

The effects of chronic testosterone administration on body weight, food intake, and adipose tissue are changed by estrogen treatment in female rats

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Abstract

In females, estrogens play pivotal roles in preventing excess body weight (BW) gain. On the other hand, the roles of androgens in female BW, appetite, and energy metabolism have not been fully examined. We hypothesized that androgens' effects on food intake (FI) and BW regulation change according to the estrogens' levels. To evaluate this hypothesis, the effects of chronic testosterone administration in ovariectomized (OVX) female rats with or without estradiol supplementation were examined in this study. Chronic testosterone administration decreased BW, FI, white adipose tissue (WAT) weight, and adipocyte size in OVX rats, whereas it increased BW, WAT weight, and adipocyte size in OVX with estradiol-administered rats. In addition, chronic testosterone administration increased hypothalamic CYP19a1 mRNA levels in OVX rats, whereas it did not alter CYP19a1 mRNA levels in OVX with estradiol-administered rats, indicating that conversion of testosterone to estrogens in the hypothalamus may be activated in testosterone-administered OVX rats. Furthermore, chronic testosterone administration decreased hypothalamic TNF- α mRNA levels in OVX rats, whereas it increased hypothalamic IL-1 β mRNA levels in OVX with estradiol-administered rats. On the other hand, IL-1 β and TNF- α mRNA levels in visceral and subcutaneous WAT and liver were not changed by chronic testosterone administration in both groups. These data indicate that the effects of chronic testosterone administration on BW, FI, WAT weight, and adipocyte size were changed by estradiol treatment in female rats. Testosterone has facilitative effects on BW gain, FI, and adiposity under the estradiol-supplemented condition, whereas it has inhibitory effects in the non-supplemented condition.

Differences in the responses of hypothalamic factors, such as aromatase and inflammatory cytokines, to testosterone might underlie these opposite effects.

Introduction

Energy balance and reproductive function are closely linked in most species. The sex hormones, estrogens and androgens, are particularly related in the regulation of food intake (FI), energy metabolism, and body weight (BW) in mammals and humans (Hirschberg, 2012). Ovariectomy increases FI and BW in female animals, and these effects are prevented by estradiol replacement, indicating that estrogens play pivotal roles in preventing excess BW gain in females (Asarian and Geary, 2013; Blaustein and Wade, 1976; Hirschberg et al., 2012). These estrogens' effects on BW are primarily mediated by estrogen receptor- α (ER- α) in the hypothalamic area and brain stem. Estradiol injection into the hypothalamic paraventricular nucleus decreases BW and FI in ovariectomized rats (Palmer et al., 1986; Butera et al., 1989). Mutations of ER- α genes induce obesity in humans and mice, and deletion of ER- α blocks the effects of estradiol on BW (Heine et al., 2000; Okura et al., 2003; Gao et al., 2007).

On the other hand, the roles of androgens in female FI, energy metabolism, and BW regulation have not been fully examined, although some studies have shown that androgens increase FI and fat weight in females (Hirschberg et al., 2004; Naessen et al., 2007; Lim et al., 2009; Hirschberg, 2012) and increase the risk of visceral obesity in women and experimental animals (Evans et al., 1988; Dieudonne et al., 1998; Ibanez et al., 2003; Gambineri et al., 2004; Nohara et al., 2014). Androgen-administered animals have been used as a model of polycystic ovary syndrome (PCOS), which is one of the common causes of anovulation and metabolic abnormalities in women of reproductive age, because these animals show disruption

of ovulation, as well as increased BW and fat mass (Walters et al., 2012). However, the physiological mechanisms by which androgens disturb FI and BW regulation in females at this age have not been fully evaluated. Interestingly, it has been reported that the effects of androgens on energy metabolism and BW in males are different from those in females of reproductive age. Deficiency of testosterone, one of the most important androgens, increases the amount of visceral adipose tissue and insulin resistance in males, and it increases the risk of diabetes and metabolic syndrome (Navarro et al., 2015). These data suggest that testosterone plays pivotal roles in preventing excess BW gain and obesity in males.

In a previous study, we evaluated the effects of chronic testosterone administration on BW, FI, and body composition in female rats of different reproductive ages and gonadal status, i.e., pre-pubertal age, ovarian-intact reproductive age, and ovariectomized reproductive age (Iwasa et al., 2016). We found that BW gain and FI were increased by chronic testosterone administration in pre-pubertal and ovarian-intact reproductive age rats, but not in ovariectomized rats. In this study, all ovariectomized rats showed undetectable levels of serum estradiol, whereas 25% of pre-pubertal rats showed detectable levels of serum estradiol (unpublished data). In addition, because the ovaries were not removed in pre-pubertal rats, other kinds of estrogen may be produced and secreted into the plasma. Thus, it is speculated that pre-pubertal rats may have higher estrogen levels than ovariectomized rats. Therefore, we hypothesized that testosterone's effects on FI and BW regulation may be changed by the estrogen level.

To further investigate this hypothesis, we evaluated the effects of chronic administration of testosterone in ovariectomized female rats with or without estradiol supplementation in this study. Because estradiol could be stably supplemented, the interaction between testosterone and estradiol may be more precisely evaluated in this model. The effects on BW, FI, body composition, histological findings of fat and liver were evaluated. In addition, the peripheral and hypothalamic orexigenic factor, neuropeptide Y (NPY), and anorexigenic factors, leptin, proopiomelanocortin (POMC), and leptin receptor (OBRb), were evaluated, because it has been reported that sex steroids affect serum and hypothalamic levels and actions of these factors (Kimura et al., 2002; Anukulitch et al., 2007; Santollo and Eckel, 2008; Li et al., 2016). Hypothalamic estrogen and androgen receptors and aromatase gene expressions were also measured, because these factors also play pivotal roles in the regulation of BW and FI (Jones et al., 2000; Wu et al., 2009; Hirschberg, 2012). Furthermore, gene expressions of peripheral and hypothalamic pro-inflammatory cytokines were also measured, because these factors were involved in the disruption of the regulation of BW and FI in the obesity models, including the model induced by ovariectomy (Carvalheira et al., 2003; De Souza et al., 2005; Rogers et al., 2009; Thaler et al., 2013).

Materials and Methods

Animals

Eight-week-old Wistar female adult rats (200-230 g) were purchased from

Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and housed in a room under controlled light (12 h light, 12 h darkness; lights turned on at 0800 and turned off at 2000) and temperature (24°C) with free access to food and water. In total, 32 rats were used in this study. All animal experiments were conducted in accordance with the ethical standards of the institutional Animal Care and Use Committee of the University of Tokushima. Ovariectomy was carried out under sodium pentobarbital (60-80 mg/kg, intraperitoneal, i.p.), and tube implantations and decapitation were carried out under sevoflurane anesthesia. At nine weeks of age, rats were ovariectomized bilaterally and individually housed after surgery. Water containing ibuprofen (0.1 mg/ml) was provided during the three days after surgery to reduce the rats' postoperative pain.

Effects of chronic testosterone administration in ovariectomized (OVX) rats not administered estradiol

Four weeks after ovariectomy (13 weeks of age), rats were randomly divided into either the testosterone-administered (Testosterone) or the without testosterone-administered (Control) group (n = 8 per group). In the Testosterone group, rats were implanted with a silastic tube filled with crystalline testosterone (inner diameter 3 mm, outer diameter 5 mm, length of the filling part 30 mm) (As One Co., Ltd., Tokyo, Japan) (De Vries et al., 1994). In the Control group, rats were implanted with an empty tube. At 16 days after implantation, the rats were sacrificed by decapitation after measurement of BW and cumulative FI. The fixed amount of normal diet (type

MF; Oriental Yeast Co. Ltd., Tokyo, Japan) (359 kcal/100 g) was placed in the food space of the wire mesh top, and the remaining food weight was measured 16 days after implantation. Wood chip bedding was changed eight days after implantation, and broken food in the bedding was collected at this time point and 16 days after implantation, and their weights were added to the remaining food weight. Brain, blood, visceral fat (parametrial, perirenal, and mesenteric depots, subcutaneous fat (inguinal depot), liver, and uterus were collected. Weights of visceral fat, subcutaneous fat, and uterus were measured immediately after removal, and tissue samples (around 300-400-mm³) of visceral (parametrial) and subcutaneous fat were dissected. Similarly, the same sizes of tissue samples were dissected from the liver. Serum was separated by centrifugation and stored at -20°C, and tissue samples were stored at -80°C. Other tissue samples of visceral and subcutaneous fat and liver were fixed in 4% paraformaldehyde.

Effects of chronic testosterone administration in ovariectomized with estradiol-administered (OVX+E) rats

Four weeks after ovariectomy (13 weeks of age), all rats were implanted with a silastic tube filled with crystalline estradiol (length of the filling part, 3 mm) (Le et al., 2014). At the same time, rats were divided into either the Testosterone or the Control group, as described above (n = 8 per group). At 16 days after implantation, the rats were sacrificed by decapitation after measurement of BW and cumulative FI. Tissue weight were measured, and samples were collected and stored or fixed, as

described above.

Hormone assay

Serum estradiol and testosterone levels were measured by a commercial laboratory (SRL, Tokyo, Japan) using an electrochemiluminescence immunoassay (ECLIA; Roche Diagnostics GmbH, Mannheim, Germany). Serum leptin levels were measured using radioimmunoassay kits (multi-species leptin RIA kit, Linco Research Inc., MO, USA). The sensitivity of the assay was 1.0 ng/ml, and its inter- and intra-assay coefficients of variation were 3.2% and 7.8%, respectively.

Quantitative real-time polymerase chain reaction

Whole hypothalamic explants were dissected from the frozen brains, as described previously (Iwasa et al., 2016). Briefly, the brain sections were dissected out via an anterior coronal cut at the posterior border of the mammillary bodies, parasagittal cuts along the hypothalamic fissures, and a dorsal cut 2.5 mm from the ventral surface. Total RNA was isolated from hypothalamic explants and visceral fat using a TRIzol® reagent kit (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy® mini kit (Qiagen GmbH, Hilden, Germany). Then, cDNA was synthesized with oligo (deoxythymidine) primers at 50°C using the SuperScript III first-strand synthesis system for the real-time polymerase chain reaction (PCR; Invitrogen Co.). The PCR analysis was performed using the StepOnePlus™ real-time PCR system (PE Applied Biosystems, Foster City, CA, USA) and FAST SYBR® green. The ER- α

androgen receptor (AR), CYP19a1, NPY, POMC, OBRb, interleukin-1 β (IL-1 β), and tumor necrosis factor- α (TNF- α) mRNA levels in the hypothalamus, leptin, IL-1 β , and TNF- α mRNA levels in visceral and subcutaneous WAT, and IL-1 β and TNF- α mRNA levels in the liver were measured. The mRNA expression level of each factor was normalized to that of GAPDH or the 18S rRNA level. Dissociation curve analysis was also performed for each gene at the end of PCR. Each amplicon generated a single peak. Primer sequences, product size, and annealing temperatures are shown in Table 1. The PCR conditions were as follows: initial denaturation and enzyme activation at 95°C for 20 s, followed by 45 cycles of denaturation at 95°C for 3 s, and annealing and extension for 30 s.

Histology

Fixed visceral and subcutaneous WAT and liver were dehydrated with ethanol and xylene and sliced into sections after being embedded in paraffin. Serial 4- μ m-thick sections were stained with hematoxylin and eosin (H&E), and histological findings were captured by a Zeiss Imager M2 microscope with AxioVision version 4.8 acquisition software (Zeiss). The mean adipocyte area of 50 randomly selected adipocytes per specimen was determined using ImageJ software.

Statistical analyses

All results are presented as means \pm SEM values. Student's *t*-test or the Mann-Whitney *U* test was used for comparisons between the control and testosterone-

administered groups within the OVX and OVX+E groups. *P*-values of < 0.05 were considered significant. Two-way analysis of variance (ANOVA) was used for comparison of the effects of testosterone administration between the OVX and OVX+E groups. Cohen's *d* (small effect = 0.2, medium effect = 0.5, large effect = 0.8), *r* (small effect = 0.1, medium effect = 0.3, large effect = 0.5), and Eta squared (η^2) (small effect = 0.2, medium effect = 0.5, large effect = 0.8) are reported when analyses were undertaken by Student's *t*-test, the Mann-Whitney *U* test, and ANOVA, respectively (small effect size = 0.2, medium = 0.5, large = 0.8).

Results

Serum estradiol and testosterone levels in each group

Serum estradiol levels were lower in the OVX groups than in the OVX+E groups ($p < 0.01$, $r = 0.85$) (Table 2). Serum testosterone levels were higher in the Testosterone-administered groups than in the Control groups. On the other hand, serum estradiol levels were not different between the OVX-Control and OVX-Testosterone groups. Similarly, serum estradiol levels were not different between the OVX+E-Control and OVX+E-Testosterone groups. Serum testosterone levels were not different between the OVX-Testosterone and OVX+E-Testosterone groups.

These results indicate that each hormone was administered adequately in this experimental protocol.

Effects of chronic testosterone administration on BW, FI, WAT weight, and uterine weight in OVX and OVX+E rats

In the OVX group, BW change (% of initial BW) after implantation was significantly smaller in the Testosterone group than in the Control group ($p = 0.03$, $d = 1.21$) (Fig. 1A). Cumulative FI (g / 100 g initial BW) after implantation was significantly less in the Testosterone group than in the Control group ($p < 0.01$, $d = 1.94$) (Fig. 1B). The weights (g) of visceral WAT, subcutaneous WAT, and total WAT were significantly lower in the Testosterone group than in the Control group (visceral WAT, $p < 0.01$, $d = 1.51$; subcutaneous WAT, $p = 0.01$, $d = 1.41$; total WAT, $p < 0.01$, $d = 1.66$) (Fig. 1C-E). On the other hand, uterine weight (mg) was significantly greater in the Testosterone group than in the Control group ($p < 0.01$, $d = 1.84$) (Fig. 1F).

In the OVX+E group, BW change (% of initial BW) after implantation was significantly higher in the Testosterone group than in the Control group ($p = 0.02$, $d = 1.36$) (Fig. 1G). On the other hand, cumulative FI (g/100 g initial BW) after implantation in the Testosterone group was not different from that in the Control group (Fig. 1H). The weights (g) of subcutaneous WAT and total WAT were significantly greater in the Testosterone group than in the Control group (subcutaneous WAT, $p = 0.04$, $d = 1.14$; total WAT, $p = 0.05$, $d = 1.07$) (Fig. 1J, K). Uterine weights (mg) were not different between the Testosterone group and the Control group (Fig. 1L).

The effects of testosterone administration on BW change (% of initial BW) and

cumulative FI (g/100 g initial BW) were significantly different between the OVX and OVX+E groups (BW change, $F(1,31) = 126.9, p < 0.01, \eta^2 = 0.75$, FI; $F(1,31) = 75.5, p < 0.01, \eta^2 = 0.67$). Similarly, the effects of testosterone administration on the weight of subcutaneous WAT, visceral WAT, and total WAT were significantly different between the OVX and OVX+E groups (subcutaneous WAT, $F(1,31) = 42.8, p < 0.01, \eta^2 = 0.50$; visceral WAT, $F(1,31) = 89.3, p < 0.01, \eta^2 = 0.36$; total WAT, $F(1,31) = 14.2, p < 0.01, \eta^2 = 0.26$). The effects of testosterone administration on uterine weight were significantly different between the OVX and OVX+E groups ($F(1,31) = 162.4, p < 0.01, \eta^2 = 0.69$).

Effects of chronic testosterone administration on the mRNA levels of hypothalamic factors in OVX and OVX+E rats

In the OVX group, hypothalamic mRNA levels of CYP19a1 and NPY were significantly higher in the Testosterone group than in the Control group (CYP19a1, $p < 0.01, d = 1.56$; NPY, $p = 0.04, d = 1.15$) (Fig. 2, upper row). On the other hand, hypothalamic ER- α and TNF- α mRNA levels were significantly lower in the Testosterone group than in the Control group (ER- α , $p = 0.01, d = 7.61$; TNF- α , $p = 0.04, d = 1.08$). Hypothalamic mRNA levels of AR, POMC, OBRb, and IL-1 β were not different between the Testosterone and Control groups.

In the OVX+E group, hypothalamic ER- α and AR mRNA levels were significantly lower in the Testosterone group than in the Control group (ER- α , $p =$

0.03, $r = 0.55$; AR, $p < 0.01$, $r = 0.84$) (Fig. 2, lower row). On the other hand, the hypothalamic IL-1 β mRNA level was significantly higher in the Testosterone group than in the Control group ($p = 0.01$, $d = 1.43$). Hypothalamic mRNA levels of CYP19a1, NPY, POMC, OBRb, and TNF- α were not different between the Testosterone and Control groups.

The effects of testosterone administration on the hypothalamic IL-1 β and TNF- α mRNA levels were significantly different between the OVX and OVX+E groups (IL-1 β , $F(1,31) = 7.62$, $p = 0.01$, $\eta^2 = 0.16$; TNF- α , $F(1,31) = 7.22$, $p = 0.01$, $\eta^2 = 0.17$). By contrast, the effects of testosterone administration on ER- α , AR, CYP19a1, IL-1 β , and TNF- α were not significantly different between the OVX and OVX+E groups.

Effects of chronic testosterone administration on serum leptin levels in OVX and OVX+E rats

In the OVX group, serum leptin levels were not different between the Testosterone and Control groups (Fig. 3A). The serum leptin level per unit of WAT (ng/mL/g) was significantly higher in the Testosterone group than in the Control group ($p = 0.03$, $d = 1.23$) (Fig. 3B).

In the OVX+E group, serum leptin levels were not different between the Testosterone and Control groups (Fig. 3C). The serum leptin level per unit of WAT was significantly lower in the Testosterone group than in the Control group ($p = 0.04$, $d = 1.08$) (Fig. 3D).

The effects of testosterone administration on the leptin level per unit of WAT were not significantly different between the OVX and OVX+E groups ($F(1,31) = 9.57, p < 0.01, \eta^2 = 0.20$).

Effects of chronic testosterone administration on the mRNA levels of inflammatory factors and adipocyte size in visceral WAT in OVX and OVX+E rats

In the OVX group, visceral WAT mRNA levels of leptin, IL-1 β , and TNF- α were not different between the Testosterone and Control groups (Fig. 4A). Mean adipocyte size (μm^2) was not different between the Testosterone and Control groups (Fig. 4B, C).

In the OVX+E group, visceral WAT mRNA levels of leptin, IL-1 β , and TNF- α were not different between the Testosterone and Control groups (Fig. 4D). Mean adipocyte size was significantly larger in the Testosterone group than in the Control group ($p < 0.01, r = 0.07$) (Fig. 4E, F).

The effects of testosterone administration on adipocyte size of visceral WAT were significantly different between the OVX and OVX+E groups ($F(1,1599) = 89.9, p < 0.01, \eta^2 = 0.05$).

Effects of chronic testosterone administration on the mRNA levels of inflammatory factors and adipocyte size in subcutaneous WAT in OVX and OVX+E rats

In the OVX group, subcutaneous WAT mRNA levels of leptin, IL-1 β , and TNF- α were not different between the Testosterone and Control groups (Fig. 5A). Mean adipocyte size (μm^2) was significantly smaller in the Testosterone group than in the Control group ($p < 0.01$, $r = 0.30$) (Fig. 5B, C).

In the OVX+E group, subcutaneous WAT mRNA levels of leptin, IL-1 β , and TNF- α were not different between the Testosterone and Control groups (Fig. 5D). Mean adipocyte size was not different between the Testosterone group and the Control group (Fig. 5E, F).

The effects of testosterone administration on adipocyte size of subcutaneous WAT were significantly different between the OVX and OVX+E groups ($F(1,1599) = 825.6$, $p < 0.01$, $\eta^2 = 0.33$).

Effects of chronic testosterone administration on the mRNA levels of inflammatory factors in liver in OVX and OVX+E rats

In the OVX group, IL-1 β and TNF- α mRNA levels in liver were not different between the Testosterone and Control groups (Fig. 6A). There were no obvious differences in microscopic findings of the liver between the Testosterone and Control groups (Fig. 6B).

In the OVX+E group, IL-1 β and TNF- α mRNA levels in the liver were not different between the Testosterone and Control groups (Fig. 6C). There were no obvious differences in the microscopic findings of the liver between the Testosterone and Control groups (Fig. 6D).

Discussion

In the present study, chronic testosterone administration decreased BW, FI, and WAT weight in OVX rats, whereas it increased BW and WAT weight in OVX+E rats. Similarly, chronic testosterone administration decreased subcutaneous adipocyte size in OVX rats, whereas it increased visceral adipocyte size in OVX+E rats. These results indicate that the effects of testosterone on BW and adipocytes in OVX rats were completely opposite to those in OVX+E rats. Sex hormones play pivotal roles in the regulation of FI, energy metabolism, and BW in mammals and humans (Hirschberg, 2012). Many studies have indicated that estrogens prevent excess BW gain mainly through the modification of central and peripheral factors, such as increased leptin secretion and hypothalamic OBRb and POMC gene expressions and decreased hypothalamic NPY gene expression and action (Kimura et al., 2002; Blaustein and Wade, 1976; Anukulitch et al., 2007; Santollo and Eckel, 2008; Hirschberg et al., 2012; Santollo et al., 2012; Asarian and Geary, 2013), whereas the roles of androgens in the regulation of BW, FI, and energy metabolism and their mechanisms have not been fully examined. Some studies have suggested that chronic administration of androgens, such as testosterone, increases BW and body fat in female experimental animals at reproductive age (Walters et al., 2012), and that hyperandrogenism is related to the dysfunctions of BW and metabolic regulation in women (Golden et al., 2004; Coviello et al., 2006; Navarro et al., 2015). On the other hand, as noted above, the effects of androgens on BW and energy metabolism in males

do not correspond to those in females. Androgens prevent excess BW gain, obesity, and insulin resistance, and these actions decrease the risk of diabetes and metabolic syndrome in males (Navarro et al., 2015). Thus, we hypothesized that testosterone, one of the important androgens, prevents the inhibitory action of estrogen on BW gain, FI, and adiposity, and that it induces facilitative effects on them only in the presence of estrogen. Our previous data, which showed that the effects of chronic testosterone administration on BW and FI were changed by reproductive age and gonadal status, partially support this hypothesis (Iwasa et al., 2016). However, because gonadal-intact rats were used and serum estrogens levels were not evaluated in that study, the possibility that testosterone only suppressed estrogens secretion from the ovary, but not estrogens' actions, could not be excluded. Therefore, ovariectomized with estradiol-supplemented rats were used in the present study, and serum estradiol levels were not different between control rats and testosterone-administered rats; nevertheless, chronic testosterone administration increased BW and WAT weight. These results may support our hypothesis that testosterone has facilitative effects on BW gain and adiposity by preventing the inhibitory action of estrogens.

On the other hand, there are some discrepancies in the results between the present and our previous studies. For example, chronic testosterone administration did not affect FI in OVX+E rats in the present study, whereas it increased FI in gonadal intact female rats in a previous study (Iwasa et al., 2016). Because the mean serum estradiol level in OVX+E rats used in this study (143.9 ± 29.2 pg/mL) was higher than that in gonadal intact female rats used in a previous study (45.9 ± 7.8 pg/mL, unpublished

data), it may be assumed that the administered testosterone could not overcome the anorectic effects of estradiol in the present study. Besides this, differences in the study protocol, such as the duration of estradiol/testosterone administration, may have induced the discrepant results between the present and previous studies.

As mentioned above, chronic testosterone administration decreased BW, FI, and WAT weight in OVX rats in the present study. Because testosterone administration increased uterine weight in OVX rats, but not in OVX+E rats, conversion of testosterone to estrogens may be activated in OVX rats. Although serum estradiol levels in testosterone-administered OVX rats were not different from those in OVX rats not administered testosterone, other kinds of estrogens might be increased by testosterone administration. If so, it is possible that converted estrogens attenuate BW gain and WAT accumulation.

In the present study, chronic testosterone administration increased hypothalamic CYP19a1 mRNA levels in OVX rats, whereas it did not alter CYP19a1 mRNA levels in OVX+E rats. Thus, these results indicate that conversion from testosterone to estradiol in the brain may be increased in OVX rats compared with OVX+E rats. It has been reported that hypothalamic estrogens that are converted from testosterone by aromatase play roles in preventing BW gain, and that knockout of the aromatase gene induces obesity in male and female rodents (Jones et al., 2000; Wu et al., 2009). Therefore, we hypothesize that brain aromatase might be activated under the chronic testosterone-administered condition in OVX rats, and that converted estrogens acted to suppress the BW gain and FI and prevented accumulation of WAT in the present

study.

In the present study, chronic testosterone administration decreased hypothalamic AR mRNA levels in OVX+E rats, whereas it did not alter AR mRNA levels in OVX rats. It remains unclear whether this AR change is involved in the BW gain and increased WAT weights in testosterone-administered OVX+E rats. No study has evaluated the effects of exogenous testosterone on the hypothalamic AR levels in both sexes. On the other hand, some studies have shown that AR-knockout male and female mice show late-onset obesity and diet-induced obesity by modulation of lipid metabolism, respectively (Sato et al., 2003; Fagman et al., 2015). In addition, it has been shown that aromatase activity and mRNA in the brain are regulated primarily by androgens via AR in mammals, and that a potential androgen-responsive element is present at brain specific aromatase (Abdelgadir et al., 1994; Honda et al., 1994; Veney and Rissman, 2000; Roselli et al., 2001, 2009). Given this background, one possibility is that a decrease of AR itself is related to the increases of BW gain and WAT weight in the OVX+E rats in the present study. Another possibility is that decreased AR disrupts the response of aromatase to administered testosterone in OVX+E rats and induces the changes noted above.

In the present study, chronic testosterone administration decreased hypothalamic TNF- α mRNA levels in OVX rats, whereas it increased hypothalamic IL-1 β mRNA levels in OVX+E rats. On the other hand, IL-1 β and TNF- α mRNA levels in visceral and subcutaneous WAT and liver were not changed by chronic testosterone administration in both groups. It has been reported that systemic low-grade

inflammation is observed in obese individuals, and that these alterations are related in some metabolic diseases (Bastard et al., 2006; Rogers et al., 2009). In particular, inflammation in the hypothalamus produces defective FI and obesity through hypothalamic insulin and leptin resistance (Carvalho et al., 2003; De Souza et al., 2005; Thaler et al., 2013). Thus, testosterone-induced alterations in hypothalamic cytokine levels, i.e., decreases in OVX rats and increases in OVX+E rats, might be partially involved in the changes of BW, FI, and WAT weight.

In the present study, chronic testosterone administration did not alter the serum leptin levels in both OVX rats and OVX+E rats. However, serum leptin levels per unit WAT were increased or decreased by chronic testosterone administration in OVX rats and OVX+E rats, respectively. These results show that testosterone alters leptin secretion from WAT, and that its actions are changed according to the estrogen treatment. Interestingly, it has been reported that leptin levels per unit WAT are also decreased in high-fat diet-induced obese rats (Ainslie et al., 2000), indicating that the mechanisms by which testosterone induces BW and FI under the presence of estradiol may be similar to those acting in diet-induced obesity. In this study, chronic testosterone administration increased hypothalamic NPY mRNA levels in OVX rats. Although it is possible that this alteration may be one of the counter regulatory actions to increase FI and BW gain, the precise physiological role of this change remains unclear.

The limitations of the present study are as follows. Serum testosterone levels in the testosterone-administered groups were near the physiological range of male rats

(De Vries et al., 1994), and they were supra-physiological levels for female rats. Therefore, it remains unclear whether the results of the present study reproduce the pathophysiology of women with hyperandrogenism. In addition, it needs to be carefully considered whether these results reflect the efficacy of treatments, such as hormone replacement therapy for menopausal women and anti-androgen therapy for PCOS women.

In summary, the effects of chronic testosterone administration on BW, FI, WAT weight, and adipocyte size changed according to estrogen treatment in female rats. Testosterone has facilitative effects on BW gain and adiposity under the estradiol-supplemented condition, whereas it has inhibitory effects under the non-supplemented condition. In other words, the beneficial effects of testosterone administration on BW and adiposity in OVX rats are prevented by estradiol administration. Differences in the responses of hypothalamic factors, such as aromatase and inflammatory cytokines, to testosterone might underlie these opposite effects. These results may support our hypothesis that testosterone has unfavorable effects on BW and energy metabolism by preventing the favorable actions of estrogens.

Conflict of interest

The authors indicate no potential conflicts of interest.

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Figure legends

Fig. 1

(A, G) Body weight (BW) change (% of initial BW), (B, H) cumulative food intake (FI) (g/100 g initial BW), (C, I) visceral white adipose tissue (WAT) weight (g/100 g BW), (D, J) subcutaneous WAT weight (g/100 g BW), (E, K) total WAT weight (g/100 g BW), and (F, L) uterine weight in control (□) and testosterone-administered (■) female rats. The upper row shows the results in ovariectomized (OVX) rats, and the lower row shows those in estradiol-administered OVX (OVX+E) rats. $n = 8$ per group. Data are expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$.

Fig. 2

Hypothalamic mRNA levels of ER- α , AR, CYP19a1, NPY, POMC, OBRb, IL-1 β , and TNF- α in control (□) and testosterone-administered (■) female rats. The upper row shows the results in ovariectomized (OVX) rats, and the lower row shows those in estradiol-administered OVX (OVX+E) rats. The mRNA expression levels of control rats are expressed as 1.0. $n = 8$ per group. Data are expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$.

Fig. 3

(A, C) Serum leptin levels and (B, D) serum leptin/total white adipose tissue (WAT) weight in control (□) and testosterone-administered (■) female rats. The upper row shows the results in ovariectomized (OVX) rats, and the lower row shows those in

estradiol-administered OVX (OVX+E) rats. n = 8 per group. Data are expressed as means \pm SEM. * $P < 0.05$.

Fig. 4

(A, D) Visceral adipose mRNA levels of leptin, IL-1 β , and TNF- α , (B, E) representative photomicrographs of visceral adipose tissues with H&E staining, and (C, F) area of adipocytes in control (\square) and testosterone-administered (\blacksquare) female rats. The upper row shows the results in ovariectomized (OVX) rats, and the lower row shows those in OVX with estradiol-administered (OVX+E) rats. n = 8 per group. Data are expressed as means \pm SEM. * $P < 0.01$. Bars in B and E = 100 μ m. Images captured at 40 X.

Fig. 5

(A, D) Subcutaneous adipose mRNA levels of leptin, IL-1 β , and TNF- α , (B, E) representative photomicrographs of visceral adipose tissues with H&E staining, and (C, F) area of adipocytes in control (\square) and testosterone-administered (\blacksquare) female rats. The upper row shows the results in ovariectomized (OVX) rats, and the lower row shows those in estradiol-administered OVX (OVX+E) rats. n = 8 per group. Data are expressed as means \pm SEM. * $P < 0.01$. Bars in B and E = 100 μ m. Images captured at 40 X.

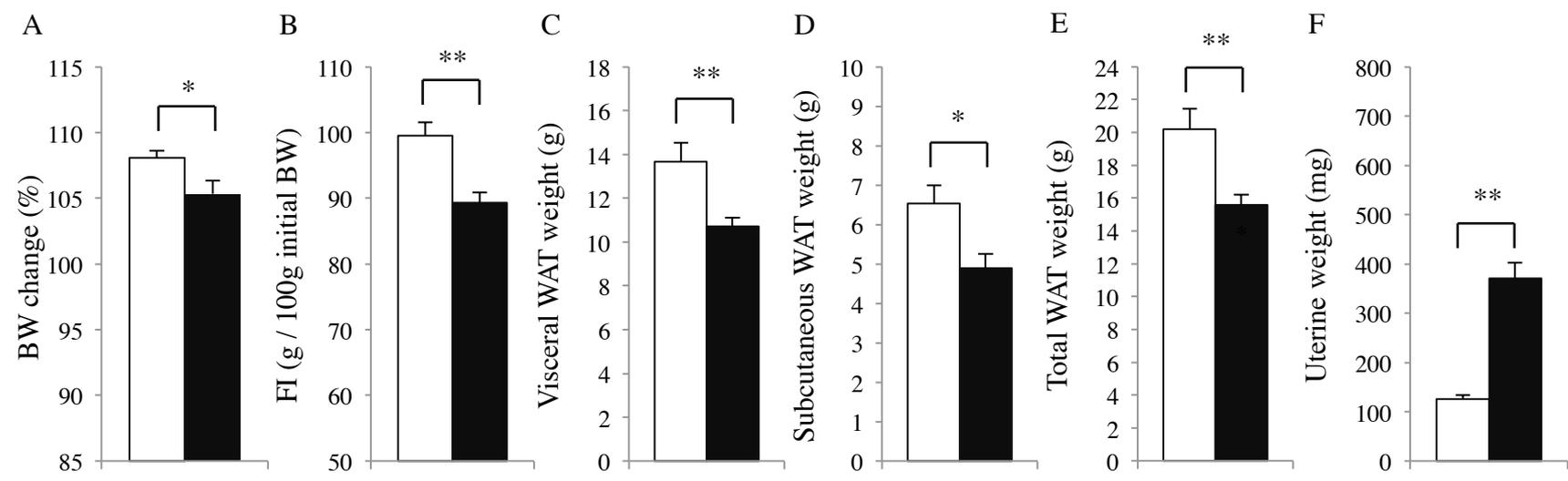
Fig. 6

(A, C) Hepatic mRNA levels of IL-1 β and TNF- α , and (B, D) representative photomicrographs of hepatic tissues with H&E staining in control (\square) and testosterone-

administered (■) female rats. The upper row shows the results in ovariectomized (OVX) rats, and the lower row shows those in estradiol-administered OVX (OVX+E) rats. n = 8 per group. Data are expressed as means \pm SEM. Bars in B and E = 100 μ m. Images captured at 40 X.

Fig. 1

OVX



□ Control
■ Testosterone

OVX+E

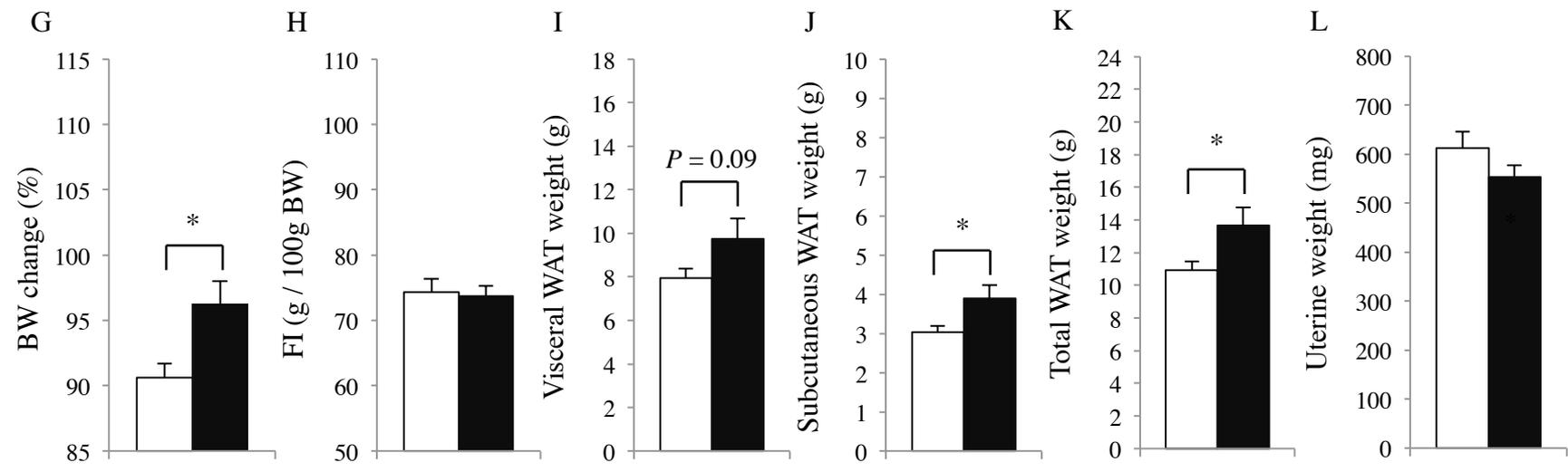


Fig. 2

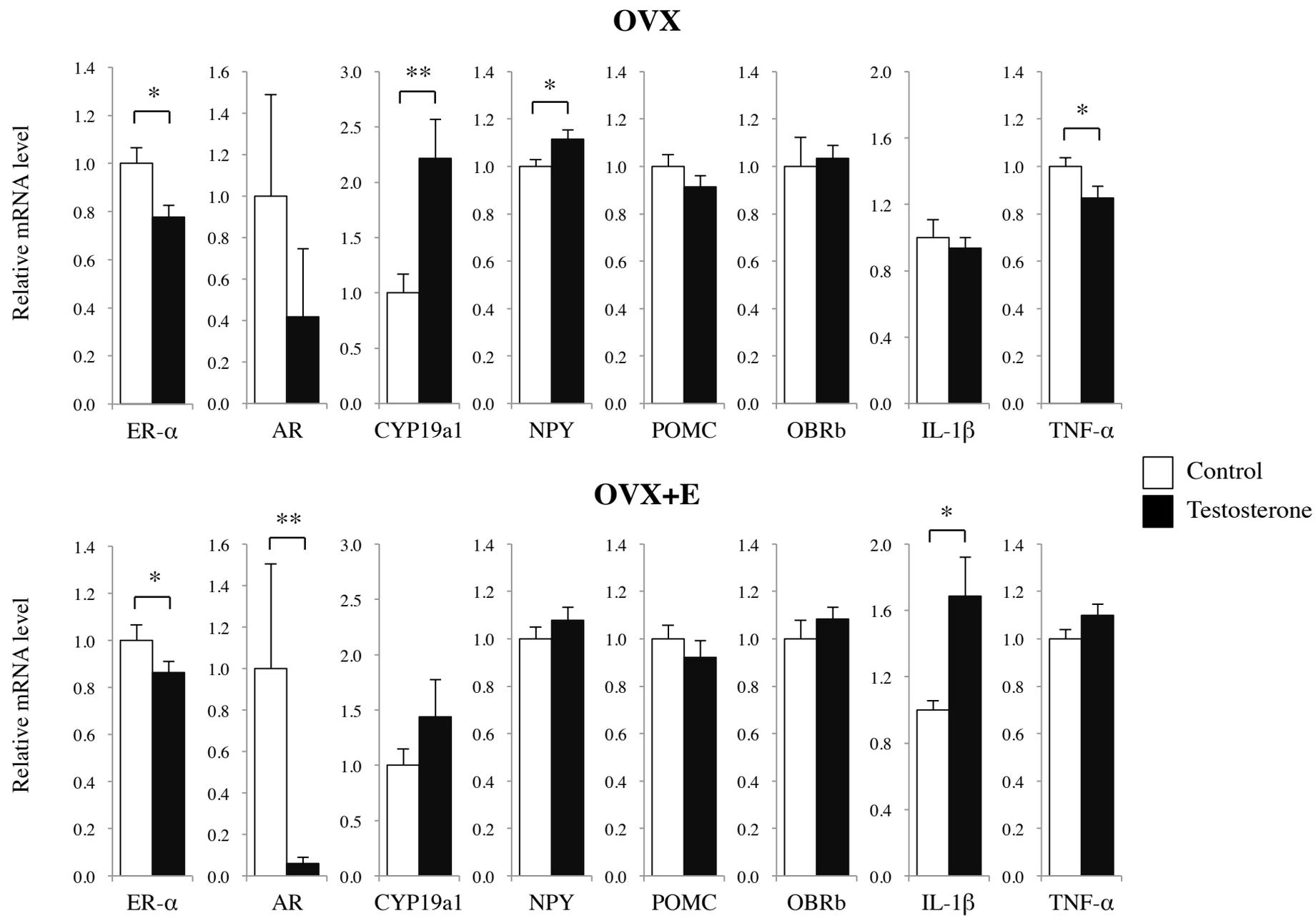


Fig. 3

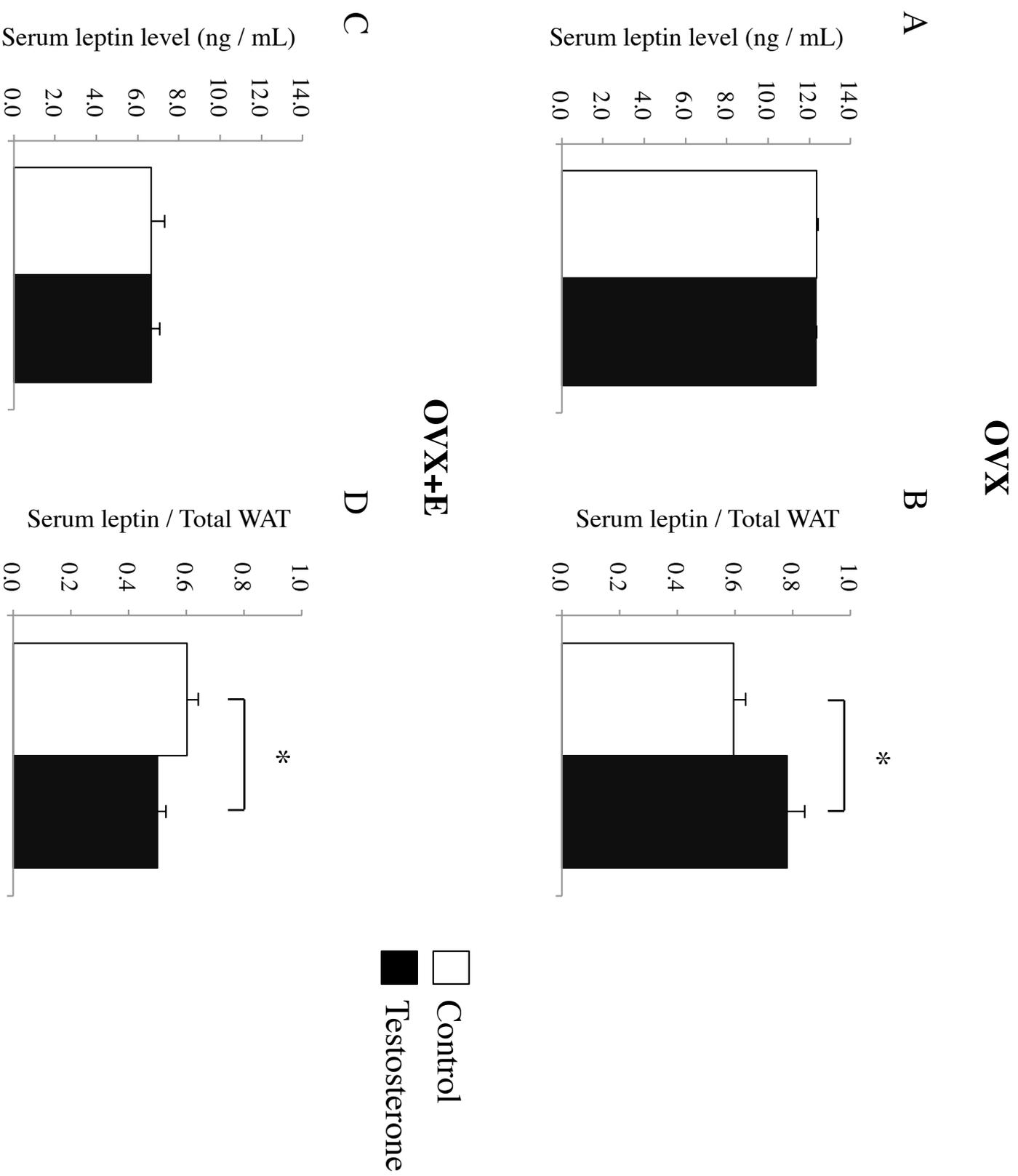
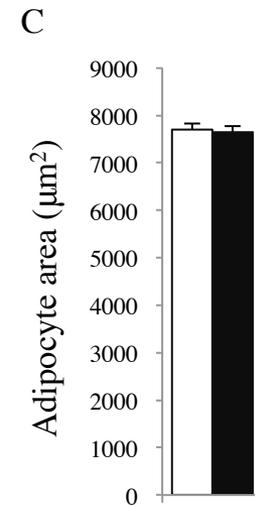
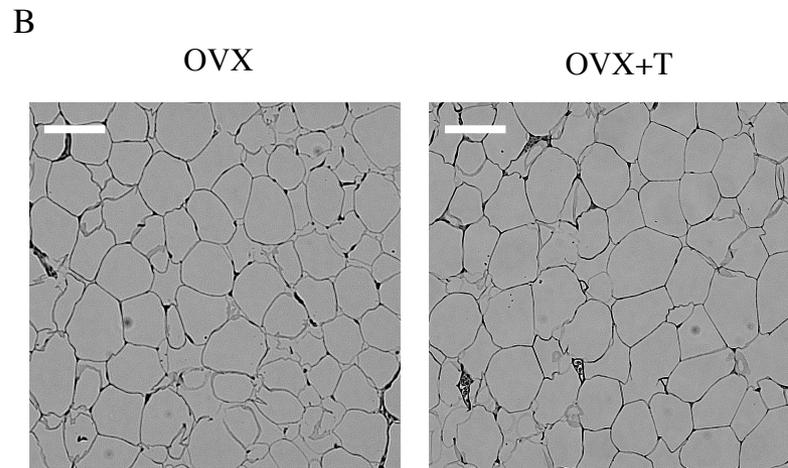
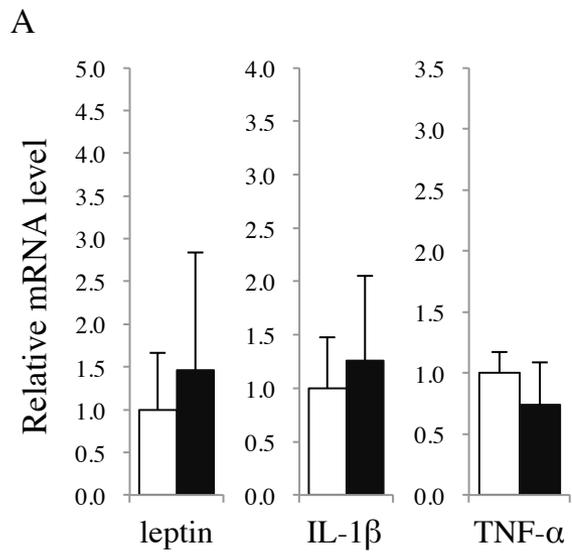
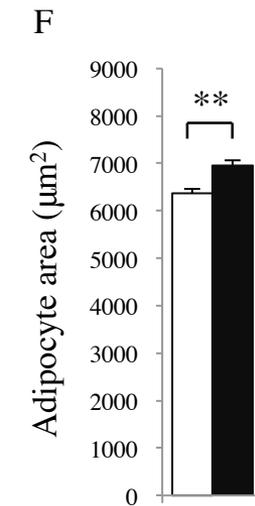
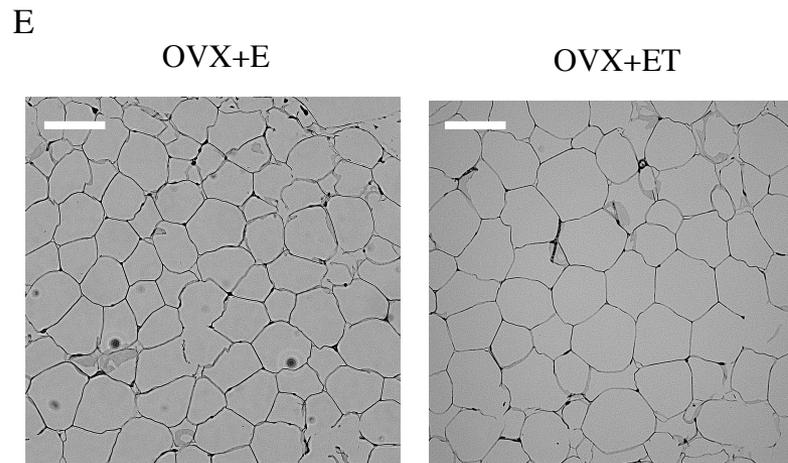
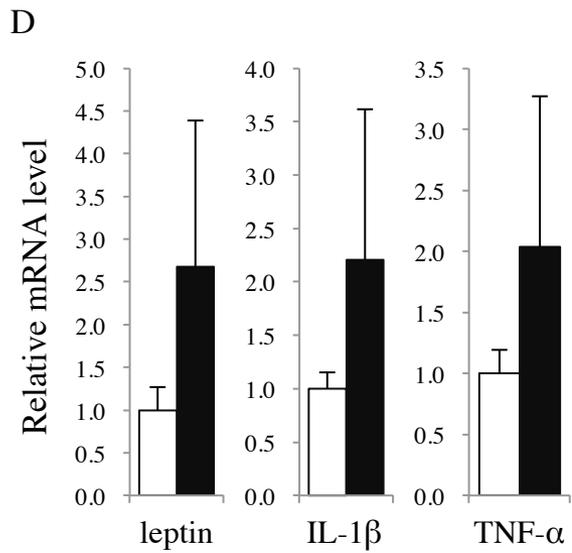


Fig. 4

OVX



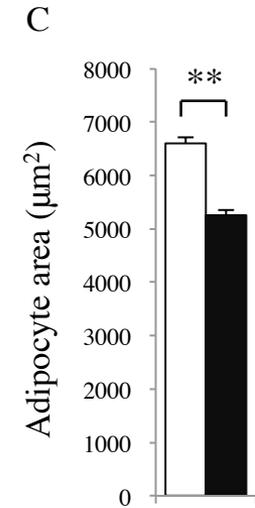
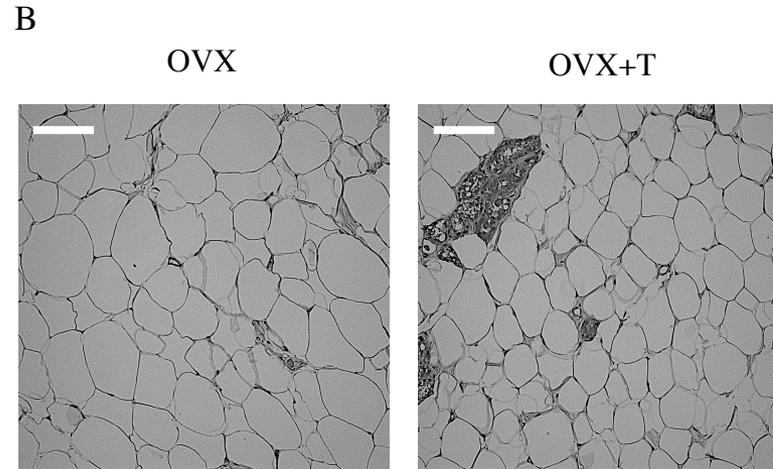
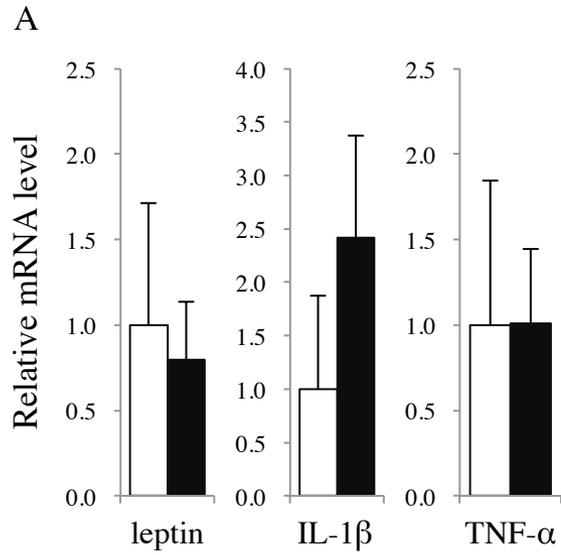
OVX+E



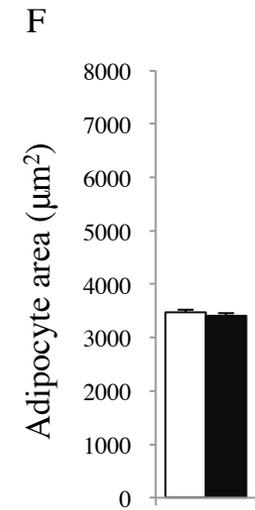
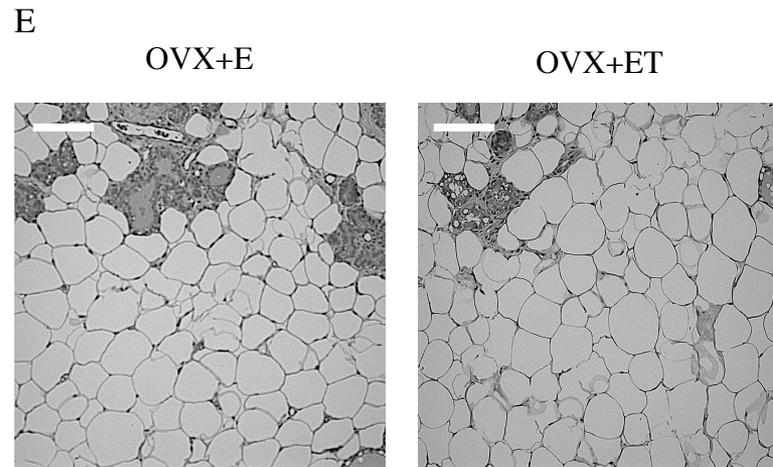
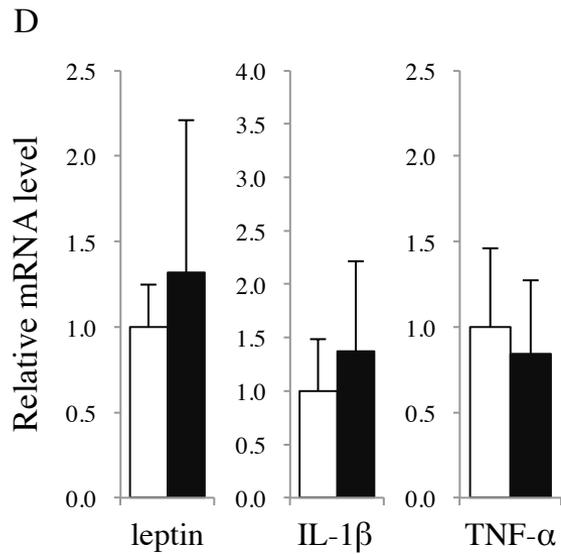
□ Control
■ Testosterone

Fig. 5

OVX



OVX+E



□ Control
■ Testosterone

Fig. 6

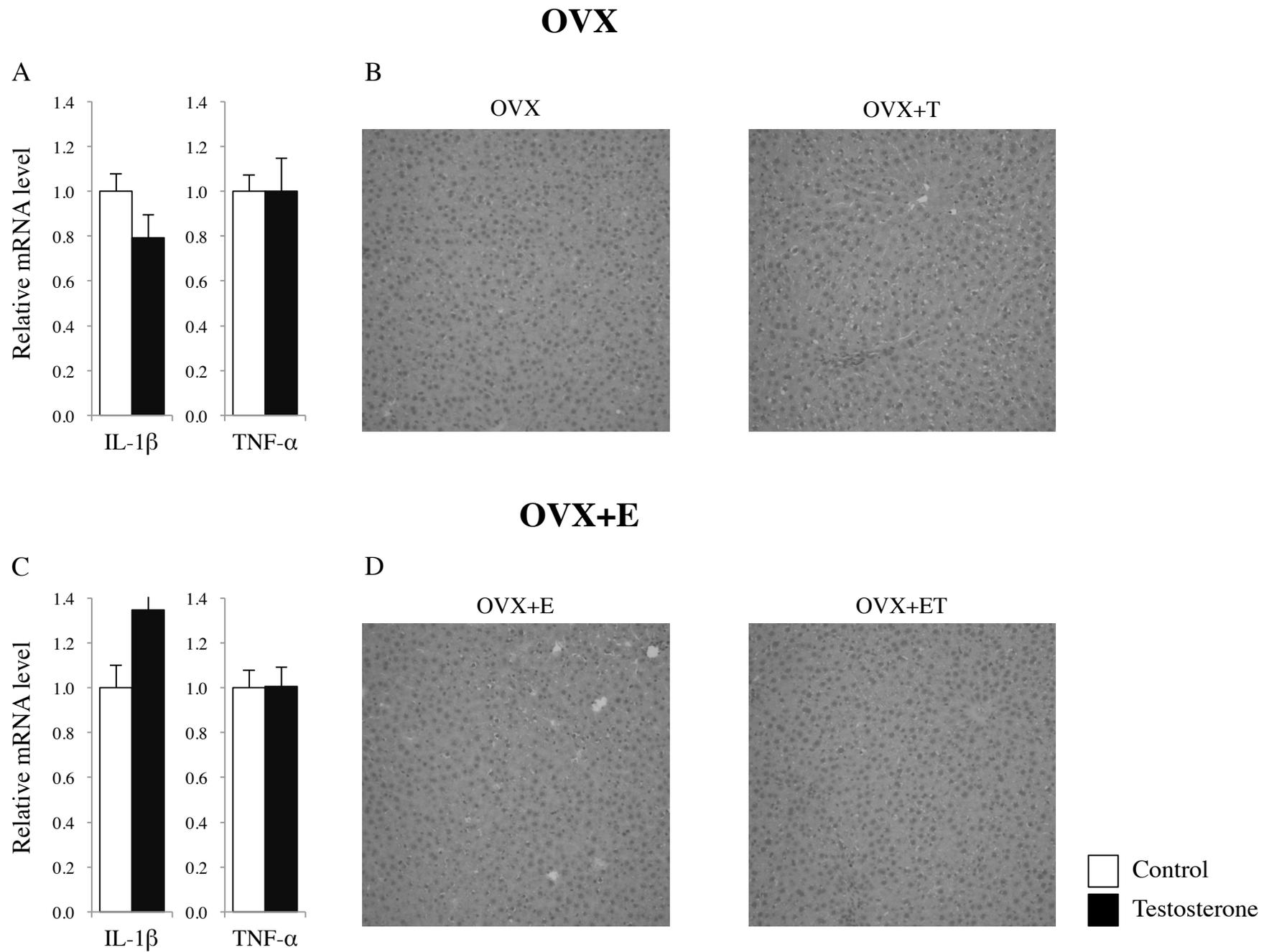


Table 1 Primer sequences, product sizes, and annealing temperatures

Primer	Sequence	Product size (bp)	Annealing T (°C)
ER- α forward	CAT CGA TAA GAA CCG GAG GA	128	62
ER- α reverse	TCT GAC GCT TGT GCT TCA AC		
AR forward	CCC ATC GAC TAT TAC TTC CC	58	63
AR reverse	TTA CGA GCT CCC AGA GTC AT		
CYP19a1 forward	CTC CTC CTG ATT CGG AAT TGT	<95	63
CYP19a1 reverse	TCT GCC ATG GGA AAT GAG AG		
NPY forward	GGG GCT GTG TGG ACT GAC CCT	148	66
NPY reverse	GAT GTA GTG TCG CAG AGC GGA G		
POMC forward	CCT CAC CAC GGA AAG CA	104	66
POMC reverse	TCA AGG GCT GTT CAT CTC C		
OBRb forward	GCA GCT ATG GTC TCA CTT CTT TTG	113	63
OBRb reverse	GTT CCC TGG GTG CTC TGA		
IL-1 β forward	GCT GTG GCA GCT ACC TAT GTC TTG	120	61
IL-1 β reverse	AGG TCG TCA TCA TCC CAC GAG		
TNF- α forward	AGC CCT GGT ATG AGC CCA TGT	109	65.5
TNF- α reverse	CCG GAC TCC GTG ATG TCT AAG T		
leptin forward	GGT CAC CGG TTT GGA CTT CAT	64	66
leptin reverse	CTG GTC CAT CTT GGA CAA ACT CA		
GAPDH forward	ATG GCA CAG TCA AGG CTG AGA	64	70
GAPDH reverse	CGC TCC TG GAA GAT GGT GAT		
18S rRNA forward	GAC GGA CCA GAG CGA AAG C	<65	64
18S rRNA reverse	AAC CTC CGA CTT TCG TTC TTG A		

Table 2 Serum estradiol and testosterone levels in each group

	Estradiol (pg / mL)	Testosterone (ng / mL)
OVX-Control	17.3 ± 1.7	UD
OVX-Testosterone	17.1 ± 1.8	2.4 ± 0.16
OVX+E ₂ -Control	174.4 ± 55.6	UD
OVX+E ₂ -Testosterone	113.5 ± 17.6	2.4 ± 0.22

Values are expressed as mean ± SEM

UD : undetectable