Ectopic lipid deposition and the metabolic profile of skeletal muscle in ovariectomized mice


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We previously demonstrated that a decrease in circulating estrogens in ovariectomized female mice (OVX) results in an increased basal lipolytic rate of visceral fat, and subsequent increases in circulating nonesterified free fatty acids (NEFAs) (56). Under these conditions, increases in circulating NEFAs provoke an increase in demand for clearance of these moieties from circulation (2, 4). Skeletal muscle has the capacity to oxidize or store substantial amounts of NEFA and, therefore, serves as a critical disposal site for circulating NEFAs. In skeletal muscle, NEFAs that do not undergo oxidation are stored as triglycerides, creating a pool of intramyocellular lipid (IMCL), thus the fate of the NEFA is largely dependent upon skeletal muscle energetic demands (18, 37). Although IMCLs do not typically impair skeletal muscle metabolic function, excessive accumulation of IMCLs is associated with increased insulin resistance of skeletal muscle, due to increases in lipid intermediates and subsequent activation of specific inflammatory or stress signaling complexes (14). Our data suggest that under conditions of reduced estrogen function, skeletal muscle would be challenged by excess circulating NEFAs, which would potentially affect vital metabolic processes within the muscle cell.

In women, circulating estrogens appear to play a critical role in defining the capacity to utilize lipids as a source of energy. For example, a number of publications have found that premenopausal women oxidize more lipid than men in response to an acute bout of exercise (51). Furthermore, genetic ablation of the α-form of the estrogen receptor (ERKO) results in a significant reduction in the ability of mitochondria to oxidize lipid (46). These data support the theory that repetitive, but cyclic, estrogen exposure in women induces a metabolic profile that encourages lipid oxidation. Although largely untested, under conditions of reduced ovarian function, it would be predicted that skeletal muscle has increased IMCL content. Indeed, some investigations have suggested that reduced estrogen function leads to enhanced triacylglycerol (TAG) storage within whole muscle lysates (28, 46). However, it is unclear...
whether the increased TAG is due to increases in extramuscular or intramuscular TAG (i.e., IMCL) storage.

The goal of this study was to utilize an integrative metabolic approach to identify potential areas of metabolic dysfunction in skeletal muscle under conditions of reduced ovarian function. We employed multiple physiological approaches coupled with a nonbiased, comprehensive, metabolic profiling approach in skeletal muscle from aged match female mice with and without bilateral OVX to identify potential mechanisms. We hypothesized that a loss of ovarian function would result in a metabolic phenotype that would encourage increases in intracellular lipid storage in muscle. Currently, there is a poor understanding of changes induced by the removal of this critically important endocrine organ (i.e., ovary) in women. Therefore, it is important that we begin to address not only the effects of ovarian hormones on nonreproductive tissue but also consider the effects of absence of ovarian hormone exposure on tissue function. The OVX model is critically important for women’s health, in that a significant number of women undergo prophylactic removal of their ovaries (i.e., oophorectomy) for a variety of clinical reasons (37, 62). Because the ovary cyclically secretes numerous endocrine hormones in addition to estrogens, it is critical to recognize that the ovary may play a larger role in regulating peripheral tissue function beyond 17β-estradiol (i.e., the dominant form of estrogen in circulation). Further, current literature, including 17β-estradiol supplementation, provides 17β-estradiol at supraphysiological supplementation, provides 17β-estradiol at supraphysiological levels and does not mimic the cyclic nature of circulating 17β-estradiol. Some studies using 17β-estradiol supplementation also demonstrate that 17β-estradiol does not fully attenuate all consequences of the loss of functional ovaries (60). The experiments described here will hopefully provide critical direction for investigators examining the role of female sex steroids in the regulation of metabolic function.

METHODS

Animals. Prior to beginning this study, all aspects were approved by the University of Maryland Institutional Animal Care and Use Committee Review Board. Eight to ten-week-old virgin female C57BL6 mice were utilized in this study. The mice were divided into two groups: sham and OVX, where OVX mice underwent bilateral ovariectomy, and the sham group was anesthetized but ovaries were left intact. We have previously shown that OVX surgery results in an ~70% reduction in circulating estrogens within 48 h (51). Loss of circulating ovarian hormones due to ovariectomy was confirmed in OVX animals by a significant decrease in uterine weights compared with sham animals (Table 1). Utilizing uterine mass as indicator of reduced estrogen function is employed since most methods for quantifying murine derived estrogens are not considered accurate.

All mice were housed individually in a standard mouse cage in a temperature-controlled room with a 12:12-h light-dark cycle and were provided with ad libitum access to standard rodent chow (Purina Laboratory Rodent Diet 5001: 23% protein, 4.5% fat, and 6% fiber) and water. Unlike rats, mice do not become hyperphagic in response to ovariectomy and were, therefore, preferentially chosen as the animal model for this study (22, 34, 59). To confirm this finding, food intake was measured in a separate cohort of age-matched animals, as previously described (57). All tissue was collected 8–10 wk after the OVX surgery. All animals were age-matched, and the food was removed 4–5 h prior to the tissue collection, with the food removed at 0500 and tissue collected at ~1000. We have previously observed that increases in visceral adiposity and loss of regulatory control of lipolytic function occurs at 8 wk postsurgery (60).

**IMCL quantification.** Lipid droplets (LD) were visualized and quantified using BODIPY (493/503) (Invitrogen, Carlsbad, CA), as previously described by our group (52, 63).

**Muscle cross-sections.** The soleus and plantaris muscles from sham and OVX mice were mounted using Tissue-Tek O.C.T. Compound (Andwin Scientific, Schaumburg, IL) and sectioned transversely through the midbelly of the muscle. The cross sections were mounted on glass slides and remained at room temperature for 10 min prior to fixation in 10% paraformaldehyde for 5 min. Sections were then rinsed 3 times using 1× PBS solution and then were exposed to BODIPY 493/503 (1 μg/ml) in 1× PBS for 30 min. Sections were then rinsed 4 times for 10 min using 1× PBS to remove excessive BODIPY stain. Vectashield (Vector Laboratories, Burlingame, CA) containing DAPI was then placed on each section followed by cover slips. Sections were imaged using a Nikon Eclipse 50i (Nikon Instruments Melville, NY) at 20× and 40× magnification. Pictures were taken with a Photometrics CoolSnap camera (Tucson, AZ) and processed using Image Pro-Express 6.3 software. Images were quantified using Image J (developed by National Institutes of Health) using the same criteria previously described for Oil Red O procedures (12). A total of 50 fibers were quantified per muscle section.

**Single muscle fibers.** Intact single skeletal muscle fibers were enzymatically isolated from the flexor digitorium brevis (FDB) muscle from sham and OVX animals. In brief, surgically excised FDB muscles were incubated in dissociation media (DM) containing DMEM (Invitrogen), gentamycin (50 μg/ml), FBS (2%, no. 30–2020; American Type Culture Collection, Rockville, MD), and collagenase A (4 mg/ml; Roche, Indianapolis, IN) in an incubator (37°C, 5% CO2) for 1.5–2 h. Following the dissociation, muscles were placed in a new 35-mm plate with warmed media containing gentamycin and FBS but without collagenase. FDB muscles were triturated with a small bore (~1 mm) fire-polished glass transfer pipette to yield single FDB myofibers. Following trituration, large debris (nerve, undigested FDB muscle) was removed with forceps. The single fibers were then placed onto an extracellular matrix (ECM)-coated glass bottom plate (MatTek, Ashland, MA), according to previously described techniques (14). Fibers were allowed to adhere, rinsed with Ringer buffer to remove all media, and stained for 30 min with BODIPY 493/503 and 4,6-diamidino-2-phenylindole (DAPI) to label myonuclei (Invitrogen, Cambridge, MA) (14). After 30 min, the dyes were removed by rinsing fibers 3× with fresh Ringer buffer. Fibers were imaged using a Zeiss AxioObserver Z1 fluorescent microscope (Carl Zeiss Micro-Imaging, Jena, Germany).

**Immunoblot procedures.** Plantaris muscles were used to determine the protein content of CD36/FAT and FATPpm in the sham and OVX mice, according to previously described methods (3). The soleus muscle was not used because we were unable to extract a sufficient amount of total protein to reliably measure the CD36/FAT or FATPpm. Muscles were homogenized, proteins separated by SDS-PAGE, and the transport.

### Table 1. Anatomical characteristics of sham and OVX animals

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>BM, g</th>
<th>SE</th>
<th>VF, g</th>
<th>SE</th>
<th>IF, g</th>
<th>SE</th>
<th>Uterus, g</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>4 mo</td>
<td>23.46</td>
<td>0.526</td>
<td>0.262</td>
<td>0.03</td>
<td>0.175</td>
<td>0.009</td>
<td>0.070</td>
<td>0.009</td>
</tr>
<tr>
<td>OVX</td>
<td>4 mo</td>
<td>28.37</td>
<td>0.998</td>
<td>1.296</td>
<td>0.18</td>
<td>0.575</td>
<td>0.134</td>
<td>0.019*</td>
<td>0.005</td>
</tr>
</tbody>
</table>

BM, body mass; VF, visceral fat; IF, inguinal fat. *Statistically significant difference from sham, P < 0.05.
ers were detected through immunoblotting using antibodies specific for CD36/FAT or FABPpm, as previously described (3). Equal quantities of total protein were loaded (20 μg) on each gel, and Ponceau S staining on the membranes was used to confirm equal loading.

Mitochondria isolation and immunoblot procedure. Mitochondria were isolated from gastrocnemius muscles from sham and OVX muscles, as previously described (10). Isolated mitochondria were used to determine the protein content of mitochondrial enzymes very long-chain, long-chain, and medium-chain acyl CoA dehydrogenases (VLCAD, LCAD, and MCAD) and mitochondrial complexes I-V, as previously described (23, 26, 31, 45). Immunoblotting was conducted using antibodies specific for VLCAD, LCAD, and MCAD (kindly provided by Dr. Jerry Vockley, University of Pittsburgh), and aspects of mitochondrial complexes I-V were detected using an antibody cocktail (AbCam, Cambridge, MA).

**Metabolic profiling.** All procedures were performed as previously described (25). Briefly, the whole gastrocnemius muscle was isolated from sham and OVX animals and frozen in liquid nitrogen. The tissue was ground in a liquid nitrogen-chilled mortar and pestle, and ~25 mg was suspended in water, homogenized on ice, sonicated, and then spun for 15 min at 4°C, 14,000 rpm. Data are normalized to the total protein content in each sample, as determined by BCA protein assay (Pierce Thermo Fisher Scientific, Austin, TX). Measurement of free carnitine, acylcarnitines, and amino acids in muscle was completed by direct-injection electrospray tandem mass spectrometry (MS/MS), using a Micromass Quattro Micro LC-MS system (Waters-Micromass, Milford, MA) equipped with a model HTS-PAL 2777 autosampler (Leap Technologies, Carrboro, NC), a model 1525 HPLC solvent delivery system (Agilent Technologies, Palo Alto, CA), and a data system running MassLynx 4.0 software (Waters, Milford, MA) at the Sarah W. Steedman Nutrition and Metabolism Center Mass Spectrometry Laboratory. Organic acids in muscle were quantified using methods described previously employing Trace Ultra GC coupled to a Trace DSQ MS operating under Eccalibur 1.4 (Thermo Fisher Scientific, Austin, TX).

**Skeletal muscle malonyl CoA measures.** Malonyl CoA measures were performed on the skeletal muscle from sedentary sham and OVX mice, as previously described (36). Single muscle fiber-based microplate respirometry. Bioenergetic analyses of isolated FDB muscle fibers were performed using an XF24-3 Extracellular Flux Analyzer (Seahorse Bioscience), as previously described by our group with slight modifications (50). After euthanasia, both FDB muscles were removed from OVX mice and SHAM animals (n = 3/group). Individual fibers were isolated and plated on ECM (ECM; EW1270; Sigma, St. Louis, MO)-coated V7 microplate (Seahorse Bioscience, Billerica, MA) overnight, according to our previously described methods (50). After calibration of the XF24-3 Extracellular Flux Analyzer, the microplate containing the sham and OVX single muscle fibers was placed in the analyzer. Basal oxygen consumption rate (OCR; pmol/min) were initially quantified across both groups in assay measurement buffer (MB) at ~37°C contained 120 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl2, 0.4 mM KH2PO4, 1 mM MgCl2, 5 mM HEPES (pH 7.4) supplemented with 2.5 mM d-glucose (Sigma G7528) and 0.5 mM t-carnitine (Sigma C0158). Mitochondrial respiration was induced with either albumin (03117405001; Roche, Indianapolis, IN) conjugated sodium palmitate (CO158). Mitochondrial respiration was induced with either albumin (03117405001; Roche, Indianapolis, IN) conjugated sodium palmitate (CO158). Mitochondrial respiration was induced with either albumin (03117405001; Roche, Indianapolis, IN) conjugated sodium palmitate (CO158). Mitochondrial respiration was induced with either albumin (03117405001; Roche, Indianapolis, IN) conjugated sodium palmitate (CO158). Mitochondrial respiration was induced with either albumin (03117405001; Roche, Indianapolis, IN) conjugated sodium palmitate (CO158). 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Radiation that reflect shifts in substrate availability and/or flux limitations at specific catabolic enzymes.

Acylcarnitine intermediate profile. Acylcarnitine species levels were quantified in gastrocnemius muscles from the sham and OVX groups to assess specific steps of β-oxidation. This approach provides the investigator a snapshot of substrate flux and can provide the investigator specific experimental direction for identifying potential metabolic limitations. For example, high

Fig. 1. IMCLs quantified using direct fluorescent visualization were significantly elevated in ovariectomized (OVX) female mice in both the plantaris (A) and soleus (B) muscle groups compared with age-matched sham surgery (sham) female mice. n = 3 animals/group and 50 fibers/muscle quantified. *Significant difference from Sham (P < 0.05).

Fig. 2. Representative images of IMCL through BODIPY (493/503) staining of neutral lipid droplets and nuclei (DAPI-blue) within skeletal muscle cross sections of soleus and plantaris muscle (A) and single muscle fibers from sham and OVX age-matched female mice (B).
4.

Mitochondrial oxygen consumption. To determine whether any of these alterations in metabolite levels was associated with compromised mitochondrial function, we assessed mitochondrial oxygen consumption induced by either palmitate or pyruvate in intact single FDB muscle fibers isolated from sham
levels of LC might suggest that movement of fatty acids via mitochondrial carnitine palmitoyltransferase I (CPT-1) exceeds flux through β-oxidation enzymes, such as long-chain (LC) acyl-CoA dehydrogenase and/or β-OH-acyl-CoA dehydrogenase (56).

Odd-chain acyl-carnitine species. Odd-chain acyl-carnitine species propionylcarnitine (C3), isovaleryl carnitine (C5), and tiglyl carnitine (C5:1) are products of BCAA catabolism (32, 38). Because of the observed decline in skeletal muscle amino acids in OVX mice, we measured C3, C5, and C5:1 species in sham and OVX mice. We detected a reduction in C3 species in OVX mice compared with sham (P < 0.05) and no difference in C5 species (Fig. 8). However, we observed a reduction in the unsaturated C5:1 acyl-carnitine species (P < 0.05) (Fig. 8). No significant differences in hydroxylated odd-chain acyl carnitine species ratio C5-OH/C3-OH were detected. Collectively, these data indicate a decline in catabolism of BCAs and likely a reduced pool of available amino acids to the muscle in the OVX condition (38).

Amino acid profile. It is well established that amino acids play an integral role in skeletal muscle metabolism by serving as substrates for catabolic and anabolic processes that directly contribute to overall function of the muscle. In addition, previous work has shown that the branched-chain amino acid (BCAA) profile is a potential contributor to insulin resistance in the obese state (41). Therefore, we evaluated the skeletal muscle amino acid profile in the OVX mice compared with sham mice (Fig. 7, A–D). In OVX mice, we identified significant decreases in the BCAAs, leucine and isoleucine; however, there was no significant difference in valine compared with sham mice (P < 0.05) (Fig. 7, B and C). We also found significant decreases in the concentrations of alanine, glutamine/glutamic acid, proline, serine, and histidine in OVX mice compared with SHAM (Fig. 7, A–C).

Glycolytic and TCA cycle metabolites. Previous data in other models of obesity have shown that accumulation of LC-acylcarnitines species was associated with a significant reduction in Krebs cycle intermediates, reflecting a form of mitochondrial dysfunction. We found no differences in either pyruvate or lactate levels between OVX and sham animals (Fig. 6A). However, citrate and succinate levels were significantly (P < 0.05) higher in skeletal muscle from OVX compared with sham (Fig. 6B and D). Finally, no significant differences were detected in TCA cycle intermediates, α-ketoglutarate, fumarate, and malate (Fig. 6, C and E).

Serum metabolites. Changes in serum metabolites were assayed for evidence of dysregulation of both lipid and carbohydrate metabolism. No differences between OVX and sham animals were detected for either triglycerides or glucose levels. However, the acetone:total ketone body ratio was significantly higher in OVX compared with sham (0.881 ± 0.0822 vs. 0.0822 ± 0.05) (Fig. 8). There were no differences detected in alanine, glutamine, pyruvate, alanine, or oxaloacetate levels between sedentary sham and OVX animals (Fig. 8).

Skeletal muscle malonyl CoA and CPT-1 content. The reduced levels of acetyl-carnitine coupled with the reduced free carnitine content might suggest an impaired flux through β-oxidation as a result of reduced fatty acid transport through CPT-1. We measured CPT-1 mRNA levels and detected no differences between sham and OVX animals (CPT-1/18S: sham = 0.881 ± 0.03 vs. OVX = 0.0822 ± 0.02). CPT-1 activity is negatively regulated by allosteric interactions with malonyl CoA; therefore, the observed reductions in acylcarnitines in the OVX animals could be the result of increased malonyl CoA content (5, 44, 48). However, no differences were detected in skeletal muscle malonyl CoA content between sedentary sham and OVX animals (Fig. 5).

Collectively, these data imply that reductions in flux through β-oxidation could contribute to a decline in skeletal muscle lipid catabolism under conditions of reduced estrogenic function.

Skeletal muscle malonyl CoA and CPT-1 content. The reduced levels of acetyl-carnitine coupled with the reduced free carnitine levels might suggest an impaired flux through β-oxidation as a result of reduced fatty acid transport through CPT-1. We measured CPT-1 mRNA levels and detected no differences between sham and OVX animals (CPT-1/18S: sham = 0.881 ± 0.03 vs. OVX = 0.0822 ± 0.02). CPT-1 activity is negatively regulated by allosteric interactions with malonyl CoA; therefore, the observed reductions in acylcarnitines in the OVX animals could be the result of increased malonyl CoA content (5, 44, 48). However, no differences were detected in skeletal muscle malonyl CoA content between sedentary sham and OVX animals (Fig. 5).

On the basis of previous results in other models of obesity and because of the accumulation of IMCL in the OVX muscles, we hypothesized that LC in the muscle from OVX muscles would be elevated compared with the sham animals (25, 53, 56). Surprisingly, we found total LC species to be reduced in the OVX group compared with the sham group (Fig. 4A). No differences in medium-chain acylcarnitines were identified (Fig. 4B), but we did detect significantly lower levels of short-chain acylcarnitines (SC) in the OVX group compared with the sham group (Fig. 4C). These data suggest a reduced flux through the β-oxidation pathway in the muscle from the OVX group compared with the sham group. Coupled with the reduction in acylcarnitines, we determined that skeletal muscle free carnitine levels were significantly lower (P < 0.05) in OVX mice compared with sham mice (Fig. 4D). A number of previous publications have found that a reduction in free carnitine levels correlates with decreases in skeletal muscle lipid metabolism (24, 42, 58).
and OVX mice. The advantage to this approach is that we can assess mitochondrial function with no disruption to the integrity of the muscle fiber or the organelle, thereby allowing measures to be taken in the most native form of the mitochondria. Basal OCR was significantly lower in fibers isolated from the OVX animals compared with the sham animals (Fig. 9A). To determine the response to substrate exposure, we normalized the OCR to the baseline OCR values, since the basal OCR measures were different between the sham and OVX prior to substrate exposure. Palmitate exposure stimulated significant increases in both normalized OCR from baseline in sham and OVX fibers, with no significant differences between groups (Fig. 9B). Pyruvate stimulated normalized OCR was increased from baseline in both OVX and sham; however, there was no significant difference between the OVX and sham groups (Fig. 9C). To assess substrate-specific maximal electron transport activity (ETC) activity, we added the uncoupling agent, FCCP and found significant increases in normalized OCR in the OVX and sham compared with baseline OCR with both palmitate and pyruvate substrates (Fig. 9, B and C). However, regardless of substrate, normalized FCCP-stimulated OCRs were significantly lower in the fibers from the OVX group compared with the Sham group, indicating a potential impairment in electron transport chain function (Fig. 9, B and C). Calculation of the spare respiratory capacity (SRC), as previously described by our group (50), suggests a reduced ability of mitochondria from the OVX fibers compared with sham fibers to respond to stimuli that would activate mitochondria (Fig. 9, D and E).

Mitochondrial enzyme content. To determine whether our observations of decreased long-chain acylcarnitines and diminished FCCP stimulated respiration were due to differences in mitochondrial enzyme content, we measured mitochondrial enzyme proteins in skeletal muscle from sham and OVX mice. No differences in VLCAD, LCAD, and MCAD protein content or ETC mitochondrial protein content were observed between sham and OVX animals (Fig. 10).

DISCUSSION

Reductions in circulating estrogens in women, due to either the onset of age-induced menopause or menopause as a result of ovariectomy, correlate with a robust increase in visceral fat mass (15, 18, 29). Our data demonstrate that compared with the sham group, OVX mice exhibit increased adiposity that is coupled with significant accumulation of IMCL, CD36/FAT, and FABPpm protein content within the skeletal muscle. To identify potential metabolic mechanisms, we employed a metabolic profiling approach in the skeletal muscle from these animals. When comparing data from OVX and sham animals, the results suggest that reduced substrate flux through β-oxidation in the OVX animals may contribute to increased IMCL content. Finally, we determined that there are specific deficits in basal and maximal stimulated mitochondrial oxygen consumption in single muscle fibers from OVX animals compared with sham animals that are not explained by reductions in mitochondrial protein content. Collectively, the data indicate that loss of ovarian function leads to visceral adiposity and IMCL accumulation, and our experiments document the metabolic profile of skeletal muscle under this condition.

Consistent with other studies, we found that ovariectomy resulted in significant increases in both visceral and subcutaneous fat mass compared with the sham mice (14, 23). We previously reported that increases in visceral fat mass in the OVX model are associated with an enhanced lipolytic rate, yielding an increase in circulating NEFAs (60) that suggests skeletal muscle in the OVX animals is exposed to higher levels
of NEFA. On the basis of experiments in cultured skeletal muscle cells, exposure of the cells to exogenous NEFA results in increased IMCL content (27). OVX mice exhibited significantly more IMCL than sham animals, which was associated with markedly higher protein content of two key fatty acid sarcolemmal transporters FABPpm and FAT/CD36. Although other models of obesity have demonstrated similar findings (4, 21), to our knowledge, this is the first time these increases have been documented in the OVX model. The observed increase in FAT/CD36 and FABPpm in the OVX group is likely the result of an enhanced demand for clearance of NEFAs from circulation and corresponds with an increase in IMCL deposition into skeletal muscle.

Because of the metabolic complexity of skeletal muscle, we employed a targeted metabolic profiling approach to generate a comprehensive nonbiased analysis of skeletal muscle in the Sham and OVX groups (25). Since the OVX group exhibited significant increases in adiposity coupled with high IMCL, we hypothesized that, in a similar fashion to other obesity models (29, 33), we would find substantial increases in LC in the OVX group compared with the Sham group. Surprisingly, we found relative decreases in the LC levels in the OVX, which could suggest impaired transport of LCFA into the mitochondria. The enzyme CPT-1 combines cytosolic free carnitine with long-chain fatty acids, producing an acyl-carnitine that is then transported into the mitochondria (6). CPT-1 activity is reduced through allosteric inhibition by malonyl CoA and a lack of free carnitine (20, 24, 58). Therefore, because we detected no differences in malonyl CoA levels or CPT-1 mRNA levels but did detect a reduction in free carnitine levels in the OVX group, we suspect that transport of LCFA into the mitochondria via CPT-1 is a potential point of limitation for lipid oxidation under conditions of reduced ovarian function. In agreement with this conclusion, others have shown in the OVX rat model that CPT-1 activity in skeletal muscle is significantly lower than in sham controls (7).

The observed decline in SC in response to ovariectomy might suggest reduced flux of fatty acids through β-oxidation. This theory is further supported by evidence in humans that suggests numerous points in the β-oxidation and other lipid
metabolism pathways are sensitive to fluctuations in estrogens (11, 30, 55). However, we found no differences in the mitochondrial protein content of VLCAD, LCAD, or MCAD in the muscle from the sham and OVX animals. On the basis of our data, it seems critical to measure SCAD as well; however, we were unable to obtain an antibody specific to murine tissue. Collectively, these data indicate that under conditions of reduced estrogen function, there are critical changes that may be affecting flux through β-oxidation and contributing to alterations in lipid metabolism.

Upon entry into the cell, NEFAs are routed either toward reesterification and subsequently synthesized into IMCL, or if energetic demand is elevated, shuttled into the mitochondria for oxidation (21). Our data demonstrate that skeletal muscle IMCL is robustly increased in both the plantaris and soleus muscles in the OVX group, suggesting NEFA flux into the muscle is elevated. To determine whether alterations in mitochondrial function are contributing to the increased IMCL, we measured mitochondrial oxygen consumption (OCR) of intact single muscle fibers from sham and OVX animals. Under basal conditions (i.e., low glucose), mitochondrial OCR was significantly reduced in the muscle fibers from the OVX fibers compared with sham fibers. However, when we added substrate (i.e., Pyr or PA), the OCR increased by the same percent in both groups. These findings suggest that under conditions of low energetic demand, skeletal muscle mitochondria in the OVX animals respond in a similar fashion to sham animals to increased substrate delivery. When stimulated with FCCP to induce mitochondrial uncoupling, the fibers from the OVX animals demonstrated a reduced ability to respond to increased oxygen consumption compared with the sham animals. Further, the lower SRC capacity of the fibers from OVX animals suggests that mitochondria have a reduced ability to respond to a maximal stimulus that would enhance oxidation of metabolic substrates, such as lipid or glucose. However, since mitochondria rarely operate in these maximal ranges, it seems unlikely that reduced SRC capacity is a limiting factor that explains the IMCL accumulation in the OVX model. Because the fibers from the OVX animals were able to increase their OCR to the same magnitude as the fibers from the sham animals in response to the PA exposure, it also seems unlikely that the mitochondria are the limiting factor to explain the IMCL buildup. We also detected no differences in the protein content of various mitochondrial proteins, making it unlikely that a loss of mitochondrial content is a contributing factor. Using FCCP to drive mitochondrial oxygen consumption is a common experimental procedure; however, it is unclear whether lower

Fig. 7. OVX animals exhibited significantly lower levels of various amino acid species in the gastrocnemius muscle compared with age-matched sham animals. A: OVX mice had significantly lower levels of alanine and no difference in glycine levels compared with sham animals. B: OVX mice had significantly lower levels of serine, leucine/isoleucine, and glutamate in skeletal muscle compared with sham animals. C: OVX mice had significantly lower levels of proline and histidine, and no differences in valine, aspartate, or arginine levels compared with sham animals. D: no differences were detected methionine, phenylalanine, tyrosine, ornithine, or citrulline in the skeletal muscle between the OVX and sham groups. Amino acid abbreviations are as follows: alanine (Ala), serine (Ser), leucine/isoleucine (Leu/Ile), glutamate (Glu), proline (Pro), histidine (His), glycine (Gly), valine (Val), aspartate (Asx), arginine (Arg), methionine (Met), phenylalanine (Phe), tyrosine (Tyr), ornithine (Orn), and Citrulline (Cit). *Significantly different from the sham group (P < 0.05); n = 6 animals/group.

Fig. 8. Significant decreases in odd-chain acylcarnitine species propionylcarnitine (C3) and unsaturated isovalerylcaritnine (C5:1) in the whole gastrocnemius muscle from the OVX compared with age-matched sham mice. No significant differences were detected in isovalerylcaritnine (C5) acylcarnitine species or in the hydroxylated species ratio C5-OH/C3-OH. *Significantly different from sham group (P < 0.05); n = 6 animals/group.
FCCP-driven OCR rates would translate to a measurable physiological deficiency (i.e., reduced exercise capacity) in skeletal muscle of the OVX mice. For example, we have found little evidence indicating enhanced in vitro or in situ fatigue development in skeletal muscle from OVX animals (61); however, it is well documented that OVX animals often exhibit poor exercise performance. Specifically, we and others previously reported a decline in voluntary wheel running in OVX animals (14, 23). In agreement with our findings, Rogers et al. (47) found a decline in nocturnal ambulatory activity levels in OVX mice, as well as decreased oxygen consumption compared with sham. Therefore, the enhanced deposition of IMCL in skeletal muscle is not solely a response of reduced mitochondrial function in the OVX fibers. Considering the number of in vitro and in vivo studies showing that estrogens influence mitochondrial biogenesis and function in tissues other than skeletal muscle (33, 54, 64–66), it remains plausible that optimal mitochondrial function in skeletal muscle is influenced by estrogen function; however, it is likely that other unknown factors are contributing to the increased IMCL.

Skeletal muscle amino acid metabolism is not well characterized with regard to estrogenic influences. Recent evidence in other obesity models has suggested that elevated concentrations of BCAA are a significant contributor to the development of insulin resistance (41). Surprisingly, we found that concentrations of the

\[\text{Fig. 9. Basal and stimulated oxygen consumption rates (OCR) of cultured single skeletal muscle fibers (isolated from the flexor digitorum brevis) from OVX and age-matched sham mice. A: basal OCR was significantly lower in OVX compared with sham animals. *Significantly different from sham animals (P < 0.05). B, C: maximal OCR rates induced by FCCP (calculated as percent change in OCR from baseline) in the presence of either palmitate (PA, 50 µM) or pyruvate (Pyr; 10 mM) were significantly reduced in skeletal muscle fibers from OVX compared with sham animals. However, no differences were detected in OCR responses to PA stimulation or Pyr stimulation alone. #Significantly different from sham basal (P < 0.05). $Significantly different from OVX basal (P < 0.05), *Significantly different from sham FCCP (P < 0.05). D, E: in response to PA or Pyr stimulation, spare respiratory capacity (absolute OCR_{FCCP}/absolute OCR_{basal}) was significantly reduced in OVX single muscle fibers compared with age-matched sham single-muscle fibers. *Significantly different from the sham group (P < 0.05); n = 5 animals/group.}\]

\[\text{Fig. 10. Mitochondrial enzyme content did not differ in the whole gastrocnemius muscle between sham and OVX mice. A: mitochondrial enzymes responsible for the initial steps of \(\beta\)-oxidation of fatty acids VLCAD, LCAD, and MCAD did not differ between sham and OVX animals. B: mitochondrial respiratory chain complexes I-V did not differ between sham and OVX animals. n = 5 animals/group.}\]
majority of amino acids were significantly reduced in the OVX group compared with the sham groups, with even the nonsignificant differences tending to decrease in the OVX group. The decreases in amino acid levels may suggest enhanced metabolic amino acid catabolism or a loss of the available amino acid pool. The former is a perplexing finding, since we detected decreases in odd-chain acetylcarnitine species, and our previous research has not identified losses in skeletal muscle mass of the OVX animals compared with the sham animals (60). However, an accelerated decline in skeletal muscle mass has been observed in postmenopausal women (1), which may suggest that if OVX animals were allowed to remain in the reduced estrogen condition longer, reductions in muscle mass would become apparent. Our data may also suggest there is a reduction in the amino acid pool within the muscle of the OVX animals, which leads to a reduction in the availability of amino acids for protein translation. This finding may explain previous results in which we and others have shown that OVX animals have reduced mTOR signaling response coupled with lower muscle growth with loading of the skeletal muscle compared with sham animals (35, 51). However, this hypothesis would need to be further tested.

Potential limitations. It should be noted that using the metabolic profiling approach does not elucidate the mechanism that may be disrupted in the experimental model, but it does provide direction and insight into potential mechanisms that would require further in-depth studies. The data collected in these studies resulted in a number of unexpected findings that we are currently following up on. We chose to employ the OVX model as a means to define metabolic function under conditions of reduced female sex steroid concentrations. Although it is likely that decreases in estrogens are contributing to a number of these effects, we cannot rule out the possibility that other ovarian hormones are playing a critical role in defining these phenotypes. Since estrogens affect multiple tissues, it is difficult to assess which effects are a direct result of reduced estrogen levels and which are secondary effects due to changes in other nonskeletal muscle tissues. It is critical to develop experimental models to specifically isolate estrogen signaling in skeletal muscle to determine the primary role for estrogens in skeletal muscle metabolism.

Overall, utilization of the metabolic profiling approach in the OVX model demonstrates that metabolic disturbances in the OVX animal do not recapitulate the same metabolic profile observed in other rodent models of obesity. Two frequently observed results include an accumulation of LC (25) and an increase in skeletal muscle BCAA levels in both human and animal models of obesity (19). However, the OVX mice do not exhibit this metabolic profile, suggesting that more studies are needed to critically examine the mechanistic role of female sex steroids in the regulation of peripheral metabolism. In summary, our data suggest that reduced ovarian function results in an increase in IMCL content that is not entirely explained by a reduction in the capacity of the mitochondria to utilize fatty acids. Because of the critical relationship between insulin signaling and IMCL content, it will be important to examine alternative explanations for the accumulation of lipid in the skeletal muscle of the OVX animals.

Perspectives and Significance

The results from this study provide a critical base of knowledge in an understudied area of women’s health, and we hope the data will provide critical direction for additional mechanistic studies to be conducted. The OVX model is the most frequently used murine model to study menopause in women; however, it is likely most representative of women undergoing surgical-induced menopause prior to the onset of age-induced menopause. Thus, the resulting data are also likely most relevant to women experiencing premature ovarian failure or being treated for estrogen-positive cancers. Previous publications have shown that the surgical removal of ovaries or the onset of ovarian failure in women leads to adiposity, particularly in the visceral region, which corresponds with glucose intolerance and insulin resistance (8). In addition, there are indications of altered responses to muscle loading and reduced force output by the muscle (9, 16). We and others have observed many of the same effects in the OVX model, indicating the importance of the OVX model for issues relevant to women’s health (23, 39, 47, 51). The novel aspect of our study is that we now demonstrate a more comprehensive metabolic phenotype of skeletal muscle in the OVX model, which will help to elucidate the underlying mechanisms responsible for changes induced under conditions of estrogen signaling disruption. In conclusion, the data demonstrate the importance of considering each model of obesity independently and highlight the need for more research to understand the role of female sex steroids in the regulation of metabolic function. Specific metabolic profiles likely exist across multiple models of metabolic disease, and each model should be evaluated individually when assessing the mechanisms behind the development of metabolic dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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LOSS OF OVARY FUNCTION INFLUENCES MUSCLE METABOLISM


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