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Effects of ER β and ER α on OVX-induced changes in adiposity and insulin resistance

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Abstract

Loss of ovarian hormones leads to increased adiposity and insulin resistance (IR), increasing the risk for cardiovascular and metabolic diseases. The purpose of this study was to investigate whether the molecular mechanism behind the adverse systemic and adipose tissue-specific metabolic effects of ovariectomy requires loss of signaling through estrogen receptor alpha (ER α) or estrogen receptor β (ER β). We examined ovariectomized (OVX) and ovary-intact wild-type (WT), ER α -null (α KO), and ER β -null (β KO) female mice (age ~49 weeks; $n = 7$ –12/group). All mice were fed a phytoestrogen-free diet (<15 mg/kg) and either remained ovary-intact (INT) or were OVX and followed for 12 weeks. Body composition, energy expenditure, glucose tolerance, and adipose tissue gene and protein expression were analyzed. INT α KO were ~25% fatter with reduced energy expenditure compared to age-matched INT WT controls and β KO mice (all $P < 0.001$). Following OVX, α KO mice did not increase adiposity or experience a further increase in IR, unlike WT and β KO, suggesting that loss of signaling through ER α mediates OVX-induced metabolic dysfunction. In fact, OVX in α KO mice (i.e., signaling through ER β in the absence of ER α) resulted in reduced adiposity, adipocyte size, and IR ($P < 0.05$ for all). β KO mice responded adversely to OVX in terms of increased adiposity and development of IR. Together, these findings challenge the paradigm that ER α mediates metabolic protection over ER β in all settings. These findings lead us to suggest that, following ovarian hormone loss, ER β may mediate protective metabolic benefits.

Key Words

- ▶ menopause
- ▶ metabolism
- ▶ glucose metabolism
- ▶ estrogen receptors
- ▶ adipose tissue

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Introduction

Estrogen (17 β -estradiol (E₂)) deficiency in menopausal women and ovariectomized (OVX) rodents is associated with reduced energy expenditure, metabolic disturbances (i.e., insulin resistance (IR)), increased adiposity, and white adipose tissue (WAT) dysfunction (Lovejoy *et al.* 2008, Bluher 2009, 2013, Vieira-Potter *et al.* 2015).

Thus, E₂ deficiency increases the risk for developing obesity-related cardiovascular and metabolic diseases, such as type 2 diabetes (Stefanska *et al.* 2015, Razmjou *et al.* 2016). E₂ signaling occurs primarily via the classical genomic pathway through nuclear receptors, estrogen receptor alpha (ER α) and beta (ER β) (Nilsson *et al.* 2001,

Barros & Gustafsson 2011). There is mounting evidence that a relationship exists between ER α expression and WAT function in both humans and rodents (Nilsson *et al.* 2007b, Lizcano & Guzman 2014, Monteiro *et al.* 2014). Young female-knockout mice lacking ER α (α KO) have increased adiposity, impaired glucose tolerance (Heine *et al.* 2000), and increased WAT inflammation (Ribas *et al.* 2010), whereas mice with functional ER α , but not ER β (β KO), at least prior to OVX, do not show this adverse phenotype (Ohlsson *et al.* 2000). Thus, ER α appears critical for providing protection against systemic metabolic and adipose dysfunction prior to OVX. However, whether this dichotomy noted in young ovary-intact females also occurs in older OVX females has not been adequately studied. Davis *et al.* (2013) examined the specific effects of ER α in visceral WAT by selective deletion via viral knockdown of ER α to a single perigonadal fat pad *in vivo* and reported increased adipocyte size and WAT inflammation compared to the untreated fat pad of the same animals. Those data, in particular, provide strong evidence that E₂ signaling via ER α in WAT is required for WAT immune and metabolic health. Moreover, recent data indicate that direct application of E₂ on 3T3-L1 adipocytes induces translocation of ER α , but not ER β , from the nucleus to the plasma membrane (Campello *et al.* 2017), which associated with enhanced insulin-stimulated phosphorylation of Akt and GLUT4 translocation. These effects were mimicked by PPT (4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol), an ER α agonist, and were blocked by the ER α antagonist, MPP (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenyl]-1H-pyrazole dihydrochloride), suggesting that ER α signaling directly affects adipocyte insulin signaling (Campello *et al.* 2017). Insulin sensitivity of adipose tissue is critical to whole-body glucose regulation, particularly among women and older individuals who have a greater relative abundance of adipose tissue. However, insulin sensitivity of adipose tissue also enhances efficient energy storage. Thus, when combined with a state of low total energy expenditure, as is observed following OVX in rodents and menopause in humans, this may increase obesity susceptibility.

E₂ supplementation (i.e., hormone replacement therapy (HRT)) has been shown to attenuate weight gain and improve glucose tolerance in both postmenopausal women (Ryan *et al.* 2002, Musatov *et al.* 2007) and OVX rodents (Xu *et al.* 2011, Stubbins *et al.* 2012a). Prior studies investigating the role of skeletal muscle showed that E₂ directly lowers hepatic glucose production presumably due to greater glucose uptake into muscle via activation

of Akt and induction of GLUT4 expression (Moreno *et al.* 2010, Gorres *et al.* 2011). On the other hand, liver-specific knockdown of ER α increases hepatic glucose production leading to hyperglycemia (Bryzgalova *et al.* 2006, Qiu *et al.* 2017). Recently, we found that liver expression of ER α is required for optimal metabolic improvements following exercise (Winn *et al.* 2019). A recent study further showed that E₂'s ability to suppress hepatic glucose output requires its ability to suppress Foxo1 via a mechanism involving ER α and activation of Akt (Yan *et al.* 2019). Unfortunately, clinical evidence suggests that HRT increases cardiovascular risks (i.e., stroke) in postmenopausal women (Nelson *et al.* 2012, Sood *et al.* 2014) and is generally not prescribed. Since the detrimental cardiovascular and cancer-provoking effects of HRT are likely mediated through ER α (Justenhoven *et al.* 2012, Drew *et al.* 2015), therapeutic strategies that target the beneficial effects of E₂ but do not activate ER α are necessary to reduce metabolic disturbances seen in this population. Here, we tested the systemic and tissue-specific metabolic effects of OVX in aged female mice null for either ER α or ER β to assess how adipose tissue E₂ signaling via these predominant receptors affect metabolic health following ovarian hormone loss. We hypothesized that, in the setting of advanced age, adipose tissue ER β signaling would play a protective role against OVX-induced weight gain. As such, we predicted that ER β -null mice would respond more adversely to OVX compared to WT and ER α -null mice. The results support that hypothesis.

Materials and methods

Study design

Randomly cycling female (*Mus musculus* on C57BL/6J background) α KO and β KO mice ($n=7-12$ /group) were pair-housed in a thermoneutral environment (26–28°C) on a 12-h light/12-h dark cycle. ER α WT controls were used to validate replication of previous studies (Davis *et al.* 2013, Naaz *et al.* 2002) and underwent all treatments similar to the α KO and β KO mice. All mice were initially provided with a low phytoestrogen content (i.e., 'washout') diet (150–250 mg/kg; #2018; Teklad Diets, Madison, WI, USA) and water *ad libitum*. One week post OVX, mice were placed on a phytoestrogen-free (<15 mg/kg phytoestrogens) diet purchased from Harlan Laboratories (Madison, WI, USA). Body weight (BW) and food intake were monitored and recorded weekly. Ovary-intact (INT) WT, α KO, and β KO mice were examined as separate cohorts serving as INT control groups; these animals did not undergo OVX surgery but

Figure 1

Overview of study design. Wildtype (WT), ER α KO (α KO) and ER β KO (β KO) female mice were either left ovary-intact (INT) or ovariectomized (OVX). INT groups were assessed for all indicated outcomes at 49 weeks of age. OVX groups were assessed for body weight and body composition at 49 weeks, ovariectomized, then followed for an additional 12 weeks and assessed for all outcomes as indicated above.

were age matched to represent the 'Pre-OVX' condition. For OVX mice, assessments (other than endpoints taken after killing) were made prior to OVX, in addition to 12 weeks post OVX. At the study conclusion, tissues were harvested from all animals for further analyses as described below. National Institutes of Health guidelines were strictly followed and all procedures were approved by the Institutional Animal Care and Use Committee at the University of Missouri prior to study initiation. α KO mice have been validated to not have functional ER α whereas β KO mice have been validated to exhibit functional ER α (i.e., ER α positive) (Lubahn *et al.* 1993). The overall study design is provided in Fig. 1.

Ovariectomy surgeries

For ovariectomy (OVX) surgery, mice (~49 weeks old) were anesthetized and maintained under 2% isoflurane. A single 1 cm dorsal midline incision was made in the skin along the mid-lumbar region followed by two bilateral muscular incisions to expose the ovaries. OVX included removal of the whole ovary, ovarian bursa and part of the oviduct. After ovary removal, the skin incision was closed using skin sutures (4-0 Vicryl; Ethicon) and adhesive (3M VetBond Tissue Adhesive). Buprenorphine (Patterson Veterinary, Devens, MA, USA) was administered subcutaneously at a dose of 0.05 mg/kg immediately after surgery and post-operatively as needed. OVX effectiveness was determined at the conclusion of the study via verification of uterine atrophy; uterine weights are provided herein for INT and OVX WT, α KO, and β KO.

Body composition

Body fat and lean mass were measured by a non-invasive nuclear MRI whole-body composition analyzer

(EchoMRI® 4in1/1100; Echo Medical Systems, Houston, TX, USA) as previously described (Clookey *et al.* 2018). EchoMRI® was performed on conscious mice ($n=7-12$ /group) to gather pre-surgery data (week 0), mid-way through the study (week 7), and one day prior to killing (12 weeks).

Glucose tolerance and surrogate markers of insulin resistance

An intraperitoneal glucose tolerance test (GTT) was performed on INT mice and at 8 weeks post OVX for the OVX groups, as previously described (Vieira-Potter *et al.* 2015, Wainright *et al.* 2015). Briefly, following a 6-hour fast, baseline blood (time 0) was collected from a nick in the tail vein and sampled by a hand-held glucometer (AlphaTRAK; Abbott Laboratories). Then, an intraperitoneal injection of a sterile solution of 50% dextrose (2 g/kg BW) was administered. Blood glucose measurements were taken at 15, 30, 45, 60, and 120 min following injection. Glucose area under the curve (AUC) above baseline was calculated. Homeostasis model assessment of IR (HOMA-IR) and adipocyte-IR (Adipo-IR) were used as surrogate measures of IR (Matthews *et al.* 1985, Sondergaard *et al.* 2017) and calculated using the following equations: HOMA-IR (fasting insulin (ng/mL) $\times$ fasting glucose (mg/dL)/405) and Adipo-IR (fasting insulin (ng/mL) $\times$ NEFA (mmol/L)).

Indirect calorimetry and physical activity

Indirect calorimetry with a multidimensional beam break monitoring system (Promethion, Sable Systems International, Las Vegas, NV, USA) was used to assess total energy expenditure (TEE), resting energy expenditure (REE), and spontaneous physical activity (SPA) for ovary-intact and 10 weeks post-OVX mice. This system captures synchronized metabolic and behavioral information using a shared gas analyzer chain which pairs individual metabolic cage units with their own flow generator and gas analyzer. Manufacturer-constructed data analysis macros (Expedata Software, Sable Systems International) were used to gather averaged 12-h light or 12-h dark cycles. Animals were singly housed in the monitoring system and were allowed to acclimate to the environment 24 h prior to data collection. SPA was calculated as the sum of the X, Y, and Z beam breaks. TEE and REE are presented as kcal/kg BW/12-h cycle as indicated in the figures. Because light REE is most reflective of true resting conditions, we chose to report light cycle REE and dark cycle TEE.

Fasting circulating markers

Blood was collected upon killing via cardiac puncture and placed into 2.0 mL 7.5% EDTA-treated microcentrifuge tubes (Covidien Monoject). The tubes were then centrifuged to obtain plasma, which was stored in the -80°C freezer until further analysis. Commercial assays for fasting insulin, glucose, cholesterol, triglycerides (TG), low- and high-density lipoproteins (LDL, HDL) and non-esterified fatty acids (NEFA) were performed by a diagnostic laboratory (Comparative Clinical Pathology Services, Columbia, MO, USA) on an Olympus AU680 automated chemistry analyzer (Beckman-Coulter, Brea, CA, USA) according to manufacturer's guidelines. Plasma E_2 was measured via RIA at the Vanderbilt University Medical Center Hormone Assay Analytical Core (Vanderbilt University, Nashville, TN, USA) on KO mice. E_2 was measured via ELISA (CalBioTech, El Cajon, CA, USA).

Histological analyses

Formalin-fixed samples ($n=4-5/\text{group}$) were processed through paraffin embedment, sectioned at 5 μm , and stained with hematoxylin and eosin for morphometric determinates, as previously described (Thyfault *et al.* 2009). Sections were examined using an Olympus BX60 photomicroscope (Olympus) and photographed at 20 $\times$ magnification with a Spot Insight digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). An investigator, blinded to experimental conditions, collected four fields of view from each sample photograph. A representative photograph from one animal per group was selected. For cell size analysis, ~ 100 random adipocytes, representing at least four fields of view, were analyzed per animal using ImageJ software (National Institutes of Health).

RNA extraction and real-time PCR

Samples were homogenized in a Qiazol solution (Qiagen Catalog #79306) using a tissue homogenizer (TissueLyser LT, Qiagen). Total RNA from WAT samples from retroperitoneal (i.e., visceral) and subcutaneous

(inguinal region) depots was isolated using the Qiagen's RNeasy Lipid Tissue Kit and assayed using a Nanodrop spectrophotometer (Thermo Scientific) to assess purity and concentration. First-strand cDNA was synthesized from total RNA using the High-Capacity cDNA RT kit (Applied Biosystems). Quantitative real-time PCR was performed as previously described using the ABI StepOne Plus sequence detection system (Applied Biosystems) (Padilla *et al.* 2013). Primer sequences were designed using the NCBI Primer Design tool (Primers: IDT, Coralville, IA, USA) (Table 1). A 20- μL reaction mixture containing 10 μL iTaq UniverSYBR Green SMX (BioRad) and the appropriate concentrations of gene-specific primers plus 4 μL of cDNA template were loaded in each well of a 96-well plate. All PCR were performed in duplicate. PCR was conducted with thermal conditions as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. A dissociation melt curve analysis was performed to verify the specificity of the PCR products. 18S primers were used to amplify the endogenous control product (i.e., HK gene). mRNA expression values are presented as $2^{\Delta\text{CT}}$ whereby $\Delta\text{CT} = \text{HK CT} - \text{gene of interest CT}$; data are expressed as fold difference relative to the respective control group.

Western blot analysis

Western blotting was performed as previously described (Rector *et al.* 2008, Thyfault *et al.* 2009). WAT samples were pulverized and homogenized in Triton X-100 containing protease and phosphatase inhibitors using a tissue homogenizer (TissueLyser LT, Qiagen). WAT protein homogenates (5 μg protein/sample) and Laemmli buffer were then separated by SDS-PAGE using the Criterion vertical gel electrophoresis cell (BioRad), transferred to polyvinylidene difluoride membranes utilizing the Criterion blotter wet tank (BioRad), then subsequently blocked overnight at 4°C in 5% non-fat dry milk. PVDF membranes were washed with TBS-T then probed with primary antibodies (1:250–1:2000 in 5% bovine serum) overnight at 4°C . Finally, membranes were washed again

Table 1 q-rtPCR primer sequences.

	5'-3' primer sequence forward	5'-3' primer sequence reverse	Product length (bp)	NCBI reference sequence
CD11c	ATGCCACTGTCTGGCTTCAT	GAGCCAGGTCAAAGGTGACA	84	NM_0213334.3
TNF α	AGCCGATGGGTTGTACCTTG	ATAGCAAATCGGCTGACGGT	99	NM_013693.3
Adiponectin	GCACTGGCAAGTTCTACTGCAA	GTAGGTGAAGAGAACGGCCTTGT	122	NM_009605.5
Leptin	CCTATTGATGGGTCTGCCCA	TGAGCGCTACCTGCATAGAC	95	NM_008493.3
18s	TCAAGAACGAAAGTCGGAGG	GGACATCTAAGGGCATCAC	488	NR_003278.3

with TBS-T then incubated in secondary antibody for 1 h at room temperature. Individual protein bands were quantified via chemiluminescence using the FluoroChem HD2 (AlphaView, version 3.4.0.0) and normalized to total protein. Antibodies were purchased from Cell Signaling: GLUT4 (1:1000; #2213), phosphorylated Akt (pAkt) (1:250; #4060), and Akt (1:500; #4691).

Statistics

Determination of sample size was done via power analysis based on our initial studies using $\beta=0.80$ and $\alpha=0.05$ when determining differences between INT and OVX WT mice on the major outcomes, perigonadal fat pad mass and glucose AUC, which indicated a minimum sample size of 5 and 11, respectively. The sample sizes varied from 7 to 12/group and are provided in the figure legends. Normality was tested using standard assessments (Kolmogorov–Smirnov and Shapiro–Wilk); *P* values were all >0.05 allowing us to determine that the data were normally distributed. Where appropriate, main effects of genotype (WT, α KO, β KO) and OVX (OVX vs INT) were determined via 2×3 ANOVA. When a significant genotype $\times$ OVX interaction was observed, a *post hoc* Tukey test was used to detect where differences occurred. *Post hoc* Tukey's tests were used to determine which genotypes differed, and which were affected by OVX, where appropriate. For mRNA expression of the retroperitoneal WAT depot, we were not able to run 2×3 ANOVA because the INT and OVX samples were processed separately and differences in housekeeping gene expression did not allow for INT vs OVX comparison. Thus, we utilized 1-way ANOVA within each condition (i.e., INT and OVX) followed by *post hoc* Tukey's where appropriate for those analyses. Data were analyzed using SPSS v24 and are presented as mean $\pm$ S.E.M. where $P \leq 0.05$ was considered statistically significant.

Results

Genotype-specific body composition changes following OVX

INT α KO mice weighed $\sim 25\%$ more than WT and β KO controls (Fig. 2A; $P < 0.01$), whereas β KO and WT did not differ. Following OVX, weight differences no longer existed because both WT (+25%) and β KO (+31%) mice demonstrated a significant increase in body mass compared to their respective pre-OVX body weights, while α KO did not change; thus, WT and β KO had significantly

Figure 2

Genotype-specific body composition changes following ovariectomy. (A) Body weight (BW) in INT versus OVX; (B) change in BW 12 weeks after OVX; (C) % body fat in INT versus OVX; (D) change in % body fat 12 weeks after OVX. Ovary-intact (INT); ovariectomized (OVX); wildtype (WT); estrogen receptor alpha knockout (α KO); estrogen receptor beta knockout (β KO). * $P < 0.05$ compared to INT within genotype; # $P < 0.05$ compared to other genotypes within INT or OVX state. Values are expressed as mean $\pm$ S.E.M.

greater OVX-associated body weight gain than α KO (Fig. 2B, $P < 0.01$). These body weight differences were due to differences in adiposity; INT α KO mice had significantly greater percent fat mass than WT and β KO ($P < 0.01$) (Fig. 2C). Similarly, percent fat increased in both WT (+66%) and β KO (+60%) following OVX (both $P < 0.05$), while it did not increase in α KO (–9%) (NS) (Fig. 2C). This resulted in OVX-associated body fat increases only in the WT and β KO (Fig. 2D), whereas α KO moved in the opposite direction following OVX. Similarly, following OVX, β KO had significantly lower percent lean mass than α KO due to reductions in percent lean mass in WT (–16%; $P < 0.05$) and β KO (–18%; $P < 0.05$) and non-significant gain in the α KO (+8%) (NS) (data not shown).

Visceral adiposity (i.e., assessed here via perigonadal fat pad weights, Fig. 3A; and mean adipocyte size, Fig. 3B and C) between the INT groups did not differ. Following OVX, visceral adiposity of α KO did not change ($P = 0.89$), whereas WT ($P = 0.02$) and β KO ($P = 0.015$) both experienced OVX-induced increases, resulting in lower visceral adiposity in α KO compared to the other groups. Representative histological images of perigonadal adipose

Figure 3

Genotype-specific visceral adiposity changes following ovariectomy. (A) Perigonadal (PGAT) fat pad weights in INT versus OVX; (B) mean PGAT adipocyte cell size in INT and OVX mice; and (C) representative histological images of PGAT in INT and OVX mice. Ovary-intact (INT); ovariectomized (OVX); wildtype (WT); estrogen receptor α knockout (α KO); estrogen receptor β knockout (β KO). * $P < 0.05$ compared to INT within genotype; # $P < 0.05$ compared to α KO within INT or OVX state. Values are expressed as mean $\pm$ s.e.m. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0321>.

tissue reflect these differences, revealing that adipocyte size only increased following OVX in the WT and β KO groups (Fig. 3C).

Genotype-specific differences in white adipose tissue gene expression

White adipose tissue (WAT) from subcutaneous and visceral (from retroperitoneal region) depots were assessed for inflammatory cytokines, adipokines, and markers of mitochondrial activity and endocrine function. Interestingly, in the INT state, mRNA expression of the insulin-sensitizing adipokine, adiponectin in the retroperitoneal WAT was ~3-fold higher in the β KO mice compared to WT and α KO mice (Fig. 4A; $P < 0.001$), whereas leptin expression was not different between genotypes. This resulted in the adiponectin-to-leptin ratio to be higher in the β KO compared to the α KO mice ($P = 0.04$). In addition, gene expression of the inflammatory macrophage marker, CD11c was increased in both α KO and β KO compared to WT (both $P < 0.05$ compared to WT).

Unfortunately, due to timing differences of mRNA isolation resulting in housekeeping gene differences between INT and OVX mice, we were not able to compare INT to OVX gene expression for this WAT depot. Among OVX groups, no statistically significant differences were observed between groups (Fig. 4B). Thus, the increase in adiponectin gene expression in β KO was dependent on ovarian status (i.e., only observed in the INT state).

In examining mRNA differences in subcutaneous WAT across INT and OVX groups, no significant differences were observed. Since we had sufficient tissue in this depot, we also analyzed aromatase and uncoupling protein 1 (UCP1) gene expression (we were not able to measure these in the retroperitoneal depot due to insufficient tissue). No differences between groups were observed. Insulin signaling proteins, IRS1 and GLUT4, were both reduced by OVX independent of genotype (main effect of OVX, $P < 0.05$ for both genes) (Fig. 4C).

Genotype-specific changes in indicators of insulin resistance following OVX

GTT curves are presented for INT (Fig. 5A) and OVX states (Fig. 5B) across the three genotypes. While both WT and β KO experienced an OVX-induced increase in GTT glucose area under the curve (AUC), this was not observed in α KO mice (Fig. 5C, genotype $\times$ OVX $P < 0.05$). Similarly, the surrogate indices of insulin resistance, HOMA-IR (Fig. 5D) and Adipo-IR (Fig. 5E), both increased following OVX in the WT and β KO mice, but decreased in the α KO (all genotype $\times$ OVX interactions, $P < 0.05$). In comparing genotypes in the INT state for basal insulin signaling in WAT (subcutaneous depot), as determined by phosphorylated Akt (i.e., pAKT relative to total Akt), β KO had significantly higher levels of pAKT compared to both WT and α KO (Fig. 5F; $P < 0.001$). However, following OVX, whereas both α KO and WT experienced increases in pAKT, β KO did not change (genotype $\times$ OVX interaction, $P < 0.05$). There were no differences across OVX groups for GLUT4 protein in the basal/fasting condition, indicating that the apparent greater basal insulin stimulation (reflected by pAkt) did not result in increased glucose uptake into WAT (Fig. 5G).

Fasting blood biochemistry, including blood lipids, are provided in Table 2. Coinciding with their glucose intolerance, INT α KO mice had higher levels of fasting insulin compared to WT (non-significant trend, $P = 0.12$) and β KO mice ($P = 0.043$); β KO mice were similar to WT. As far as blood lipids, the only significant differences that were observed were for the β KO mice, who experienced

Figure 4

Genotype-specific differences in white adipose tissue gene expression in intact and ovariectomized states. (A) Visceral white adipose tissue (WAT) inflammatory gene expression in ovary-intact (INT) mice; (B) visceral WAT inflammatory gene expression in ovariectomized (OVX) mice; and (C) subcutaneous WAT gene expression in INT versus OVX mice. Ovary-intact (INT); ovariectomized (OVX); wildtype (WT); estrogen receptor alpha knockout (α KO); estrogen receptor beta knockout (β KO). * $P < 0.05$ compared to INT within genotype; # $P < 0.05$ compared to α KO within INT or OVX state; \$ $P < 0.05$ compared to WT within INT or OVX state. Values are expressed as mean $\pm$ S.E.M.

increases in total, HDL, and LDL cholesterol with OVX compared to the INT state (all $P < 0.05$). Thus, OVX β KO mice had significantly greater total and LDL cholesterol than OVX WT mice (both $P < 0.05$). Circulating levels of E_2 were not different than INT WT and β KO, and α KO mice had significantly higher estradiol levels than the other groups ($P < 0.05$), which is typical for this model (Islander *et al.* 2003). Liver mass and triglyceride content were evaluated as additional indirect indicators of insulin sensitivity and overall metabolic health. Liver mass was not different based on genotype ($P = 0.234$) or OVX state ($P = 0.716$); yet, liver TG content was almost twice as high in the β KO compared to WT and α KO ($P < 0.05$); OVX did not significantly affect liver TG content in either

genotype ($P = 0.218$). As included in Table 2, ovarian mass differences validated successful OVX surgery in all genotypes ($P < 0.001$).

Genotype-specific changes in energy expenditure and physical activity following OVX

INT α KO mice had significantly lower total energy expenditure (TEE) compared to age-matched WT in both the 12-h light (inactive) and dark (active) cycles (dark cycle shown, Fig. 6A; $P < 0.05$). β KO mice were not different from WT controls in either cycle. These differences coincided with similar differences in resting energy expenditure (REE) (Fig. 6B; $P < 0.05$).

Figure 5

Genotype-specific changes in indicators of insulin resistance following ovariectomy. (A) Glucose tolerance test (GTT) curves of ovary-intact (INT) mice; (B) GTT curves of ovariectomized (OVX) mice; (C) GTT area under the curve (AUC) in INT versus OVX mice; (D) homeostatic assessment of insulin resistance (HOMA-IR) in INT and OVX mice; (E) adipocyte insulin resistance (Adipo-IR) in INT and OVX mice; (F) white adipose tissue (WAT) (from subcutaneous depot) pAkt/Akt ratio protein levels in INT and OVX mice; and (G) WAT (from subcutaneous depot) GLUT4 protein levels in INT and OVX mice (representative blot images shown to the right). Ovary-intact (INT); ovariectomized (OVX); wildtype (WT); estrogen receptor alpha knockout (α KO); estrogen receptor beta knockout (β KO). * $P < 0.05$ compared to INT within genotype; # $P < 0.05$ compared to other genotypes within INT state. Values are expressed as mean $\pm$ S.E.M.

Table 2 Fasting metabolic parameters and organ weights in ovary-intact (INT) and ovariectomized (OVX) WT, α KO and β KO mice.

	WT-INT	WT-OVX	α KO-INT	α KO-OVX	β KO-INT	β KO-OVX
Insulin (ng/mL)	0.73 $\pm$ 0.21	0.79 $\pm$ 0.17	1.29 $\pm$ 0.25	0.77 $\pm$ 0.18	0.68 $\pm$ 0.08 [#]	1.27 $\pm$ 0.27 ^{#*}
Glucose (mg/dL)	155.7 $\pm$ 38.3	212.7 $\pm$ 29.9	219.3 $\pm$ 31.8	242.9 $\pm$ 16.8	208.5 $\pm$ 14.2	213.7 $\pm$ 15.8
Cholesterol (mg/dL)	56.3 $\pm$ 7.9	62.1 $\pm$ 6.4	68.7 $\pm$ 10.8	80.0 $\pm$ 6.1	74.3 $\pm$ 3.5	86.4 $\pm$ 5.6 ^{*\$}
LDL (mg/dL)	4.83 $\pm$ 1.08	5.57 $\pm$ 0.90	5.14 $\pm$ 0.59	7.14 $\pm$ 1.06	4.80 $\pm$ 0.20	8.80 $\pm$ 0.79 ^{*\$}
HDL (mg/dL)	25.2 $\pm$ 2.5	32.9 $\pm$ 2.8	32.1 $\pm$ 5.4	41.3 $\pm$ 2.2	34.9 $\pm$ 1.3 [#]	39.4 $\pm$ 2.0 [*]
Triglycerides (mg/dL)	133.5 $\pm$ 5.4	153.6 $\pm$ 9.9	133.57 $\pm$ 10.0	149.3 $\pm$ 5.9	140.0 $\pm$ 5.5	148.6 $\pm$ 9.3
NEFA (mmol/L)	0.85 $\pm$ 0.15	0.88 $\pm$ 0.13	0.70 $\pm$ 0.12	0.77 $\pm$ 0.07	0.74 $\pm$ 0.07	0.88 $\pm$ 0.07
Estradiol (pg/mL)	13.91 $\pm$ 1.76	14.22 $\pm$ 1.44	34.8 $\pm$ 11.7 ^{#\$}	9.97 $\pm$ 2.45 [^]	10.0 $\pm$ 1.8	9.17 $\pm$ 2.38
Uterine weight (mg)	9.17 $\pm$ 1.08	2.23 $\pm$ 0.18 [*]	8.00 $\pm$ 1.62	2.44 $\pm$ 0.65 [*]	10.10 $\pm$ 1.14	4.79 $\pm$ 1.24 [*]
Liver weight (g)	1.14 $\pm$ 0.12	1.12 $\pm$ 0.07	1.36 $\pm$ 0.14	1.17 $\pm$ 0.13	1.27 $\pm$ 0.08	1.32 $\pm$ 0.07
Liver TG (mgTAG/g)	47.19 $\pm$ 7.30	36.23 $\pm$ 4.99	42.40 $\pm$ 8.80	44.40 $\pm$ 10.70	77.70 $\pm$ 4.43 [#]	61.08 $\pm$ 9.01 ^{\$}

Two (i.e., INT vs OVX) by three (i.e., WT vs α KO vs β KO) ANOVA were performed to determine main effects and interaction effects, which were followed by *post hoc* Tukey's tests where appropriate. Values are expressed as mean $\pm$ S.E.M.

* $P < 0.05$; [^] $P < 0.06$ different from INT within genotype, [#] $P < 0.05$ compared to α KO within INT or OVX state; ^{\$} $P < 0.05$ different from WT within INT or OVX state.

Spontaneous physical activity (SPA) during the dark cycle tended to be lower in α KO (Fig. 6C; $P = 0.052$). These data confirm what has been demonstrated in previous studies using younger α KO mice compared to their WT littermates (Heine *et al.* 2000, Naaz *et al.* 2002). Following OVX, WT experienced a reduction in SPA ($P < 0.05$) but β KO and α KO did not significantly change; this resulted in no differences in SPA following OVX among the three genotypes. Comparisons between the INT and OVX state within genotype also revealed significant reductions in TEE during the dark (active) cycle ($P < 0.01$) and REE during the light (inactive) cycle ($P < 0.01$) in both WT and β KO following OVX, but were not evident in α KO mice following OVX. There were also no differences among the genotypes following OVX in TEE, REE, or food intake (data not shown).

Discussion

The purpose of the current study was to use age-appropriate mouse models to determine whether the molecular mechanisms for OVX-mediated systemic and WAT-specific metabolic dysfunction require loss of signaling through either ER α or ER β . In order to investigate which ER is responsible for OVX-mediated metabolic changes, we used aged female WT, whole body α KO, and whole body β KO mice and compared them before (i.e., ovary-intact (INT) state) and after OVX. WAT is a tissue with high expression of both ER α and ER β (Stubbins *et al.* 2012b, Gao & Dahlman-Wright 2013) and estrogen is produced in adipose tissue and modulates adipose tissue metabolism both before and after OVX (Stubbins *et al.* 2012b). OVX of these ER α and ER β KO mouse models provides a unique

opportunity to investigate the role of adipose tissue local estrogen signaling on metabolic manifestations in the setting of low circulating estrogen. The major findings are that: (1) obesity and systemic IR caused by OVX is likely attributed to loss of central signaling through ER α and (2) the presence of ER β signaling, combined with the absence of ER α signaling in WAT, may play a protective role in preventing OVX-mediated obesity and IR.

First, we compared the WT, α KO, and β KO mice in the INT state at the age that we ovariectomized an additional cohort from each group (i.e., ~49 weeks). This allowed us to gain age-appropriate insight about the effect ovarian hormone loss has on women approaching menopause. We found that these older female α KO mice display phenotypic characteristics of metabolic dysfunction compared to WT controls, similar to those differences previously observed in younger α KO mice (Heine *et al.* 2000, Ogawa *et al.* 2003, Clookey *et al.* 2019). Compared to WT, they were obese and displayed IR. The obesity was not driven by differences in food intake (data not shown – no differences), but rather, reduced energy expenditure in the α KO. Further, the reduced energy expenditure was driven by a trend toward lower cage physical activity, as well as lower resting metabolic rate. Although other studies have shown significant differences in spontaneous physical activity between younger WT and α KO mice, our findings of more modest physical activity differences may be explained by age-related reduction in physical activity, as observed in other models. These data confirm that ER α plays a protective metabolic role on systemic health prior to ovarian insufficiency, due in part to its role in increasing energy expenditure. Similarly, as previously shown in younger animals (Heine *et al.* 2000, Gao & Dahlman-Wright 2013, Clookey *et al.* 2019),

Figure 6

Genotype-specific changes in energy expenditure and spontaneous physical activity following ovariectomy. (A) Total energy expenditure (TEE); (B) resting energy expenditure (REE); and (C) 12-h dark cycle spontaneous physical activity (SPA). Ovary-intact (INT); ovariectomized (OVX); wildtype (WT); estrogen receptor alpha knockout (α KO); estrogen receptor beta knockout (β KO); * P < 0.05 compared to INT within genotype; # P < 0.05 compared to other genotypes within INT or OVX state. Values are expressed as mean $\pm$ S.E.M.

the INT β KO studied here did not display an adverse metabolic profile compared to WT and were not different in indices of obesity, IR, or energy expenditure compared to WT. One exception was that, compared to the α KO, the β KO had greater visceral WAT content, and greater WAT adiponectin gene expression. This may be due to ER β -mediated antagonization of PPAR λ . Previously published *in vitro* studies by Foryst-Ludwig and colleagues showed that ER β inhibits ligand-mediated PPAR λ transcriptional activity, which results in abrogation of PPAR λ -induced adipocyte proliferation (Foryst-Ludwig *et al.* 2008). Other studies support an important relationship between ER β and PPAR λ (Velickovic *et al.* 2014, Wang *et al.* 2015).

As predicted, OVX did not exacerbate the metabolic phenotype in α KO mice, who exhibited pronounced metabolic dysfunction even in the INT state. However, this group exhibited apparent reductions in adiposity and adipocyte size, as well as apparent improvements in insulin sensitivity, indicated predominantly by lower levels of fasting insulin. Others had previously demonstrated that OVX in young α KO mice reduces body weight, visceral adiposity, and glucose intolerance (Naaz *et al.* 2002); we confirm here that this phenomenon is also true in older animals. This was in stark contrast to WT and β KO mice, who experienced increases in adiposity and IR following OVX. This may implicate ER β (possibly via ligand activation by local estrogen produced in WAT) as having a protective metabolic role in the setting of low circulating estrogen (e.g., following OVX). Importantly, estrogen production in adipose tissue is the major source of estrogen following OVX, where estrogen signaling in WAT remains or is increased (Iyengar *et al.* 2013). Unfortunately, we were not able to measure aromatase in visceral WAT. We were only able to measure aromatase gene expression in subcutaneous WAT and found no differences in INT or OVX states. Future studies should measure aromatase activity and protein content, and look at depots other than subcutaneous WAT.

It has recently been suggested that the balance between ER α and ER β protein in the WAT of menopausal women may be an important determinant for estrogen-mediated improvements in WAT insulin sensitivity, such that women with a higher ER α /ER β ratio respond more robustly to estrogen in terms of adipocyte insulin sensitivity (Shin *et al.* 2007, Park *et al.* 2017). It has also been reported that a greater density of ER α over ER β associates with obesity in rodents (Shin *et al.* 2007), and human studies reveal strong links between polymorphisms in the ESR2 gene (i.e., encoding ER β) and obesity, whereas polymorphisms in the ESR1 gene (i.e., encoding ER α) do not associate with obesity in humans (Nilsson *et al.* 2007a). This may be due to the fact that activation of ER α improves WAT insulin sensitivity, thus facilitating efficient fat storage. On the other hand, activation of ER β reduces adipogenesis and adiponectin expression, possibly via a mechanism involving inhibition of PPAR λ as described above (Foryst-Ludwig *et al.* 2008), which may help explain its recently documented adiposity-reducing effect (Ponnusamy *et al.* 2017a; Sasayama *et al.* 2017). This may also explain why INT β KO in the present study had significantly greater WAT adiponectin expression prior to OVX and tended to gain more adiposity following OVX compared to the other groups. Adiponectin mRNA

expression in the visceral WAT of the INT β KO mice was ~3-fold higher compared to both WT and α KO, while the adiponectin-to-leptin ratio (indicative of adipocyte insulin sensitivity) was ~2-fold higher compared to α KO mice. Adiponectin is a potent anti-inflammatory and insulin-sensitizing adipokine secreted from the adipose tissue (Yamauchi *et al.* 2001). Premenopausal women, who exhibit greater adiposity yet are metabolically protected against metabolic dysfunction compared to age-matched males, secrete greater levels of adiponectin (Kern *et al.* 2003). Our data may suggest that ER α signaling is mechanistically responsible for that protective increase in adiponectin. Supporting this, young ER β -null mice exhibit improved insulin sensitivity despite weight gain, presumably related to their greater adiponectin synthesis (Foryst-Ludwig *et al.* 2008).

Thus, it appears that most of the insulin-sensitizing effect of estrogen signaling is through ER α . Yet, we show that, following OVX, mice lacking ER α (i.e., forcing the majority of the available estrogen to activate ER β) respond with improved insulin sensitivity, adipocyte size, and adiposity. To interrogate potential WAT-specific mechanisms for this paradoxical finding, we investigated insulin signaling genes and proteins in WAT. In the INT state, WAT protein levels of pAKT relative to total AKT, indication of basal levels of insulin signaling, were greater in the β KO mice compared to WT and α KO mice. Moreover, OVX significantly increased pAKT only in WT and α KO, bringing their levels to that of the INT β KO (Fig. 5F). Although comparisons of pAKT in response to acute insulin stimulation may be predicted to be lower in settings of IR, here we only measured basal (i.e., non-insulin stimulated) levels. Thus, these higher levels likely reflect greater IR and greater lipid storage, rather than enhanced glucose clearance. Indeed, these differences did not associate with increases in GLUT4, the insulin-dependent glucose transporter in WAT. In fact, gene expression levels of GLUT and IRS1 were significantly suppressed following OVX, supporting OVX-associated disruption in glucose clearance. Future studies should more precisely measure adipocyte insulin signaling in these models. Although no other studies (to our knowledge) have assessed how OVX affects pAkt in WAT in α KO or β KO mice, cell signaling studies have shown that ER β mediates Akt activation (Lin *et al.* 2017) supporting the hypothesis that ER β may actually facilitate insulin-stimulated glucose uptake.

Additional research into potential mechanisms by which ER β signaling affects WAT insulin sensitivity and metabolism is clearly required. These findings raise questions about selective activation of ER α vs ER β ,

particularly following menopause. Previous work has shown that the selective ER α agonist, PPT, in aged OVX mice attenuated weight gain, enhanced insulin sensitivity, and increased energy expenditure compared to controls (Hamilton *et al.* 2016) confirming ER α 's beneficial role in systemic metabolism in aged mice; however, those authors did not assess ER β 's role. Since selective agonization of ER α is not recommended in postmenopausal women who have an elevated risk for breast cancer, our findings that ER β may also mediate beneficial effects following hormone loss, has important clinical relevance. In the present study, OVX in both WT and β KO mice (i.e., two genotypes with normal ER α expression) resulted in weight gain via increased adiposity, reduced energy expenditure, and glucose intolerance. However, OVX in β KO mice led to increased IR compared to the INT condition. Together, these data support a possible protective metabolic role for ER β following OVX.

Since OVX causes WAT inflammation, which precedes the onset of systemic IR (Vieira Potter *et al.* 2012), and visceral WAT-specific ER α knockdown also increases inflammation (Davis *et al.* 2013), it seems that loss of ER α would result in increased inflammation. Indeed, gene expression of the inflammatory macrophage marker known to associate with systemic IR, CD11c, was significantly elevated in visceral WAT of INT α KO compared to INT WT. However, and INT β KO also had greater levels compared to WT, suggesting that in aged mice, ER β may also buffer inflammation (Fig. 4A). Interestingly, INT β KO was unique in having significantly higher visceral WAT adiponectin expression, a difference that diminished following OVX (Fig. 4B). This may have protected the INT β KO from IR (Fig. 5D and E). Although it was surprising that no other differences in inflammatory markers were found in either WAT depot of either genotype, robust increases in WAT inflammation may require chronic high-fat feeding or longer duration following OVX. Or, it may be that all animals experienced age-induced WAT inflammation thus preventing differences between groups from being determined. We also quantified immune cells (i.e., from the stromal vascular fraction) from visceral WAT from a subset of these mice and phenotyped T cells and macrophages – no differences were found, so those data were not included. Future studies should compare old and younger animals in this regard in order to interrogate aging effects from effects attributed to hormone loss *per se*.

Although it is tempting to attribute the robust OVX-associated changes observed in WT and β KO mice to reduction in circulating E₂, no differences in circulating E₂ between INT and OVX β KO or between INT and OVX WT

mice were found. There are a few potential explanations for this. One is that the robust adiposity increases in the WT and β KO mice, but not ER α , resulted in increases in extragonadal E₂ production (i.e., from adipose tissue), which may have been detected in the circulation. Indeed, high-fat diet-induced obesity is known to increase circulating E₂ in OVX mice, whereas this obesity-induced E₂ increase does not appear to be protective (at least on bone). It is not known if ovarian vs extra-ovarian estrogens have differential effects on metabolism. Additional studies are necessary to further interrogate how ovarian vs extra-ovarian estrogens affect systemic and adipose tissue metabolism. Another possibility is that ovarian hormones other than E₂ may play an important role in OVX-mediated changes, while only E₂ was measured here. Future studies should measure testosterone, progesterone, and additional forms of estrogen (e.g., estrone). It may be that the ratio of E₂ to these other hormones is more important than total circulating E₂, *per se*. Finally, we did not measure sex hormone-binding globulin (SHBG); thus, it may be that OVX increased SHBG, making the level of bioavailable E₂ much less following OVX; we measured total E₂ only. Future studies should thus measure SHBG in addition to other ovarian hormones.

Strengths and limitations

Use of older female α KO and β KO mice provided an additional stress not previously investigated in these models following OVX. This approach is more representative of age-related hormonal changes typically observed in women. However, future studies should utilize WAT-specific knockout models to further interrogate the adipose tissue-specific effects of signaling through ER α and ER β on responses to selective ligands following OVX. We recently demonstrated by adipose tissue-specific activation (i.e., via selective beta 3 adrenergic receptor ligand) completely restored the systemic and adipose tissue metabolic phenotype induced by loss of ER α signaling (Clookey *et al.* 2019). The role of aging and ovarian hormone state on the potential efficacy of specific ER α versus ER β ligands requires more intense investigation. Nevertheless, based on our primary findings that (1) forced' signaling through ER β (owing to the absence of ER α) results in reductions in adiposity, adipocyte size, and IR following OVX and (2) suppressed signaling through ER β exacerbates adiposity gain following OVX, we conclude that selective activation of ER β may be an important metabolic therapeutic in settings of low circulating estrogen. Previous studies have shown that ER β -null mice display

increased susceptibility to OVX (Seidlova-Wuttke *et al.* 2012) and we confirm that here. Although mechanisms are not fully understood, accumulating evidence indicates that systemic activation of ER β reduces adiposity by activating adipocyte mitochondrial activity (Ponnusamy *et al.* 2017a). We hypothesize that local adipose tissue estrogen may be protective following OVX by activating ER β and improving mitochondrial activity. On the other hand, activation of ER α may enhance adipocyte insulin sensitivity and facilitate lipid storage.

As summarized in Fig. 7, following OVX, a reduction in systemic energy expenditure, driven by reduced central ER α signaling, associates with 'healthy' deposition of

Figure 7

Proposed model by which a predominance of ER α signaling in white adipose tissue (WAT) may enable ovariectomy (OVX)-induced obesity. A greater α KO/ β KO ratio favors insulin-sensitive WAT creating an obesity phenotype in the setting of low energy expenditure; this is due to increased insulin-mediated lipid deposition. Estrogen receptor alpha (ER α); estrogen receptor beta (ER β). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0321>.

adipose tissue due to its high insulin sensitivity, which is attributed largely to ER α signaling in adipose tissue. Thus, in the ovary-intact (and premenopausal) condition, estrogen (via ER α) both increases systemic energy expenditure and enhances adipocyte insulin sensitivity – this leads to a lean and insulin-sensitive phenotype. However, following OVX (and menopause), a reduction in centrally mediated energy expenditure combined with high adipocyte insulin sensitivity (due to greater ER α / ER β signaling in adipose tissue combined with local adipose tissue estrogen production) leads to more efficient fat storage and positive energy balance (i.e., obesity). We hypothesize that a greater ER β / ER α ratio may offset this by reducing fat storage and adipocyte size. Mechanistically, this may occur via ER β -mediated increased adipocyte energy expenditure and suppressed adipocyte proliferation. This hypothetical model requires further testing, but support for it would implicate ER β ligands as potential therapeutics to reduce obesity following menopause. Indeed, recent evidence indicates that activation of ER β exerts potent anti-obesity effects (Miao *et al.* 2016, Ponnusamy *et al.* 2017a; Ponnusamy *et al.* 2017b; Sasayama *et al.* 2017, Gonzalez-Granillo *et al.* 2019). Importantly, unlike ER α , adipose tissue expression of ER β increases following OVX in the setting of aging (Tomicek *et al.* 2011), revealing a window of opportunity to protect postmenopausal women via selective ER β activation. Another important fact to note is that the metabolic actions of ER α are mediated through two distinct activation functions – AF1 and AF2. In 2017, Guillaume *et al.* demonstrated that the effects of tamoxifen (used to activate ER α in specific tissues) to protect against IR and obesity requires AF1 but not AF2 (Guillaume *et al.* 2017). Our studies cannot distinguish which aspects of ER α (and/or ER β) signaling were responsible for the differential effects following OVX, but future studies should determine the specific receptor-mediated mechanisms. Finally, a critical adverse consequence of menopause is reduced bone density due to the decline in circulating estrogens, and reduction in estrogen/ER-mediated effects are largely responsible for this. This has been shown in OVX animals and postmenopausal women. Thus, an important future direction is to determine the role of reduced ER signaling on bone-specific responses to OVX. In conclusion, the findings presented here led us to suggest that the systemic metabolic effects of OVX are mediated through central loss of ER α signaling, while the activation of ER β in adipose tissue by locally produced estrogen may be protective. Thus, ER β may be an attractive therapeutic target to improve metabolism following ovarian hormone loss.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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