Opposite effects of 17-β estradiol and testosterone on mitochondrial biogenesis and adiponectin synthesis in white adipocytes

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Abstract

Sexual dimorphism has been found in both mitochondrial functionality and adiponectin expression in white adipose tissue, with female rats presenting more functional mitochondria than males and greater adiponectin expression. However, little is known about the role of sex hormones in this dimorphism. The aim was to elucidate the role of sex hormones in mitochondrial biogenesis and dynamics and in adiponectin synthesis in white adipocytes, and also to provide new evidence of the link between these processes. 3T3-L1 preadipocytes were differentiated and treated either with 17-β estradiol (E2; 10 nM), progesterone (Pg), testosterone (1 mM both), or a combination of Pg or testosterone with flutamide (FLT; 10 μM) or E2 (1 μM). The markers of mitochondrial biogenesis and dynamics and adiponectin expression were analyzed. E2 induced mitochondrial proliferation and differentiation in 3T3-L1, although testosterone showed opposite effects. Pg treatment stimulated proliferation but impaired differentiation. In concerns mitochondrial dynamics, these hormones promoted fusion over fission. FLT treatment indicated that Pg elicits its effects on mitochondrial dynamics through the androgen receptor. E2 coadministration with testosterone or Pg reversed its effects. In conclusion, our results show that E2 induces stimulation of mitochondrial biogenesis in white adipocytes in vitro, especially in situations that imply an impairment of mitochondrial function, whereas testosterone would have opposite effects. Moreover, testosterone and Pg alter mitochondrial dynamics by promoting fusion over fission, while E2 stimulates both processes. All these alterations run in parallel with changes in adiponectin expression, thus suggesting the existence of a link between mitochondrial biogenesis and dynamics and adiponectin synthesis in white adipocytes.

Key Words
- sex hormones
- 17-β estradiol
- progesterone
- testosterone
- mitochondrial biogenesis
- mitochondrial dynamics
- WAT
- adiponectin

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Introduction

Mitochondrial biogenesis is a complex event that includes both mitochondrial proliferation (increase in mitochondrial mass) and differentiation (improvement of mitochondrial capacities) (Attardi & Schatz 1988), whose regulation requires the coordinated contribution of both mitochondrial and nuclear genome (Fernández-Silva et al. 2003). The family of the peroxisome proliferator-activated receptor γ coactivator 1 (PGC1) plays a central role in the regulatory network governing the transcriptional control of mitochondrial biogenesis (Scarpulla 2011), and among them, PGC1a is the master regulator of the mitochondrial biogenesis process (Wu et al. 1999), but other members such as PGC1b function in the same or along similar pathways (Handschin & Spiegelman 2006). In short, PGC1a exerts its effects by activating other transcription factors, such as nuclear respiratory factors (NRFs; Wu et al. 1999), which in turn regulate the expression of nuclear genes involved in mitochondrial biogenesis, including mitochondrial transcription factor A (Tfam), a crucial factor for proper mitochondrial DNA (mtDNA) transcription and replication (Scarpulla 1997, Garstka et al. 2003, Maniura-Weber et al. 2004).

Mitochondrial function is also regulated by changes in mitochondrial morphology, which is the result of the balance between fusion and fission processes. Mammalian mitochondrial fusion is mediated by mitofusins (MFN) and optic atrophy protein 1 (OPA1), while mitochondrial fission is mediated mainly by dynamin-related protein 1 (DRP1) and fission protein 1 (FIS1) (Liesa et al. 2009). Alterations in these mitochondrial dynamics induce changes in mitochondrial shape and size: a shift toward fusion enables the cell to build extended, interconnected mitochondrial networks; whereas when fission prevails mitochondria are mainly small, spherical organelles (Chen & Chan 2004). This plasticity enables mitochondrial recruitment to critical subcellular compartments, mitochondria shape control, content exchange between mitochondria, mitochondrial communication with the cytosol, and mitochondrial quality control (Lee et al. 2004, Chen et al. 2005, 2010, Twig et al. 2008, Liesa et al. 2009).

White adipose tissue (WAT) is more than a simple fat deposit, as it is an endocrine organ that contributes to energy homeostasis mechanisms by releasing proteins that are commonly referred to as adipokines (Harwood 2012, Henry et al. 2012). Adiponectin is an insulin-sensitizing adipokine that modulates energy expenditure and caloric intake, and there is an inverse correlation between the levels of circulating adiponectin and the risk of obesity, insulin resistance, type 2 diabetes, and cardiovascular disease (Ronti et al. 2006, Trujillo & Scherer 2006, Fonseca-Alaniz et al. 2007, Galic et al. 2010). Adiponectin also exerts autocrine effects in WAT, promoting adipocyte differentiation through the activation of peroxisome proliferator-activated receptor γ (PPARγ), a key transcriptional factor to initiate and maintain the fully differentiated adipocyte phenotype (Spiegelman et al. 1993, Fu et al. 2005). Mitochondrial function is an important factor in adipocyte differentiation (Lu et al. 2010), and an impairment of mitochondrial function has been related to alterations in adipokine synthesis, including adiponectin, in WAT (Koh et al. 2007, Wang et al. 2013).

Several studies suggest that sex steroids may be regarded as critical modulators of preadipocyte proliferation and/or differentiation, as well as of the metabolism of mature adipocytes (Roncari & Van 1978, Anderson et al. 2001). In this context, previous research by our group demonstrates that there is a sexual dimorphism in mitochondrial function and biogenesis in WAT, in that mitochondria of female rats are more functional than those of males, and this is accompanied by greater adiponectin expression (Amengual-Cladera et al. 2012a,b). Although estrogens are known to modulate both mitochondrial biogenesis (Mattingly et al. 2008) and adipokine secretion (Hong et al. 2007), our previous studies in ovariectomized rats pointed out that 17β estradiol (E2) would not be the only factor responsible for the differences between males and females in WAT (Amengual-Cladera et al. 2012c).

Thus, the aim of this in vitro study was to elucidate whether sex hormones, specifically E2, mediate changes in the expression of mitochondrial biogenesis and dynamics markers and adiponectin, thus providing new evidence of both the link between mitochondrial function and adiponectin production in WAT and the role of sex hormones in the modulation of this link.

Subjects and methods

Cell culture

Murine 3T3-L1 preadipocytes (American Type Culture Collection, Barcelona, Spain) were routinely stored at 37 °C in a humidified atmosphere of 5% CO2. Fetal bovine serum (FBS), charcoal-stripped FBS, and penicillin–streptomycin were from Biological Industries (Beit-Haemek, Israel), and...
all DMEM were from Gibco (Gibco by Invitrogen). The cells were grown for 2 days after confluence in DMEM/high-glucose medium supplemented with 10% FBS and 1% penicillin–streptomycin. Then, differentiation was induced by incubating for 2 days in the same medium supplemented with 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 μM insulin, and for 2 more days supplemented only with 5 μM insulin. This was followed for 3 days in complete medium, which was replaced 24 h before treatment by phenol red-free DMEM/high-glucose medium supplemented with 1% penicillin–streptomycin and 10% charcoal-stripped FBS to avoid the interference of lipid compounds from the serum. For all the experiments, cell line was between 16 and 21 passages.

Treatments and sample collection

Previous experiments were carried out in order to determine the suitable concentration of each compound (data not shown). To test the possible toxic effects of the treatments, cell death was assessed by the LDH-Cytotoxicity Assay Kit II (BioVision, Inc., San Francisco, CA, USA). None of the treatments induced cell death (data not shown).

Hormones and flutamide (FLT) were purchased from Sigma–Aldrich. They were dissolved in ethanol and added to the cell culture plates for 24 h to a final concentration of 10 nM E2, 1 μM progesterone (Pg), and testosterone. When corresponded, 0.5 μM FLT, an androgen receptor (AR) blocker (Labrie 1993), was added to the plate 1 h before Pg or testosterone. In sex hormone-combined treatments, all concentrations were set at 1 μM. An equivalent volume of ethanol was added to the untreated control plates.

The cells were harvested with TriPure Isolation Reagent (Roche Diagnostics) and DNA, RNA, and protein were isolated following the manufacturer’s instructions, quantified using the Gene5 Take3 Module with the Powerwave XS Reader (Bio-Tek, Winooski, VT, USA), and stored for future use. For immunoblot analysis, cells were harvested in solubilization buffer (50 mM Tris, pH 8.8, 1 mM EGTA, 1% Igepal, 10 mM iodoacetamide, 1 mM leupeptin, 1 mM pepstatin, and 1 mM sodium orthovanadate), incubated for 1 h at 4 °C by shaking, and centrifuged at 15,000 g for 30 min. The supernatant was collected, and the protein content was assessed by a BCA protein Assay Kit (Pierce, Bonn, Germany) and then stored for future use. Protein concentration within culture media was assessed by colorimetric assay (Bradford 1976). To determine enzymatic activities, cells were harvested with sterile water. Cell disruption was carried out mechanically by passing the cells through a pipette. Cell lysate was sonicated at 20 W and centrifuged at 500 g to remove membranes. Citrate synthase (CS) activity was determined spectrophotometrically (Nakano et al. 2005), and protein content was measured using the BCA Kit (Pierce).

Real-time PCR

One microgram of RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U MuLV reverse transcriptase in 10 μl of retrotranscription reaction mixture containing 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 each dNTP in a GeneAmp 9700 Thermal Cycler (Applied Biosystems). Each cDNA was diluted to 1/10. Real-time PCR was carried out for 18 target genes, and 18S (Rnl18s) rRNA was used as a housekeeping gene (Table 1). Real-time PCR was carried out using LightCycler 480 SYBR Green I Master technology on a LightCycler 480 System II rapid thermal cycler (Roche Diagnostics). Each reaction contained 5 μl of LightCycler 480 SYBR Green I Master (containing FastStart Taq DNA polymerase, dNTP mix, reaction buffer, MgCl2, and SYBR Green I dye), 0.5 μM of the sense and antisense specific primers, and 2.5 μl of the cDNA dilution in a final volume of 10 μl. The amplification program consisted of a preincubation step for the denaturation of the template cDNA (2 min, 94 °C), followed by 40 cycles consisting of a denaturation step (95 °C, 10 s), annealing (primer-dependent temperature, 10 s), extension (72 °C, 12 s), and fluorescence capture (Table 1). Product specificity was confirmed routinely by melting curve analysis and agarose gel electrophoresis. A negative control without cDNA was run in each assay.

mtDNA quantification

mtDNA content relative to nuclear DNA was assessed by quantitative real-time PCR, using diluted total DNA as template and primers for cytochrome c oxidase subunit 2 (Cox2 (mtCo2)) as a mitochondrial gene and 18S as a nuclear gene (Table 1). Reactions were carried out by real-time PCR (see above).

Western blot analysis of protein levels

A volume of 30 μl of conditioned culture medium or 30 μg of purified cell protein (for medium and cell adiponectin determination respectively) or 30 μg of protein from homogenate (for estrogen receptor (ER) determination)
Table 1  Oligonucleotide primer sequences and conditions used in real-time PCR amplification in 3T3-L1 adipocytes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primers (5’→3’)</th>
<th>Annealing temperature (°C)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>CTCCTCAATGGATCCTCGT</td>
<td>AAGGGCTACCACTCATCCA</td>
<td>54</td>
<td>160</td>
</tr>
<tr>
<td>Cox2</td>
<td>AAGACGCACACTCCCTATT</td>
<td>CTCAGTATCATTGGTGCCCTT</td>
<td>53</td>
<td>291</td>
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<tr>
<td>Cox4</td>
<td>AGAGGCGGCTGAGGAGGAAGAGGA</td>
<td>CCCCATGCCGGAGGAGGTGA</td>
<td>58</td>
<td>385</td>
</tr>
<tr>
<td>Cs</td>
<td>GTTGCTGGAGACCCTTT</td>
<td>AGAGGCTGGAGGAAGAAC</td>
<td>53</td>
<td>157</td>
</tr>
<tr>
<td>Nrf1</td>
<td>CCTCTGTGCTTTGCTTGCTGCT</td>
<td>TTATCTGCTTTGCTTGCTGATT</td>
<td>55</td>
<td>71</td>
</tr>
<tr>
<td>Gabpa</td>
<td>GGAGGCAAGAACAGAGGAAA</td>
<td>CCGTATGCAAGGGATTAAGTT</td>
<td>60</td>
<td>189</td>
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<tr>
<td>Pgc1a</td>
<td>ATCTACCTCCCTGGGAGCCTTTT</td>
<td>ATGTGCTGCTTTGCTTGGTCT</td>
<td>62</td>
<td>179</td>
</tr>
<tr>
<td>Pgc1b</td>
<td>ACTATGATCCCACTGGTGAGATGCTGTC</td>
<td>CTTCGGCTGAGGTAGTTGAGATT</td>
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<tr>
<td>Tfat</td>
<td>AGCCAGGTCAAGCTACTCTACT</td>
<td>AAACCCAGAAAGGATCGT</td>
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<td>165</td>
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<tr>
<td>Mfn1</td>
<td>GCTGTCAGAGCCCATCTTTTCT</td>
<td>CAGCCCATGTTTTTCCAAAT</td>
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<td>198</td>
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<td>Fis1</td>
<td>AAGAGTATGGCAGGAGGCTGTT</td>
<td>AAGGCACGGTCAAGGTAGTCC</td>
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<tr>
<td>Drp1</td>
<td>AGAAAGAAGTCCTCCGGCCGAGA</td>
<td>GCTGCCCCTACCAGGTCTCCTC</td>
<td>53</td>
<td>169</td>
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<tr>
<td>Opa1</td>
<td>GATGACACGCTCTCCAGTGA</td>
<td>TCGGGCTAAAGCTACACC</td>
<td>55</td>
<td>176</td>
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<tr>
<td>Adiponectin</td>
<td>GTGCGACGACTCTCCCTGCTC</td>
<td>AGCCGGCTGAGGTAGTCTGACTCT</td>
<td>55</td>
<td>192</td>
</tr>
<tr>
<td>AdipoR2</td>
<td>CCAACACCTGCTTCACTACCC</td>
<td>AGCAACACTCTGCTGCTGAC</td>
<td>54</td>
<td>118</td>
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<tr>
<td>AdipoR1</td>
<td>AGATGGGAGAGGATCTGTTAGAAG</td>
<td>ATGATGCAAGGTATGCTGTC</td>
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<td>100</td>
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<tr>
<td>Resistin</td>
<td>CCAAGGGTCAGCTCCCTCCCGG</td>
<td>AGGGTTGTGTGGGAAATGTG</td>
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<td>169</td>
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<tr>
<td>Pparg</td>
<td>TTTTCAAGGGTGGCAGGTTCTTC</td>
<td>AATCTTGGGACCCCTGTGAGAT</td>
<td>56</td>
<td>173</td>
</tr>
<tr>
<td>ALBPlaP2</td>
<td>CGGACCCCTGACCTTGATGACTTCA</td>
<td>GCACCCCTGACCTTGATGACTTCA</td>
<td>56</td>
<td>234</td>
</tr>
</tbody>
</table>

Cox2, cytochrome c oxidase subunit 2; Cox4, cytochrome c oxidase subunit 4; Cs, citrate synthase; Nrf1, nuclear respiratory factor 1; Gabpa, GA-binding protein α; Pgc1a, peroxisome proliferator-activated receptor coactivator 1α; Pgc1b, peroxisome proliferator-activated receptor coactivator 1β; Tfat, mitochondrial transcription factor A; Mfn1, mitofusin 1; Opa1, optic atrophy protein 1; Fis1, fission protein 1; Drp1, dynamin-related protein; AdipoR2, adiponectin receptors 1 and 2; Pparg, peroxisome proliferator-activated receptor γ; ALBPlaP2, adipocyte lipid-binding protein αP2. 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, 10 s), and annealing (primer-dependent temperature, 10 s), extension (72 °C, 12 s), and fluorescence capture step. Product length was assessed by agarose electrophoresis.

was fractioned by SDS–PAGE and electrotransferred onto a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with blocking solution (5% nonfat powdered milk in PBS, pH 7.5, and containing 0.1% Tween 20) and were incubated with the corresponding antibody: adiponectin was purchased from ProSci Incorporated (Poway, CA, USA), and ERs (ERα and ERβ), tubulin, and BSA (loading controls) were from Santa Cruz Biotechnology. Development of the immunoblots was carried out using an ECL Kit (LAS, Wetzlar, Germany), and processed using ImageJ 1.45 Software, version Advanced Fluorescence 2.3.6 build 5381 (LAS, Wetzlar, Germany), and processed using ImageJ 1.45 (http://imagej.nih.gov/ij/; NIH, Bethesda, MA, USA).

Analysis of cardiolipin content

Cardiolipin content was assayed using nonyl acridine orange (NAO). The cells were seeded in a fluorescence plate, differentiated, and treated with either vehicle, E2, Pg, or testosterone for 24 h. The culture medium was aspirated and NAO (250 nM) dissolved in PBS–glucose (20 mM) was added. The plate was incubated in the dark for 30 min at 37 °C and fluorescence was quantified using a microplate fluorescence reader FLx800 (Bio-Tek) set at 485 nm excitation and 528 nm emission. The fluorescence was corrected by cell density assessed by a violet crystal staining. Briefly, 20 μl of violet crystal (0.5% w/v in 30% acetic acid) was added in all wells and incubated for 10 min. Four washes with distilled water were done and the remaining violet crystal was solubilized in 100 μl of methanol. Absorbance was determined at 570 nm in a spectrophotometer Powerwave XS reader (Bio-Tek).

Laser scanning confocal microscopy

The cells were seeded in cover slips placed in the culture plate and differentiated to adipocytes. Then, cells were treated as described above and, at the time of the analysis, 0.5 μM MitoTracker Green (MTG; Invitrogen) was added. The cells were incubated for 1 h and washed twice with fresh media. Fluorescence was viewed using a Leica confocal microscope and images were acquired using a 63× objective lens and the Leica Application Suite (LAS) Software, version Advanced Fluorescence 2.3.6 build 5381 (LAS, Wetzlar, Germany), and processed using ImageJ 1.45 (http://imagej.nih.gov/ij/; NIH, Bethesda, MA, USA).

Statistical analysis

All data are expressed as mean values ± S.E.M. of at least three independent experiments carried out in duplicate.
The Ct values of the real-time PCR were analyzed using GenEx Standard Software (MultiD Analyses, Goteborg, Sweden). The efficiency of the reaction was taken into account for each gene. Statistical differences between groups were stated either by Student’s t-test, when two groups were compared, or by one-way ANOVA followed by least-significant difference (LSD) post-hoc analysis, when three or more groups were compared. A P value <0.05 was considered statistically significant. All statistical analyses were carried out using the SPSS 17.0 for Windows, Inc. software package.

**Results**

**Effect of hormonal treatments on mitochondrial biogenesis and function markers**

E2 treatment increased the expression of Pgc1a (Pparγc1a), Pgc1b (Pparγc1b), and Tfam, which are the key regulators of mitochondrial content and functional capacity (Table 2). Cox4 mRNA levels, mtDNA, cardiolipin content (an indicator of inner mitochondrial membrane quantity), and mitochondrial mass measured by MTG fluorescence intensity (Fig. 1) were also increased by E2 treatment. Pg treatment also increased mtDNA, MTG intensity, and cardiolipin content, whereas Cox4, Pgc1b, Nrf1, and Gabpa mRNA levels were decreased. Testosterone treatment decreased Cox4, Pgc1b, and Gabpa expression, as well as CS activity and mitochondrial mass measured by MTG fluorescence (Fig. 1).

**Effect of hormonal treatments on markers of mitochondrial dynamics**

The expression of genes encoding proteins involved in fusion and fission processes in mitochondria is shown in Figs 2A, 3A and 4A. E2 treatment induced an increase in Opa1 and in Drp1 (Dnm1l) mRNA levels. Pg upregulated Mfn1 expression and decreased Fis1 and Drp1 mRNA levels. The addition of E2 to Pg-treated cells returned Fis1 and Drp1 levels to control values. FLT addition had the same effect on Drp1 levels and increased Fis1 expression, although without achieving control values in the latter. Testosterone treatment induced an increase in Mfn1 mRNA levels that was reversed to control values by FLT or E2 supplementation. The expression of Fis1 and Drp1 was also decreased by testosterone. FLT and E2 returned Drp1 mRNA levels of testosterone-treated cells to control values and the same happened with Fis1 mRNA when FLT was added. However, Fis1 levels increased over control values in the testosterone + E2 treatment.

**Effect of hormonal treatments on adipokine and adiponectin receptor levels**

Effects of E2, Pg, and testosterone on adipokines and adiponectin receptor expression are shown in Figs 2A, B, 3B, D and 4B, D respectively. E2 induced an increase in adiponectin protein content of 3T3-L1 adipocytes, which was reflected in the culture media levels. Neither adiponectin, nor resistin, nor adiponectin receptor mRNA levels

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**Table 2** Effects of sex hormones treatment on the markers of mitochondrial biogenesis and function in 3T3-L1 adipocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>E2</th>
<th>Pg</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pgc1a mRNA (AU)</td>
<td>1.48 ± 0.17</td>
<td>2.04 ± 0.19*</td>
<td>1.12 ± 0.06</td>
<td>1.96 ± 0.25</td>
</tr>
<tr>
<td>Pgc1b mRNA (AU)</td>
<td>2.42 ± 0.07</td>
<td>3.73 ± 0.43*</td>
<td>1.75 ± 0.24*</td>
<td>2.21 ± 0.06*</td>
</tr>
<tr>
<td>Nrf1 mRNA (AU)</td>
<td>3.28 ± 0.18</td>
<td>3.33 ± 0.38</td>
<td>1.73 ± 0.30*</td>
<td>3.52 ± 0.50</td>
</tr>
<tr>
<td>Gabpa mRNA (AU)</td>
<td>2.80 ± 0.09</td>
<td>2.97 ± 0.32</td>
<td>1.34 ± 0.11*</td>
<td>1.53 ± 0.06*</td>
</tr>
<tr>
<td>Tfam mRNA (AU)</td>
<td>1.55 ± 0.20</td>
<td>2.80 ± 0.39*</td>
<td>1.35 ± 0.11</td>
<td>1.91 ± 0.28</td>
</tr>
<tr>
<td>mtDNA (AU)</td>
<td>5.91 ± 2.37</td>
<td>49.9 ± 17.7*</td>
<td>22.3 ± 9.9*</td>
<td>3.89 ± 1.09</td>
</tr>
<tr>
<td>Cardiolipin content (%)</td>
<td>100 ± 2</td>
<td>112 ± 5*</td>
<td>115 ± 3*</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>Cox4 mRNA (AU)</td>
<td>2.92 ± 0.25</td>
<td>3.44 ± 0.08*</td>
<td>1.73 ± 0.32*</td>
<td>2.19 ± 0.30*</td>
</tr>
<tr>
<td>Cs mRNA (AU)</td>
<td>2.41 ± 0.54</td>
<td>3.17 ± 0.36</td>
<td>2.16 ± 0.23</td>
<td>2.58 ± 0.16</td>
</tr>
<tr>
<td>CS activity (IU/mg DNA)</td>
<td>4.75 ± 0.14</td>
<td>5.08 ± 0.27</td>
<td>4.64 ± 0.41</td>
<td>4.21 ± 0.09*</td>
</tr>
</tbody>
</table>

E2, 17-β estradiol; Pg, progesterone; Pgc1a, peroxisome proliferator-activated receptor γ coactivator 1α; Pgc1b, peroxisome proliferator-activated receptor γ coactivator 1β; Nrf1, nuclear respiratory factor 1; Gabpa, GA-binding protein α; Tfam, mitochondrial transcription factor A; mtDNA, mitochondrial DNA; Cox4, cytochrome c oxidase subunit 4; Cs, citrate synthase. Results are expressed in arbitrary units (AU) or in international units (IU). 3T3-L1 were treated with E2 (10 nM) and Pg and testosterone (1 μM both) for 24 h. Control cells were treated with the equivalent volume of vehicle. Values are expressed as the mean ± S.E.M. of three independent experiments (n=6). The values for vehicle-treated cells were set as 100% for the determinations of cardiolipin. For mRNA levels, GenEx Software was used to analyze the Ct values normalized to 18S Ct. *Difference vs control cells (Student’s t-test, P<0.05).
were affected by E2 treatment. Pg-treated cells showed lower adiponectin mRNA and protein levels as well as lower adiponectin receptor expression than control cells. Combination of Pg and E2 treatments induced a greater decrease in adiponectin expression than Pg alone. Only AdipoR1 (Adipor1) mRNA levels recovered control values when FLT was added to Pg treatment. Pg + E2 treatment restored AdipoR2 (Adipor2) expression to control levels and increased AdipoR1 expression over control values. Resistin mRNA levels increased with Pg treatment, but the combination with either FLT or E2 returned it to control values. Testosterone treatment induced a decrease in adiponectin mRNA and protein levels and also in adiponectin receptor expression. FLT and E2 prevented this testosterone-associated decrease, and in the specific case of AdipoR1, the response overcame control levels. Resistin mRNA levels increased with testosterone treatment and decreased with both FLT and E2 addition.

Figure 1
Mitochondrial mass measured as MTG intensity and representative confocal images. E2, 17ß-estradiol; Pg, progesterone; FLT, flutamide; MTG, Mitotracker Green. 3T3-L1 cell lines were treated with E2 (10 nM), Pg and testosterone (1 µM both), Pg or testosterone (1 µM) plus E2 (1 µM), and Pg or testosterone (1 µM) plus FLT (10 µM) for 24 h. Control cells were treated with the equivalent volume of vehicle. Values are expressed as the mean ± S.E.M. of nine regions of a field of view. Values of control cells were set as 100. ImageJ Software was used to quantify MTG intensity.

(A) The effects of E2 treatments. *Significant difference vs control cells (Student’s t-test, P < 0.05). (B and C) The effects of Pg and testosterone treatments alone and combined with FLT or E2. One-way ANOVA (P < 0.05) and post-hoc analysis (LSD-test, P < 0.05): a, significant difference vs control cells; and c, significant difference vs testosterone-treated cells. (D) Confocal images of 3T3-L1 stained with MTG 0.5 µM. The fluorescence was monitored with a Leica confocal microscope using 63X lens.
Figure 2
Effects of 17β-estradiol on the expression levels of mitochondrial dynamics and adipogenic markers, adipokines and adiponectin receptors on 3T3-L1 adipocytes (A), and on intracellular and secreted adiponectin protein levels (B). E2, 17β-estradiol; Mfn1, mitofusin 1; Opa1, optic atrophy protein 1; Fis1, fusion protein 1; Drp1, dynamin-related protein; Adipor1 and Adipor2, adiponectin receptors 1 and 2; Pparg, peroxisome proliferator-activated receptor γ; ALBP/aP2, adipocyte lipid-binding protein aP2; Ad, adiponectin. 3T3-L1 adipocytes were treated with E2 (10 nM) for 24 h. Control cells were treated with the equivalent volume of vehicle. Values are expressed as the mean ± S.E.M. of three independent experiments performed in duplicate (n = 6). Values of control cells were set as 100.

GenEx Software was used to analyze the Ct values normalized to 18S Ct (GenEx Software, MultiD Analyses, Goteborg, Sweden). Two representative immunoblots are shown below the corresponding graphs, and when immunoblots have been grouped from different parts of the same gel it is indicated by lines in the figure. *Significant difference vs control cells (Student’s t-test, P < 0.05).

Effect of hormonal treatments on the mRNA levels of adipogenic markers and on ER protein ratio

The expression of the adipogenic marker ALBP/aP2 (Fabp4) was enhanced in E2-treated cells (Fig. 2A). Pparg expression was not affected by E2 treatment but was downregulated by Pg (Fig. 3C). The addition of E2 induced an increase that resulted in higher levels than those in control cells. ER protein ratio (ERα:ERβ) was unaffected by E2 and Pg treatments, whereas it was decreased by testosterone treatment (Fig. 5).

Discussion

Mitochondrial biogenesis is aimed at maintaining optimal mitochondrial function (Lee & Wei 2005). Our study reveals that E2 induces mitochondrial proliferation in white adipocytes – as indicated by the rise in mtDNA and in mitochondrial mass – through the increase in both Pgc1a and Pgc1b and also in Tfam expression. PGC1α is set as the master regulator of mitochondrial biogenesis. It activates NRFs that bind and upregulate the expression of nuclear genes encoding mitochondrial proteins, as well as the expression of Tfam, a factor involved in the regulation of the transcription and replication of the mitochondrial genome (Clayton 1992, Dairaghi et al. 1995, Puigserver & Spiegelman 2003). Along these lines, E2-induced Pgc1a upregulation also enhances mitochondrial differentiation or maturation in 3T3-L1 adipocytes, as revealed by the rise in Cox4 expression and in cardiolipin content. These results agree with our previous in vivo studies, in which we reported that the WAT of female rats showed a higher mitochondrial content than that of males, which was also associated with an increase in both Pgc1a and Tfam expression (Amengual-Cladera et al. 2012a,b).

Interestingly, the effects of testosterone on mitochondrial biogenesis are opposite to those observed with E2. This androgen promotes a decrease in mitochondrial mass (measured by MTG and by CS activity) in 3T3-L1 adipocytes in relation to control cells that is accompanied by a decrease in Pgc1b and Gabpa, but not in Pgc1a, thus indicating that E2 and testosterone effects in mitochondrial biogenesis are mediated by different pathways. Furthermore, the testosterone-induced decrease in ER ratio (ERα:ERβ) would be in agreement with the negative effects of testosterone in mitochondrial biogenesis, because ERβ, which is known to be activated by testosterone metabolites (Hiroi et al. 2013), represses NRFs expression and a subset of mitochondrial respiratory chain genes (O’Lone et al. 2007).

These opposite effects of E2 and testosterone in mitochondrial biogenesis are also evident in mitochondrial dynamics. Along with mitochondrial biogenesis, mitochondrial dynamics (i.e. fission and fusion processes) is crucial for proper mitochondrial function,
and even mild defects in mitochondrial dynamics are associated with pathologies such as neuropathies, non-alcoholic fatty liver disease progression, and type 2 diabetes (Liesa et al. 2009, Zorzano et al. 2009, Galloway & Yoon 2012). We found that E₂, which enhances mitochondrial biogenesis, stimulates both mitochondrial fusion and fission by upregulating Opa1 and Drp1 expression respectively. Thus, E₂ helps to maintain active, balanced mitochondrial dynamics in white adipocytes that is a key to ensuring proper mitochondrial and, therefore, cell function. Meanwhile, Pg and testosterone upregulate fusion marker Mfn1 expression and impair fission through the decrease in Fis1 and Drp1 mRNA levels. This predominance of mitochondrial fusion over fission induced by testosterone treatment could be understood as a mechanism to counteract the impaired mitochondrial function promoted by this hormone, as enhanced fusion is known to preserve mitochondrial function by favoring content exchange between mitochondria (Detmer & Chan 2007).

The controversial effects of Pg on mitochondrial functionality are remarkable, because it promotes mitochondrial proliferation and fusion, and impairs mitochondrial maturation. It has been reported that progestins in high dosages can bind AR, given that it is closely related to the Pg receptor (Bardin et al. 1983). Pg can also be converted to testosterone in adipocytes, and thus can bind AR. In fact, we found that both Pg and testosterone would act over mitochondrial fission markers through AR, while fusion marker Mfn1 regulation seems to

Figure 3
Effects of progesterone and its combined treatments with flutamide or E₂ on the expression levels of mitochondrial dynamics (A) and adipogenic markers (C), adipokines and adiponectin receptors on 3T3-L1 adipocytes (B), and on intracellular and secreted adiponectin protein levels (D). E₂, 17-β estradiol; Pg, progesterone; FLT, flutamide; Mfn1, mitofusin 1; Opa1, optic atrophy protein 1; Fis1, fission protein 1; Drp1, dynamin-related protein; AdipoR1 and AdipoR2, adiponectin receptors 1 and 2; Pparg, peroxisome proliferator-activated receptor γ; ALBP/aP2, adipocyte lipid-binding protein aP2; Ad, adiponectin. 3T3-L1 adipocytes were treated with Pg (1 μM) alone or in combination with FLT (10 μM) or with E₂ (1 μM) for 24 h. Control cells were treated with the equivalent volume of vehicle. Values are expressed as the mean ± S.E.M. of three independent experiments performed in duplicate (n = 6). Values of control cells were set as 100. GenEx Software was used to analyze the Ct values normalized to 18S Ct. Two representative immunoblots are shown below the corresponding graphs, and when immunoblot bands have been grouped from different parts of the same gel it is indicated by lines in the figure. One-way ANOVA (P < 0.05) and post-hoc analysis (LSD-test, P < 0.05): a, significant difference vs control cells and b, significant difference vs Pg-treated cells.
be AR independent, as the addition of FLT, an AR blocker (Labrie 1993), does not prevent the increase in Mfn1 mRNA levels. It is of great interest that E\textsubscript{2} acts by rebalancing mitochondrial dynamics when administered with either testosterone or Pg, with all markers reaching the control levels. In 3T3-L1 adipocytes, E\textsubscript{2} could act by counteracting stimuli that impair mitochondrial function. Thus, E\textsubscript{2} effects on mitochondrial dynamics would be stronger in situations that imply an impairment of mitochondrial function than in control situations with full mitochondrial functionality. These findings reinforce our previous reported results in a rat model of high-fat, diet-induced mitochondrial dysfunction, in which WAT mitochondrial dynamics was impaired in males but not in females, which the hormonal milieu would protect from the negative effects of an obesogenic diet (Amengual-Cladera et al. 2012b).

Adiponectin is an insulin-sensitizing hormone that modulates energy expenditure and caloric intake (Gallic et al. 2010), and its synthesis has been related to mitochondrial function in WAT (Koh et al. 2007, Wang et al. 2013). In agreement with this idea, the increase in both mitochondrial proliferation and maturation induced by E\textsubscript{2} runs parallel to an increase in adiponectin synthesis and secretion in 3T3-L1 adipocytes. In our study, E\textsubscript{2} also induces an increase in the differentiation status (reflected by the increase in the adipogenic marker ALBP/aP\textsuperscript{2}), which could be related to the increase in adiponectin expression, as adiponectin is known to exert autocrine actions in white adipocytes, regulating cytokine...
secretion (Dietze-Schroeder et al. 2005) and acting as a differentiation factor (Fu et al. 2005). A link between mitochondrial function and adiponectin synthesis is also suggested by testosterone treatment, as we observe a testosterone-induced decrease in adiponectin receptors and adiponectin expression that is consistent with the reported reduction in mitochondrial biogenesis. Such a decrease in adiponectin expression could be mediated by the lowering of Pparg mRNA levels, as adiponectin promoter presents PPAR-responsive elements, and PPARg is a key factor of the adipogenic program (Iwaki et al. 2003, Fu et al. 2005). Indeed, testosterone exerts direct effects on adiponectin secretion, as serum total and high molecular weight adiponectin concentrations are high in castrated rodents and hypogonadal men, and decrease after testosterone therapy (Nishizawa et al. 2002, Xu et al. 2005). As E2 and testosterone coadministration reverts the fall in the expression of both adiponectin and its receptors to the control situation, E2 is acting as a balancing factor in a situation of impaired cell function, as happened with mitochondrial dysfunction.

In conclusion, our study demonstrates the involvement of E2 in the stimulation of mitochondrial biogenesis in white adipocytes, whereas testosterone would have quite opposite effects. These alterations in mitochondrial capacities are paralleled in both cases by changes in adiponectin expression, thus suggesting a link between mitochondrial biogenesis and adiponectin synthesis in white adipocytes, but additional experiments would be necessary to demonstrate this. These data support and extend our previous in vivo observations, in which we reported higher WAT mitochondrial function in female rats than in males which was accompanied by greater adiponectin expression (Amengual-Cladera et al. 2012a, b, c). E2 also enhances mitochondrial dynamics, while testosterone stimulates fusion over fission, probably in an attempt to counteract the impairment of mitochondrial biogenesis promoted by this androgen. Furthermore, E2 stands out as an equilibrator of mitochondrial biogenesis and dynamics in situations that imply mitochondrial dysfunction, such as testosterone treatment, which is in agreement with our in vivo results indicating that mitochondrial dysfunction induced by high-fat diet had fewer detrimental effects in the adiponectin profile of female rats than in male rats (Amengual-Cladera et al. 2012a, b).

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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