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Estradiol Regulates Insulin Signaling and Inflammation in Adipose Tissue

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Abstract

Background—Obesity-associated low-grade inflammation at white adipose tissue (WAT) leads to metabolic defects. Sex steroid hormone estrogen may be protective against high-fat diet (HFD)-induced obesity and insulin resistance. This has been tested by many previous studies utilizing rodent models of ovariectomy (OVX) and/or treatment of estradiol (E2), the major biologically active form of estrogen. Body weight and adiposity are increased by OVX and reduced following E2 treatment however. Thus, the protective roles of E2 may be secondary effects to the changes of body weight and adiposity. We hypothesize that E2 directly prevents inflammation and maintains insulin sensitivity in WAT independent of energy status using mice with similar body weights and adiposity.

Materials and methods—Four groups of female C57BL/6 mice were used, including sham-operated mice treated with vehicle for E2 and fed with either a low-fat diet (LFD; Sham-Veh-LFD) or a HFD (Sham-Veh-HFD), and HFD-fed OVX mice treated with either vehicle (OVX-Veh-HFD) or E2 (OVX-E2-HFD). Body weight and abdominal parametrial WAT mass, insulin signaling and expression levels of genes related to low-grade inflammation in WAT were compared among these groups pair-fed with equal amounts of calories for a period of four days.

Results—Body weights and WAT mass were similar among all four groups. OVX-Veh-HFD mice had impaired insulin signaling associated with rapid activation of inflammation, whereas OVX-E2-HFD group maintained insulin sensitivity without showing inflammation in WAT.

Conclusions—E2 directly contributed to the maintenance of insulin sensitivity during early phase of development of metabolic dysfunction, possibly via preventing low-grade inflammation in WAT.

Keywords

Estradiol; Insulin sensitivity; Low-grade inflammation; Ovariectomy; Pair-feeding

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Introduction

The prevalence of obesity and its related metabolic disorders including non-insulin dependent type 2 diabetes mellitus (T2DM) [1] have reached epidemic proportion with enormous costs in both human lives and healthcare dollars spent [2]. High-fat content in typical Western diets is an important factor leading to obesity and its related insulin resistance [3, 4]. Low-grade inflammation activated by macrophages of white adipose tissue (WAT) is one of the key factors for developing metabolic dysfunctions [5, 6]. Previous studies suggest that dietary fat rapidly induces inflammation and insulin resistance in WAT. Adipocyte insulin resistance [7] and low-grade inflammation in WAT [8] are rapidly induced following high-fat diet (HFD) feeding for a short-term of three days, prior to the development of insulin resistance and tissue inflammation in skeletal muscle and the liver [7, 9].

Premenopausal women have much less metabolic disorders than men [10–12]. Interestingly, the prevalence of metabolic disorders increases dramatically in postmenopausal women with deficiency in endogenous estrogen [13]. Sex steroid hormone estrogen may be protective against the development of metabolic syndrome and may contribute to the regulation of insulin sensitivity. Indeed, hormone replacement therapy or administration with estradiol (E2), the major biologically active form of estrogen, improves insulin sensitivity and lowers blood glucose levels [14–16], and thus reduce incidence of diabetes in postmenopausal women [17–19] or in men with congenital aromatase deficiency [20]. Estrogen deficiency also contributes to the development of insulin resistance in rodents [21]. Many previous studies have employed rodent models of ovariectomy (OVX) and/or E2 treatment to investigate the roles of estrogen in the regulation of glucose metabolism. OVX rodents with low levels of endogenous estrogen [22] or aromatase knockout mice with a genetic impairment in estrogen synthesis [23] exhibit insulin resistance. All these previous studies collectively suggest that estrogen contributes to the maintenance of insulin sensitivity and its deficiency leads to the development of insulin resistance. Because estrogen has been implicated in glucose regulation, it has enormous potential as a therapeutic agent for use in the prevention or treatment of T2DM, although the specific underlying mechanisms are not completely clear.

It is noteworthy that body weight and adiposity are increased by estrogen deficiency and reduced following long-term estrogen treatment [24]. Thus, the protective roles of estrogen may be secondary effects to the changes of energy status. We hypothesize that estrogen directly contributes to the maintenance of insulin sensitivity in adipose tissue during an early phase of the development of metabolic dysfunction independent of energy status. To understand the roles of estrogen in the regulation of tissue inflammation and insulin signaling, expression levels of genes related to low-grade inflammation in adipose tissue and activation of Akt, a key mediator of insulin signaling in adipocytes [25], were compared among groups of sham-operated, OVX, and E2-treated OVX mice fed with identical calories of a low-fat diet (LFD) or a HFD for a short period of four days and thus with similar body weights and adiposity.

Materials and Methods

Animals and diets

Ten weeks-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were single-housed (12 h light-dark cycle, lights on at 0600) and fed a standard LFD (Teklad, Madison, WI) during acclimation when their body weight and daily food intake were monitored, and their ovarian cycles were tracked by predominant cell types of vaginal cytology samples. Forty female mice that displayed normal cyclicity with repeating estrus every 4–5 days were used in this study. Daily food intake of each mouse was calculated individually by the difference of food weights over 24 hours and corrected for spillage. After acclimation, mice were grouped into four groups (n=10 per group) with matched average body weight and daily LFD intake. Two groups received OVX and the other two groups received sham surgeries. Three days after surgeries, mice were injected with either 17β-estradiol-3-benzoate (E2; Sigma-Aldrich, St. Louis, MO) dissolved in sesame oil (Sigma-Aldrich) or oil vehicle once every four days (see below for detailed procedure). Two weeks after surgeries, mice were fed daily with equal amount of calories of either LFD (3.003 kcal/g; 14% fat) or HFD (4.728 kcal/g; 45% fat; Research Diets, Inc., New Brunswick, NJ, D12451) for four days. The LFD and HFD had closely matched amounts of proteins (LFD: 0.243 g/g, HFD: 0.237 g/g) and carbohydrates (LFD: 0.402 g/g, HFD: 0.414 g/g), but different amounts of fat (LFD: 0.047 g/g, HFD: 0.236 g/g). Two groups of sham-operated mice were injected with oil vehicle and were fed with either LFD (Sham-Veh-LFD) or HFD (Sham-Veh-HFD), and two groups of OVX mice were fed with HFD and were injected with either oil vehicle (OVX-Veh-HFD) or E2 (OVX-E2-HFD). HFD induces insulin resistance and tissue inflammation. LFD OVX group was not included in the current experiment, because OVX-Veh-LFD and OVX-E2-LFD groups were expected to have normal metabolic profiles as LFD-fed male and female mice respectively [26]. The key questions asked were if estrogen deficiency would lead to insulin resistance and tissue inflammation when estrogen-deficient mice were fed a HFD, and if such malfunction would be corrected by estrogen treatment. All procedures were approved by the Institutional Animal Care and Use Committee at Miami University Ohio.

Experimental procedure

Female's metabolism and energy balance were influenced by estrogen [27–30], especially during proestus-estrus when endogenous estrogen is high and has greatest physiological effects [31]. Thus, Sham-Veh-LFD and Sham-Veh-HFD groups began the four-day feeding experiment during their estrus on Day 1 and terminated during the proestrus-estrus on Day 5. Injections of oil vehicle or E2 were made a day before feeding on Day 0 and again four days later on Day 4. OVX induces hyperphagia whereas E2 treatment suppresses caloric intake [32–34]. In order to examine the effects of E2 on metabolism without a potential confound of changes in caloric intake and body weight due to OVX and E2 treatment, all groups were fed equal amounts of calories daily, same as the average amount of daily calories consumed by Sham-Veh-LFD group post-surgically. HFD groups were given less food than LFD groups to equalize caloric intake. Detailed experimental procedure was listed in the Table 1.

OVX and sham surgeries

OVX was performed to decrease endogenous estrogen and disrupt the ovarian cycles of female mice. Under isofluorane anesthesia (Butler Schein Animal Health, Dublin, OH) bilateral skin and muscle incisions on the dorsolateral flank were made paralleling the spinal column. In the OVX procedure, bilateral ovaries were removed without disturbing the uterus, oviduct, or gonadal parametrial WAT. In the sham procedure, the ovaries were visualized but no tissue was removed. After all surgeries, the muscle was sutured with sterile absorbable vicryl sutures, and the skin was closed with sterile wound clips. Success OVX was confirmed by loss of the estrous phase and reduced plasma E2 levels.

Cyclic oil or E2 treatment

Cyclic oil vehicle or E2 treatment was started three days after surgeries when plasma E2 levels should have significantly decreased due to rapid degradation of E2 by steroidal esterases [35]. The OVX-E2 mice were injected subcutaneously with 0.5 μg E2/100 μl oil, a dose has been described to decrease LFD intake in OVX mice [36]. Such result was not duplicated in this study however. Injection of a higher dose of 4 μg E2/100 μl reduced LFD intake during the second and third days after injections in a similar manner as cycling female mice [37] (see Results). Cyclic E2 treatment at a dose of 4 μg every four days was used in the OVX-E2 group. Injection of 100 μl oil alone to OVX-Veh and Sham-Veh mice was used for control treatment.

Sample collection

Blood samples were collected by the tail bleeding method before and after vehicle or E2 injections in OVX mice, and plasma E2 was measured by radioimmunoassay (Diagnostic Systems Laboratories Inc., Webster, TX, DSL-39100). The intra- and inter-assay coefficients of variation are 3.9–4.1%, and the sensitivity is 1.5 pg/ml. At the end of feeding on Day 5 mice were injected intraperitoneally with saline or insulin (1 mU/g; Sigma-Aldrich) and sacrificed 15 min after injection. Abdominal parametrial WAT was dissected, weighed, and frozen at −80°C until extractions for protein and total RNA. Parametrial WAT has greater expression levels of inflammation-related genes, such as macrophage infiltration marker monocyte chemoattractant protein-1 (*Mcp-1*) and a macrophage-specific marker *Cd68* [6], and contains less connective tissue and fewer vessels than subcutaneous and visceral WAT, assuring accuracy in analysis of protein activity and gene expression. Gene expression levels were not significantly different between saline- and insulin-injected groups within each treatment, analyzed by unpaired two-tailed t tests ($P>0.05$), and thus data were combined.

Insulin signaling measured by western blot

Protein was extracted by homogenizing using lysis buffer with sodium orthovanadate, phenylmethylsulfonyl fluoride, protease inhibitor (Santa Cruz Biotechnology, Santa Cruz, CA) and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein lysates were resolved in 4–15% tris-glycine gels and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Activity of kinase Akt indicates stimulated insulin signaling [38]. Phosphorylated and total Akt (Ser^{473} pAkt and tAkt; 1:1000; Cell Signaling, Danvers, MA) were detected by

immunoblotting via chemiluminescence (Amersham™ ECL™ Prime, GE Healthcare) and visualized using autoradiography film. Density was quantified using ImageQuant software (Amersham Biosciences). pAkt measurements were normalized to tAkt (pAkt/tAkt). Activation of insulin signaling was indicated by pAkt/tAkt % difference between insulin- and saline-injected mice.

Gene expression measured by quantitative PCR

Total RNA was isolated from WAT and cDNA was synthesized from DNAase (Ambion, Austin, TX)-treated RNA using an iScript kit (Bio-Rad). Expressions levels of *Mcp-1* and *Cd68* implicated in low-grade inflammation were analyzed. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA levels were similar among all groups and *Gapdh* was used as a reference gene (Table 2). Quantitative PCR was run in triplicates using iQ SYBR Green Supermix (Bio-Rad) and an iCycler (Bio-Rad) with 40 cycles of amplification (95 °C for 10 s) and annealing (58 °C for 30 s). The amplified products were confirmed via gel electrophoresis and melt curve analysis. Results were calculated by a $2^{-\Delta\Delta Ct}$ method and presented using Sham-Veh-LFD group as 100%.

Statistical analysis

Data were presented as mean±SEM and were analyzed using Prism 5 (La Jolla, CA). Food intake, plasma E2 concentrations, and body weight were analyzed by a two-way (time x injection) repeated-measures ANOVA followed by Bonferroni posttest. WAT mass, insulin sensitivity, and gene expression levels were analyzed by a one-way ANOVA followed by Tukey's multiple comparison tests. A test with $P<0.05$ was considered statistically significant.

Results

Food intake and circulating E2 levels following injections

Injection of 0.5 μg E2 on Day 0 did not reduce caloric intake. Daily (Fig 1A) or cumulative (Fig 1B) LFD intake were similar between 0.5 μg E2- and oil vehicle-injected OVX mice. In contrast, injection of 4 μg E2 on Day 0 reduced LFD intake on Day 2 [t=3.78, $P<0.001$] (Fig 1C). As a result, daily intake was lower in E2- than oil vehicle-injected group on Day 2 [t=2.79, $P<0.05$] and Day 3 [t=2.68, $P<0.05$] (Fig 1C). Additionally, cumulative LFD intake was lower in E2- than oil vehicle-injected OVX mice on Day 3 [t=3.61, $P<0.01$] and Day 4 [t=4.19, $P<0.001$] (Fig 1D). Plasma E2 levels of OVX mice were similar before oil or E2 injections on Day 0. E2 injection on Day 0 increased E2 levels on Day 1 [t=8.16, $P<0.001$], Day 2 [t=4.49, $P<0.001$], and Day 3 [t=2.34, $P<0.05$]. Additionally, E2 levels were significantly higher in E2- than oil vehicle-injected OVX mice during the first two days after injection [Day 1: t=8.17, Day 2: t=4.58, $P<0.001$], but were not significantly different between E2- and oil vehicle-injected groups on Day 3 or Day 4 [$P>0.05$] (Fig 1E).

Body weight and WAT mass

During the first two weeks following the surgeries, the daily average LFD intake was less in Sham-Veh mice (n=20; 3.45±0.10 g) than OVX-Veh mice (n=10; 3.98±0.15) [$t_{28}=3.00$, $P=0.0056$], whereas their body weights were similar [$P>0.05$] (Fig 2A). This is consistent

with our previous experiments showing similar body weights between sham- and OVX-operated rodents within the first two weeks post-surgically [39]. During the four-day experiment period, each mouse was fed 10.36 kcal daily (*i.e.* 3.45 g LFD or 2.19 g HFD). Effort was made to accurately weigh food with less than ±0.10 g of variation to ensure that all four groups of mice consumed equal amounts of calories. The majority of food was consumed every day, with a little food occasionally remaining when next meal was provided for a few mice. At the end of the four-day feeding experiment, body weight [$F_{(3,72)}$=0.03, P=0.99] (Fig 2A) and WAT mass [$F_{(3,36)}$=0.97, P=0.42] (Fig 2B) were similar among all groups.

Insulin sensitivity

Insulin sensitivity in WAT was indicated by pAkt/tAkt% difference between insulin- and saline-injected mice within each group. pAkt/tAkt% was greater than 100% in Sham-Veh-LFD [t=7.46, P<0.001], Sham-Veh-HFD [t=5.09, P<0.001], and OVX-E2-HFD [t=4.81, P<0.001] mice, indicating increased Akt activity and activated insulin signaling following insulin injections compared with saline injections in these groups. In contrast, pAkt/tAkt% of OVX-Veh-HFD mice was not different from 100% [t=0.039, P>0.05], indicating similar Akt activity between insulin- and saline-injected OVX-Veh-HFD mice. Additionally, pAkt/tAkt% was lower in OVX-Veh-HFD mice than Sham-Veh-LFD [t=7.50, P<0.001], Sham-Veh-HFD [t=5.13, P<0.05], and OVX-E2-HFD [t=5.13, P<0.05] groups (Fig 3). These data suggested that insulin signaling was similar among Sham-Veh-LFD, Sham-Veh-HFD, and OVX-E2-HFD groups, but was impaired in WAT of OVX-Veh-HFD mice.

Gene expression levels involved in inflammation

Defective insulin signaling might be related to low-grade inflammation in WAT. Transcript levels of *Cd68* and *Mcp-1* in WAT were similar between Sham-Veh-LFD and Sham-Veh-HFD groups. In contrast, mRNA levels of *Cd68* [t=4.30, P<0.05] (Fig 4A) and *Mcp-1* [t=4.52, P<0.05] (Fig 4B) were higher in OVX-Veh-HFD mice than OVX-E2-HFD mice.

Discussion and Conclusions

Sex steroid hormone estrogen plays critical roles in the regulation of glucose metabolism and maintenance of insulin sensitivity, and deficiency of estrogen may lead to the development of insulin resistance [40]. Postmenopausal women with deficiency in endogenous estrogen have increased risk of developing T2DM [13], while hormone replacement therapy or treatment with E2 improves insulin sensitivity and lowers blood glucose levels [14, 16], and reduce incidence of diabetes [17, 18]. The changes in insulin sensitivity however, could be secondary responses to the increase in body fat when estrogen levels are low and the decrease in body fat following estrogen treatment, instead of direct effects of estrogen. OVX in rodents provides a good model of postmenopausal estrogen deficiency and development of insulin resistance [21]. Loss of ovarian hormones by OVX induces an increase in body weight along with elevated plasma glucose levels, impaired glucose tolerance, and decreased insulin response to glucose [41], all of which can be reversed through treatment with estrogen in OVX animals [41, 42]. Again, OVX is associated with increased body weight and adiposity whereas E2 treatment has opposite

effects. Thus, the challenge remains in rodent OVX model to distinguish whether insulin resistance is primarily mediated by loss of estrogen or is developed secondary to adipose deposition.

The current study is the first study to our knowledge that compared the effects of E2 on insulin signaling in OVX mice with matched body weights and adiposity using a short-term pair-feeding regimen. Although all groups consumed equal amounts of calories and thus had similar WAT mass and body weights, the OVX-Veh-HFD mice with estrogen deficiency developed defective insulin signaling and low-grade inflammation in WAT, whereas the OVX-E2-HFD mice treated cyclically with E2 maintained insulin sensitivity without developing inflammation in WAT. These findings suggested that E2 directly regulated insulin sensitivity independent of energy balance and adiposity. Therefore beneficial effects of E2 in prevention of inflammation and maintenance of insulin sensitivity were corroborated.

Insulin signaling of abdominal adipocytes was similar between sham-operated and E2-treated OVX mice, whereas vehicle-treated OVX mice with lower circulating E2 than sham-operated or E2-treated OVX mice had defective insulin signaling (Fig 3). This finding is consistent with previous studies showing greater insulin sensitivity in female adipocytes compared with male adipocytes [43, 44]. Female abdominal adipocytes have a robust increase in insulin-induced phosphorylation of Akt and extracellular signal-related kinase when stimulated by low physiological concentrations of insulin [44]. In contrast, adipocytes of males with similar adiposity as females show activation of insulin signaling only when they are stimulated at much higher insulin concentrations [44]. Furthermore, female adipocytes have higher mRNA and protein levels of several genes involved in glucose and lipid metabolism, including glucose transporters and key lipogenic enzymes fatty acid synthase and acetyl CoA carboxylase, than male adipocytes [44], suggesting that the sex differences in insulin sensitivity at adipocytes are attributable at least partially to increased glucose transporters and lipogenic enzyme levels. Additionally, adipocytes of OVX females have lowered insulin sensitivity [44], suggesting that insulin sensitivity of adipocytes is regulated by physiological levels of estrogen. The adipose tissues of females were collected at proestus-estrus of their ovarian cycles, a phase when female's metabolism is mostly influenced by endogenous estrogen [31]. Besides its roles in reproduction and sexual development, estrogen regulates insulin sensitivity by increasing phosphorylation of insulin receptor substrate and Akt in adipose tissue [45, 46], which could account for maintaining insulin sensitivity in the Sham-Veh-HFD group. In contrast, insulin resistance developed in OVX-Veh-HFD mice. These findings are consistent with a recent study showing that HFD-fed males with similarly lower levels of endogenous estrogen as OVX mice [47], but not females with normal ovarian cycles, had defective insulin signaling in adipocytes [26].

Low-grade inflammation associated with HFD consumption is a crucial factor for obesity-induced insulin resistance [6]. Gene expression analysis of this study indicated that rapid recruitment of macrophages and elevated macrophage infiltration occurred in WAT of OVX-Veh-HFD females, whereas E2 treatment in OVX-E2-HFD mice reversed such increase, possibly due to estrogens' roles in suppressing inflammatory signaling in macrophages [48]. Our findings in the OVX-Veh-HFD group are consistent with previous

studies using male mice showing rapid activation in low-grade inflammation and increased macrophage content in WAT after 4 days of HFD feeding [26, 49] and increased inflammation in WAT after 3 days of HFD feeding [8].

Environmental factors including availability of palatable and high-energy content food greatly impact on the prevalence of obesity and its related metabolic syndrome. Interestingly, sex differences exist in the regulation of glucose metabolism [40]. There is active search for the mechanisms of estrogen in the regulation of glucose homeostasis. Short-term HFD feeding induced adipocyte insulin resistance and rapid activation of low-grade inflammation in females with low levels of estrogen. In contrast, female groups with either normal endogenous estrogen or exogenously replaced estrogen via E2 treatment maintained insulin sensitivity in adipose tissues without displaying tissue inflammation. It is possible that estrogen regulates glucose homeostasis via its estrogen receptor α, which is expressed in most metabolic tissues including adipocytes, liver cells, pancreatic β cells, and skeletal muscle cells [50] to allow for coordination of signals regulating metabolic homeostasis in response to hormonal milieu. Further studies are needed to test specific roles of estrogen receptors.

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Abbreviations

E2	Estradiol
HFD	High-fat diet
LFD	Low-fat diet
Mcp-1	Monocyte chemoattractant protein-1
OVX	Ovariectomy
T2DM	Type 2 diabetes mellitus
WAT	White adipose tissue

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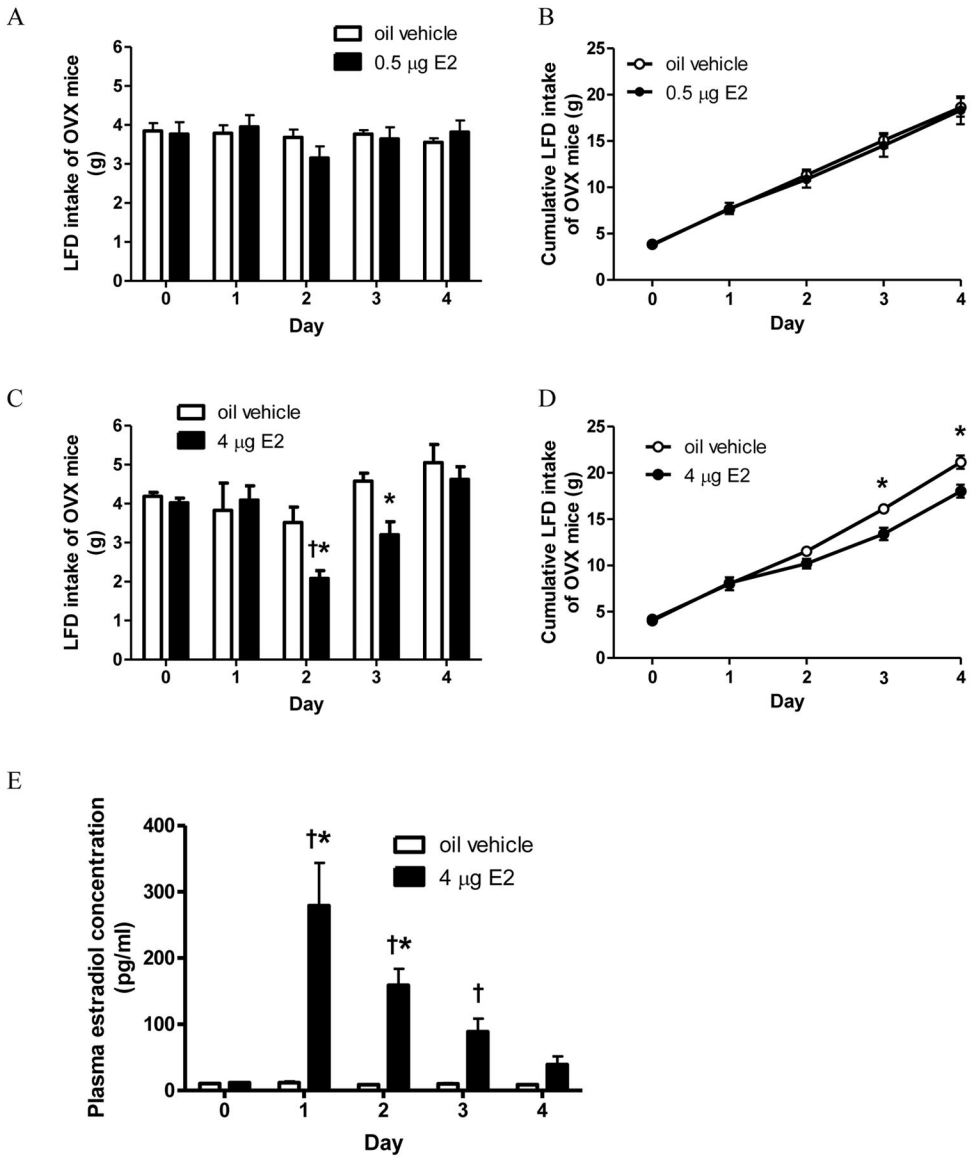

Figure 1.

Low-fat diet (LFD) daily and cumulative intake and circulating estradiol (E2) levels of oil vehicle- and E2-treated mice after ovariectomy (OVX) surgeries.

Daily (A) and cumulative (B) LFD intake after oil or 0.5 μg E2 injections, daily (C) and cumulative (D) LFD intake after oil or 4 μg E2 injections, and plasma E2 levels were analyzed by a two-way (time x injection) repeated-measures ANOVA followed by Bonferroni posttest.

*: Significantly different between oil vehicle and E2 injections.

†: Significantly different comparing to Day 0 of the same treatment.

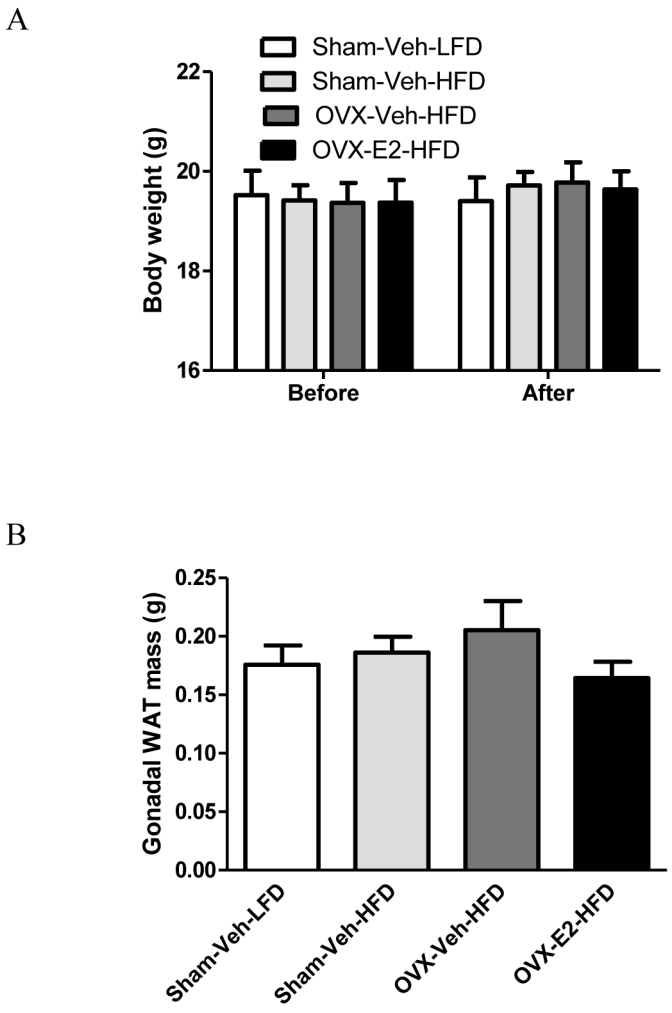

Figure 2.

Body mass and gonadal parametrial white adipose tissue (WAT) mass of four groups of mice.

Body mass before four-day feeding (A) were analyzed by a two-way (time x injection) repeated-measures ANOVA followed by Bonferroni posttest. WAT mass after four-day feeding (B) was analyzed by a one-way ANOVA followed by Tukey's multiple comparison tests.

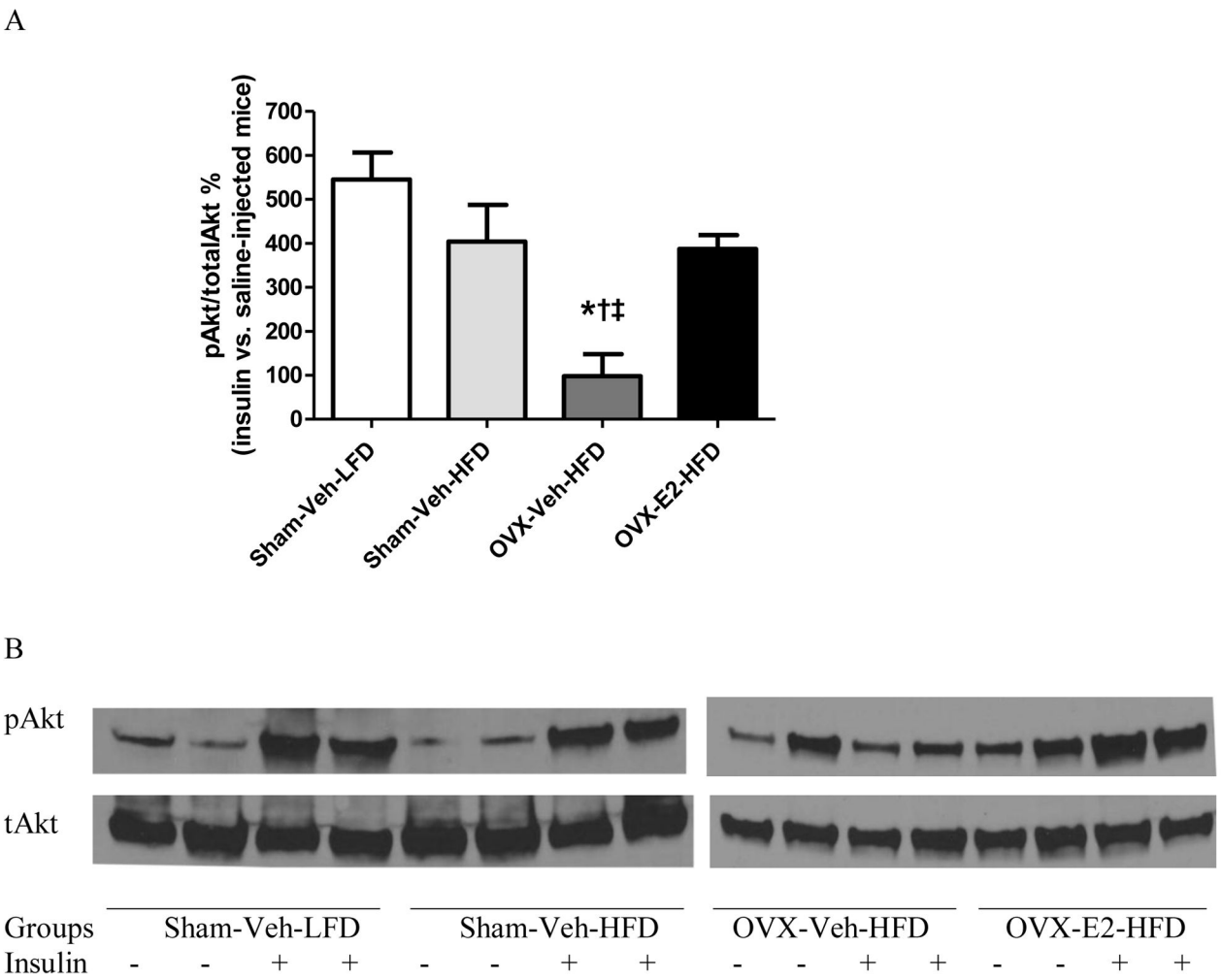

Figure 3.

Analysis of Akt activation at gonadal parametrial white adipose tissue of four groups of mice.

Percentage of pAkt/tAkt ratio of insulin- vs. saline-treated mice (pAkt/tAkt %; A) was analyzed by a one-way ANOVA followed by Tukey's multiple comparison tests.

pAkt/tAkt % = (average pAkt/tAkt [insulin]) / (average pAkt/tAkt [saline]) x 100%

*: Significantly different from Sham-Veh-LFD group.

†: Significantly different from Sham-Veh-HFD group.

‡: Significantly different from OVX-E2-HFD group.

Representative western blot analysis of pAkt and tAkt expression from one experiment was shown (B).

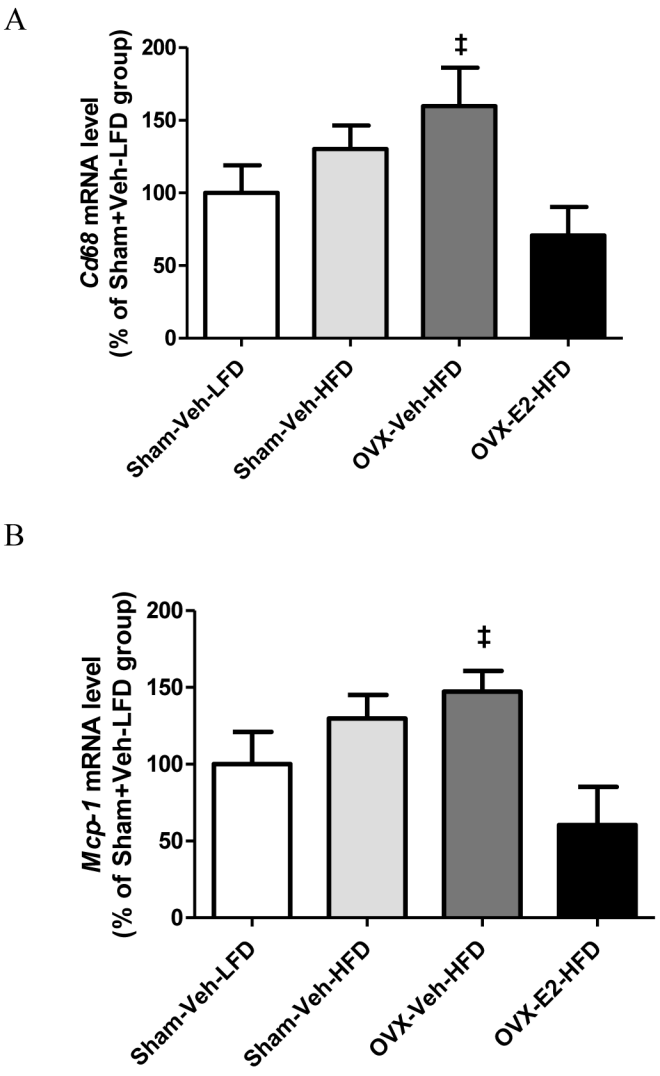

Figure 4.

Inflammatory gene expression levels measured at gonadal parametrial white adipose tissue. Gene expression levels of Sham-Veh-LFD group were set at 100%. Gene expression levels of a macrophage marker (*Cd68*, A) and an inflammation marker for macrophage infiltration monocyte chemoattractant protein-1 (*Mcp-1*, B) were analyzed by a one-way ANOVA followed by Tukey's multiple comparison tests.

‡: Significantly different from OVX-E2-HFD group.

Table 1

Experimental procedures.

Groups (n=10)	After surgeries	Day 0	Four-day feeding experiment				
			Day 1	Day 2	Day 3	Day 4	Day 5
Sham-Veh-LFD	Veh injection every 4 days; Monitor daily LFD intake	Proestrus; Veh injection; LFD	Estrus; 3.45 g LFD	Metestrus; 3.45 g LFD	Diestrus; 3.45 g LFD	Diestrus-proestrus; Veh injection; 3.45 g LFD	Proestrus-estrus; Saline or insulin injection
Sham-Veh-HFD	Veh injection every 4 days; Monitor daily LFD intake	Proestrus; Veh injection; LFD	Estrus; 2.19 g HFD	Metestrus; 2.19 g HFD	Diestrus; 2.19 g HFD	Diestrus-proestrus; Veh injection; 2.19 g HFD	Proestrus-estrus; Saline or insulin injection
OVX-Veh-HFD	Veh injection every 4 days; Monitor daily LFD intake	Veh injection; LFD	2.19 g HFD	2.19 g HFD	2.19 g HFD	Veh injection; 2.19 g HFD	Saline or insulin injection
OVX-E2-HFD	E2 injection every 4 days; Monitor daily LFD intake	E2 injection; LFD	2.19 g HFD	2.19 g HFD	2.19 g HFD	E2 injection; 2.19 g HFD	Saline or insulin injection

Table 2

Quantitative PCR primer sequences.

Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), macrophage-specific marker (*Cd68*), and monocyte chemoattractant protein-1 (*Mcp-1*).

Genes	GenBank accession number	Forward and reverse primer sequences
Gapdh	NC_000072	Forward: 5′-GCGACTTCAACAGCAACTC-3′ Reverse: 5′-GCCTCTCTTGCTCAGTGTCC-3′
Cd68	NM_009853	Forward: 5′-TTCTGCTGTGGAAATGCAAG -3′ Reverse: 5′-AGAGGGGCTGGTAGGTTGAT-3′
Mcp-1	NM_011333	Forward: 5′-CCCAATGAGTAGGCTGGAGA-3′ Reverse: 5′-TCTGGACCCATTCCTTCTTG-3′