Oestrogen-mediated cardioprotection following ischaemia and reperfusion is mimicked by an oestrogen receptor (ER) agonist and unaffected by an ERβ antagonist

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Abstract

Oestrogen protects the heart from ischaemic injury. The current study aims to characterise two novel oestrogen receptor (ER) ligands, an ERα agonist ERA-45 and an ERβ antagonist ERB-88, and then use them to investigate the roles of ERα and ERβ in mediating the cardioprotection by E from ischaemia–reperfusion injury in the rat. The ER ligands were characterised by gene transactivation assay using ER–transfected Chinese hamster ovary (CHO) cells and in bioavailability studies in vivo. Female rats (n=48) were ovariectomised and implanted with 17β-oestradiol (17βE2) releasing or placebo pellets. ERA-45, ERB-88 or vehicle was administered for 5 days prior to ischaemia–reperfusion studies. Necrosis, neutrophil infiltration (myeloperoxidase activity) and oxidant stress production (electron paramagnetic resonance) from the area-at-risk were measured to assess reperfusion injury. The ERα agonist ERA-45 showed more than 35-fold selectivity for ERα compared with ERβ gene transactivation. In vitro, the ERβ antagonist ERB-88 inhibited transactivation by 17βE2 via ERβ with 46-fold selectivity relative to inhibition via ERα. In vivo, 17βE2 significantly reduced neutrophil infiltration, oxidant stress and necrosis following ischaemia and reperfusion. Cardioprotection by 17βE2 was not inhibited by ERB-88 but was completely reproduced by ERA-45. In conclusion, protection of the rat heart after ischaemia–reperfusion by 17βE2 is achieved through the reduction of cardiomyocyte death, neutrophil infiltration and oxygen-free radical availability. The results of this study indicate that these effects are primarily mediated via activation of ERα.

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Introduction

Premenopausal women have a lower incidence of cardiovascular disease compared with postmenopausal women or age-matched men (reviewed in Gray et al. 2001, Jeanes et al. 2007). The production of oestrogen is thought to confer this protective effect in premenopausal women. Experimentally, administration of 17β-oestradiol (17βE2) reduces infarct size (Hale et al. 1996, Patten et al. 2004, Kim et al. 2006) and the inflammatory response associated with myocardial ischaemia–reperfusion injury in ovariectomised rats (Squadrito et al. 1997, Jeanes et al. 2007).

There are two known oestrogen receptors (ERs), ERα and ERβ. The receptors are members of the steroid hormone receptor family with distinct tissue distribution and transcriptional activity (Mendelsohn & Karas 2005). ERα is expressed in human cardiomyocytes (Mendelsohn & Karas 2005) while ERβ has been identified in cardiomyocytes and fibroblasts (Taylor et al. 2000). A similar ER distribution has been reported in the rat heart (Grohe et al. 1997, 1998, Jankowski et al. 2001, Yang et al. 2004). Experiments using the non-selective ER antagonist ICI 182 780 have demonstrated that the 17βE2–induced reduction in infarct size is ER mediated (Hayashi et al. 1995, Dubey et al. 2001, Booth et al. 2003). However, the lack of selective ligands has prevented identification of specific receptors involved. Attempts have been made to investigate the roles of ERα and ERβ using mice genetically altered to ablate the ERα (ERKO) or ERβ (βERKO). These mouse studies have failed to provide a clear answer as the protective effects of E in ischaemia reperfusion are reported to be lost in both ERKO (Zhai et al. 2000a, Wang et al. 2006) and βERKO mice (Gabel et al. 2005).

Neutrophils invade the myocardium during reperfusion and are an important source of oxidant stress causing injury (Reimer et al. 1989). In vivo, the inhibition of myocardial adhesion molecule expression by oestrogen reduces neutrophil infiltration into the reperfused myocardium (Delyani et al. 1996). In vitro, oestrogen has been shown to inhibit neutrophil activation (Squadrito et al. 1997). Isolated neutrophils express both ERα and ERβ (Stygar et al. 2006), but the receptor
mediating the effects of oestrogen in reperfusion injury has not been identified.

The aim of the current study was to investigate the roles of ERα and ERβ in mediating the chronic effects of 17βE2 on infarct size, neutrophil infiltration and oxidative stress following ischaemia and reperfusion in ovariectomised rats. The study used an ERα-selective agonist ERA-45 and a novel ERβ-selective antagonist ERB-88.

This study is the first to use a selective ERβ antagonist to investigate the effects of 17βE2 in vivo. The antagonist did not inhibit the reduction in infarct size, neutrophil infiltration and free radical release in hearts from 17βE2-treated rats. However, these effects of 17βE2 were all reproduced by the selective ERα agonist. Together, these observations suggest that the cardioprotective effects of oestrogen in this rat model of ischaemia and reperfusion are mediated via activation of the ERα.

Materials and Methods

The investigation conforms with the UK Home Office guidelines as outlined in the Animals (Scientific Procedures) Act 1988 and was approved by the University of Edinburgh Ethical Review Committee.

In vitro characterisation of compound activity

An ERα-selective agonist ERA-45 and an ERβ antagonist ERB-88 were used for investigation of the roles of ERα and ERβ. The activity of these compounds was first tested using in an in vitro gene transactivation assay (De Gooyer et al. 2003). CHO cells stably transfected with human ERα, the rat oxytocin promoter (RO) with firefly luciferase reporter gene (LUC) hERα-RO-LUC or human ERβ-RO-LUC was cultured in medium with 5% charcoal-treated supplemented-defined bovine calf serum. For the assay, 5 × 10⁴ cells/well were seeded into a 96-well plate and incubated with compounds (final ethanol content: 1% v/v) for 16 h in medium with 5% charcoal-treated supplemented-defined bovine calf serum at 37 °C in a humidified atmosphere of air supplemented with 5% CO2. Of the total 250 µl incubation volume, 200 µl were removed and 50 µl LucLite were added for cell lysis and luciferase measurement. Luciferase activity was measured in a Topcount luminescence counter (Canberra Packard, Schwadorf, Austria). Full agonist curves were constructed for oestradiol and the ER ligands using cells expressing either ERα or ERβ. Oestrogenic antagonist curves (10⁻¹¹–10⁻⁶ M) for ERB-88 and the non-selective ER antagonist ICI 164 384 (De Gooyer et al. 2003) were determined in CHO cells as described above, in the presence of 0·1 × 10⁻⁷ M or 0·4 × 10⁻⁷ M 17βE2 for ERα and ERβ respectively.

Pharmacokinetic studies in vivo

To confirm that appropriate concentrations of compounds were achieved in vivo, pharmacokinetic studies were carried out in female rats. The ERα-selective agonist ERA-45 (prepared in 5% (w/v) mannitol 0·5% (w/v) gelatin) was administered at a dose of 3·4 mg/kg p.o. Compound concentration in plasma was assessed by LCMS-MS in samples taken from the jugular vein at hourly intervals after administration. The ERβ antagonist ERB-88 (also prepared in 5% (w/v) mannitol 0·5% (w/v) gelatin) was administered s.c. at a dose of 0·68 mg/kg and the samples were subsequently taken for analysis of plasma concentration as described above.

Experimental design

Two studies were carried out. The first (n = 30) was designed to investigate the influence of the selective ERα agonist and ERβ antagonist on ischaemia–reperfusion injury in the rat in vivo including the area-at-risk (AAR), necrotic area and also free radical production from the AAR. In placebo-treated ovariectomised rats, the ERα agonist ERA-45 or vehicle (5% (w/v) mannitol 0·5% (w/v) gelatin) was administered twice daily for 5 days prior to the experiment by gavage at a dosage of 75 µg/kg. The ERβ antagonist or vehicle (PBS) was administered at a dose of 1 mg/kg to ovariectomised rats treated with 17βE2 or placebo, for 5 days prior to the experiment by s.c. injection. In both cases, a final dose was given 2–3 h before the experiment.

The second study (n = 18) aimed to investigate the effects of the selective ERα agonist on neutrophil infiltration, assessed by myeloperoxidase (MPO) activity. For these studies, ERA-45 was administered as mentioned above.

All studies used female Wistar rats (170–200 g; Charles River, Marlow, UK). Rats were ovariectomised under isoflurane anaesthesia (4% induction and 2·5% maintenance) and implanted with pellets (Innovative Research of America, Sarasota, FL, USA) releasing 17βE2 (2 µg/day for 21 days) or placebo. Preliminary experiments confirmed that this method achieved a plasma concentration of oestrogen in the physiological range (6·1·42 ± 7·7 pg/ml, n = 6, RIA, Bio-Stat Diagnostics, Stockport, UK). The rats received 17βE2 or placebo for a minimum of 14 days prior to experimental use.

Ischaemia–reperfusion protocol

Rats were anaesthetised with 60 mg/kg sodium pentobarbitone i.p. (Sagatal, Rhone Merieux, UK). The procedures were carried out as described previously (Jeannes et al. 2006). Briefly, the carotid artery and jugular vein were cannulated for the measurement of mean arterial blood pressure (MABP) and administration of anaesthetic respectively. The rats were intubated and ventilated with oxygen-enriched room air (60 strokes/min; 0·1 ml/g). Body temperature was maintained by a thermostatically controlled under blanket. The chest was then opened at the fourth intercostal space, the heart was temporarily removed from the chest and the left coronary artery (LCA) ligated (5/0 Mersilk, Ethicon, UK). Once the heart was replaced in the chest, it was allowed a
20-min stabilisation period prior to the LCA being reversibly occluded through the formation of a snare. After 45 min of ischaemia, the snare was released and a 2-h reperfusion period followed. At the end of the 2 h of reperfusion, the LCA was permanently occluded and 1 ml Evans blue dye (1% in saline) was administered via the jugular vein. The heart was then removed from the chest and rapidly frozen for determination of the necrotic area and AAR. For MPO activity, the right ventricle and atria were removed from separate hearts and the ischaemic and non-ischaemic areas of the left ventricle separated prior to rapid freezing. For experiments aimed at determination of oxidative stress production, the heart was removed from the chest prior to injection of Evans blue and set on the Langendorff perfusion set-up. In all experiments, the uterus was removed and weighed for confirmation of hormone deficiency or delivery.

**MPO activity**

Neutrophil infiltration into the ischaemic myocardium was assessed through measurement of MPO activity, as previously described (Jeanes et al. 2006). Briefly, the ischaemic area was homogenised in buffer containing 20 mM sodium phosphate (pH 4.7) 1.5×10⁻² M EDTA and 10⁻¹ M sodium chloride and then centrifuged at 10 000 g at 4°C for 15 min. The resulting pellet was resuspended and homogenised in sodium phosphate buffer (pH 5-4) containing 0-5% hexadecyltrimethylammonium bromide. Homogenisation was followed by four cycles of freeze-thaw, a brief sonication and 15-min centrifugation at 10 000 g at 4°C. The resulting supernatant (30 μl) was mixed with 200 μl citrate phosphate buffer containing o-dianisidine dihydrochloride and 0.0015% hydrogen peroxide. The absorbance was measured over 10 min at 405 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol peroxide per min at 25°C, expressed in units per gram of weight.

**Electron paramagnetic resonance (EPR)**

EPR was used to measure the free radical production from hearts that had undergone ischaemia–reperfusion in vivo. Hearts were removed after 2 h of reperfusion and rapidly perfused in retrograde mode with warmed and gassed Krebs–Henseleit solution, as described below. After a short stabilisation period, the reversible snare was re-tied and 10 μl 10⁻¹ M CP-H (1-hydroxy-3-carboxy-pyrrolidine; Axxora Ltd, Nottingham, UK) spin trap was injected into the perfusate above the heart via an injection arm. A 500 μl aliquot of the resulting perfusate was collected from the base of the heart (drops 6–11 after the injection of spin trap). This was repeated four times to obtain an average measurement for each heart. The snare around the LCA was then released to allow perfusion of the entire myocardium and the procedure of perfusion and administration of CP-H spin trap was repeated. Finally, to determine the contribution of superoxide radicals to the oxidant signal generated by the heart 500 U superoxide dismutase (SOD) was perfused through the entire heart along with the CP-H spin trap. The collected samples were incubated at 37°C for 20 min and then the concentration of the free radical was measured using the EPR magnet (Magnettech MiniScope MS200 and MiniScope Control 6.51 software, Magnettech, Berlin, Germany), with the settings below: B0 field: 3357 G; Sweep: 44 G; Sweep time: 30 s; No. of Passes: 1; Modulation Amplitude: 1500 mG; Microwave Power: 20 mW). The results are expressed as an estimate of the signal generated from the ischaemic myocardium, calculated by subtracting the signal obtained from the non-ischaemic myocardium from the signal obtained from the entire heart.

**Measurement of infant size**

The extent of myocardial damage was assessed using the triphenyltetrazolium chloride–Evans blue technique, as described previously (Jeanes et al. 2006). The heart was cut into 2–3 mm slices from apex to base and then incubated in 2,3,5-triphenyltetrazolium chloride (1% (w/v) in saline) at 37°C for 15 min. The heart was then fixed in 10% formalin for 10 min and rinsed in 0-9% saline. Three distinct areas of the heart result, the Evans blue-stained non-ischaemic myocardium, the red ischaemic non-necrotic tissue and the white necrotic tissue were separated, blotted dry and weighed. The AAR was taken as the red ischaemic non-necrotic tissue and the white necrotic tissue; this was expressed as a percentage of the total heart weight. The necrotic tissue was expressed as a percentage of the AAR.

**Statistical analysis**

Statistical analysis was performed using PRISM (GraphPad, San Diego, CA, USA). Two- and one-way ANOVAs with Bonferroni post hoc or paired and unpaired two-tailed Student’s t-tests were used where appropriate. Statistical difference was taken as P<0.05.

**Chemicals**

ERα agonist ERA-45 and ERβ antagonist ERB-88 were supplied by Organon. All other chemicals were from Sigma–Aldrich unless stated otherwise.

**Results**

**Characterisation of compounds: in vitro transactivation assay**

The ERα agonist ERA-45 activated luciferase expression in CHO cells transfected with recombinant human ERα with an EC₅₀ of 3.7×10⁻¹⁰ M (95% confidence limits 3.2 and 4.4×10⁻¹⁰ M, n=4; Fig. 1a). The EC₅₀ for activation of human ERβ in the same cell system was 1.3×10⁻⁸ M (95% confidence limits 0.9 and 1.7×10⁻⁸ M, n=4; Fig. 1b).
17βE2 activated ERα with an EC50 of 2.6 × 10⁻¹¹ M (95% confidence limits 1.7 and 4.1 × 10⁻¹¹ M; Fig. 1a) and ERβ with an EC50 of 6.5 × 10⁻¹¹ M (95% confidence limits 4.9 and 8.7 × 10⁻¹¹ M; Fig. 1b). The non-selective ER antagonist ICI 164 384 inhibited activation by 17βE2 in cells expressing both ERα (EC50 of 5.7 × 10⁻⁹ M, 95% confidence limits 4.1 and 8.7 × 10⁻⁹ M; Fig. 1c) and ERβ (EC50 of 2.6 × 10⁻⁹ M, 95% confidence limits 1.8 and 3.6 × 10⁻⁹ M; Fig. 1d). By contrast, ERB-88-inhibited gene transactivation induced by 17βE2 in cells expressing recombinant human ERβ with an IC50 of 2.1 × 10⁻⁹ M (95% confidence limits 0.8 and 5.7 × 10⁻⁹ M; Fig. 1d) and did not inhibit ERα-mediated gene activation up to 10⁻⁶ M (Fig. 1c). The ERα agonist ERA-45, at concentration up to 10⁻⁷ M, did not inhibit activation of either receptor by 17βE2 (data not shown).

Characterisation of compounds: in vivo bioavailability

Plasma concentration rose to a peak of 63 nM at 30 min after dosing of female rats with ERA-45 (3.4 mg/kg p.o.) and dropped to a minimum of 3.6 nM at 12 h after dosing, the half-life of ERA-45 was 5.5 ± 0.78 h (n = 3). The target plasma concentration to achieve selective activation of ERα was 1 nM, based on in vitro studies. A dose of 0.75 mg/kg p.o. was selected for the in vivo ischaemia–reperfusion study, this was administered twice daily to ensure that plasma levels were maintained at a suitable level for activation of ERα.

Subcutaneous administration of ERB-88 at a dose of 0.68 mg/kg achieved a peak plasma concentration of 241 × 10⁻⁹ M at 2 h, after administration, this fell to 41 × 10⁻⁹ M at 7 h and to below 10 × 10⁻⁹ M by 24 h (n = 2). The target plasma concentration to achieve maximal selective blockade of ERβ was 10⁻⁸ M. An s.c. dose of 1 mg/kg was selected for the in vivo ischaemia–reperfusion study. The final dose of ERB-88 was administered 2–3 h before the experiment began.

Body weight and uterine weights

The body weights of all the animals were recorded at the time of ovariectomy and of ischaemia–reperfusion. There was no significant difference in body weight at the time of ovariectomy. At the time of ischaemia–reperfusion, rats that received 17βE2 or the ERα agonist ERA-45 weighed significantly less than placebo/vehicle-treated rats (Table 1). Treatment with ERβ antagonist ERB-88 did not have any influence on weight when given to rats receiving placebo or...
17βE2 (Table 1). The uterine weights of all animals were recorded at the experimental end point to confirm the effective removal of the ovaries and successful hormone delivery in appropriate animals. Animals receiving placebo/vehicle had a significantly reduced uterine weight compared with those receiving either 17βE2 or placebo in combination with selective ERα agonist (Table 1). The selective ERα agonist appeared to induce less proliferation than 17βE2 (Table 1). The ERβ antagonist ER-B-88 had no significant effect on the uterine weight when given to rats receiving placebo or 17βE2 (Table 1). These data confirm successful activation of the ERα by ERA-45 at the dose chosen and a lack of effect of the chosen dose of ERβ antagonist on ERα-mediated uterine proliferation.

In vivo ischaemia–reperfusion

The MABP in the placebo/vehicle-treated group before ischaemia was 73 ± 13 mmHg (n – 5). This dropped by about 41% at the onset of ischaemia and then recovered during the initial 15 min of ischaemia to a pre-ischaemic level that was maintained until the end of the experiment. Treatment with 17βE2 or selective ER ligands did not have a significant effect on this pattern or on the MABP at the end of the ischaemia or reperfusion periods (data not shown). The heart rate did not change significantly throughout the protocol in any of the treatment groups (data not shown).

The total AAR in the placebo/vehicle-treated group was 57.1 ± 3.8% of the total heart. The AAR in the other treatment groups did not differ significantly from this (Table 1). 17βE2 significantly (P < 0.05) reduced the size of the necrotic zone within the AAR, compared with placebo/vehicle-treated ovariectomised rats (Fig. 2). Treatment with the ERβ antagonist ERB-88 did not significantly alter this 17βE2-induced decrease in necrotic tissue (Fig. 2). By contrast, placebo-treated rats receiving the ERα agonist ERA-45 also displayed a significant reduction in necrotic tissue compared with placebo/vehicle-treated rats (P < 0.01; Fig. 2). Treatment with ERB-88 after placebo treatment had no significant effect on infarct size compared with vehicle (43 ± 3% AAR versus 42 ± 3% AAR, n = 6).

Neutrophil infiltration in vivo

Tissue MPO activity was measured as a marker of neutrophil infiltration following reperfusion. Supplementation with 17βE2 after ovariectomy significantly (P < 0.05) reduced the amount of MPO activity detected in the AAR compared with that in hearts from placebo/vehicle-treated rats (Fig. 3). To determine whether the reduction in infarct size associated with ERα agonist was linked to a reduction in neutrophil infiltration, MPO activity was also assessed in this group. Dosing placebo-treated rats with the selective ERα agonist also significantly (P < 0.05) reduced the level of MPO activity within the AAR, reproducing the effect of 17βE2 (Fig. 3).

Free radical production after in vivo ischaemia–reperfusion

Neutrophils are a major source of oxidant stress following reperfusion. Any change in neutrophil infiltration should therefore be correlated with a reduction in free radical production from the AAR. EPR was used to detect the influence of 17βE2 treatment on free radical production by the reperfused AAR of the myocardium following the in vivo ischaemia–reperfusion protocol. In hearts from placebo/vehicle-treated animals,

Table 1 The influence of 17β-oestradiol (17βE2) and selective oestrogen receptor (ER) drugs on the body weight, uterine weight and the area-at-risk after ischaemia reperfusion in the rat. Data shown are mean ± S.E.M.

| Hormone treatment                  | Body weight (BW, g) | Uterine weight/BW (%) | Area-at-risk (% total heart) |
|-----------------------------------|---------------------|-----------------------|-----------------------------|
| Placebo + vehicle                 | 278 ± 6             | 0.04 ± 0.01           | 57 ± 4                      |
| 17βE2 + vehicle                   | 221 ± 7*            | 0.25 ± 0.03*          | 54 ± 2                      |
| 17βE2 + ERβ antagonist            | 217 ± 4*            | 0.23 ± 0.02*          | 59 ± 2                      |
| Placebo + ERα agonist            | 233 ± 5*            | 0.12 ± 0.03*          | 55 ± 2                      |
| Placebo + ERβ antagonist          | 279 ± 9             | 0.05 ± 0.01           | 56 ± 3                      |

A one-way ANOVA with a Bonferroni post hoc test; *P < 0.001 compared with placebo; †P < 0.001 compared with 17βE2 + vehicle; n = 6–8. BW, body weight.
significantly more free radicals were detected in the perfusate collected from the whole heart than from the non-ischaemic myocardium alone (3524 ± 834 vs 1055 ± 395 arbitrary units, \( P = 0.005, n = 5 \)), demonstrating production from the AAR. The majority of the oxidant stress could be accounted for by superoxide radicals as administration of SOD along with the spin trap significantly decreased free radical production from the reperfused heart by 53 ± 10·3\% (\( P = 0.04, n = 7 \)).

Significantly fewer free radicals were generated from the AAR of hearts from animals that received 17βE2 than those that received placebo/vehicle following ovariectomy (\( P < 0.01 \); Fig. 4). This influence of 17βE2 was not modified in hearts from ERβ receptor antagonist-treated rats (\( P < 0.01 \); Fig. 4). However, rats that received placebo pellets and the ERα agonist ERA-45 also had significantly decreased free radical production compared with the rats treated with placebo/vehicle (\( P < 0.01 \); Fig. 4).

**Discussion**

The aim of this study was to identify the roles of ERα and ERβ in mediating the cardioprotection provided by chronic administration of 17βE2 in a model of ischaemia–reperfusion. This was achieved using two novel ER ligands that we show, using a gene transactivation assay, to be a selective agonist at ERα (ERA-45) and a selective antagonist at ERβ (ERB-88). The results from the current study suggest that ERα is the predominant ER involved in cardioprotection by 17βE2. Chronic administration of ERB-88 to ovariectomised rats receiving 17βE2 had no significant influence on infarct size or oxidant stress production. In comparison, the cardioprotective effects of 17βE2 were not completely reproduced by ERA-45.

It is well established that 17βE2 provides protection in experimental models of ischaemia–reperfusion (Stumpf et al. 1977, Squadrito et al. 1997, Zhai et al. 2000b). This protection is mediated through reduction of ischaemia-induced cell death (Patten et al. 2004, Patten & Karas 2006), inhibition of neutrophil infiltration (Squadrito et al. 1997, Jeanes et al. 2006) and reduction of oxidative damage to the myocardium (Kim et al. 1998, 2006, Mchugh et al. 1998, Urata et al. 2006). In the present study, we confirmed that both infarct size and neutrophil infiltration, assessed by myocardial MPO activity, were reduced in 17βE2-treated animals.

Oxidant stress is an important cause of injury following tissue reperfusion. Therefore, in the present study, we additionally aimed to measure free radical release from the reperfused myocardium using EPR. This technique has previously been used to detect the brief oxidative burst that occurs following ischaemia–reperfusion in vivo in isolated buffer-perfused hearts (Zweier et al. 1989). However, this is the first time that it has been used to assess free radical production from hearts that have undergone in vivo ischaemia–reperfusion. In vitro perfusion of these hearts with the CP-H spin trap was successful in detecting free radical release. Free radical detection was significantly reduced when perfusion through the AAR was prevented, identifying the AAR as the major site of free radical production in these hearts. The most likely source of oxidative stress within the AAR is neutrophils that have infiltrated following reperfusion (Mccord 1985). In the present study, the reduction in EPR signal after dual perfusion of spin trap and SOD demonstrates that the majority of free radicals produced in the myocardium are superoxide anions, the predominant free radical released from neutrophils (Tauber & Babior 1977). Treatment with 17βE2 clearly reduced the oxidant signal generated in the myocardium after ischaemia reperfusion compared with placebo/vehicle-treated rats. This reduction corresponds to the reduction in neutrophil infiltration in 17βE2-treated animals. In the
reperfused heart, damaged mitochondria are also a source of oxidant stress through the electron transfer chain (Lesnáski et al. 2001) and may also contribute here. 17βE2 has previously been shown to reduce the oxidative stress from isolated rat mitochondria (Sturone et al. 2005) and this may be an additional protective mechanism of 17βE2 in the current model. Free radical scavenging is also likely to be enhanced following chronic treatment with 17βE2, as it is known to upregulate endogenous antioxidant systems, such as glutathione peroxidase and SOD (Kim et al. 1998, Borras et al. 2005, Urata et al. 2006).

The principal aim of this study was to identify the roles of ERα and ERβ in mediating the cardioprotective effects of oestrogen using a selective ERα agonist and a novel ERβ antagonist. In vitro gene transactivation studies showed that the ERα agonist ERA-45 has ~35-fold greater potency for gene transactivation via ERα, compared with ERβ. The dose of ERA-45 selected for the current study was based on these in vitro data and extrapolation from parallel in vivo bioavailability studies. The chosen dose was expected to achieve a plasma concentration of around 1 nM. At this concentration, we would expect 70–80% activation of the ERα but minimal, if any, activation of ERβ. In rats, ERα is responsible for the stimulation of epithelial cell proliferation in the uterus that results in the observed increase in uterine weight when treated with 17βE2 or ERα agonist (Frasor et al. 2003). ERα also mediates the 17βE2-induced attenuation of food intake and weight gain (Roesch 2006). In the present study, administration of the ERα agonist ERA-45 increased uterine weight and decreased body weight, consistent with successful activation of this receptor. The increase in uterine proliferation with ERA-45 was less than that achieved with 17βE2 suggesting that the selected dose was achieving a less than maximal effect, as expected from the pharmacokinetic data. The ERβ antagonist ERβ-88 also showed clear efficacy and selectivity in gene transactivation studies. In ERα-transfected cells activation by 17βE2 was inhibited by the established ERα/β antagonist ICI 164 384 (Zwart et al. 2007), but ERβ-88 had no influence until 10⁻⁶ M. By contrast, at ERβ, activation by 17βE2 was inhibited by both ICI 164 384 and ERβ-88. Based on bioavailability data, the dose used in the present study should be sufficient to block 80–90% of ERβ without influencing ERα. While there is no easily quantifiable measure of ERβ activation in vivo to confirm blockade of this receptor, the lack of influence on uterine or body weight confirms a lack of effect on ERα.

The ERβ antagonist ERβ-88 did not attenuate the effects of 17βE2 on infarct size, or free radical production. ERβ has been detected in the rat heart (Grohe et al. 1998, Jankowski et al. 2001, Yang et al. 2004) and it was implicated in mediating cardioprotection following trauma–haemorrhage in experiments using an ERβ agonist (Yu et al. 2006). However, it does not appear to have a role in mediating cardioprotection by 17βE2 following ischaemia–reperfusion in the rat. The results of the present study suggest that 17βE2 is more likely to act through stimulation of ERα in the rat heart. This conclusion is supported by the fact that the ERα agonist ERA-45 completely reproduced the cardioprotective effects of 17βE2 and the effects of 17βE2 on neutrophil infiltration and oxidant stress production. In another study, acute administration of the selective ERα agonist PPT (4,4,4-propyl-(1H)-pyrazole 1,3,5-triyltriphenol-PPT) 30 min before ischaemia and reperfusion also reduced infarct size in the rabbit, while the ERβ agonist DPN (2,3-bis(4-hydroxy-phenyl propionitrile-DPN) failed to provide any cardioprotection (Booth et al. 2005). In this study, cardioprotection via stimulation of ERα was linked to reduction of C-reactive protein (CRP) deposition within the infarct area. We have recently shown that CRP can enhance infarct damage following permanent ligation of the coronary artery in the rat (Pepys et al. 2006). Reduction of CRP deposition may also be a feature of protection following chronic stimulation of ERα in the present study. However, additional mechanisms will also contribute, for example, enhanced expression of endothelial nitric oxide synthase and reduced expression of adhesion molecules, effects of E that reduce neutrophil infiltration (Delyani et al. 1996, Squadrito et al. 1997), and have previously been linked to activation of ERα (Tan et al. 1999, Schrepfer et al. 2006). Rapid activation of Akt, resulting in a reduction in cardiomyocyte apoptosis following ischaemia has also been linked to ERα in vitro (Patten et al. 2004, Patten & Karas 2006). Further studies are required to confirm the cellular mechanisms involved in reduction of infarct size, neutrophil infiltration and free radical release following stimulation of ERα in myocardial ischaemia–reperfusion injury in vivo. In a more recent study, activation of the ERβ, using the ERβ agonist DPN, has been shown to preserve cardiac contractility following ischaemia and reperfusion in the mouse (Nikolic et al. 2007), although neither infarct size nor inflammatory cell infiltration was assessed as end points, making direct comparison with the present study difficult. Species differences may explain the apparent variability in contribution of ERα and ERβ to cardioprotection in the rabbit (Booth et al. 2005), rat (present study) and mouse (Nikolic et al. 2007). It is also possible that the ERβ has a specific role in the preservation of contractile function that was not identified in the present study. Alternatively, selective activation of either receptor may not reflect the actual contributions of the receptors to the effects of 17βE2 that can activate both receptors. In this sense, the antagonist data generated in the current study are important in demonstrating that removal of the ERβ does not prevent reduction of infarct size and oxidant stress generation by 17βE2. Studies using an ERα antagonist would provide further evidence for a role of ERα, and also reveal whether ERβ can have a role when it is activated by 17βE2 in the absence of the ERα.

In conclusion, the current study is the first study to demonstrate, using a combination of ERα agonist and ERβ antagonist, that the cardioprotective properties of 17βE2 in a rat model of ischaemia–reperfusion are primarily mediated via stimulation of the ERα.
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Disclosure

The authors declare that there are no conflicts of interest that would prejudice the impartiality of this research.

References

Booth EA, Marchest M, Kilbourne EJ & Lucchesi BR. 2003 17Beta-estradiol as a receptor-mediated cardioprotective agent. Journal of Pharmacology and Experimental Therapeutics 307 395–401.

Booth EA, Obead NR & Lucchesi BR. 2005 Activation of estrogen receptor-beta protects the in vivo rabbit heart from ischemia-reperfusion injury. American Journal of Physiology. Heart and Circulatory Physiology 289 H1209–H1204.

Boras C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann GE & Vina J 2005 17Beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2 [MAPK]/NFκappaB cascade. Aging Cell 4 113–118.

Delyani JA, Murohara T, Nossuli TO & Lefer AM 1996 Protection from myocardial reperfusion injury by acute administration of 17 beta-estradiol. Journal of Molecular and Cellular Cardiology 28 1001–1008.

Dubey RK, Jackson EK, Keller PJ, Imthurn B & Rosselli M 2001 Estradiol metabolites inhibit endothelin synthesis by an estrogen receptor-independent mechanism. Hypertension 37 640–644.

Fraser J, Barnett DH, Dames JN, Hess R, Parlow AF & Katzenellenbogen BS 2003 Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by E2beta in the uterus. Endocrinology 144 3159–3166.

Gabel SA, Walker VR, London RE, Steenbergen C, Korach KS & Murphy E 2005 Estrogen receptor beta mediates gender differences in ischemia/reperfusion injury. Journal of Molecular and Cellular Cardiology 38 289–297.

de Gooyer ME, Deckers GH, Schoonen WG, Verheul HA & Kloosterboer HJ 2003 Receptor profiling and endothrine interactions of tuboline. Steroids 68 21–30.

Gray GA, Sharif I, Webb DJ & Seckl JR. 2001 Oestrogen and the cardiovascular system: the good, the bad and the puzzling. Trends in Pharmacological Sciences 22 152–156.

Grohe C, Kahler S, Lobbert K, Stimpel M, Karas RH, Vetter H & Neyses L 1997 Cardiac myocytes and fibroblasts contain functional estrogen receptors. FEBS Letters 416 107–112.

Grohe C, Kahler S, Lobbert K & Vetter H 1998 Expression of oestrogen receptor alpha and beta in rat heart: role of local oestrogen synthesis. Journal of Endocrinology 156 R1–R7.

Hale SL, Birnbaum Y & Kloner RA 1996 beta-Estradiol, but not alpha-estradiol, reduced myocardial necrosis in rabbits after ischemia and reperfusion. American Heart Journal 132 258–262.

Hayashi T, Yamada K, Eski T, Kuzuya M, Satake S, Ishikawa T, Hidaka H & Iguchi A 1998 Estrone increases endothelial nitric oxide by a receptor-mediated system. Biochemical and Biophysical Research Communications 214 847–855.

Jankowski M, Rachelska G, Donghao W, McCann SM & Gutkowska J 2001 Estrogen receptors activate atrial natriuretic peptide in the rat heart. PNAS 98 11765–11770.

Jeans H, Newby D & Gray GA 2007 Cardiovascular risk in women: the impact of hormone replacement therapy and prospects for new therapeutic approaches. Expert Opinion on Pharmacotherapy 8 279–288.

Kim YD, Farhat MY, Myers AK, Koutets P, DeGroot KW, Pacquing A, Ramnall PW, Snyderhoud JP & Lees DE 1998 17-Beta estradiol regulation of myocardial glutathione and its role in protection against myocardial stunning in dogs. Journal of Cardiovascular Pharmacology 32 457–465.

Kim JK, Pedram A, Razandi M & Levin ER. 2006 Estrogen prevents cardiomyocyte apoptosis through inhibition of reactive oxygen species and differential regulation of p38 kinase isomers. Journal of Biological Chemistry 281 6760–6767.

Klesnyk EJ, Moghadss A, Tandler B, Kerner J & Hoppel CL 2001 Mitochondrial dysfunction in cardiac disease: ischemia-reperfusion, aging, and heart failure. Journal of Molecular and Cellular Cardiology 33 1065–1089.

McCord JM 1985 Oxygen-derived free radicals in postischemic tissue injury. New England Journal of Medicine 312 159–163.

McHugh NA, Merrill GF & Powell SR. 1998 Estrogen diminishes postischemic hydroxyl radical production. American Journal of Physiology 274 H1950–H1954.

Mendelsohn ME & Karas RH 2005 Molecular and cellular basis of cardiovascular gender differences. Science 308 1583–1587.

Nikolic I, Liu D, Bell JA, Collins J, Steenbergen C & Murphy E 2007 Treatment with an estrogen receptor-beta-selective agonist is cardioprotective. Journal of Molecular and Cellular Cardiology 42 769–780.

Patterson RD & Karas RH 2006 Estrogen replacement and cardiomyocyte protection. Trends in Cardiovascular Medicine 16 69–75.

Patterson RD, Pourati I, Aronovitz MJ, Baur J, Celestin F, Chen X, Michael A, Haq S, Nuedling S, Grohe C et al. 2004 17Beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phosphoinoside-3 kinase/Akt signaling. Circulation Research 95 692–699.

Peps MB, Hirschfield GM, Tenent GA, Gallimore JR, Kahan MC, Bellotti V, Hawkins PN, Myers RM, Smith MD, Polara A et al. 2006 Targeting C-reactive protein for the treatment of cardiovascular disease. Nature 440 1217–1221.

Reiner KA, Murr CE & Richard VJ 1989 The role of neutrophils and free radicals in the ischemic-reperfusion heart: why the confusion and controversy? Journal of Molecular and Cellular Cardiology 21 1225–1239.

Roeck DM 2006 Effects of selective estrogen receptor agonists on food intake and body weight gain in rats. Physiology and Behavior 87 39–44.

Schreper P, Deuse T, Munzel T, Schafer H, Braendle G & Reichenpurner H 2006 The selective estrogen receptor-beta agonist biochanin A shows vasculoproteective effects without uterotrophic activity. Menopause 13 489–499.

Squiroli D, Flavilla D, Squiroli R, Campo GM, Arlotta M, Arcoraci V, Minutoli L, Serrano M, Saita A & Caputi AP 1997 17Beta-estradiol reduces cardiac leukocyte accumulation in myocardial ischaemia reperfusion injury in rat. European Journal of Pharmacology 335 185–192.

Strone C, Duskic SP, Krause DN & Prooccupo V 2005 Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels. Molecular Pharmacology 68 959–965.

Stumpf WE, Sar M & Aumann G 1977 The heart: a target organ for estradiol. Sirner 196 319–321.

Stygar D, Westlund P, Eriksson H & Sahlin L 2006 Identification of wild type and variants of oestrogen receptors in polymorphonuclear and mononuclear leukocytes. Clinical Endocrinology 64 74–81.

Tan E, Gurjar MV, Sharma RV & Bhulla RC 1999 Estrone receptor-alpha gene transfer into bovine aortic endothelial cells induces eNOS gene expression and inhibits cell migration. Cardiovascular Research 43 788–797.

Tauber AI & Babior BM 1977 Evidence for hydroxyl radical production by human neutrophils. Journal of Clinical Investigation 60 374–379.

Taylor AH, Fox-Robichaud AE, Egan C, Dione J, Lawless DE, Raymond J, Romney J & Wong NC 2000 Oestradiol decreases rat allopriptide A1 transcription via promoter site B. Journal of Molecular Endocrinology 25 207–219.

Urata Y, Ibara Y, Murata H, Goto S, Koji T, Yodoi J, Inoue S & Kondo T 2006 17Beta-estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardic H9c2 cells. Journal of Biological Chemistry 281 13092–13102.
Wang M, Crisostomo P, Wairiuko GM & Meldrum DR 2006 Estrogen receptor-alpha mediates acute myocardial protection in females. *American Journal of Physiology. Heart and Circulatory Physiology* **290** H2204–H2209.

Yang SH, Liu R, Perez EJ, Wen Y, Stevens SM Jr, Valencia T, Brun-Zinkernagel AM, Prokai L, Will Y, Dykens J et al. 2004 Mitochondrial localization of estrogen receptor beta. *PNAS* **101** 4130–4135.

Yu HP, Shimizu T, Choudhry MA, Hsieh YC, Suzuki T, Bland KI & Chaudry IH 2006 Mechanism of cardioprotection following trauma-hemorrhagic shock by a selective estrogen receptor-beta agonist: up-regulation of cardiac heat shock factor-1 and heat shock proteins. *Journal of Molecular and Cellular Cardiology* **40** 185–194.

Zhai P, Eurell TE, Cooke PS, Lubahn DB & Gross DR 2000a Myocardial ischemia-reperfusion injury in estrogen receptor-alpha knockout and wild-type mice. *American Journal of Physiology. Heart and Circulatory Physiology* **278** H1640–H1647.

Zhai P, Eurell TE, Cotthaus R, Jeffery EH, Bahr JM & Gross DR 2000b Effect of estrogen on global myocardial ischemia-reperfusion injury in female rats. *American Journal of Physiology. Heart and Circulatory Physiology* **279** H2766–H2775.

Zwart W, Griekspoor A, Rondaij M, Verwoerd D, Neeffes J & Michalides R 2007 Classification of anti-estrogens according to intramolecular FRET effects on phospho-mutants of estrogen receptor alpha. *Molecular Cancer Therapeutics* **6** 1526–1533.

Zweier JL, Kuppusamy P, Williams R, Rayburn BK, Smith D, Weisfeldt ML & Flaherty JT 1989 Measurement and characterization of postischemic free radical generation in the isolated perfused heart. *Journal of Biological Chemistry* **264** 18890–18895.

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