Estradiol stimulates mitochondrial biogenesis and adiponectin expression in skeletal muscle

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Abstract

Sexual dimorphism has been found in mitochondrial features of skeletal muscle, with female rats showing greater mitochondrial mass and function compared with males. Adiponectin is an insulin-sensitizing adipokine whose expression has been related to mitochondrial function and that is also expressed in skeletal muscle, where it exerts local metabolic effects. The aim of this research was to elucidate the role of sex hormones in modulation of mitochondrial function, as well as its relationship with adiponectin production in rat skeletal muscle. An *in vivo* study with ovariectomized Wistar rats receiving or not receiving 17 β -estradiol (E₂) (10 μ g/kg per 48 h for 4 weeks) was carried out, in parallel with an assay of cultured myotubes (L6E9) treated with E₂ (10 nM), progesterone (Pg; 1 μ M), or testosterone (1 μ M). E₂ upregulated the markers of mitochondrial biogenesis and dynamics, and also of mitochondrial function in skeletal muscle and L6E9. Although *in vivo* E₂ supplementation only partially restored the decreased adiponectin expression levels induced by ovariectomy, these were enhanced by E₂ and Pg treatment in cultured myotubes, whereas testosterone showed no effects. Adiponectin receptor 1 expression was increased by E₂ treatment, both *in vivo* and *in vitro*, but testosterone decreased it. In conclusion, our results are in agreement with the sexual dimorphism previously reported in skeletal muscle mitochondrial function and indicate E₂ to be its main effector, as it enhances mitochondrial function and diminishes oxidative stress. Moreover, our data support the idea of the existence of a link between mitochondrial function and adiponectin expression in skeletal muscle, which could be modulated by sex hormones.

Key Words

- ▶ 17 β -estradiol
- ▶ testosterone
- ▶ ovariectomy
- ▶ mitochondrial biogenesis
- ▶ mitochondrial dynamics
- ▶ skeletal muscle
- ▶ adiponectin
- ▶ L6E9

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Introduction

Sexual dimorphism in mitochondrial functionality has been described in many rat tissues such as liver, adipose tissue, brain, and skeletal muscle (Colom *et al.* 2007a,b, Valle *et al.* 2007a,b, Gómez-Pérez *et al.* 2008, 2012,

Guevara *et al.* 2009, Nadal-Casellas *et al.* 2011a, 2012, Amengual-Cladera *et al.* 2012b). In all these tissues, female rats present higher oxidative capacity than males, which is associated with greater mitochondrial biogenesis and

with morphological differences consisting of larger mitochondria with greater cristae size and density. Oxidative stress status associated with physiopathological situations such as caloric restriction, high-fat-diet feeding, or aging, also shows sexual dimorphism, as female rats show lower oxidative damage than males, a fact that has been related to their higher antioxidant defences (Colom *et al.* 2007b, Valle *et al.* 2007b, Gómez-Pérez *et al.* 2008, Viña *et al.* 2011, Amengual-Cladera *et al.* 2012b). In this sexual dimorphism, 17 β -estradiol (E_2) would be a key factor, as it plays a relevant role in mitochondrial modulation in tissues such as adipose tissue, liver, and brain (Mattson *et al.* 1997, Chen *et al.* 1999, Rodríguez-Cuenca *et al.* 2007, Moreira *et al.* 2011, Nadal-Casellas *et al.* 2011b, Amengual-Cladera *et al.* 2012a,c). As E_2 is known to activate the expression of specific proteins of the mitochondrial machinery and of genes controlling mitochondrial biogenesis and mitochondrial DNA transcription (Klinge 2008, Mattingly *et al.* 2008), this hormone ameliorates mitochondrial functionality, although its effects are tissue-specific (Moreira *et al.* 2011). The role of E_2 in sexual dimorphism of skeletal muscle mitochondrial function remains to be fully elucidated.

E_2 also plays a role in the regulation of body weight, and a loss of E_2 is associated with the development of obesity (Chen *et al.* 2009), which is related to a decrease in adiponectin levels in serum. Adiponectin is an insulin-sensitizing hormone secreted mainly by white adipose tissue (WAT; Harwood 2012). Circulating levels of adiponectin show sexual dimorphism in both humans and rodents, with females presenting higher serum concentrations than males (Riestra *et al.* 2013). Adiponectin expression in WAT is also greater in females than in males (Amengual-Cladera *et al.* 2012a,b,c), a fact that in humans has been associated with sex hormone milieu (Riestra *et al.* 2013, Wildman *et al.* 2013). Proper mitochondrial function is essential for the production of adiponectin in adipocytes (Koh *et al.* 2007), so impaired mitochondrial function in WAT also has negative effects on the insulin response in other tissues through a decrease in the expression and secretion of adiponectin (Wang *et al.* 2013). Furthermore, adiponectin plays an important role in the regulation of mitochondrial content and function in other tissues such as skeletal muscle (Civitarese *et al.* 2006). Although it was originally proposed that adiponectin was expressed and secreted only in adipose tissue, it has been shown to be also expressed in non-adipose tissues such as liver and muscle (Ding *et al.* 2007, Krause *et al.* 2008, Uribe *et al.* 2008).

In skeletal muscle, adiponectin can be detected within the myofibers and is involved in muscle contractile function, phenotype, and metabolism (Krause *et al.* 2008, Liu *et al.* 2009a).

As skeletal muscle from female rats exhibits greater mitochondrial mass, antioxidant protection, and oxidative-phosphorylative capacities than that from males (Colom *et al.* 2007a, Català-Niell *et al.* 2008, Gómez-Pérez *et al.* 2008), and E_2 has been shown to play a role in mitochondrial modulation in other tissues (Mattson *et al.* 1997, Chen *et al.* 1999, Rodríguez-Cuenca *et al.* 2007, Moreira *et al.* 2011, Nadal-Casellas *et al.* 2011b, Amengual-Cladera *et al.* 2012a,c), the aim of the present study was to elucidate the role of sex hormones in these differences, as well as their relationship with adiponectin production in skeletal muscle. This was accomplished by using a model of ovariectomized (OVX) rats to test the effects of a decrease in ovarian hormone levels and of E_2 replacement, combined with *in vitro* experiments with L6E9 myotubes, which were treated with E_2 , progesterone (Pg), and testosterone.

Materials and methods

Animals and treatments

Animal experiments were carried out in accordance with general guidelines approved by our institutional ethics committee and EU regulations (2010/63/UE). At 10 weeks of age, Wistar rats (Charles River Laboratories, Barcelona, Spain) were distributed into three experimental groups: control female ($n=6$), OVX ($n=6$), and OVX supplemented with E_2 (OVX + E_2 , $n=6$). Ovariectomy and sham surgery were performed at 5 weeks of age at Charles River Laboratories. Animals were housed in a controlled environment (22 °C and 65 $\pm$ 3% humidity) under a 12 h light:12 h darkness cycle with free access to water and pelleted standard diet (A04, Panlab, Barcelona, Spain). Every week, animals were weighed and food intake was measured. For the OVX + E_2 group, E_2 (10 μ g/kg per 48 h) was dissolved in corn oil and administered via s.c. injection for the 4 weeks preceding killing, whereas the OVX group was treated only with the vehicle. At 14 weeks of age and after a period of 12 h fasting, rats were killed by cervical decapitation. Trunk blood was collected and white gastrocnemius muscle (WGM) was quickly removed, frozen in liquid N₂, and stored at -80 °C until analysis was performed. The estrous cycle stage was regularly determined by measuring the vaginal wall impedance (Impeast, Cibertec, Madrid, Spain) and by microscopic

evaluation of the vaginal smears. All the control animals were in diestrus phase at the time they were killed.

Serum and blood parameters

Blood was allowed to clot for 20 min and then it was centrifuged (1000 g, 20 min, 4 °C) to obtain serum, which was immediately used to assay total antioxidant capacity using the TAC Assay Kit (BioVision, Inc., San Francisco, CA, USA). The remaining serum was aliquoted and stored at –20 °C until analyzed. Nonesterified fatty acid (NEFA) levels were determined spectrophotometrically using a kit from Wako Diagnostics (Richmond, VA, USA). Enzyme immunoassay kits were used for measuring serum E₂ and Pg (DRG Instruments, Marburg, Germany), adiponectin (Merck-Millipore, Billerica, MA, USA), and resistin (USCN Life Science, Houston, TX, USA). Before E₂ analysis, the serum organic fraction was concentrated using a sample extraction procedure (Dighe & Sluss 2004).

WGM homogenate preparation and determinations

WGM portions were homogenized with a disperser (IKA T10 basic ULTRA-TURAX) in the solubilization buffer (250 mM sucrose, 20 mM Tris-HCl, 40 mM KCl, and 2 mM EGTA, pH 7.4). The homogenates were sonicated at 20 W, centrifuged (at 600 g, 4 °C, for 10 min), and immediately used to assess the enzymatic activities of citrate synthase (CS) (Nakano *et al.* 2005), glutathione peroxidase (GPx) (Smith *et al.* 2001), superoxide dismutase (SOD) (Quick *et al.* 2000), and cytochrome *c* oxidase (COX) (Chrzanowska-Lightowler *et al.* 1993) by using spectrophotometric methods. The remaining volume was stored at –20 °C with phosphatase and protease inhibitors (1 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride, 0.1 mg/ml aprotinin, 10 µM leupeptin) until analysis. Triglycerides were measured spectrophotometrically using a commercial kit (Spinreact, Girona, Spain), DNA measurement was carried out by a fluorimetric method based on the 3,5-diaminobenzoic acid dihydrochloride reaction (Thomas & Farquhar 1978), and total protein content was measured by Bradford's protein-dye binding assay (Bradford 1976). Protein carbonyl groups, an index of protein oxidation, were determined in the homogenates by immunoblotting using the Oxyselect Protein Carbonyl Immunoblot Kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol with minor modifications. 4-Hydroxynoneal (HNE) was used as a lipid damage marker, and was assessed by western blot (see below) using the anti-HNE antibody from Alpha Diagnostics (San Antonio, TX, USA).

Western blot analysis

Briefly, 30 µg protein from the homogenates were fractioned by using SDS-PAGE (10–12% acrylamide) and electrotransferred onto a nitrocellulose membrane. The membranes were blocked with blocking solution (5% nonfat powdered milk in PBS, pH 7.5, containing 0.1% Tween 20) and were incubated with the corresponding primary antibody. The antibodies for mitofusin 1 (MFN1, 86 kDa), mitofusin 2 (MFN2, 80 and 86 kDa), optic atrophy protein 1 (OPA1, 120 kDa), dynamin-related protein 1 (DRP1, 80 kDa), uncoupling protein 3 (UCP3, 34 kDa), and α tubulin (55 kDa) were from Santa Cruz; COX subunit 4 (COX4, 16 kDa) was from MitoSciences (Eugene, OR, USA); and adiponectin receptor 1 (AdipoR1, 49 kDa) from Alpha Diagnostics. Development of the immunoblots was carried out using an enhanced chemiluminescence kit (Immun-star Western Chemiluminescence kit, Bio-Rad). Immunoblot bands were visualized using the ChemiDoc XRS system (Bio-Rad) and analyzed using the image analysis program, Quantity One (Bio-Rad). Precision Plus Protein (TM) Dual Color Standard (Bio-Rad) was used as a molecular weight marker. The band density of each protein was quantified in relation to the loading control (actin) from its own gel. Representative immunoblots are shown in Fig. 1.

Detection of H₂O₂ production in permeabilized myofibers

Myofibers were obtained from WGM and permeabilized following a protocol published previously (Burelle & Hochachka 2002). The rate of H₂O₂ production was determined by measuring the oxidation of the fluorogenic indicator, Amplex Red (Molecular Probes, Paisley, UK), in the presence of HRP. WGM myofibers (4–5 mg) were placed in buffer Z (110 mM K-methanesulfonate, 35 mM KCl, 1 mM EGTA, 3 mM MgCl₂, 10 mM K₂HPO₄, pH 7.1) supplemented with 5 mg/ml fatty-acid-free BSA. Amplex Red reagent (10 mM) and HRP (10 U/ml) were added and then incubated at 37 °C for 5 min. H₂O₂ production was initiated by the addition of substrates (glutamate/malate, 5 mM/2.5 mM) and fluorescence was recorded in a microplate reader (FLx800; Bio-Tek Instruments, Winooski, VT, USA) with 530 nm excitation and 590 nm emission wavelengths. Rates were determined by converting fluorescence readings, using standard curves generated over a range of H₂O₂ concentrations. Myofibers were recovered and placed into 500 µl solubilization buffer for determination of CS activity. H₂O₂ production was expressed per unit of CS activity in relation to mitochondrial mass.

Figure 1

Representative immunoblots from white gastrocnemius muscle. C, control; OVX, ovariectomized rats; OVX + E₂, ovariectomized rats treated with 17β-estradiol; COX4, cytochrome c oxidase subunit 4; UCP3, uncoupling protein 3; MFN1, mitofusin 1; MFN2, mitofusin 2; OPA1, optic atrophy protein 1; DRP1, dynamin-related protein 1; *AdipoR1*, adiponectin receptor 1. When immunoblot bands or marker are grouped from different parts of the same gel, this is indicated by lines in the figure. Each protein was quantified in relation to the loading control from its gel, but only one representative immunoblot for actin is shown in this figure.

Measurement of O₂ consumption in permeabilized myofibers

Mitochondrial O₂ consumption in WGM permeabilized myofibers was measured polarographically. Myofibers (10 mg) were incubated in a water thermostatically regulated chamber with a computer-controlled Clark-type O₂ electrode (Oxygraph; Hansatech, Norfolk, UK) in 500 µl of buffer B (2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 1.38 mM MgCl₂, 3 mM K₂HPO₄, 0.50 mM dithiothreitol, 20 mM imidazole, 100 mM K-methanesulfonate, 20 mM taurine, pH 7.3) supplemented with fatty-acid-free BSA (2 mg/ml). Glutamate and malate (5 and 2.5 mM respectively) were added in the absence of ADP and the decrease of O₂ concentration in the chamber was recorded.

O₂ consumption was expressed per unit of CS activity in relation to mitochondrial mass.

Analysis of gene expression in WGM

RT-PCR was used to determine the gene expression of adiponectin, *AdipoR1*, adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1 (*Appl1*), carnitin-palmitoyl transferase 1m (*Cpt1m* (*Cpt1b*)), nuclear respiratory factor 1 (*Nrf1*), peroxisome proliferator-activated receptor coactivator 1α (*Pgc1a* (*Ppargc1a*)), sirtuins 1 and 3 (*Sirt1* and *Sirt3*), mitochondrial transcription factor A (*Tfam*). *18S* (*Rn18s*) was used as a house-keeping gene for WGM and actin for L6E9 myotubes. Total RNA was obtained from 0.1 g WGM using TriPure Isolation Reagent (Roche Diagnostics) following the manufacturer's instructions, and quantified using the Take3 Microplate in a PowerwaveXS spectrophotometer (BioTek, Winooski, VT, USA). A 1 µg sample of total RNA was reverse transcribed to cDNA using 25 U MuLV reverse transcriptase in 5 µl retrotranscription mixture (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 2.5 µM random hexamers, 10 U RNase inhibitor, and 500 µM of each dNTP) for 60 min at 42 °C in a Gene Amp 9700 thermal cycler (Applied Biosystems). cDNA solutions were diluted 1/10, and aliquots were frozen (−20 °C) until analyzed. Real-time PCR was carried out using SYBR Green technology in a LightCycler rapid thermal cycler (Roche Diagnostics). The amplification program consisted of a preincubation step for denaturation of template cDNA (95 °C, 10 min) followed by 45 cycles consisting of a denaturation, an annealing, and an extension step under the conditions given in Table 1. After each cycle, fluorescence was measured at 72 °C. Product specificity was confirmed in the initial experiments by agarose gel electrophoresis and then routinely by melting curve analysis.

Cell culture of L6E9 myotubes

Rat L6E9 myoblasts, a subclone of the L6 cell line obtained from the muscle of newborn rats (Yaffe 1968), were routinely maintained at 37 °C in a humidified atmosphere of 5% CO₂ in DMEM/high-glucose medium (Gibco by Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, both from Biological Industries (Beit-Haemek, Israel). After the cells reached confluence, myotube formation was induced by restricting serum in media (2% FBS). Differentiation medium was replaced 24 h before treatment by phenol-red-free

Table 1 Oligonucleotide primer sequences and conditions used in real-time PCR amplification in white gastrocnemius muscle and in L6E9 myotubes. The amplification program consisted of a preincubation step for denaturation of the template cDNA (2 min, 94 °C), followed by 40 cycles consisting of a denaturation step (95 °C, 10s), annealing (primer-dependent temperature, 10s), extension (72 °C, 12s), and fluorescence capture step. Product length was assessed by agarose gel electrophoresis

Gene	Forward primer (5'→3')	Reverse primers (5'→3')	Annealing temperature (°C)	Product length (bp)
18S	CCTCCAATGGATCCTCGT	AAACGGCTACCACATCCA	54	160
Actin	GAGACCTTCAACACCCC	GTGGTGGTGAAGCTGTAGCC	56	219
Adiponectin	CTGGCTCCAAGTGTATGGGG	AAGCCTGTCGCCTGTTCTT	55	161
<i>AdipoR1</i>	GTCGACAGGCCTAAGTGTC	CTAGAAGGAGCCCCATTGCC	60	165
<i>Appl1</i>	AGTGCAGCAACTCACCTGAC	GGAACATCATGGCATCAGCG	60	159
<i>Cpt1m</i>	TTTGAGATGCACGGCAAGAC	CTGGACAAGAGGCGAACACA	59	64
<i>Nrf1</i>	CCTCTGATGCTTGCCTCGTCT	TACTCTGCTGTGGCTGATGG	55	87
<i>Pgc1a</i>	ATCTACTGCCTGGGGACCTT	ATGTGTCGCCTTCTTGCTCT	60	179
<i>Sirt1</i>	CAGTGTCATGGTTCCTTTGC	CACCGAGGAACCTACCTGAT	56	103
<i>Sirt3</i>	AGGCCCATATCCCTCTCTGT	ACTCCCTGGGGATCTGAAGT	55	185
<i>Tfam</i>	GCTAAACACCCAGATGCAAAA	CGAGGTCTTTTGGTTTTCC	53	248

AdipoR1, adiponectin receptor 1; *Appl1*, adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1; *Cpt1m*, carnitin-palmitoyl transferase 1m; *Nrf1*, nuclear respiratory factor 1; *Pgc1a*, peroxisome proliferator-activated receptor coactivator 1 α ; *Sirt1* and 3, sirtuins 1 and 3; *Tfam*, mitochondrial transcription factor A.

DMEM/high-glucose medium (Gibco by Invitrogen) supplemented with 2% charcoal-stripped FBS (Biological Industries) and 1% penicillin-streptomycin. Preliminary experiments were carried out to determine the appropriate concentration for each compound, and cell death was ruled out by the LDH Cytotoxicity Assay Kit II (Biovision, Inc., San Francisco, CA, USA). The cells were treated with either E₂ (1 nM), Pg, or testosterone, both at 10 μ M, or the vehicle (ethanol) for 18 (for mRNA determination) or 24 h (for confocal analysis). For all the experiments, the cell line had undergone between 15 and 20 passages.

Analysis of L6E9 mRNA levels by real-time reverse transcriptase PCR

mRNA of L6E9 rat myotubes was obtained from a six-well plate using 1 ml TriPure Isolation Reagent (Roche Diagnostics) per well and following the manufacturer's instructions. It was quantified with the Nanodrop system (BioTek). RT and real-time PCR were carried out as described earlier for *Pgc1a*, *Tfam*, adiponectin, and *AdipoR1*.

Confocal microscopy

L6E9 were seeded, differentiated, and treated as described previously for 24 h in Nunc Lab-Tek II Chamber Slide (Thermo-Fisher Scientific, Madrid, Spain). At the time of the analysis, 0.5 μ M MitoTracker Green (MTG, Invitrogen) was added. The cells were incubated for 1 h and then washed three times with fresh media. Fluorescence was viewed using a Leica confocal microscope and images were

acquired using a 40 $\times$ objective lens and the Leica Application Suite (LAS) software, version Advanced Fluorescence 2.3.6 build 5381.

Statistical analysis

All data from *in vivo* experiments are expressed as the mean $\pm$ S.E.M. of six animals per group. Hormone effects were analyzed by one-way ANOVA. Least significant difference (LSD) was applied as a *post hoc* analysis of hormone effects. *In vitro* data were analyzed from three individual experiments carried out in duplicate ($n=6$) and statistical differences between groups were assessed by independent-samples Student's *t*-test.

All statistical analyses were carried out using a statistical software package (SPSS 21.0 for Windows, Inc.), and a *P* value <0.05 was considered statistically significant. Ct values of the real-time PCR were analyzed using GenEx Standard Software 5.3.6 (MultiD Analyses, Goteborg, Sweden), and the efficiency of the reaction was taken into account for each gene.

Results

Effects of ovariectomy and E₂ replacement on serum levels of sex hormones, adipokines, and NEFA

Ovariectomy induced a decrease in E₂ and Pg serum levels, although only the latter reached statistical significance (Table 2). With the administration of E₂, the levels of both hormones increased, but while Pg did not reach control

Table 2 Effects of ovariectomy and 17 β -estradiol replacement on serum levels of sex hormones, adipokines, and NEFA. E₂ concentration is expressed in arbitrary units (AU) with control values set as 100. Values are expressed as the mean $\pm$ S.E.M. of six animals per group

	Control	OVX	OVX + E ₂	ANOVA
17 β -estradiol (AU)	100 $\pm$ 24	61.3 $\pm$ 9.7	240 $\pm$ 103 ^{a,b}	H
Progesterone (ng/ml)	24.6 $\pm$ 2.1	3.42 $\pm$ 1.28 ^a	10.7 $\pm$ 2.3 ^{a,b}	H
Adiponectin (μ g/ml)	39.9 $\pm$ 4.7	44.4 $\pm$ 3.4	22.6 $\pm$ 0.3 ^{a,b}	H
Resistin (ng/ml)	137 $\pm$ 16	250 $\pm$ 20 ^a	177 $\pm$ 23 ^b	H
NEFA (mmol/l)	0.891 $\pm$ 0.034	0.777 $\pm$ 0.018	0.886 $\pm$ 0.105	NS

OVX, ovariectomized rats; OVX + E₂, ovariectomized rats treated with 17 β -estradiol; NEFA, nonesterified fatty acids. Hormone effects were analyzed by one-way ANOVA ($P < 0.05$): H indicates hormone effect and NS stands for nonsignificant. LSD was applied as a *post hoc* analysis of hormone effects ($P < 0.05$).

^aSignificant difference from control.

^bSignificant difference from OVX.

values, E₂ exceeded them more than twofold. Ovariectomy did not induce changes in adiponectin levels, whereas resistin concentration was increased. E₂ replacement decreased both adiponectin and resistin serum levels. NEFA levels were not affected either by OVX or E₂ administration.

Effects of ovariectomy and E₂ replacement on body and gastrocnemius weight, adiposity index, and WGM composition

Ovariectomy induced 36 and 39% increases in body and gastrocnemius weight, respectively, compared with control rats (Table 3). Although E₂ replacement produced a 19% decrease in both parameters compared with the OVX group, the values remained higher than those of controls. The adiposity index also rose with ovariectomy, and E₂ treatment reversed this increase. Triglyceride and DNA levels were not modified by ovariectomy or by E₂ administration, and protein content increased with E₂ administration.

Effects of ovariectomy and E₂ replacement on markers of mitochondrial oxidative capacity in WGM

Myofiber oxygen consumption determined using glutamate and malate was lower in the OVX group compared with the control group (Table 4), but administration of E₂ reversed this decrease, although it did not reach statistical significance ($P = 0.050$). COX and CS activities, as well as COX4 (COX4I1) protein levels (Table 4, representative immunoblots are shown in Fig. 1), were not modified by ovariectomy but were increased over control values by E₂ administration. *Cpt1m* mRNA levels were unaffected by hormonal manipulation.

Effects of ovariectomy and E₂ replacement on oxidative stress markers in WGM

The H₂O₂ generation of WGM-permeabilized myofibers was measured in state 4 using glutamate/malate as a substrate (Table 5). Myofibers from OVX groups generated

Table 3 Effects of ovariectomy and 17 β -estradiol replacement on body and gastrocnemius weight, adiposity index, and white gastrocnemius muscle composition. Gastrocnemius weight represents the total weight of gastrocnemius muscle (white plus red). Adiposity index is the sum of gonadal, mesenteric, and retroperitoneal depot weights relative to 100 g of body weight. Values are expressed as the mean $\pm$ S.E.M. of six animals per group

	Control	OVX	OVX + E ₂	ANOVA
BW (g)	219 $\pm$ 5	298 $\pm$ 6 ^a	242 $\pm$ 5 ^{a,b}	H
Adiposity index (%)	4.80 $\pm$ 0.68	7.27 $\pm$ 0.44 ^a	5.30 $\pm$ 0.28 ^b	H
Gastrocnemius weight (g)	2.64 $\pm$ 0.05	3.67 $\pm$ 0.05 ^a	2.94 $\pm$ 0.02 ^{a,b}	H
(g/100 g BW)	1.22 $\pm$ 0.01	1.24 $\pm$ 0.01	1.25 $\pm$ 0.03	NS
Protein (mg/g tissue)	43.3 $\pm$ 1.1	42.6 $\pm$ 0.7	47.8 $\pm$ 0.9 ^{a,b}	H
TG (mg/g tissue)	16.5 $\pm$ 2.7	31.3 $\pm$ 4.8	23.2 $\pm$ 4.4	NS
DNA (mg/g tissue)	0.298 $\pm$ 0.031	0.281 $\pm$ 0.027	0.374 $\pm$ 0.039	NS

OVX, ovariectomized rats; OVX + E₂, ovariectomized rats treated with 17 β -estradiol; BW, body weight; TG, triglycerides. Hormone effects were analyzed by one-way ANOVA ($P < 0.05$): H indicates hormone effects and NS stands for nonsignificant. LSD was applied as a *post hoc* analysis of hormone effects ($P < 0.05$).

^aSignificant difference from control.

^bSignificant difference from OVX.

Table 4 Effects of ovariectomy and 17 β -estradiol replacement on markers of mitochondrial oxidative capacity in white gastrocnemius muscle. Glutamate/malate was used as a substrate for O₂ consumption measurement. CS activity is expressed as international units (μ mol/min). For mRNA levels, GenEx software was used to analyze the Ct values normalized to 18S Ct. Values are expressed as the mean $\pm$ s.e.m. of six animals per group

	Control	OVX	OVX + E ₂	ANOVA
O ₂ consumption (VO ₂ /min·IU CS)	137 $\pm$ 17	44.9 $\pm$ 3.9 ^a	85.6 $\pm$ 20.9 ^a	H
CS activity (mIU/g tissue)	3.00 $\pm$ 0.22	3.08 $\pm$ 0.19	3.86 $\pm$ 0.09 ^{a,b}	H
COX activity (AU/mg tissue)	100 $\pm$ 13	113 $\pm$ 12	158 $\pm$ 12 ^{a,b}	H
COX4 (%)	100 $\pm$ 18	84.9 $\pm$ 12.6	152 $\pm$ 7 ^{a,b}	H
<i>Cpt1m</i> mRNA (normalized data)	1.61 $\pm$ 0.17	1.49 $\pm$ 0.13	2.07 $\pm$ 0.12	NS

OVX, ovariectomized rats; OVX + E₂, ovariectomized rats treated with 17 β -estradiol; CS, citrate synthase; COX, cytochrome c oxidase; COX4, cytochrome c oxidase subunit 4; *Cpt1m*, carnitin-palmitoyl transferase 1m; AU, arbitrary units. Hormone effects were analyzed by one-way ANOVA ($P < 0.05$): H indicates hormone effect and NS stands for nonsignificant. LSD was applied as a *post hoc* analysis of hormone effects ($P < 0.05$).

^aSignificant difference from control.

^bSignificant difference from OVX.

more H₂O₂ than those of controls, and this increase was accompanied by higher GPx activity. WGM SOD activity and serum TAC were not altered by hormonal manipulations. Protein carbonyl groups and lipid peroxides (HNE) decreased in the OVX + E₂ group when comparing with OVX animals. UCP3 protein levels decreased with ovariectomy and returned to control values after E₂ administration (Table 5, representative immunoblots are shown in Fig. 1).

Effects of ovariectomy and E₂ replacement on the expression of the markers of mitochondrial function, biogenesis, and dynamics in WGM

Ovariectomy decreased mRNA levels of the main markers of mitochondrial biogenesis, *Pgc1a* and *Tfam* (Table 6). Ovariectomy also reduced the protein levels of mitochondrial dynamics markers MFN1, MFN2, and OPA1, as

well as DRP1 (DNM1L) (representative immunoblots are shown in Fig. 1), although it did not reach statistical significance ($P = 0.054$) (Table 6). This decrease was reversed by E₂ administration for all the biogenesis and dynamics parameters except for OPA1. The expression of *Nrf1*, *Sirt1*, and *Sirt3* was unaffected by OVX but increased with E₂ administration.

Effects of hormonal treatments on the markers of mitochondrial biogenesis in L6E9 myotubes

Intensity of MTG staining was used as an indicator of mitochondrial mass in L6E9 myotubes (Fig. 2). E₂ and Pg treatments increased both mitochondrial mass (Fig. 2A and B) and *Pgc1a* expression (Fig. 2C). *Tfam* mRNA levels also increased in E₂-treated myotubes (Fig. 2D). No effect of testosterone treatment on any of these parameters was observed.

Table 5 Effects of ovariectomy and 17 β -estradiol replacement on oxidative stress markers in white gastrocnemius muscle. GPx and SOD activities are expressed as international units (μ mol/min). Glutamate/malate was used as a substrate for H₂O₂ production. Values are expressed as the mean $\pm$ s.e.m. of six animals per group

	Control	OVX	OVX + E ₂	ANOVA
H ₂ O ₂ production (pmolH ₂ O ₂ /IU CS·min)	0.035 $\pm$ 0.008	0.116 $\pm$ 0.006 ^a	0.122 $\pm$ 0.032 ^a	H
TAC (nmol Trolox/ μ l serum)	81.4 $\pm$ 4.4	78.8 $\pm$ 1.7	84.9 $\pm$ 5.4	NS
GPx (IU/g tissue)	0.363 $\pm$ 0.036	0.490 $\pm$ 0.041 ^a	0.521 $\pm$ 0.041 ^a	H
SOD (IU/g tissue)	1.08 $\pm$ 0.11	0.969 $\pm$ 0.049	1.12 $\pm$ 0.02	NS
4HNE (%)	100 $\pm$ 8	112 $\pm$ 4	94.6 $\pm$ 2.7 ^b	H
Protein carbonyl groups (%)	100 $\pm$ 4	104 $\pm$ 12	91.9 $\pm$ 0.45 ^b	H
UCP3 (%)	100 $\pm$ 10	56.3 $\pm$ 6.4 ^a	84.7 $\pm$ 4.6 ^b	H

OVX, ovariectomized rats; OVX + E₂, ovariectomized rats treated with 17 β -estradiol; CS, citrate synthase; GPx, glutathione peroxidase; SOD, superoxide dismutase; TAC, total antioxidant capacity; 4HNE, 4-hydroxynonenal; UCP3, uncoupling protein 3. Hormone effects were analyzed by one-way ANOVA ($P < 0.05$): H indicates hormone effect and NS stands for nonsignificant. LSD was applied as a *post hoc* analysis of hormone effects ($P < 0.05$).

^aSignificant difference from control.

^bSignificant difference from OVX.

Table 6 Effects of ovariectomy and 17 β -estradiol replacement on the expression of markers of mitochondrial function, biogenesis, and dynamics in white gastrocnemius muscle. GenEx software was used to analyze the Ct values normalized to 18S Ct. Protein levels are expressed in arbitrary units per mg of protein and control values were set as 100. Values are expressed as the mean $\pm$ S.E.M. of six animals per group

	Control	OVX	OVX + E ₂	ANOVA
mRNA levels (normalized data)				
<i>Pgc1a</i>	2.01 $\pm$ 0.23	1.44 $\pm$ 0.18 ^a	2.17 $\pm$ 0.19 ^b	H
<i>Nrf1</i>	1.50 $\pm$ 0.08	1.51 $\pm$ 0.13	1.97 $\pm$ 0.15 ^{a,b}	H
<i>Tfam</i>	5.52 $\pm$ 0.03	3.07 $\pm$ 0.57 ^a	4.96 $\pm$ 0.50 ^b	H
<i>Sirt1</i>	1.61 $\pm$ 0.10	1.42 $\pm$ 0.12	2.18 $\pm$ 0.09 ^{a,b}	H
<i>Sirt3</i>	1.37 $\pm$ 0.06	1.24 $\pm$ 0.07	1.58 $\pm$ 0.07 ^{a,b}	H
Protein levels (arbitrary units)				
MFN1	100 $\pm$ 3	54.4 $\pm$ 10.5 ^a	114 $\pm$ 4 ^{a,b}	H
MFN2	100 $\pm$ 12	59.8 $\pm$ 10.5 ^a	103 $\pm$ 11 ^b	H
OPA1	100 $\pm$ 3	79.3 $\pm$ 7.1 ^a	84.9 $\pm$ 2.1	H
DRP1	100 $\pm$ 25	37.6 $\pm$ 5.9	141 $\pm$ 16 ^b	H

OVX, ovariectomized rats; OVX + E₂, ovariectomized rats treated with 17 β -estradiol; *Pgc1a*, peroxisome proliferator-activated receptor- γ coactivator 1 α ; *Tfam*, mitochondrial transcription factor A; *Nrf1*, nuclear respiratory factor 1; *Sirt1* and 3, sirtuin 1 and 3; MFN1 and 2, mitofusin 1 and 2; OPA1, optic atrophy protein 1; DRP1, dynamin-related protein 1. Hormone effects were analyzed by one-way ANOVA ($P < 0.05$): H indicates hormone effect and NS stands for nonsignificant. LSD was applied as a *post hoc* analysis of hormone effects ($P < 0.05$).

^aSignificant difference from control.

^bSignificant difference from OVX.

Effects of hormonal manipulation on adiponectin, AdipoR1, and Appl1 levels in WGM and in L6E9 myotubes

Ovariectomy decreased adiponectin expression in WGM (Fig. 3A). ADIPOR1 protein levels from WGM also decreased in OVX rats and they were fully restored in OVX + E₂ animals (Fig. 3B). *Appl1* mRNA levels decreased with ovariectomy, although this decrease did not reach statistical significance ($P = 0.052$), and E₂ replacement returned *Appl1* expression to control levels (Fig. 3C). In L6E9 myotubes, E₂ and Pg administration increased adiponectin expression (Fig. 3D). *AdipoR1* mRNA levels were also increased by E₂ treatment but they decreased in

testosterone-treated cells (Fig. 3E). *Appl1* mRNA levels were also decreased by testosterone treatment, but E₂ treatment had no effect (Fig. 3F).

Discussion

Previous studies have reported that skeletal muscle of female rats shows greater mitochondrial differentiation and content accompanied by a higher OXPHOS capacity and better antioxidant response than that of males, which could explain the greater capacity of females to adapt to altered metabolic energy situations (Colom *et al.* 2007a, Gómez-Pérez *et al.* 2012). In addition, this sexual

Figure 2

Mitochondrial mass and biogenesis markers in L6E9 myotubes treated with vehicle, E₂ (10 nM), Pg, or testosterone (1 μ M both). (A) Mitochondrial staining of L6E9 myotubes treated with E₂, Pg, or testosterone (T). (B) Green fluorescence quantification in L6E9 myotubes. Fluorescence of control cells was set as 100 (C and D) mRNA levels of *Pgc1a* and *Tfam* in L6E9 myotubes. GenEx software was used to analyze the Ct values normalized to 18S Ct.

MTG, Mitotracker Green; C, control; E₂, 17 β -estradiol; Pg, progesterone; *Pgc1a*, peroxisome proliferator-activated receptor coactivator 1 α ; *Tfam*, mitochondrial transcription factor A. Values are expressed as the mean $\pm$ S.E.M. of three independent experiments carried out in duplicate ($n = 6$). Student's *t*-test ($P < 0.05$). *Significant difference vs control. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-14-0008>.

Figure 3

Effects of ovariectomy and E₂ supplementation on adiponectin (A) and on *App1* (C) mRNA and on ADIPOR1 protein levels (B) in white gastrocnemius muscle. Effects of sex hormone administration on adiponectin (D), *AdipoR1* (E), and *App1* (F) mRNA levels in L6E9 myotubes. OVX, ovariectomized rats; OVX + E₂, ovariectomized rats treated with 17β-estradiol; C, control; E₂, 17β-estradiol; Pg, progesterone; *AdipoR1*, adiponectin receptor 1; *APPL1*, adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1. Protein levels are expressed in arbitrary units per mg of protein. α-tubulin was used as a loading control. For mRNA, GenEx software was used to analyze the Ct values normalized to 18S Ct. Values in (A), (B), and (C) are expressed as the mean ± s.e.m. of six animals per group. Hormone effects were analyzed by one-way ANOVA ($P < 0.05$): H indicates hormone effect. LSD was applied as a post hoc analysis of hormone effects ($P < 0.05$): ^asignificant difference from control; ^bsignificant difference from OVX. In (D), (E), and (F) L6E9 cells were treated with either E₂ (10 nM), Pg, or testosterone (1 μM both). Control cells were treated with the equivalent volume of vehicle. Values are expressed as the mean ± s.e.m. of three independent experiments carried out in duplicate ($n = 6$). Student's *t*-test ($P < 0.05$). *Significant difference from control.

dimorphism in mitochondrial functionality has also been associated with the greater susceptibility of male rats to development of skeletal muscle insulin resistance in response to both aging (Gómez-Pérez *et al.* 2011) and high -fat-diet intake (Gómez-Pérez *et al.* 2012). In the present study, we used OVX rats to evaluate the effects of both a decrease in ovarian hormone levels and E₂ replacement on skeletal muscle mitochondrial function and adiponectin expression. In order to identify the specific role of sex hormones, we treated cultured L6E9 myotubes with E₂, Pg, or testosterone. Our results confirm the role of E₂ in the maintenance of proper mitochondrial content and function in skeletal muscle, and support the

idea of E₂-mediated modulation of adiponectin expression, a fact that indicates the existence of a link between mitochondrial function and adiponectin expression in skeletal muscle.

In the present study, more than 2 months separated ovariectomy from killing, which could explain the lack of a significant decrease in E₂ serum levels for OVX animals, a fact that has been previously described (Pantaleao *et al.* 2010, Nadal-Casellas *et al.* 2011b, Amengual-Cladera *et al.* 2012a,c). It is plausible to consider that compensatory mechanisms that alleviate the effects of ovariectomy could have been activated in OVX females. In this sense, it is worth noting that estrogen production has been reported to shift from ovaries to extragonadal sites when ovarian function fails in young females (Simpson 2003). Moreover, results described in previous reports have shown that circulating estrogen concentration increases gradually with time after ovariectomy in rats, which has been attributed to greater aromatase activity in adipose tissue, among other factors (Zhao *et al.* 2005).

Mitochondrial biogenesis involves both mitochondrial proliferation (increase in mitochondrial content) and differentiation (improvement of mitochondrial capacities) (Attardi & Schatz 1988). Our data show E₂-induced enhancement of mitochondrial biogenesis in rat skeletal muscle. We found that E₂ replacement in OVX rats upregulates mRNA levels of *Pgc1a*, a master regulator of mitochondrial biogenesis (Puigserver & Spiegelman 2003), contrary to results reported for smooth muscle in a previous study (Macari *et al.* 2010). In accordance with the increase in *Pgc1a*, *Tfam* and *Nrf1* mRNA levels were also upregulated by E₂. *Tfam* products have been determined to drive transcription and replication of mitochondrial DNA (Ventura-Clapier *et al.* 2008), and *Tfam* is activated by *Pgc1a* through the induction of the expression of *Nrf1* (Wu *et al.* 1999). This stimulatory role of E₂ replacement in mitochondrial proliferation is supported by our *in vitro* results, because E₂ treatment of L6E9 myotubes increases mitochondrial mass, probably through the upregulation of *Pgc1a*, as both parameters show the same profile. Pg treatment brings about the same effects as E₂, although to a lesser extent, whereas testosterone does not seem to be involved in the enhancement of skeletal muscle mitochondrial proliferation.

The E₂-induced increase in mitochondrial proliferation runs in parallel with a higher oxidative capacity in E₂-treated animals (indicated by COX and CS activities and by COX4 protein levels), which is accompanied by a slight recovery of oxygen consumption. These results support the idea that E₂ also increases mitochondrial

differentiation in skeletal muscle. This increase in oxidative capacity might be due to the aforementioned rise in *Nrf1* mRNA levels, as this factor triggers the expression of nuclear genes encoding mitochondrial proteins (Scarpulla 2011), and also due to the enhanced mRNA expression of *Sirt3* that we found in OVX+E₂ rats, as this protein is involved in the regulation of mitochondrial respiratory chain subunits through the reversible acetylation of lysine residues (Schwer *et al.* 2006, Lombard *et al.* 2007).

E₂ replacement also induced a reduction in oxidative stress markers compared with OVX animals, in spite of their higher H₂O₂ production. This fact could be attributed to the maintenance of both SOD and GPx activities, and also to the E₂-associated reestablishment of UCP3 protein levels with E₂ replacement. In fact, UCP3 has been proposed to play a relevant role in protection against ROS production in skeletal muscle (Vidal-Puig *et al.* 2000, Brand *et al.* 2002), and females have been shown to have a better ability to counteract oxidative-stress-associated pathologies induced by high-fat diet in part through greater UCP3 expression (Gómez-Pérez *et al.* 2008). Moreover, the intrinsic antioxidant role of E₂ (Ayres *et al.* 1996) cannot be ruled out, especially considering the high E₂ serum levels reached by OVX+E₂ animals. Mitochondrial function and the response to oxidative stress can be strongly affected by fiber composition (Anderson & Neufer 2006, Picard *et al.* 2008, 2012). Ovariectomy has been reported to modify muscle contractile properties, phenotype, metabolism, and force-generating capacity (Moran *et al.* 2006, Liu *et al.* 2009c), whereas estradiol replacement reverses these alterations (Moran *et al.* 2007, Liu *et al.* 2009b, Cavalcanti-de-Albuquerque *et al.* 2014). In this sense, it is not possible to rule out the hypothesis that the changes observed in white gastrocnemius mitochondrial function and oxidative stress response from OVX and OVX+E₂ rats are mediated by a change in fiber metabolism or phenotype; further studies would be necessary.

Mitochondrial biogenesis requires a continuous cycle of fusion and fission events (Ventura-Clapier *et al.* 2008) to maintain proper mitochondrial dynamics (Chen & Chan 2004). E₂ replacement enhances both fusion and fission processes, as shown by the upregulation of mitofusins and DRP1, paralleling the profile observed for mitochondrial biogenesis markers. This is in agreement with the existence of a connection between both mitochondrial dynamics and biogenesis, which would be modulated by E₂.

Mitochondrial function is linked with adiponectin synthesis in adipocytes (Koh *et al.* 2007, Wang *et al.* 2013). Adiponectin expressed in skeletal muscle can be post-translationally modified within the myotubes, allowing

the formation of biologically active forms that exert local metabolic effects that are independent of circulating levels of adiponectin (Liu *et al.* 2009a). Although *in vivo* E₂ replacement only partially reinstated the decrease in the levels of expression of adiponectin induced by ovariectomy, *in vitro* E₂ and Pg treatments (but not testosterone administration) clearly enhance adiponectin expression in cultured myotubes, as occurred with mitochondrial biogenesis. This indicates a relevant role of E₂ in the stimulation of adiponectin expression in skeletal muscle. This stimulatory effect of E₂ on adiponectin expression in skeletal muscle is not reflected in levels of circulating adiponectin, which is not surprising taking into account that the main contributor to serum levels of adiponectin is WAT. In fact, the serum adiponectin profile parallels noticeably the profile that we have previously observed in the expression of adiponectin mRNA in retroperitoneal WAT (Amengual-Cladera *et al.* 2012a,c). These results indicate that regulation of skeletal muscle adiponectin is independent of circulating levels.

AdipoR1 is the main adiponectin receptor in skeletal muscle (Yamauchi *et al.* 2003), and its action is mediated through APPL1, which acts as a positive regulator of adiponectin signaling in muscle cells (Deepa & Dong 2009). Our *in vitro* results indicate that E₂ stimulates both adiponectin and *AdipoR1* expression, whereas testosterone treatment has the opposite effects and impairs the expression of both adiponectin and key elements of its signaling pathway. The effects of E₂ observed *in vitro* are in agreement with the results obtained in WGM, where the decrease in estrogen levels produced by ovariectomy induces impairment of adiponectin expression and its signaling pathway, which are restored by administration of E₂. However, the E₂-associated restoration of *Appl1* mRNA levels in WGM of OVX rats is not observed in L6E9 myotubes. This lack of response to the sex hormone could be attributed to the absence of stimulation of the adiponectin signaling pathway by the adipokine.

As *AdipoR1* has been reported to mediate the autocrine effects of adiponectin (Jortay *et al.* 2012, Patel *et al.* 2012), our results indicate a role of E₂ in the enhancement of not only skeletal muscle adiponectin production but also of its local action. Moreover, the E₂-mediated increase in *Sirt1* expression at the mRNA level further supports the idea that this hormone could play a role in the stimulation of adiponectin signaling in skeletal muscle, given that *Sirt1* is a downstream effector of the adiponectin signaling pathway that promotes PGC1α activation (Iwabu *et al.* 2010), which induces an improvement in mitochondrial function and biogenesis. These results lead to the idea of a potential link

between adiponectin signaling and mitochondrial function in skeletal muscle in a similar way to that described for WAT (Koh *et al.* 2007, Wang *et al.* 2013) and that is supported by the similarities we observed in this study between the profiles of mitochondrial biogenesis and adiponectin expression under E_2 stimulation.

On the whole, our data indicate that E_2 can be established as a relevant stimulator of the mitochondrial biogenesis process in skeletal muscle, because it enhances mitochondrial proliferation and dynamics, as well as mitochondrial oxidative capacities, whereas oxidative stress is reduced. These results support a role of E_2 in the sexual dimorphism in rat skeletal muscle that we have previously reported, whereby female rats show higher mitochondrial mass, greater oxidative capacity, and also a better antioxidant response than their male counterparts (Colom *et al.* 2007a, Gómez-Pérez *et al.* 2008, 2012). Furthermore, we demonstrate that ovarian hormones play a relevant role in adiponectin synthesis in skeletal muscle, and that E_2 could be a key effector in the stimulation of skeletal muscle adiponectin sensitivity. In contrast, testosterone does not seem to play a relevant role in mitochondrial biogenesis or in modulation of adiponectin expression under the studied conditions, although it decreases adiponectin sensitivity. Finally, the similarities observed between E_2 -induced stimulation of mitochondrial function and biogenesis and enhanced adiponectin expression indicates the existence of a link between mitochondrial function and adiponectin expression in skeletal muscle, which could be modulated by sex hormones.

Declaration of interest

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

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