively similar.

Statistical analysis

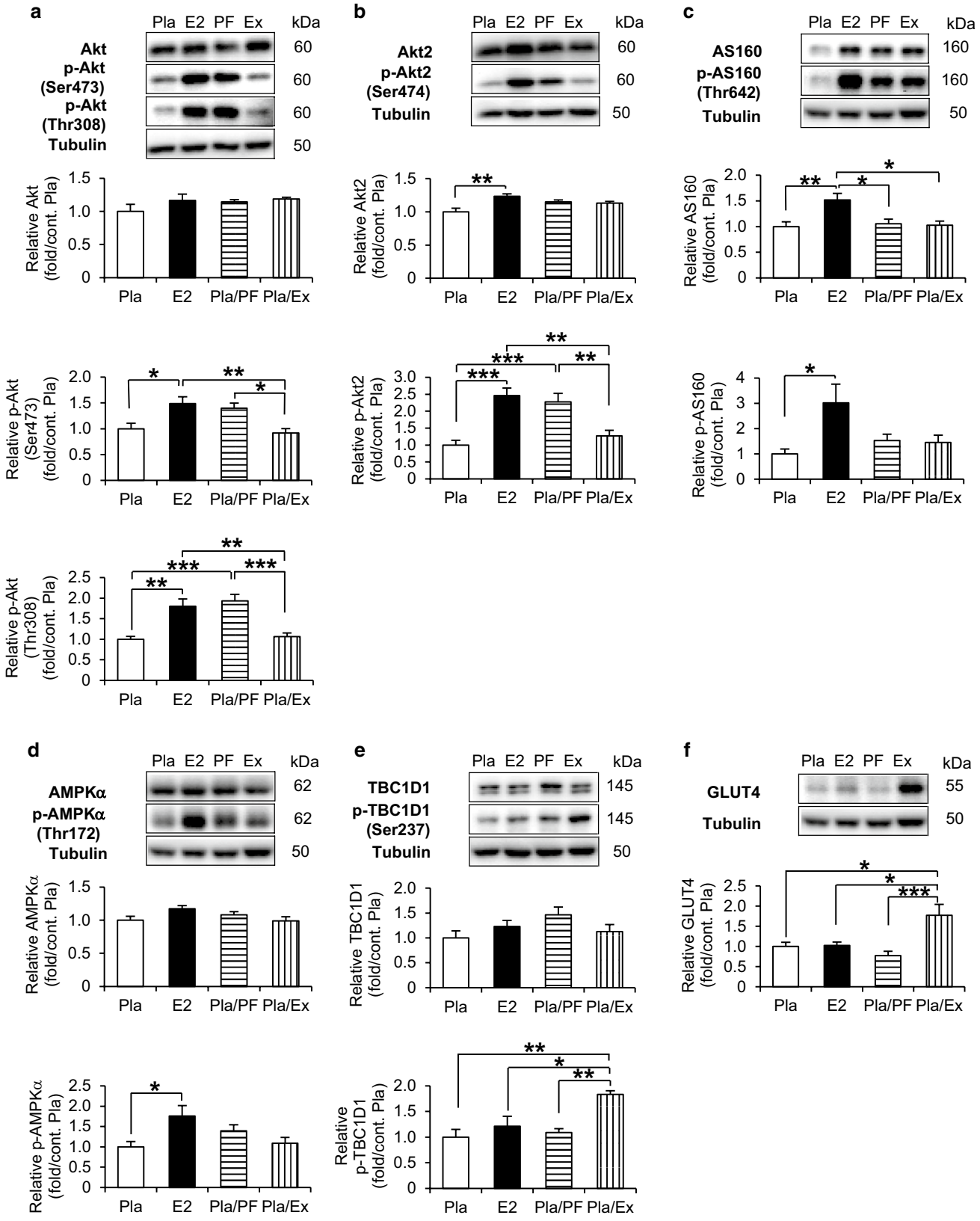
All values were expressed as means ± SE. Two-way repeated-measures ANOVA for each pair-wise comparison among four groups was used to analyze the effects of E2, PF, and Ex on body weight and food intake. One-way ANOVA was used for the comparison of the adipose tissue weight, plasma E2 and glucose concentrations, insulin concentrations, HOMA-IR, and signaling protein and mRNA levels among the four groups, and was followed by a post hoc Tukey's HSD test. We considered a value of $P<0.05$ to be statistically significant.

Results

Characterization of rats studied

As shown in Fig. 1a, food intake in the E2 group was markedly decreased at 14 and 15 weeks of age, 1–2 weeks after E2 pellet implantation, compared with that at 13 weeks ($P<0.001$) or the Pla group ($P<0.001$ and $P<0.01$, respectively). After that, the intake in the E2 groups came to be similar to the Pla group at 16 weeks of age. In contrast, food intake in the Pla/Ex group was increased at 15 weeks of age compared with 14 weeks ($P<0.05$), and returned to the same level as the Pla group.

The body weight in the E2 group was significantly lighter than that in the Pla group at 15–17 weeks of age (Fig. 1b). In contrast, the Pla/PF group showed heavier body weight than the E2 group, resulting in a significant difference in the time course of body weight between the E2 and Pla/PF groups (interaction: $P<0.05$), though they were still lighter than those in the Pla group. In addition, body weights in the


a
Pla E2 PF Ex kDa
Akt 60
p-Akt (Ser473) 60
p-Akt (Thr308) 60
Tubulin 50
Relative Akt (fold/cont. Pla)
Relative p-Akt (Ser473) (fold/cont. Pla)
Relative p-Akt (Thr308) (fold/cont. Pla)
b
Pla E2 PF Ex kDa
Akt2 60
p-Akt2 (Ser474) 60
Tubulin 50
Relative Akt2 (fold/cont. Pla)
Relative p-Akt2 (fold/cont. Pla)
c
Pla E2 PF Ex kDa
AS160 160
p-AS160 (Thr642) 160
Tubulin 50
Relative AS160 (fold/cont. Pla)
Relative p-AS160 (fold/cont. Pla)
Pla E2 Pla/PF Pla/Ex
d
Pla E2 PF Ex kDa
AMPKα 62
p-AMPKα (Thr172) 62
Tubulin 50
Relative AMPKα (fold/cont. Pla)
Relative p-AMPKα (fold/cont. Pla)
e
Pla E2 PF Ex kDa
TBC1D1 145
p-TBC1D1 (Ser237) 145
Tubulin 50
Relative TBC1D1 (fold/cont. Pla)
Relative p-TBC1D1 (fold/cont. Pla)
f
Pla E2 PF Ex kDa
GLUT4 55
Tubulin 50
Relative GLUT4 (fold/cont. Pla)

◂**Fig. 3** Representative blots and relative values of protein kinase B (Akt) and phospho (p)-Akt Ser^{473}, and p-Akt Thr^{308} (**a**), Akt2, and p-Akt2 Ser^{474} (**b**), Akt substrate of 160 kDa (AS160) and p-AS160 Thr^{642} (**c**), AMPKα and p-AMPKα Thr^{172} (**d**), TBC1D1 and p-TBC1D1 Ser^{237} (**e**), and GLUT4 (**f**) in the gastrocnemius of rats in the placebo (Pla, $n=6$)-, the 17β-estradiol (E2, $n=6$)-treated, the placebo/pair-feeding (Pla/PF, $n=6$), and the placebo/exercise (Pla/Ex, $n=6$) groups. Data are expressed as means ± SE and were analyzed by one-way ANOVA. This was followed by a post hoc Tukey's HSD test. *$P<0.05$, **$P<0.01$, and ***$P<0.001$, differences between the two groups

Pla/Ex group were similar to the Pla group, but heavier than both the E2 and Pla/PF groups (Fig. 1b).

The wet weights of total visceral (the sum of mesenteric, kidney-genital, and retroperitoneal) adipose tissues per body weights were significantly lighter in the E2 and Pla/PF groups than in the Pla group (Fig. 1c). The weights of inguinal subcutaneous adipose tissues per body weights were significantly lighter in the E2, Pla/PF, and Pla/Ex groups than the Pla group (Fig. 1d). Plasma E2 concentrations were significantly higher in the E2 group than in the other Pla groups (Fig. 1e).

Effects of E2, PF, and Ex on plasma glucose, insulin, and HOMA-IR

Fasting plasma glucose concentration was significantly lower in the E2, Pla/PF, and Pla/Ex groups than in the Pla group (Fig. 2a). In contrast, there was no significant difference in fasting plasma insulin among the Pla, E2, Pla/PF, and Pla/Ex groups (Fig. 2b). HOMA-IR indices were significantly lower in the E2, Pla/PF, and Pla/Ex groups than in the Pla group (Fig. 2c).

Effects of E2, PF, and Ex on insulin signaling and AMPK pathway in basic condition

To reveal the molecular mechanism accounting for the effects of E2, PF, and Ex on plasma glucose and insulin, we investigated signaling pathway components mediating glucose transport, the Akt/AS160, and AMPK/TBC1D1 pathways, as well as GLUT4, in the gastrocnemius muscle (Fig. 3) and mesenteric adipose tissue (Fig. 4).

The quantity of Akt protein in the muscle was similar between the four groups (Fig. 3a). The relative levels of p-Akt Ser^{473} and p-Akt Thr^{308} were significantly higher in the E2 group than those in the Pla and Pla/Ex groups, but were not different between the Pla and the Pla/Ex groups. In addition, p-Akt Thr^{308} was higher in the Pla/PF group than the Pla and Pla/Ex groups. Figure 3b shows that Akt2 and p-Akt2 Ser^{474} protein levels in the muscle were increased in E2 group compared to the Pla group ($P<0.01$ and $P<0.001$, respectively). In contrast, PF increased only p-Akt2 Ser^{474} ($P<0.001$), but Ex had no effects on Akt2 and p-Akt2 Ser^{474}. Furthermore, Fig. 3c shows that AS160 and p-AS160 Thr^{642} protein levels were increased in the E2 group compared with the Pla, Pla/PF, and Pla/Ex groups, and compared with the Pla group, respectively. Moreover, p-AMPKα Thr^{172} in the muscle was increased in the E2 group compared to the Pla group, with no change in the protein level (Fig. 3d). Interestingly, p-TBC1D1 Ser^{237} in the Pla-Ex group was higher than in the Pla, E2, and Pla/PF groups, with no differences in TBC1D1 protein levels among the four groups (Fig. 3e). In addition, GLUT4 protein level was significantly higher in the Pla/Ex group than in any other group (Fig. 3f).

In the mesenteric adipose tissue, the amounts of Akt and Akt2 proteins, as well as their phosphorylated protein levels, were similar among the four groups (Fig. 4a, b). AS160 and p-AS160 Thr^{642} protein levels were increased in the E2 group compared with the Pla and Pla/Ex groups (Fig. 4c). The p-AMPKα Thr^{172} levels were higher in the E2, Pla/PF, and Pla/Ex groups than in the Pla group, with no differences in AMPKα protein levels among the four groups (Fig. 4d). In contrast, TBC1D1 and p-TBC1D1 were not different among the four groups (Fig. 4e). GLUT4 was not detected in the mesenteric adipose tissue of any group.

AS160 and GLUT4 mRNA levels in the gastrocnemius muscle and mesenteric adipose tissue

The levels of AS160 and GLUT4 mRNAs in the gastrocnemius muscle or mesenteric adipose tissue of the four groups were determined by RT-qPCR. As shown in Fig. 5a, the relative level of AS160 mRNA in the muscle was higher in the E2 group than those in the Pla ($P<0.01$), Pla/PF, and Pla/Ex groups. In contrast, the relative GLUT4 mRNA in the muscle and AS160 mRNA levels in the mesenteric adipose tissues were similar among the four groups (Fig. 5b, c).

Discussion

The present study demonstrated that endurance running Ex training improved hyperglycemia by the activation of the TBC1D1/GLUT4 pathway in the muscle of OVX rats. The mechanism varied from that of E2 replacement, which restored hyperglycemia via the activated Akt2/AS160 pathway in the muscle, or from that of PF of the E2 replaced rats.

Endurance Ex training did not affect body weight gain in the OVX rats despite a decrease in inguinal fat accumulation. It is likely that Ex training might increase lean body mass instead of subcutaneous adipose tissues. In contrast, the E2 replacement suppressed body weight compared with the OVX rats by reducing both visceral and inguinal fat accumulations. In addition, PF partially compensated the

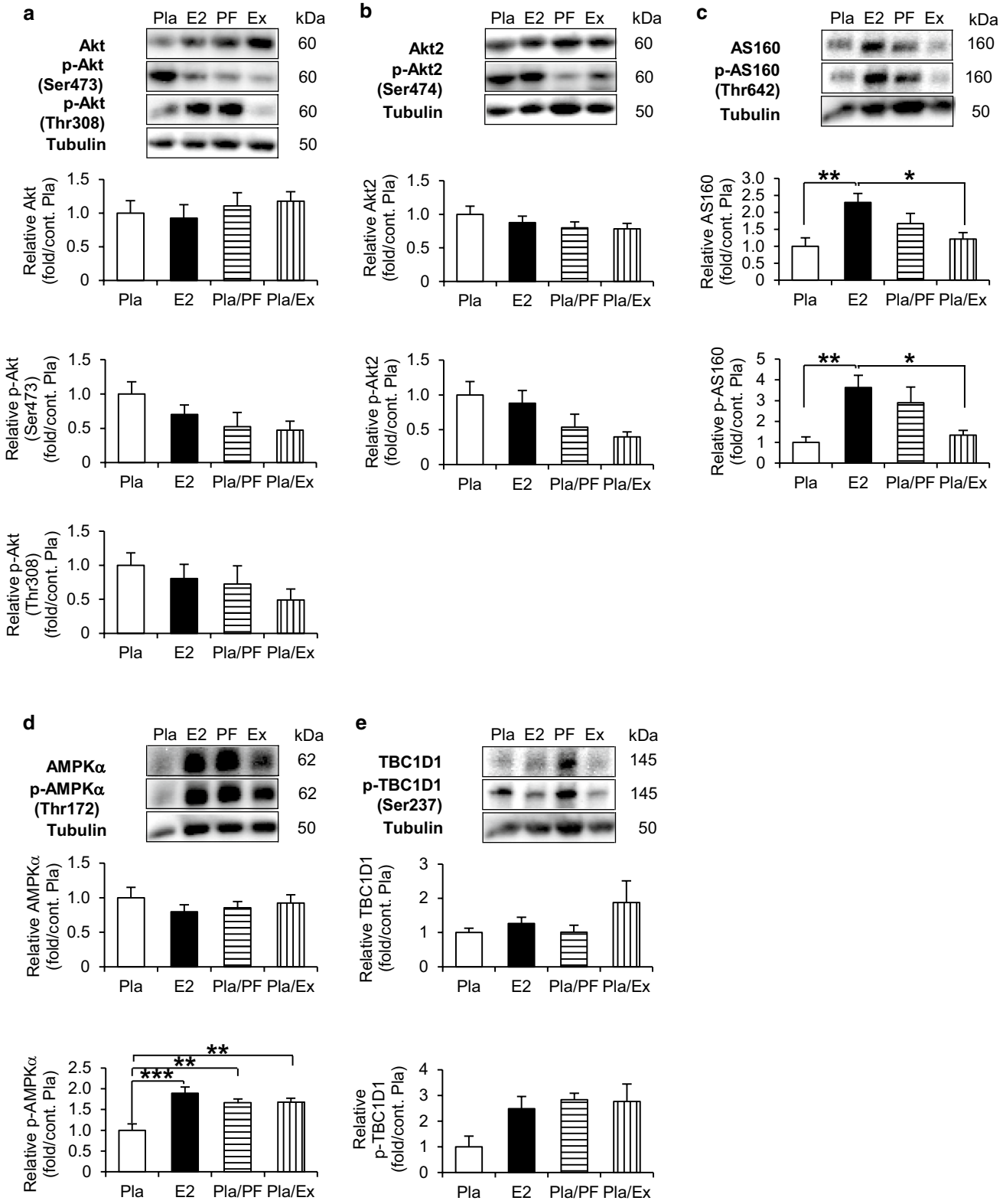


Fig. 4 Representative blots and relative values of protein kinase B (Akt) and phospho (p)-Akt Ser^{473}, and p-Akt Thr^{308} (**a**), Akt2 and p-Akt2 Ser^{474} (**b**), Akt substrate of 160 kDa (AS160) and p-AS160 Thr^{642} (**c**), AMPKα and p-AMPKα Thr^{172} (**d**), and TBC1D1 and p-TBC1D1 Ser^{237} (**e**) in the mesenteric adipose tissues of rats in the placebo (Pla, $n=6$)-, the 17β-estradiol (E2, $n=6$)-treated, the placebo/pair-feeding (Pla/PF, $n=6$), and the placebo/exercise (Pla/Ex, $n=6$) groups. Data are expressed as means ± SE and were analyzed by one-way ANOVA. This was followed by a post hoc Tukey's HSD test. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$, differences between the two groups

suppressive effect of E2 replacement on body weight gain in the OVX rats. In our previous study using a radiotelemetry system [32, 33], we confirmed that the 24-h locomotor activities of freely moving rats did not differ between Pla and E2 groups (24-h average: 2.40 ± 0.39 counts/min vs. 2.31 ± 0.14 counts/min in Pla and E2 groups, respectively). Further study is required to confirm the locomotor activity of rats in the PF or Ex group. Therefore, E2 replacement may suppress body weight gain not only by reducing energy intake, but also by enhancing the energy metabolism in OVX rats. These findings are at least partially consistent with several previous studies that demonstrated a direct effect of estrogen on the energy metabolism [40–42], and with some other studies, showing that the anorexigenic effect of estrogen was a major contributor to the suppression of adiposity and body weight [43, 44].

The present study shows that 4-week E2 replacement or 5-week Ex training in OVX rats reduced the basal level of plasma glucose without affecting plasma insulin levels. This result was inconsistent with previously reported findings that resting basal levels of both insulin and glucose were not different among OVX, E2-treated, and endurance Ex-trained OVX rats [17, 27]. In contrast, our previous study using male rats showed that the resting levels of blood glucose in Ex-trained rats were lower than those in untrained rats [45]. These discrepancies may depend on experimental conditions: notably, intensity and duration of Ex training, conditions for blood sampling, dose of estrogen replacement, or period after OVX. In our study design, an intensity of the Ex training on a treadmill (17 m/min) might be moderate, because previous investigations have chosen low-intensity (8 m/min) or high-intensity (28 m/min) treadmill running to train female Sprague-Dawley rats [35] based on the finding that a running speed at 8 m/min and 28 m/min in female rats elicited ~ 45% and ~ 75% of maximal O_2 uptake, respectively [34]. Additionally, in this study, blood was collected under 16-h fasting conditions from cardiac puncture 4 weeks after E2 replacement and 5 weeks after Ex training started in the OVX rats. Therefore, the duration of each intervention and the moderate intensity of Ex training may be appropriate to cause differences in basal plasma glucose levels.

In our study design, a 3-week-recovery duration was required after OVX and before the Ex training to achieve stable low levels of plasma E2. This was needed to evaluate the effects of Ex training in the OVX rats characterized by low plasma E2 levels, similar to postmenopausal women. Therefore, the present results suggest that Ex training can restore the developed hyperglycemia in the OVX rats. These findings showed the effectiveness of Ex training as an alternative treatment for postmenopausal women. In contrast, rats in the E2 group were administered E2 replacement for 4 weeks after a 4-week-recovery period from OVX to ensure that the plasma levels were stabilized at moderately high levels of E2 (136.9 ± 25.4 pg/ml), as seen in a postmenopausal model replaced by E2, which were within the physiological range for intact female rats in proestrus reported in previous studies [46, 47].

To assess the anorexigenic effect of E2 replacement on glucose homeostasis, we included a Pla/PF group of rats in our experiments. Food restriction by PF in the Pla/PF group ameliorated hyperglycemia in the OVX rats, but failed to mimic the effects of E2 replacement on signal pathway components mediating glucose transport. E2 increased Akt2 and AS160 protein levels, their phosphorylation, and AS160 mRNA level, but PF increased only phospho-Akt2. These findings show that the effects of E2 replacement on the transcriptional upregulation of AS160 were not mediated by PF-induced metabolic changes in OVX rats, suggesting direct E2 action, most likely via the estrogen receptor. On the other hand, a previous study reported that even in obese male Zucker rats, food restriction throughout the first year of life did not alter the development of hyperplastic obesity and insulin resistance [48].

Our study did not determine how OVX induces glucose intolerance, as our experiment did not include a group of sham-operated rats. However, the fact that E2 replacement restored the Akt2/AS160 pathway suggests that OVX impairs the signal pathway that mediates glucose transport. Unlike E2 replacement, Ex had no activating effect on the Akt/AS160 pathway in the OVX rats. Alternatively, the present study revealed that Ex training enhanced the TBC1D1/GLUT4 pathway in the muscle of the OVX rats, and improved hyperglycemia similar to E2 replacement.

Recent studies have reported the effects of Ex training on the signal pathway components, especially GLUT4 in OVX rats [17, 26, 27]. These findings were inconsistent, because it was reported that chronic Ex increased the GLUT4 protein levels of skeletal muscles from OVX rats [17], that Ex reduced the mRNA expression of GLUT4 in gastrocnemius [27], or that it had no effects on GLUT4 protein level in hindlimb muscles of OVX rats [26].

Here, we have provided evidence for the first time that Ex training enhances basal levels of phosphorylated TBC1D1 Ser^{237}, as well as GLUT4 protein, in the gastrocnemius muscle of OVX rats (18 h after final training session). Actually, TBC1D1 abundances did not differ from AS160 among multiple rat muscles with divergent fiber type profiles, including the soleus, EDL, and tibialis anterior muscles [19].

We did not clarify why GLUT4 protein was increased in the Pla/Ex group without increased mRNA levels. There is some controversy as to the mechanism for the Ex-induced increase of GLUT4 protein levels; however, the majority of studies reported increased GLUT4 protein levels rather than mRNA levels. Gurley et al. reported that voluntary wheel running Ex increased muscle GLUT4 protein levels and improved fasting plasma insulin, but did not increase muscle GLUT4 mRNA in high-fat diet-induced obese mice, suggesting that

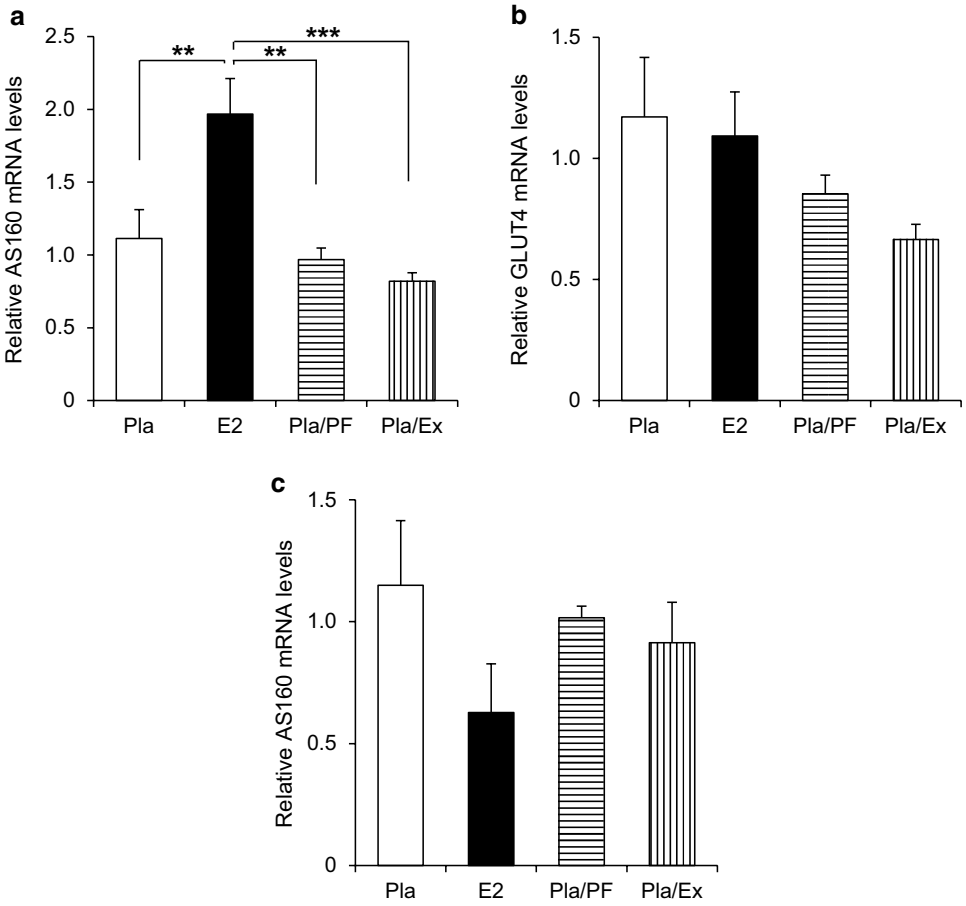


Fig. 5 The relative values of Akt substrate of 160 kDa (AS160) (**a**) and GLUT4 (**b**) mRNA levels in the gastrocnemius, and AS160 mRNA levels in the mesenteric adipose tissue (**c**) of rats in the placebo (Pla, $n=6$)-, the 17β-estradiol (E2, $n=6$)-treated, the placebo/pair-feeding (Pla/PF, $n=6$), and the placebo/exercise training (Pla/Ex, $n=6$) groups. Data are evaluated as $2^{-\Delta\Delta C_t}$ using β-2M as a housekeeping gene and expressed as means ± SE. One-way ANOVA followed by a post hoc Tukey's HSD test. $^{**}P<0.01$ and $^{***}P<0.001$, difference between the two groups

a post-transcriptional mechanism regulated muscle GLUT4 protein expression in response to Ex [49]. Similarly, a post-transcriptional mechanism might explain our results, showing an Ex training-induced increase in muscle GLUT4 protein expression in OVX rats. Our data suggest the E2 upregulates AS160 gene expressions most likely by the transcriptional activation function of estrogen receptor (ER) and at least partially by autoregulation of ER mRNA stabilities [50]. Taken together, the cellular mechanism underlying the beneficial effects of endurance Ex on the plasma glucose level might be distinct from that of E2 replacement.

In summary, this is a report showing endurance running Ex training which improves OVX-induced hyperglycemia and HOMA-IR, an indicator for insulin resistance, via activation of the TBC1D1/GLUT4 pathway in gastrocnemius by an alternative mechanism from action of E2 replacement or PF diet. Further study is required to identify the effects of endurance Ex training on insulin- and contraction-stimulated glucose uptake and signaling pathways, on the basis of comparison with the effects of E2 replacement. Our results provide insights into the alternative effects of endurance Ex training on glucose metabolism under reduced estrogen function in postmenopausal women.

Author contribution Concept/design: KM and MK; acquisition of data: MK, KM, and NY-N; data analysis and interpretation: KM and MK; drafting of the manuscript: KM, MK, and AT; critical revision of the manuscript: KM, MK, and AT; approval of the article: all authors.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study were in accordance with the guidelines on the use and care of laboratory animals as put forward by the Physiological Society of Japan and under the control of the Ethics Committee of Animal Care and Experimentation, Nara Women's University, Japan.

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