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

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Abstract

Disruptions of ovarian function in women are associated with increased risk of metabolic disease due to dysregulation of peripheral glucose homeostasis in skeletal muscle. Our previous evidence suggests that alterations in skeletal muscle lipid metabolism coupled with altered mitochondrial function may also develop. The objective of this study was to use an integrative metabolic approach to identify potential areas of dysfunction that develop in skeletal muscle from ovariectomized (OVX) female mice compared to age-matched ovary intact adult female mice (SHAM). The OVX mice exhibited significant increases in body weight, visceral and inguinal fat mass compared to SHAM mice. OVX mice also had significant increases in skeletal muscle intramyocellular lipids (IMCL) compared to the SHAM animals, which corresponded to significant increases in the protein content of the fatty acid transporters CD36/FAT and FABPpm. A targeted metabolic profiling approach identified significantly lower levels of specific acyl carnitine species and various amino acids in skeletal muscle from OVX mice compared to the SHAM animals, suggesting a potential dysfunction in lipid and amino acid metabolism, respectively. Basal and maximal mitochondrial oxygen consumption rates were significantly impaired in skeletal muscle fibers from OVX mice compared to SHAM animals. Collectively, these data indicate that loss of ovarian function results in increased IMCL storage that is coupled with alterations in mitochondrial function and changes in the skeletal muscle metabolic profile.
It is well established that loss of ovarian function in women is associated with an increase in fat mass, primarily in the region of the visceral organs, without large changes in overall weight gain (13, 15, 26). This effect extends to female animal models of reduced ovarian function (12, 20, 43). Unlike diet-induced obesity, the accumulation of visceral fat mass after loss of ovarian function is not entirely due to alterations in activity level or eating behaviors, and can occur independent of age (15). The accumulation of visceral fat mass is strongly associated with multiple deleterious metabolic conditions, including peripheral insulin resistance, cardiovascular disease, and the metabolic syndrome in various models of menopause (7, 11, 40, 45).

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 non-esterified 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 oxidization are stored as triglyceride 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). Further, genetic ablation of the alpha 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 if 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 non-biased, 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 non-reproductive 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). Since 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 supra-physiological 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 (IACUC) Review Board. Eight-ten week old virgin female C57/BL6 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 anaesthetized but ovaries were left intact. We have previously shown that OVX surgery results in an approximate ~70% reduction in circulating estrogens within 48 hours (51). Loss of circulating ovarian hormones due to ovariectomy was confirmed in OVX animals by a significant decrease in uterine weights compared to 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 hour light/dark cycle and were provided with ad libitum access to standard rodent chow (Purina Laboratory Rodent Diet 5001: 23% protein, 4.5% fat, 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.
All tissue was collected 8-10 weeks after the OVX surgery. All animals were age-matched and the food was removed 4-5 hrs prior to the tissue collection, with the food removed at 05:00 and tissue collected at ~10:00. We have previously observed that increases in visceral adiposity and loss of regulatory control of lipolytic function occurs at eight weeks post surgery (60).

Intramyocellular lipid (IMCL) quantification: Lipid droplets (LD) were visualized and quantified using BODIPY (493/503) (Invitrogen, 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 mid-belly of the muscle. The cross-sections were mounted on glass slides and remained at room temperature for 10 mins prior to fixation in 10% paraformaldehyde for 5 mins. Sections were then rinsed 3 times using 1x PBS solution and were then exposed to BODIPY 493/503 (1 μg/mL) in 1xPBS for 30 mins. Sections were then rinsed 4 times for 10 mins using 1X PBS to remove excessive BODIPY stain. Vectashield (Vector Labs, Burlingame, CA) containing DAPI was then placed on each section followed by cover slips. Sections were imaged using a Nikon Eclipse 50i (Nikon Instruments Inc. Melville, NY) at 20x and 40x 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%, ATCC, #30-2020 Rockville, MD), and collagenase A (4 mg/ml, Roche, Indianapolis, IN,) in an incubator (37°C, 5% CO2) for 1.5-2 hours. 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 (~ 1mm) fire polished
glass transfer pipette to yield single FDB myofibers. Following trituration, large debris (nerve,
un-digested FDB muscle) was removed with forceps. The single fibers were then placed onto an
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 mins with BODIPY 493/503 and 4,6-diamidino-2-phenylindole (DAPI) to
label myonuclei (Invitrogen, Cambridge, MA) (14). After 30 minutes, the dyes were removed by
rinsing fibers 3X with fresh Ringer buffer. Fibers were imaged using a Zeiss AxioObserver Z1
fluorescent microscope (Carl Zeiss MicroImaging, 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 transporters were detected through
immunoblotting using antibodies specific for CD36/FAT or FATPpm 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, 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, USA). 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, USA) equipped with a model HTS-PAL 2777 auto sampler (Leap Technologies, Carrboro, NC, USA), a model 1525 HPLC solvent delivery system (Agilent Technologies, Palo Alto, CA, USA) and a data system running MassLynx 4.0 software (Waters Corporation, Milford, MA) at the Sarah W. Stedman Nutrition and Metabolism Center Mass Spectrometry Lab. Organic acids in muscle were quantified using methods described previously employing Trace Ultra GC coupled to a Trace DSQ MS operating under Excalibur 1.4 (Thermo Fisher Scientific, Austin, TX, USA).

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 flexor digitorum brevis (FDB) muscles were removed from OVX mice and SHAM animals (n = 3/group). Individual fibers were isolated and plated on extracellular matrix (ECM; Sigma EW1270, 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, pmoles/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 L-carnitine (Sigma CO158). Mitochondrial respiration was induced with either albumin (Roche, Indianapolis, IN, 03117405001) conjugated sodium palmitate (Sigma P9767; 50μM) or sodium pyruvate (10 mM, Sigma P8574) and OCR was measured. A second identical treatment of substrate was initiated after 20 mins and OCR was again recorded. Following the last OCR measure induced by the second exposure of substrate, 400nM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma C2920) was injected to induce maximal mitochondrial oxygen consumption and OCR was measured. At the conclusion of the last FCCP measure Antimycin A (Sigma A8674; 1 μM), a known inhibitor of mitochondrial complex III, was injected to assess non-mitochondrial OCR measures. OCR measures presented are the average values detected after the OCR reaches a steady state following the introduction of the substrate or FCCP. Basal OCR values presented are taken immediately prior to the first injection of either pyruvate or palmitate. The mean derived for each group was determined by collecting the average OCR values from 7-10 different wells per muscle from each animal. This results in approximately 25-30 independent measures per group as we previously described (28). The single fibers were seeded so that each well is ~60% confluent.

Statistics: All data are expressed as means ± SE. Statistical significance was determined using a t-test for all measures. A P value of ≤ 0.05 was considered significant.

**Results**

Anatomical Characteristics: OVX mice have significantly greater body mass compared to age-matched SHAM animals (Table 1). The OVX mice also exhibited significant increases in visceral (omentum/mesenteric) and subcutaneous (inguinal) fat mass compared to the SHAM mice (Table 1). As expected, uterine mass was significantly decreased in OVX mice compared to
SHAM mice indicating a successful reduction in circulating estrogens (Table 1). OVX mice also ate significantly less food per day over the 8 weeks, even though they continued to gain body mass (Table 2). When comparing feed efficiency as calculated body weight per kcal consumed, the OVX animals had increased ability to store energy as body weight (Table 2).

**IMCL Accumulation:** Based on our previous studies demonstrating that OVX animals exhibit significant increases in basal lipolytic rates of visceral fat and elevations in circulating NEFA, we sought to determine if OVX mice have increased IMCL in their muscle compared to SHAM animals. IMCL levels were significantly elevated in both the soleus and plantaris muscles from the OVX mice compared to SHAM mice (Figures 1A-B and 2A). In addition, we observed IMCL within the single muscle fibers to confirm that the IMCL elevations in the muscle from the OVX were due to increased LD frequency within the myoplasm and not between the muscle fibers (Fig 2B). In the SHAM animals, we found very few LD within the isolated single muscle fibers (Fig 2B).

**Fatty Acid Transporter protein content:** Since IMCL content was elevated in the OVX groups compared to the SHAM animals, we measured the protein content of the two primary sarcolemmal fatty acid transporters in skeletal muscle. We found that both FAT/CD36 (Figure 3A) and FABPpm (Figure 3B) were significantly increased in OVX mice compared to SHAM mice.

**Metabolic Profiling:** Due to the metabolic complexity of skeletal muscle, we assessed the metabolic profile of skeletal muscle from OVX animals compared to SHAM animals using a targeted metabolomics approach. The purpose of these experiments was to identify potential pathways or targets that could account for the metabolic dysfunction that appears in the OVX animals. These metabolites are byproducts of fuel degradation 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 \( \beta \)-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 levels of long chain acylcarnitines (LC) might suggest that movement of fatty acids via mitochondrial Carnitine palmitoyltransferase I (CPT-1) exceeds flux through \( \beta \)-oxidation enzymes, such as long-chain (LC) acyl-CoA dehydrogenase and/or \( \beta \) -OH-acyl-CoA dehydrogenase (56). Based on previous results in other models of obesity and due to the accumulation of IMCL in the OVX muscles, we hypothesized that LC in the muscle from OVX muscles would be elevated compared to the SHAM animals (25, 53, 56). Surprisingly, we found total LC species to be reduced in the OVX group compared to the SHAM group (Figure 4A). No differences in medium chain (MC) acylcarnitines were identified (Figure 4B), but we did detect significantly lower levels of short chain (SC) acylcarnitines in the OVX group compared to the SHAM group (Figure 4C). These data suggest a reduced flux through the \( \beta \)-oxidation pathway in the muscle from the OVX group compared to 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 to SHAM mice (Figure 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). Collectively, these data imply that reductions in flux through \( \beta \)-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 content might suggest an impaired flux through \( \beta \)-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 (Figure 5).

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 (Figure 6A). However, citrate
and succinate levels were significantly (p < 0.05) higher in skeletal muscle from OVX compared
to SHAM (Figure 6B,D). Finally, no significant differences were detected in TCA cycle
intermediates, α-ketoglutarate (αKG), fumarate, and malate (Figure 6C,E).

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 to the
SHAM mice (Figure 7A-D). In OVX mice, we identified significant decreases in the BCAAs,
leucine and isoleucine, however there was no significant difference in valine as compared to
SHAM mice (p < 0.05) (Figure 7B,C). We also found significant decreases in the concentrations
of alanine, glutamine/glutamic acid, proline, serine, and histidine in OVX mice compared to
SHAM (Figure 7A,B,C).

Odd Chain Acyl-Carnitine Species: Odd chain acyl-carnitine species propionylcarnitine (C3),
isovalerylcaritnine (C5), and tiglyl carnitine (C5:1) are products of BCAA catabolism (32, 38).
Due to 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 to SHAM (p<0.05) and no difference in C5 species (Figure 8). However, we observed
a reduction in the unsaturated C5:1 acyl-carnitine species (p<0.05) (Figure 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 BCAAs and likely a reduced pool of
available amino acids to the muscle in the OVX condition (38).

Mitochondrial Oxygen Consumption: To determine if 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 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 to the SHAM animals (Figure 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 (Figure 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 (Figure 9C).
To assess substrate specific maximal ETC activity, we added the uncoupling agent, FCCP, and
found significant increases in normalized OCR in the OVX and SHAM compared to baseline
OCR with both palmitate and pyruvate substrates (Figure 9B and C). However, regardless of
substrate, FCCP stimulated normalized OCR were significantly lower in the fibers from the OVX
group compared to the SHAM indicating a potential impairment in electron transport chain
function (Figure 9B,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 to SHAM fibers to respond to stimuli that would activate mitochondria (Figure 9 D,E).

**Mitochondrial Enzyme Content:** To determine if 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, MCAD protein content or ETC mitochondrial protein content were observed between SHAM and OVX animals (Figure 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 to 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 the OVX animals compared to the SHAM 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 to 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. Based on 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.

Due to the metabolic complexity of skeletal muscle, we employed a targeted metabolic
profiling approach to generate a comprehensive non-bias 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 to 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. Based on 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 towards re-esterification 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 if 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 to 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 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 to 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. Since, 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 if lower 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 \textit{in vitro} or \textit{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. found a decline in nocturnal ambulatory activity levels in OVX mice as well as decreased oxygen consumption compared to SHAM (47). 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 \textit{in vitro} and \textit{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 regards 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 majority of amino acids were significantly reduced in the OVX group compared to the SHAM groups, with even the non-significant
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 to 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 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 to 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 provides 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 steroids 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 non-skeletal 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 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. Due to 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 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 pre-mature 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 in order 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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with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal


66. **Zhai P, Eurell TE, Cotthaus RP, Jeffery EH, Bahr JM, Gross DR.** Effects of dietary phytoestrogen on global myocardial ischemia-reperfusion injury in isolated female rat
### Table 1. Anatomical characteristics of SHAM and OVX animals

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>BM (g)</th>
<th>SEM</th>
<th>VF (g)</th>
<th>SEM</th>
<th>IF (g)</th>
<th>SEM</th>
<th>Uterus (g)</th>
<th>SEM</th>
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<tbody>
<tr>
<td>SHAM</td>
<td>4 mos</td>
<td>23.46</td>
<td>0.526</td>
<td>0.262</td>
<td>0.03</td>
<td>0.175</td>
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<td>0.070</td>
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<tr>
<td>OVX</td>
<td>4 mos</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; * Indicates statistically different from SHAM p<0.05

### Table 2. Daily Food Consumption

<table>
<thead>
<tr>
<th></th>
<th>Food consumption (g/day)</th>
<th>SEM</th>
<th>Feed Efficiency Body mass (g)/Food Intake (Kcal*day)</th>
<th>SEM</th>
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<tbody>
<tr>
<td>SHAM</td>
<td>4.943</td>
<td>0.096</td>
<td>1.139</td>
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</tr>
<tr>
<td>OVX</td>
<td>4.228*</td>
<td>0.33</td>
<td>1.457*</td>
<td>0.008</td>
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</tbody>
</table>
Figure Legends

**Figure 1 (A-C)**: IMCLs quantified using direct fluorescent visualization were significantly elevated in ovariectomized (OVX) female mice in both the (A) plantaris and (B) soleus muscle groups compared to age matched sham surgery (SHAM) female mice. N=3 animals/group and 50 fibers/muscle quantified. * indicates significant difference from SHAM (p<0.05)

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

**Figure 3 (A-B)**: OVX female mice demonstrated significant elevations in (A) CD36/FAT protein and (B) FABPpm in the plantaris muscle compared to age matched SHAM animals. Example western blots are presented for each target. N=5 animals/group. * indicates significant difference from SHAM (p<0.05)

**Figure 4 (A-D)**: OVX animals exhibited significant reductions in various acylcarnitine species coupled with lower carnitine levels in the whole gastrocnemius muscle compared to age-matched SHAM animals. (A) OVX female mice demonstrated a trend (p=0.06) for lower levels of long chain (LC; C16-C18) acylcarnitine species compared to age matched SHAM animals. (B) No differences between OVX and age-matched SHAM mice were detected for medium chain (MC, C10-12) acylcarnitine species. (C) OVX female mice had significantly lower levels of short chain (SC; C2-C5) acylcarnitine species compared to age matched SHAM animals. (D) OVX female mice had significantly lower levels of free carnitine compared to age matched SHAM animals. N=6 animals/group. * indicates significantly different from SHAM (p<0.05).
Figure 5: No differences in the whole gastrocnemius skeletal muscle malonyl CoA content was detected between SHAM and OVX age matched female mice. N=5 animals/group.

Figure 6 (A-E): OVX mice exhibit no changes in whole gastrocnemius muscle pyruvate or lactate levels, but exhibit selective differences in Krebs cycle intermediates compared to age-matched SHAM mice. (A) OVX mice exhibited no differences in skeletal muscle lactate and pyruvate levels compared to age matched SHAM animals. (B) OVX animals had significantly higher levels of citrate levels compared to age matched SHAM animals. (C) No significant differences in alpha ketogluterate were detected in the gastrocnemius muscle between age matched OVX and SHAM female mice. (D) OVX mice demonstrated significant elevations in skeletal muscle succinate levels compared to age matched SHAM female mice. (E) No significant differences were detected in skeletal muscle fumarate or malate levels between OVX and SHAM groups. N=6 animals/group. * indicates significantly different from SHAM (p<0.05).

Figure 7 (A-D): OVX animals exhibited significantly lower levels of various amino acid species in the gastrocnemius muscle compared to age matched SHAM animals. (A) OVX mice had significantly lower levels of alanine, and no difference in glycine levels compared to SHAM animals. (B) OVX mice had significantly lower levels of serine, leucine/isoleucine, glutamate in skeletal muscle compared to SHAM animals. (C) OVX mice had significantly lower levels of proline and histidine, and no differences in valine, aspartate, or arginine levels compared to 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 (Glx), proline (Pro), histidine (His), glycine (Gly), valine (Val), aspartate (Asx), arginine (Arg), methionine (Met), phenylalanine (Phe), tyrosine (Tyr), ornithine (Orn), and Citrulline (Cit). N=6 animals/group. * indicates significantly differs from SHAM (p<0.05).
Figure 8: Significant decreases in odd chain acylcarnitine species propionylcarnitine (C3) and unsaturated isovalerylcarnitine (C5:1) in the whole gastrocnemius muscle from the OVX compared to age matched SHAM mice. No significant differences were detected in isovalerylcarnitine (C5) acylcarnitine species or in the hydroxylated species ratio C5-OH/C3-OH. N=6 animals/group. * indicates significantly differs from SHAM (p<0.05).

Figure 9 (A-E):
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 to SHAM animals. * indicates significant difference from SHAM (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, 50μM) or pyruvate (Pyr, 10mM) were significantly reduced in skeletal muscle fibers from OVX compared to SHAM animals. However, no differences were detected in OCR responses to PA stimulation or Pyr stimulation alone. # indicates significantly different from SHAM basal (p<0.05), $ indicates significantly different from OVX basal (p<0.05), + indicates 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 to age matched SHAM single muscle fibers. N=5 animals/group. * indicates significantly different from SHAM (p<0.05)

Figure 10 (A-B): 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 β-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.
Figure 1. (A-B)

A. Plantaris

B. Soleus

Arbitrary Units

0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8

SHAM OVX

*
Figure 2. (A-B)
Figure 2 (A-B)
Figure 3. (A-B)

(A) CD36/FAT protein content (% of SHAM)

(B) FABPpm protein content (% of SHAM)
Figure 4 (A-D)

A.  

P = 0.06

B.  

C.  

D.

- **SHAM**
- **OVX**

* * P = 0.06
Figure 5

Malonyl-CoA (pmoles/mg muscle)

Sham  | O VX
Figure 6 (A-E)

A.

B.

C.

D.

E.

SHAM  
OVX  
Lactate  
Pyruvate  
Citrate  
alpha-KG  
Succinate  
Fumarate  
Malate
Figure 7 (A-D)

(A) SHAM and OVX comparison for Gly and Ala.

(B) SHAM and OVX comparison for Ser, Leu/Ile, and Glx.

(C) SHAM and OVX comparison for Pro, Val, His, Asx, and Arg.

(D) SHAM and OVX comparison for Met, Phe, Tyr, Orn, and Cit.
Figure 8
Figure 9 (A-E)

A.

B.

Palmitate

SHAM | OVX
---|---
Basal | Basal | PA | FCCP

B.

Palmitate

SHAM | OVX
---|---
Basal | Basal | PA | FCCP

C.

Pyruvate

SHAM | OVX
---|---
Basal | Basal | Pyr | FCCP

D.

Palmitate

SHAM | OVX
---|---
Spare Respiratory Capacity

E.

Pyruvate

SHAM | OVX
---|---
Spare Respiratory Capacity
Figure 10 (A-B)

A.

<table>
<thead>
<tr>
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<tr>
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</tr>
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B.

<table>
<thead>
<tr>
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