Role of estrogen on skeletal muscle mitochondrial function in ovariectomized rats: a time course study in different fiber types

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1Laboratório de Fisiologia Endócrina Doris Rosenthal, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; 2Laboratório de Biofísica do Exercício, Escola de Educacao Física e Desportos e Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; and 3Laboratório de Bioenergética e Fisiologia Mitocondrial, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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Cavalcanti-De-Albuquerque JP, Salvador IC, Martins EL, Jardim-Messeder D, Werneck-De-Castro JP, Galina A, Carvalho DP. Role of estrogen on skeletal muscle mitochondrial function in ovariectomized rats: a time course study in different fiber types. J Appl Physiol 116: 779–789, 2014. First published January 23, 2014; doi:10.1152/japplphysiol.00121.2013.—Postmenopausal women are prone to develop obesity and insulin resistance, which might be related to skeletal muscle mitochondrial dysfunction. In a rat model of ovariectomy (OVX), skeletal muscle mitochondrial function was examined at short- and long-term periods after castration. Mitochondrial parameters in the soleus and white gastrocnemius muscles fiber types were analyzed. Three weeks after surgery, there were no differences in coupled mitochondrial respiration (ATP synthesis) with pyruvate, malate, and succinate; proton leak respiration; or mitochondrial reactive oxygen species production. However, after 3 wk of OVX, the soleus and white gastrocnemius muscles of the OVX animals showed a lower use of palmitoyl-carnitine and glycerol-phosphate substrates, respectively, and decreased peroxisome proliferator-activated receptor-γ coactivator-1α expression. Estrogen replacement reverted all of these phenotypes. Eight weeks after OVX, ATP synthesis was lower in the soleus and white gastrocnemius muscles of the OVX animals than in the sham-operated and estrogen-treated animals; however, when normalized by citrate synthase activity, these differences disappeared, indicating a lower muscle mitochondria content. No differences were observed in the proton leak parameter. Mitochondrial alterations did not impair the treadmill exercise capacity of the OVX animals. However, blood lactate levels in the OVX animals were higher after the physical test, indicating a compensatory extramitochondrial ATP synthesis system, but this phenotype was reverted by estrogen replacement. These results suggest early mitochondrial dysfunction related to lipid substrate use, which could be associated with the development of the overweight phenotype of ovariectomized animals.

Bilateral ovariectomy (OVX) is a good animal model to study the impact of estradiol deficiency on energy balance and body mass (48). Previous studies demonstrated that OVX rats have an increase in body mass that could be a consequence of a hyperphagic state (32, 33), a decrease in energy expenditure (EE) (50), or both. Thus these animals show an increase in adipose tissue (43), fat content in the liver (43, 44), heart and skeletal muscle (28), dyslipidemia (28, 53), insulin resistance (46, 51), and changes in the serum cytokine profile (37), which are characteristics of a metabolic syndrome. These metabolic disturbances that lead to changes in body composition and insulin resistance are also detected in postmenopausal women (29, 31, 59a).

Skeletal muscle metabolism is the major determinant of total EE (60), and OVX causes a decrease in the expression of important muscle genes, such as major histocompatibility complex IIx (42), and the genes involved in energy metabolism (49), such as the evident decrease in AMP-activated protein kinase (AMPK) signaling, which regulates EE (14, 25). Additionally, OVX induces a time-dependent decrease in muscle glucose uptake (46, 51).

Although these metabolic parameters are associated with mitochondrial metabolism (20, 34), little is known about the effects of estrogen on skeletal muscle mitochondrial function and whether mitochondrial dysfunction precedes weight gain. Thus the aim of this study was to assess the short-term (to determine possible causal factors leading to weight gain) and the long-term (changes that are most likely secondary to the overweight phenotype) effects of rat OVX and estrogen replacement on the mitochondria electron transport system in skeletal muscle fibers, using complex I [pyruvate/malate (PM)] and complex II [PM + succinate (PMS)] substrates and by determining the lipid metabolite use capacity [palmitoyl-carnitine (PC) and glycerol-phosphate (GP)]. We conclude that skeletal muscle mitochondria dysfunction related to lipid use occurs early after OVX and might correspond to the underlying mechanism related to weight gain in OVX rats.

MATERIALS AND METHODS

Animals. Female Wistar rats (8-12 wk old), donated by the Vital Brazil Institute (Niteroi, Rio de Janeiro, Brazil), were used in these experiments. The animals were housed in a temperature-controlled environment (22 ± 2°C) with a light/dark cycle of 12 h. The animals had ad libitum access to commercial rat chow (Nova Lab, Rio de Janeiro, Brazil) and tap water. The Rio de Janeiro Federal University Institutional Committee for Evaluation of Animal Use in Research

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were put in metabolic cages and were killed 24 h later.

Electrical stimulus to keep the rats running. (30). After adaptation (3–5 days, 5 min/day at a speed of 10 m/min) (AVS Project, São Paulo, Brazil), running as described previously 16 h of measurements—8 h in each phase of the dark-light cycle.

Whole-body oxygen consumption. The animals were allocated into metabolic cages (PhysioGage; Panlab, Harvard Apparatus, Barcelona, Spain) to measure whole-body oxygen consumption (LE 405 gas analyzer; Panlab, Harvard Apparatus) during 24 consecutive hours. The first and last 4 h of the measurement were discarded, resulting in 16 h of measurements—8 h in each phase of the dark-light cycle.

Treadmill exercise. We adapted a ramp test for maximum treadmill (AVS Project, São Paulo, Brazil), running as described previously (30). After adaptation (3–5 days, 5 min/day at a speed of 10 m/min) to the apparatus, each rat was tested for maximum endurance running capacity. The trial began at a starting speed of 10 m/min and a constant slope of 10°. The treadmill speed was then increased by 1.2 m/min every 2 min until exhaustion. Stainless-steel grids provided an electrical stimulus to keep the rats running.

Experimental Design

First Protocol – 3 weeks of surgery

<table>
<thead>
<tr>
<th>20° Day – Metabolic Cage</th>
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<tbody>
<tr>
<td>Days 0 3 6 9 12 15 18 21</td>
</tr>
<tr>
<td>Sacrifice</td>
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Second Protocol – 8 weeks of surgery

<table>
<thead>
<tr>
<th>21° Day – Physical Test 55° day – Metabolic Cage Physical Test</th>
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<tbody>
<tr>
<td>Weeks 0 1 2 3 4 5 6 7 8</td>
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<tr>
<td>Sacrifice</td>
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</table>

Fig. 1. Experimental design. Rats were evaluated 3 and 8 wk after surgery. In the 1st protocol, the animals were maintained in individual cages and were weighed every 3 days until the end of the experiment. In the 2nd protocol, the animals were maintained in collective cages and were weighed every 3 days until the end of the experiment. The animals were allocated in metabolic cages for in vivo oxygen consumption 1 day before death.

Lactate measurement. Ten minutes before and right after exercise, 25 µl blood was collected from the tail, and it was mixed with 50 µl 1% NaF and stored at –20°C. After 1 wk, the samples were thawed, and 25 µl was analyzed using an automatic lactate analyzer (YSI 1500 series; YSI Life Sciences, Yellow Springs, OH) after system calibration. The results are expressed in millimeters and were corrected for the dilution factor (1:3).

Death. The animals were killed after 4 h fasting. After decapitation, the soleus and white gastrocnemius muscle fibers were removed and snap frozen in liquid nitrogen for further analysis of mRNA expression and citrate synthase (CS) activity. Contralateral muscles were used for high-resolution oxygraphy experiments. Visceral retroperitoneal and subcutaneous inguinal adipose tissue and the uterus were removed and weighed.

Preparation of permeabilized muscle fibers. Mitochondrial respiration was studied in situ using saponin-permeabilized fibers, as described previously (27). We used the saponin-permeabilized fiber protocol, because, compared with traditional skeletal muscle mitochondria isolation, it results in a better recovery of soluble proteins and other molecules from the mitochondrial matrix. This protocol also preserves the mitochondrial morphology and representative mitochondrial populations and protects against matrix protein degradation (41).

After excision, the muscles were immersed immediately in cold BIOPS solution [10 mM EGTA, 0.1 µM potassium 2-(N-morpholino)ethanesulfonate, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, and 15 mM phosphocreatine, pH 7.1]. Muscle fibers were separated using two forceps and permeabilized with iced BIOPS solution containing 50 µg/ml saponin for 30 min. After permeabilization, the fibers were washed for 10 min in cold mitochondrial respiration solution MIR05 (0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurin, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, and 1 g/l BSA, pH 7.1) and were immediately frozen.

High-resolution respirometry. Muscle fibers (1.5–2.5 mg soleus and 3.5–4.5 mg white gastrocnemius) were dried on filter paper, weighed, and placed in the Oxygraph 2k chamber (Oroboros Instruments, Innsbruck, Austria) with 2 ml MIR05 at 37°C. To assess O2 flux, the substrates were added sequentially as follows: 5 mM pyruvate and 5 mM malate, 3 mM ADP, 10 µM cytochrome c, 10 mM succinate, 1 µM oligomycin, and 10 mM KCN. To evaluate the participation of complex I (PM) or complex II (PMS) in the coupled respiration of the fibers, we used a titration protocol with pyruvate, malate, and succinate (38). Proton leak and ATP synthesis, related to O2 consumption, were calculated using the following equations: oligomycin O2 flux = KCN O2 flux to calculate proton leak and PMS O2 flux – oligomycin O2 flux to calculate O2 consumption by ATP synthesis. For the experiments involving lipid oxidation, we used PC (5–75 µM) and GP (0.5–20 mM) in the presence of 3 mM ADP and 5 mM malate (45). Each step was interspersed with a period of stabilization between the injections. The oxygen flux/mass [pmol O2/(s · mg)] was recorded online using the DatLab software (Oroboros Instruments).

Reactive oxygen species production. Mitochondrial H2O2 production was measured as a surrogate for reactive oxygen species (ROS) production. H2O2 was detected by measuring the rate of resorufin emission, which is a product from the reaction of H2O2 and Amplex red. We used a Cary Eclipse Fluorescence Spectrophotometer (Varian Instruments, Mulgrave, Victoria, Australia) at an excitation wavelength of 563 nm and an emission wavelength of 587 nm and specific software. The samples were prepared as described above, and the measurements were performed as described previously (1, 39). After the reaction was initiated, the substrates were added in the following order: 5 mM pyruvate and 5 mM malate, 20 mM succinate, 3 mM ADP, 1 µg/ml oligomycin, 50 mM trilactolactylglycine phenylhydrazone (FCCP), and 10 mM KCN. H2O2 emission was expressed as [pmol/(min·mg)].

Real-time quantitative PCR. RNA was extracted from the frozen muscle using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions, and was treated with
DNase. Total RNA (0.5 μg) was reverse transcribed using a high-
capacity reverse-transcription kit (Applied Biosystems, Foster City, 
CA), following the instructions of the manufacturer. Real-time am-
plification reactions were performed using an ABI 7500 PCR System 
(Applied Biosystems) in a reaction containing diluted cDNA (1:20), 
12.2 μl Master Mix SYBR, and 150 nM primers. The follow-
ing primers were used: peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α), forward (5′ ACCAAACCAG-
CAAGGACAGC 3′) and reverse (5′ GGTCAGAGGAGAGATA-
AAATTTG 3′); nuclear respiratory factor 1 (NRF1), forward (5′
CACCCAGATGCAAAAGTTTCAG 3′) and reverse (5′
CACCTTTCTACATAATGCCTAGTCAAGCATTACGGAC 3′); NRF2, forward (5′
GCTATTTTCATTCCCGAGTTAC 3′) and reverse (5′
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Effect of Estrogen on Skeletal Muscle Mitochondria Function • Cavalcanti-de-Albuquerque JPA et al. 781

SO, sham-operated; OVX, ovariectomized; E2, ovariecto-
mized treated with 1,3,5 estratriene-3,17-β-diol 3-benzoate (estradiol benzoate). *P < 0.05, **P < 0.01, and ***P < 0.001 SO vs. OVX.

RESULTS

Body mass, fat compartments, uterus weight, and estradiol
levels. The uterus weight was lower in the OVX rats than in
the SO rats, and estrogen replacement was able to reverse this
OVX effect completely. OVX rats had a larger body mass than

<table>
<thead>
<tr>
<th>Table 1. Body mass, fat-pads content, uterus weight, and serum estradiol levels at 3 and 8 wk after sham operation, ovariectomy, or ovariectomy followed by estrogen replacement in rats</th>
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</thead>
<tbody>
<tr>
<td><strong>3 wk</strong></td>
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<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>Initial body mass, g</td>
</tr>
<tr>
<td>Final body mass, g</td>
</tr>
<tr>
<td>Body mass gain, g</td>
</tr>
<tr>
<td>Food ingestion, g</td>
</tr>
<tr>
<td>Subcutaneous fat mass, g</td>
</tr>
<tr>
<td>Visceral fat mass, g</td>
</tr>
<tr>
<td>Uterus, g</td>
</tr>
<tr>
<td>Estradiol, pg/ml</td>
</tr>
</tbody>
</table>

SO, sham-operated; OVX, ovariectomized; E2, ovariectomized treated with 1,3,5 estratriene-3,17-β-diol 3-benzoate (estradiol benzoate). Data are presented as mean ± SE. *P < 0.001, +P < 0.05, †P < 0.01 vs. SO.
the SO rats, 3 and 8 wk after surgery, whereas estrogen replacement decreased the final body mass by 20% compared with the SO group after 8 wk. Subcutaneous and visceral fat compartments were larger in the OVX rats than the SO rats at both times after castration. These data confirm the effects of OVX and estrogen replacement on body mass (Table 1).

OVX rats maintained in individual cages were already significantly heavier than the SO animals, 9 days after surgery (Fig. 2A). On the other hand, OVX rats in collective cages were significantly heavier only 2 wk after surgery (Fig. 2B). There were no differences in the in vivo oxygen consumption among the groups, either 3 or 8 wk after surgery (Fig. 3, A and B, respectively). However, the OVX rats ingested significantly more food during the first 3 wk of castration (Table 1).

**Soleus and white gastrocnemius O$_2$ flux consumption.** To analyze mitochondrial function, we used a multisubstrate protocol to measure complex I (PM) and complex I + II (PMS) participation in the O$_2$ consumption rate of the permeabilized fibers of the soleus and white gastrocnemius muscles. The differences between the fiber types are shown in Fig. 4. No differences in any parameter (PM or PMS) were detected 3 wk after surgery in the soleus or white gastrocnemius fibers (Fig. 4, A and B, respectively). In contrast, 8 wk after surgery, the coupled oxygen consumption of the soleus and white gastrocnemius of the OVX rats was lower in the presence of PM and PMS (Fig. 5, C and D). Furthermore, after succinate was included in the reaction medium, a significant decrease was detected in the soleus fibers of the OVX rats when compared with the SO rats, and estrogen replacement re-established the O$_2$ consumption to SO levels (Fig. 5C). As expected, oligomycin and KCN decreased the mitochondrial O$_2$ consumption rate to basal levels (Fig. 5).

CS, a classical mitochondrial content marker, was significantly higher in the soleus than in the white gastrocnemius muscles, as expected (Table 2). However, 8 wk after castration, when the O$_2$ consumption values were normalized by the average of the CS activities, the decrease in mitochondrial respiration that was detected in the soleus of the OVX rats disappeared (Fig. 6A). These data show that there was a decrease in the skeletal muscle mitochondria content induced by the absence of gonadal hormones.

The proton leak rate of oxygen consumption is an indirect measure of uncoupled oxidative phosphorylation (OXPHOS) routes of proton transport through the inner mitochondrial membrane and may be calculated as the difference of the oligomycin values minus the KCN values of each analysis using high-resolution respirometry. The values were significantly different between the soleus and the white gastrocnemius, but no differences were detected among the groups, 3 wk after OVX (Table 3). However, after 8 wk, the proton leak values of the white gastrocnemius almost doubled compared with the 3-wk values (Table 3). No significant difference was observed in the soleus muscle after 8 wk (Table 3).

The oxygen consumption, coupled exclusively to ATP synthesis, may be estimated by calculating the difference between the respiration induced by PMS and ADP minus the oligomycin-insensitive oxygen consumption rate. After 3 or 8 wk, the
O2 consumption by ATP synthesis was higher in the soleus than in the white gastrocnemius (Fig. 7). Moreover, O2 consumption of the OVX soleus and white gastrocnemius muscles at 8 wk was lower than in the SO animals, and E2 treatment prevented this decrease (Fig. 7B).

Muscle lipid oxidative capacity. GP and PC, which are lipid metabolism intermediates, were evaluated 3 wk after OVX to analyze the contribution of these substrates to the mitochondrial respiration of the white gastrocnemius and soleus muscles, respectively (Fig. 8, A and C). There was a decrease in the O2 consumption rate measured in the presence of 20 mM GP (Fig. 8B) and 75 μM PC (Fig. 8D) in OVX rats compared with SO rats. The Vmax of GP use was lower in the muscles from the OVX rats than in the E2 rats (SO, 34.9 ± 1; OVX, 24.8 ± 2*).

Table 2. Citrate synthase activity (citrate nmol · min⁻¹ · mg protein⁻¹) in skeletal muscles obtained at 3 and 8 wk after sham operation, ovariectomy, or ovariectomy followed by estrogen replacement in rats

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Time</th>
<th>SO</th>
<th>OVX</th>
<th>E2</th>
<th>SO</th>
<th>OVX</th>
<th>E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>3 wk</td>
<td>178 ± 27</td>
<td>183.6 ± 25</td>
<td>184 ± 25</td>
<td>191 ± 15</td>
<td>143 ± 13</td>
<td>170 ± 9</td>
</tr>
<tr>
<td>White gastrocnemius</td>
<td>3 wk</td>
<td>87 ± 4*</td>
<td>83 ± 5*</td>
<td>89 ± 11*</td>
<td>87 ± 7*</td>
<td>64 ± 5*</td>
<td>99 ± 14*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. *P < 0.001 vs. white gastrocnemius.
E2, 39.1 ± 4, *P < 0.05 vs. E2). The catalytic efficiency (V_{max}/K_m) was lower in the OVX group compared with the SO group (SO, 5.8 ± 0.7; OVX, 3.2 ± 0.4**; E2, 4.4 ± 0.5, **P < 0.05 vs. SO). The V_{max} of PC use was lower in the OVX soleus compared with the SO soleus muscle (SO, 22.9 ± 1; OVX, 11.7 ± 2**; E2, 20.4 ± 3. **P < 0.05 vs. SO). However, there were no differences in V_{max}/K_m among the groups.

Expression of mitochondrial biogenesis and lipid metabolism genes. To explain the mitochondrial dysfunctions observed in the skeletal muscles of ovariectomized rats (Figs. 5, 7, and 8), we decided to check the expression of certain genes related to mitochondrial biogenesis (PGC-1α, NRF1, NRF2, and TFAM) and lipid metabolism (CPT and GPD2), 3 wk after surgery (Fig. 9). Both the soleus and white gastrocnemius muscles of the OVX rats exhibited a lower expression level of PGC-1α mRNA than the SO rats (Fig. 9, A and B). However, there were no differences in the expression of the other genes evaluated (Fig. 9). With the exception of TFAM expression and the expression of PGC-1α in the soleus, which was restored to basal levels by estrogen replacement, estrogen treatment led to an increase in the expression of all other mitochondrial biogenesis genes in both muscles (Fig. 9). Furthermore, CPT-1 and GPD2 expression was lower in the OVX soleus and white gastrocnemius, respectively (Fig. 9, A and B), and estrogen replacement prevented these decreases.

Mitochondrial H_{2}O_{2} production. Mitochondrial ROS production was measured in the muscle fibers of the OVX animals, 3 wk after surgery. As expected, PMS and oligomycin increased H_{2}O_{2} production, whereas ADP and FCCP decreased it. H_{2}O_{2} production was higher in the soleus than in the white gastrocnemius when stimulated with PMS [42.9 ± 9 vs. 16.4 ± 3 pmol/(min·mg), P < 0.05] or oligomycin [37.3 ± 6 vs. 13.5 ± 3 pmol/(min·mg), P < 0.05]. However, no difference was observed when the data were normalized by CS activity [PMS - 0.2 ± 0.1 vs. 0.19 ± 0.1; oligo - 0.2 ± 0.1 vs. 0.18 ± 0.01 [pmol/(min·mg)-CS1], there were no differences in mitochondria ROS production among the groups (data not shown).

Physical test parameters and blood lactate levels. Despite the mitochondrial alterations described here, there were no differences in the physical test parameters (distance and work rate) among the groups, 3 or 8 wk after surgery (data not shown). However, the OVX animals had a higher blood lactate level at the end of the physical tests, 8 wk after surgery, and E2 replacement reverted this increase (SO, 6.8 ± 0.5; OVX, 8.9 ± 0.6**; E2, 7.4 ± 0.9 mM/L, **P < 0.05 vs. SO; Table 4).

**DISCUSSION**

With the corroboration of a previous study from our laboratory (37), we showed that OVX rats have a faster body mass growth compared with their controls. Furthermore, the physical tests showed that the OVX group had a higher blood lactate level 8 wk after surgery, and E2 replacement reversed this increase (SO, 6.8 ± 0.5; OVX, 8.9 ± 0.6**; E2, 7.4 ± 0.9 mM/L, **P < 0.05 vs. SO; Table 4).

Table 3. Oxygen flux relative to proton leak in skeletal muscles obtained at 3 and 8 wk after sham operation, ovariectomy, or ovariectomy followed by estrogen replacement in rats

<table>
<thead>
<tr>
<th>Muscle</th>
<th>3 wk</th>
<th>8 wk</th>
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<tbody>
<tr>
<td></td>
<td>SO (10)</td>
<td>OVX (8)</td>
</tr>
<tr>
<td>SO</td>
<td>16.9 ± 3</td>
<td>21.4 ± 1</td>
</tr>
<tr>
<td>White gastrocnemius</td>
<td>12.1 ± 0.8*</td>
<td>12.2 ± 1*</td>
</tr>
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</table>

Data are presented as mean ± SE. *P < 0.001 vs. white gastrocnemius.
gain in the first 2 wk after castration in both protocols used in this study (Fig. 2). The increase in body mass was associated with an increased visceral adipose tissue accumulation (Table 1). Although the increase in body mass could be caused by the hyperphagic state of the OVX rats, as reported previously (36, 48), some recent studies suggest that the reduction of EE could also contribute to obesity (14, 19, 24). However, we could not detect any decrease in total body oxygen consumption among the groups, after 3 or 8 wk of castration (Fig. 3). It is important to notice that there are fundamental species-specific differences in the food-consumption response to the OVX, since mice do not become hyperphagic (22, 23, 58).

Classically, estrogen replacement prevents body mass gain (35, 48). As shown herein, estrogen replacement decreased the food ingestion of the OVX rats (Table 1). It is known that some hypothalamic regions that control energy homeostasis, such as the arcuate nucleus, the ventromedial hypothalamus, and the preoptic area, express both estrogen and leptin receptors (15). Estrogen potentiates leptin action and most likely promotes an increase in STAT3 and phosphoinositide 3-kinase signaling (11, 16, 17) and a decrease in phosphorylated AMPK (32). However, because no ER-α expression has been detected in orexigenic neurons [neuropeptide Y/Agouti-related peptide (AgRP) and pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript], estrogen could activate ER-α in the paraventricular nucleus and might regulate AgRP neurons through the presynaptic neurons (33).

Some previous studies have shown that OVX leads to obesity, even in pair-fed animals (14). Additionally, the loss of ER-α (19) or the deficiency of aromatase (24) both promote increases in the number and size of white adipose cells without increasing food ingestion. This increase in fat content is observed not only in white adipose tissue but also in the liver (43, 44), heart, and skeletal muscle (23, 28). However, the exact mechanisms underlying the organ lipid accumulation that follows estrogen deficiency are largely unknown.

Skeletal muscle is the predominant tissue in the body, and it is responsible for ~40% of the total EE (60). AMPK is an enzyme that participates in the metabolic shift of the cell (54). When it is phosphorylated, inhibition of acetyl-CoA carboxylase occurs, causing a decrease in malonyl-CoA levels and an increase in CPT activity. Thus AMPK activation favors an increase in fatty acid use by the mitochondria (54). In skeletal muscle, estrogen increases p-AMPK levels compared with ovariectomized animals (13, 14, 25). Furthermore, estrogen replacement increased the expression of PPAR-α, CPT-1, and PGC-1α (7, 8). Sixteen days after OVX, the CPT-1 and β-3-hydroxyacyl-CoA dehydrogenase (β-HAD) activities of the soleus were decreased, and estrogen replacement increased the CPT-1 activity to the levels found in the control group. β-HAD expression in white gastrocnemius muscle followed the same profile, but no differences were found between the CS activity (7). Thus the lack of estrogen could lead to a decrease in lipid metabolism use and a change in mitochondrial enzyme function and mitochondria content in skeletal muscles, leading to increased fat compartments.

Jackson et al. (23) demonstrated that lipid accumulation could be associated with a decrease in substrate flux to β-oxidation and a lower mitochondrial oxidative profile in single muscle fiber isolated after 8–10 wk postsurgery. Thus the goal of our study was to verify further whether OVX-induced obesity was associated with early alterations in muscle mitochondrial metabolism, using a different methodological approach with saponin-permeabilized fibers.

A time-dependent muscle lipid accumulation in the OVX animals (33) could be responsible for the mitochondrial dysfunction, as demonstrated previously for insulin resistance (12, 22). The hydrolysis of triglycerides generates fatty acids and GP. Mitochondria can use these substrates through β-oxidation and the GP shuttle, respectively (45). However, the different fiber types have different capabilities in regard to using these substrates, and for this reason, we chose the soleus muscle to investigate fatty acid (PC) metabolism and the white gastrocnemius to analyze GP mitochondrial metabolism. Interestingly, 3 wk after surgery, OVX animals showed a lower potential to use both substrates, and treatment with estrogen reversed this condition (Fig. 8). These results could be a consequence of a decrease in lipid metabolism gene expression, as was shown for CPT-1 in the soleus and for mitochondrial GPD2 (Fig. 9). These findings of short-term mitochondria lipid use dysfunction might correspond to an underlying mechanism related to body mass gain, since estrogen-treated OVX rats do not develop overweight, which parallels the normalization of the mitochondrial changes observed in muscles.
The normal CS activity found after 3 wk of OVX does not initially appear to agree with the lower PGC-1α expression demonstrated by our group and other groups (14, 26). However, we cannot exclude the possibility that the decrease in CS activity occurs later than the decrease in PGC-1α expression. Thus we decided to evaluate a longer period after OVX to investigate the possible repercussions of the PGC-1α decrease. Eight weeks after castration, we found a clear decrease in the mitochondrial respiration capacity in both fiber types, culminating in a reduction of the mitochondria maximal respiration in the soleus. This decrease of mitochondria respiration capacity was observed a long period after castration and occurred together with a lower ATP synthase respiration rate, which could be explained by a decrease in the number of mitochondria. Accordingly, although the CS activity was not significantly different among the groups, when we normalized the O2 flux by the average CS activity, the changes disappeared (Fig. 6). Furthermore, there were no differences in the proton leak values, suggesting that the OVX animals had a higher percentage of proton leak than the SO and E2 rats, 8 wk after surgery. This finding might correspond to a reduction in the mitochondria membrane potential, culminating in a decrease in ROS production. However, this late alteration did not lead to changes in the total in vivo basal oxygen consumption. These animals might have insulin resistance, as described by others (46, 51), so our data could be related to the findings of Boushel et al. (5), who showed that diabetic patients have decreased muscle respiration that disappeared when normalized by the amount of mitochondrial DNA.

Estrogen treatment prevented the decrease in PGC-1α expression in the soleus and led to an increase in almost all of the mitochondrial biogenesis markers analyzed in both muscles (Fig. 9). The interpretation of how protein expression of the mitochondrial biogenesis markers is regulated by estrogen is not obvious, but ERs are expressed in skeletal muscles (2– 4). In addition, progesterone and estrogen have some antagonist actions (7, 8) that could explain the significant increase in the mitochondrial gene expression in the E2-treated OVX rats that have low progesterone levels (Fig. 9).

In the present study, we demonstrated that the muscle mitochondrial H2O2 production was higher in the soleus than in the white gastrocnemius in the presence of PMS and oligomycin. These differences disappeared when data were normalized by CS activity, suggesting that these differences were relative to the mitochondrial amount in the fibers. As expected (47), ADP and FCCP decreased H2O2 production by decreases...
ing the membrane potential. Our data are in apparent contrast with data reported by Picard and colleagues (40), who showed a higher ROS production in the soleus than in the white gastrocnemius muscle. The explanation for these differences was related to the different contents of the antioxidant enzyme systems (40). However, in contrast to our H2O2 generation protocol that used both complex I and complex II substrates, their experiments evaluated H2O2 generation only with complex I (glutamate/malate) or complex II (succinate) substrates. Furthermore, in Picard’s study (40), male rats were used, in contrast to our study that used only female and ovariectomized rats, to test the muscle mitochondrial H2O2 production.

All of these mitochondrial alterations, particularly the decrease in ATP synthesis, 8 wk after OVX, suggest that OVX rats could have a lower physical exercise performance. We used a maximal exercise test to evaluate the rat fatigue resistance (30). Although no clear information about the physical capacity of OVX and estrogen-replenished rats is available, we did not see any differences in the maximal distance achieved and the work among the groups. These results could be explained by the type of test used. With higher intensity exercises, there is a shift in substrate use, with a higher use of carbohydrates than lipids to maintain the ATP levels needed for contraction (6). Furthermore, there is no consensus as to whether OVX could lead to skeletal muscle fatigue. One of the studies showed that OVX induced a decrease of muscle fatigue index (21), whereas the other demonstrated an increase (59), and others did not detect differences (18, 55, 59). Further physical evaluations are necessary to detect the relationship between the mitochondrial alterations and muscle fatigue.

However, 8 wk after surgery, the OVX rats had higher blood lactate levels than the SO rats, which could be associated with a compensatory higher glycolytic pathway to maintain the ATP levels in the absence of part of the oxidative mitochondrial system. This observation is in line with the well-known effects of estrogen on glucose transport, glycolysis, the tricarboxylic acid cycle, the mitochondrial respiratory chain, and fatty acid synthesis (9, 10, 26).

We concluded that shortly after castration, skeletal muscle mitochondrial alterations that were related to lipid substrate use were present. However, the OXPHOS capacity and the CS activity were preserved. Furthermore, after a long period of castration, mitochondrial dysfunction occurred at the OXPHOS level, and this dysfunction was associated with a decrease in mitochondria content. Estrogen replacement restored all of these disorders. Early events related to estrogen deficiency seem to be responsible for the delayed decrease in mitochon-

Table 4. Blood lactate (mmol/l) before and after the maximal exercise test

<table>
<thead>
<tr>
<th></th>
<th>BS</th>
<th>AE</th>
<th></th>
<th>BS</th>
<th>AE</th>
<th></th>
<th>BS</th>
<th>AE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BE</td>
<td>AE</td>
<td></td>
<td>BE</td>
<td>AE</td>
<td></td>
<td>BE</td>
<td>AE</td>
</tr>
<tr>
<td>SO</td>
<td>1.4 ± 0.1</td>
<td>7.6 ± 0.4*</td>
<td>1.4 ± 0.1</td>
<td>7.4 ± 0.6*</td>
<td>1.4 ± 0.1</td>
<td>6.8 ± 0.5*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>1.4 ± 0.1</td>
<td>7.6 ± 0.8*</td>
<td>1.6 ± 0.1</td>
<td>7.7 ± 0.2*</td>
<td>1.7 ± 0.1</td>
<td>8.9 ± 0.6*†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>1.5 ± 0.1</td>
<td>6.5 ± 0.2*</td>
<td>1.8 ± 0.2</td>
<td>7.3 ± 0.8*</td>
<td>1.5 ± 0.1</td>
<td>7.4 ± 0.9*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BS, before surgery; BE, before exercise; AE, after exercise. Data are presented as mean ± SE. *P < 0.05 vs. BE; †P < 0.05 vs. SO.
drial biogenesis, such as decreased PGC-1α expression and lipid use disruption.

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DISCLOSURES

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS


REFERENCES


