BJP British Journal of Pharmacology

British Journal of Pharmacology (2017) **174** 4329–4344 4329

RESEARCH PAPER

Activation of G protein-coupled oestrogen receptor 1 at the onset of reperfusion protects the myocardium against ischemia/reperfusion injury by reducing mitochondrial dysfunction and mitophagy

Correspondence Jean C Bopassa, Department of Cellular and Integrative Physiology, School of Medicine, The University of Texas Health at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229, USA. E-mail: bopassa@uthscsa.edu

Received 10 April 2017; **Revised** 29 August 2017; **Accepted** 30 August 2017

Yansheng Feng[1,2], Ngonidzashe B Madungwe[1,3], Carolina Victoria da Cruz Junho[1,4] and Jean C Bopassa[1]

[1]Department of Cellular and Integrative Physiology, School of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA, [2]Department of Pathophysiology, Xinxiang Medical University, Xinxiang, China, [3]Department of Biomedical Engineering, University of Texas at San Antonio, San Antonio, TX, USA, and [4]Center of Natural and Human Sciences, Federal University of ABC, Sao Paulo, Brazil

BACKGROUND AND PURPOSE

Recent evidence indicates that GPER (G protein-coupled oestrogen receptor 1) mediates acute pre-ischaemic oestrogen-induced protection of the myocardium from ischaemia/reperfusion injury *via* a signalling cascade that includes PKC translocation, ERK1/2/ GSK-3β phosphorylation and inhibition of the mitochondrial permeability transition pore (mPTP) opening. Here, we investigated the impact and mechanism involved in post-ischaemic GPER activation in ischaemia/reperfusion injury. We determined whether GPER activation at the onset of reperfusion confers cardioprotective effects by protecting against mitochondrial impairment and mitophagy.

EXPERIMENTAL APPROACH

In vivo rat hearts were subjected to ischaemia followed by reperfusion with oestrogen (17β-oestradiol, E2), E2 + G15, a GPER antagonist, or vehicle. Myocardial infarct size, the threshold for the opening of mPTP, mitophagy, mitochondrial membrane potential, ROS production, proteins ubiquitinated including cyclophilin D, and phosphorylation levels of ERK and GSK-3β were measured.

RESULTS

We found that post-ischaemic E2 administration to both male and female ovariectomized-rats reduced myocardial infarct size. Post-ischaemic E2 administration preserved mitochondrial structural integrity and this was associated with a decrease in ROS production and increased mitochondrial membrane potential, as well as an increase in the mitochondrial Ca^{2+} load required to induce mPTP opening *via* activation of the MEK/ERK/GSK-3β axis. Moreover, E2 reduced mitophagy *via* the PINK1/Parkin pathway involving LC3I, LC3II and p62 proteins. All these post-ischaemic effects of E2 were abolished by G15 suggesting a GPER-dependent mechanism.

CONCLUSION

These results indicate that post-ischaemic GPER activation induces cardioprotective effects against ischaemia/reperfusion injury in males and females by protecting mitochondrial structural integrity and function and reducing mitophagy.

Abbreviations

b.w., body weight; CRC, calcium retention capacity; CypD, cyclophilin D; E2, 17β-oestradiol or oestrogen; ER, oestrogen receptor; GPER, G protein-coupled oestrogen receptor 1; GSK-3β, glycogen synthase kinase 3β; I/R, ischaemia/reperfusion;

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DOI:10.1111/bph.14033

LC3, microtubule-associated protein light chain 3; MEK, MAPK kinase; I, myocardial infarction; MMP, mitochondrial membrane potential; mPTP, mitochondrial permeability transition pore; p62, ubiquitin-binding protein; TTC, triphenyltetrazolium chloride

Introduction

Cardiovascular disease remains the leading cause of death in the United States. Currently, there is no effective post-ischaemic pharmacological agent for protecting the heart against the detrimental effects of lethal myocardial ischaemia/reperfusion (I/R) injury. Therefore, the search for clarification of the molecular mechanisms underlying the progression and development of coronary artery disease that can protect the myocardium against the detrimental effect of I/R injury has become an area of intense research. With this in mind, increased attention has been focused on the sex hormone oestrogen for its ability to induce cardioprotective effects against I/R injury and atherosclerosis. Several studies have demonstrated that **oestrogen** (E2, 17β-oestradiol) exerts protective effects against I/R injury on the myocardium (Luo *et al.,* 2016; Sivasinprasasn *et al.,* 2016) and in the brain after stroke (Carpenter *et al.,* 2016). The effects of oestrogen are mediated by three types of oestrogen receptor (ER), **ERα** (*Esr1*), **ERβ** (*Esr2*) and the seven-transmembrane **G protein-coupled oestrogen receptor** (GPER, also referred to as GPR30). This hormone activates complex pathways involving both genomic and non-genomic targets within cells. Although the genomic mechanisms are relatively well characterized, being mediated by classical ERs (Klinge, 2001), the non-genomic ones still need to be explored (Filardo *et al.,* 2000). In direct genomic regulation, binding of E2 to the ERs promotes homo/hetero dimers that translocate to the nucleus to bind directly to gene oestrogen response elements, regulating gene transcription (Skavdahl *et al.,* 2005). ERs at the plasma membrane or in the cytosol can also interact with non-genomic targets like kinases and scaffolding molecules modulating multiple signal pathways (Stoica *et al.,* 2003), thus inducing cardio-protective effects after I/R (Patten *et al.,* 2004).

In addition to the classic nuclear ER isoforms, several studies have demonstrated that GPER can mediate the rapid effects of oestrogen (Carmeci *et al.,* 1997; Martensson *et al.,* 2009). It has been proposed that GPER binds oestrogen, resulting in intracellular calcium mobilization and synthesis of **phosphatidylinositol (3,4,5)-triphosphate** (PIP_3). GPER has been shown to play a role in the rapid non-genomic signalling events of E2 widely observed in cells and tissues (Revankar *et al.,* 2005). Despite past studies having questioned the involvement of the hitherto orphan GPR30 receptor (GPER) in mediating oestrogen actions, it should be noted that there is now consensus on the role of GPER as an ER (Pedram *et al.,* 2006; Otto *et al.,* 2009). Using isolated perfused hearts subjected to I/R, we and others have shown that acute (~1 h) pretreatment with the GPER agonist, **G1** (Bologa *et al.,* 2006; Noel *et al.,* 2009), confers cardioprotective effects against I/R injury *via* the **MEK**/**ERK** (MAPK kinase) and **PI3K** pathways (Deschamps and Murphy, 2009; Bopassa *et al.,* 2010). These observations were later confirmed by Maggiolini's group using male spontaneously hypertensive rats (SHRs). Indeed, De Francesco *et al* have found that G1 treatment to isolated perfused hearts from SHRs induces negative inotropic and lusitropic effects compared to normotensive Wistar Kyoto rats, effects that were mediated by the activation of ERK1/2, **Akt**, **GSK-3β**, c-Jun and eNOS. In addition, after hypoxia, the same team has reported an increased expression of GPER along with that of the hypoxic mediator HIF-1a and the fibrotic marker CTGF (De Francesco *et al.,* 2013), suggesting a potential role of GPER in hypertensive disease. More recently, using perfused hearts from male knockout mice, we have shown that GPER, but not ERα nor ERβ, plays a key role in mediating acute pre-ischaemic E2-induced cardioprotection against I/R injury (Kabir *et al.,* 2015). These observations were similar to those indicating that acute E2 treatment induces similar reno-protective effects in WT, $Esr1^{-/-}$ and $Esr2^{-/-}$ mice after cardiac arrest and cardiopulmonary resuscitation, suggesting a mechanism independent of these two ERs. We have further shown that acute pre-ischaemic E2-GPER cardioprotective effects involve regulation of mitochondrial permeability transition pore (mPTP) opening (Bopassa *et al.,* 2010), known to play a critical role in the initiation of the cell death after I/R. These observations indicated that pre-ischaemic GPER activation preserves mitochondrial quality and function. The opening of the mPTP is well recognized to play a crucial role in the mechanism of cell death after I/R (Lim *et al.,* 2007). However, although several proteins have been reported to contribute to mPTP formation, its molecular identity and mechanism is highly debated and incompletely understood (Bernardi, 2013). Nevertheless, there is a consensus on the role of cyclophilin D (a peptidyl-prolyl cis-trans isomerase, CypD) as a regulator of the mPTP opening (Baines *et al.,* 2005). Mitochondrial quality control depends upon a balance between biogenesis and autophagic destruction (Gottlieb and Gustafsson, 2011). It is now well known that in response to various environmental stresses, an intracellular self-digesting process to remove abnormal organelles occurs, a mechanism referred to as macroautophagy (Saito and Sadoshima, 2015). In fact, in severe conditions like during I/R injury, anomalous mitochondria are cleared through selective autophagy, a process named mitophagy (Takagi *et al.,* 2007). It is now well established that the cytosolic E3 ubiquitin ligase, Parkin, and the outer mitochondrial membrane kinase, **PTEN-induced putative kinase 1** (PINK1), are the two main regulators of mitophagy in mammalian cells (Vasquez-Trincado *et al.,* 2016).

In the present study, we investigated whether GPER activation at the onset of reperfusion, or 'post-ischaemia' induces cardioprotective effects against I/R injury in intact animals (*in vivo*).

Methods

Experimental protocols

Protocols followed the Guide for the Care and Use of Laboratory Animals (US Department of Health, NIH) and received

UT Health Science Centre at San Antonio Institutional Animal Care and Use Committee (IACUC) institutional approval. Animals were housed in the animal-specific pathogen-free facility at UTHSCSA main campus in cages with standard wood bedding and space for two rats. The animals had free access to food and drinking water and a 12 h shift between light and darkness. The animals were selected randomly, and the data analysis was performed by a blinded investigator. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Left anterior descending coronary artery occlusion and measurement of infarct size

Male and OVX-female Sprague–Dawley rats (250–300 g) were anaesthetised with ketamine (80 mg·kg^{-1} i.p.) and xylazine (8 mg·kg^{-1} i.p.). Depth of anaesthesia was monitored by toe pinch reflex and palpebral reflex. OVX-female rats were used to avoid confounding study results with endogenous oestrogen. The rats were intubated and ventilated with a ventilator (CWE SAR-830/P). The hearts were exposed through a left thoracotomy in the fourth intercostal space. The pericardium was opened, and a 5.0 Prolene suture was tightened around the proximal left anterior descending (LAD) coronary artery. Ischaemia was confirmed by ST elevation in the electrocardiogram. The heart was subjected to 35 min of ischaemia, followed by 3 h of reperfusion, which was achieved by releasing the tension on the ligature. An E2 bolus [0.05-mg·kg^{-1} body weight (b.w.)] (Samantaray *et al.,* 2016) was applied *via* the jugular vein 5 min before reperfusion and **G15** (0.3 mg·kg^{-1}) (Serizawa *et al.,* 2017), a GPER antagonist, was given 10 min before E2. The same volume of PBS was given to the control group. At 3 h of reperfusion, the left anterior descending artery (LAD) was briefly re-occluded, and Evans blue dye (2.5 mL of a 2% solution) was infused into the jugular vein to delineate the area at risk (AAR) (Ovize *et al.,* 1994), the region lacking blue staining. Hearts were excised, and atrial and right ventricular tissues were trimmed off. The ventricles of the hearts were sliced transversely into 2 mm thick slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37°C for 15 min to identify the non-infarcted and infarcted areas. The infarcted area was displayed as the area unstained by TTC. Infarct size was expressed as a percentage of the AAR.

Ca^{2+}-induced opening of the mitochondrial permeability transition pore (mPTP)

Mitochondria isolation. After 10 and 30 min of reperfusion, hearts were excised while still beating and immediately placed in cold Krebs–Henseleit buffer. The right ventricle was discarded, and the AAR of the myocardium was harvested for mitochondria isolation. All procedures were carried out at 4°C as previously described in Bopassa *et al.* (2010). Briefly, myocardial AAR was placed in isolation buffer A (mM): 70 sucrose, 210 mannitol, 1 EDTA and 50 Tris–HCl, pH 7.4. The tissue was finely minced with scissors and homogenized in the same buffer A (1 mL buffer/0.15 g of tissue) using Kontes and Potter-Elvehjem tissue grinders. The homogenate was centrifuged at 1300× *g* for 3 min, and the supernatant was filtered through cheesecloth and centrifuged at 10 000× *g* for 10 min. The supernatant was discarded, and the pellet was gently washed three times with 500 μL of buffer B (in mM): 150 sucrose, 50 KCl, 2 KH_2PO_4, 5 succinic acid and 20 Tris/HCl, pH 7.4, and resuspended with 50 μL of the same buffer. Mitochondrial protein concentration was assayed using the Bradford method and adjusted to a final concentration of 25 mg·mL^{-1}.

Calcium retention capacity (CRC). The method used to assess CRC was adapted from that previously described by Ichas *et al.* (1994). CRC was defined as the amount of Ca^{2+} required to trigger a massive Ca^{2+} release by mitochondria, which corresponds to mPTP opening. Mitochondrial CRC was measured spectrofluorometrically using calcium green-5 N (Invitrogen, Carlsbad, CA, USA) and excitation and emission wavelengths set at 500 and 530 nm respectively. Isolated mitochondria (250 μg of mitochondrial protein mL^{-1}) were added to a spectrofluorometer cuvette containing 2 mL of buffer B supplemented with 0.5 μM calcium green-5 N under constant stirring. Upon addition of mitochondria, there is a progressive reduction of the Ca^{2+} in the media due to mitochondrial Ca^{2+} uptake reaching a quasi-steady-state in ~90 s. At this time, Ca^{2+} pulses of 20 nmol·mg^{-1} of mitochondrial protein were added every 60 s to the cuvette. The Ca^{2+} pulses induced a peak in the extra-mitochondrial Ca^{2+} concentration that returns to near-baseline levels as Ca^{2+} enters the mitochondrial matrix. With increasing mitochondria calcium loading, extra-mitochondrial Ca^{2+} starts accumulating until the addition of Ca^{2+} leads to a sustained Ca^{2+} increase indicating a massive release of mitochondrial Ca^{2+}.

Western blot analysis

Equal amounts of protein were loaded into each well of 4–20% Tris-glycine gels (Bio-Rad, Hercules, CA, USA) as recently described in Kabir *et al.* (2015). After electrophoresis for 90 min at 125 V of constant voltage, the gel was blotted onto a nitrocellulose membrane by electrophoretic transfer at 90 V of constant voltage for 1.5 h. The membrane was washed, blocked with 5% blocking solution and probed with various primary antibodies at 4°C overnight. Antibodies: ubiquitin-binding protein (p62) (Cell Signalling Technology, Danvers, MA, USA, 5114, 2 μg·mL^{-1}), LC3 (Novus biologicals, Littleton, CO, USA, NB100–2220, 2 μg·mL^{-1}), GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA, sc-32 233, 2 μg·mL^{-1}), VDAC1 (Santa Cruz Biotechnology, sc-390 996, 2 μg·mL^{-1}), $ERK_{1/2}$ (Cell Signalling Technology, 9102, 19 ng·mL^{-1}), p-$ERK_{1/2}$ (Cell Signalling Technology, 9101, 0.31 μg·mL^{-1}), GSK-3β (Cell Signalling Technology, 9315, 24 ng·mL^{-1}), p-GSK-3β (Cell Signalling Technology, 9336, 67 ng·mL^{-1}), ubiquitin (Thermo Fisher Scientific, Waltham, MA, USA, PA5-17067, 2 mg.mL^{-1}), cyclophilin D (Novus Biologicals, 455 900, 1 μg·mL^{-1}), PINK1 (Cell Signalling Technology, 6946, 2 μg·mL^{-1}), Parkin (Santa Cruz Biotechnology, sc-32 282, 2 μg·mL^{-1}), pAkt (Ser^{473}; 33 ng·mL^{-1}) and Akt (38-ng·mL^{-1}). After being washed, membranes were incubated for 1 h at room temperature with the corresponding fluorophore-conjugated secondary antibodies (goat anti-rabbit Alexa 680, 20 ng·mL^{-1}; goat anti-mouse IR Dye 800CW, 10 ng·mL^{-1}). Then after a further wash, bands were visualized using an infrared fluorescence system (Odyssey Imaging System, Li-COR Biosciences).

Flow cytometry to detect mitophagy

Mitophagy in mitochondria was detected by use of a Mitophagy Detection Kit according to the manufacturer (Dojindo Molecular Technology, Rockville, MD, USA) as described in Liu *et al*. (2016). This kit is composed of Mtphagy Dye, reagent for detection of mitophagy, and Lyso Dye, reagent for staining of lysosomes allowing accurate quantification of the damaged mitochondria fusing to the lysosomes. The signal was detected in isolated cardiomyocytes as described previously (Singh *et al.,* 2013) using flow cytometry analysis at the following wavelengths: Mtphagy Dye: 561 nm (Ex) and 650 nm (Em); Lyso Dye: 488 nm (Ex) and 550 nm (Em).

Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was qualitatively assessed using tetramethylrhodamine methyl ester (TMRM) fluorescence intensity (excitation/emission 550/575 nm) in isolated mitochondria. Isolated mitochondria (0.125 mg·mL^{-1}) from different groups were added to 2.0 mL of buffer containing: 70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM Tris-phosphate, 5 mM succinate, adjusted to pH 7.4 at 30°C with continuous stirring, in the presence of TMRM (200 nM), and potential was spectrofluorometrically assessed by quenching of the fluorescent signal and carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (CCCP) was used to depolarize mitochondria.

Mitochondrial ROS production

Mitochondrial ROS production was assessed as described in Madungwe *et al*. (2016). Mitochondrial ROS generation was measured spectrofluorometrically (560 nm excitation and 590 nm emission) in 0.125 mg·mL^{-1} of mitochondrial protein incubated in a solution containing: 20 mM Tris, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA and 0.15% BSA adjusted to pH 7.4 at 30°C with continuous stirring. ROS was measured with the H_2O_2-sensitive dye Amplex red (10 μM) according to the manufacturer's instructions (Invitrogen). H_2O_2 levels were measured from a calibration curve obtained from the fluorescence emission intensity as a function of H_2O_2 concentration. The sodium salt of glutamate/malate (3 mM) was used to activate complex I of the mitochondrial electron transport chain.

Co-immunoprecipitation assay

Heart lysates obtained after I/R were lysed in lysis buffer [150 mM NaCl, 50 mM Tris, 5 mM EDTA, 10 mM HEPES, 0.1% octylphenyl-polyethylene glycol (IGEPAL CA-630), 0.25% sodium deoxycholate, pH 7.4, plus Complete Protease Inhibitor Cocktail Tablets]. Lysates were centrifuged (10 min, 13 000× *g*, 4°C), and the supernatants were precleared with 10 μL protein A/G resin·mg^{-1} protein (1 h, 4°C, shaking) (Pierce Biotechnology, Inc.) and centrifuged 2 min at 2000× *g*. The precleared lysates (1 mg protein) were incubated overnight at 4°C with 10 μL antibody saturated protein A/G resin (2 μg Ab/10 μL resin, 2 h at 4°C, shaking) in a final volume of 500 μL lysis buffer. Samples were centrifuged 2 min at 2000× *g* and washed five times with Buffer A. The immunoprecipitated proteins were eluted from the beads with 30 μL 3× Laemmli sample buffer (37°C, 1 h). After centrifugation (3 min, 13 000× *g*, 4°C), immunoprecipitated proteins as well as lysates were analysed by SDS/PAGE and immunoblotting (Western blot).

Electron microscopy

The rat hearts were imaged by electron microscopy to observe mitochondrial quality and morphology. Hearts from all groups were immediately fixed in 2.5% (wv^{-1}) glutaraldehyde (Fluka, Mexico City, Mexico) and stored in the same solution at 4°C overnight. The dissected heart tissues were washed with PBS, post-fixed in 2% (wv^{-1}) osmium tetroxide for 2 h at room temperature. Fixed dissected tissues were dehydrated in a graded alcohol series and embedded in Eponate 12 medium. The blocks were cured at 60°C for 48 h, and sections (70 nm) were cut with an RMC Boeckeler, Tucson, AZ, USA ultramicrotome and mounted on Formvar-coated grids. The sections were double-stained with uranyl acetate and lead citrate and finally examined and imaged with a 100CX JEOL transmission electron microscope.

Statistical analysis

Error bars represent ±SEM for a minimum of five independent hearts. For cardiac infarct size and mitochondrial CRC, means were compared between groups using one-way ANOVA. Under the ANOVA model, pair wise mean comparisons were judged significant using the Tukey's studentized range criterion. SPSS, version 13.0 (SPSS Inc, Chicago, IL, USA), was used to carry out the computations. Because all outcomes were continuous, results were summarized with means ± SEMs. $P < 0.05$ was considered statistically significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al*., 2015).

Nomeclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.,* 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.,* 2015a,b).

Results

Post-ischaemic GPER activation protects the myocardium against ischaemia/reperfusion injury in the in vivo rat model

Using the isolated perfused heart model, we recently showed that pre-ischaemic treatment with oestrogen can protect the heart against I/R injury in WT mice hearts but not *Gper1*$^{-/-}$ hearts (Kabir *et al.,* 2015). Although this *ex vivo* model is reliable, it is not possible to fully test how the alterations observed in isolated organs would affect the rest of the organism's body. Therefore, we studied whether post-ischaemic administration of E2 to intact male or ovariectomized female rats (*in vivo*, a more clinically relevant model) can also protect the heart against I/R injury. The LAD artery was ligated for 35 min followed by 3 h of reperfusion. One single bolus of PBS or E2 (0.05 mg·kg^{-1}) (Ablove *et al.,* 2009; Samantaray *et al.,* 2016) was applied through the femoral vein 5 min before reperfusion (Figure 1A). The AAR to left ventricle (LV)

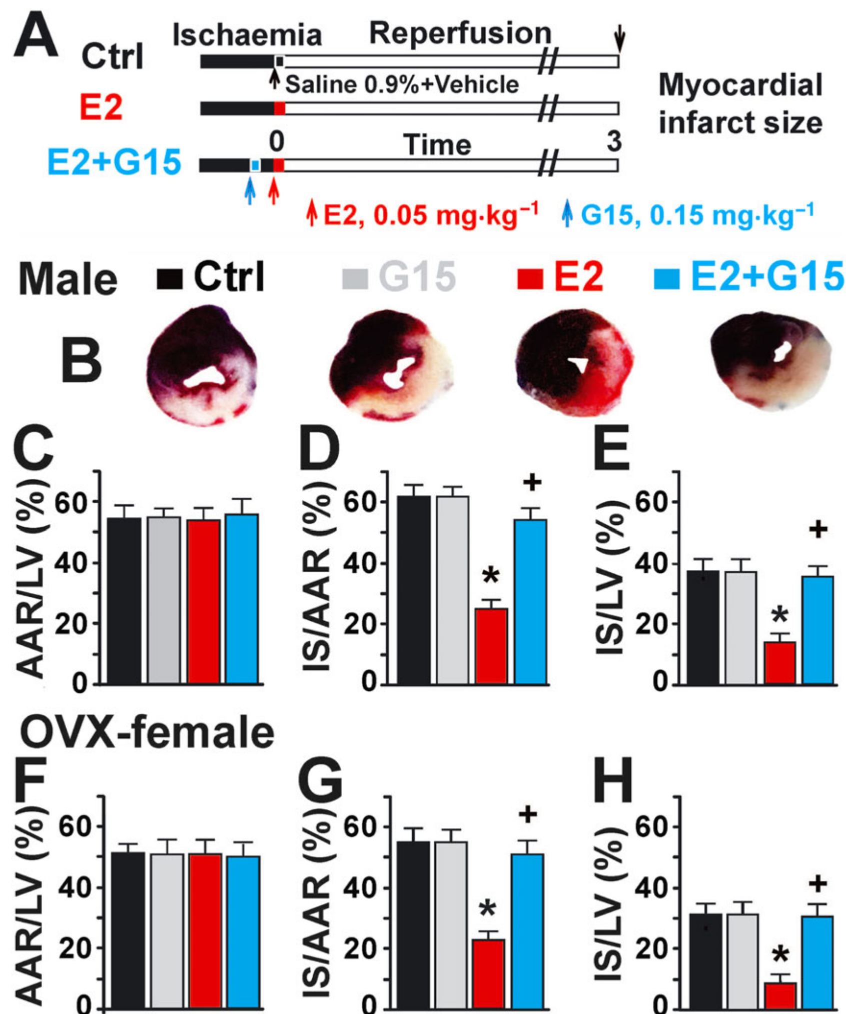

Figure 1

Post-ischaemic GPER activation induces cardioprotective effects against I/R injury in male and OVX-female rats. (A) Protocol showing administration of a single bolus of saline (0.9%) for control, E2 or G15. (B) Example of images showing post-ischaemic E2-induced reduction in myocardial infarct size (IS) prevented by pre-administration of G15 in male and OVX-female rats. (C, F) Graphs showing the % of area at risk (AAR) in the LV. (D, G) Graph showing the percentage of myocardial IS in the AAR and in the LV. (E, H) $^{*}P < 0.05$ E2-treated versus control. $^{+}P < 0.05$ E2 versus E2 + G15-treated group ($n = 6$ hearts per group). Note that while the areas at risk are similar in all groups, myocardial IS as a function of the LV and AAR is reduced in E2-treated group versus both control and E2 + G15-treated groups indicating a GPER-dependent mechanism.

ratio was similar in all the groups ($n = 6$ per group), indicating that all these groups were subjected to a comparable degree of ischaemic risk (Figures 1C, F). However, the infarct size was significantly smaller in the E2 group versus control. In male, the ratio of infarct size to AAR was 21 ± 2 in E2 versus 62 ± 5 in control (Figures 1D, G), and the ratio of infarct size to LV was 12 ± 2 in E2 versus 38 ± 2 in control (Figures 1E, H). These E2 effects were abolished by co-administration with the GPER antagonist G15 (0.15-mg·kg^{-1}) (Figures 1D, G and E, H), while G15 alone did not change myocardial infarct size compared to control. These data demonstrate that administration of only one bolus of E2 at the onset of reperfusion is sufficient to protect the myocardium against I/R injury *in vivo* and this action is mediated *via* GPER activation.

Post-ischaemic GPER activation increases mitochondrial Ca^{2+} retention capacity

We have shown that pre-ischaemic treatment with the GPER agonist, G1, induced cardioprotective effects against I/R injury and the mechanism involves an increase in the Ca^{2+}-load required to induce the mPTP opening (Bopassa *et al.*, 2010). Therefore, we postulated that if indeed GPER is essential for post-ischaemic E2 protective action, administration of E2 should exhibit an increased tolerance to Ca^{2+} overload or augmented mitochondrial Ca^{2+} retention capacity (CRC). To test this argument, mitochondria were isolated in the areas at risk at 30 min of reperfusion. Figure 2A shows that post-ischaemic E2 treatment increased mitochondrial CRC, reflecting an inhibition of the mPTP opening in both male and OVX-female animals. Indeed, mitochondria from

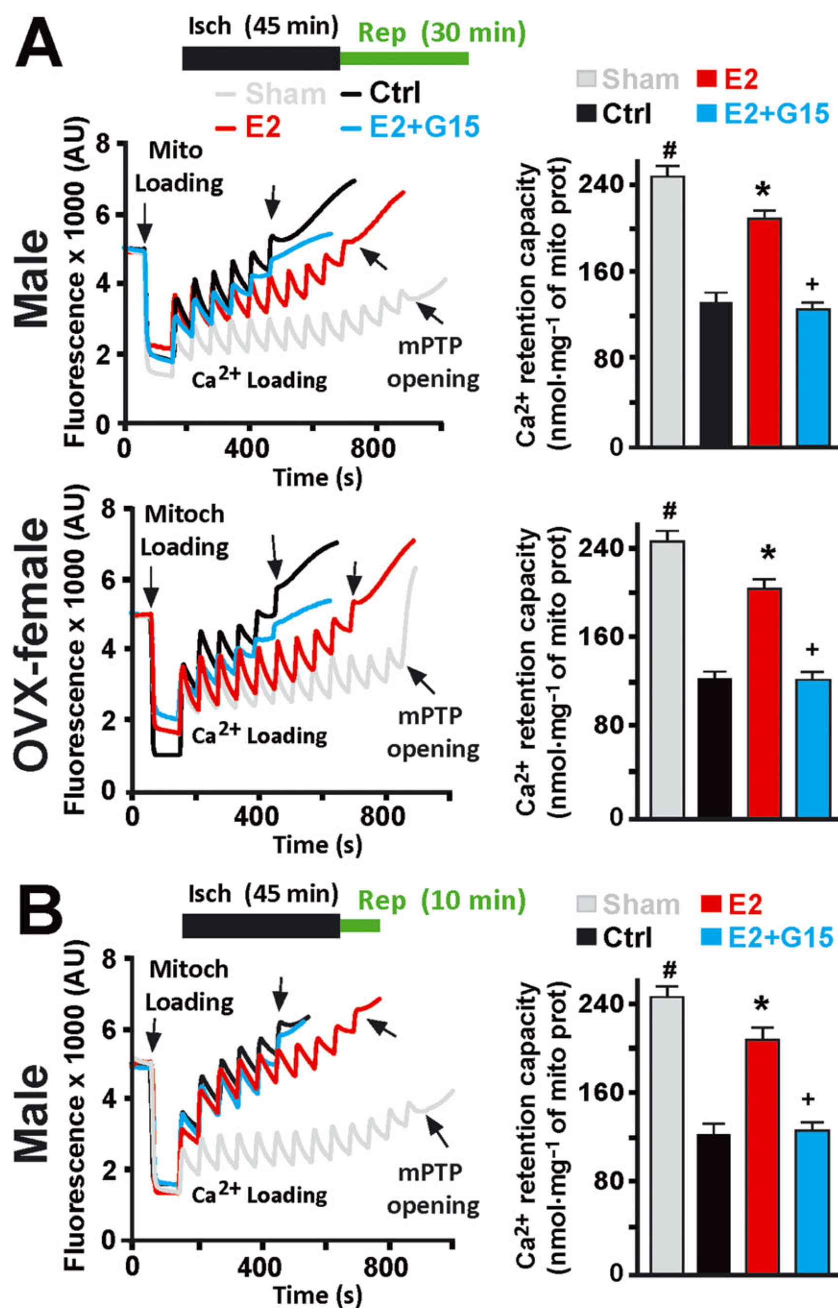

Figure 2

Post-ischaemic GPER activation decreases mitochondrial sensitivity to Ca^{2+} overload in male and OVX-female rats. In (A), the data were obtained after 35 min ischaemia followed by 30 min reperfusion, and in (B), the results were obtained after only 10 min reperfusion. Left: spectrofluorometric recordings of Ca^{2+} overload in mitochondria isolated from hearts subjected to I/R in vehicle group (Ctrl), E2, E2 + G15 and sham group in male (top) and female (bottom) rats. Arrowheads mark the time of mitochondria addition and the initial mitochondrial Ca^{2+} uptake. Subsequent 20-nmol·mg^{-1} of protein Ca^{2+} pulses were delivered until a spontaneous massive release was observed, presumably to the opening of mPTP (arrows). Only mitochondria from the E2-treated group kept their ability to endure higher Ca^{2+} overload similar to non-ischaemic group (sham) much more than both control and E2 + G15-treated groups indicating a GPER-dependent mechanism. Right: graphs of mean Ca^{2+} retention capacity values (amount of Ca^{2+} load needed to induce mPTP opening) in male (top) and female (bottom) rats. Values are expressed as mean ± SEM; $^{*}P < 0.05$ control versus sham group, $^{+}P < 0.05$ control versus E2-treated group and $^{\#}P < 0.05$ E2 versus E2 + G15-treated group ($n = 5$ per group).

control and control + G15 hearts required an average of five to seven Ca^{2+} pulses (20 nmol·mg^{-1} of protein each) to trigger the mPTP opening (arrows) (Figure 2A). This number was increased to between 10 and 12 when hearts were reperfused with E2, and pre-administration with G15 prevented this E2-induced increase in mitochondrial CRC (Figure 2A). Mean values for mitochondrial CRC in each animal model in the absence (control, ctrl) or presence of E2

are given in Figure 2A. The control, control + G15 and E2 + G15 displayed similar values: control = 130 ± 10, control + G15 = 132 ± 7, nmol·mg^{-1} of mitochondrial protein (n = 5). In contrast, post-ischaemic E2 treatment produced a significant increase of this mitochondrial CRC to 205 ± 6 nmol·mg^{-1} of protein (n = 5), but the E2 + G15 CRC was similar to control (E2 + G15 = 127 ± 10 nmol·mg^{-1} of protein). Due to reports indicating that the opening of the mPTP occurs during the early moments of reperfusion (Griffiths and Halestrap, 1995), we confirmed in mitochondria isolated at 10 min reperfusion that post-ischaemic E2 administration increases mitochondrial CRC versus control (Figure 2B) and this effect was also prevented by the pre-administration of G15. Together, these results indicate that GPER activation mediates E2-induced increase in mitochondrial CRC and the GPER-mediated cardioprotective effect of post-ischaemic E2 is related to a decrease in mPTP sensitivity to Ca^{2+} overload.

Post-ischaemic GPER activation reduces the level of mitophagy

As we found similar effects of post-ischaemic GPER activation on myocardial infarct size and mitochondrial integrity and function in male and OVX-female animals, we focused on investigating the molecular mechanisms involved in post-ischaemic GPER using only male animals. We found that post-ischaemic GPER activation delays the mPTP opening indicating a better state of mitochondria. Thus, we studied

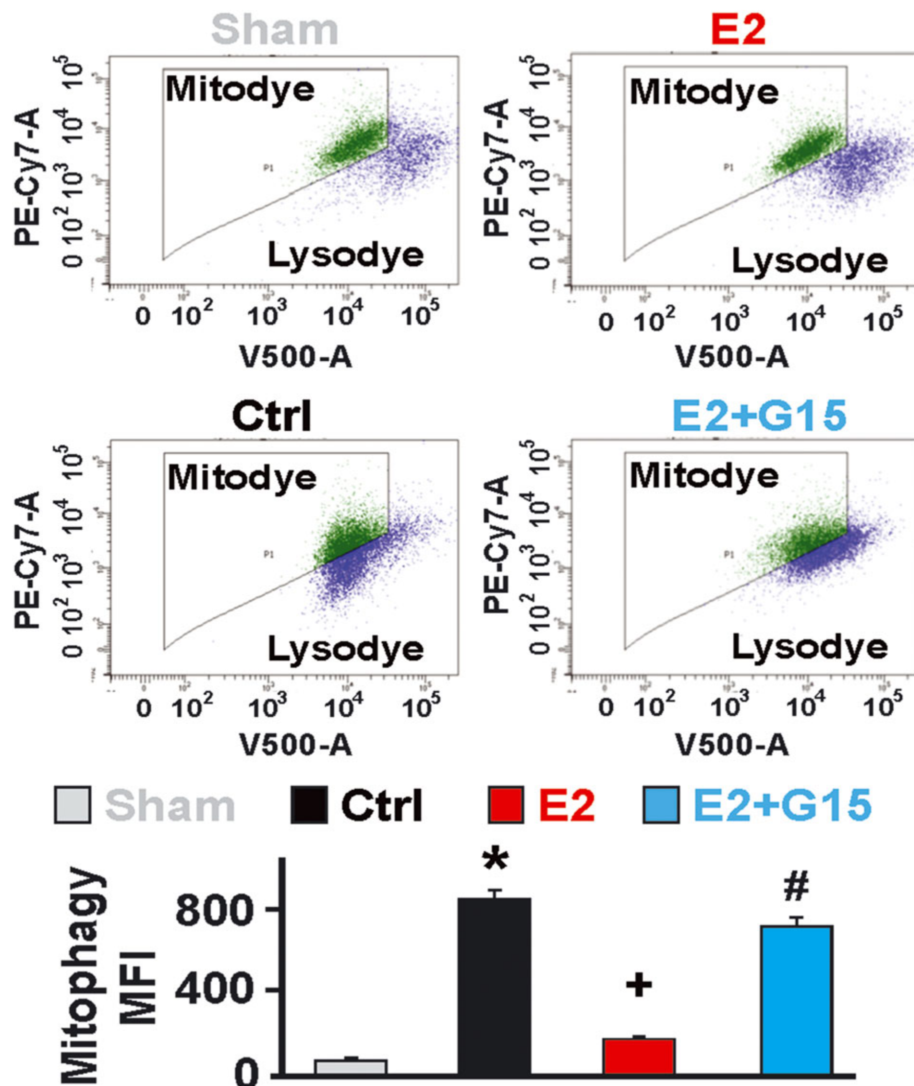

Figure 3

Post-ischaemic GPER activation reduces mitophagy after I/R injury. Top: flow cytometry analysis of mitophagy with Mitodye (green) and mitochondria fused to lysosomes with Lysodye (blue) in all the groups. Bottom: graph showing reduction in mitophagy in E2-treated group similar to sham versus control and E2 + G15-treated groups. Note that the number of mitochondria fused to lysosomes is significantly reduced in the E2-treated group versus control and E2 + G15-treated groups suggesting a GPER-dependent mechanism. Values are expressed as mean ± SEM; *$P < 0.05$ control versus sham groups, $^{+}P < 0.05$ control versus E2-treated group and $^{\#}P < 0.05$ E2 versus E2 + G15-treated group (n = 5 per group).

whether the protection of mitochondria during E2-GPER action is due to the reduction of mitophagy. To this end, we isolated cardiomyocytes from hearts subjected to 35 min ischaemia followed by 30 min reperfusion. Mitophagy was evaluated by flow cytometry analysis using mitodye and lysodye. As shown in Figure 3, the level of mitophagy was dramatically reduced in hearts treated with E2 as compared to control, and this E2 effect was prevented by the addition of G15. In fact, the mean fluorescence intensity that corresponds to the level of mitophagy was dramatically higher in control and E2 + G15 groups, than in sham and E2-treated groups Figure 3. To determine the mechanism by which post-ischaemic GPER induces reduction of mitophagy, we investigated the impact of the PINK1/Parkin pathway, which is known to be involved in mitophagy *via* autophagy adaptors (Kubli and Gustafsson, 2012), in E2-GPER action. Using Western blot analysis, we found that in the post-ischaemic E2 group, Parkin expression was lower in the mitochondrial fraction and higher in the cytosolic fraction similar to sham, as compared to control. Addition of the GPER antagonist G15 abolished this E2 effect by increasing Parkin translocation to the mitochondria (Figure 4). We also found that post-ischaemic E2 treatment reduced LC3I and LC3II and increased p62 and PINK1 levels versus control, effects that were prevented by G15, indicating a GPER-dependent mechanism (Figure 4). These results indicate that the mechanism by which post-ischaemic GPER activation reduces mitophagy involves deactivation of the PINK1/Parkin pathway. Using electron microscopy, we observed that mitochondrial structural integrity was preserved with post-ischaemic E2 treatment as compared to both control and E2 + G15. We also noticed that although the number of mitochondria was not very different between the groups, mito-phagosomes were more frequently observed after I/R in control and E2 + G15 groups versus E2-treated and sham groups (Figure 5). These data support the postulate that post-ischaemic GPER action involves the reduction of mitophagy after I/R.

Post-ischaemic GPER activation reduces myocardial infarct size via activation of the MEK/ERK/GSK-3β pathway

To define the mechanisms involved in post-ischaemic GPER-induced cardioprotection against myocardial I/R injury, we examined the impact of the MEK/ERK and PI3K/Akt pathways whose action is known to deactivate GSK-3β leading to the delay of the mPTP opening (Rahman *et al.*, 2011). We found that administration of E2 significantly increases the level of the phosphorylation (p) of ERK1/2 and GSK-3β but not Akt as compared to control, and addition of G15 (Figure 6A) or **U0126** (MEK/ERK inhibitor)(Figure 6B) blocked the E2-induced up-regulation of pERK1/2 and pGSK-3β (Figure 6). Note that the control value was set at 100% and values were obtained as (p)form/(t)form, in which phosphorylated forms (p) of Akt, ERK1/2, and GSK-3β were normalized to total form of Akt, ERK and GSK-3β, respectively, to account for changes in the phosphorylated form that are caused by regulation of the total form of the protein. These results suggest that the post-ischaemic GPER-mediated cardioprotective effect is related to activation of the MEK/ERK, but not PI3K/Akt pathway, leading to deactivation of GSK-3β.

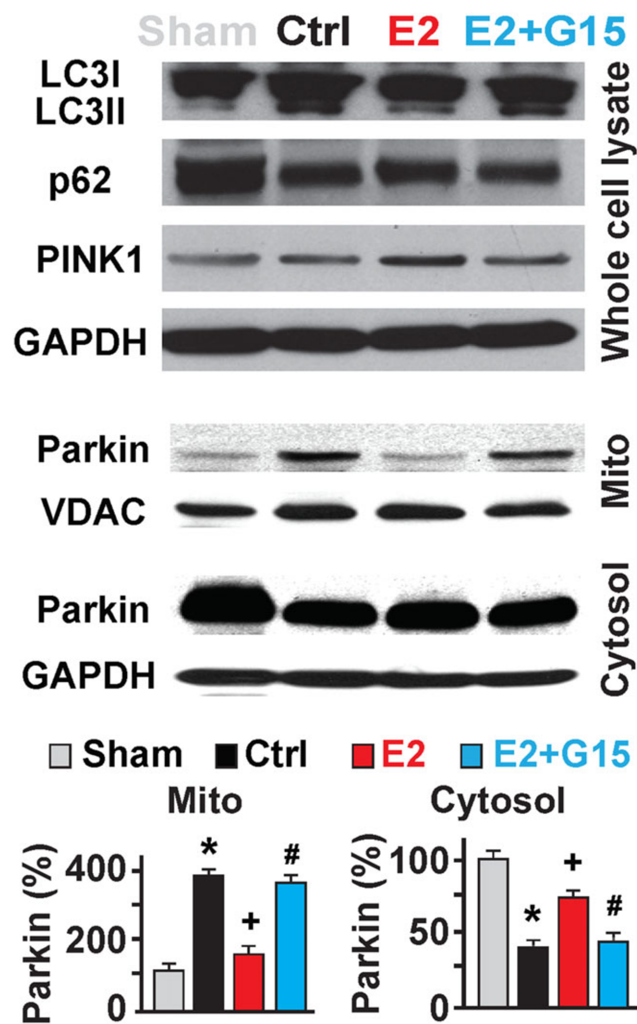

Figure 4

Post-ischaemic GPER activation reduces mitophagy *via* the PINK1/Parkin mechanism. In the whole-cell lysate, post-ischaemic E2 treatment decreased the protein levels of LC3I and LC3II and increased p62 and PINK1 expression versus control. In mitochondria, the Parkin level is reduced, while it is increased in the cytosol in E2 group versus control group indicating a reduction of Parkin translocation into mitochondrial. Note that all these E2 effects were prevented by addition of G15 suggesting a GPER-dependent mechanism. Values are expressed as mean ± SEM; *$P < 0.05$ control versus sham groups, $^{+}P < 0.05$ control versus E2-treated group and $^{\#}P < 0.05$ E2 versus E2 + G15-treated group (n = 5 per group).

Inhibition of mitophagy prevented post-ischaemic GPER-induced cardioprotection

We further investigated whether inhibition of autophagy plays a critical role in post-ischaemic GPER activation-induced reduction of myocardial infarct size. We used two well-known inhibitors of autophagy: U0126 (5 $mg{\cdot}kg^{-1}$ b.w.) (Korkmaz *et al.*, 2011) that acts *via* the MEK/ERK pathway (Pattingre *et al.*, 2003) and 3-Methyladenine (3-MA, 0.3 $mg{\cdot}kg^{-1}$ b.w.; Xia *et al.*, 2016), also well known as a class I and III inhibitor of PI-3K that inhibits autophagy (Wu *et al.*, 2010) as shown in Figure 7A. We found that

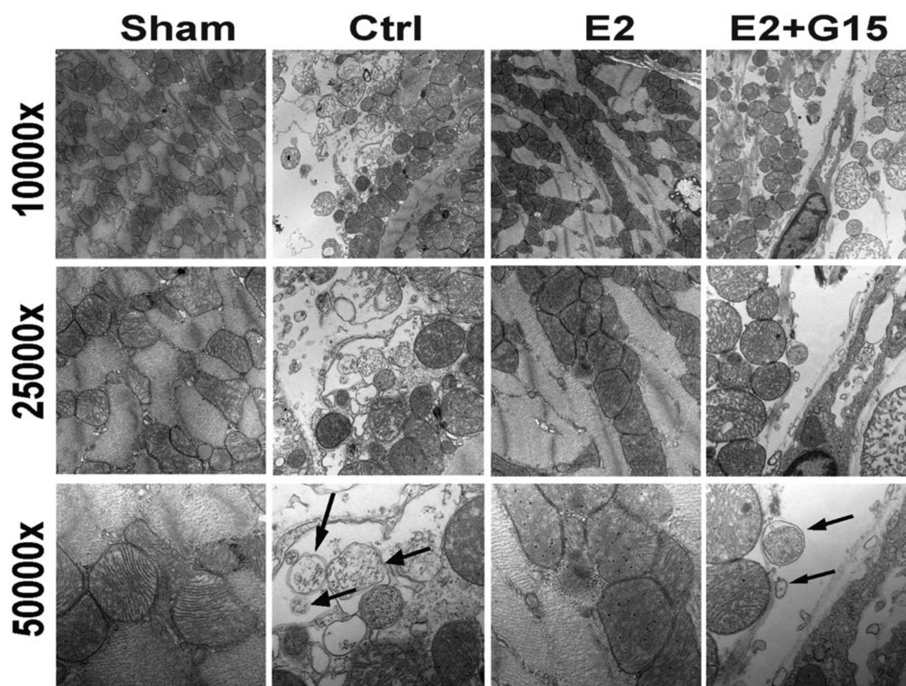

Figure 5

GPER activation preserves mitochondrial integrity. Electron microscopy images of mitochondria in tissue (magnification 10 000×, 25 000× and 50 000×) from non-ischaemic (sham), after I/R control, E2 and E2 + G15 treatment hearts showing a better mitochondrial structure integrity in E2-treated and sham groups in which mitochondrial cristae are intact versus control and E2 + G15-treated groups in which mitochondrial cristae morphology is drastically disrupted and damaged. Note that post-ischaemic E2-induced protection of mitochondrial structural integrity and decrease in the number of mito-phagosomes are abolished by the addition of G15 suggesting a GPER-dependent mechanism.

addition of U0126 slightly reduced myocardial infarct (MI) size, while 3-MA infusion decreased MI as compared to control Figure 7B–E. This result confirms the two different pathways of autophagy. We also found that while pre-administration of U0126 prevented E2-induced reduction of myocardial infarct size, 3-MA did not have any effect on this E2 protective effect (Figure 7B–E). These results indicate that E2 reduces only mitophagy that is mediated by the MEK/ERK pathway but not that acting *via* the PI-3K/Akt axis.

Post-ischaemic GPER activation reduces mitochondrial dysfunction

To study the morphology of mitochondria, we imaged mitochondria with electron microscopy in all the groups. Images revealed that post-ischaemic E2 treatment preserved mitochondrial structural integrity as cristae are well organized, being more similar to the sham group versus the vehicle group, in which cristae are disrupted (Figure 5). We also observed that mitochondria from the E2 + G15 group were similar to control suggesting the protective mechanism is *via* GPER. Mitochondrial protein ubiquitination in response to PINK1/Parkin pathway activation has been implicated in mitophagy (Kubli and Gustafsson, 2012). We, therefore, studied the involvement of mitochondrial protein ubiquitination in post-ischaemic E2 action. We found that post-ischaemic E2 treatment reduced mitochondrial protein ubiquitination versus untreated samples, and addition of G15 prevented these E2 effects (Figure 8A).

We found that post-ischaemic GPER activation decreases the mitochondrial sensitivity to Ca^{2+} overload required to induce the mPTP opening during I/R (Figure 2). As, currently, there is a consensus regarding the role of cyclophilin D as a regulator of the mPTP opening (Baines *et al.,* 2005), we determined whether post-ischaemic GPER delay of the mPTP opening mechanism involves preservation of cyclophilin D by reducing its ubiquitination caused by I/R insult. To this end, we performed IP with anti-ubiquitin pAb and detected cyclophilin D in the pull-down by Western blot analysis. We found that mitochondria from E2-treated hearts exhibited much less ubiquitination of cyclophilin D versus control and this E2 effect was prevented by G15 (Figure 8B). This finding indicates that post-ischaemic GPER action reduces cyclophilin D ubiquitination and hence prevents its degradation. This effect might play an important role in the mechanism of GPER-induced inhibition of the mPTP opening.

It is known that under normal conditions, the E3 ubiquitin ligase Parkin is present in the cytosol and only rapidly translocates into mitochondria upon loss of MMP (Suen *et al.,* 2010). To determine whether the loss of MMP might act as of trigger of mitophagy and play an important role in post-ischaemic E2 action, we isolated mitochondria from hearts subjected to 35 min ischaemia followed by 30 min reperfusion. Mitochondrial MMP was measured

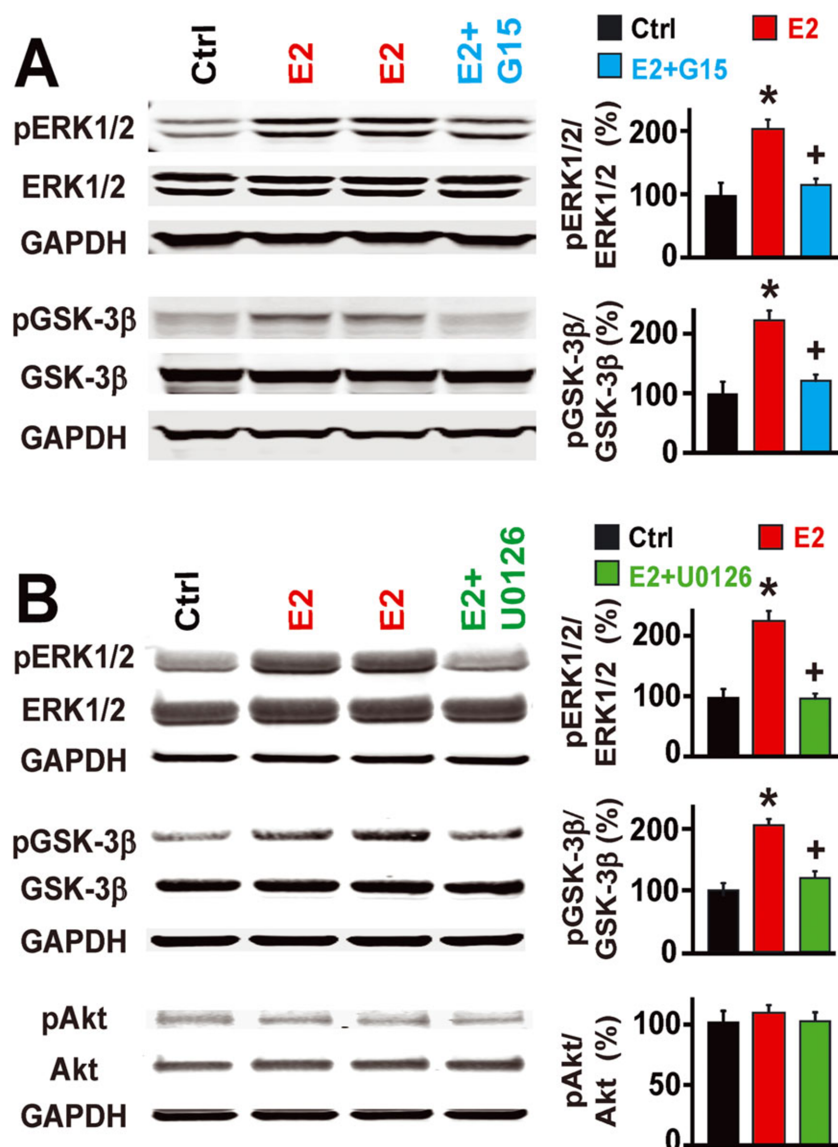

Figure 6

Post-ischaemic GPER activation increases the phosphorylation of ERK1/2 and GSK-3β. Immunoblots and corresponding bar graphs show post-ischaemic E2 treatment increasing pERK1/2/ERK1/2 and pGSK-3β/GSK-3β ratios versus control. These E2 effects were prevented by addition of G15 (A) suggesting a GPER-dependent mechanism and abolished by addition of U0126, a MEK/ERK pathway inhibitor (B). Values are expressed as mean ± SEM; $^{*}P < 0.05$ E2-treated versus control group and $^{+}P < 0.05$ E2 versus E2 + G15-treated groups (n = 5 per group).

spectroflourometrically using TMRM dye (Floryk and Houstek, 1999) and in the presence of CCCP, which depolarizes mitochondria. We found that mitochondria from control hearts were more uncoupled than those treated with E2, and this E2 effect was abolished by G15, the GPER antagonist (Figure 9A). Note that in this study, the sham group was set at 100% as the maximum value of MMP in normal conditions, and the values measured after I/R in all the groups were normalized to sham to determine the degree of mitochondrial depolarization compared to sham group. To further determine the degree of mitochondrial dysfunction, we measured their ability to produce ROS. Mitochondrial ROS generation was measured using the H_2O_2-sensitive dye Amplex red reagent in the presence of horseradish peroxidase. Reduction of molecular oxygen (O_2) produces superoxide ($^{\cdot}O^{-2}$), which is the precursor of most other ROS. Dismutation of superoxide produces hydrogen peroxide (H_2O_2) (Turrens, 2003), making its generation a good index of ROS production in a biological trial. Glutamate/malate was used to activate respiratory complex I. As shown in Figure 9B, ROS production was reduced in the post-ischaemic E2 group as compared to control, and this E2 effect was also prevented by G15. Together, these results indicate that post-ischaemic GPER activation protects the myocardium against mitochondrial dysfunction caused by I/R stress resulting in reduction of mitochondrial protein ubiquitination and ROS production, as well as preservation of mitochondria membrane potential.

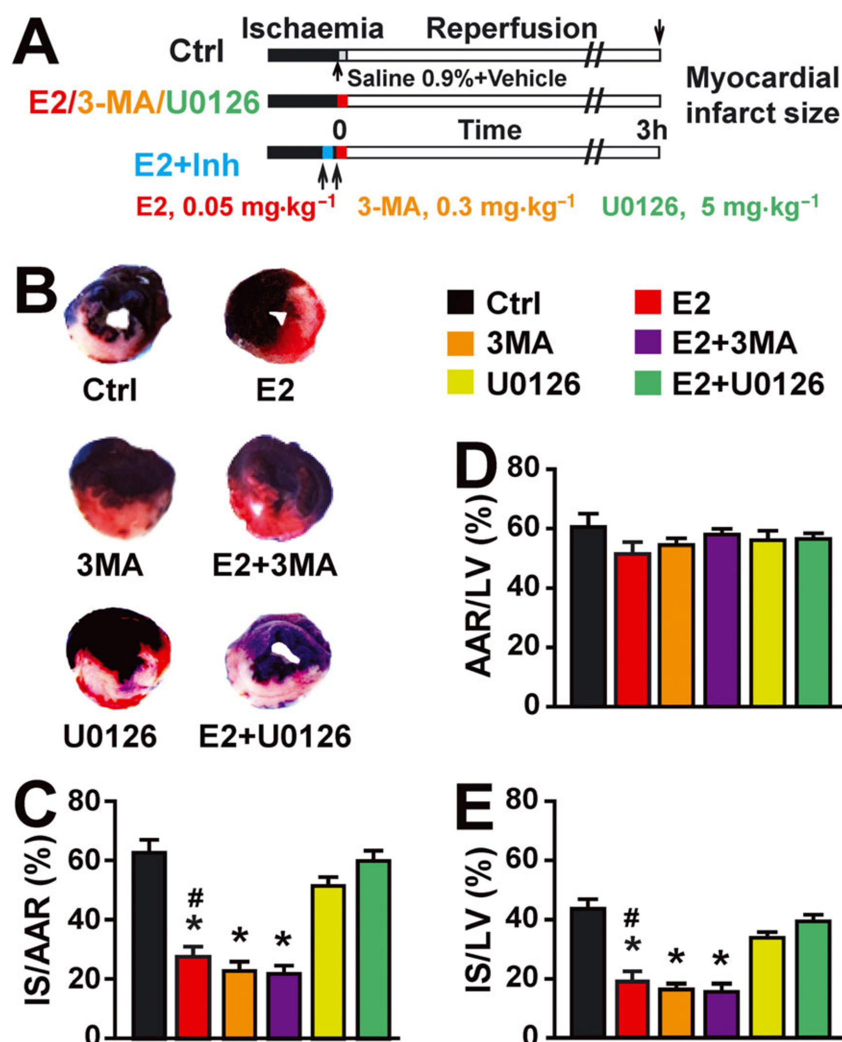

Figure 7

Inhibition of mitophagy prevents post-ischaemic GPER activation reduction of myocardial infarct size (IS). (A) Protocol showing administration of a single bolus of saline (0.9%) for control, E2, U0126 or 3-MA as well as co-addition of E2 + U0126 and E2 + 3-MA. (B) Images showing post-ischaemic E2-induced reduction in myocardial IS prevented by pre-administration of U0126 but not 3-MA in male rats. (C) Graphs showing % AAR in the LV. (D) Graph showing the percentage of myocardial IS in the AAR and in the LV. (E) $^{*}P < 0.05$ E2-treated versus control. $^{+}P < 0.05$ E2 versus E2 + U0126-treated group (n = 5 hearts per group). $^{\#}P < 0.05$ E2 versus E2 + 3-MA-treated group (n = 5 hearts per group). Note that while the areas at risk are similar in all groups, myocardial IS as a function of the LV and AAR is reduced in the E2-treated group (red) versus both control and E2 + U0126-treated (green) groups but not in the E2 + 3-MA group (purple) indicating a MEK/ERK-dependent mechanism of mitophagy.

Discussion

In this study, we discovered that in intact male and OVX-female rats subjected to LAD artery occlusion that post-ischaemic GPER activation induces cardioprotective effects against I/R injury by reducing mitochondrial dysfunction, mitophagy and regulation of the mPTP opening.

Pharmacological pre-ischaemic stimulation of GPER with its agonist G1 has been shown to induce cardioprotective effects against I/R injury in rat and mouse models (Deschamps and Murphy, 2009; Bopassa *et al.*, 2010). Recently, using isolated perfused hearts from animals genetically modified and subjected to I/R, we have shown that GPER activation is essential for the cardioprotective action of acute pre-ischaemic E2 treatment and that ERα (*Esr1*) and ERβ (*Esr2*) are not needed for this effect (Kabir *et al.*, 2015). As administration of drugs at the onset of the ischaemia is difficult to practice in a clinical setting, in this study, we investigated the effects of acute post-ischaemic E2 administration in intact rats (*in vivo*) subjected to LAD artery occlusion followed by reperfusion. We found that administration of E2 at the onset of reperfusion reduces myocardial infarct size as compared to vehicle. These results indicate that acute post-ischaemic E2 administration to the intact animal induces cardioprotective effects against I/R injury. These observations also confirm those obtained with the phytoestrogen (genistein) when treated during reperfusion to the isolated perfused heart (Tissier *et al.*, 2007). However, in these studies using genistein, the observed effects were not related to any ERs. To determine the molecular mechanism by which post-ischaemic

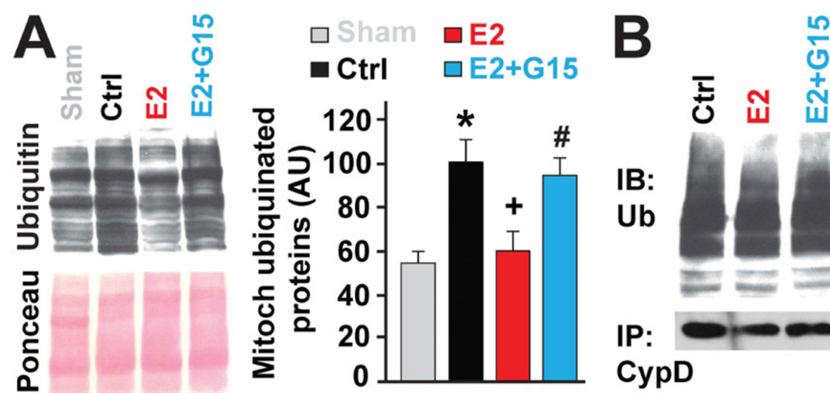

Figure 8

Post-ischaemic GPER activation reduces mitochondrial protein ubiquitination including that of cyclophilin D (CypD). (A) Immunoblot and graph showing post-ischaemic E2 treatment reduced mitochondrial ubiquitinated proteins versus control. Note that this E2 effect is prevented by addition of G15 suggesting a GPER-dependent mechanism. Values are expressed as mean ± SEM; $^{*}P < 0.05$ control versus sham groups, $^{+}P < 0.05$ control versus E2-treated group and $^{\#}P < 0.05$ E2 versus E2 + G15-treated group (n = 5 per group). (B) To determine whether cyclophilin D is ubiquinated, IP was performed with anti-ubiquitin polyclonal antibody and Western blot analysis with anti-cyclophilin monoclonal antibody. The result indicates that post-ischaemic E2 treatment reduces the cyclophilin D ubiquitination level versus control, and this effect is abolished by G15, a GPER antagonist.

E2 administration induces cardioprotection, we studied the role of GPER, using its selective antagonist, G15. Our results indicate that pre-administration of G15, 10 min before E2, prevents acute post-ischaemic E2-induced reduction of myocardial infarct size. In fact, co-administration of G15 with E2 also displayed similar myocardial infarct size as compared to vehicle, suggesting an abolishment of E2 action. These results indicate that GPER activation plays a key role in mediated acute post-ischaemic E2 cardioprotection against I/R injury *in vivo*.

Several studies have shown a central role of the mPTP opening in the process of the lethal myocardial reperfusion injury (Gateau-Roesch *et al.,* 2006). Therefore, we determined the impact of the mPTP opening in acute post-ischaemic E2 action. We used mitochondrial Ca^{2+} retention capacity (CRC) as an indicator of the mPTP opening after Ca^{2+} overload (Baumgartner *et al.,* 2009). A higher CRC indicates better mitochondrial integrity, more resistance to injury and lowered probability to mPTP opening. We found that acute post-ischaemic E2 treatment delays the mPTP opening after I/R. In fact, the mitochondrial Ca^{2+} load required to induce the mPTP opening is higher in acute post-ischaemic E2-treated mitochondria as compared to vehicle, and pretreatment with G15 prevented this E2 effect on mitochondrial CRC indicating a GPER-dependent mechanism. These results indicate that post-ischaemic E2 action *via* GPER might protect mitochondrial structural integrity that results in the decrease in mitochondrial sensitivity to calcium overload during reperfusion. To confirm this statement, we imaged mitochondria with electron microscopy and revealed that acute post-ischaemic E2 treatment preserved mitochondrial integrity as cristae are well organized, being more similar to the non-ischaemic (sham) group than to the vehicle-treated group, in which cristae are disrupted. We also observed that mitochondria from the E2 + G15 group were similar to control indicating that the protective mechanism is *via* GPER. These findings support the hypothesis that acute E2 treatment preserves mitochondrial integrity from degradation caused by I/R stress, which presumably may result in the protection of mitochondrial function. These results may also explain the protection of mitochondrial membrane potential and reduction of mitochondrial ROS observed in the post-ischaemic E2-treated group versus vehicle and E2 + G15 groups, confirming a GPER-dependent mechanism.

The decrease in mitochondrial membrane potential observed after I/R injury has been shown to trigger mitophagy *via* autophagy adaptors (Takagi *et al.,* 2007). We therefore determined whether the protection of mitochondrial integrity and function observed in post-ischaemic E2-GPER action might result in reduction of excessive mitophagy caused by I/R stress. Using a mitophagy detection kit, we found a dramatic reduction of mitophagic flux in the E2-treated group versus control and E2 + G15 groups. Furthermore, using electron microscopy, we observed that mitophagosomes were more abundant after I/R in control and E2 + G15 groups as compared to both E2-treated and sham groups. These observations are similar to those revealed recently by Wu *et al.* (2014) using wild-type mouse embryonic fibroblasts treated with carbonyl cyanide m-chlorophenyl hydrazine or hypoxia. These findings support a reduction of mitochondria elimination by auto-phagosomes during post-ischaemic insult.

It is now well established that the E3 ubiquitin ligase, Parkin, plays a key role in mitophagy *via* autophagy adaptors. In fact, being mostly present in the cytosol under normal conditions, Parkin rapidly translocates into mitochondria upon loss of mitochondrial membrane potential (Suen *et al.,* 2010). In mitochondria, Parkin promotes protein ubiquitination, which triggers mitophagy (Geisler *et al.,* 2010). To determine the mechanism underlying the reduction of mitophagy in E2-GPER action, we measured the levels of key proteins known to play a key role in autophagy including LC3I, LC3II, p62, Parkin and PINK1. In mitophagy *via* autophagy, the p62 protein binds to ubiquitinated proteins *via*

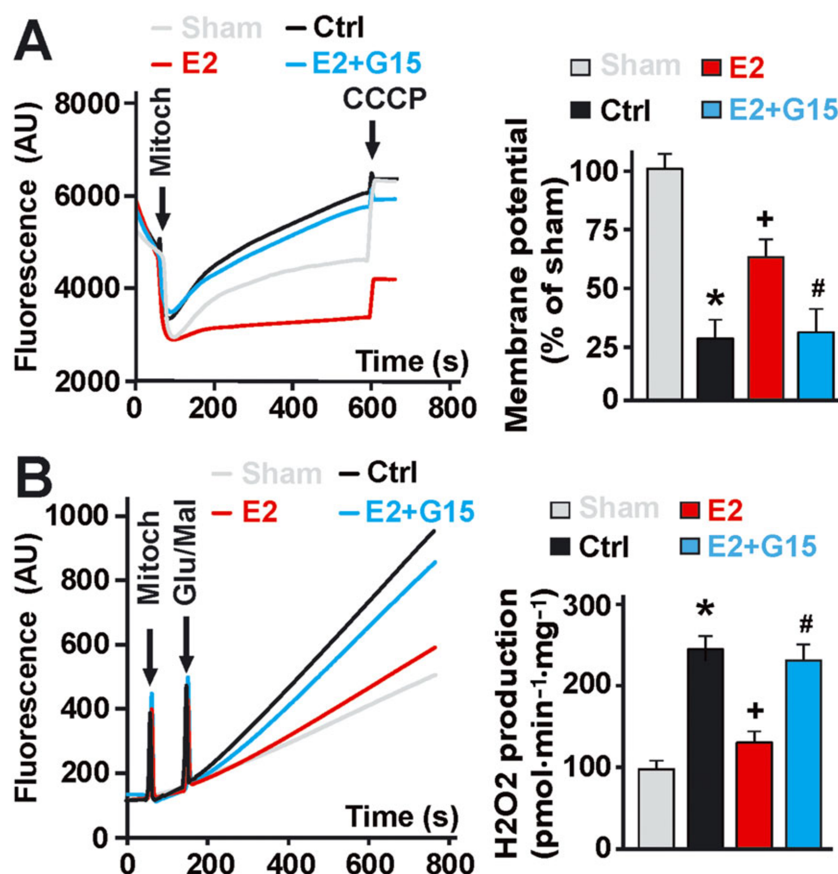

Figure 9

Post-ischaemic GPER activation preserves the mitochondrial membrane potential (MMP) and reduces ROS production. (A) MMP was measured spectrofluorometrically. Left: recordings of fluorescence in the presence of TMRM in mitochondria isolated from hearts subjected to I/R in vehicle group (Ctrl), E2, E2 + G15 and sham group. Arrowheads mark the time of mitochondria and CCCP addition. Note that addition of CCCP induces mitochondrial uncoupling, which attenuates the MMP. Right: the graph represents mean MMP values measured after CCCP addition in all the groups showing increased MMP in mitochondria treated with E2 versus both control and E2 + G15-treated groups. Values are expressed as mean ± SEM; *$P < 0.05$ control versus sham group, $^{+}P < 0.05$ control versus E2-treated group and $^{\#}P < 0.05$ E2 versus E2 + G15-treated group (*n* = 5 hearts per group). (B) Left: recording of mitochondrial ROS production using amplex red in the presence of horseradish peroxidase after stimulation of complex I with glutamate/malate. Right: graph showing reduction in ROS production in post-ischaemic E2-treated group versus control and E2 + G15-treated groups. Values are expressed as mean ± SEM; *$P < 0.05$ control versus sham group, $^{+}P < 0.05$ control versus E2-treated group and $^{\#}P < 0.05$ E2 versus E2 + G15-treated group (*n* = 5 per group).

its ubiquitin-associated domain (Seibenhener *et al.*, 2004) and to LC3 *via* its LC3-interacting region (Pankiv *et al.*, 2007). LC3I and LC3II are known to interact with p62 to tether mitochondria to the autophagosome (Ashrafi and Schwarz, 2013). Our results indicate that E2-GPER action reduces Parkin translocation from the cytosol to mitochondria. We found that post-ischaemic E2 action *via* GPER reduces LC3I and LC3II and increases p62 and PINK1 protein expression. These findings point to the involvement of the PINK1/Parkin pathway in E2-GPER-induced reduction of mitophagy. As E2-GPER action leads to the delay of the opening of mPTP that is known to initiate mitochondrial depolarization and the subsequent sequestration of mitochondria into autophagosomes (Elmore *et al.*, 2001), our findings suggest that post-ischaemic GPER activation reduces excessive mitophagy caused by I/R *via* inhibition of the PINK1/Parkin pathway.

Ubiquitination is an enzymatic post translational modification in which a ubiquitin protein is attached to a substrate protein. Ubiquitination tags proteins for degradation *via* proteasomes. As protein ubiquitination in response to PINK1/Parkin pathway activation has been implicated in mitophagy (Gegg *et al.*, 2010; Shiba-Fukushima *et al.*, 2014), we therefore determined the pool of mitochondrial ubiquitinated proteins in post-ischaemic E2 group versus vehicle. We found that post-ischaemic E2 treatment reduces mitochondrial ubiquitinated proteins versus untreated and pretreatment with G15 prevented these E2 effects suggesting a GPER-dependent mechanism. Cyclophilin D has emerged as a regulator of the mPTP opening (Baines *et al.*, 2005), and we, thus, questioned whether E2-GPER action on the mPTP opening is associated to cyclophilin D ubiquitination, which reduces cyclophilin D expression during I/R. To this end, we performed immunoprecipitation with anti-ubiquitin pAb and detected cyclophilin D in the pull-down by Western blot analysis. We found that with the same amount of protein (1 mg), the level of ubiquitinated cyclophilin D is reduced

in E2-treated mitochondria versus untreated and E2 + G15. This finding indicates that E2-GPER action reduces cyclophilin D ubiquitination and might protect it from degradation during I/R, thus regulating the mPTP opening.

Activation of the MEK/ERK axis phosphorylates GSK-3β at the Ser 9 site leading to GSK-3β deactivation and, thus, reduction of protein ubiquitination (Liu *et al.,* 2014). We recently showed that acute pre-ischaemic E2-GPER action induces cardioprotective effects against I/R injury *via* MEK/ERK/GSK-3β signalling but not the PI3K/Akt/GSK-3β axis (Kabir *et al.,* 2015). In the present study, we also report that activation of the MEK/ERK/GSK-3β pathway, instead of the PI3K/Akt pathway, is involved in the post-ischaemic E2-GPER mechanism. We therefore postulate that post-ischaemic GPER activation acts *via* the MEK/ERK/GSK-3β pathway to reduce mitochondrial protein ubiquitination that leads to the inhibition of the mPTP opening and the reduction of mitophagy by protecting the mitochondrial membrane potential from dissipating. However, the control of the quality of mitochondria depends upon a balance between biogenesis and autophagic destruction, hence it will be interesting to investigate insights into mitochondrial biogenesis, fission, fusion and autophagy in future studies to determine the progression of mitochondrial quality control during E2-GPER action.

To examine the upstream mechanism leading to inhibition of mitophagy during post-ischaemic-GPER action, we used two inhibitors of autophagy: U0126 that acts *via* the MEK/ERK/GSK-3β pathway and 3-MA, an inhibitor of classes I and III of PI3K. We found that U0126 alone slightly reduced myocardial infarct size versus control. However, pre-administration of U0126 prevented E2-induced reduction of myocardial infarct size. We also found that administration of 3-MA alone reduced myocardial infarct size versus control, a result similar to that obtained by Xia *et al*. (2016) but opposite to the report from Ling *et al*. (2016), who used 50 times our dose. But pre-administration of 3-MA did not have any effect on E2-induced cardioprotection. Together, these findings indicate that post-ischaemic GPER activation reduces mitophagy *via* the MEK/ERK pathway but not *via* the PI-3K/Akt axis. U0126 has been reported to display conflicting effects: it decreases autophagy, an effect that was associated with a reduction of myocardial infarct size after I/R (Wang *et al.,* 2016), and in contrast, it also prevents cardioprotection induced by melatonin after I/R (Yu *et al.,* 2016). Our results support the observations by Yu *et al*. (2016) suggesting that U0126 prevents the reduction of autophagy by inhibiting the activation of the MEK/ERK pathway resulting in increased myocardial infarct size.

In conclusion, we report here that acute post-ischaemic GPER activation induces cardioprotective effects against I/R injury by protecting mitochondrial structural integrity. Post-ischaemic stimulation of GPER activates the MEK/ERK pathway that deactivates GSK-3β leading to the reduction of mitochondrial protein ubiquitination and protection of mitochondrial membrane potential dissipation. These effects cause the inhibition of the mPTP opening, as well as reduction of excessive mitophagy caused by I/R stress, processes that result in reduction of harmful mitochondrial ROS generation and subsequent reduction of myocardial infarct size, therefore proving the cardioprotection. The mechanism underlying post-ischaemic GPER-actions in reducing mitophagy involves a decrease in Parkin's translocation into mitochondria.

Acknowledgements

This work was supported by the American Heart Association (AHA) grant 17SDG33100000 (J.C.B.); NIH grant HL138093 and Max and Minnie Tomerlin Voelcker Fund (J.C.B.). The authors thank Dr Mihaela Lie for her help in the study.

Author contributions

Y.F. and J.C.B. conceived and designed the experiments. Y.F. performed the experiments. Y.F., N.B.M. and J.C.B. analysed the data. J.C.B. drafted the manuscript. Y.F., N.B.M., C.V.C. J. and J.C.B. revised the paper. J.C.B. supervised the project.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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