Estradiol replacement improves high-fat diet-induced obesity and inslin resistance in postmenopausal model rats

Contents

Chapter 3

Effects of estradiol replacement on the AMP kinase pathway in skeletal muscle of ovariectomized rats fed on high-fat diet

General abstract

Menopause is a risk factor for metabolic syndrome and type 2 diabetes. However, the role of estrogen in this context remains unclear. This study aims to investigate the effects of estrogen replacement on high-fat diet (HFD; 60.0% energy from fat, predominantly lard)-induced obesity and insulin resistance in postmenopausal model rats. These thesis consists of 3 chapters.

In chapter 1, I investigated the effects of 17β-estradiol (E2) replacement on the orexigenic action of ghrelin in ovariectomized (OVX) obese rats fed with a HFD. Several studies have reported that the regulatory effects of estrogen on food intake, energy balance, and fat accumulation are mediated through its interaction with orexigenic and anorexigenic hormones. I have focused on the effects of E2 on ghrelin action via ghrelin receptor (growth hormone secretagogue receptor; GHSR) in the stomach, especially under HFD feeding in OVX rats. In this study, I used OVX Wistar rats subcutaneously implanted with either E2 or placebo (Pla) pellets and started them on HFD feeding 4 weeks before the experiments. HFD increased energy intake and body weight in the Pla group, while it temporarily reduced these in the E2 group. Growth hormone-releasing peptide-6, a GHSR agonist injected intraperitoneally, enhanced HFD intake and activated neurons in the hypothalamic arcuate nucleus only in the Pla group. Furthermore, gastric ghrelin and GHSR protein levels were lower in the E2 group than in the Pla group, but plasma acyl ghrelin levels were similar in both groups. These results suggest that E2 replacement improves obesity by inhibiting the orexigenic action of ghrelin via downregulation of ghrelin and its receptor in stomach in HFD-fed OVX rats.

In chapter 2, I examined the effects of E2 replacement on whole-body insulin sensitivity and insulin signaling in HFD-fed OVX rats. HFD is associated not only with obesity but also with insulin resistance. However, the mechanisms underlying HFD-

impaired insulin sensitivity are not fully understood. Moreover, our previous study showed that E2 replacement restored the insulin sensitivity by enhancing activation of the protein kinase B (Akt) 2 and its substrate of 160 kDa (AS160) pathway in the gastrocnemius muscle of OVX rats fed a normal standard diet (SD). However, it remains unclear whether HFD impairs the insulin sensitivity and Akt2/AS160 pathway more than SD or whether E2 restores the HFD-induced impairment in OVX rats. I evaluated the effects of E2 replacement on whole-body insulin sensitivity using an intravenous glucose tolerance test under free-moving conditions and *in vivo* insulin-stimulated activation of the Akt2/AS160 pathway in the gastrocnemius muscles and livers of HFD-fed OVX rats. The results showed that HFD reduced insulin sensitivity only in the Pla groups, resulting in the difference between the Pla and E2 groups of HFD-fed rats. In addition, HFD disturbed the insulin-stimulated phosphorylation of Akt2 and AS160 in the muscles of rats in the Pla group, but not of rats in the E2 group. These findings suggest that E2 replacement improves HFD-impaired insulin sensitivity by activating the Akt2/AS160 pathway in the insulin-stimulated muscles of OVX rats.

In chapter 3, I investigated the protective effects of E2 replacement on AMP kinase (AMPK)-TBC1 (Tre2, BUB2, CDC16) domain family member 1 (TBC1D1)/glucose transporter 4 (GLUT4) pathway in gastrocnemius muscles of HFD-fed obese OVX rats. It is presently unclear whether HFD influence the AMPK signaling pathway, primarily recognized as insulin-independent glucose uptake pathway, or whether E2 replacement increases the signaling proteins and their activations in the muscle of OVX rats. The E2 replacement and HFD feeding did not affect the AMPKα, TBC1D1, and GLUT4 protein levels and the phosphorylation of AMPKα or TBC1D1. These results suggest that E2 replacement and HFD intake have no effect on the AMPK pathway in the muscle of OVX

rats.

In conclusion, the present studies revealed that E2 replacement improved HFD-induced obesity by inhibiting the orexigenic action of ghrelin via downregulation of ghrelin and its receptor in stomach, and restored HFD-induced impairment of insulin sensitivity by activating Akt2/AS160 pathway in the muscles in HFD-fed OVX rats. These results provide insight into the beneficial effects of E2 replacement against HFD-induced obesity and insulin resistance in postmenopausal women.

Chapter 1

Estradiol replacement improves high-fat diet-induced obesity
by suppressing the action of ghrelin in ovariectomized rats

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Abstract

This study aims to investigate the effects of estradiol replacement on the orexigenic action of ghrelin in ovariectomized (OVX) obese rats fed with a high-fat diet (HFD). Four weeks after OVX at 9 weeks of age, Wistar rats were subcutaneously implanted with either 17β-estradiol (E2) or placebo (Pla) pellets and started on HFD feeding. After 4 weeks, growth hormone-releasing peptide (GHRP)-6, a growth hormone secretagogue receptor (GHSR) agonist injected intraperitoneally, induced changes in HFD intake, and c-Fos-positive neurons in the hypothalamic arcuate nucleus (ARC) were measured in both groups. The ghrelin protein and mRNA levels, as well as GHSR protein in stomach, were analyzed by Western blotting and real-time PCR. HFD increased energy intake and body weight in the Pla group, while it temporarily reduced these in the E2 group. GHRP-6 enhanced HFD intake and activated neurons in the ARC only in the Pla group. Furthermore, gastric ghrelin and GHSR protein levels were lower in the E2 group than in the Pla group, but plasma acyl ghrelin levels were similar in both groups. Our results suggest that E2 replacement improves obesity by inhibiting the orexigenic action of ghrelin via downregulation of ghrelin and its receptor in stomach in HFD-fed OVX rats.

1. Introduction

Obesity, defined as excessive fat accumulation, has become a worldwide epidemic [1]. Overweight and fat accumulation are associated with many adverse health outcomes, including type 2 diabetes, stroke, and heart disease [2]. Some studies have shown that dietary composition, particularly high-fat diet (HFD), promotes obesity in humans [3] and mice [4]. Furthermore, ovarian hormones play an important role in the control of food intake and body weight in female mammals [5, 6]. The onset of menopause is accompanied by increases in energy intake and appetite and a decrease in energy consumption, followed by an increase in total body fat and visceral adipose tissue mass [7, 8]. Hormone replacement therapy (HRT) attenuates body weight gain and fat accumulation in postmenopausal women [9, 10]. However, to our knowledge, the effect of menopause or HRT in women consuming HFD remains unclear. Moreover, previous studies using ovariectomized (OVX) rats, an animal model widely used for studying the pathology of human menopause, showed that 17β-estradiol (E2) replacement suppresses body weight gain and fat accumulation and enhanced whole-body energy consumption not only under a normal standard diet (SD) [11, 12] but also under HFD [13]. HFD-induced obesity in OVX rats shares several features with diet-related obesity in postmenopausal women. It is plausible that E2 replacement improves obesity through its regulatory effects on food intake, even under conditions where HFD is easily accessible. However, the mechanism underlying the anorexigenic effect of E2 replacement on HFD intake is yet to be clarified.

Recently, several studies have reported that the regulatory effects of E2 on food intake, energy balance, and fat accumulation are mediated through its interaction with orexigenic and anorexigenic hormones [14, 15]. Ghrelin is a candidate whose orexigenic

action is regulated by E2 [16]. Ghrelin, a brain-gut peptide hormone secreted from the stomach, stimulates food intake via the action on its receptor, the growth hormone secretagogue receptor (GHSR) [17, 18]. Ghrelin is mainly synthesized in X/A-like endocrine cells of the gastric oxyntic mucosa [19]. GHSR is produced in the neurons of the vagus nerve ganglion and transported to nerve endings in the stomach, where it binds to ghrelin [20, 21]. Orexigenic signals from ghrelin are transmitted to the brain via either a neural or a hormonal pathway, or through both [22]. The neural pathway consists of afferent vagal fibers that pass through the nodose ganglia and terminate in the nucleus tractus solitarius located in the brainstem to transmit their signals ultimately to the hypothalamus. A previous study suggested that the vagal afferent nerve-dependent pathway was involved in peripherally injected ghrelin-induced increase in food intake and neural activation in the hypothalamic arcuate nucleus (ARC), a key center regulating food intake and satiety [20]. ARC contains orexigenic neurons expressing the neuropeptide Y (NPY) and agouti-related protein (AgRP) and anorexigenic neurons expressing proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) [23]. Peripheral ghrelin increases the number of c-Fos-positive neurons in the NPY/AgRP neurons in the ARC in rodents [24, 25]. Because intranuclear c-Fos is the product of the immediate-early-gene c-fos, an established marker of changes in neuronal activity in response to stimuli in rodents [26, 27], these findings indicate that ghrelin activates orexigenic neurons in ARC, resulting in an increase in food intake. The hormonal pathway also may reach the hypothalamus directly via the blood circulation, but details of the route remain unclear.

A recent study reported that female GHSR-null mice accumulated less food intake, body weight, and adiposity than wild-type mice when given HFD [28]. Additionally,

OVX increased food intake and body weight in wild-type female mice, but these effects were not observed in the GHSR-null mice [16]. A previous study also reported that 7-days subcutaneous treatment with a GHSR antagonist, [D-Lys^3]-growth hormone-releasing peptide (GHRP)-6, decreased food intake in OVX mice fed with either HFD or SD, and the antagonistic effect was reduced after E2 supplementation [29]. Taken together, GHSR might be involved in the inhibitory effects of E2 on food intake and body weight gain. In contrast, it has been reported that a change in plasma estrogen level during estrous cycle had no effect on GHSR mRNA expression at least in the ARC of intact female rats, despite the fact that the orexigenic effect and the c-Fos-inducing effect of intracerebroventricular ghrelin were influenced by the estrous cycle phase [30]. Therefore, the mechanism accounting for the anorexigenic effects of E2 associated with GHSR or the action site of E2 regulating GHSR remains unclear. We have focused on the effects of E2 on ghrelin action via GHSR in the stomach, especially under HFD feeding in OVX rats. Most of the previous studies, which demonstrated the inhibitory effects of estrogen on ghrelin's eating-stimulatory action, were performed using SD-fed rodents. To our knowledge, no previous report has investigated the mechanisms accounting for the inhibitory effect of E2 replacement on HFD-induced obesity in OVX rats.

The aim of this study is to determine whether chronic E2 replacement in physiological dosage attenuates HFD intake by suppressing the orexigenic action of ghrelin through the ghrelin-GHSR-ARC pathway in HFD-fed OVX rats. Moreover, we examined the effects of E2 replacement on intraperitoneal (IP) GHSR agonist, GHRP-6-induced orexigenic response, and neural activation in ARC, on plasma acyl ghrelin concentrations, and on ghrelin protein, ghrelin mRNA, and GHSR protein levels in the stomach in order to clarify the underlying mechanism of estrogen action in OVX rats.

2. Materials and Methods

2. 1. Animals and Diets

Animal procedures were approved by The Nara Women's University Committee on Animal Experiments (No. 17-02) and were conducted in accordance with the Standards relating to the Care and Keeping and Reducing Pain of Laboratory Animals (Notice of the Ministry of the Environment, Government of Japan) and ARRIVE guidelines. In total, 88 female Wistar rats (CLEA Japan, Inc., Tokyo, Japan) were used in this study. The rats were individually housed in standard polycarbonate cages containing paper bedding under controlled temperature and light conditions (26 ± 1 °C, a 12:12-h light-dark cycle, with lights on at 7:00 a.m.). Tap water and rodent chow were provided ad libitum. All surgeries were performed while the rats were under anesthesia (pentobarbital sodium; 25-40 mg/kg IP or isoflurane; 1.5-2.0% in oxygen).

Nine-week-old female rats fed on SD (MF; Oriental Yeast, Tokyo, Japan) were ovariectomized followed by E2 or placebo (Pla) replacement as previously described [12, 31]. In brief, after a 4-week recovery period, the rats were assigned randomly to either the Pla (n = 48)- or the E2 (n = 40)-treated group and were subcutaneously implanted with either E2 (1.5 mg/60-day release) or Pla pellets (Innovative Research of America, Sarasota, Florida, USA). Additionally, HFD (F2HFD2; Oriental Yeast) containing 515.7 kcal per 100 g (60.7% energy from fat, predominantly lard) was started the day after the replacement and was continued until the cessation of the experiments for HFD-fed rats in both groups. The SD-fed rats continued to receive SD, which contained 360.0 kcal per 100 g (13.2% energy from fat). Food intake and body weight were measured daily during the experiments.

2. 2. Experimental Protocols

After 4 weeks of replacement therapy, the subsets of 17-week-old rats were used for IP injection of GHRP-6 (Sigma-Aldrich, St. Louis, Missouri, USA) (Pla, n = 13; E2, n = 14). One week after the experiment, the rats were used in immunohistochemical analysis (Pla, n = 9; E2, n = 9). A separate set of 17-week-old rats was used for blood sampling for measurements of plasma acyl ghrelin and for stomach sampling for ghrelin protein, ghrelin mRNA, and GHSR protein analyses (Pla, n = 9-11; E2, n = 8-10). Furthermore, the wet weights of the intra-abdominal (mesenteric, kidney-genital, and retroperitoneal) and subcutaneous (inguinal) adipose tissues were measured (Pla, n = 34; E2, n = 26).

2. 3. IP Injection of GHRP-6

The experiment to examine the effects of GHRP-6 was performed 4 weeks after the replacement using satiated rats with free access to HFD. HFD intake was measured hourly from 2 h before and up to 4 h after GHRP-6 injection. The rats were injected intraperitoneally either with GHRP-6 dissolved in saline (80 nmol/kg or 400 nmol/kg) or saline at 11:00 a.m. The animals had free access to water during the experiments.

2. 4. Immunohistochemistry

The rats aged 18 weeks were injected intraperitoneally either with GHRP-6 dissolved in saline (400 nmol/kg) or saline followed by 3-h fasting. At 3 h after the injection, the rats were deeply anesthetized with sodium pentobarbitone (45 mg/kg, IP) and transcardially perfused with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in a 0.1 M-phosphate buffer (pH 7.4) for fixation. The removed brains were post-fixed in fixative at 4 °C and then immersed in PBS containing 15% sucrose for

1 day, followed by 25% sucrose for 2 days for cryoprotection. Then, 30-μm coronal sections of the brain were prepared using a cryostat (Leica CM3050 S; Leica Biosystems, Wetzlar, Germany). c-Fos immunohistochemical staining in the ARC was performed using a rabbit c-Fos antibody (dilution 1:4000, Sc52; Santa Cruz Biotechnology, Dallas, Texas, USA) as previously described [32]. The sections were incubated in biotinylated goat anti-rabbit IgG (dilution 1:400, BA-100; Vector Laboratories, Burlingame, California, USA), followed by ABC Elite Kit solution (dilution 1:400, Vector Laboratories). Visualization of the antibodies was performed using 0.02% 3,3-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) and 0.01% H_2O_2 in 50 mM Tris HCl buffer (pH 7.4). The sections were mounted on gelatin-coated glass slides, dehydrated with graded ethanol, cleared with Lemosol®, and coverslipped. Four consecutive sections containing ARC (in between −2.16 to −2.28 mm from the bregma) were observed using a microscope (Olympus BX-50; Olympus Corporation, Tokyo, Japan), and images were obtained using a cooled charge-coupled device camera (Micropublisher RTV 5.0; QImaging, Surrey, British Columbia, Canada). The ARC region was identified using the rat brain stereotaxic atlas [33]. The number of c-Fos-positive neurons in the ARC was counted for each of the four consecutive sections using image analysis software (ImageJ; NIH, Bethesda, Maryland, USA). The results were expressed as the average number of c-Fos-positive neurons per bilateral sections in the ARC.

2. 5. Sampling of Gastric Mucosae and Measurement of Adipose Tissues

After 16 h of fasting, a subset of Pla and E2 rats were euthanized by a pentobarbital sodium overdose. Mucosa from upper and middle portions of the stomach was harvested

between 10:00 a.m. and 2:00 p.m. The mucosae for GHSR and ghrelin protein analysis were immediately frozen in liquid nitrogen before being stored at −50 °C until further processing. The tissues were stored in RNA stabilization solution until RT-qPCR analysis for ghrelin was performed. Upon completion of the experiments, the rats were sacrificed by a pentobarbital sodium overdose, and wet weights of the intra-abdominal (mesenteric, kidney-genital, and retroperitoneal) and subcutaneous (inguinal) adipose tissues were measured. The total weight of the visceral adipose tissues was calculated as the sum of the intra-abdominal fat weights.

2. 6. Immunoblotting

Isolated gastric mucosae were immediately homogenized in homogenization buffer, as previously described [12]. The homogenates were centrifuged at 15,000× *g* for 30 min at 4 °C. Sodium dodecyl sulfate (SDS) samples containing equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and immunoblotted using a PVDF membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) with the following antibodies: Antibodies for ghrelin (1:2000) and the β-actin (1:2000) from Sigma-Aldrich, and GHSR (1:1000) from Abcam (Cambridge, Massachusetts, USA). The primary antibodies were detected with anti-rabbit (Promega, Madison, Wisconsin, USA) or anti-chicken (Abcam) IgG horseradish peroxidase-conjugated secondary antibody. An enhanced chemiluminescence (ECL; GE Healthcare Life Sciences) system was used for protein detection. Imaging and densitometry were performed using the Ez-Capture imaging system (ATTO, Tokyo, Japan) and the CS Analyzer image processing program (ATTO).

2. 7. RNA Isolation and RT-qPCR

Total RNA was extracted using the TRI Reagent Solution (Ambion, Austin, Texas, USA) according to the manufacturer's protocol. The amount of total RNA extracted was determined, and its purity (absorption ratio of optical density at 260 and 280 nm >1.9) was verified spectrophotometrically using a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, Delaware, USA). Then, the cDNA was synthesized using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, California, USA). RT-qPCR was performed using a StepOne Software v2.1 system (Applied Biosystems). The commercially available TaqMan Gene Expression Assay (Applied Biosystems) for ghrelin (Rn00572319_m1) and β-actin (Rn00667869_m1) was used in this study. For the analysis, gene expression levels of ghrelin were normalized using β-actin 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 60 °C for 20 s. No amplification of fragments occurred in the control samples without reverse transcriptase. The mRNA quantity was calculated using the ΔΔCt (comparative Ct) method under the assumption that the primer efficiencies were relatively similar.

2. 8. Assessment of Plasma Acyl Ghrelin Level

Freely moving rats aged 16 weeks in separate sets of Pla (n = 9) and E2 groups (n = 8) were catheterized with venous cannula, as previously described [34]. Blood sampling was performed on the 5th-day post-cannulation. After 16 h of fasting and 1 h of rest after connecting the cannula with the extension tube that allowed free movement, a blood sample (0.2 ml) was collected from the cannula between 11:00 a.m. and 1:00 p.m. and

harvested in plastic tubes containing 50 mg/ml ethylenediamine tetraacetic acid. The plasma was immediately treated with 1/10 volume of 1 mol/l HCl and stored at −45 °C until assayed for acyl ghrelin using an active ghrelin ELISA kit that recognizes *n*-octanoylated ghrelin (Mitsubishi Chemical Medience Corporation, Tokyo, Japan).

2. 9. Statistical Analyses

All values are expressed as means ± standard error (SE). Two-way repeated measures ANOVA for each pair-wise comparison among the total four (Pla-SD, Pla-HFD, E2-SD, and E2-HFD) groups, followed by a Tukey-Kramer post hoc test was used to analyze the effect of E2 or HFD on the course of change in food intake, energy intake, body weight, and the effect of E2 or GHRP-6 on the course of change in HFD and cumulative HFD intakes. Two-way factorial ANOVA followed by a Tukey-Kramer post hoc test was used to analyze the effect of E2 on HFD-induced change in wet weights of adipose tissues and the effect of E2 on GHRP-6-induced change in the number of c-Fos-positive neurons between the groups. The ghrelin, GHSR proteins, and ghrelin mRNA levels in the stomach, and plasma acyl ghrelin between the Pla and E2 (HFD-fed rats) groups were compared using unpaired *t*-test. We considered a value of $p < 0.05$ to be statistically significant.

3. Results

3. 1. Characterization of the Studied Rats

Regardless of diet, food intake remarkably decreased in the E2 group compared with the Pla group at 14 weeks of age one week after the E2 replacement and returned to control levels as indicated by the Pla group at 17 weeks of age (Figure 1A). HFD

decreased food intake between 14-17 weeks of age, compared with SD, only in the E2 group. In contrast, HFD increased energy intake between 14-16 weeks of age, compared with SD, in the Pla group. Therefore, the energy intake from HFD during this period was significantly higher in the Pla group compared to the E2 group, but not in the two SD-fed groups (Figure 1B).

HFD increased body weight compared with SD in the Pla group between 15-17 weeks of age, but not in the E2 group, resulting in the difference between the two groups (Figure 1C). The wet weights of total visceral and inguinal subcutaneous adipose tissues (Figure 1D) were significantly increased by HFD in the Pla group, but not in the E2 group. Taken together, E2 replacement suppressed body weight gains and fat accumulation by adjusting the energy intake.

3. 2. Effect of the IP GHRP-6 Injection on HFD Intake

GHRP-6 at a dose of 80 nmol/kg had no effect on HFD intake in the Pla and E2 groups (Figure 2A). However, GHRP-6 at a dose of 400 nmol/kg enhanced HFD intake at 60 min after the injection compared with saline only in the Pla group but not in the E2 group ($p < 0.01$) (Figure 2B). Figure 2C shows that IP injection of GHRP-6 at a dose of 400 nmol/kg enhanced cumulative HFD intake for 120 min after the injection only in the Pla group ($p < 0.01$) but not in the E2 group, resulting in a difference in the GHRP-6-induced response between the two groups (interaction, $p < 0.01$).

3. 3. In vivo Effects of E2 Replacement on Ghrelin and GHSR in the Stomach

To examine the molecular mechanism accounting for the inhibitory effect of E2 on the orexigenic action of ghrelin in the OVX rats, we investigated ghrelin and GHSR

protein levels in the gastric mucosa. The E2 group showed significantly suppressed ghrelin ($p < 0.05$) and GHSR ($p < 0.01$) protein levels compared with the Pla group (Figure 3A, B). In contrast, the levels of ghrelin mRNA in the gastric mucosa were not different between the two groups (Figure 3C). As shown in Figure 3D, the plasma acyl ghrelin concentrations after 16 h of fasting at 17-weeks-old were similar between the two groups.

3. 4. c-Fos-positive Neurons in the ARC Induced by IP Injection of GHRP-6

The injection of GHRP-6 at a dose of 400 nmol/kg enhanced the number of the c-Fos-positive neurons in the ARC in the Pla group compared with the injection of saline ($p < 0.05$), but it did not reach significance ($p = 0.05$) in the E2 group. Furthermore, the number of c-Fos-positive neurons after the injection was significantly lower in the E2 group compared with the Pla group ($p < 0.05$; Figure 4).

4. Discussion

The present study shows that E2 replacement reduces energy intake from HFD by suppressing orexigenic effects of ghrelin via downregulations of ghrelin and GHSR proteins levels in the stomach of HFD-fed OVX rats.

In this study, E2 replacement suppressed the increase in energy intake and body weight gain in HFD-fed OVX rats much more than in SD-fed rats. To our knowledge, our study is the first to show strongly suppressive effects of E2 replacement on HFD-induced energy intake increase and obesity compared with SD-fed OVX rats. On the contrary, HFD-fed OVX mice, despite being more obese, showed slightly lower food intake than the E2-replaced mice [35] or showed similar body weight to the E2-replaced mice under

1-month HFD feeding [36]. These discrepancies may be attributed to the differences in species between mice and rats. In our previous study, we showed that OVX increased food intake and intra-abdominal fat accumulation, which were improved by E2 replacement in the SD-fed OVX rats [12]. In addition, Witte et al. reported that OVX led to hyperphagia in rats but did not in mice and that OVX-induced weight gain in female mice was mediated by reduced locomotor activity and metabolic rate [37]. Therefore, we used rats as experimental animals to investigate the effects of E2 on food intake via ghrelin action.

IP injection of GHRP-6 at a dose of 400 nmol/kg increased the cumulative HFD intake for 120 min by approximately 2.5 times and enhanced the number of c-Fos-positive neurons in the ARC by about two times compared with saline injection in OVX rats. The dose is comparable to one-third of the daily dosage in a previous study showing that the chronic GHRP-6 infusion by a pump increased food intake in male rats [38]. Additionally, the administration of 3 nmol/rat (250-300 g) ghrelin (IP) increased food intake threefold [39] and the number of c-Fos-positive neurons in the ARC twofold compared with saline in male rats [40]. Therefore, a GHRP-6 dose of 400 nmol/kg may induce a comparable response in OVX rats to approximately 10 nmol/kg ghrelin in male rats. In contrast, a GHRP-6 dose of 80 nmol/kg had no effect on food intake in both groups. This dose is likely insufficient to produce an orexigenic neural activation in the ARC. In addition, the 400 nmol/kg dose-induced increase of food intake in the OVX group was inhibited completely in the E2 group. Therefore, we used only the 400 nmol/kg dose of GHRP-6 for the immunohistochemical analysis to evaluate the neural activation indicated by the increased number of c-Fos-positive neurons in the ARC, which regulate food intake.

Our results suggest that E2 replacement may regulate GHSR protein in the stomach

in OVX rats. A recent study using GHSR-null mice reported that the abundance of GHSR-expressing neurons in the nodose ganglion is critical for peripheral ghrelin administration-induced hyperphagia via the vagus nerve [41]. A presumable target site of E2 may be the cell bodies of the vagal nodose ganglion, but no report has investigated the relationship between E2 and GHSR. Clegg et al. [16] showed that the increases in feeding and body weight in wild-type OVX mice were blunted in GHSR-null mice. This finding suggests a pivotal role of GHSR in OVX-related body weight gain, though the target site of ovarian hormones remains unclear. The present study demonstrates for the first time that E2 replacement suppresses energy intake and obesity via the reduction of GHSR protein level in the stomach of HFD-fed OVX rats. A previous report has shown that the estrogen receptor (ER) exists in vagal nodose ganglionic neurons of the rat [42]. Therefore, estrogen may have affected GHSR gene expression through ER in the vagal afferent neurons. Further studies are required to elucidate whether the ERs co-localize with GHSR in the nodose ganglionic neurons of vagal afferent nerve and downregulate GHSR mRNA followed by reduction of GHSR protein level in the stomach. Additionally, we consider the possibility that the reduction of GHSR level in the stomach of E2-replaced rats is a secondary effect caused by the decreased gastric ghrelin levels observed in this study.

Previous studies have suggested that gastric vagal afferent is the major pathway involved in peripherally injected ghrelin-induced increase in food intake and c-Fos immunoreactivity in the ARC [20, 43]. Most of the ghrelin actions in ARC are realized via the NPY/AgRP neurons pathway [18], and simultaneously, ghrelin suppresses anorexigenic POMC/CART neurons [44]. The present study has shown that E2 replacement suppressed IP GHRP-6-induced HFD intake and simultaneous neuronal

activation in the ARC of OVX rats. It is plausible that the c-Fos-positive cells in which the numbers were increased by GHRP-6 in ARC are orexigenic NPY/AgRP neurons. However, further studies are necessary to clarify whether c-Fos-positive cells, which were downregulated by E2 replacement in HFD-fed OVX rats, are NPY/AgRP neurons or others such as tyrosine hydroxylase neurons [45].

Interestingly, E2 replacement reduced ghrelin protein levels in the stomach of OVX rats in the present study. This finding is likely related to the mechanism of suppressive effects of E2 replacement on HFD intake in OVX rats. In contrast, E2 replacement had no effect on the ghrelin mRNA in the stomach of OVX rats. Consequently, these results suggest that the reduction of ghrelin protein is mediated by a post-transcriptional mechanism. In previous studies, Northern blot analysis showed that ghrelin mRNA expression in the stomach increased 3 days after OVX in female rats aged 4 weeks, but not in rats aged 9 weeks [46] and that it was not affected by OVX in adult rats [47]. Another study reported that E2 replacement had no effect on gastric ghrelin mRNA levels in the 4-week-old OVX rats, although OVX increased gastric ghrelin secretion in female rats, which was restored by E2 replacement [48]. Therefore, our results are consistent with these findings, suggesting no effects of OVX or E2 on gastric ghrelin mRNA at least in adult female rats, although the animal models used were different. To our knowledge, this is the first data to show that E2 replacement attenuates ghrelin protein levels in the stomach of the postmenopausal model rats.

The other route for conveying the gastric-derived ghrelin signal to the brain is the blood circulation [22]. Our results suggest that the E2 replacement had no effect on the plasma acyl ghrelin level, although it decreased gastric ghrelin proteins in OVX rats. Whereas, ghrelin is widely expressed in peripheral tissues such as the intestine and

pancreas [22] in addition to the stomach, plasma ghrelin might not depend on the gastric ghrelin level as shown in our result. In addition, the proportion of acyl ghrelin to that of total ghrelin has been reported to be far smaller proportion at 2-5% in the plasma than that at 20-40% in the stomach in rodents [49, 50]. These findings may be reasons why E2 had an inhibitory effect on the gastric ghrelin protein, but not on plasma acyl ghrelin in this study. Moreover, Cleggs et al. [16] found that plasma ghrelin and food intake were transiently increased 2 to 4 weeks after OVX in SD-fed rats. Another study in female rats showed that the plasma ghrelin level increased 3 days after OVX but returned to the basal level 7 days after OVX in 4-week-old rats [46]. Our results showing that 4-week E2 replacement to rats 4 weeks after OVX had no effect on plasma ghrelin level are consistent with these findings. This may be another reason for no clear effects of E2 on plasma acyl ghrelin in this study.

Furthermore, we have to take into account the possibility that the inhibitory effects of E2 on ghrelin action are intermediated by other peptides regulating food intake such as leptin because a conditional deletion of leptin receptor from the vagal afferent neurons failed to protect exogenous ghrelin-induced hyperphagia in female mice [51].

In this study, the rats in the E2 group are considered postmenopausal models replaced with E2 because they were used for the experiment 4 weeks after E2 replacement in a 4-week post-OVX. In addition, the plasma E2 concentration in the E2 group might be within the accepted physiological range, as shown in our previous study (44.1 ± 10.3 and 9.2 ± 0.5 pg/ml in the E2 and Pla groups, respectively) using E2 pellets containing the same dosage as in the present study [12]. Therefore, our results provide the animal data to support that E2 replacement reverses the postmenopausal obesity exacerbated by HFD. Menopause was associated with an increase in total body fat and visceral abdominal fat

[7]. The percentage of energy intake from fat significantly increased in the postmenopausal years, though the decrease in this variable was observed during the menopausal transition [8]. Our results suggest that postmenopausal women need to avoid a HFD and control dietary fats appropriately to prevent obesity.

5. Conclusion

In conclusion, the present study suggests that E2 replacement reverses the HFD-induced increase in energy intake and fat accumulation by suppressing orexigenic ghrelin action via the reduction of GHSR and ghrelin in the stomach of OVX rats. Our findings may provide insight into the mechanism of diet-induced postmenopausal obesity as well as the beneficial effects of HRT against obesity in postmenopausal women. Ghrelin and GHSR in the stomach may be new targets for strategies in medicines and dietary habits to improve postmenopausal obesity.

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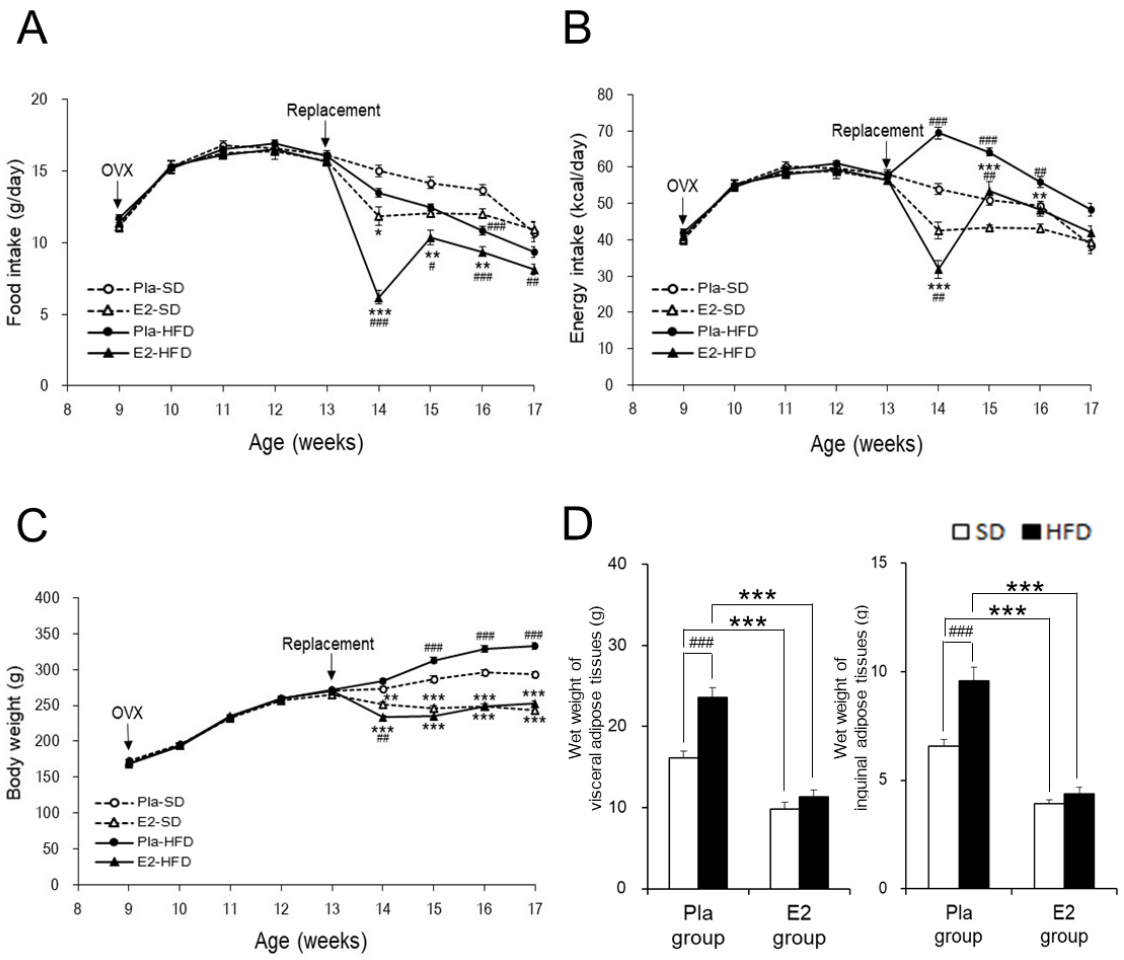

Figure 1. Characterization of rats studied. Data are expressed as means ± SE. Line graphs represent the course of change in mean food intake (A), energy intake (B), and body weight (C) in the placebo (Pla) standard diet (SD) ($n = 18$), 17β-estradiol (E2)-SD ($n = 14$), Pla-high-fat diet (HFD) ($n = 30$), and E2-HFD ($n = 26$) groups of ovariectomized (OVX) rats. Two-way repeated measures ANOVA for each pair-wise comparison among the four groups revealed significant differences in food intake, energy intake, and body weight. There was an interaction of age and group effects in food intake ($p_{\text{Age}\times\text{Group}} < 0.001$: E2-HFD vs. E2-SD or Pla-HFD), energy intake ($p_{\text{Age}\times\text{Group}} < 0.001$: Pla-HFD vs. Pla-SD or E2-HFD) and body weight ($p_{\text{Age}\times\text{Group}} < 0.05$: Pla-SD vs. Pla-HFD, $p_{\text{Age}\times\text{Group}} < 0.01$: Pla-SD vs. E2-SD, and $p_{\text{Age}\times\text{Group}} < 0.001$: PlaHFD vs. E2-HFD). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, differences between the E2 and Pla groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, differences between the SD and HFD groups. Bar graphs represent wet weights of visceral (the sum of the mesenteric, kidney-genital, and retroperitoneal adipose tissue weights) and inguinal adipose tissues (D) at 17 week of age in the Pla-SD ($n = 18$), the Pla-HFD ($n = 16$), the E2-SD ($n = 14$), and the E2-HFD ($n = 12$) groups. Data were analyzed by two-way factorial ANOVA, followed by a post hoc Tukey-Kramer test. There was an interaction of diet and group effects in the wet weights of visceral ($p_{\text{Diet}\times\text{Group}} < 0.01$) and inguinal adipose tissues ($p_{\text{Diet}\times\text{Group}} < 0.01$). $^{***}p < 0.001$, differences between the E2 and Pla groups; $^{\#\#\#}p < 0.001$, differences between the SD and HFD groups.

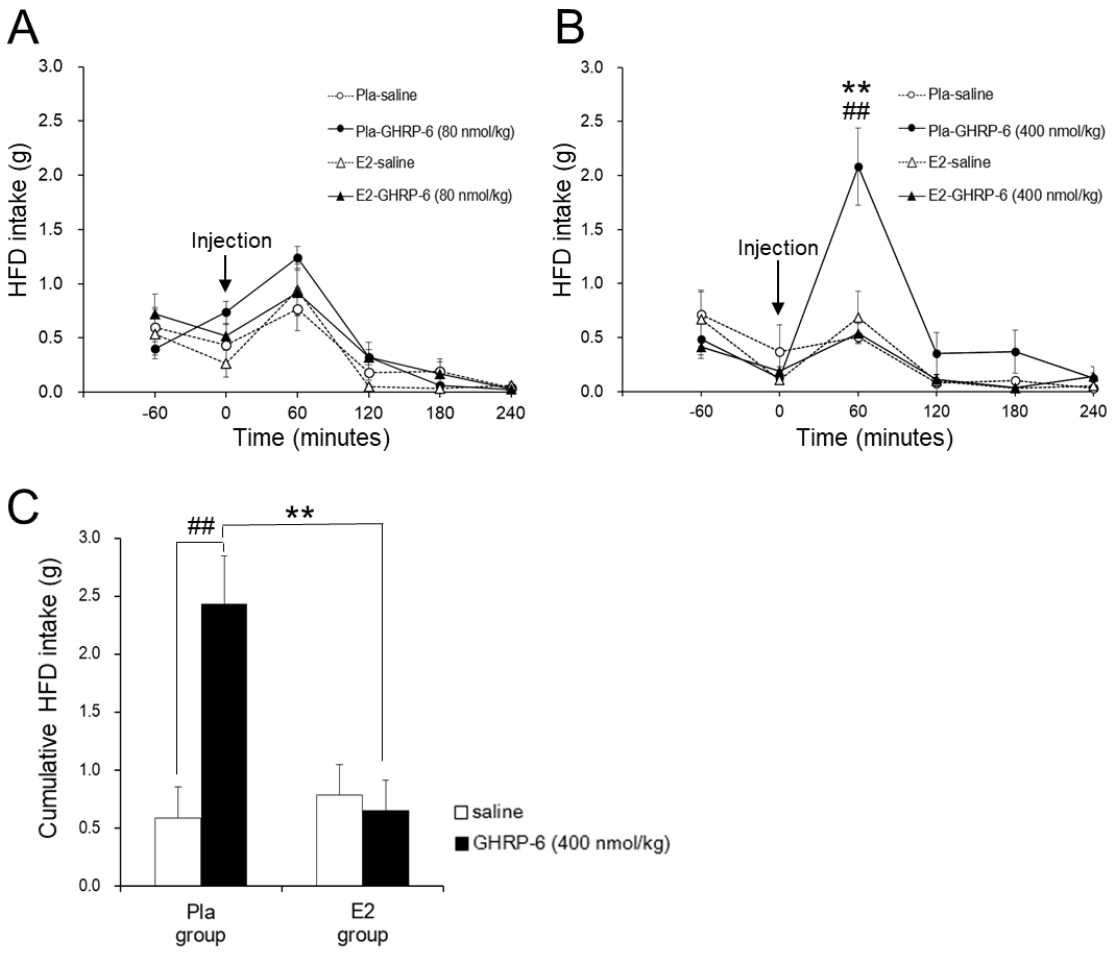

Figure 2. Effect of intraperitoneally (IP) GHRP-6 injection on high-fat diet (HFD) intake. Line graphs represent changes in HFD intake after IP injection of GHRP-6 (A: 80 nmol/kg, B: 400 nmol/kg) or saline in the placebo (Pla) ($n = 13$), 17β-estradiol (E2) ($n = 14$) groups of ovariectomized rats fed HFD. Data are expressed as means ± SE and were analyzed by two-way repeated-measures ANOVA for each pairwise comparison among the four groups. There was an interaction of time and group effects in the HFD intake after GHRP-6 injection at dose of 400 nmol/kg ($p_{\text{Time}\times\text{Group}} < 0.05$: Pla-GHRP-6 vs. Pla-saline or E2-GHRP-6). Significant differences were observed at 60 min after GHRP-6 injection at dose of 400 nmol/kg between the Pla-GHRP-6 and the E2-GHRP-6 groups ($^{**}p < 0.01$), and between the Pla-saline and the Pla-GHRP-6 ($^{\#\#}p < 0.01$). Bar graphs on bottom represent the cumulative HFD intake for 2 h after GHRP-6 injection at a dose of 400 nmol/kg in the Pla and E2 groups (C). Two-way repeated measures ANOVA showed an interaction of injection and group effects in the HFD intake ($p_{\text{Injection}\times\text{Group}} < 0.01$). Significant differences in HFD intakes were observed between the Pla-saline and the Pla-GHRP groups ($^{\#\#}p < 0.01$), and between the Pla-GHRP-6 and the E2-GHRP-6 groups ($^{**}p < 0.01$).

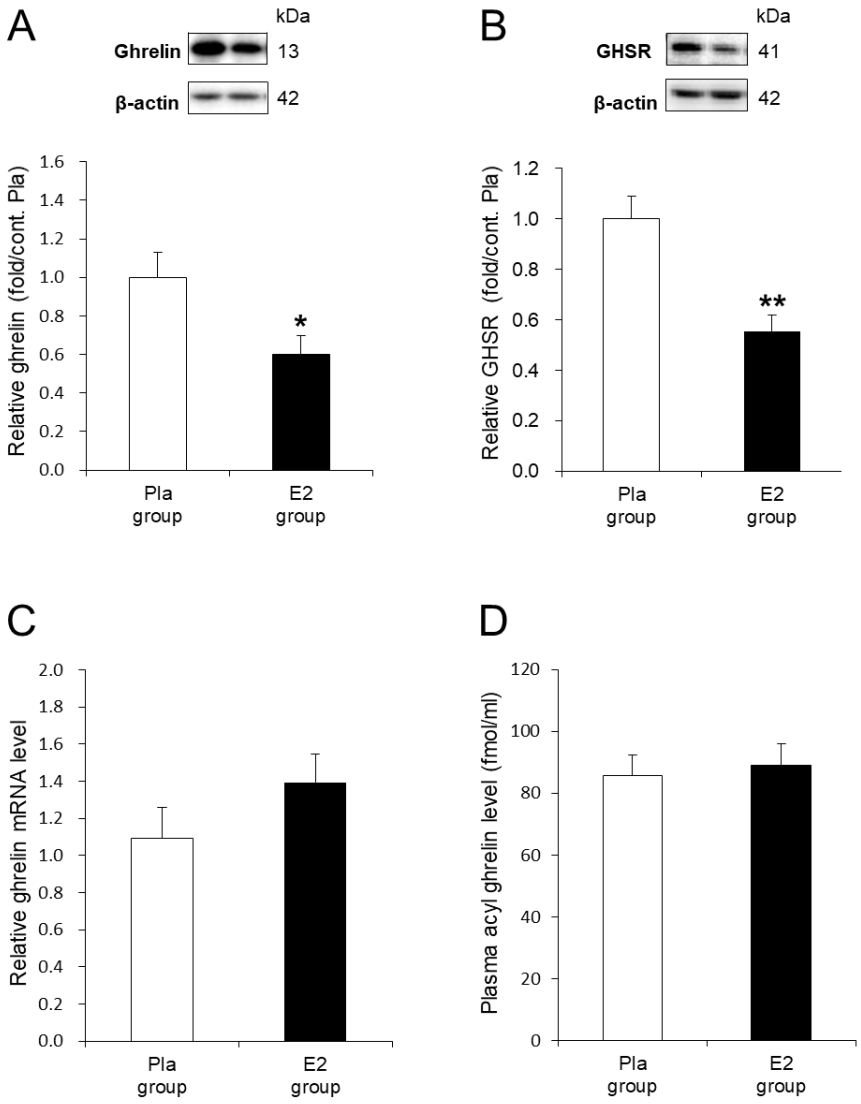

Figure 3. *In vivo* effects of 17β-estradiol (E2) replacement on ghrelin and growth hormone secretagogue receptor (GHSR) in the stomach. (A) Representative blots and relative values of ghrelin protein level in the gastric mucosa of HFD-fed rats in the placebo (Pla, $n = 9$)- and E2 ($n = 8$)-treated groups. (B) Representative blots and relative values of GHSR protein level in stomach of rats in the Pla ($n = 11$) and E2 ($n = 10$) groups. (C) Relative values of ghrelin mRNA level in gastric mucosa of rats in the Pla ($n = 8$) and the E2 ($n = 8$) groups. Data are evaluated as $2^{-\Delta\Delta Ct}$ using β-actin as a housekeeping gene. (D) Plasma acyl ghrelin level after a 16-h fasting in the Pla ($n = 9$) and the E2 ($n = 8$) groups at 17 weeks of age. Data are expressed as means ± SE and were analyzed by unpaired *t*-test. $^{*}p < 0.05$ and $^{**}p < 0.01$, differences between E2 and Pla groups.

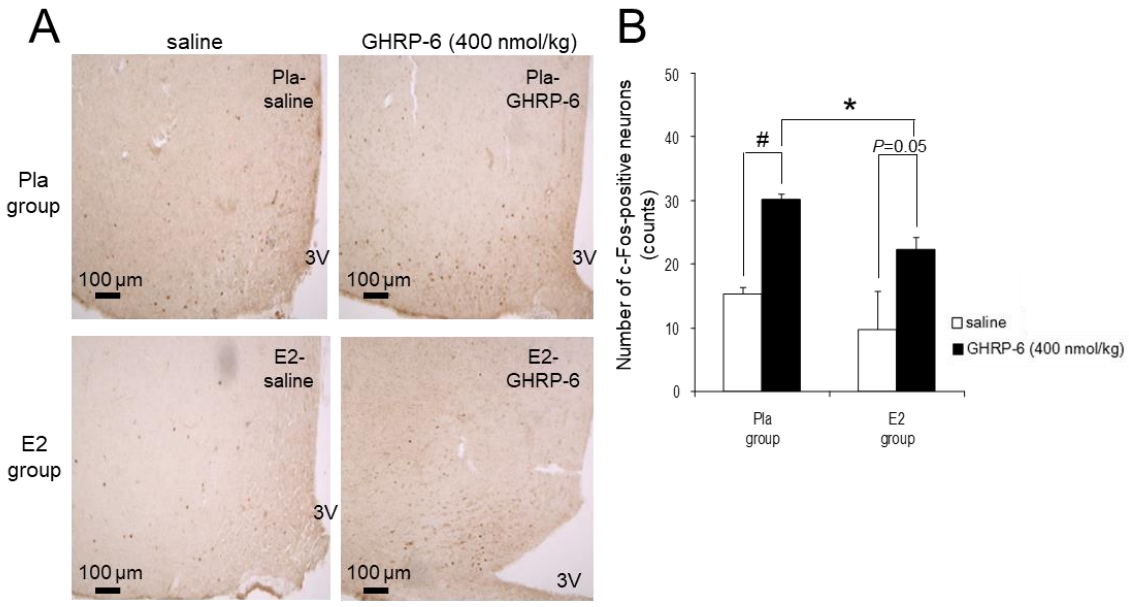

Figure 4. c-Fos-positive neurons in the arcuate nucleus (ARC) induced by intraperitoneal (IP) injection of growth hormone-releasing peptide (GHRP)-6. (A) Representative images of c-Fos-positive neurons after IP injection of 400 nmol/kg GHRP-6 or saline in the ARC of high-fat diet (HFD)-fed rats in the placebo (Pla)-saline, Pla-GHRP-6, 17β-estradiol (E2)-saline, and E2-GHRP-6 groups. 3V, third ventricle. Scale bars: 100 μm. (B) The number of the c-Fos-positive neurons in the ARC after IP injection of 400 nmol/kg GHRP-6 or saline in the Pla-saline ($n = 4$), Pla-GHRP-6 ($n = 5$), E2-saline ($n = 4$) and E2-GHRP-6 ($n = 5$) groups. Values are shown as the mean ± SE. Data were analyzed by two-way factorial ANOVA, followed by a post hoc Tukey-Kramer test. $^{*}p < 0.05$, differences between the E2 and Pla groups. $^{\#}p < 0.05$, differences between the GHRP-6 and saline groups.

Chapter 2

Effects of estradiol replacement on insulin resistance
in high-fat diet-induced obesity in ovariectomized rats.

Nutrients. submitted

Abstract

Menopause is a risk factor for metabolic syndrome and type 2 diabetes. However, the role of estrogen in this context remains unclear. This study examined the effects of chronic estrogen replacement on insulin sensitivity and signaling in ovariectomized (OVX) rats fed high-fat or standard diet (HFD and SD, respectively). Four weeks after ovariectomy, Wistar rats were subcutaneously implanted with either 17β-estradiol (E2) or placebo (Pla) pellets and started on HFD feeding. The intravenous glucose tolerance tests were later performed to assess insulin sensitivity. Following insulin injection into the rats' portal veins, the livers and gastrocnemius muscles were dissected for insulin signaling analysis. The expressions of key enzymes related to insulin signaling in these organs were examined. HFD increased energy intake and body weight in the Pla group, but temporarily reduced these measures in the E2 group. The accumulation of visceral and inguinal adipose tissues was larger in HFD-fed rats than in SD-fed rats in the Pla group, but not in the E2 group. HFD reduced insulin sensitivity only in the Pla group. *In vivo* insulin stimulation increased protein kinase B (Akt) isoform 2 (Akt2) phosphorylation to a similar degree in the muscles of SD-fed rats in the Pla and E2 groups. In contrast, HFD disturbed the insulin-stimulated phosphorylation of Akt2 and its substrate of 160 kDa (AS160) in the muscles of rats in the Pla group but not of rats in the E2 group. These results suggest that E2 replacement improves HFD-impaired insulin sensitivity by activating the Akt2/AS160 pathway in the insulin-stimulated muscles of OVX rats.

1. Introduction

Obesity and overweightness have been well-established as major risk factors for several diseases, including type 2 diabetes, metabolic syndrome, and cardiovascular disease [1]. Estrogen plays a pivotal role in the energy metabolism, appetite, and body weight regulation in females [2, 3]. In humans, estrogen deficiency in the postmenopausal period is associated with visceral fat accumulation, impaired glucose tolerance, and insulin resistance [4, 5]. Similarly, ovariectomized (OVX) rats, an animal model widely used for studying the pathology of human menopause, develop additional body weight, visceral fat accumulation, and impairment of whole-body glucose homeostasis [2, 3, 6].

It has been claimed that dietary fat intake is responsible for this increase in adiposity. Human studies have shown that high-fat diet (HFD) (≥30% of energy from fat) can easily induce obesity [7, 8]. Diets rich in fat not only induce obesity in humans but also cause obesity in animals. In both rats [9, 10] and mice [11, 12], a positive relationship was found between the level of fat in the diet and body weight gain or fat gain. Furthermore, HFD is associated with elevated basal plasma insulin levels and resistance to the metabolic effects of insulin [13]. Animal studies have shown that hyperinsulinemia and insulin resistance are induced by the feeding of HFD [14, 15]. In female mice fed HFD with 10% to 60% dietary fat for 15 weeks, a linear relationship was found between the percentage of dietary fat and glucose intolerance [12].

Insulin resistance has been suggested as a consequence of defects in the insulin signaling cascade [16, 17]. It is now well-established that the binding of insulin to its receptor, insulin receptor substrate (IRS) proteins, and phosphatidylinositol (PI) 3-kinase proteins leads to phosphorylation and activation of protein kinase B (Akt) [18, 19, 20]. Furthermore, experiments using genetically modified cells and muscles have

demonstrated that Akt2, the Akt isoform that is highly expressed in skeletal muscle, was the most important factor for insulin-stimulated glucose transport [21, 22, 23]. An Akt substrate of 160 kDa, AS160 (a Rab-GTPase activating protein), is phosphorylated by Akt at Thr^{642} and regulates the trafficking of glucose transporter 4 (GLUT4) [24, 25]. AS160 is highly expressed in muscles, the heart, and adipose tissues but minimally expressed in the liver [26, 27].

Some previous studies using OVX rodents have reported the effects of estrogen on insulin signaling intermediates related to insulin sensitivity [6, 28, 29, 30]. A previous study showed that OVX may cause insulin resistance—as evaluated by the simple surrogate homeostatic model assessment (HOMA) index—at least in part, by decreased PI 3-kinase and Akt2 protein expression in the skeletal muscle of female rats; this can be reversed by administration of estrogen [31]. Recently, we found that 17β-estradiol (E2) replacement restored the impairment of insulin sensitivity by enhancing activation of the Akt2/AS160 pathway in the gastrocnemius muscle of OVX rats fed a normal standard diet (SD) [6]. Moreover, several studies examined E2 effects on HFD-induced insulin resistance and reported that E2-replete female rodents are protected against HFD-induced impairment [28, 32, 33], although a 40% to 50% reduction in insulin-mediated glucose disposal is consistently observed in male mice after HFD feeding [34, 35]. Additionally, a previous study using HFD-fed OVX mice showed that E2 improves insulin-stimulated Akt phosphorylation and AMP-activated kinase-α activation in skeletal muscles [28]. However, it remains unclear whether HFD impairs the downstream of Akt in the insulin signaling cascade or whether E2 restores the impairment in OVX rats, although male or intact female rodent studies have reported HFD-induced impairment in Akt isoforms and their downstream signaling pathways. It has been reported that HFD reduced cytosolic

insulin-stimulated Akt2 activities, while Akt2 protein concentrations were unaltered in skeletal muscle of male rats [36]. Furthermore, a 4-week HFD induced whole-body insulin resistance and decreased insulin-stimulated phosphorylation of AS160 Thr^{642} in the soleus muscles of male mice [37]. Another study showed that Thr^{642} phosphorylation of AS160 in the muscle was significantly lower in HFD-fed female rats than in low-fat-diet-fed rats [38]. However, no previous report has investigated the effective strategy of E2 replacement against HFD-induced insulin resistance and glucose intolerance via the Akt2/AS160 pathway in the gastrocnemius muscles of OVX rats.

This study was designed to test the hypothesis that chronic E2 replacement, at a physiological dose, improves the HFD-induced impairment in whole-body insulin sensitivity via insulin-induced activation of the Akt2/AS160 pathway in OVX rats. We evaluated the effects of E2 replacement on whole-body insulin sensitivity using an intravenous glucose tolerance test (IGTT) under free-moving conditions and *in vivo* insulin-stimulated activation of the Akt2/AS160 pathway in the gastrocnemius muscles and livers of SD- and HFD-fed OVX rats.

2. Materials and Methods

2. 1. Animals and diets

Animal procedures were approved by the Nara Women's University Committee on Animal Experiments (No. 17-02) and were conducted in accordance with the Standards relating to the Care and Keeping and Reducing Pain of Laboratory Animals (Notice of the Ministry of the Environment, Government of Japan) and ARRIVE guidelines. In total, 64 female Wistar rats (CLEA Japan, Inc., Tokyo, Japan) were used in this study. The rats were individually housed in standard polycarbonate cages containing paper bedding

under controlled temperature and light conditions (26 ± 1°C, a 12:12-h light-dark cycle, with lights on at 7:00 a.m.). Tap water and rodent chow were provided ad libitum. All surgeries were performed while the rats were under anesthesia (pentobarbital sodium; 25-40 mg/kg IP or isoflurane; 1.5-2.0% in oxygen).

Nine-week-old female rats fed using SD (MF; Oriental Yeast, Tokyo, Japan) underwent ovariectomies followed by E2 or placebo (Pla) replacements as previously described [6, 39]. In summary, after a 4-week recovery period, the rats were randomly assigned to either the Pla- or the E2-treated group (n = 34 and n = 30, respectively) and were subcutaneously implanted with either E2 (1.5 mg/60-day release) or Pla pellets (Innovative Research of America, Sarasota, Florida, USA). Additionally, HFD (modified F2HFD2; Oriental Yeast) containing 498.9 kcal per 100 g (60.0% energy from fat, predominantly lard) was started the day after the replacement and was continued until the cessation of the experiments for HFD-fed rats in both groups. The SD-fed rats continued on SD, which contained 360.0 kcal per 100 g (13.2% energy from fat). The rats' food intakes and body weights were measured daily during the experiments.

2.2. Assessment of insulin sensitivity by IGTT using free-moving rats

Four weeks after replacement, rats aged 16 weeks in both the Pla and E2 groups were implanted with intravenous catheters in the superior vena cava for the IGTT as previously described [6]. IGTTs were performed on the rats capable of delivering blood from an unobstructed catheter (Pla-SD, n = 7; Pla-HFD, n = 11; E2-SD, n = 12; E2-HFD, n = 12) 5 days after cannulation. In preparation for the experiments, the rats were housed individually in experimental plastic cages (length, 26 cm; width, 17 cm; depth, 30 cm) and were acclimated to the experimental procedures the day before the experiment, when the rats were adapted to be equipped with an extension tube for blood sampling.

Following 16 h of fasting and 1 h of rest after connecting the catheter with the extension tube, which allowed for free movement, a blood sample (0.3 ml) was collected from each catheter and harvested in a plastic tube containing 50 mg/ml ethylenediamine tetraacetic acid. Subsequently, glucose (1 g/kg) was injected into the venous catheters. Blood samples were obtained at 5, 10, 30, and 60 min. To prevent compensatory sympathoadrenal activation due to blood loss caused by blood sampling, we returned red blood cells in saline to the rats after each collection. The collected blood samples were centrifuged, and the plasma was stored at −45°C for the analysis of plasma glucose or insulin concentration.

2.3. Sampling for estimation of insulin signaling analysis

After a 16-h fast, the rats (Pla-SD, $n = 12$; Pla-HFD, $n = 12$; E2-SD, $n = 11$; E2-HFD, $n = 11$) at 17 weeks of age were used for the insulin signaling analysis experiment under anesthesia (isoflurane; 1.5-2.0% in oxygen). Through an abdominal incision, 10 ml/kg of physiological saline (0.9% NaCl) with or without 10^{-5} mol/l insulin (Novolin R 100 IU/ml; Novo Nordisk, Bagsværd, Denmark) was injected into each rat's portal vein as a single bolus. Livers and gastrocnemius muscles were harvested 30 and 90 s after the injection, respectively, as described by Ogihara et al. [40]. Tissues were then dissected, immediately frozen in liquid nitrogen, and stored at −50°C until further processing was performed.

Upon completion of the experiments, the rats were sacrificed by a pentobarbital sodium overdose, and wet weights of the intra-abdominal (mesenteric, kidney-genital, and retroperitoneal) and subcutaneous (inguinal) adipose tissues were measured. The total weight of each rat's visceral adipose tissues was calculated as the sum of the intra-

abdominal fat weights.

2.4. Analytical methods for plasma glucose and insulin

Plasma glucose concentration was measured by a glucose oxidase method using a glucose assay kit (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin concentrations were assayed using a rat insulin ELISA kit (AKRIN-010T, Shibayagi, Gunma, Japan). The insulin or glucose response during the IGTT was used for calculating the incremental plasma its value integrated over the 60-min period following the glucose injection (incremental area under the curve). To assess insulin resistance, the glucose-insulin index was calculated as the product of the areas under the glucose and insulin curves following the glucose challenge.

2.5. Immunoblotting

Isolated livers and muscles 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% Triton X-100]. The homogenates were centrifuged at 15,000 × *g* for 30 min at 4°C. Sodium dodecyl sulfate (SDS) samples containing equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and immunoblotted using a PVDF membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) with the following antibodies: antibodies for Akt (1:5000 or 1:3000), phospho (p)-Akt Ser^{473} (1:1000), Akt2 (1:1000), p-Akt2 Ser^{474} (1:1000), and p-AS160 Thr^{642} (1:500) from Cell Signaling

Technology (Beverly, MA, USA). The AS160 antibody (1:3000) was from MILLIPORE (Temecula, CA, USA), and the β-actin antibody (1:2000) was from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was obtained from Promega (Madison, WI, USA). An enhanced chemiluminescence (ECL; GE Healthcare Life Sciences) system was used for protein detection. Imaging and densitometry were performed using the imaging system Ez-Capture (ATTO, Tokyo, Japan) and the image processing program CS Analyzer (ATTO) as described previously [6, 39]. We did not examine Akt2 or AS160 in the liver tissues by Western blotting, since previous studies have reported that liver expresses little AS160 [26, 41].

2.6. Statistical analyses

All values are expressed as means ± standard errors (SE). Two-way repeated measures analysis of variance (ANOVA) for each pair-wise comparison among the 4 groups (Pla-SD, Pla-HFD, E2-SD, and E2-HFD), followed by a Tukey-Kramer post-hoc test, was used to analyze the effect of E2 or HFD on the change in food intake, energy intake, body weight, and plasma glucose and insulin concentrations. Two-way factorial ANOVA, followed by a Tukey-Kramer post hoc test, was used to analyze the effect of E2 on HFD-induced changes in the wet weights of adipose tissues, glucose area under the curve (AUC), insulin AUC, glucose-insulin index during the IGTT, and comparison of the responses to insulin by the insulin signaling proteins between the groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of the rats studied

HFD had no effect on food intake in rats 14-15 weeks of age in the Pla group, but HFD-fed rats demonstrated gradually decreased food intake at 16-17 weeks of age when compared with SD-fed rats (Figure 1A). In contrast, HFD-fed rats demonstrated decreased food intake between 14 and 17 weeks of age when compared with SD-fed rats in the E2 group. Therefore, HFD did not affect energy intake in rats in the E2 group but enhanced it in rats 14 and 15 weeks of age in the Pla group, resulting in a difference in energy intake between the Pla and E2 groups of only HFD-fed rats (Figure 1B).

Rats between 15 and 17 weeks of age showed higher body weights when fed HFD compared to rats fed SD in the Pla group. However, this was not true in the E2 group, resulting in a difference in body weight between the two groups (Figure 1C). Rats fed HFD showed significantly increased wet weights of total visceral and inguinal subcutaneous adipose tissues (Figure 1D) in the Pla group, but not in the E2 group. In summary, E2 replacement suppressed body weight gain and fat accumulation by adjusting the energy intake in HFD-fed OVX rats.

3.2. Assessments of insulin sensitivity by IGTT using free-moving rats

Fasting glucose levels were higher in HFD-fed rats in the Pla group than in HFD-fed rats in the E2 group ($p < 0.01$). Following glucose injections, plasma glucose concentrations increased rapidly, reached peak levels at 5 min, and then gradually returned to basal levels over a 30-min period. Glucose levels at 5 and 10 min did not differ between the groups, although significant differences were observed at 30 and 60 min in HFD-fed rats between the two groups ($p < 0.05$ and $p < 0.01$, respectively) (Figure 2A).

As shown in Figure 2a, E2 replacement and HFD had no effect on the glucose AUC in SD-fed OVX rats, but E2 attenuated the AUC in HFD-fed OVX rats ($p < 0.01$).

As shown in Figure 2B, plasma insulin levels increased rapidly after glucose injections and showed peak values at 5 min in the Pla group and at 10 min in the E2 group. There was a significant difference in plasma insulin levels at 5 min between the two HFD-fed groups ($p < 0.01$). The insulin response over 60 min was significantly greater in the Pla group than in the E2 group regardless of the type of diet (interaction, $p < 0.05$: Pla-SD vs. E2-SD and $p < 0.01$: Pla-HFD vs. E2-HFD) (Figure 2B). Additionally, HFD augmented the insulin AUC in the Pla group ($p < 0.05$) but not in the E2 group (Figure 2b).

The glucose-insulin index established for the index of insulin resistance was enhanced in HFD-fed rats when compared with SD-fed rats only in the Pla group ($p < 0.05$). Moreover, the index in HFD-fed rats in the Pla group was higher than that of those in the E2 group ($p < 0.05$) (Figure 2C).

3.3. In vivo effect of estrogen on insulin signaling

To reveal the molecular mechanism accounting for the beneficial effect of E2 on insulin sensitivity in HFD-fed OVX rats, we investigated the key enzymes of the insulin signaling cascade in the gastrocnemius muscles and livers.

As shown in Figure 3A, the amounts of Akt proteins in the rats' muscles were similar between the Pla and E2 groups, regardless of whether they were fed SD or HFD. Insulin injection increased phosphorylation of Akt Ser^{473} in the muscles of SD- and HFD-fed rats to a similar extent in both the Pla and E2 groups. Figure 3B shows that Akt2 protein levels were higher in the E2 group when compared with the Pla group in the SD-fed rats. Insulin increased phosphorylation of Akt2 Ser^{474} in the muscles of SD-fed rats in both the Pla and

E2 groups. In contrast to SD-fed rats, E2 had no effect on Akt2 protein level in HFD-fed rats, but increased p-Akt2 level in insulin-stimulated muscles ($p < 0.01$: Pla-HFD vs. E2-HFD). An interaction between insulin and group effects was observed in the insulin-induced phosphorylation of Akt2 Ser^{474} in HFD-fed rats ($p < 0.05$). Figure 3C shows that insulin enhanced the phosphorylation of AS160 Thr^{642} in the muscles of rats only in the E2 group, regardless of whether they were fed SD or HFD ($p < 0.05$). Additionally, the levels of phosphorylated AS 160 were higher in HFD-fed rats from the E2 group than in HFD-fed rats from the Pla group ($p < 0.05$).

Figure 4 shows Akt and p-Akt Ser^{473} protein levels in the livers of SD- and HFD-fed rats in the Pla and E2 groups. Insulin increased Akt phosphorylation in the two groups fed SD. However, the insulin-induced phosphorylation was no longer statistically significant in the HFD-fed rats in the two groups.

4. Discussion

We have shown that E2 replacement in physiological doses improves HFD-induced impairment in insulin sensitivity by activating the Akt2/AS160 insulin-signaling pathway in the muscles of OVX rats.

In this study, E2 replacement suppressed increases in energy intake and body weight in HFD-fed OVX rats more strongly than in SD-fed rats, as already shown in our previous report [39]. Contrarily, a previous study reported that HFD-fed OVX mice, despite being more obese, demonstrated slightly lower food intakes than E2-replaced mice [42], and another showed that E2-replaced mice and OVX mice demonstrated similar body weights when fed HFD diets for 1 month [28]. Furthermore, a previous study using OVX Sprague-Dawley rats reported that those fed HFD demonstrated similar body weights and daily

energy intakes when compared with those fed SD [43]. These discrepancies may be attributed not only to the difference in rodent species but also to the difference in the strain of rats (i.e., Wistar or Sprague-Dawley). This idea is supported by a study showing that the metabolic effects caused by HFD seemed to be more pronounced in Wistar rats than in Sprague-Dawley rats [44].

We found that E2 replacement restored the impaired insulin sensitivity in HFD-fed OVX rats. Our results are similar to those of a previous study reporting the protective effect of E2 treatment on HFD-induced insulin resistance evaluated simply by the HOMA index in OVX rats [33]. In contrast, there is increasing evidence that E2 improves HFD-induced insulin resistance in OVX mice [28, 42]. Additionally, the effect of E2 on insulin sensitivity was reported to be dose-dependent, since an excess or a lack of E2 was associated with insulin resistance in OVX rats [45]. The HFD generally used in rodent studies contain the amount of dietary fat that is 45-60% of the rodents' total energy intake [15, 46]. Furthermore, some studies have reported that different fatty acid (FA) contents in dietary fat lead to different metabolic outcomes in HFD-fed rats [46, 47, 48]. In rats, diets high in saturated FAs were reported to cause a greater degree of insulin resistance than diets high in polyunsaturated FAs [47, 48]. Because the HFD used in our study contained predominantly saturated and monounsaturated fatty acids (24% energy saturated; 27% energy monounsaturated; 8% energy polyunsaturated), our results are consistent with these previous findings. In addition to the composition and fat content of HFD, the dose of E2 treatment or the parameter for assessment of insulin sensitivity may also influence the effects of E2 on insulin sensitivity. In addition, the postmenopausal HFD-induced obese rat model in the present study advantageously has greater relevance to women, wherein overconsumption of calories after menopause leads to increased fat

mass and insulin resistance when compared with genetically obese rodent models [49].

To clarify the mechanism of action of estrogen, we studied a gastrocnemius muscle that was predominantly composed of fast-twitch (type II) fibers and had previously been used to examine the insulin signaling pathway [50, 51] and liver. Sufficient activation of the PI 3-kinase/Akt pathway is a key mechanism for insulin-stimulated glucose uptake by skeletal muscle. A great deal of recent evidence supports the idea that the insulin-stimulated increase in AS160 phosphorylation at Thr^{642} and Ser^{588} downstream of the PI 3-kinase/Akt pathway is important for increased GLUT4 translocation and glucose transport in muscles [24, 27]. In the present study, we demonstrated for the first time that HFD impaired insulin-stimulated phosphorylation of Akt2 Ser^{474} in the gastrocnemius muscles of OVX rats and that E2 replacement restored the phosphorylation of Akt2 Ser^{474} and increased the p-AS160 Thr^{642}. In summary, E2 replacement seems to maintain glucose uptake in the muscle by sustaining insulin-stimulated phosphorylation in the Akt2/AS160 pathway under HFD-feeding in OVX rats.

The association between lipid oversupply and insulin resistance is well-established, and evidence for mechanisms through which lipids may play a causative role in the generation of muscle insulin resistance have been reported [52]. When the amount of circulating lipids chronically exceeds the capacity of white adipose tissue for uptake and storage, FAs accumulate in other tissues such as the liver and skeletal muscles. In muscles, FAs accumulate intracellularly in myocytes, primarily as long-chain fatty acyl-CoA [52, 53]. Among the FA derivatives, high intramyocellular levels of diacylglycerol (DAG) and ceramides are directly associated with insulin resistance [52]. Notably, increased intracellular levels of DAG in muscles have been shown to activate the signaling of protein kinase C (PKC), leading to serine phosphorylation of IRS-1. This inhibits the

phosphorylation of tyrosine residues on IRS-1, resulting in a defect in canonical PI 3-kinase/Akt signaling and consequently reducing insulin-stimulated glucose uptake in muscles [52]. A similar mechanism may exist in the present study, although it has not yet been revealed how HFD cause impairment of insulin signaling in the muscles of OVX rats. In HFD-fed OVX mice, a recent study showed that E2 replacement reduced DAG content in both the liver and muscles, reduced PKC activation, and promoted insulin-stimulated Akt2 phosphorylation [42]. Further studies are required to elucidate the mechanisms underlying HFD-induced insulin resistance, which may be improved by E2, as demonstrated in our rat model.

In the context of the present study, it is plausible that the effect of E2 replacement on insulin resistance in HFD-fed OVX rats is directly mediated by estrogen receptors (ER). Additionally, the effects of E2 on insulin sensitivity may be mediated at least partly by E2-induced inhibition of abdominal obesity. Previously, Riant et al. showed that in HFD-fed female mice, the beneficial effect of E2 on insulin sensitivity was mediated through ERα activation, because this action was abolished in ER-deficient mice [28]. To our knowledge, the present study is the first to show HFD-induced impairment and E2-induced improvement of *in vivo* insulin-stimulated Akt2/AS160 pathways in HFD-fed OVX rats. Only one previous report using SD-fed OVX rats has shown that the ERα agonist results in increased insulin-stimulated glucose uptake into the skeletal muscle by potentiating the insulin-stimulated phosphorylation of Akt and increasing GLUT4 protein but not insulin-stimulated phosphorylation of AS160 [30]. ERα and ERβ are expressed in rodent and human skeletal muscles [30, 54, 55]; therefore, it is likely that the ERs are involved in the E2-induced activation of the Akt2/AS160 pathway, although our study did not show a direct link between ERs and activation of the pathway.

A previous study reported that whole-body insulin resistance was detected rapidly 1 week after initiation of HFD due to hepatic insulin resistance, while skeletal muscle displayed insulin resistance at 3 weeks in male mice [56]. However, another study found that insulin resistance in skeletal muscle, adipose tissue, and liver developed simultaneously after 3 weeks of HFD-feeding in male mice [57]. These findings show that the 4 weeks of HFD-feeding in our study may be a sufficient duration for rodents to develop insulin resistance. Our data also showed that 4 weeks of HFD-feeding inhibits insulin-induced Akt activation in the liver, which is not restored by E2 replacement, and disturbs the muscular Akt2/AS160 pathway, which is improved by E2. Distinct patterns of tissue-specific E2 effects on HFD-induced impairment may be related to the differing onsets and durations of insulin resistance in the livers and muscles.

In this study, the rats in the E2 group were considered postmenopausal models replaced with E2, because they underwent the experiment on the fourth week of E2 replacement (which was started 4 weeks after OVX). In addition, the plasma E2 concentration in the E2 group may be within the accepted physiological range, as shown in our previous study (44.1 ± 10.3 pg/ml and 9.2 ± 0.5 pg/ml in the E2 and Pla groups, respectively) using E2 pellets containing the same dosage as in the present study [6]. Therefore, our results provide animal data to support the idea that E2 replacement restores the insulin sensitivity impaired by HFD in postmenopausal women. Menopause is associated with an increase in metabolic syndrome or type 2 diabetes [4]. The percentage of energy intake from fat significantly increases in the postmenopausal years [5]. Our results suggest that postmenopausal women should avoid HFD and control dietary fat intake appropriately in order to prevent insulin resistance.

5. Conclusion

In conclusion, this *in vivo* study suggests that E2 replacement improves HFD-induced deterioration of whole-body insulin sensitivity by enhancing activation of the Akt2/AS160 pathway in insulin-stimulated gastrocnemius muscles of OVX rats. Our results provide insight into the protective effects of E2 replacement against HFD-induced insulin resistance in postmenopausal women.

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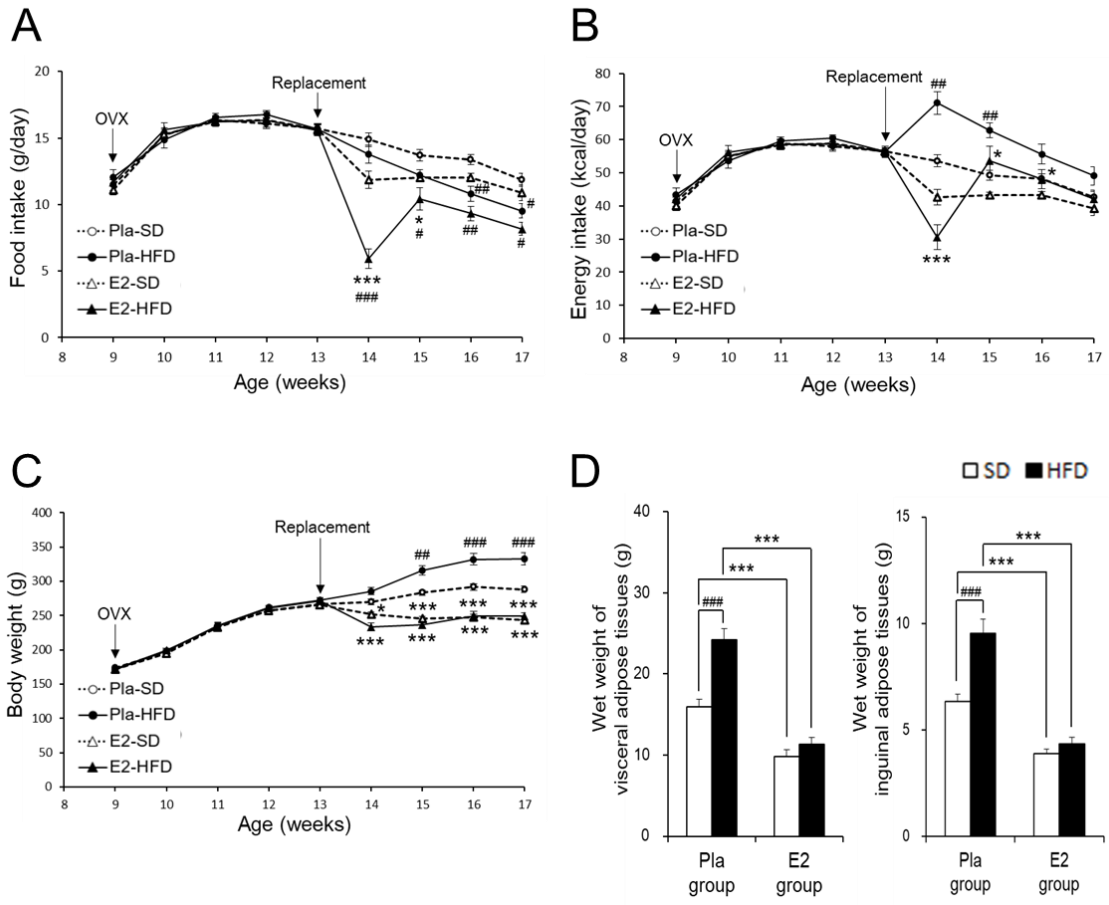

Figure 1. Characterization of rats studied. Data are expressed as means ± SE. Line graphs represent the courses of change in mean food intake (A), energy intake (B), and body weight (C) in the placebo (Pla)-standard diet (SD) ($n = 14$), Pla-high-fat diet (HFD) ($n = 13$), 17β-estradiol (E2)-SD ($n = 14$), and E2-HFD ($n = 12$) groups of ovariectomized (OVX) rats. Two-way repeated measures ANOVA for each pair-wise comparison among the 4 groups revealed significant differences in food intake, energy intake, and body weight. There was an interaction of age and group effects in food intake ($p_{\text{Age}\times\text{Group}} < 0.05$: Pla-SD vs. Pla-HFD, and $p_{\text{Age}\times\text{Group}} < 0.001$: Pla-HFD or E2-SD vs. E2-HFD), energy intake ($p_{\text{Age}\times\text{Group}} < 0.01$: Pla-SD vs. Pla-HFD, and $p_{\text{Age}\times\text{Group}} < 0.001$: Pla-HFD vs. E2-HFD) and body weight ($p_{\text{Age}\times\text{Group}} < 0.01$: E2-SD vs. E2-HFD, and $p_{\text{Age}\times\text{Group}} < 0.001$: Pla-SD vs. Pla-HFD or E2-SD and Pla-HFD vs. E2-HFD). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, differences between the Pla and E2 groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, differences between the SD and HFD groups. Bar graphs represent wet weights of visceral (the sum of the mesenteric, kidney-genital, and retroperitoneal adipose tissue weights) and inguinal adipose tissues (D) at 17 weeks of age in the Pla-SD, Pla-HFD, E2-SD, and E2-HFD groups. Data were analyzed by two-way factorial ANOVA, followed by a post-hoc Tukey-Kramer test. There was an interaction of diet and group effects in the wet weights of visceral ($p_{\text{Diet}\times\text{Group}} < 0.01$) and inguinal adipose tissues ($p_{\text{Diet}\times\text{Group}} < 0.01$). $^{***}p < 0.001$, differences between the Pla and E2 groups; $^{\#\#\#}p < 0.001$, differences between the SD and HFD groups.

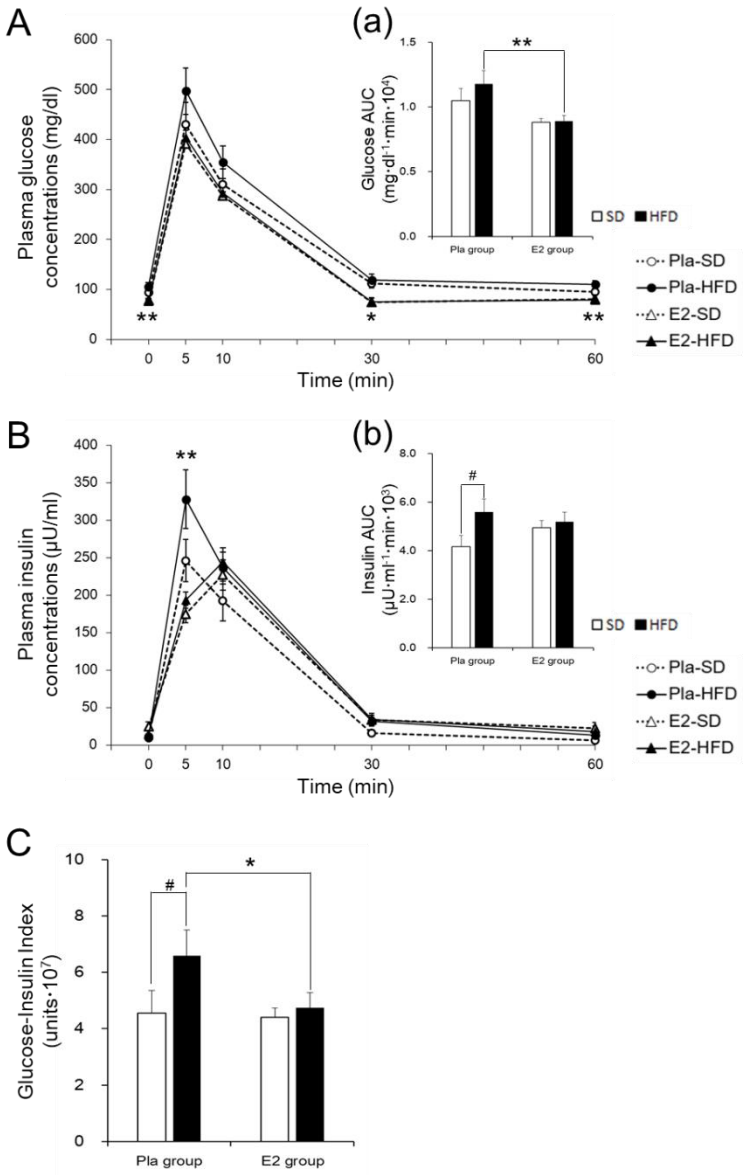

Figure 2. Data are expressed as means ± SE. Line graphs represent the data for the intravenous glucose tolerance tests in in the placebo (Pla)-standard diet (SD) (n = 7), Pla-high-fat diet (HFD) (n = 11), 17β-estradiol (E2)-SD (n = 12), and E2-HFD (n = 12) groups of ovariectomized (OVX) rats. Blood samples (0.3 ml) were collected before and at 5, 10, 30, and 60 min after glucose injection (1 g/kg). Data were analyzed by two-way repeated-measures ANOVA for each pair-wise comparison among the 4 groups. $*p < 0.05$ and $**p < 0.01$, differences between the Pla and E2 groups. A: changes in mean plasma glucose concentration after glucose injection. Significant differences were observed at 0, 30, and 60 min after the injection between the Pla-HFD and E2-HFD groups ($p < 0.05$ or 0.01), but no interactions between the time and group effects were detected. B: changes in mean plasma insulin concentration after glucose injection. A significant difference was observed between the Pla-HFD and E2-HFD groups at 5 min after the injection ($p < 0.01$). There was an interaction of time and group effects in the plasma insulin response ($p_{\text{Time}\times\text{Group}} < 0.05$: Pla-SD vs. E2-SD and $p_{\text{Time}\times\text{Group}} < 0.01$: Pla-HFD vs. E2-HFD). Bar graphs represent the area under the curve (AUC) for glucose (a) and insulin (b) concentrations over 60 min after the glucose injection in each group. The glucose-insulin index (C) was calculated by the glucose AUC multiplied by insulin AUC for the evaluation of insulin sensitivity. Data were analyzed by two-way factorial ANOVA, followed by a post-hoc Tukey-Kramer test. $*p < 0.05$ and $**p < 0.01$, differences between Pla and E2 groups; $^{\#}p < 0.05$, differences between the SD and HFD groups.

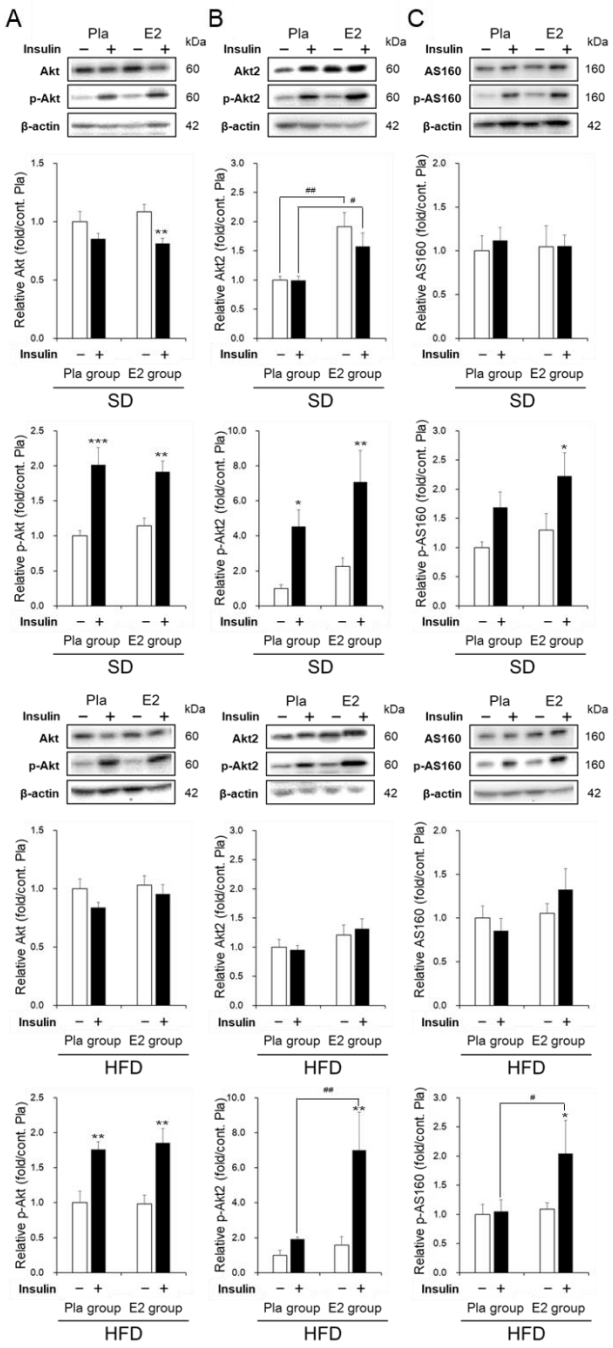

Figure 3. Representative blots and relative values of protein kinase B (Akt) and phospho (p)-Akt Ser^{473} (A), Akt2 and p-Akt2 Ser^{474} (B), and Akt substrate of 160 kDa (AS160) and p-AS160 Thr^{642} in the gastrocnemii of rats in the placebo (Pla)-standard diet (SD) (n = 12), Pla-high-fat diet (HFD) (n = 12), 17β-estradiol (E2)-SD (n = 11), and E2-HFD (n = 11) groups of ovariectomized (OVX) rats. Data are expressed as means ± SE, with (+) or without (-) insulin stimulation by injection of 10^{-5} mol/l insulin or saline in the portal vein, and were analyzed by two-way ANOVA using the following factors: replacement (p_{Replace}: Pla or E2 group), insulin (p_{Insulin}: saline or insulin injection), and interaction of replacement and insulin injection ($p_{\text{Replace}\times\text{Insulin}}$). This was followed by a post-hoc Tukey-Kramer test.
$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, difference between saline and insulin injections.
$^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, difference between the Pla and E2 groups.

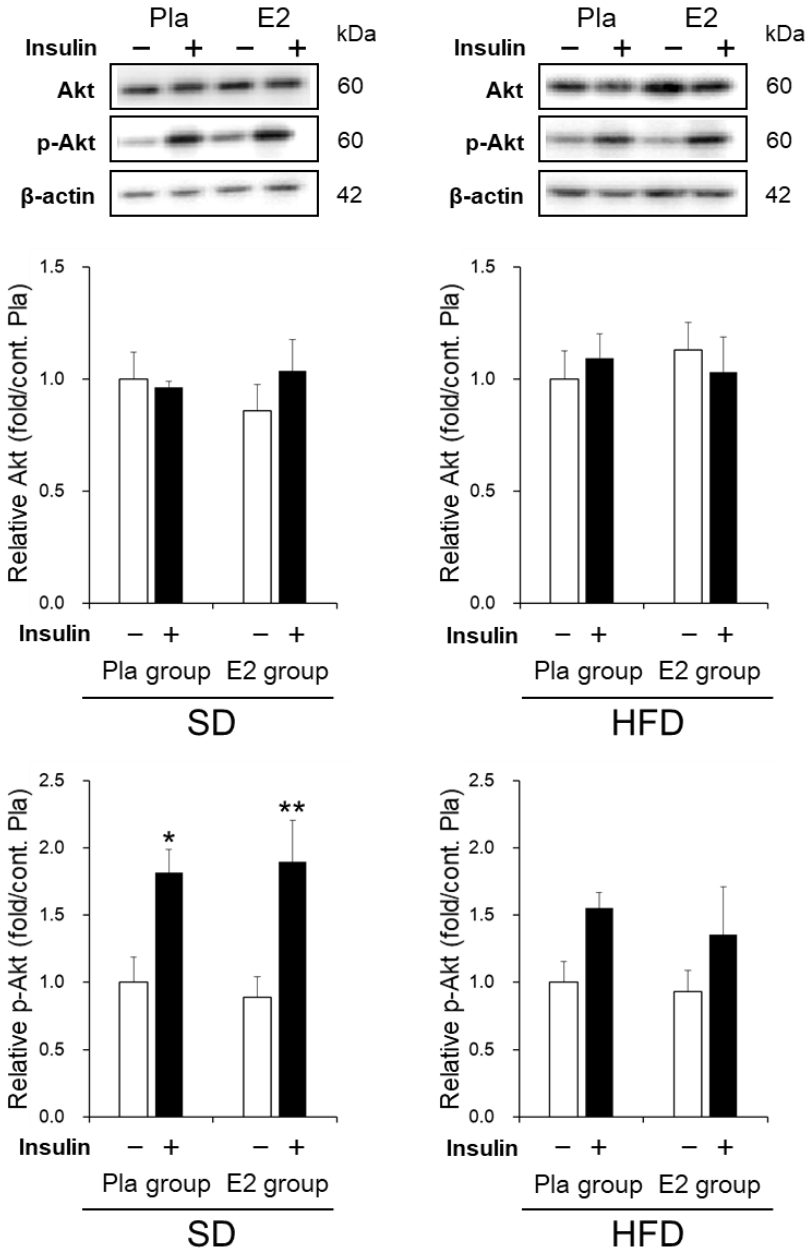

Figure 4. Representative blots and relative values of protein kinase B (Akt) and phospho (p)-Akt Ser^{473} in the livers of rats in the placebo (Pla)-standard diet (SD) (n = 12), Pla-high-fat diet (HFD) (n = 12), 17β-estradiol (E2)-SD (n = 11), and E2-HFD (n = 11) groups of ovariectomized (OVX) rats. Data are expressed as means ± SE, with (+) or without (-) insulin stimulation by injection of 10^{-5} mol/l insulin or saline in the portal vein, and were analyzed by two-way ANOVA using the following factors: replacement ($p_{Replace}$: Pla or E2 group), insulin ($p_{Insulin}$: saline or insulin injection), and interaction of replacement and insulin injection ($p_{Replace \times Insulin}$). This was followed by a post-hoc Tukey-Kramer test. *$p < 0.05$ and **$p < 0.01$, difference between saline and insulin injections.

Chapter 3

Effects of estradiol replacement on the AMP kinase pathway in skeletal muscle of ovariectomized rats fed on high-fat diet

Abstract

Menopause predisposes women to impaired glucose metabolism, but the role of estrogen in this context remains unclear. This study was designed to investigate in protective effects of 17β-estradiol (E2) replacement on glucose metabolism via the AMP kinase (AMPK) pathway in muscle of ovariectomized (OVX) rats fed with a high-fat diet (HFD; 60.0% energy from fat) or standard diet (SD).

Four weeks after OVX at 9 weeks of age, Wistar rats were subcutaneously implanted with either E2 or placebo (Pla) pellets and started on HFD feeding. After 4 weeks, the levels of key enzymes relating AMPK pathway in the gastrocnemius muscle were assayed by Western blotting.

The amount of AMPKα proteins and phosphorylation of AMPKα at Thr^{172}, as a marker for AMPKα activation, were not altered by E2 replacement and HFD feeding. In addition, the amount and phosphorylation of TBC1 (Tre2, BUB2, CDC16) domain family member 1 were similar between the Pla and E2 groups regardless of SD or HFD. Similarly, E2 replacement and HFD intake had no effect on glucose transporter 4 protein level in OVX rats.

These results suggest that E2 replacement and HFD intake had no effect on the AMPK signaling pathway in muscle of OVX rats.

1. Introduction

Estrogen deficiency in the postmenopausal period is at a greater risk for impairing glucose metabolism such as type 2 diabetes [1]. In humans, estrogen replacement therapy can ameliorate the menopause-induced increase in type 2 diabetes risk [2]. Similarly, ovariectomized (OVX) rats, an animal model widely used for studying the pathology of human menopause, was shown to impair insulin sensitivity and glucose metabolism, and these deleterious metabolic effects were reversed by the chronic administration of estrogens [3, 4].

In both human and animals, high-fat diet (HFD) (≥30% of energy from fat) is not only induced obesity [5, 6, 7, 8, 9] but also associated with elevated basal plasma glucose levels and resistance to the contraction- or insulin-stimulated glucose uptake in the muscle [9, 10, 11, 12]. However, the mechanisms underlying HFD-induced impairment of glucose uptake in muscle remains unclear.

Skeletal muscle is the major tissue responsible for uptake of glucose from the blood, accounting for 70-85% of whole body glucose disposal [13]. There are two widely studied physiological stimuli that increase muscle glucose uptake, insulin and exercise/muscle contraction, and both of these stimuli increase glucose uptake via the activation of intracellular signaling cascades [14, 15, 16]. The signaling mechanism by which insulin stimulates muscle glucose uptake is relatively well known and involves binding of insulin to the insulin receptor, phosphorylation of the serine/threonine kinase, Akt, and phosphorylation of the Rab-GTPase activating protein (Rabs), an Akt substrate of 160 kDa (AS160) [17, 18]. Our previous study showed that E2 replacement improves HFD-induced insulin resistance by activating the Akt2/AS160 pathway in the insulin-stimulated muscle of OVX rats [9]. In contrast, the signaling mechanisms by which

muscle contraction acts is not fully understood, although studies have shown that activation of the energy sensing kinase, AMP-activated protein kinase (AMPK), has been positively correlated with increases in muscle glucose uptake [19]. In addition, the downstream regulators of AMPK are still debated, while AS160 or TBC1 (Tre2, BUB2, CDC16) domain family member 1 (TBC1D1), another Rabs of AS160 (also known as TBC1D4), is reported as a glucose uptake regulator in exercise/muscle contraction [14, 16, 20]. Intriguingly, recent study has shown that acute stimulation of skeletal muscle cells with physiological doses of 17β-estradiol (E2), increased the phosphorylation of Akt [21] and AMPK [22, 23], suggesting that the beneficial effect of estrogen replacement therapy on type 2 diabetes. Furthermore, it has been shown to bind to and activate gene transcription via the estrogen receptor (ER) in estrogen-sensitive tissues and cell lines [21, 22]. In addition, it has been revealed that ER-α is a positive regulator of glucose transporter 4 (GLUT4) expressions in skeletal muscle contributing to the insulin resistance [24]. However, it is presently unclear whether HFD influence insulin-independent AMPK-TBC1D1/GLUT4 pathway, or whether the activation of these signaling proteins by E2 replacement increases in skeletal muscle of OVX rats.

Thus, this study was designed to investigate in protective effects of estrogen replacement on glucose metabolism through the AMPK-TBC1D1/GLUT4 pathway in skeletal muscle of HFD-induced obese ovariectmized (OVX) rats.

2. Materials and Methods

2. 1. Animals and diets

Animal procedures were approved by the Nara Women's University Committee on Animal Experiments (No. 17-02) and were conducted in accordance with the Standards relating to the Care and Keeping and Reducing Pain of Laboratory Animals (Notice of the Ministry of the Environment, Government of Japan) and ARRIVE guidelines. In total, 50 female Wistar rats (CLEA Japan, Inc., Tokyo, Japan) were used in this study. The rats were individually housed in standard polycarbonate cages containing paper bedding under controlled temperature and light conditions (26 ± 1°C, a 12:12-h light-dark cycle, with lights on at 7:00 a.m.). Tap water and rodent chow were provided ad libitum. All surgeries were performed while the rats were under anesthesia (pentobarbital sodium; 25-40 mg/kg IP or isoflurane; 1.5-2.0% in oxygen).

Nine-week-old female rats fed using SD (MF; Oriental Yeast, Tokyo, Japan) underwent ovariectomies followed by E2 or placebo (Pla) replacements as previously described [9, 25, 26]. In brief, after a 4-week recovery period, the rats were assigned randomly assigned to either the Pla- or the E2-treated group (n = 26 and n = 24, respectively) and were subcutaneously implanted with either E2 (1.5 mg/60-day release) or Pla pellets (Innovative Research of America, Sarasota, Florida, USA). Additionally, HFD (modified F2HFD2; Oriental Yeast) containing 498.9 kcal per 100 g (60.0% energy from fat, predominantly lard) was started the day after the replacement and was continued until the cessation of the experiments for HFD-fed rats in both groups. The SD-fed rats continued on SD, which contained 360.0 kcal per 100 g (13.2% energy from fat).

2. 2. Sampling for estimation of signaling pathway

After 16-h fast, the rats (Pla-SD, *n* = 13, Pla-HFD, *n* = 13; E2-SD, *n* = 12, E2-HFD, *n* = 12) at 17 weeks of age were used for the insulin-independent signaling analysis experiment under anesthesia (isoflurane; 1.5-2.0% in oxygen). Through an abdominal incision, 10 ml/kg of physiological saline (0.9% NaCl) with or without 10^{-5} mol/l insulin (Novolin R 100 IU/ml; Novo Nordisk, Bagsværd, Denmark) was injected into each rat's portal vein as a single bolus. Gastrocnemius muscles were harvested 90 s after the injection, respectively, as described by Ogihara et al. [27]. Tissues were then dissected, immediately frozen in liquid nitrogen, and stored at −50°C until further processing was performed.

2. 3. Immunoblotting

Isolated muscles 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% Triton X-100]. The homogenates were centrifuged at 15,000 × *g* for 30 min at 4°C. Sodium dodecyl sulfate (SDS) samples containing equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and immunoblotted using a PVDF membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) with the following antibodies: antibodies for AMPKα (1:5000) and phospho (p)-AMPKα Thr^{172} (1:1000) from Cell Signaling Technology (Beverly, MA, USA). The TBC1D1 (1:1000) and GLUT4 antibody (1:500) were from Abcam (Cambridge, MA, USA), and the p-TBC1D1 Ser^{237} antibody (1:500) was from

MILLIPORE (Temecula, CA, USA). Furthermore, the β-actin antibody (1:2000) was from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was obtained from Promega (Madison, WI, USA). An enhanced chemiluminescence (ECL; GE Healthcare Life Sciences) system was used for protein detection. Imaging and densitometry were performed using the imaging system Ez-Capture (ATTO, Tokyo, Japan) and the image processing program CS Analyzer (ATTO) as described previously [9, 25, 26, 28].

2.4. Statistical analyses

All values are expressed as means ± standard errors (SE). Two-way factorial ANOVA, followed by a Tukey-Kramer post hoc test, was used to analyze the effect of E2 on HFD-induced changes in food intake, energy intake, body weight, the wet weights of adipose tissues, and the AMPK-TBC1D1 and GLUT4 pathway between the groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

HFD-fed rats showed lower food intake rather than SD-fed rats in both the Pla and E2 groups. Although, HFD had no effect on energy intake in the two groups, energy intake was lower in the HFD-fed rats in the E2 group than in the Pla group. Rats fed HFD showed significantly increased body weight and wet weights of adipose tissues in the Pla group but not in the E2 group. Therefore, there were significant differences in body weight and wet weights of adipose tissues between the Pla and E2 groups regardless of the type of diet (Table).

To reveal the molecular mechanism accounting for the beneficial effect of E2 on the

AMPK signaling cascade in the HFD-fed OVX rats, we investigated the AMPK-TBC1D1 and GLUT4 in the gastrocnemius muscles.

As shown in Figure 1, the amounts of AMPKα proteins and phosphorylation of AMPKα Thr^{172}, as a marker for AMPKα activation, were not altered by E2 replacement or HFD.

Figure 2 shows that the amounts of TBC1D1 proteins were similar between the Pla and E2 groups, regardless of whether they were fed an SD or HFD. In addition, *in vivo* experiment of insulin injection to the rats revealed that the insulin stimulation did not enhanced phosphorylation of TBC1D1 Ser^{237} in both the groups regardless of the type of diet. Moreover, E2 replacement and HFD had no effect on TBC1D1 phosphorylation in the muscle.

Similarly, GLUT4 protein levels were not influenced by E2 replacement and HFD (Figure 3).

4. Discussion

We have shown that E2 replacement at physiological doses and HFD intake have no effect on the AMPK pathway in muscle of OVX rats. In our previous study using HFD-fed OVX rats, we found that fasting blood glucose levels was suppressed by E2 replacement [9], but these results seem not to be related to the AMPK pathway.

Obesity can be characterized as a state of overnutrition, and it is therefore logical to hypothesize that the activity of the energy sensing pathway, AMPK, could be affected by obesity. Some studies suggest that AMPK activity is reduced in obese rodents and humans compared to lean [12, 29, 30]. In addition, previous study reported that HFD inhibit activation of AMPKα in muscles of male rats [11]. In contrast, our results have shown

that HFD had no effect on both the amounts of AMPKα proteins and phosphorylation of AMPKα Thr^{172}, although HFD significantly increased energy intake in OVX rats compared with E2 replaced rats at 17 week of age. Our results are consistent with several studies, which reported that in both rodents and humans obesity was generally not associated with reductions in AMPK protein levels in skeletal muscle [12, 30, 31]. Furthermore, it is well known that estrogen has effects on physical activity [32]. E2 replacement may affect AMPK protein levels and its activation indirectly by enhancing physical activity. However, we confirmed that the 24-h locomotor activities of freely moving rats fed on SD 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) in previous studies using a radiotelemetry system [33, 34]. Further study is required to confirm the locomotor activity of HFD-fed rats as demonstrated in our rat model.

Available data suggest that AMPK is not essential for whole-body or muscle insulin sensitivity under normal resting conditions, although AMPK activation may be able to induce insulin sensitivity in some situations [19]. In fact, our previous report using SD-fed rat showed that insulin has not stimulated phosphorylation of AMPKα Thr^{172} in both the OVX and E2-replaced groups [25]. However, TBC1D1 is targeted by multiple kinases on multiple sites, and the individual role of this phosphorylation and even phosphorylation in general is largely unknown. In this study, we revealed that the insulin stimulation did not enhanced phosphorylation of TBC1D1 Ser^{237}. Moreover, HFD did not affect the amounts of TBC1D1 proteins and TBC1D1 phosphorylation, and E2 replacement also had no effect on them in the muscle of OVX rats regardless of the type of diets. Similarly, GLUT4 protein levels were not influenced by the HFD and E2 replacement. Our previous study revealed that chronic running training enhanced the

phosphorylated TBC1D1 and GLUT4 protein levels in the muscle of OVX rats fed with SD, and improved hyperglycemia as much as E2 replacement [28]. In the present study, we found that chronic HFD feeding and E2 replacement had no effect on the TBC1D1/GLUT4 pathway in OVX rats, unlike exercise training. In addition, the effect of a HFD on GLUT4 protein expression is equivocal with some studies demonstrating a decrease or no change in GLUT4 protein expression as a result of HFD feeding [11, 35, 36, 37]. Furthermore, a recent study using female rats subject to OVX or sham surgery reported that a 6-week HFD had no effect on GLUT4 protein expression in either the soleus or extensor digitorum longus muscle [38]. Previous study reported HFD impaired GLUT4 translocation to the cell surface, which played a major role in the decrease in insulin- or contraction-stimulated glucose transport in male rats [39]. Furthermore, another study showed that E2 replacement enhanced insulin-induced GLUT4 translocation to the plasma membrane in the cerebral cortex of OVX rats [40]. Further study is required to investigate the effects of HFD or E2 replacement on the contraction- or insulin-stimulated GLUT4 translocation to the cell surface using our rat model.

5. Conclusion

In conclusion, this *in vivo* study suggests that E2 replacement and HFD intake had no effect on pathway of AMPK-TBC1D1-GLUT4 in skeletal muscle of OVX rats.

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Table. Characterization of rats studied at 17 weeks of age.

Group	Pla		E2	
Diet	SD	HFD	SD	HFD
Food intake (g/day)	12.1 ± 0.4	9.5 ± 0.5$^{\#\#}$	10.9 ± 0.6	8.2 ± 0.5$^{\#\#}$
Energy intake (kcal/day)	43.7 ± 1.5	49.1 ± 2.8	39.2 ± 2.1	42.1 ± 2.5*
Body weight (g)	287 ± 5	332 ± 9$^{\#\#\#}$	244 ± 4***	249 ± 5***
Wet weight of adipose tissues (g)				
Visceral	16.0 ± 1.0	24.2 ± 1.4$^{\#\#\#}$	9.8 ± 0.8***	11.3 ± 0.9***
Inguinal	6.5 ± 0.4	9.5 ± 0.7$^{\#\#\#}$	3.9 ± 0.2***	4.4 ± 0.3***

Table. Food intake, energy intake, body weight, and wet weights of visceral (the sum of the mesenteric, kidney-genital, and retroperitoneal adipose tissue weights) and inguinal adipose tissues (D) at 17 weeks of age in the placebo (Pla)-standard diet (SD) (n = 13), Pla-high-fat diet (HFD) (n = 13), 17β-estradiol (E2)-SD (n = 14), and E2-HFD (n = 12) groups of ovariectomized (OVX) rats. Data are expressed as means ± SE, and were analyzed by two-way factorial ANOVA, followed by a post-hoc Tukey-Kramer test. There was an interaction of diet and group effects in body weight ($p_{\text{Diet}\times\text{Group}} < 0.01$), and the wet weights of visceral ($p_{\text{Diet}\times\text{Group}} < 0.01$) and inguinal adipose tissues ($p_{\text{Diet}\times\text{Group}} < 0.01$). *$p < 0.05$, and ***$p < 0.001$, differences between the Pla and E2 groups; $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, differences between the SD and HFD groups.

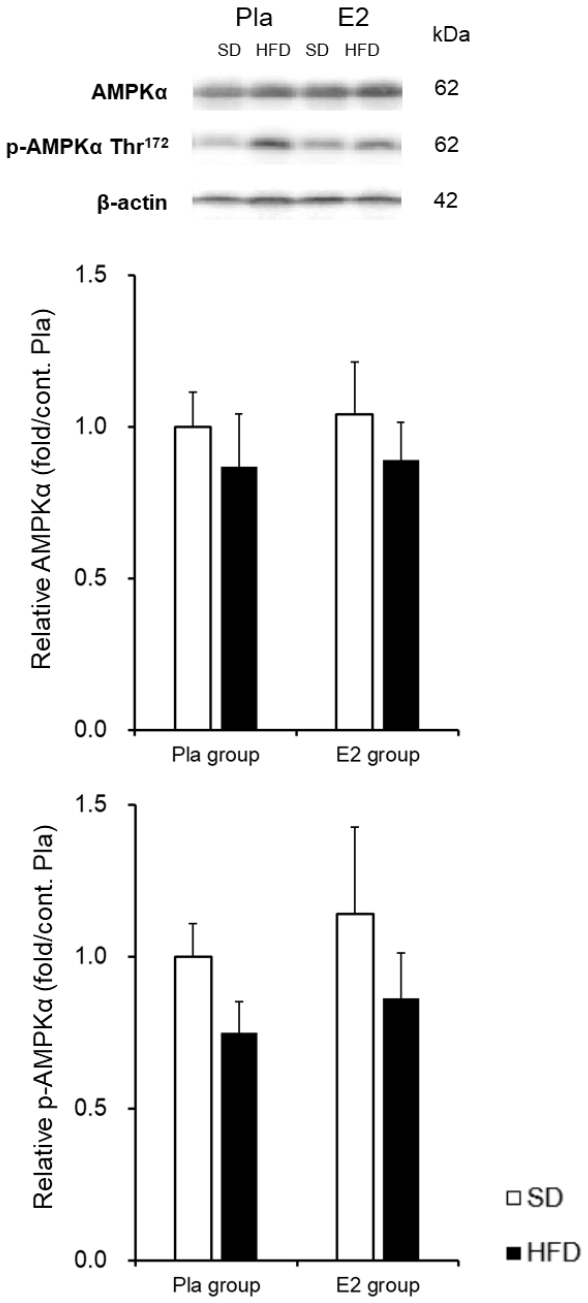

Figure 1. Representative blots and relative values of AMP kinase (AMPK)α and phospho (p)-AMPKα Thr^{172} in the gastrocnemii of rats in the placebo (Pla)-standard diet (SD) (n = 12), Pla-high-fat diet (HFD) (n = 13), 17β-estradiol (E2)-SD (n = 12), and E2-HFD (n = 12) groups of ovariectomized (OVX) rats. Data are expressed as means ± SE, and were analyzed by two-way ANOVA using the following factors: replacement (p_{Replace}: Pla or E2 group), diet (p_{Diet}: SD or HFD), and interaction of replacement and insulin injection ($p_{\text{Replace}\times\text{Diet}}$). This was followed by a post-hoc Tukey-Kramer test.

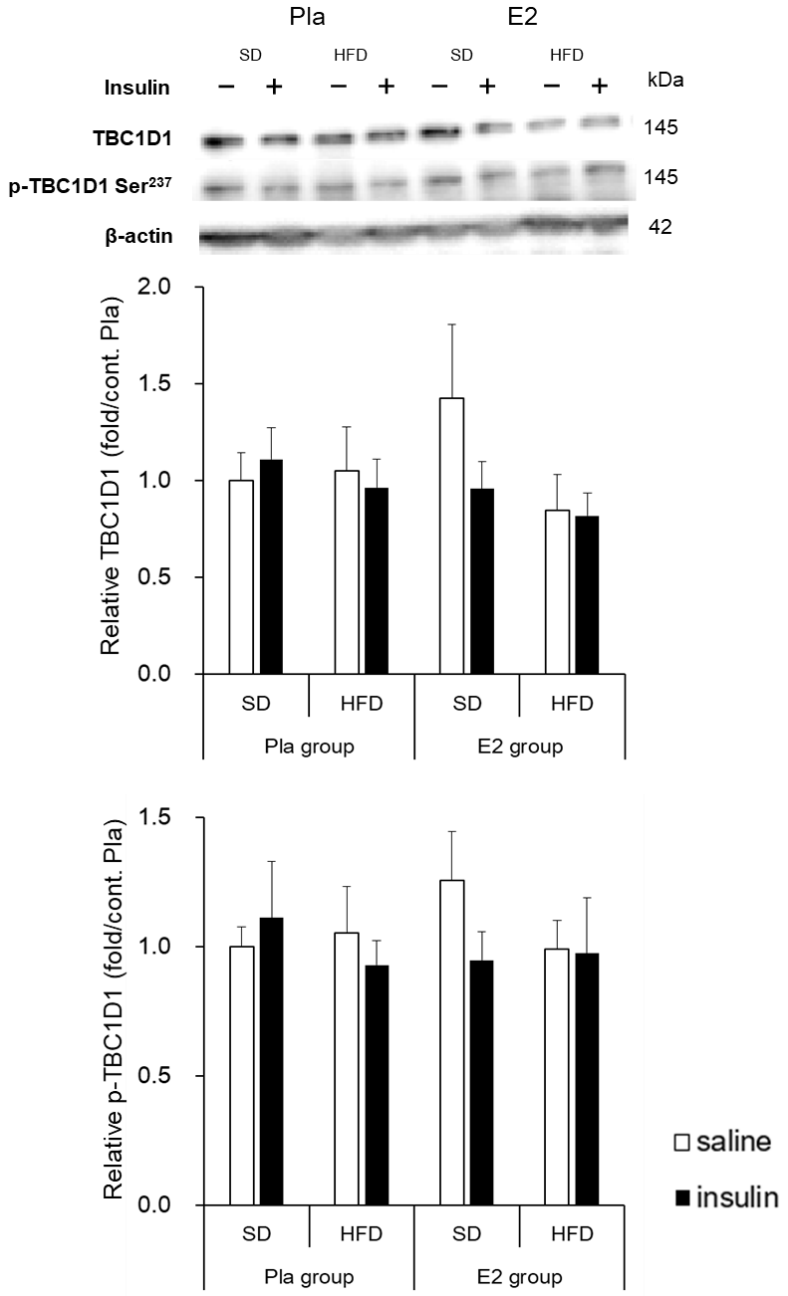

Figure 2. Representative blots and relative values of TBC1 (Tre2, BUB2, CDC16) domain family member 1 (TBC1D1) and phospho (p)-TBC1D1 Ser^{237} in the gastrocnemii of rats in the placebo (Pla)-standard diet (SD) (n = 13), Pla-high-fat diet (HFD) (n = 13), 17β-estradiol (E2)-SD (n = 12), and E2-HFD (n = 11) groups of ovariectomized (OVX) rats. Data are expressed as means ± SE, with (+) or without (-) insulin stimulation by injection of 10^{-5} mol/l insulin or saline in the portal vein, and were analyzed by two-way ANOVA using the following factors: group (p_{Group}: Pla-SD, Pla-HFD, E2-SD or E2-HFD group), insulin ($p_{Insulin}$: saline or insulin injection), and interaction of replacement and insulin injection ($p_{Group \times Insulin}$). This was followed by a post-hoc Tukey-Kramer test.

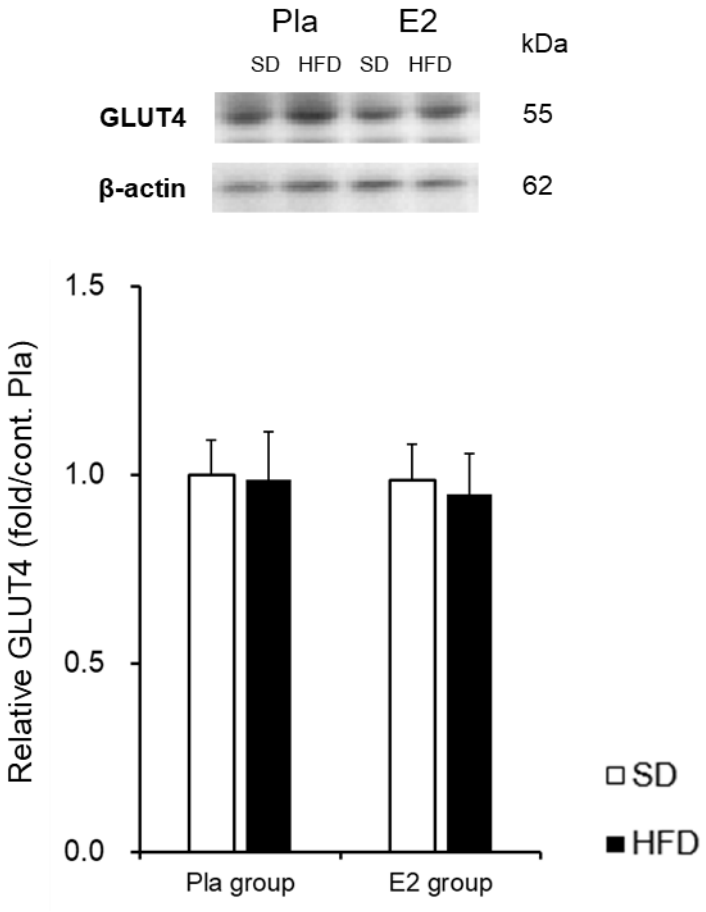

Figure 3. Representative blots and relative values of glucose transporter 4 (GLUT4) in the gastrocnemii of rats in the placebo (Pla)-standard diet (SD) (n = 12), Pla-high-fat diet (HFD) (n = 13), 17β-estradiol (E2)-SD (n = 12), and E2-HFD (n = 11) groups of ovariectomized (OVX) rats. Data are expressed as means ± SE, and were analyzed by two-way ANOVA using the following factors: replacement (p_{Replace}: Pla or E2 group), diet (p_{Diet}: SD or HFD), and interaction of replacement and insulin injection ($p_{\text{Replace}\times\text{Diet}}$). This was followed by a post-hoc Tukey-Kramer test.

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Naoko Yokota-Nakagi

List of Publications

Doctoral thesis

- **Yokota-Nakagi N**, Takahashi H, Kawakami M, Takamata A, Uchida Y, Morimoto K. Estradiol replacement improves high-fat diet-induced obesity by suppressing the action of ghrelin in ovariectomized rats. *Nutrients.* **2020**, *12*, 907. [Chapter 1]
- **Yokota-Nakagi N**, Omoto S, Tazumi S, Takamata A, Morimoto K. Effects of estradiol replacement on high-fat diet-induced insulin resistance in ovariectomized rats. *Nutrients.* submitted. [Chapter 2]

Other papers

- Kawakami M, **Yokota-Nakagi N**, Takamata A, Morimoto K. Endurance running exercise is an effective alternative to estradiol replacement for restoring hyperglycemia through TBC1D1/GLUT4 pathway in skeletal muscle of ovariectomized rats. *J Physiol Sci.* **2019**, *69*, 1029–1040.
- Kawakami M, **Yokota-Nakagi N**, Uji M, Yoshida KI, Tazumi S, Takamata A, Uchida Y, Morimoto K. Estrogen replacement enhances insulin-induced AS160 activation and improves insulin sensitivity in ovariectomized rats. *Am J Physiol Endocrinol Metab.* **2018**, *315*, E1296–E1304.
- Tazumi S, Omoto S, Nagatomo Y, Kawahara M, **Yokota-Nakagi N**, Kawakami M, Takamata A, Morimoto K. Estrogen replacement attenuates stress-induced pressor responses through vasorelaxation via β_2-adrenoceptors in peripheral arteries of ovariectomized rats. *Am J Physiol Heart Circ Physiol.* **2018**, *314*, H213–H223.
- Tazumi S, **Yokota N**, Kawakami M, Omoto S, Takamata A, Morimoto K. Effects of estrogen replacement on stress-induced cardiovascular responses via renin-angiotensin system in ovariectomized rats. *Am J Physiol Regul Integr Comp Physiol.* **2016**, *311*, R898–R905.