17β-estradiol improves hepatic mitochondrial biogenesis and function through PGC1B

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Abstract

Sexual dimorphism in mitochondrial biogenesis and function has been described in many rat tissues, with females showing larger and more functional mitochondria. The family of the peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1) plays a central role in the regulatory network governing mitochondrial biogenesis and function, but little is known about the different contribution of hepatic PGC1A and PGC1B in these processes. The aim of this study was to elucidate the role of 17β-estradiol (E2) in mitochondrial biogenesis and function in liver and assess the contribution of both hepatic PGC1A and PGC1B as mediators of these effects. In ovariectomized (OVX) rats (half of which were treated with E2) estrogen deficiency led to impaired mitochondrial biogenesis and function, increased oxidative stress, and defective lipid metabolism, but was counteracted by E2 treatment. In HepG2 hepatocytes, the role of E2 in enhancing mitochondrial biogenesis and function was confirmed. These effects were unaffected by the knockdown of PGC1A, but were impaired when PGC1B expression was knocked down by specific siRNA. Our results reveal a widespread protective role of E2 in hepatocytes, which is explained by enhanced mitochondrial content and oxidative capacity, lower hepatic lipid accumulation, and a reduction of oxidative stress. We also suggest a novel hepatic protective role of PGC1B as a modulator of E2 effects on mitochondrial biogenesis and function supporting activation of PGC1B as a therapeutic target for hepatic mitochondrial disorders.

Introduction

Sexual dimorphism in mitochondrial biogenesis and function has been reported in many rat tissues, pointing to sex hormones as relevant candidates in the modulation thereof. Female rats show larger mitochondria and higher levels of mitochondrial DNA (mtDNA) and mitochondrial respiratory chain proteins, which are consequently
associated with greater oxidative capacity and increased ATP content in many tissues (Justo et al. 2005a, Stirono et al. 2005, Gómez-Pérez et al. 2008, Guevara et al. 2009, Nadal-Casellas et al. 2010, 2013, Amengual-Cladera et al. 2012, Gaignard et al. 2015). Research using cell cultures treated with 17β-estradiol (E2) also supports these previous findings in rats, denoting a main role of this hormone in modulating mitochondrial biogenesis and function (Chen et al. 1998, Mattingly et al. 2008, Caplonch-Amer et al. 2013, 2014, Guo et al. 2013, Sbert-Roig et al. 2016). In postmenopausal women and in ovariectomized (OVX) rats, estrogen deficiency is associated with body weight gain and abdominal fat deposition (Tchesnoff et al. 2000, Moreno et al. 2015), hepatic triglyceride accumulation (Völzke et al. 2007, Moreno et al. 2015), and increased insulin resistance (Szmuiłowicz et al. 2009). In this same line, men usually show greater hepatic fat accumulation than premenopausal women, a profile that is inverted when the comparison is made with women after menopause (Park et al. 2006). Moreover, increased defense against oxidative stress has also been reported in females of many mammalian species, which may also corroborate a protective role of estrogen (Borrás et al. 2003, 2010, Viña et al. 2005). All in all, estrogen seems to positively influence mitochondrial function and energy homeostasis.

The peroxisome proliferator-activated receptor gamma coactivator-1 (PGC1) family of transcriptional coactivators plays a central role in regulating mitochondrial biogenesis and function (Scarpulla 2011). This regulation is achieved by both PGC1A and PGC1B through coactivation of nuclear respiratory factors 1 (Nrf1) and 2 (Nrf2) and estrogen-related receptor alpha (ERR-alpha) (Wu et al. 1999, Schreiber et al. 2004, Shao et al. 2010). These transcription factors regulate the expression of mitochondrial transcription factor A (Tfam) and mitochondrial subunits of the electron transport chain complex (Wu et al. 1999). PGC1A was originally discovered as a cold-inducible coactivator of adaptive thermogenesis and mitochondrial biogenesis, in both brown adipose tissue and skeletal muscle, where it is abundantly expressed (Puigserver et al. 1998, Liu & Lin 2011). PGC1A levels in liver are minimal under fed condition but a dramatic increase is observed during fasting and under diabetes, thereby increasing gluconeogenesis, has been reported (Herzig et al. 2001). PGC1B regulates hepatic fatty acid metabolism by controlling gene expression such as those regulating both b-oxidation (Lin et al. 2003) and de novo synthesis of fatty acids (Chambers et al. 2012). In contrast, PGC1B, which shares sequence similarities with PGC1A, is a poor inducer of hepatic gluconeogenic genes (Lin et al. 2003).

Table 1 Oligonucleotide primer sequences and conditions used in real-time PCR amplification in liver and HepG2 hepatocytes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′ → 3′)</th>
<th>Reverse primer (5′ → 3′)</th>
<th>Annealing temperature (°C)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pgc1a (R)</td>
<td>ATCTACTGCTGGGAGCCCT</td>
<td>ATGTGTGGCCTCCTGCTCT</td>
<td>60</td>
<td>180</td>
</tr>
<tr>
<td>Pgc1b (R)</td>
<td>ATCATGATCCACGTCGAGGAGGTC</td>
<td>CTTGCTGAGGATTTGGGATTC</td>
<td>55</td>
<td>152</td>
</tr>
<tr>
<td>Tfm (R)</td>
<td>GCTAAACCCCAGTGCAAAAA</td>
<td>CAGGCTCCTTTTGGTTTCCC</td>
<td>60</td>
<td>269</td>
</tr>
<tr>
<td>Nrf1 (R)</td>
<td>TTTACTCGTGCTGCTGATGG</td>
<td>CTCAGTGCTGCTGCTGCTT</td>
<td>55</td>
<td>92</td>
</tr>
<tr>
<td>mtDNA (R)</td>
<td>TACAGCTGAGGACCAAAAA</td>
<td>GGTAGGGGAGTGTGAGGAG</td>
<td>60</td>
<td>162</td>
</tr>
<tr>
<td>Pepck (R)</td>
<td>GGGGTTGGTTACTGGAAGAGG</td>
<td>GGGGCTCAATAATGGGAGAAGGAC</td>
<td>60</td>
<td>161</td>
</tr>
<tr>
<td>Ppara (R)</td>
<td>TCTCGCCTGGTGAAGATG</td>
<td>CCTCGAATTGGGGAGAGAGAGG</td>
<td>60</td>
<td>151</td>
</tr>
<tr>
<td>Cpt1a (R)</td>
<td>CGCACTTAGACGGAGAGGAGAG</td>
<td>CTTGCGACCACTGCCCTG</td>
<td>60</td>
<td>184</td>
</tr>
<tr>
<td>Srebp1c (R)</td>
<td>CGCTACCCTGTCCTTCTAATGAGC</td>
<td>AGTCTTCTGGTGTGCTGGTTGAAG</td>
<td>60</td>
<td>140</td>
</tr>
<tr>
<td>Gapdh (R)</td>
<td>ATCTTGCGATGTCGAGGAGG</td>
<td>CGGTCACGCTGAGGATGAC</td>
<td>60</td>
<td>178</td>
</tr>
<tr>
<td>PGC1A (H)</td>
<td>CACTCCCTCCTACAAAGCCAA</td>
<td>GGACCTGCGTGCTTGAGAAGAA</td>
<td>60</td>
<td>190</td>
</tr>
<tr>
<td>PGC1B (H)</td>
<td>GCTGACAAAGAATAAGAGAGG</td>
<td>TGAATTGGAATCAGTGCAGT</td>
<td>60</td>
<td>184</td>
</tr>
<tr>
<td>TFAM (H)</td>
<td>GTGTTTCTTACTCTTCTTGGC</td>
<td>ACTCGCCCTATAAGCATCTT</td>
<td>60</td>
<td>200</td>
</tr>
<tr>
<td>NRF1 (H)</td>
<td>TGACCCACATTGGCTGATG</td>
<td>CTCAGTGCTGCTGCTGCT</td>
<td>60</td>
<td>150</td>
</tr>
<tr>
<td>COX1 (H)</td>
<td>TACGTGTTAGCAGCCCTTCCACT</td>
<td>GGAAGGCGAGAAATGGGTTG</td>
<td>60</td>
<td>189</td>
</tr>
<tr>
<td>PEPCK (H)</td>
<td>AGTCAAGAGATGGGGAAACC</td>
<td>CCAAGAAGACTCTAGTCG</td>
<td>60</td>
<td>196</td>
</tr>
<tr>
<td>PPARA (H)</td>
<td>CGGTGACATTTCTGCTGCTT</td>
<td>CGGTGACATTTCTGATACAC</td>
<td>60</td>
<td>184</td>
</tr>
<tr>
<td>CPT1A (H)</td>
<td>TGCCCTGGTACGTCCCTG</td>
<td>AGATAAAGCAGCAGGAGG</td>
<td>60</td>
<td>172</td>
</tr>
<tr>
<td>SREBP1C (H)</td>
<td>TAAGTCTGGCGACTCTGTCG</td>
<td>TGAAAGGTTGAGCCAGCATC</td>
<td>60</td>
<td>157</td>
</tr>
<tr>
<td>GAPDH (H)</td>
<td>CTTGTTGCGACGGGTCTCTA</td>
<td>CCACCTCGCCACCTTTGACG</td>
<td>60</td>
<td>156</td>
</tr>
</tbody>
</table>

Cox1, cytochrome c oxidase subunit 1; Cpt1a, carnitine palmitoyltransferase 1a (liver); Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; H, human; mtDNA, mitochondrial DNA; Nrf1, nuclear respiratory factor 1; Pepck, phosphoenolpyruvate carboxykinase; Pgc1a, peroxisome proliferator-activated receptor coactivator 1a; Pgc1b, peroxisome proliferator-activated receptor coactivator 1b; Ppara, peroxisome proliferator-activated receptor alpha; R, rat; Srebp1c, sterol regulatory element-binding transcription factor 1c; Tfam, mitochondrial transcription factor A.
Table 2  Effects of ovariectomy and 17β-estradiol replacement on body and liver weights, liver composition and serum levels of estradiol and insulin.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OVX</th>
<th>OVX+E2</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>218 ± 5</td>
<td>298 ± 6a</td>
<td>242 ± 5ab</td>
<td>H</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>5.84 ± 0.11</td>
<td>6.64 ± 0.17a</td>
<td>6.08 ± 0.16a</td>
<td>H</td>
</tr>
<tr>
<td>TG (mg/g tissue)</td>
<td>20.9 ± 0.5</td>
<td>24.2 ± 0.6a</td>
<td>19.6 ± 0.6b</td>
<td>H</td>
</tr>
<tr>
<td>E2 (%)</td>
<td>100 ± 24</td>
<td>61.3 ± 9.7</td>
<td>240 ± 103ab,b</td>
<td>H</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.40 ± 0.01</td>
<td>0.51 ± 0.04a</td>
<td>0.40 ± 0.02ab</td>
<td>H</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. of six animals per group. Hormone effect is analyzed by one-way ANOVA (P < 0.05): H indicates hormone effect. Least significant difference (LSD) is applied as a post hoc analysis of hormone effect (P < 0.05).

The aim of the present study was to clarify the contribution of E2 to the differences observed in hepatic mitochondrial biogenesis and function between male and female rats. Furthermore, the involvement of both PGC1A and PGC1B were assessed as mediators of these effects. To accomplish this, studies were performed in vivo using OVX rats with E2 replacement, which were combined with in vitro studies silencing PGC1A or PGC1B in HepG2 cells treated with E2.

Materials and methods

Animals and treatments

Animal experiments were performed in accordance with general guidelines approved by EU regulations (2010/63/UE) and our institutional ethics committee. Wistar female rats both control (n=6) and OVX (n=12) were purchased from Charles River, where OVX rats had been ovariectomized or sham operated at 5 weeks of age to suppress endogenous ovarian steroid production. Animals were kept in a controlled environment of 22°C and 65 ± 3% humidity on a 12-h light–darkness cycle with free access to water and pelleted standard diet (A04, SAFE, Paris, France). At ten weeks of age, OVX rats were divided into two experimental groups: OVX (n=6) and OVX treated with E2 (OVX+E2) (n=6). OVX+E2 rats...
were given a subcutaneous injection of 10 µg/kg of E2 dissolved in corn oil (vehicle) every 48 h for 4 weeks prior to killing, whereas OVX group was treated only with the vehicle. The estrous cycle was regularly determined by measuring vaginal wall impedance with the estrous cycle monitor Impeast (Cibertec, Madrid, Spain) and confirmed by microscopic examination of fresh vaginal smears, so all the control animals were in the diestrous phase at the time they were killed. At 14 weeks of age and after a 12-h period of fasting, rats were killed by decapitation. Trunk blood was collected and the liver was rapidly removed and weighed, and a piece was frozen at −80°C until analysis. Serum levels of E2 and insulin were determined by ELISA kits provided by DRG Instruments (Marburg, Germany) and Mercodia (Uppsala, Sweden), respectively. Before E2 analysis, the serum organic fraction was concentrated using a sample extraction procedure (Dighe & Sluss 2004).

Hepatic homogenate preparation and determinations
Fresh liver was homogenized with a disperser (IKA T10 basic ULTRA-TURRAX) in a proportion of 0.1 g in 1 mL STE buffer (250 mM sucrose, 20 mM Tris–HCl, 40 mM KCl, 2 mM EDTA, pH 7.4) and immediately used to assay the activities of cytochrome c oxidase (COX) (Chrzanowska-Lightowlers et al. 1993), citrate synthase (CS) (Nakano et al. 2005), and superoxide dismutase (SOD) (Quick et al. 2000) by spectrophotometric methods. Hepatic triglycerides were measured spectrophotometrically using a commercial kit (Spinreact, Girona, Spain). The remaining volume was stored at −20°C with phosphatase and protease inhibitors (1 mM sodium orthovanadate, 1 mM PMSF, 10 µM leupeptin and 10 µM pepstatin) until analysis. Protein carbonyl groups, an index of protein oxidation, were determined in the homogenates by immunoblotting using the OxiSelect Protein Carbonyl Immunoblot Kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer’s protocol. Isolation and
semi-quantification of mtDNA, an index of mitochondrial content, was carried out in tissue homogenates as previously described (Justo et al. 2005b). Briefly, real-time PCR was performed to amplify the mitochondrial gene NADH dehydrogenase subunit 4, which is exclusive of mtDNA. The oligonucleotide sequences and annealing step conditions used in real-time PCR are detailed in Table 1. RNA was obtained using TriPure Isolation Reagent (Roche Diagnostics).

HepG2 cell culture

Human hepatocellular carcinoma cell line HepG2 (American Type Culture Collection, Manassas, USA) was routinely maintained at 37°C in a humidified atmosphere of 5% CO₂ in MEM containing 5.6 mM glucose (Biowest, Nuaillé, France), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, from Biowest and Biological Industries (Beit-Haemek, Israel), respectively. All experiments were conducted between sixth and twelfth passages. Cells were seeded in six well plates until 70% confluence and then the medium was replaced 24 h before treatment by phenol red-free MEM 5.6 mM glucose (Biological Industries) supplemented with 10% charcoal-stripped FBS (Biological Industries) and 1% penicillin-streptomycin (Biowest). Afterward, cells were treated with 10nM E2 or the vehicle (ethanol) for 40 h. Preliminary experiments were carried out to determine the appropriate concentration of each compound, and cell death was assessed in 96 well plates using crystal violet nuclear staining assay (Nagamine et al. 2009). Briefly, cells were stained with 0.5% (p/v) crystal violet in 30% (v/v) acetic acid for 10 min. After washing, the dye was solubilized in 100μl methanol and absorbance was measured photometrically at 595 nm to determine cell viability. RNA and protein were obtained using TriPure Isolation Reagent (Roche Diagnostics).

Table 3  Effects of ovariectomy and 17β-estradiol replacement on oxidative stress markers in liver.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OVX</th>
<th>OVX+E2</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (IU/g protein)</td>
<td>10.0 ± 0.3</td>
<td>8.25 ± 0.46</td>
<td>12.9 ± 1.2</td>
<td>H</td>
</tr>
<tr>
<td>HNE (au)</td>
<td>100 ± 5</td>
<td>124 ± 4</td>
<td>115 ± 2</td>
<td>H</td>
</tr>
<tr>
<td>Protein carbonyl groups (au)</td>
<td>100 ± 6</td>
<td>129 ± 11</td>
<td>77 ± 19</td>
<td>H</td>
</tr>
</tbody>
</table>

SOD activity is expressed as international units (μmol/min). Protein levels were normalized to GAPDH intensity. Values for control group were set as 100 for the determination of 4HNE and protein carbonyl groups. Values are mean±S.E.M of six animals per group. Hormone effect is analyzed by one-way ANOVA (P<0.05): H indicates hormone effect. Least significant difference (LSD) is applied as a post hoc analysis of hormone effect (P<0.05).

Western blot analysis

About 30–40μg protein from the hepatic homogenates or 20μg from HepG2 cells were fractioned using SDS-PAGE (10–12% acrylamide) and electrotransferred onto a nitrocellulose membrane. The membranes were blocked

Table 4  Effects of ovariectomy and 17β-estradiol replacement on mRNA levels of markers of glucose and lipid metabolism in liver.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OVX</th>
<th>OVX+E2</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepck (au)</td>
<td>1.00 ± 0.03</td>
<td>1.58 ± 0.11</td>
<td>1.14 ± 0.09</td>
<td>H</td>
</tr>
<tr>
<td>Ppara (au)</td>
<td>1.00 ± 0.07</td>
<td>0.99 ± 0.10</td>
<td>1.38 ± 0.05</td>
<td>H</td>
</tr>
<tr>
<td>Cpt1a (au)</td>
<td>1.00 ± 0.11</td>
<td>1.75 ± 0.05</td>
<td>1.59 ± 0.04</td>
<td>H</td>
</tr>
<tr>
<td>Srebp1c (au)</td>
<td>1.00 ± 0.08</td>
<td>1.46 ± 0.08</td>
<td>0.88 ± 0.15</td>
<td>H</td>
</tr>
</tbody>
</table>

GenEx software was used to analyze the Cₐ values normalized to Gadph. Cₐ Values of control group were set as 1. Values are mean±S.E.M of six animals per group. Hormone effect is analyzed by one-way ANOVA (P<0.05): H indicates hormone effect. Least significant difference (LSD) is applied as a post hoc analysis of hormone effect (P<0.05).

*Significant difference from control. ©Significant difference from OVX. OVX, ovariectomized; OVX+E2, OVX treated with 17β-estradiol; Pepck, phosphoenolpyruvate carboxykinase; Ppara, peroxisome proliferator-activated receptor alpha; Cpt1a, carnitine palmitoyltransferase 1a (liver); Srebp1c, sterol regulatory element-binding transcription factor 1c.
Mitochondrial mass was determined by quantifying the band density of each protein in relation to the loading control (GAPDH).

### Analysis of gene expression

About 1 μg RNA was reverse transcribed to cDNA using 25 U MuLV reverse transcriptase in 10 μL retrotranscription mixture (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 2.5 mM random hexamers, 10 U RNase inhibitor, and 500 mM of each dNTP) for 60 min at 42°C in a GeneAmp 9700 thermal cycler (Applied Biosystems). cDNA solutions were diluted with RNase-free water to a final volume of 100 μL, and aliquots were frozen at −20°C until analyzed. Real-time PCR was performed using 25 U MuLV reverse transcriptase in 10 μL. The amplification program consisted of a pre-incubation step for denaturation of template DNA (95°C, 2 min), followed by 40 cycles consisting of a denaturation (95°C, 10 s), annealing (primer-dependent temperature, 10 s), and extension steps (72°C, 10 s). The oligonucleotide sequences and annealing step conditions used in real-time PCR are detailed in Table 1. After each cycle, fluorescence was measured at 72°C. Product specificity was confirmed in initial experiments by agarose gel electrophoresis and routinely by melting curve analysis.

### Measurement of mitochondrial mass, mitochondrial membrane potential and ATP content in HepG2 cells

For all determinations, HepG2 cells were seeded in 96 well plates and treated with E2 as previously described. Values were normalized per number of viable cells determined by crystal violet nuclear staining assay (Nagamine et al. 2009). Mitochondrial mass was assessed using the fluorescent probe N-nonyl acridine orange (NAO) as it specifically stains mitochondrial phospholipid cardiolipin (Petit et al. 1992). Briefly,
0.5 μM NAO were added to HepG2 cells for 30 min at 37°C in darkness and fluorescence measurement was performed in a FLX800 microplate fluorescence reader (Bio-Tek) with excitation at 485 nm and emission at 528 nm. Mitochondrial membrane potential (MMP) was tested using tetramethylrhodamine methylester (TMRM) as a lipophilic cationic dye that accumulates within mitochondria according to the membrane potential (Scaduto & Grotyohann 1999). Cells were dyed with 0.5 μM of TMRM for 15 min at 37°C in darkness and fluorescence intensity was measured with excitation at 552 nm and emission at 576 nm. Finally, ATP was measured with an ATP bioluminescent assay kit (BioVision) following the manufacturer’s instructions.

Statistical analysis

All data from in vivo experiments are expressed as the mean ± S.E.M. of six animals per group. The effects of ovariectomy and E2 supplementation (hormone effect) were analyzed by one-way ANOVA. Least significant difference (LSD) was applied as a post hoc analysis when hormone effect was present. In vitro data from cell viability, cardiolipin levels, MMP, and ATP content are expressed as mean ± S.E.M. (n = 8) and statistical differences were assessed by Student’s t-test. In vitro data from silencing experiments were analyzed from three independent experiments carried out in duplicate (n = 6) and statistical differences were assessed by Student’s t-test. All statistical analyses were performed using a statistical software package (SPSS 20.0 for Mac OSX), and a P value <0.05 was considered statistically significant.

Ct values of 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.
Table 6 mRNA and protein levels of PGC1A and PGC1B after silencing by specific siRNA in HepG2 cells.

<table>
<thead>
<tr>
<th></th>
<th>Control siRNA</th>
<th>Pgc1a siRNA</th>
<th>Pgc1b siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGC1A mRNA levels (au)</td>
<td>– E2</td>
<td>1.00 ± 0.02</td>
<td>0.66 ± 0.02b</td>
</tr>
<tr>
<td></td>
<td>+ E2</td>
<td>0.99 ± 0.07</td>
<td>0.65 ± 0.03b</td>
</tr>
<tr>
<td>PGC1A protein levels (vs GAPDH)</td>
<td>– E2</td>
<td>100 ± 1.35</td>
<td>65.8 ± 9.27b</td>
</tr>
<tr>
<td></td>
<td>+ E2</td>
<td>96.6 ± 6.97</td>
<td>57.0 ± 4.34b</td>
</tr>
<tr>
<td>PGC1B mRNA levels (au)</td>
<td>– E2</td>
<td>1.00 ± 0.03</td>
<td>1.04 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>+ E2</td>
<td>1.22 ± 0.05a</td>
<td>1.16 ± 0.03a</td>
</tr>
<tr>
<td>PGC1B protein levels (vs GAPDH)</td>
<td>– E2</td>
<td>100 ± 2.38</td>
<td>82.5 ± 11.7</td>
</tr>
<tr>
<td></td>
<td>+ E2</td>
<td>119 ± 6.80a</td>
<td>117 ± 4.30a</td>
</tr>
</tbody>
</table>

GenEx software was used to analyze the C t values normalized to Gapdh C t and protein levels were normalized to GAPDH intensity. Values of control group for mRNA levels were set as 1 and for protein levels as 100. Cells were silenced using 20 nM of specific siRNA for 24 h and subsequently either treated with E2 (10 nM) or left untreated for another 40 h. Values are mean ± S.E.M. of three independent experiments performed in duplicate (n=6). Statistically significant differences were determined using Student’s t-test (P<0.05).

*Significant difference from E2 control. **Significant difference from siControl.

PGC1A, peroxisome proliferator-activated receptor coactivator 1A; PGC1B, peroxisome proliferator-activated receptor coactivator 1B.

Results

Effects of ovariectomy and E2 replacement on body and liver weights, hepatic triglycerides and serum levels of E2 and insulin

Body and liver weights as well as hepatic triglycerides increased in OVX rats (Table 2). E2 replacement resulted in a decrease of these parameters, but only body weight remained higher than that of controls. Ovariectomy decreased E2 serum levels and E2 treatment increased the levels of this hormone. Ovarian withdrawal increased serum levels of insulin, while E2 replacement restored control values.

Effects of ovariectomy and E2 replacement on markers of mitochondrial biogenesis and function in liver and HepG2 cells

Ovariectomy induced an impairment of mitochondrial biogenesis and function, assessed by lower PGC1B, TFAM and ATPase mRNA or protein levels (Fig. 1A and B). The OVX group supplemented with E2 exhibited increased levels of markers of mitochondrial biogenesis and function, such as PGC1B, NRF1, COX1 and ATPase mRNA or protein levels. PGC1A protein levels were not modified under any condition, although mRNA levels rose with E2 administration. mtDNA levels increased in response to E2 treatment compared with control and OVX rats, but the decrease with ovariectomy did not reach statistical significance (P=0.077) (Fig. 1A). COX activity was not altered by ovariectomy but E2 administration enhanced it, whereas ovarian withdrawal diminished CS activity and E2 administration did not restore control values (Fig. 1C).

Accordingly, protein and mRNA levels of PGC1B were enhanced in HepG2 cells in response to E2 treatment, while PGC1A did not suffer any modification (Fig. 2A and B). TFAM, NRF1, COX1, COX4 and ATPase mRNA or protein levels also rose with E2 treatment. Furthermore, E2-treated HepG2 cells exhibited higher mitochondrial mass and MMP as well as upper levels of ATP content (Fig. 3).

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

Ovariectomy was associated with decreased SOD activity, which was increased with E2 replacement even in comparison with control rats (Table 3). Lipid peroxides (HNE) rose with ovariectomy, although E2 treatment did not fully revert this condition. Although levels of carbonyl groups (a marker of protein damage) did not increase with ovariectomy, E2 administration led to a significant reduction compared with OVX group.

Effects of ovariectomy and E2 replacement on glucose and lipid metabolism markers in liver and HepG2 cells

Concomitant with hormone withdrawal, phosphoenolpyruvate carboxykinase (Pepck) mRNA levels rose, whereas E2 supplementation restored them to control values (Table 4). Both carnitine palmitoyltransferase 1a (Cpt1a) and sterol regulatory element-binding transcription factor 1c (Srebp1c) were increased with ovariectomy. E2 treatment restored only Srebp1c to control values. Gene expression of peroxisome proliferator-activated receptor alpha (Ppara) increased with E2 replacement in comparison with control and OVX rats.
In HepG2 cells, E2 treatment led to a decrease in PEPCK expression, while mRNA levels of both PPARA and CPT1A were increased (Table 5). SREBP1C was not altered by E2.

**Effects of PGC1A or PGC1B knockdown and E2 treatment on markers of mitochondrial biogenesis and function**

Cell viability was assessed in silencing experiments, showing that transfection with control siRNA or Pgc1a decreased viability by 30–40% compared to non-transfected hepatocytes whereas Pgc1b knockdown significantly decreased cell viability by 65% (Fig. 4).

PGC1A and PGC1B inhibition by siRNA was determined by measuring mRNA and protein levels after transfection and treatment with E2 (Fig. 5 and Table 6). siRNA specifically knocked down its target genes by 34–50% compared to hepatocytes transfected with a non-target siRNA, both for mRNA and protein levels. Silencing efficiency was not statistically different between both PGC1A and PGC1B knockdown.

HepG2 cells treated with siRNA targeting PGC1B reduced TFAM and ATPase levels by 25% compared to basal conditions and remained lower with E2 treatment (Figs 5 and 6). PGC1A downregulation did not produce a reduction of these parameters either under basal conditions or E2 treatment, compared to its control.

**Discussion**

Previous studies have reported the existence of sexual dimorphism in mitochondrial biogenesis and function in rat tissues, which could be attributed to sex hormones (Justo et al. 2005a, Stirone et al. 2005, Gómez-Pérez et al. 2008, Guevara et al. 2009, Nadal-Casellas et al. 2010, 2013, Amengual-Cladera et al. 2012, Gaignard et al. 2015). In the present study, we demonstrate the involvement of E2 in maintaining energy homeostasis both in liver of OVX rats and in HepG2 cells. We also provide evidence for an active role of hepatic PGC1B in mediating the effects of E2 in mitochondrial function using HepG2 cells transfected with specific siRNA targeting PGC1A or PGC1B.

Ovariectomy-induced impairment of mitochondrial biogenesis and function led to a decline in the main markers of mitochondrial differentiation and proliferation (Tfam and mtDNA) and a decrease in oxidative capacity, as reflected by the decrease in CS activity and ATPase levels, suggesting that ovarian hormone deprivation is associated with an impairment of hepatic mitochondrial function. These results further support the role of ovarian hormones in mitochondrial function enhancement, which is in agreement with studies in rat liver showing that females exhibit greater oxidative capacity and mitochondrial differentiation compared to males (Nadal-Casellas et al. 2010). Furthermore, we meaningfully demonstrate that E2 may be a key factor in mitochondrial function, as this hormone restored hepatic mitochondrial function in OVX rats through an enhancement of mitochondrial content, which ran in parallel with improved oxidative capacity. Consistently with *in vivo* findings, HepG2 cells treated with E2 increased mitochondrial mass and MMP, thus increasing ATP content and levels of essential proteins involved in oxidative metabolism and mitochondrial biogenesis. OVX rats also exhibited greater hepatic oxidative stress, indicated by reduced SOD activity and higher lipid peroxidation, in agreement with previous studies (Moreno et al. 2015). Treatment with E2 increased antioxidant response, thus lowering protein oxidation damage in accordance with E2 involvement in defense against oxidative stress (Borrás et al. 2003, 2010, Viña et al. 2005).

We also reported defective glucose and lipid hepatic metabolism in OVX rats, suggested by the increase in Pepck expression and triglyceride accumulation, both of which were restored with E2 treatment. These results are in agreement with previous studies that connect estrogens with insulin sensitivity (Szmulowicz et al. 2009, Nadal-Casellas et al. 2012), hepatic gluconeogenesis (Ahmed-Sorour & Bailey 1981, Bryzgalova et al. 2006), and lipid metabolism (D’Eon et al. 2005, Völzke et al. 2007, Paquette et al. 2008, 2009, Suzuki & Abdelmalek 2009). However, the higher Pepck mRNA levels in the liver of OVX rats, which point to an induction of hepatic gluconeogenesis, may be attributed to posttranslational modifications of PGC1A, such as phosphorylation or deacetylation, which increase its activity (Rodgers et al. 2005, Jäger et al. 2007), as levels of this coactivator remained unaltered with ovariectomy. In addition, the impaired oxidative capacity described above in combination with increased lipogenesis, such as the higher Srebplc mRNA levels shown, caused hepatic triglyceride accumulation. In contrast, E2 replacement ameliorated mitochondrial function and biogenesis of OVX rats, accompanied by diminished lipogenesis (*Srebplc*) and increased fatty acid oxidation (*Ppara* and *Cpt1a*), leading to a reduction of hepatic fat accumulation. Taken all together, E2 seems to contribute to the normal functioning of hepatocytes, as...
ovarian hormonal withdrawal produces an impairment of mitochondrial function and metabolism profile, which is reversed by E2 administration.

Both PGC1A and PGC1B play a pivotal role in regulating mitochondrial biogenesis and function (Scarpulla 2011). Our results suggest that PGC1B could be involved in regulating E2 effects in promoting mitochondrial function. In both in vivo and in vitro studies, the changes in mitochondrial function took place in parallel with changes in PGC1B protein levels, while those of PGC1A remained unaltered despite hormone manipulations. Thus, we aimed to look into the involvement of both PGC1A and PGC1B in the regulation of mitochondrial biogenesis and function in HepG2 cells using specific siRNA. Consistently, downregulation of PGC1B in HepG2 cells decreased TFAM and ATPase protein levels both in basal and E2-treated conditions. However, PGC1A downregulation did not diminish these markers in any condition. Our results provide strong evidence of the effects of E2 in mitochondrial function enhancement through PGC1B, as mitochondrial function did not ameliorate in hepatocytes treated with E2 when PGC1B was knocked down. On the contrary, TFAM and ATPase protein levels were increased by E2 treatment despite PGC1A knockdown. Effects of E2 on ameliorating mitochondrial function through PGC1B rather than PGC1A have already been suggested in some mice tissues (Pardo et al. 2011, Kemper et al. 2013) and in 3T3-L1 adipocytes (Pardo et al. 2011), but as far as we are concerned it has not been yet described in liver. However, although the involvement of PGC1A in hepatic mitochondrial function and biogenesis cannot be entirely ruled out, PGC1B focusing attention on these processes has to be taken into account.

The PGC1B role on the protection of liver from steatosis has been associated with a combined and balanced effect on lipid synthesis and secretion, and on mitochondrial biogenesis and function (Sonoda et al. 2007, Chambers et al. 2012, Bellafante et al. 2013). Our study suggests that E2 preserved hepatocytes from steatosis through an induction of oxidative phosphorylation, fatty acid b-oxidation, and a decrease in oxidative stress, and that PGC1B may contribute to these actions by inducing mitochondrial biogenesis. Although PGC1B has been described to enhance lipogenesis through Srebp1c in response to excess energy intake (Lin et al. 2005), we found no association between higher lipogenesis and PGC1B enhancement under E2 treatment. On the other hand, a role for estrogens in the prevention of hepatic fat accumulation has been described in OVX rats through an upregulation of Srebp1c that was restored by E2 treatment (Paquette et al. 2008), so it is plausible to consider that E2 signaling may prevent hepatic lipogenesis despite PGC1B enhancement.

On the whole, our results demonstrate that E2 induces mitochondrial biogenesis and function in hepatocytes by means of an enhancement of mitochondrial content and oxidative capacity, which lead to lower hepatic lipid accumulation and a reduction of oxidative stress. Hence, E2 could play a pivotal role in the sexual dimorphism previously described in rat liver in mitochondrial function and biogenesis. Moreover, our study provides evidence that PGC1B may mediate hepatic mitochondrial function and be more likely to respond to hormonal milieu than PGC1A. Therefore, we suggest a novel hepatic protective role of PGC1B as a modulator of E2 effects on mitochondrial biogenesis and function supporting activation of PGC1B as a therapeutic target for hepatic mitochondrial disorders.

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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