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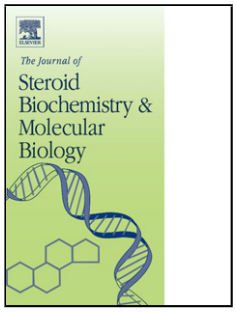

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GPER and ERα mediate estradiol enhancement of mitochondrial function in inflamed adipocytes through a PKA dependent mechanism

Short title: GPER and ERα mediate estradiol enhancement of mitochondrial function in adipocytes

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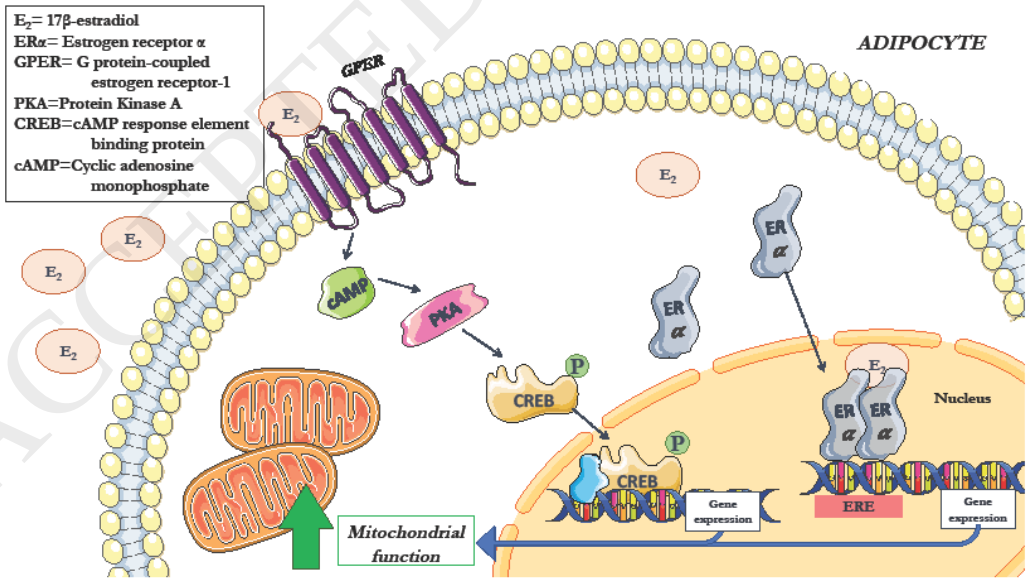

Highlights

- In adipocytes 3T3-L1, a IL6 treatment induces a profile of mitochondrial dysfunction and inflammation.
- In adipocytes 3T3-L1, estradiol ameliorates the adverse effects of inflammation on mitochondrial function through the combined activation of both GPER and ERα.
- *The effects improving the mitochondrial function of estradiol in adipocytes 3T3-L1 through ERα and GPER is dependent on protein kinase A (PKA).

1. Abstract

Obesity is associated with inflammation, dysregulated adipokine secretion, and disrupted adipose tissue mitochondrial function. Estradiol (E2) has been previously reported to increase mitochondrial function and biogenesis in several cell lines, but neither the type of oestrogen receptor (ERα, ERβ and GPER) involved nor the mechanism whereby such effects are exerted have been fully described. Considering the anti-inflammatory activity of E2 as well as its effects in enhancing mitochondrial biogenesis, the aim of this study was to investigate the contribution of ERα, ERβ, and GPER signaling to the E2-mediated enhancement of adipocyte mitochondrial function in a pro-inflammatory situation.

3T3-L1 cells were treated for 24h with ER agonists (PPT, DPN, and G1) and antagonists (MPP, PHTPP, and G15) in the presence or absence of interleukin 6 (IL6), as a pro-inflammatory stimulus. Inflammation, mitochondrial function and biogenesis markers were analyzed. To confirm the involvement of the PKA pathway, cells were treated with a GPER agonist, a PKA inhibitor, and IL6. Mitochondrial function markers were analyzed.

Our results showed that activation of ERα and GPER, but not ERβ, was able to counteract the proinflammatory effects of IL6 treatment, as well as mitochondrial biogenesis and function indicators. Inhibition of PKA prevented the E2- and G1-associated increase in mitochondrial function markers.

In conclusionE2 prevents IL6 induced inflammation in adipocytes and promotes mitochondrial function through the combined activation of both GPER and ERα. These findings expand our understanding of ER interactions under inflammatory conditions in female rodent white adipose tissue.

Key words: 17beta-estradiol, oestrogen receptor alpha; G protein-coupled oestrogen receptor adipocyte; mitochondrial function, PKA.

2. Introduction

Women have a longer life expectancy than men in most developed countries and this difference has been attributed to the protective effects of oestrogens, in particular estradiol (E2) [1]. Oestrogen decline in serum after menopause results in adverse effects such as increased and redistribution of adiposity, insulin resistance, and type 2 diabetes [2,3]. Many of these comorbidities are associated with the impairment of the capacity of adipose tissue to expand in association with inflammation, fibrosis, hypoxia, dysregulated adipokine secretion, and disrupted mitochondrial function [4,5].

Mitochondrial dysfunction refers to the failure of mitochondria to generate sufficient ATP levels to meet energy demands [6]. Moreover, mitochondrial dysfunction is associated with increased ROS production, alterations of mitochondrial shape (fragmented state or ball-shaped instead of elongate and interconnected network[7]), and apoptosis [8]. In terms of adipocyte biology, one or more of these manifestations may severely tip the balance from lipid storage to oxidation and contribute directly to the development of insulin resistance and obesity [4]. Proper function of mitochondria is key to avoiding inflammation, since it has been seen that the increase in ROS production associated with the impairment of mitochondrial function is a potential inducer of the up-regulation of inflammatory cytokines and the innate immune response [9,10]. Depending on the pathophysiological scenario, mitochondrial dysfunction could be offset by increasing mitochondrial biogenesis, which involves both mitochondrial proliferation (increase of mitochondria number) and differentiation (improvement of mitochondrial function) [11]. Both processes are tightly regulated, and such regulation requires the coordinated contribution of both mitochondrial and nuclear genomes [12].

Previous studies from our group have shown that E2 is able to increase mitochondrial function by inducing mitochondrial biogenesis in different types of cells and tissues [13–15]. Nevertheless, the mechanisms by which oestrogens achieve their physiological and pharmacological effects on mitochondrial dynamics are complex and not fully understood.

Nuclear oestrogen receptors alpha (ERα) and beta (ERβ), as well as G protein-coupled oestrogen receptor (GPER) mediate E2 effects in a wide range of cell types [16]. Whereas ERα and ERβ act as transcription factors, [17] GPER initiates rapid non genomic intracellular signalling cascades such as adenylyl cyclase/cAMP and EGFR/MAPK that may or may not lead to gene expression regulation [18–21]. Specifically, MAPK pathway activation regulates gene expression through activation of transcriptional factors such as cAMP-response-element binding protein (CREB) [22], an upstream regulator of mitochondrial biogenesis, which could be another route whereby estradiol could exert its genomic effects on mitochondrial biogenesis. One of the physiological effects of ERα activation is the enhancement of the mitochondrial biogenesis programme by increasing the expression of nuclear respiratory factor 1 (NRF1), which promotes the transcription of mitochondrial DNA (mtDNA) [23,24]. Interestingly, there are some studies proposing that the physiological role of ERβ might be opposite to that of ERα [25–29]. However, the possibility of an indirect inhibition of ERα-mediated gene expression by ERβ has not yet been confirmed. In the adipocyte, ERα has been found to be more highly expressed than the ERβ variant [30].

Estradiol also exerts anti-inflammatory effects through the activation of ERα [31]. Multiple mechanisms aimed to shorten the pro-inflammatory phase have been reported, including decreasing the synthesis of pro-inflammatory compounds [32,33] and impairing NF-κB transcriptional activity by preventing its nuclear translocation in the presence of strong inflammatory stimuli through direct interaction with phosphatidylinositol 3-kinase [34]. Moreover, E2 enhances progression of the inflammatory response through the E2-ERα complex that facilitates STAT3 phosphorylation [33].

Considering the anti-inflammatory activity of E2 as well as its effects in enhancing mitochondrial biogenesis, the aim of this study was to elucidate the effects of E2 on the inflammation-associated impairment of adipocyte mitochondrial function, as well as dissecting the relative contribution/role of ERα, ERβ and GPER signaling.

3. Materials and Methods

3.1. Materials

3T3-L1 cells were obtained from ATCC (Manassas, VA, USA). Cell culture consumables, insulin, 3-isobutyl-1-methyl-xanthine, bovine calf serum, and primers were purchased from Sigma (St. Louis, MO, USA). MitoTracker® Green FM**,** CM-H_2DCFDA, SYBR green reagent and BCA protein assay kit were from Thermo Fisher Scientific (Waltham, MA, USA). TaqMan7900 sequence detection system was purchased from Applied Bio-systems (Waltham, MA, USA). M-MLV reverse transcriptase and master mix were purchased from Promega (Madison, WI, USA). XF Cell Mito Stress test kit was from Seahorse Bioscience (MA, US). Polyclonal antibodies against pCREB, CREB, pERα, ERα (Cell Signaling Technology, MA, USA), chemiluminescence kit (Bio-Rad, Hercules, CA, USA) and STAT-60 (AMS Biotech, OX, UK) were used. All other chemicals were obtained from Sigma-Aldrich (Darmstadt, Germany).

3.2. Cell Culture

3T3-L1 mouse embryonic fibroblasts cells were differentiated into mature adipocytes (day 10) accordingly to that previously described [35]. Lipid accumulation was measured by Oil Red O method [36].

3.3. Adipose tissue explant culture

C57BL/6J male mice (Charles River, Kent, UK) were used for the primary culture of adipose tissue explants. Procedures were performed under the jurisdiction of the appropriate UK Home Office project and personal animal license. Mice were placed on a normal chow diet (10% of calories derived from fat; D12450B; Research Diets, Inc) and drinking water ad libitum. Three-month-old mice were killed by cervical dislocation, and retroperitoneal adipose tissue was

extracted, weighed, and divided into 50mg pieces. Each 50mg piece was further cut into 5–10mg pieces and placed in a single well of a twelve-well tissue culture plate with 5mL Dulbecco's Modified Eagle medium (DMEM) without red phenol and supplemented with 10% fetal bovine serum, penicillin and streptomycin. Culture plates were incubated at 37°C in an atmosphere of 5% CO_2/air. Thirty minutes after the start of the culture, adipose tissues were treated with either E2, G1, IL6 or IL6 plus E2. After 24h, adipose tissue explants were collected and stored at −80°C for later RNA isolation.

3.4. Ovariectomized rats

Wistar rats of six weeks of age were purchased from Charles River (Barcelona, Spain) and were housed in pairs at 22°C and 65±3% humidity on a 12h light:12h dark cycle. Control, ovariectomized (OVX) and E2-supplemented OVX (OVX+E2) female Wistar rats (n=6) of 10 weeks of age were used. During the experiment, all animals were housed with free access to water and pelleted standard diet (A04, 2791 kcal/kg, SAFE, Augy, France). OVX and OVX+E2 rats were ovariectomized at 5 weeks of age by the supplier to supress endogenous ovarian steroid production. OVX + E2 rats were administrated a subcutaneous injection of 10µg/kg/48h of E2 dissolved in 0.1ml of corn oil (vehicle) for 4 weeks before sacrifice. In parallel, OVX rats were treated only with the vehicle. All animals were sacrificed at 14 weeks of age by decapitation and in diestrous phase, as observed in vaginal smears. The retroperitoneal white adipose tissue (WAT) depot was dissected, weighed and frozen in liquid N_2 and stored at -80°C until analysis.

Animal experiments were performed in accordance with general guidelines approved by EU regulations (2010/63/UE) and our institutional ethics committee.

3.5. RNA isolation and Real-Time PCR

RNA was extracted using STAT-60 according to manufacturer's procedures. Reverse transcription was performed using Reverse Transcriptase System according to manufacturer's

instructions. Real-time PCR was carried out using TaqMan or Sybr Green reagents using an Abi 7900 real-time PCR machine (Thermo Fisher Scientific, Waltham, MA, USA) using default thermal cycler conditions (Table 1). Several genes were tested as housekeeping genes, such as Nono, Actb, Rplp0, B2m, and 18S. However, the expression of these genes changed in response to the different treatments. Therefore, the Ct values of the real-time PCR were analyzed after being corrected by the reaction efficiency using GenEx Standard software (MultiDAnalises, Sweden). Meanwhile, samples of adipose tissue from ovariectomy experiments were normalized with 18S.

3.6. Measurements of mitochondrial content, intracellular ROS and ATP levels in 3T3-L1 adipocytes

3T3-L1 cells were differentiated in 96-well culture plates. At the end of the treatments, cells were co-incubated with MitoTracker® Green FM, a green-fluorescent mitochondrial stain that is correlated with the inner mitochondrial membrane quantity, giving information about mitochondrial mass, and with Hoechst 33342 for staining of DNA and nuclei. Mitochondrial content was assessed according to manufacturer instructions. Values were expressed as the ratio between the green fluorescence of the MitoTracker and the blue fluorescence of the Hoechst, after subtraction of the signal of not-stained cells. Values were normalized with those of untreated-cells. The ROS assay was performed as previously described [37]. Briefly, 3T3-L1 were incubated with CM-H2DCFDA and Hoechst 33342. The intracellular ROS were quantified according to manufacturer's instructions. Values were expressed as the ratio between the green fluorescence of the oxidized CM-H2DCFDA and the blue fluorescence of the Hoechst, after subtraction of the signal of not-stained cells. Values were normalized with those of untreated cells. The ATP levels assay was performed using an ATP kit in 3T3-L1 cells, according to the manufacturer's protocol. Values were normalized with those of untreated cells.

3.7. Measurement of 3T3-L1 adipocyte metabolism

3T3-L1 adipocytes were seeded in Seahorse XFe24 cell culture microplates at a density of 50,000 cells per well, before being differentiated into adipocytes as outlined above. Mitochondrial function assays and parameter calculations were performed using the Seahorse XF Cell Mito Stress test kit. Oxygen consumption rate (OCR) was measured before and after the addition of inhibitors to derive several parameters of mitochondrial respiration. Initially, baseline cellular OCR was measured, from which basal respiration could be attained by subtracting non-mitochondrial respiration. Next, oligomycin (1μM), a complex V inhibitor, was added and the resulting OCR was used to derive ATP-linked respiration (by subtracting the oligomycin rate from baseline cellular OCR) and proton leak respiration (by subtracting non-mitochondrial respiration from the oligomycin rate). Then, 400nM of carbonyl cyanide-p-trifluoromethox-yphenyl-hydrazon (FCCP), a protonophore, was added to collapse the inner membrane gradient, allowing the ETC to function at its maximal rate; and maximal respiratory capacity was obtained by subtracting non- mitochondrial respiration from the FCCP rate. Lastly, antimycin A (15μM) and rotenone (15μM), inhibitors of complex III and I, were added to shut down ETC function, revealing the non-mitochondrial respiration. Mitochondrial reserve capacity (spare capacity) was calculated by subtracting basal respiration from maximal respiratory capacity [38].

3.8. Analysis of cardiolipin content

3T3-L1 cells were seeded in cover slips placed in the culture plate and differentiated to adipocytes. After treatments, cells were fixed with 4% formaldehyde for 30min at 4ºC. Then, cardiolipin content was assayed using nonyl acridine orange (NAO, 250nM) dissolved in PBS–glucose (20 mM). The plate was incubated in the dark for 30 min at 37ºC. Cover slips were placed over a slip with a drop of fluoroshield mounting medium with DAPI. Fluorescence set at 485 nm excitation and 528 nm emission was viewed using a Leica confocal microscope and images were acquired using a 63x objective lens and the Leica Application Suite (LAS) Software, version Advanced Fluorescence 2.3.6 build 5381 (LAS, Wetzlar, Germany).

3.9. Cell lysis

3T3-L1 culture medium was removed and 0.1 ml of ice-cold RIPA buffer containing phosphatase and protease inhibitors (50mM Tris pH 7.5, 1% Triton X-100, 1mM EDTA, 150mM NaCl, 0.5% sodium deoxicolate, 0.1% SDS, 10 μM leupeptin, 10 μM pepstatin, 10μM PMSF, 1mM NaF, 1mM Na_3VO_4) was added. Cell extracts were scraped off, vortex-mixed and centrifuged (14,000g, 10min, 4°C). Cell lysis samples were stored at −20°C until further analysis. Protein concentration was measured with a BCA protein assay kit.

3.10. Western blot analysis

25μg of cell lysates were fractionated on SDS-PAGE gels and electrotransferred into a nitrocellulose filter. The membranes were blocked with blocking solution (5% non-fat powdered milk in phosphate-buffered saline, pH 7.5, containing 0.1% Tween 20) for 1h and incubated overnight with the corresponding antibodies. Polyclonal antibodies against pCREB, CREB, pERα, ERα, were used as primary antibodies. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands were visualized with the ChemiDoc XRS system (Bio-Rad, CA, USA) and analysed with the image analysis program Quantity One© (Bio-Rad, CA, USA). Bands revealed apparent molecular mass of 43 and 66 kDa for CREB and ERα, respectively.

3.11. Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was assessed using Prism 7.0 (GraphPad Inc., San Diego, CA, US). Differences between means ± S.E.M. were tested with Students t-test, one way analysis of variance or two-way analysis of variance (ANOVA). All multiple drug treatment data were analyzed with two-way ANOVA, using Ligand (understanding ligand as different oestrogen receptor agonists and antagonists) and IL6 (inflammation) as the two independent factors. Post hoc pairwise comparisons were performed using the Fisher LSD test.

4. Results

Effects of IL 6 on inflammatory and mitochondrial parameters.

IL6 treatment, used as a proinflammatory stimulus, induced a relevant increase in inflammation markers Ccl2, Cd68 and Serpine1 (Figures 1); as well as a reduction of the basal mitochondrial respiration rate and ATP production (Figures 2A-B), but not of the FCCP stimulated respiration and the spare capacity (Figures 2C-D). This IL6 induced decrease of ETC function was accompanied by increased ROS production (Figure 3C).

Protective effects of estradiol on the mitochondria

To characterize the protective effects of E2 on the mitochondrial dysfunction generated by an inflammatory stimulus in mature adipocytes, 3T3-L1 cells were treated simultaneously with E2 and IL6. E2 counteracted the deleterious effects of IL6 on the inflammation parameters studied (Figure 1), as well as normalized the ETC function by increasing basal respiration, ATP production, FCCP stimulated respiration and spare capacity (Figures 2A-D). This prevention of the decrease in ETC activity was paralleled by an increase in markers of mitochondrial biogenesis and OXPHOS, such as Atp5a1 and Cox4i1 mRNA levels (Figures 3D), and Mitochondrial mass values, (Figure 3B). E2 also increased the mitochondrial membrane lipid cardiolipin (Figure 5).

In addition, E2 treatment decreased adipocyte Esr1 (ERα) and Esr2 (ERβ) mRNA levels and increased those of Gper1 (GPER) (Figure 6). This different response of ERs to E2 was confirmed in adipose tissue of ovariectomized animals, where Gper1 mRNA was increased in response to E2 supplementation, while Esr1 (ERα) and Esr2 (ERβ) followed the opposite profile (Figure 7). When 3T3-L1 adipocytes were treated with IL6, to generate a pro-inflammatory environment, Esr1 (ERα) expression was reduced whereas that of Esr2 (ERβ) and GPER were not modified (Figure 6). E2 treatment enhanced the Gper1 mRNA expression under a pro-inflammatory environment (Figure 6).

Effects of the agonists of the different oestrogen receptors on mitochondrial parameters

To elucidate whether the enhancement of mitochondrial biogenesis by E2 was mediated by ERα, ERβ, and/or GPER, experiments were performed using selective agonists. Basal respiration and ATP production were increased by ERα, ERβ, and GPER activation, although without reaching E2 treatment values (Figures 8A-B**)**. In addition, all three agonists increased mitochondrial mass, but only the activation of ERα and GPER enhanced mitochondrial biogenesis, as shown by the increase in Ppargc1a, and Atp5a1, which in the case of GPER was also accompanied by an increase in ATP levels; whereas the activation of ERβ did not increase the expression of any of the genes measured, or ATP levels (Figure 3A-B, D). E2 stimulation combined with ERα or ERβ blockage increased mitochondrial mass in both cases, whereas the expression of Tfam mRNA levels was raised only with ERβ blockage. Meanwhile, with the blockage of GPER, ATP levels and the expression of Tfam, Atp5a1, and Cox4i1 mRNA were increased (Figures 4A-D). Under a proinflammatory stimulus, the activation of ERα increased mitochondrial mass and Ppargc1a, Ppargc1b, Tfam, Atp5a1, and Cox4i1 mRNA levels, reverted ATP levels, and decreased ROS production. ERβ activation, however, decreased ROS production but did not increase the expression of any of the mitochondrial biogenesis genes analyzed. Meanwhile, the activation of GPER reverted ATP levels, decreased ROS production, and increased the expression of all the mitochondrial biogenesis genes analyzed (Figures 3A-D). This suggests that ERα and GPER but not ERβ are involved in the regulation of mitochondrial biogenesis in the inflamed adipocyte. The contribution of both ERα and GPER to the improvement of mitochondrial function and inflammatory state became clearer when using E2 or the selective ER agonists and the chemical blockers of the other two ERs at the same time (Figures 9A-B). These results, reducing possible crosstalk effects between ERs, show that ERα and GPER activation, with both E2 and the agonist, but not ERβ, are able to decrease ROS production in IL6 treated 3T3-L1 cells and to raise mitochondrial mass in both control and inflamed adipocytes.

In the specific case of GPER activation, G1 treatment neutralized the detrimental effects of IL6 on mitochondrial function by increasing inner mitochondrial membrane quantity, basal

respiration, ATP production, FCCP stimulated respiration, and spare capacity (Figures 2A-D, 5). G1 also decreased the expression of inflammation markers such as Cd68 and Ccl2, as well as ROS production, as did E2 (Figures 1 and 3C).

Checking the possible crosstalk between GPER and ERα

Possible crosstalk between ERα and GPER was confirmed by an *in vitro* experiment in which GPER was stimulated with the agonist G1 at different times (15, 30, 45 min) and the phosphorylation of ERα at Ser-118 was analyzed (Figure 10). Ser-118 is the main site phosphorylated in ERα in response to estradiol [39], and appears to influence the recruitment of coactivators, and enhance ER-mediated transcription[40,41]. The need for ERα activation in the GPER signaling pathway was ruled out by showing the same positive effects on short-term ATP levels (15, 30, 45, 60 min) with G1 stimulation in the presence or absence of ERα blockade only under conditions of previous stimulation with IL6 (Figure 11).

Implication of the PKA pathway in the effects of GPER activation

To investigate whether PKA was involved in the improvement of mitochondrial function associated to GPER activation, an experiment using PKI, an inhibitor of PKA [42,43], was performed. PKI was able to prevent the phosphorylation of CREB (Figure 12), a transcriptional factor activated by PKA, in response to E2 or GPER agonist G1; and the increase in mitochondrial mass and ATP content, and the decrease in ROS production (Figures 13A-F). This suggested that PKA was somehow involved in the enhancement of GPER-mediated mitochondrial function.

These results were corroborated in an experiment with explants of white adipose tissue, revealing that G1 treatment brought about a greater expression of mitochondrial biogenesis markers Ppargc1a, Ppargc1b, and Nrf1 (Figures 14), but prevented a drop in Ppargc1a and Ppargc1b expression levels in the IL6 stimulus (Figures 14).

5. Discussion

The sexual dimorphism in mitochondrial function reported previously by our laboratory, in which female rats exhibit more differentiated mitochondria, is likely to be mostly mediated by oestrogens. E2 is known to enhance mitochondrial function and biogenesis in tissues such as heart [13], skeletal muscle [44], liver [15], and adipose tissues [45]. Moreover, oestrogens have been reported to attenuate the production of proinflammatory cytokines by adipocytes, and therefore to decrease inflammation [46]. This adipose-inflammation improvement has also been shown to be evident during pregnancy [47], a period in which hormones, including E2, are increased [48]. In the present study, data are provided showing that E2 enhances mitochondrial function in adipocytes exposed to a proinflammatory environment, and that this effect is mediated by ERα and GPER through signaling pathways involving PKA.

IL6 treatment induces a profile of mitochondrial dysfunction and inflammation that is reminiscent of the profile found in the adipose tissue of high-fat-diet fed rodents [49–51], type 2 diabetic mice [52], and obese humans [53]. In cultured adipocytes, E2 treatment neutralizes the deleterious effects of IL6 on 3T3-L1 cells, decreasing inflammation, and increasing mitochondrial oxidative capacity. Increased mitochondrial electron transport chain (ETC) activity is the result of enhanced mitochondrial differentiation, as reflected by the greater expression of biogenesis markers and OXPHOS proteins. Thus, E2 is able to restore mitochondrial respiration levels and, also, to decrease adipocyte ROS production. Therefore, mitochondrial function improvement would be one of the effects whereby E2 protects adipocytes from the harmful effects of ROS associated inflammation; although other pathways cannot be ruled out, since E2 has been reported to inhibit proinflammatory signals through ERα activation [33]. These effects of estradiol could contribute to the greater resistance of female rats to the deleterious effects of a high-fat diet [49,54].

The E2 effect on adipocytes is likely to be dependent on the amount and type of ERs. In fact, E2 treatment decreased the expression levels of ERα and increased those of GPER in 3T3-L1 adipocytes and WAT of OVX rats. Moreover, GPER has also been found to be more greatly expressed in adipose tissue of female rats than in males [55]. E2-induced decrease of Esr1 (ERα) mRNA levels has already been reported in several tissues [56–58] and seems to be part of

a feedback mechanism controlling the level of oestrogenic action [59–61]. It should be noted that oestrogens have been reported to induce adipocyte differentiation [62,63], a process that has been shown to increase the expression of Gper1 (GPER) and to decrease that of classic ERs [64]. Thus, it appears that GPER may be more important in mature adipocytes because of its rapid signaling events as a G-protein-coupled receptor (GPCR) [65,66], whereas classic ERs would be more important during differentiation, where greater changes in gene expression are observed [67]. Since adipogenesis also implies the formation of new mitochondria [68], GPER could play a significant role in E2-induced mitochondrial biogenesis.

In 3T3-L1 adipocytes cultured in control conditions, the ERα agonist caused an increase in all mitochondrial function markers, while the ERβ agonist was also able to increase basal respiration and ATP production (Fig 8). These results must be interpreted with caution since these agonists are selective but not specific, which implies that although they have a 100-200-fold greater affinity for their respective receptors, higher concentrations can provoke crosstalk effects. In fact, the stimulation of each type of E2 receptor while blocking the remaining two, in both control and inflammation conditions, leads us to rule out the involvement of ERβ in the increase of mitochondrial function and biogenesis and the decrease of inflammation-associated ROS production elicited by E2. Moreover, since the concentrations of agonists used modified the expression levels of genes commonly used as housekeeping genes, such as Actb (see Figure S1), it was decided not to use these

Consequently, ERα and GPER may play a key role in the improvement of adipocyte mitochondrial functionality by E2 when facing an inflammatory situation. This anti-inflammatory effect is in accordance with the obese and metabolic syndrome phenotypes shown by ERα and GPER knockout mice. [69–71].

The improvement of mitochondrial function under inflammation conditions could be achieved through both non-genomic and genomic pathways. In fact, the induction of mitochondrial biogenesis by E2 through GPER was further validated in adipose tissue explants, where G1 treatment was able to override the acute proinflammatory effects induced by IL6. Our results also show that E2 effects on mitochondrial biogenesis through GPER involve PKA

activation. Inhibition of PKA prevents the GPER-mediated actions of E2 on mitochondrial function markers (increase) and ROS production (decrease) in both control and inflammation conditions. PKA activated the transcription factor CREB, which has been shown to induce mitochondrial biogenesis through pgc1α expression [72]. This rapid signaling pathway through cAMP is characteristic of G protein receptors such as GPER [42,43], which is known to be able to phosphorylate CREB [73]. Since it has been reported that other ERs like ERα are able to induce CREB [74], crosstalk between ERα and GPER in the improvement of mitochondrial functionality through PKA cannot be ruled out.

All these results point towards ERα and GPER being involved in processes leading to the improvement of mitochondrial functionality and biogenesis in adipocytes. However, the use of pharmacological concentrations of drugs can be a limitation of the study that could, in a certain way, affect our assumptions as to the possible role of oestrogens in sex differences in the incidence and severity of inflammation-related diseases. The possible crosstalk between GPER and ERs has already been reviewed [18,75] pointing to a redundancy in the effects mediated by oestrogen receptors. This would explain at least in part why the protective/beneficial effects of oestrogen are mimicked by selective GPER agonists and are absent or reduced in GPER knockout mice, suggesting an essential or at least parallel role for GPER in the actions of oestrogen [76]. This crosstalk is likely to depend on cell type, developmental stage, and pathology [18]. In our study, GPER stimulated the phosphorylation of ERα at Ser-118 in 3T3-L1 cells in a short run (15-45min), through the activation of an unidentified kinase [77]. ERα phosphorylation by GPER could be indicative of a possible mechanism to keep ERα active, since Ser-118 is involved in ER stabilization [78] and coactivator recruitment [40,41].

In our study, G1 stimulation and simultaneous ERα and ERβ chemical blocking, from 60 min of treatment, was still able to produce positive effects on mitochondrial function, which could indicate that the action mediated by GPER would be parallel to the action mediated by ERα. As pharmacological approaches are limited by non-specific effects, other models for GPER-ERs signaling interactions cannot be ruled out. For example, the formation of a complex with both receptors, which has been shown to be able to produce anti-inflammatory effects on

human monocytes [79], or a parallel pathway activation that leads to the same effect as both receptors are capable of activating a whole series of multifunctional and ubiquitous proteins such as PI3K, MAPK or PKA [42,80–83].

In conclusion, E2 ameliorates the adverse effects of inflammation on adipocyte mitochondrial function through the combined activation of both GPER and ERα. These findings expand our understanding of ER crosstalk and suggest that both GPER and ERα might be interesting therapeutic targets against inflammation- and mitochondrial dysfunction-related diseases, such as obesity, diabetes, and cardiovascular disease.

6. Declarations 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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In loving memory of Professor Francisco José García-Palmer.

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9. Tables and Figures

Figure 1.

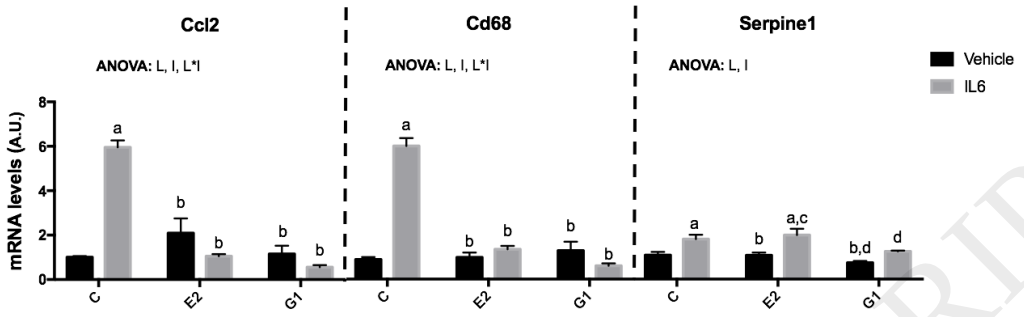


Figure 2.

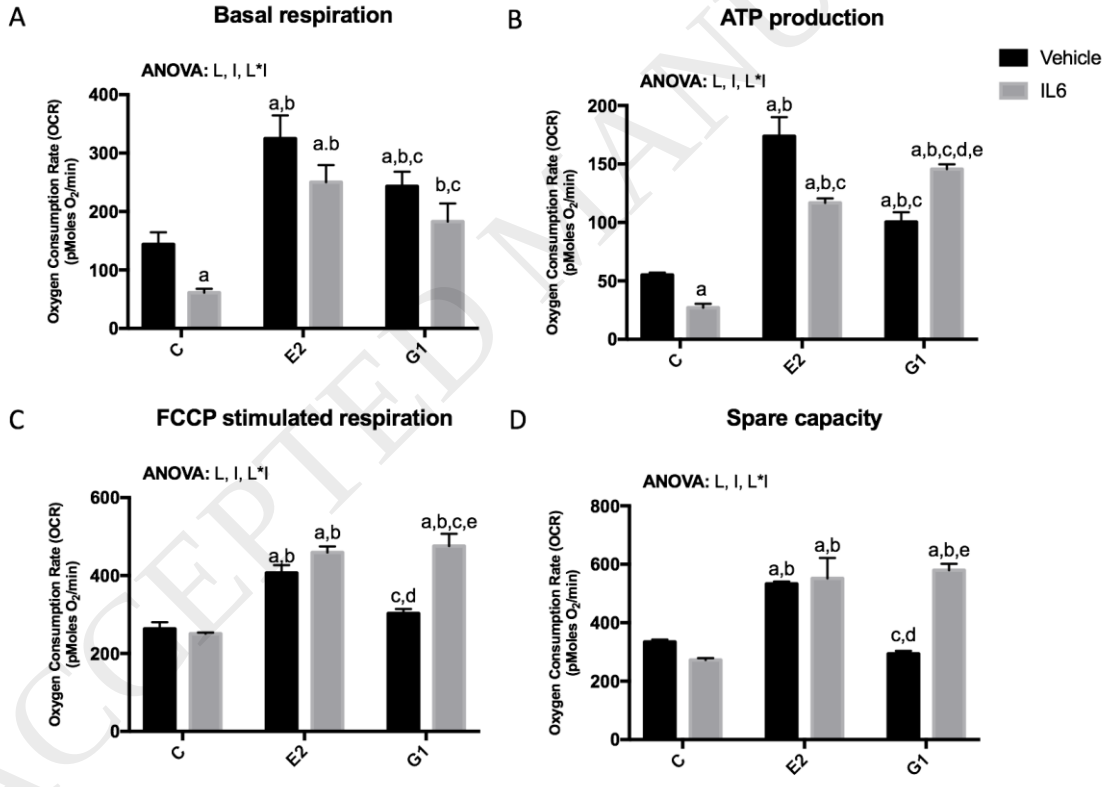


Figure 3.

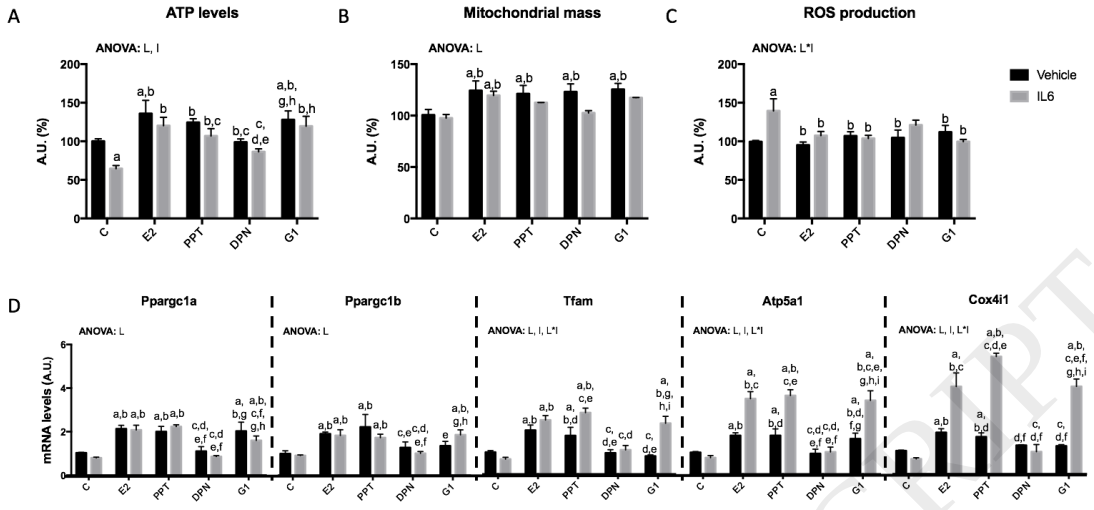
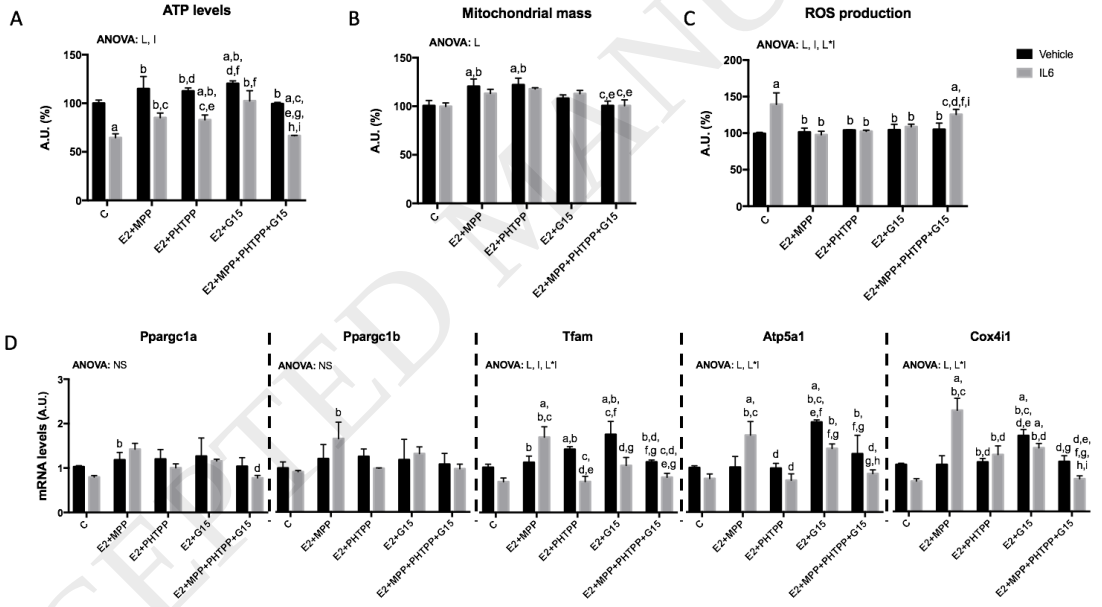


Figure 4.



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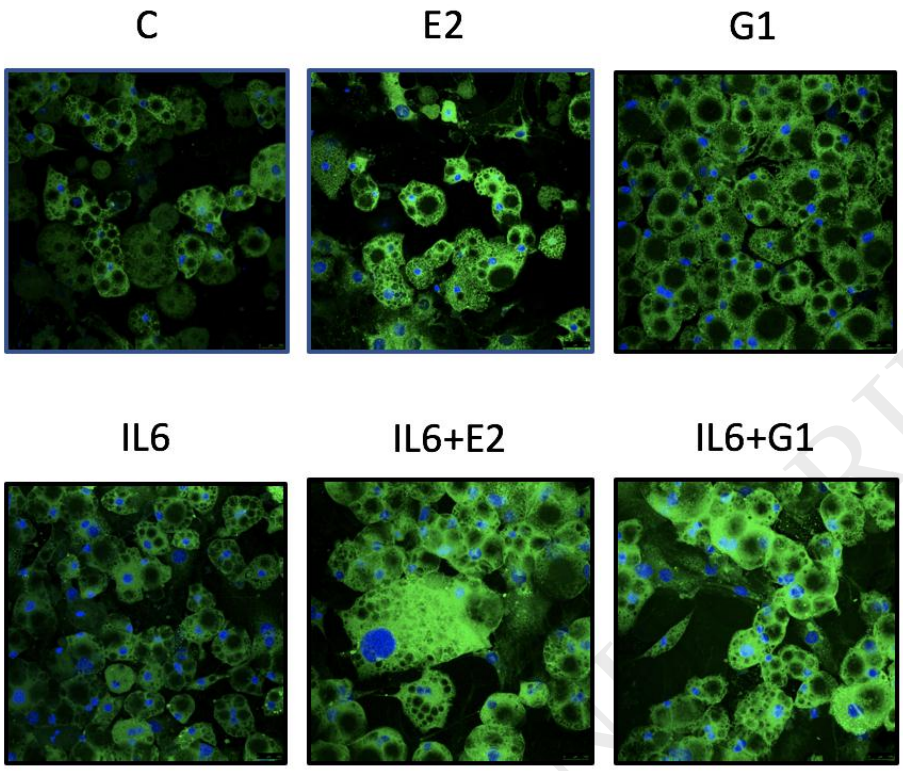

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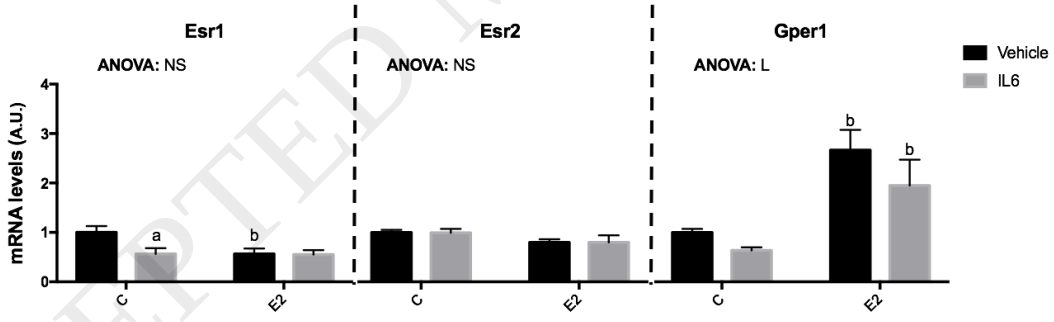

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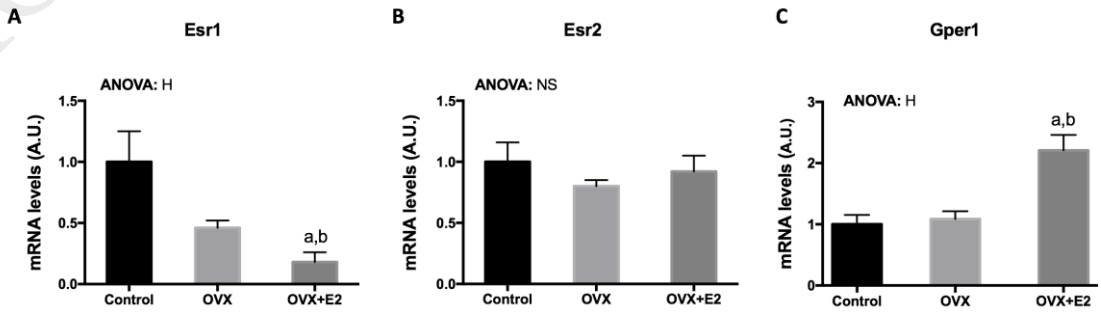

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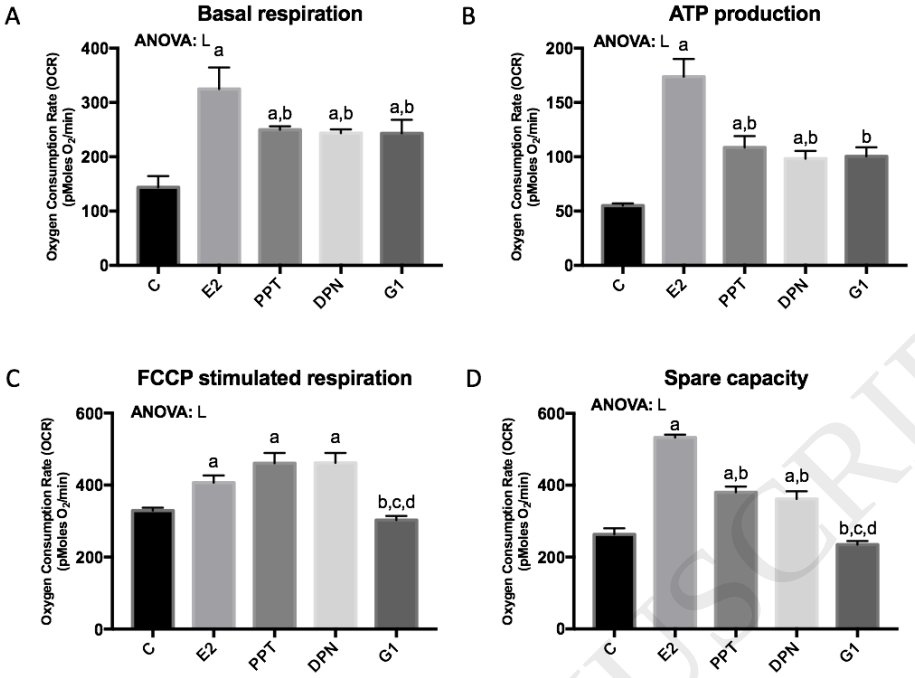

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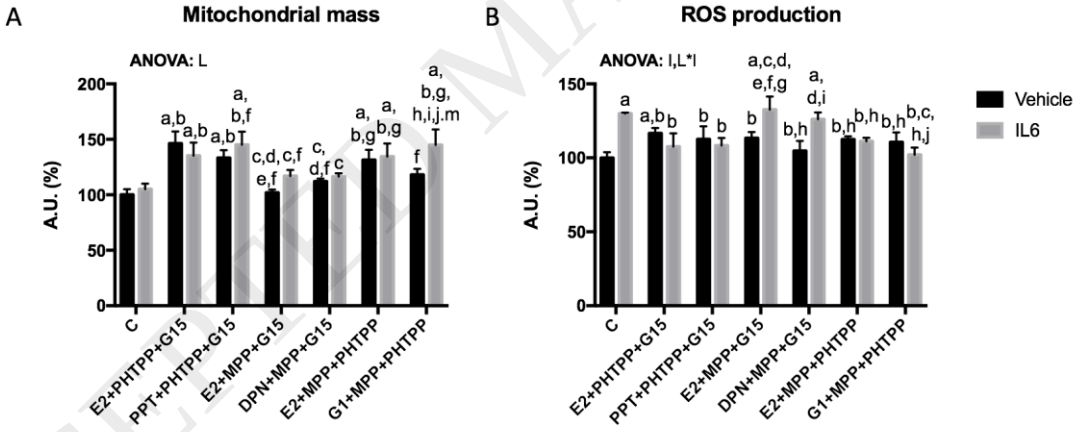

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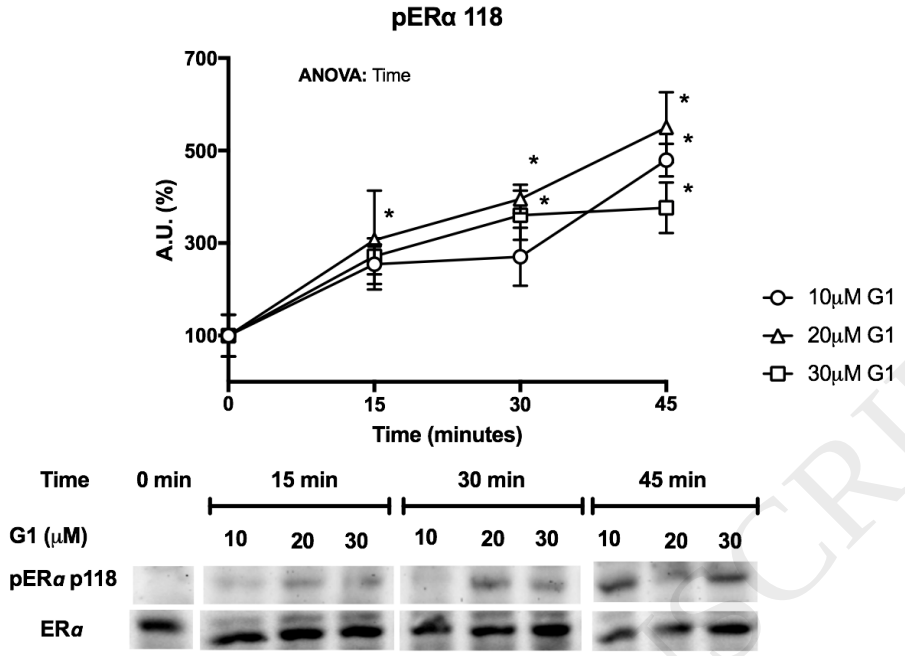

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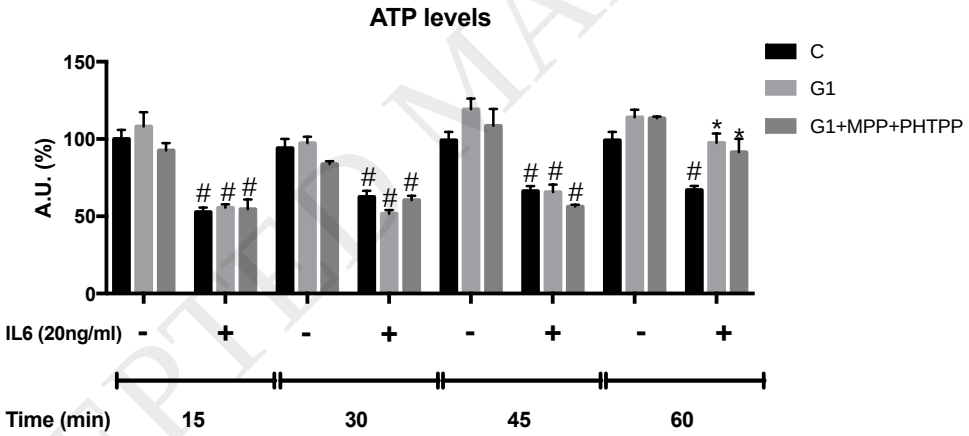

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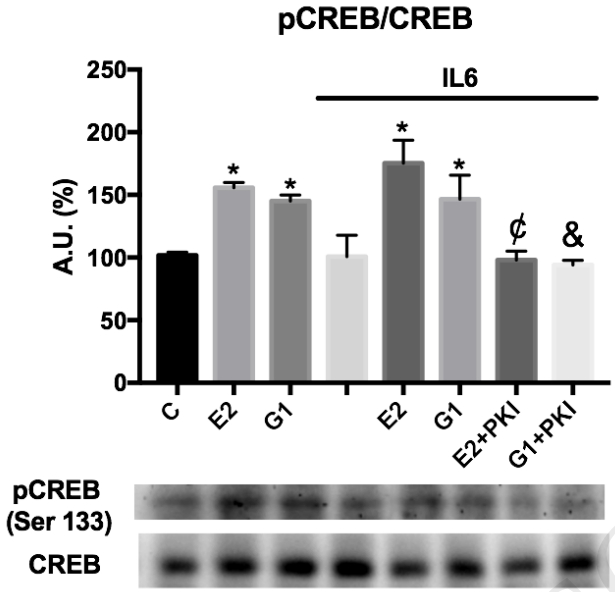

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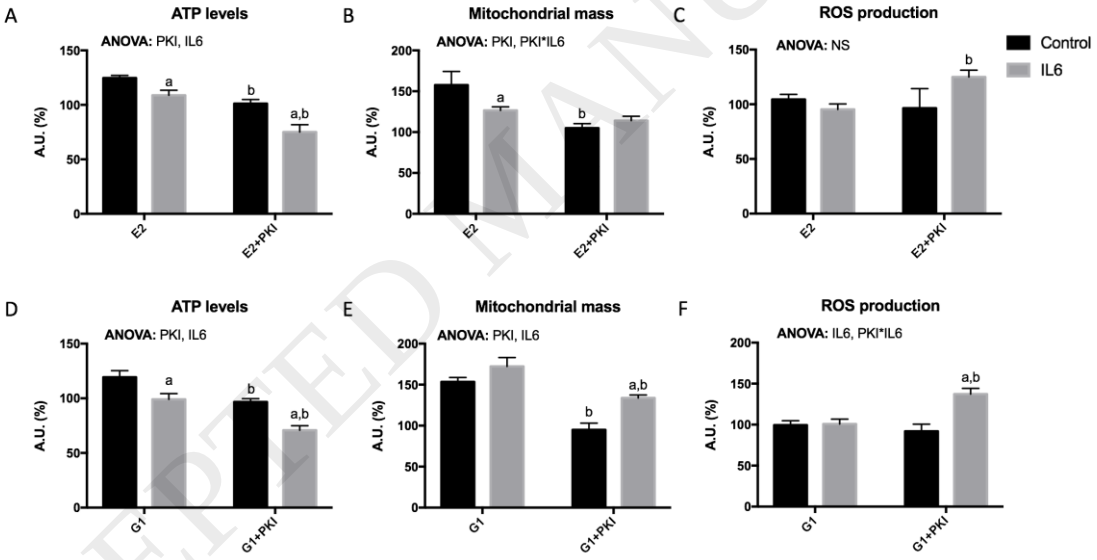

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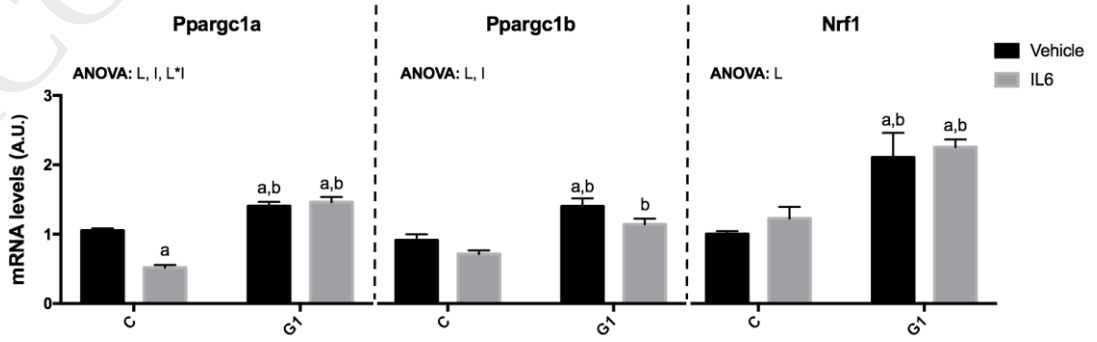

10. Legends

Figure 1.

17beta-estradiol (E2) and GPER agonist G1 decrease mRNA levels of inflammatory markers in 3T3-L1 adipocytes. *Ccl2, chemokine (C-C motif) ligand 2 alias Monocyte Chemoattractant Protein-1; Cd68, cluster of differentiation 68; Serpine1, serpin family E member 1 alias plasminogen activator inhibitor-1. Cells were treated with E2 (100nM) or G1 (1µM), combined or not with IL6 (20ng/ml) for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 1. Ligand and IL6 effect are analysed by Two-way ANOVA (P<0.05): L indicates ligand effect, I indicates IL6 effect, L*I indicates ligand and IL6 interactive effect and NS stands for non-significant. Fisher's LSD as a post hoc analysis (P<0.05): a indicates differences from control group, b indicates differences from IL6 group, c indicates differences from E2 group, d indicates differences from E2+IL6 group and e indicates differences from G1 group.*

Figure 2.

17beta-estradiol (E2) and GPER agonist (G1) increase oxygen consumption in 3T3-L1 adipocytes under inflammation conditions. *Cells were treated with E2 (1nM), or G1 (1µM) combined or not with IL6 (20ng/ml) for 24 h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6). Ligand and IL6 effect are analysed by Two-way ANOVA (P<0.05): L indicates ligand effect, I indicates IL6 effect, L*I indicates ligand and IL6 interactive effect and NS stands for non-significant. Fisher LSD as a post hoc analysis (P<0.05): a indicates differences from control group, b indicates differences from IL6 group, c indicates differences from E2 group, d indicates differences from E2+IL6 group and e indicates differences from G1 group.*

Figure 3.

17beta-estradiol (E2) and oestrogen receptor agonists increase mitochondrial function in 3T3-L1 adipocytes under inflammation conditions. *Cells were treated with E2 (100nM) or specific agonists of ERα (PPT, 1µM), ERβ (DPN, 1µM) or GPER (G1, 1µM) and IL6 (20ng/ml) for 24 h for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 100. Ligand and IL6 effect are analysed by Two-way ANOVA (P<0.05): L indicates ligand effect, I indicates IL6 effect, L*I indicates ligand and IL6 interactive effect and NS stands for non-significant. Fisher LSD as a post hoc analysis (P<0.05): a indicates differences from control group, b indicates differences from IL6 group, c indicates differences from E2 group, d indicates differences from E2+IL6 group, e indicates differences from PPT group, f indicates differences from PPT+IL6 group, g indicates differences from DPN group, h indicates differences from DPN+IL6 group and i indicates differences from G1 group.*

Figure 4.

17beta-estradiol (E2) and oestrogen receptor antagonists increase mitochondrial function in 3T3-L1 adipocytes under inflammation conditions. *Cells were treated with E2 (100 nM) combined with specific antagonists of ERα (MPP, 1 µM), ERβ (PHTPP, 1 µM), GPER (G15, 1µM) and IL6 (20ng/ml) for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 100. Ligand and IL6 effect are analysed by Two way ANOVA (P<0.05): L indicates ligand effect, I indicates IL6 effect, L*I indicates ligand and IL6 interactive effect and NS stands for non-significant. Fisher LSD as a post hoc analysis (P<0.05): a indicates differences from control group, b indicates differences from IL6 group, c indicates differences from E2+MPP group, d indicates differences from E2+MPP+IL6 group, e indicates differences from E2+PHTPP group, f indicates differences from E2+PHTPP+IL6 group, g indicates differences from E2+G15 group, h indicates differences from E2+G15+IL6 group and i indicates differences from E2+MPP+PHTPP+G15 group.*

Figure 5.

Representative confocal images of the effects of 17beta-estradiol (E2), GPER agonists (G1) and interleukin 6 (IL6) on inner mitochondrial membrane cardiolipin measured as NAO intensity*. Cells were treated with E2 (100nM) or G1 (1µM), combined or not with IL6 (20 ng/ml) for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6). Confocal images of 3T3-L1 stained with NAO 250nM (green), and DAPI (blue). The fluorescence was monitored with a Leica confocal microscope using 63X lens.*

Figure 6.

17beta-estradiol (E2) increases GPER mRNA expression in 3T3-L1 adipocytes under inflammation conditions*. Ers1, oestrogen receptor alpha; Ers2, oestrogen receptor beta; Gper1, G protein-coupled oestrogen receptor 1. Cells were treated with E2 (100nM), IL6 (20ng/ml) for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 1. Ligand and IL6 effect is analysed by Two way ANOVA (P<0.05): L indicates ligand effect, I indicates IL6 effect, L*I indicates ligand and IL6 interactive effect and NS stands for non-significant. Fisher LSD as a post hoc analysis (P<0.05): a indicates differences from control group, b indicates differences from IL6 group, c indicates differences from E2 group.*

Figure 7.

17beta-estradiol supplementation increases GPER mRNA expression in white adipose tissue of ovariectomized rats. *OVX, ovariectomized; OVX + E2, OVX treated with 17beta-estradiol. Esr1, oestrogen receptor alpha; Esr2, oestrogen receptor beta; Gper1, G protein-coupled oestrogen receptor 1. Values are means ± SEM of 6 animals per group. Hormone effect is analysed by one-way ANOVA (p < 0.05): H indicates hormone effect and NS stands for non-significant. Fisher's LSD as a post hoc analysis (p<0.05): a indicates differences from control group, b indicates differences from OVX group.*

Figure 8.

17beta-estradiol (E2) and oestrogen receptor agonists increase oxygen consumption in 3T3-L1 adipocytes. *Cells were treated with E2 (1nM), or specific agonist ERα (PPT, 1µM), ERß (DPN, 1µM) or GPER (G1, 1µM) for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6). Ligand effect is analysed by one-way ANOVA (p < 0.05): L indicates ligand effect and NS stands for non-significant. Fisher's LSD as a post hoc analysis (p<0.05): a indicates differences from control group, b indicates differences from E2 group, c indicates differences from PPT group and d indicates differences from DPN group.*

Figure 9.

17beta-oestradiol improves mitochondrial function through ERα and GPER but not ERβ in 3T3-L1 adipocytes under inflammation conditions. *Cells were treated with E2 (100nM) or specific agonists of ERα (PPT, 1µM), ERβ (DPN, 1µM) or GPER (G1, 1µM), or E2 (100nM) combined with specific antagonists of ERα (MPP, 1µM), ERβ (PHTPP, 1µM) or GPER (G15, 1µM), and IL6 (20ng/ml) for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 100. Ligand and IL6 effect is analysed by Two way ANOVA (P<0.05): L indicates ligand effect, I indicates IL6 effect, L*I indicates ligand and IL6 interactive effect and NS stands for non-significant. Fisher LSD as a post hoc analysis (P<0.05): a indicates differences from control group, b indicates differences from IL6 group, c indicates differences from E2+PHTPP+G15 group, d indicates differences from E2+PHTPP+G15+IL6 group, e indicates differences from PPT+PHTPP+G15 group, f indicates differences from PPT+PHTPP+G15+IL6 group, g indicates differences from E2+MPP+G15 group, h indicates differences from E2+MPP+G15+IL6 group, i indicates differences from DPN+MPP+G15 group, j indicates differences from DPN+MPP+G15+IL6 group, k indicates differences from E2+MPP+PHTPP group, l indicates differences from E2+MPP+ PHTPP +IL6 group and m indicates differences from G1+MPP+ PHTPP group.*

Figure 10.

GPER agonist (G1) increases oestrogen receptor alpha phosphorylation in 3T3-L1 adipocytes. *Cells were treated with G1 (µM) for 15, 30, 45 min. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 100. Statistically significant differences were determined using a two-tailed Student's t test: *p< 0.05 relative to control. G1 and time effect is analysed by Two way ANOVA (P<0.05): G1 indicates G1 concentration effect, Times indicates times effect, G1*Times indicates G1 concentration and times interactive effect and NS stands for non-significant. Fisher LSD as a post hoc analysis (P<0.05): * indicates differences from time 0 group.*

Figure 11.

GPER agonist (G1) increases ATP levels in 3T3-L1 adipocytes*. The cells were treated with G1 (1µM) or G1 with the ERα antagonist (MPP) and ERβ (PHTPP) for 15, 30, 45, 60 min in the presence or absence of interleukin 6 (IL6, 20 ng/ml) during the 24 hours before. The control cells were treated with the equivalent vehicle volume (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 100. Statistically significant differences were determined using a two-tailed Student's t test: *p< 0.05 relative to control; #p<0.05 relative to IL6*

Figure 12.

Protein kinase A inhibitor (PKI) prevents cAMP response element-binding protein phosphorylation (pCREB) by 17beta-oestradiol and GPER agonist (G1) in 3T3-L1 adipocytes under inflammation conditions. *Cells were treated with E2 (100 nM), G1 (1 µM), PKI (5µM) and IL6 (20ng/ml) for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 100. Statistically significant differences were determined using a two-tailed Student's t test: *p< 0.05 relative to control; #p< 0.05 relative to IL6; ¢p< 0.05 relative to E2+IL6; &p< 0.05 relative to G1+IL6.*

Figure 13.

Protein kinase A inhibitor (PKI) avoid the increase of mitochondrial function by 17beta-estradiol (E2) or GPER agonist (G1) in 3T3-L1 adipocytes under inflammation conditions. *Cells were treated with E2 (100nM), G1 (1µM), PKI (5µM) or IL6 (20ng/ml) for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 100. Statistically significant differences were determined using a two-tailed Student's t test: *p< 0.05 relative to control; #p< 0.05 relative to IL6; ¢p< 0.05 relative to E2+IL6; &p< 0.05 relative to G1+IL6.*

Figure 14.

17beta-estradiol (E2) and GPER agonist (G1) increase mitochondrial biogenesis markers in mouse white adipose tissue explants under inflammation conditions. *Cells were treated with E2 (100nM) or G1 (1µM), combined or not with IL6 (20ng/ml) for 24h. Control cells were treated with the equivalent volume of vehicle (ethanol). Values are expressed as mean ± SEM of three independent experiments performed in duplicate (n=6) with the values for control cells set as 1. Statistically significant differences were determined using a two-tailed Student's t test: *p< 0.05 relative to control; #p< 0.05 relative to IL6.*