Oestrogen-mediated cardioprotection following ischaemia and reperfusion is mimicked by an oestrogen receptor (ER)α 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 ($17\beta E_2$) 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 $17\beta E_2$ via ERβ with 46-fold selectivity relative to inhibition via ERα. *In vivo*, $17\beta E_2$ significantly reduced neutrophil infiltration, oxidant stress and necrosis following ischaemia and reperfusion. Cardioprotection by $17\beta E_2$ was not inhibited by ERB-88 but was completely reproduced by ERA-45. In conclusion, protection of the rat heart after ischaemia–reperfusion by $17\beta E_2$ 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 ($17\beta E_2$) 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 $17\beta E_2$–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 17βE$_2$ 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 17βE$_2$ *in vivo*. The antagonist did not inhibit the reduction in infarct size, neutrophil infiltration and free radical release in hearts from 17βE$_2$-treated rats. However, these effects of 17βE$_2$ 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, 5×10^4 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% CO_2. 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^{-9} M or 0.4×10^{-9} M 17βE$_2$ 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 17βE$_2$ 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 17βE$_2$ (2 µg/day for 21 days) or placebo. Preliminary experiments confirmed that this method achieved a plasma concentration of oestrogen in the physiological range (61.42 ± 7.7 pg/ml, $n = 6$, RIA, Bio-Stat Diagnostics, Stockport, UK). The rats received 17βE$_2$ or placebo for a minimum of 14 days prior to experimental use.

Ischaemia–reperfusion protocol

Rats were anaesthetised with 60 mg/kg sodium pentabarbitone i.p. (Sagatal, Rhone Merieux, UK). The procedures were carried out as described previously (Jeanes *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) $1·5 \times 10^{-2}$ 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-pyrolidine; 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 Mini-Scope 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 infarct 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 EC$_{50}$ of $3·7 \times 10^{-10}$ M (95% confidence limits 3·2 and $4·4 \times 10^{-10}$ M, $n=4$; Fig. 1a). The EC$_{50}$ for activation of human ERβ in the same cell system was $1·3 \times 10^{-8}$ M (95% confidence limits 0·9 and $17·7 \times 10^{-8}$ M, $n=4$; Fig. 1b).
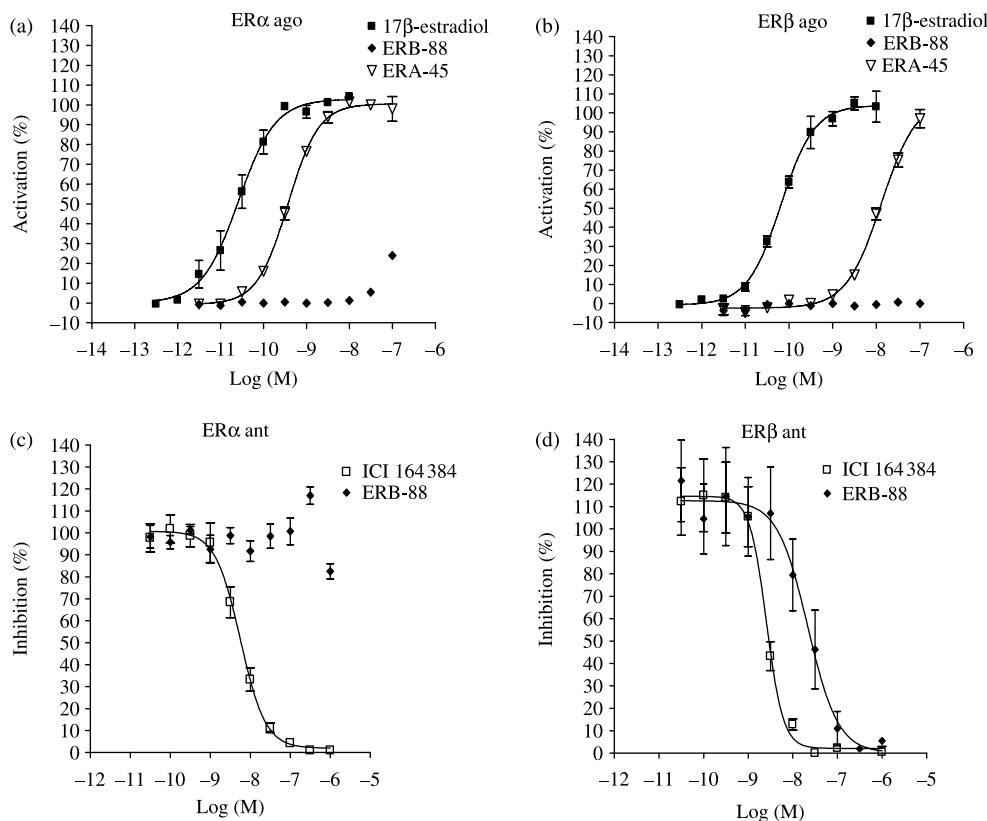
Figure 1 Characterisation of ER ligands in an *in vitro* gene transactivation assay. Activation by 17βE$_2$, the ERα agonist ERA-45 and the ERβ antagonist ERB-88 of (a) human ERα and (b) human ERβ. Antagonist activity of the ERα/ERβ antagonist ICI 164 384 and the ERβ antagonist ERB-88 at (c) human ERα and (d) human ERβ stimulated with 0·1 or 0·4 nM 17βE$_2$ respectively; $n = 5$–7 per curve.

17βE$_2$ activated ERα with an EC$_{50}$ of $2 \cdot 6 \times 10^{-11}$ M (95% confidence limits 1·7 and $4 \cdot 1 \times 10^{-11}$ M; Fig. 1a) and ERβ with an EC$_{50}$ of $6 \cdot 5 \times 10^{-11}$ M (95% confidence limits 4·9 and $8 \cdot 7 \times 10^{-11}$ M; Fig. 1b).

The non-selective ER antagonist ICI 164 384 inhibited activation by 17βE$_2$ in cells expressing both ERα (EC$_{50}$ of $5 \cdot 7 \times 10^{-9}$ M, 95% confidence limits 4·1 and $7 \cdot 7 \times 10^{-9}$ M; Fig. 1c) and ERβ (EC$_{50}$ of $2 \cdot 6 \times 10^{-8}$ M, 95% confidence limits 1·8 and $3 \cdot 6 \times 10^{-8}$ M; Fig. 1d). By contrast, ERB-88-inhibited gene transactivation induced by 17βE$_2$ in cells expressing recombinant human ERβ with an IC$_{50}$ of $2 \cdot 1 \times 10^{-9}$ M (95% confidence limits 0·8 and $5 \cdot 7 \times 10^{-9}$ 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^{-5} M, did not inhibit activation of either receptor by 17βE$_2$ (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 $5 \cdot 5 \pm 0 \cdot 78$ 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 17βE$_2$ 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

Table 1 The influence of 17β-oestradiol (17βE$_2$) 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.

	Body weight (BW, g)	Uterine weight/BW (%)	Area-at-risk (% total heart)
Hormone treatment			
Placebo+vehicle	278±6	0·04±0·01	57±4
17βE$_2$+vehicle	221±7*	0·25±0·03*	54±2
17βE$_2$+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 17βE$_2$+vehicle; $n=6–8$. BW, body weight.

17βE$_2$ (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 17βE$_2$ or placebo in combination with selective ERα agonist (Table 1). The selective ERα agonist appeared to induce less proliferation than 17βE$_2$ (Table 1). The ERβ antagonist ERB-88 had no significant effect on the uterine weight when given to rats receiving placebo or 17βE$_2$ (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 ∼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 17βE$_2$ 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). 17βE$_2$ 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 17βE$_2$-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 17βE$_2$ 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 17βE$_2$ (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 17βE$_2$ 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,
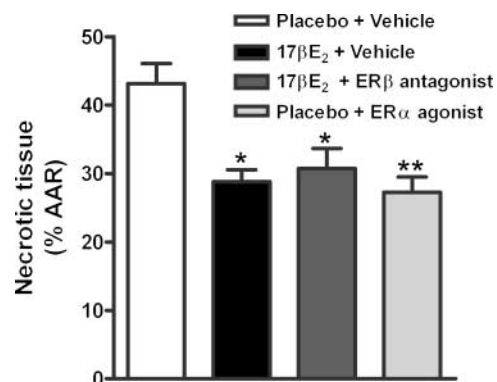
Figure 2 Myocardial necrotic tissue after *in vivo* ischaemia–reperfusion. Necrotic zone is expressed as a percentage of the total area-at-risk (AAR). Hormone treatment was placebo with vehicle, 17βE$_2$ with vehicle, 17βE$_2$ with ERβ antagonist ERB-88 or placebo with ERα agonist ERA-45. Data shown are means±S.E.M.; analysed using a two-way ANOVA and a Bonferroni *post hoc* test; **$P<0.01$, *$P<0.05$ compared with placebo; $n=6–8$.
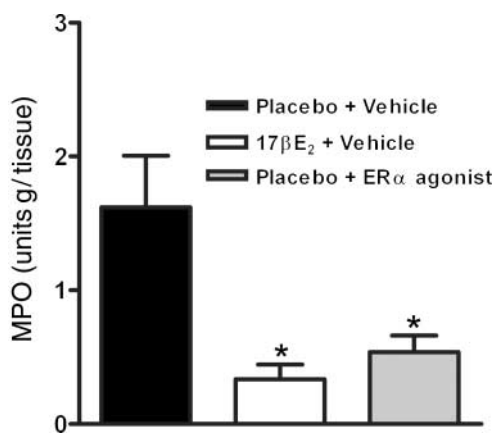
Figure 3 Myeloperoxidase activity in the area-at-risk (AAR) after ischaemia–reperfusion *in vivo* and the influence of $17\beta E_2$ or ERα agonist ERA-45 treatment. The hormone treatment prior to ischaemia–reperfusion was placebo with vehicle, $17\beta E_2$ with vehicle or placebo with ERα agonist ERA-45. Data are expressed as Ug^{-1} tissue. Data shown are mean $\pm$ S.E.M.; analysed with a one-way ANOVA and a Bonferroni *post hoc* test; *$P<0.05$ compared with placebo; $n=5$–6.

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 $53 \pm 10.3\%$ ($P=0.04$, $n=7$).

Significantly fewer free radicals were generated from the AAR of hearts from animals that received $17\beta E_2$ than those that received placebo/vehicle following ovariectomy ($P<0.01$; Fig. 4). This influence of $17\beta E_2$ was not modified in hearts
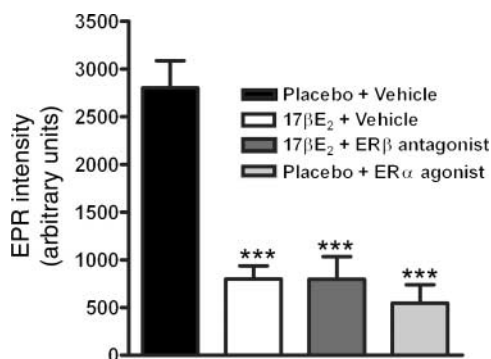

Figure 4 The effect of hormone treatment on oxidant stress generated from the reperfused myocardium. Data shown are the difference between free radicals produced from the entire heart and the ligated heart. Hormone treatment was placebo with vehicle, $17\beta E_2$ with vehicle, placebo with ERβ antagonist ERB-88, $17\beta E_2$ with ERβ antagonist ERB-88 or placebo with ERα agonist ERA-45. All data are mean $\pm$ S.E.M.; analysed using a one-way ANOVA with a Bonferroni *post hoc* test; ***$P<0.01$ compared with placebo; $n=5$–8.

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 $17\beta E_2$ 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 $17\beta E_2$. Chronic administration of ERB-88 to ovariectomised rats receiving $17\beta E_2$ had no significant influence on infarct size or oxidant stress production. In comparison, the cardioprotective effects of $17\beta E_2$ were completely reproduced by ERA-45.

It is well established that $17\beta E_2$ 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 $17\beta E_2$-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 vitro* 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 during 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 $17\beta E_2$ 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 $17\beta E_2$-treated animals. In the

reperfused heart, damaged mitochondria are also a source of oxidant stress through the electron transfer chain (Lesnefsky *et al.* 2001) and may also contribute here. $17\beta E_2$ has previously been shown to reduce the oxidative stress from isolated rat mitochondria (Stirone *et al.* 2005) and this may be an additional protective mechanism of $17\beta E_2$ in the current model. Free radical scavenging is also likely to be enhanced following chronic treatment with $17\beta E_2$, 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 $ER\alpha$ and $ER\beta$ in mediating the cardioprotective effects of oestrogen using a selective $ER\alpha$ agonist and a novel $ER\beta$ antagonist. *In vitro* gene transactivation studies showed that the $ER\alpha$ agonist ERA-45 has $\sim$35-fold greater potency for gene transactivation via $ER\alpha$, compared with $ER\beta$. 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 $ER\alpha$ but minimal, if any, activation of $ER\beta$. In rats, $ER\alpha$ is responsible for the stimulation of epithelial cell proliferation in the uterus that results in the observed increase in uterine weight when treated with $17\beta E_2$ or $ER\alpha$ agonist (Frasor *et al.* 2003). $ER\alpha$ also mediates the $17\beta E_2$-induced attenuation of food intake and weight gain (Roesch 2006). In the present study, administration of the $ER\alpha$ 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 $17\beta E_2$ suggesting that the selected dose was achieving a less than maximal effect, as expected from the pharmacokinetic data. The $ER\beta$ antagonist ERB-88 also showed clear efficacy and selectivity in gene transactivation studies. In $ER\alpha$-transfected cells activation by $17\beta E_2$ was inhibited by the established $ER\alpha/\beta$ antagonist ICI 164 384 (Zwart *et al.* 2007), but ERB-88 had no influence until 10^{-6} M. By contrast, at $ER\beta$, activation by $17\beta E_2$ was inhibited by both ICI 164 384 and ERB-88. Based on bioavailability data, the dose used in the present study should be sufficient to block 80–90% of $ER\beta$, without influencing $ER\alpha$. While there is no easily quantifiable measure of $ER\beta$ activation *in vivo* to confirm blockade of this receptor, the lack of influence on uterine or body weight confirms a lack of effect on $ER\alpha$.

The $ER\beta$ antagonist ERB-88 did not attenuate the effects of $17\beta E_2$ on infarct size, or free radical production. $ER\beta$ 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 $ER\beta$ agonist (Yu *et al.* 2006). However, it does not appear to have a role in mediating cardioprotection by $17\beta E_2$ following ischaemia–reperfusion in the rat. The results of the present study suggest that $17\beta E_2$ is more likely to act through stimulation of $ER\alpha$ in the rat

heart. This conclusion is supported by the fact that the $ER\alpha$ agonist ERA-45 completely reproduced the cardioprotective effects of $17\beta E_2$ and the effects of $17\beta E_2$ on neutrophil infiltration and oxidant stress production. In another study, acute administration of the selective $ER\alpha$ agonist PPT (4,4,4-4 propyl-(1H)-pyrazole 1,3,5-triyltrisphenol–PPT) 30 min before ischaemia and reperfusion also reduced infarct size in the rabbit, while the $ER\beta$ 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 $ER\alpha$ 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 $ER\alpha$ 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 $ER\alpha$ (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 $ER\alpha$ *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 $ER\alpha$ in myocardial ischaemia–reperfusion injury *in vivo*. In a more recent study, activation of the $ER\beta$, using the $ER\beta$ 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 $ER\alpha$ and $ER\beta$ to cardioprotection in the rabbit (Booth *et al.* 2005), rat (present study) and mouse (Nikolic *et al.* 2007). It is also possible that the $ER\beta$ 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 $17\beta E_2$ that can activate both receptors. In this sense, the antagonist data generated in the current study are important in demonstrating that removal of the $ER\beta$ does not prevent reduction of infarct size and oxidant stress generation by $17\beta E_2$. Studies using an $ER\alpha$ antagonist would provide further evidence for a role of $ER\alpha$, and also reveal whether $ER\beta$ can have a role when it is activated by $17\beta E_2$ in the absence of the $ER\alpha$.

In conclusion, the current study is the first study to demonstrate, using a combination of $ER\alpha$ agonist and $ER\beta$ antagonist, that the cardioprotective properties of $17\beta E_2$ in a rat model of ischaemia–reperfusion are primarily mediated via stimulation of the $ER\alpha$.

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Disclosure

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

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