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Increased susceptibility to OVX-associated metabolic dysfunction in UCP1-null mice

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Abstract

Premenopausal females are protected against adipose tissue inflammation and insulin resistance, until loss of ovarian hormone production (e.g., menopause). There is some evidence that females have greater brown adipose tissue (BAT) thermogenic capacity. Because BAT mass correlates inversely with insulin resistance, we hypothesized that increased uncoupling protein 1 (UCP1) expression contributes to the superior metabolic health of females. Given that UCP1 transiently increases in BAT following ovariectomy (OVX), we hypothesized that UCP1 may ‘buffer’ OVX-mediated metabolic dysfunction. Accordingly, female UCP1-knockout (KO) and WT mice received OVX or sham (SHM) surgeries at 12 weeks of age creating four groups (n = 10/group), which were followed for 14 weeks and compared for body weight and adiposity, food intake, energy expenditure and spontaneous physical activity (metabolic chambers), insulin resistance (HOMA-IR, ADIPO-IR and glucose tolerance testing) and adipose tissue phenotype (histology, gene and protein expression). Two-way ANOVA was used to assess the main effects of genotype (G), OVX treatment (O) and genotype by treatment (GxO) interactions, which were considered significant when \( P \leq 0.05 \). UCP1KO mice experienced a more adverse metabolic response to OVX than WT. Whereas OVX-induced weight gain was not synergistically greater for KO compared to WT (GxO, NS), OVX-induced insulin resistance was significantly exacerbated in KO compared to WT (GxO for HOMA-IR, \( P < 0.05 \)). These results suggest UCP1 is protective against metabolic dysfunction associated with loss of ovarian hormones and support the need for more research into therapeutics to selectively target UCP1 for prevention and treatment of metabolic dysfunction following ovarian hormone loss.

Introduction

Premenopausal females are protected against insulin resistance and adipose tissue inflammation, which are major independent predictors of metabolic disease (Regensteiner et al. 2015). However, postmenopausal women exhibit a disproportionate acceleration in metabolic disease risk relative to males, characterized

Key Words

- insulin resistance
- ovarian function
- adipose tissue
- estrogen receptor
- whole animal physiology
by increased accumulation of visceral adiposity and associated inflammation (Auro et al. 2014, Stefanska et al. 2015). Similarly, ovariectomized (OVX) rodents exhibit greater adipose tissue inflammation and systemic insulin resistance relative to ovary-intact controls (Nickelson et al. 2012, Pettersson et al. 2012, Vieira Potter et al. 2012), whereas estrogen (E$_2$) replacement therapy mitigates many of the adverse manifestations of ovarian hormone loss. Importantly, in light of the adverse side effects of E$_2$ replacement therapy, alternative therapeutic approaches are warranted (Ouyang et al. 2006).

Adipose tissue health is a major predictor of metabolic disease. Indeed, white adipose tissue inflammation has been implicated as a causal link between obesity and cardiometabolic complications (Kanda et al. 2006, Ginsberg & MacCallum 2009, Reaven 2011, Vieira-Potter 2014). However, brown adipose tissue (BAT) is metabolically protective, as its relative abundance and activity inversely associates with insulin resistance and type 2 diabetes (Manolopoulos et al. 2010, Ouellet et al. 2011). In this context, increased BAT activity has been shown to improve whole-body insulin sensitivity and glucose homeostasis in overweight adult humans (Chondronikola et al. 2014) and rodents (Stanford et al. 2012). Uncoupling protein 1 (UCP1) is the signature mitochondrial protein in BAT, which enables adaptive thermogenesis by uncoupling oxidative phosphorylation. There is evidence that UCP1 has a direct, insulin-sensitizing role, as overexpression of UCP1 protects against diet-induced obesity and insulin resistance in mice (Kopecky et al. 1996), and mice null for UCP1 are more susceptible to western diet (WD)-induced insulin resistance (Winn et al. 2017b). In considering that E$_2$ has protective actions in adipose tissue, which may contribute to the metabolic protection observed in ovary-intact females, one hypothesis is that UCP1 may be regulated by E$_2$, and this may help explain E$_2$‘s protective effects in adipose tissue. This hypothesis originates from the observations of some investigators that ovary-intact females have more BAT than age-matched males (Cypess et al. 2009, Pfannenberg et al. 2010) and/or are more sensitive to BAT activation (Queuevedo et al. 1998, van den Beukel et al. 2015). It is also known that, on a gram per gram basis, white adipose tissue from females contributes more to resting energy expenditure compared to that from males; this suggests that female adipose tissue contains more UCP1 and/or greater mitochondrial density (Nookaew et al. 2013). Whether those sex differences are at least partially explained by E$_2$ is not known. However, it is important to note that not all studies have supported that there is a sex difference in BAT content and/or activity (van der Lans et al. 2013). In fact, when we compared BAT UCP1 levels in male and female mice (housed under the same temperature conditions as the present study), we did not find an increase in UCP1 protein content (Winn et al. 2017a), although we did find greater UCP1 gene expression in females. Others have had similar findings in mice where no sex differences in BAT UCP1 content were found (Greghorst et al. 2015). However, a large survey study of adult men and women showed that young women had higher probability of having detectable BAT by positron emission tomography (Wang et al. 2015). Furthermore, others have shown that this sex difference wanes with increasing age (Ouellet et al. 2011), which lends support to the hypothesis that E$_2$ availability contributes to the sex difference, since E$_2$ declines with age in women. In fact, in high-fat diet-fed rodents, E$_2$ treatment has been shown to increase UCP1 protein and other markers of adipose tissue browning (Al-Qahtani et al. 2017). Moreover, E$_2$ signaling in the brain has been shown to increase BAT activation via the sympathetic nervous system (de Morentin et al. 2014). Taken together, those findings suggest that there may be sex differences in BAT content and/or susceptibility to its activation and these differences may be dependent on E$_2$; the major sex hormone circulating in young women that falls considerably following menopause.

Somewhat paradoxically, OVX has been shown to increase UCP1 protein expression in BAT (Nadal-Casellas et al. 2011). Similarly, we have also observed an increase in BAT UCP1 following OVX in rodents (Vieira-Potter et al. 2015), similar to increases in UCP1 observed under conditions of diet-induced obesity (Feldmann et al. 2009, Cannon & Nedergaard 2010, Yao et al. 2014, Sakamoto et al. 2016, Winn et al. 2017b). Those findings do not necessarily rule out the possibility that E$_2$ regulates UCP1, since adipose tissue continues to produce E$_2$ even following OVX or menopause via activity of aromatase, which may increase following menopause leading to increased local (i.e., adipose tissue) E$_2$ exposure. The induction of UCP1 following OVX in BAT may also be triggered by metabolic stress (i.e., similar to high-fat diet-induced UCP1 induction) and completely independent of E$_2$. Although this current study did not test those hypotheses (i.e., regarding E$_2$‘s ability to regulate UCP1 or mechanisms by which OVX, at least transiently, increases UCP1 in BAT), we took the first step of testing the hypothesis that absence of UCP1 would exacerbate OVX-associated metabolic dysfunction and adipose tissue inflammation.
Methods

Ethical approval

All animal husbandry and experimental procedures were carried out in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and approved by the University of Missouri Institutional Animal Care and Use Committee before their initiation.

Animals and experimental design

Heterozygote female UCP1+/- mice on a C57BL/6J background were purchased from Jackson Laboratory (Stock #003124) and bred at the University of Missouri Transgenic Animal Core (Columbia, MO, USA) facility to produce homozygote UCP1+/- (UCP1KO; n=20 females) and littermate WT controls (n=20 females). All mice were fed a standard chow diet (3.3 kcal/g of food, 13% kcal fat, 57% kcal carbohydrate and 30% kcal protein, 5001, LabDiet, St. Louis, MO, USA) and were housed two to three per cage (within group) in a light cycle from 07:00 to 19:00 h at 25°C. In previous studies, these conditions were shown to be adequate in preventing thermostress (Winn et al. 2017a). WT and UCP1KO female mice underwent OVX or sham (SHM) operations at 12 weeks of age creating four distinct groups (n=10/group): (1) WT SHM, (2) WT OVX, (3) KO SHM and (4) KO OVX. These animals were assessed for 14 weeks and compared for total and regional adiposity, indicators of adipose tissue inflammation and metabolism, systemic fuel metabolism and energy expenditure and insulin resistance. At 26 weeks of age, following a 5-hour fast, mice were killed and blood and tissues were collected. Tissues were harvested and either fixed in 10% formalin, snap-frozen in liquid nitrogen and stored at -80°C until analyses. Data were generated from a subset of these animals in a previous study (Winn et al. 2017a).

Body composition and tissue weights

The percent body fat (BF%) was measured by a nuclear magnetic resonance imaging whole-body composition analyzer (EchoMRI 4in1/1100; Echo Medical Systems, Houston, TX), 1 week prior to killing. Upon killing, interscapular BAT, thoracic periaortic BAT, subcutaneous (inguinal, SQAT) and visceral (perigonadal) white adipose tissue (PGAT) were extracted and tissue weights were collected.

Energy expenditure assessment

At 18 weeks of age, animals (n=10/group) were placed in indirect calorimetry chambers (Promethion; Sable Systems International, Las Vegas, Nevada) to assess metabolic activity parameters including total energy expenditure, resting energy expenditure (REE) and spontaneous physical activity (SPA) by the summation of x-, y- and z-axis beam breaks. Body weight and food intake were measured before and after each 48-h assessment. Each 48-h run captured at least two light and two darkness cycles of REE.

Fasting blood parameters

Plasma glucose, cholesterol, triglycerides and non-esterified fatty acids (NEFA) assays were performed by a commercial laboratory (Comparative Clinical Pathology Services, Columbia, MO, USA) on an Olympus AU680 automated chemistry analyzer (Beckman-Coulter, Brea, CA, USA) using assays as per manufacturer's guidelines. Plasma insulin concentrations were determined using a commercially available, mouse-specific ELISA (Alpco Diagnostics, Salem, NH, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was used as a surrogate measure of hepatic insulin resistance ((fasting insulin (µU/L)×fasting glucose (mg/dL)/405.1) (Matthews et al. 1985)) and an index of adipose tissue insulin resistance (ADIPO-IR) was calculated as the product of fasting insulin (µU/L) and fasting NEFAs (mmol/L) (Lomonaco et al. 2012). Fasting levels of circulating adipokines, leptin and adiponectin, were measured using colorimetric ELISA (#90030, #80569, Crystal Chem) and data are presented as µg/mL.

Histological assessments

Formalin-fixed samples were processed through paraffin embedment, sectioned at 5µm and stained with hematoxylin and eosin (interscapular BAT, visceral white AT). Sections were evaluated via an Olympus BX34 photomicroscope (Olympus) and images were taken via an Olympus SC30 Optical Microscope Accessory CMOS color camera. Adipocyte size was calculated from three independent regions of the same 40x objective fields for PGAT and BAT depots (50 adipocytes/animal). Cross-sectional areas of the adipocytes were obtained from perimeter tracings using ImageJ software as previously described (Wainright et al. 2015). An investigator blinded to the groups performed all procedures.
RNA extraction and quantitative real-time RT-PCR

Interscapular BAT, PGAT and SQAT samples were homogenized in TRIzol solution using a tissue homogenizer (TissueLyser LT, Qiagen). Total RNA was isolated according to the Qiagen’s RNeasy lipid tissue protocol and assayed using a Nanodrop spectrophotometer (Thermo Scientific) to assess the purity and concentration. First-strand cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative real-time PCR was performed as previously described using the ABI StepOne Plus sequence detection system (Applied Biosystems) (Roseguini et al. 2010, Padilla et al. 2013). Primer sequences were designed using the NCBI Primer Design tool. All primers were purchased from IDT (Coralville, IA, USA). The beta actin gene (Actb) was used as house-keeping control gene. Actb cycle threshold (CT) was not different among the groups of animals. mRNA expression was calculated by $2^{\Delta CT}$ where $\Delta CT = CT_{\text{gene of interest}} - CT_{\text{Actb}}$ – gene of interest CT and presented as fold-difference. mRNA levels were normalized to the of WT SHM group which was set at 1.

Western blotting

Protein content was measured as previously described (Winn et al. 2017b). Briefly, protein samples (10µg/lane were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and probed with primary antibodies, including UCP1 (#U6382, 1:1000; Sigma-Aldrich), UCP2 (#89326, 1:1000; Cell Signaling), Acetyl Co-A Carboxylase or ACC (#3662, 1:1000; Cell Signaling), Phosphorylated Acc or PACC(#3661, 1:1000; Cell Signaling), OXPHOS (#MS5604, 1:1000; MitoSciences), Ampk (#2532, 1:1000, Cell Signaling), AMPK-SER485 (4185, 1:1000, Cell Signaling), AMPK-THR172 (#2531, 1:1000, Cell Signaling), Estrogen Receptor Alpha (Yasrebi et al. 2016) (#75635, 1:1000, Abcam), Estrogen Receptor Beta (ERB) (#AB3577, 1:2000, Abcam), GLUTIV (#2213, 1:1000, Cell Signaling), AKT (#4691, 1:500, Cell Signaling). Intensity of individual protein bands was quantified using FluoroChem HD2 (AlphaView, version 3.4.0.0) and expressed as ratio to Amide Black total protein stain.

Statistical analysis

A $2 \times 2$ ANOVA was used to evaluate the effects of genotype (UCP1KO vs WT, denoted by G) and treatment (OVX vs SHM, denoted by O) and genotype by treatment interactions (denoted by GxO). When significant interactions were observed, Tukey’s post hoc tests were used to indicate significant between-group differences (* used to denote such differences). All data are presented as mean±standard error of the mean (s.e.m.). For all statistical tests, significance was accepted at $P \leq 0.05$. All statistical analyses were performed using SPSS V20.0.

Results

Effects of UCP1 ablation and OVX on adiposity

Main effects of both genotype (i.e., UCP1KO) and OVX were found for body weight gain from baseline (Fig. 1A) and final body weight (Fig. 1B), body fat percentage (Fig. 1C), total fat mass (Fig. 1D), and white adipose tissue depot weights (Fig. 1E; all $P<0.05$). Total lean mass (Fig. 1D) and BAT mass (Fig. 1E) were greater in UCP1KO animals but not affected by OVX. However, UCP1KO animals tended to be more susceptible to an OVX-induced increase in periaortic BAT (GxO, $P=0.056$). No significant GXO interactions were observed in assessments of weight gain. Actual body weight changes throughout the study for all groups is provided in Supplementary Fig. 1A (see section on supplementary data given at the end of this article).

Effects of UCP1 ablation and OVX on energy expenditure and SPA

Both KO genotype and OVX reduced total and REE relative to body weight (G, O, both $P \leq 0.05$) (Fig. 2A and C). In the dark cycle (i.e., rodent active period), OVX reduced cage physical activity (O, $P<0.001$), recapitulating findings that loss of ovarian hormones reduces voluntary physical activity (Park et al. 2016); no effect of UCP1 ablation on cage physical activity was observed (Fig. 2B). While UCP1KO animals consumed significantly less energy than WT (Fig. 2D; G, $P=0.003$), both genotypes reduced their food intake under conditions of OVX (O, $P<0.001$). Likewise, metabolic efficiency was greater in UCP1KO vs. WT animals and was also increased by OVX in both genotypes (G, O, both $P<0.05$) (Fig. 2E).

Effects of UCP1 ablation and OVX on glucose and lipid metabolism

While there were no statistically significant differences in glucose tolerance across groups (Fig. 3A), KO animals were hyperinsulinemic compared to WT (G, $P<0.001$)
Figure 1
Influence of UCP1 ablation and OVX on % weight gained, body weight, and adiposity. UCP1-null and WT female mice were subject to ovariectomy or sham surgery at 12 weeks of age, then assessed for 14 weeks for: (A) % body weight gained from baseline; (B) body weight; (C) body fat percentage; (D) body composition; (E) fat pad mass. KO, UCP1 knockout; SHM, sham surgery; OVX, ovariectomy; 2 × 2 analysis of variance (ANOVA) was performed to assess main effects of genotype (G), ovariectomy (O), and genotype and ovariectomy interactions (GxO). GxO were followed by Tukey's post hoc tests. Where such tests revealed significant differences between groups, those differences are indicated. Data are expressed as means ± standard error (s.e.); n = 10/group, significance was accepted at P<0.05.

Figure 2
Influence of UCP1 ablation and OVX on energy expenditure, spontaneous physical activity and energy intake. UCP1-null and WT female mice were subject an ovariectomy or sham surgery at 12 weeks of age, then placed in indirect calorimetry chambers and assessed for metabolic activity parameters: (A) total energy expenditure; (B) spontaneous physical activity; (C) resting energy expenditure; (D) energy intake; (E) metabolic efficiency. KO, UCP1 knockout; SHM, sham surgery; OVX, ovariectomy; 2 × 2 analysis of variance (ANOVA) was performed to assess main effects of genotype (G), ovariectomy (O), and genotype and ovariectomy interactions (GxO). GxO were followed by Tukey's post hoc tests. Where such tests revealed significant differences between groups, those differences are indicated. Data are expressed as means ± standard error (s.e.); n = 10/group, significance was accepted at P<0.05.
Table 1  Blood biochemistry in fasted female WT and UCP1 knockout mice under ovary-intact and ovariectomized conditions.

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT SHM</th>
<th>WT OVX</th>
<th>KO SHM</th>
<th>KO OVX</th>
<th>P-Value</th>
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<tbody>
<tr>
<td>NEFA (mmol/L)</td>
<td></td>
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<tr>
<td></td>
<td>0.64±0.05</td>
<td>0.63±0.04</td>
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<tr>
<td>TG (mg/dL)</td>
<td>134.60±8.80</td>
<td>136.00±5.90</td>
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<tr>
<td>LDL (mg/dL)</td>
<td>3.63±0.34</td>
<td>3.54±0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>28.50±1.00*</td>
<td>25.80±1.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>50.50±1.50</td>
<td>45.40±1.70</td>
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</table>

All data expressed as mean±s.e.; n=10/group. *P<0.05 compared to KO SHM via post hoc Tukey’s test.

G, P<0.05 main effect of genotype; GxO, P<0.05 interaction between G and O; KO, UCP1 knockout; NEFA, non-esterified fatty acids; NS, no significant differences detected; O, P<0.05 main effect of ovariectomy; OVX, ovariectomized condition; SHM, sham/ovary-intact condition; TG, triglycerides.
Effects of UCP1 ablation and OVX on BAT phenotype

Under the non-cold stressed conditions of this study, BAT phenotype was at most modestly affected based on histological analysis (Fig. 4A). UCP1 ablation did increase mean brown adipocyte size (G, \( P<0.05 \)); however, this measure was not affected by OVX (Fig. 4D). Analysis of gene expression revealed a genotype effect for an increase in the marker of mitochondrial biogenesis, mitochondrial transcription factor A (Tfam), as well as ~seven-fold increases in DNA damage-inducible transcript 3 (Ddit3) and fibroblast growth factor 1 (Fgf21) (indicators of endoplasmic reticulum (ER)/oxidative stress) in UCP1KO mice (Fig. 4B). All primer sequences are provided in Table 2. UCP1KO also had increased gene expression of E\(_2\) receptor beta (Esr2) in BAT, whereas Esr1 expression was not affected. OVX induced a decrease in glutathione peroxidase (Gpx3), an E\(_2\)-regulated protein that functions to detoxify hydrogen peroxide (Lundholm et al. 2008) and increased expression of the adipokines leptin (Lep) and adiponectin (Adipoq). In the case of Gpx3, the OVX-induced reduction was more severe in the UCP1KO, indicated by the significant GXO interaction.

Gene expression analysis of uncoupling protein 2 (Ucp2), another uncoupling protein known to be upregulated by E\(_2\) (Pedersen et al. 2001), revealed a GXO interaction in that UCP1 ablation increased, whereas OVX decreased, its expression (GxO, \( P\leq0.05 \)). Lipocalin (Lcn2), an adipokine thought to regulate E\(_2\) signaling in adipose tissue (Drew et al. 2015), was diminished in UCP1KO mice and further reduced by OVX (G, O, both \( P<0.05 \)). There was no evidence of increased inflammatory gene expression in BAT.

Recapitulating what we have previously reported in other studies comparing UCP1KO and WT mice, we observed a decrease in the mitochondrial oxidative phosphorylation proteins, cytochrome C oxidase enzyme complex (COX) 1, 2, 3 and 4 were observed (Fig. 4C and E). However, there was no evidence for changes in protein expression relevant to energy regulation, such as AMPK phosphorylation at either the Serine-491/485 (i.e., inhibitory) or Threonine-179 (i.e., activation) sites or phosphorylated acetyl-CoA carboxylase (Gambacciani et al. 1997) relative to total ACC protein, which catalyzes the committed step in fatty acid synthesis (G, \( P\leq0.05 \)). To explore potential interactions between E\(_2\) signaling...
proteins and UCP1 (Frank et al. 2017), we also measured levels of E₂ receptor alpha (Yasrebi et al. 2016) and beta (ERB). Here, we did not find significant differences in ERα or ERβ protein content in BAT with either UCP1 ablation or OVX, and there was no difference between groups in the ratio of ERα:ERB protein expression, a ratio that has been shown to correlate positively with a ‘healthy’ adipose tissue phenotype (Gao & Dahlman-Wright 2013) and E₂-mediated improvements in insulin sensitivity (Park et al. 2017) (Fig. 4C and E).

**Effects of UCP1 ablation and OVX on WAT phenotype**

OVX significantly increased PGAT (i.e., a WAT depot from the visceral region in the rodent) adipocyte size (Fig. 5A and D). In investigating gene expression in this WAT depot, OVX increased both Ddit3 and Fgf21 in each genotype (O, P ≤ 0.05), indicative of increased cellular stress. Similar to previous findings in mice (Rogers et al. 2009, Vieira Potter et al. 2012), the PGAT depot also exhibited evidence of OVX-induced inflammation, indicated by increased gene expression of Mcp1, Il6, Tnfa, Cd68 and Cdc11c (O, all P ≤ 0.05) (Fig. 5B). Unlike BAT, UCP1 ablation only modestly increased inflammation as indicated by greater Cd68 gene expression relative to WT (G, P < 0.05) with no other significant differences in inflammatory genes. Similar to BAT, OVX increased gene expression of Leptin, consistent with the phenotype of OVX-mediated adipocyte hypertrophy (Fig. 5D). In terms of GxO interactions, Ucp2 gene expression increased with OVX in WT, but not in the UCP1KO (GxO, P < 0.05). On the contrary, Pgc1a increased with OVX only in the KO (GxO, P ≤ 0.05); similarly, E₂ receptor alpha (Esr1) expression increased following OVX only in the KO (GxO, P ≤ 0.05).

In terms of protein expression, UCP1 ablation unexpectedly increased the mitochondrial subunit COX3 (G, P ≤ 0.05) but did not affect any other proteins in PGAT. On the other hand, OVX resulted in an increase in COX 5 (i.e., ATP synthase enzyme), and a decrease in COX 1, total ACC and phosphorylated ACC (pACC, the active form of the enzyme). OVX also increased the amount of pACC relative to total ACC (Fig. 5E). Since emerging evidence suggests potential cross-talk between ER signaling and proteins associated with white adipocyte browning (Grefhorst et al. 2015, Qian et al. 2016), ERs were also quantified in this depot. In this regard, there were no significant differences in Era or ERB protein expression (Fig. 5C and E). The reason we chose to comprehensively assess the phenotype of PGAT is that this depot is most susceptible to metabolic inflammation and, in females,

### Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Ucp1</td>
<td>ACTGCCACACCTGACTTATT</td>
<td>CTTGCTTCACTGAGATTG</td>
</tr>
<tr>
<td>Ucp2</td>
<td>AAGTCTTTCCTGCTCCTGACCC</td>
<td>CTAGCCTTTGACTCTCCTCCT</td>
</tr>
<tr>
<td>Gpx3</td>
<td>GTATGGAGGGCCTCCACATG</td>
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<tr>
<td>Pgc1a</td>
<td>CCGTGGCTTTCGGAAGCAC</td>
<td>TGTGGTGGTCTCGGTTTTC</td>
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<tr>
<td>Tfam</td>
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<td>Hspa5</td>
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<td>Ddit3</td>
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<td>Leptin</td>
<td>CCACTTCTAGGGCTCTTCC</td>
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<td>Adipoq</td>
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**Gene names:** Ucp1, uncoupling protein 1; Ucp2, uncoupling protein 2; Gpx3, glutathione peroxidase; Pgc1a, peroxisome proliferator-activated receptor-gamma coactivator 1-alpha; Tfam, mitochondrial transcription factor a; Mcp1, monocyte chemoattractant protein 1; Cd68, cluster of differentiation 68; IL-6, interleukin 6; Cdc11c, cluster of differentiation 11c; Tnfa, tumor necrosis factor alpha; Hspa5, heat shock protein family A member 5; Ddit3, DNA damage-inducible transcript 3 protein; Adipoq, adiponectin; Fgf21, fibroblast growth factor 21; Lcn2, lipocalin 2; Cyp19a1, aromatase, Esr1, estrogen receptor alpha; Esr2, estrogen receptor beta; Actb, beta actin.
S L Clookey et al. UCP1 knockout in response to OVX

Figure 5
Influence of UCP1 ablation and OVX on perigonadal adipose tissue immunometabolism in female mice. UCP1-null and WT female mice were subjected to an ovariectomy or sham surgery at 12 weeks of age, then sacrificed after 14 weeks. Perigonadal adipose tissue was harvested and processed for adipocyte size, gene expression and protein content: (A) perigonadal adipose tissue (PGAT) histology; (B) perigonadal gene expression; (C) western blot representative images; (D) perigonadal adipocyte size; (E) protein expression. KO, UCP1 knockout; SHM, sham surgery; OVX, ovariectomy; 2 × 2 analysis of variance (ANOVA) was performed to assess main effects of genotype (G), treatment (O), and genotype and treatment interactions (G × O). G × O were followed by Tukey’s post hoc tests. Where such tests revealed significant differences between groups, those differences are indicated. Data are expressed as means ± standard error (s.e.); n = 10/group, significance was accepted at P < 0.05.

Discussion
We sought to investigate if UCP1 plays a compensatory role in response to the metabolic dysfunction associated with ovarian hormone loss via ovariectomy (OVX) in mice. It has been observed that UCP1 is upregulated in response to metabolic stress, such as diet-induced obesity (Feldmann et al. 2009, Cannon & Nedergaard 2010, Yao et al. 2014, Sakamoto et al. 2016, Winn et al. 2017b) and OVX (Feldmann et al. 2009, Cannon & Nedergaard 2010, Crissey et al. 2014, Vieira-Potter et al. 2015). This seemingly compensatory upregulation of UCP1 led us to hypothesize that lack of UCP1 would aggravate OVX-induced metabolic dysfunction. Consistent with this idea, we found that the absence of UCP1 worsened OVX-mediated impairment in glucose metabolism, indicated by HOMA-IR scores and hyperinsulinemia. This supports the hypothesis that UCP1 upregulation following loss of ovarian hormones may mitigate metabolic dysfunction. Further, these data also support the contention that more total and/or active BAT (Cypess et al. 2009), or greater UCP1 expression, in premenopausal women compared to age-matched men and older women (Pfannenberg et al. 2010) may contribute to their metabolic protection. It should be noted that the degree of insulin resistance observed here was very modest, as neither OVX nor UCP1 ablation caused glucose intolerance. This is probably because the mice tested here were young, relatively healthy females,
known to exhibit metabolic protection. In addition, they were not under conditions of dietary stress, as they were fed a normal chow diet. Thus, despite either not expressing the protective protein, UCP1 or undergoing OVX, these mice remained relatively healthy. However, the combination of UCP1 ablation and OVX did result in a significantly worse metabolic phenotype compared to either treatment alone. Likely, OVX and UCP1 ablation-induced compensatory hyperinsulinemia resulting in apparently normal glucose tolerance test results. Future studies should employ the hyperinsulinemic–euglycemic clamp to most accurately assess insulin resistance and determine tissue-specific insulin sensitivity. Future studies should consider waiting longer for insulin resistance to develop, using older mice, challenging these groups with high-fat diet or measuring the insulin secretion response to glucose. Importantly, these current results taken together corroborate our previous findings (Winn et al. 2017b) and suggest that UCP1 improves insulin sensitivity via a mechanism independent of increased energy expenditure or fat loss.

Adipose tissue phenotype plays an important role in determining whole-body insulin sensitivity (Manolopoulos et al. 2010). This is particularly important for females who have greater adiposity and less lean mass compared to males. Importantly, while estrogen (E2) drives fat storage in adipose tissue, it has many metabolically protective effects resulting in greater insulin sensitivity in E2-sufficient females, despite their greater adiposity. However, loss of ovarian hormone production, as occurs in natural or surgical menopause, abruptly dissolves this protection, increasing metabolic disease risk (Auro et al. 2014, Stefanska et al. 2015). Similarly, UCP1 ablation induces obesity and enhances metabolic efficiency (Feldmann et al. 2009). We observed that both UCP1 ablation and OVX led to increased body weight, largely attributed to adiposity, indicated by both greater body fat percentage and adipose tissue pad weights across several depots. Those changes in body mass due to both OVX and the UCP1KO genotype were accompanied by decreases in both food intake and REE, indicating that reduced basal metabolism, and not increased food intake or decreased physical activity, caused the energy surplus that led to the increased adiposity. This is somewhat unlike our previous study, where similarly aged UCP1KO and WT females did not differ in body weight. Other studies have demonstrated that UCP1KO mice only experience enhanced susceptibility to obesity under thermoneutral conditions, thought to be ~30°C. This has been hypothesized to be driven largely by loss of diet-induced thermogenesis in UCP1KO at thermoneutral conditions, whereas under cold stress, UCPKO animals utilize other means to regulate body temperature and energy balance (e.g., increased shivering and/or cage physical activity). Thus, it is possible that the mice in our present study were exposed to a warmer temperature than those in our first study. Although we set the temperature at 25°C for both studies, it is possible that room conditions (e.g., animals housed on higher shelves in the later study, which would contribute to slightly higher temperature) contributed to those differences. Importantly, in both our previous study and in the current study, UCP1 ablation disrupted glucose homeostasis, suggesting that UCP1 has insulin-sensitizing effects that can be dissociated from its ability to reduce adiposity. Supporting that idea, the UCP1KO animals in the current study were more susceptible to OVX-induced impairment in glucose homeostasis yet did not necessarily experience an enhancement in OVX-induced weight gain. That is, while the KO/OVX animals were heavier than either the KO/SHM or WT/OVX, the effects were additive yet not synergistic (i.e., demonstrated statistically by main effects of genotype and OVX, but no significant interaction). However, HOMA-IR calculations did reveal a significant interaction between genotype and OVX, with UCP1KO/OVX animals having higher scores than all other groups. HOMA-IR is a proxy measure of insulin sensitivity, which correlates very strongly with direct measures of insulin tolerance (Antunes et al. 2016). We also observed UCP1 ablation-induced increases in ADIPO-IR scores, indicating increased adipose tissue insulin resistance. Our observations support previous findings, which illustrated a direct relationship between UCP1 expression and insulin sensitivity (Poher et al. 2015). Although studies have indicated that both UCP1 ablation (Winn et al. 2017b) and OVX (Nickelson et al. 2012, Pettersson et al. 2012, Vieira Potter et al. 2012) induce insulin resistance, this is the first time, to our knowledge, that synergistic effects of UCP1 ablation and OVX on insulin resistance have been demonstrated. These findings are important in light of the fact that aging reduces UCP1 in both sexes (Yamashita et al. 1999, Yoneshiro et al. 2013). Thus, age-related reductions in UCP1 may contribute to sex differences in age-related metabolic impairments, since women are affected by aging and ovarian hormone loss. It is also noteworthy that genetic mutations in genes that regulate UCP1 have been associated with longevity in humans (Yoneshiro et al. 2013); thus, genetic differences in UCP1 expression may make some women more or less susceptible to menopause-associated metabolic dysfunction.
Adipose tissue inflammation has been shown to induce insulin resistance (Gutierrez et al. 2009), and we previously demonstrated that adipose tissue inflammation occurs independent of adiposity increase and precedes insulin resistance following OVX in mice (Vieira Potter et al. 2012). However, our present findings do not indicate elevated adipose tissue inflammation caused the exacerbated impairment in OVX-induced insulin resistance in UCP1KO mice. It was somewhat surprising that both OVX and UCP1 ablation led to increases in circulating adiponectin. The increased adiponectin levels in the KO and OVX groups is counter-intuitive, since adiponectin is insulin sensitizing (Berg et al. 2001, Fruebis et al. 2001, Yamauchi et al. 2001). However, most studies looking at adiponectin as it relates to obesity (for example) have been conducted in male rodents. Indeed, females have a greater % body fat, better adipocyte insulin sensitivity, greater adiponectin levels and have been shown to increase adiponectin despite greater adiposity (Kern et al. 2003). Another study in rats (Garcia-Carrizo et al. 2017) showed adiponectin to decrease with obesity in males but not females, which associated with greater metabolic protection among the females. Importantly, the leptin-to-adiponectin ratio was not different between WT and KO mice and was increased with OVX in both genotypes. The lack of robust differences in insulin sensitivity among the groups studied here, all fed normal low-fat rodent chow diet, could be due to the compensatory role of adiponectin.

Mechanistically, it is unclear why the UCP1-null animals were more susceptible to OVX-induced insulin resistance. The mechanism does not appear to involve changes in adipose tissue E2 receptor expression, which has been associated with insulin resistance (Lizcano & Guzmán 2014). Nor do our data support that UCP1 protected mice from OVX-induced adipose tissue inflammation. A limitation to this study is that BAT lipid content was not quantified, although histological examination of BAT indicated subtle OVX-induced ‘whitening’, which was supported by increased leptin gene expression and larger interscapular and periaortlc BAT depot weights. However, those changes were not apparently exacerbated in UCP1KO animals. The UCP1KO did have reduced BAT mitochondrial protein content (e.g., electron transport chain proteins COX 1, 2, and 4) suggestive of BAT whitening, consistent with our previous studies (Winn et al. 2017a,b). UCP1 protein is vital for mitochondrial integrity and function, as ablation has been shown to reduce mitochondrial content and render mitochondria susceptible to reactive oxygen species-induced permeability (Kazak et al. 2017). Indeed, UCP1 activity reduces oxidative stress and improves mitochondrial function (Shabalina et al. 2006, Oelkrug et al. 2010, Stier et al. 2014). Consistently, BAT gene expression levels of Ddit3 and Fgf21, indicative of cellular stress, were significantly increased in UCP1KO mice. Although direct mitochondrial energetic assessments were not performed, our observations of reduced mitochondrial content may suggest that the UCP1KO animals had impaired mitochondrial function. Paradoxically, gene expression of the marker of mitochondrial biogenesis, Tfam was actually increased in UCP1KO. This may also be indicative of less functional mitochondria in adipose tissue of UCP1KO animals (Nadal-Casellas et al. 2011). Since OVX is known to increase oxidative stress, we hypothesized that UCP1KO animals would develop greater inflammation following OVX compared to WT. We did not find this to be true, but we did find that UCP1KO animals had increased susceptibility to OVX-induced suppression of Gpx3. Thus, it is probable that, under more adverse metabolic conditions (e.g., high-fat diet, aging), greater divergence in responsiveness to OVX between WT and KO animals would be observed.

It is critical that the findings presented here are appreciated with awareness of the import impact that environmental temperature may have had on the findings. That is, this study was conducted close to, or just below thermoneutrality. Previous studies have confirmed that the metabolic effects of UCP1 ablation differ significantly under different environmental temperature conditions (von Essen et al. 2017). Thus, an important future direction is to compare the results of the present study to those obtained when mice are housed at warmer (and colder) temperatures. Temperature-specific differences observed in how UCP1 and OVX interact may lend mechanistic insight into the relationship between ovarian hormones and UCP1.

In summary, it is apparent that ovarian hormones and UCP1 both play an integral role in the preservation of metabolic health in females. In this study, we have shown that loss of ovarian-derived hormones and UCP1 ablation cause synergistic detriment to glucose metabolism, resulting in more severe insulin resistance than either UCP1 ablation or OVX alone. Further studies are needed to examine the potential mechanistic relationships between E2 signaling and UCP1, and how their interactions may influence metabolic health. Nonetheless, our current findings suggest that UCP1 is an important candidate protein that may interact with E2 to mediate protective effects. Most important, these findings point to UCP1 as a potential therapeutic target for postmenopausal metabolic dysfunction.
Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0139.

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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Author contribution statement

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