

## Activation of estrogen receptor- $\alpha$ protects the in vivo rabbit heart from ischemia-reperfusion injury

Erin A. Booth, Nabeel R. Obeid, and Benedict R. Lucchesi

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan

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**Booth, Erin A., Nabeel R. Obeid, and Benedict R. Lucchesi.** Activation of estrogen receptor- $\alpha$  protects the in vivo rabbit heart from ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 289: H2039–H2047, 2005. First published July 1, 2005; doi:10.1152/ajpheart.00479.2005.—The estrogen receptor (ER) mediates estrogenic activity in a variety of organs, including those in the reproductive, cardiovascular, immune, and central nervous systems. Experimental studies have demonstrated that 17 $\beta$ -estradiol (E2) protects the heart from ischemia-reperfusion injury. Two estrogen receptors, ER $\alpha$  and ER $\beta$ , mediate the actions of estrogen; however, it is not certain which ER mediates the cardioprotective effects of E2. In the present study, the ER-selective agonists 4,4',4''-[4-propyl-(1H)-pyrazole-1,3,5-triyl]tris-phenol (PPT; ER $\alpha$ ) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; ER $\beta$ ) were assessed for their cardioprotective potential in an in vivo rabbit model of ischemia-reperfusion injury. Anesthetized female rabbits were administered PPT (3 mg/kg), DPN (3 mg/kg), E2 (20  $\mu$ g/rabbit), or vehicle intravenously 30 min before a 30-min occlusion of the left anterior descending coronary artery followed by 4 h of reperfusion. Acute treatment with E2 ( $17.7 \pm 2.9\%$ ;  $P < 0.001$ ) and PPT ( $18.1 \pm 2.9\%$ ;  $P < 0.001$ ), but not DPN ( $45.3 \pm 2.4\%$ ) significantly decreased infarct size as a percent of area at risk compared with vehicle ( $45.3 \pm 2.4\%$ ). Coadministration of PPT or E2 with the ER antagonist ICI-182,780 limited the infarct size-sparing effect of the compounds ( $43.8 \pm 6.6\%$  and  $40.6 \pm 5.7\%$  respectively, expressed as a percentage of risk region). PPT reduced the release of cardiac-specific troponin-I and reduced the tissue deposition of the membrane attack complex and C-reactive protein similar to that of E2. The results indicate that activation of ER $\alpha$ , but not ER $\beta$ , is required for the observed cardioprotective effects of E2.

infarct size; membrane attack complex; C-reactive protein; estrogen; 4,4',4''-[4-propyl-(1H)-pyrazole-1,3,5-triyl]tris-phenol

DESPITE OVERWHELMING DATA showing that premenopausal women have a reduced risk for cardiovascular disease (1), recent large clinical trials have failed to show cardioprotection for postmenopausal females on estrogen-progestin replacement therapy (23, 36). To date it is not understood why premenopausal women have a reduced incidence of cardiovascular disease while treatment with estrogen-progestin has had a negative outcome. Although hormone replacement therapy (HRT) was not shown to prevent cardiovascular events (14, 38), some observational trials have reported clinically relevant reductions in heart disease, and all cause mortality in women receiving HRT (17, 22). Furthermore, experimental evidence has demonstrated that the administration of 17 $\beta$ -estradiol (E2) reduces myocardial injury associated with ischemia-reperfusion (20, 42), and the cytoprotection is associated with activation of the estrogen receptor (ER) (6). Other studies

suggest that treatment with conjugated equine estrogen alone or with progesterone protects against the development of coronary atherosclerosis, although estrogen replacement does not appear to enhance the regression of the atherosclerotic plaque (7, 8, 43). The conflicting experimental and clinical data bring to light the need for a better understanding of the mechanisms responsible for estrogen's protective cardiovascular effects.

Estrogen mediates responses in a variety of organs, including those in the cardiovascular and immune systems. Estrogen-mediated events were originally believed to be regulated by the ER, which is now known to exist as two distinct subtypes, ER $\alpha$  and ER $\beta$ . The two receptors are encoded by different genes, share common structural and functional domains, bind to estrogen with high affinity, and bind estrogen response elements in a similar manner. ER $\alpha$  and ER $\beta$  differ in their tissue distribution, transcriptional activities, and phenotypes in knockout animals (26). Both receptors are expressed in vascular tissue; however, their expression and localization in cardiomyocytes remain controversial (18, 30).

At present, it is not known which receptor subtype accounts for the estrogen-mediated cardioprotective effects in hearts subjected to ischemia-reperfusion. Although antiestrogens have been available, the compounds are generally nonspecific and do not differentiate between the two receptor subtypes. The mechanism of action of antiestrogens is poorly understood, and as such, their use is limited. Recently, two putatively selective ER agonists have become available. 4,4',4''-[4-propyl-(1H)-pyrazole-1,3,5-triyl]tris-phenol (PPT) has a 410-fold binding affinity preference for ER $\alpha$  compared with ER $\beta$  (39), whereas 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) exhibits a 70-fold higher relative binding affinity and 170-fold higher relative potency with ER $\beta$  than with ER $\alpha$  in transcription assays (31). Recent studies utilizing PPT or DPN have demonstrated the dose-dependent induction of ER-dependent vascular relaxation by PPT equal to the effects of estradiol, whereas DPN was without effect, thereby demonstrating ER $\alpha$ 's importance in the vasculature (5). The cardioprotective actions of these compounds have not been determined. The aim of the present study was to investigate, for the first time, the acute effects of the ER $\alpha$  agonist PPT and the ER $\beta$  agonist DPN on cardioprotection after myocardial ischemia and reperfusion.

### METHODS

#### Guidelines for Animal Research

The procedures used in this study are in agreement with the guidelines of the University of Michigan Committee on the Use and Care of Animals. The University of Michigan Unit for Laboratory

Address for reprint requests and other correspondence: B. R. Lucchesi, Dept. of Pharmacology, Univ. of Michigan Medical School, 1301C Medical Science Research Bldg. III, Ann Arbor, MI 48109-0632 (e-mail: benluc@umich.edu).

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Animal Medicine provides veterinary care. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Health Care, and the animal care use program conforms to the standards in the *Guide for the Care and Use of Laboratory Animals* [DHEW Publication No. (NIH) 86-23]. Animal protocols were approved by the University of Michigan University Committee on Use and Care of Animals (UCUCA).

### Surgical Preparation

Female New Zealand White rabbits (2.6–3.2 kg) (Covance, Kalamazoo, MI) were anesthetized with a combination of xylazine (3.0 mg/kg) and ketamine (35 mg/kg) administered intramuscularly, followed by an intravenous injection of pentobarbital sodium (15 mg/kg). After insertion of a cuffed endotracheal tube, the animals were placed on positive-pressure ventilation using room air. The left jugular vein was isolated and cannulated for drug administration. The left carotid artery was isolated and instrumented with a Millar catheter microtip pressure transducer (Millar Instruments, Houston, TX) positioned immediately above the aortic valve to monitor aortic blood pressure. The lead II electrocardiogram was monitored throughout the experiment. A left thoracotomy and pericardiotomy were performed, followed by identification of the left anterior descending coronary artery. A silk suture (3-0; Genzyme Biosurgery, Cambridge, MA) was passed under the artery and passed through a short length of polyethylene tubing. Simultaneous downward displacement of the polyethylene tubing while applying upward traction on the suture resulted in occlusion of the coronary artery and cessation of regional myocardial blood flow. Coronary artery occlusion was maintained for 30 min after which time reperfusion was initiated by withdrawing the polyethylene tubing. Regional myocardial ischemia was verified by the presence of a zone of cyanosis in the area of distribution of the occluded vessel and by changes in the electrocardiogram consistent with the presence of transmural regional myocardial ischemia (ST segment elevation).

### Experimental Protocol

Animals were allowed to stabilize for 15 min before beginning the protocol, which involved three experimental groups. *Group 1* consisted of 34 intact female rabbits randomized equally among four treatment groups: PPT (3 mg/kg;  $n = 8$ ), DPN (3 mg/kg;  $n = 10$ ), E2 (20  $\mu$ g;  $n = 8$ ), or vehicle [1 ml of 20% DMSO-80% polyethylene glycol (PEG);  $n = 8$ ] administered 30 min before occlusion of the left anterior descending coronary artery. Female rabbits in *group 2* were treated with the ER antagonist ICI-182,780 in conjunction with either the ER $\alpha$  agonist PPT ( $n = 5$ ), E2 ( $n = 5$ ), or vehicle (20% DMSO-80% PEG;  $n = 5$ ). *Group 3* consisted of ovariectomized New Zealand White rabbits to eliminate any interference of activation of the ER by endogenous estrogens. Animals in *group 3* were randomized to five different treatment regimens: PPT (3 mg/kg;  $n = 6$ ), PPT (10 mg/kg;  $n = 6$ ), DPN (3 mg/kg;  $n = 6$ ), E2 (20  $\mu$ g;  $n = 6$ ), or vehicle (1 ml of 20% DMSO-80% PEG;  $n = 6$ ). The structures for E2 and the ER-selective agonists are shown in Fig. 1.

### Determination of Infarct Size

At the completion of the 4-h reperfusion period, the hearts were removed, the aorta was cannulated, and the coronary vascular bed was

perfused on a Langendorff apparatus with oxygenated Krebs-Henseleit buffer at a constant flow of 22–24 ml/min for 10 min to clear the vascular compartment of plasma and blood cellular elements. Forty-five milliliters of a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4, 37°C) was perfused through the heart. TTC demarcates the noninfarcted myocardium within the area at risk with a brick-red color, indicating the presence of a formazan precipitate resulting from the reduction of TTC by dehydrogenases present in viable myocardial tissue. Irreversibly injured tissue, lacking cytosolic dehydrogenases, is unable to form the formazan precipitate and appears pale yellow. On completion of the TTC infusion, the left circumflex coronary artery was ligated at the site identical to that ligated during the induction of regional myocardial ischemia. The perfusion pump was stopped, and 3 ml of a 0.25% solution of Evans blue was injected slowly through a sidearm port connected to the aortic cannula. The dye was passed through the heart for 10 s to ensure its uniform tissue distribution. The presence of Evans blue was used to demarcate the left ventricular tissue that was not subjected to regional ischemia, as opposed to the risk region. The heart was removed from the perfusion apparatus and cut into three transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded. Both surfaces of each transverse section were traced onto clear acetate sheets by the executor of the protocol in an unblinded fashion. The images were scanned and downloaded into Adobe PhotoShop (Adobe Systems Seattle, WA). The areas of the normal left ventricle nonrisk region, area at risk, and infarct region were determined by calculating the number of pixels occupying each area using the Adobe PhotoShop software. Total area at risk is expressed as the percentage of the left ventricle. Infarct size is expressed as the percentage of the area at risk.

### Biochemical Markers of Myocardial Damage

Plasma concentrations of cardiac-specific troponin I (cTnI) were determined by enzyme-linked immunosorbent assays (Life Diagnostics, West Chester, PA). Briefly, plasma was prepared from whole blood drawn at baseline, 2 h, and 4 h after the start of reperfusion (*group 1*,  $n = 6$ ; *group 2*,  $n = 5$ ; *group 3*,  $n = 5$ ). Samples were frozen immediately in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . On the day of the assay, samples were thawed over ice and diluted appropriately with diluent supplied with the assay kit. Protein concentrations were determined using the optical density of each sample compared with a standard curve.

### Immunofluorescent Detection of the Membrane Attack Complex and C-Reactive Protein

Tissue samples used for infarct size determination were fixed in 10% buffered formalin immediately after the completion of the experimental protocol. The tissue samples were embedded in paraffin blocks and cut into sections 2- $\mu\text{m}$  thick, which were mounted on glass slides. Two consecutive sections (mirror images) from a single heart slice were mounted on each slide. The slides were deparaffinized in three washes of xylene and rehydrated in an ethanol gradient. To remove any residual formalin and paraffin, antigen unmasking was performed using a commercially available antigen unmasking solution (Vector Laboratories, Burlingame, CA) and a pressure cooker (Fagor

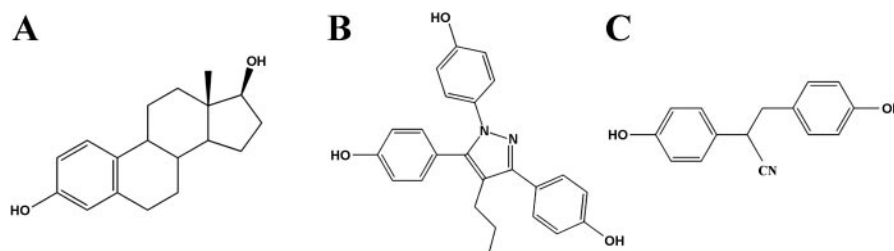


Fig. 1. Chemical structures of the compounds 17 $\beta$ -estradiol (E2; A), 4,4',4''-[4-propyl-(1H)-pyrazole-1,3,5-triyl]tris-phenol (PPT; B), and 2,3-bis(4-hydroxyphenyl)-propanitrile (DPN; C).

America, Lyndhurst, NJ). Slides were placed in a boiling solution of the diluted unmasking agent. The pressure cooker was sealed, and slides were incubated for 1 min once the cooker reached maximal pressure (~15 psi). The slides were cooled immediately in a tap water bath and blocked with 5% milk for 45 min. Primary antibodies were incubated at room temperature in a humidity chamber for 45 min. One section per slide was incubated with a chicken anti-rabbit C-reactive protein (CRP) antibody (5  $\mu$ g/ml final concentration; Immunology Consultants Laboratory, Newberg, OR). The opposing transverse heart section was incubated with a chicken anti-rabbit membrane attack complex (MAC) antibody (1:2,500 final dilution, developed in conjunction with Lampire Biological Laboratories, Popersville, PA). After three consecutive washes in PBS/1% milk each section was incubated with a goat anti-chicken biotinylated secondary antibody (1.5  $\mu$ g/ml final concentration; Vector Laboratories) for 30 min. After three PBS washes, the slides were incubated with fluorescein and Texas red (MAC and CRP sections, respectively)-labeled streptavidin (Fluorescent Streptavidin Kit; Vector Laboratories) to visualize the proteins. The fluorescent streptavidin reagents were allowed to incubate for 10 min followed by two washes in PBS. ProLong Gold antifade (Molecular Probes, Eugene, OR) and coverslips were used to preserve the sections. For comparison, images were captured using a digital camera (Sony DKC5000; Sony of America, New York) connected to a Leica fluorescent stereoscope (Leica MX FLIII) and the accompanying software (Leica, Wetzlar, Germany). Images were analyzed using IP Lab (Scanalytics, Fairfax, VA) software to determine mean fluorescence intensity per heart section. The sections were normalized to the amount of background on each slide. The mean intensities for three hearts in each treatment group were averaged and compared.

### Materials

The ER $\alpha$ -specific agonist PPT and the ER antagonist ICI-182,780 were provided by Wyeth Pharmaceuticals (Collegeville, PA). The DPN was purchased from Tocris (Ellisville, MO). All other materials were purchased from Sigma Chemical (St. Louis, MO).

### Statistical Analysis

The data are expressed as means  $\pm$  SE. Differences between control and experimental groups were determined using a one-way ANOVA for multiple groups or repeated measures. Differences between groups were determined using Bonferroni post hoc test. For cTnI, differences within each time point were compared using the Student's *t*-test for unpaired comparisons. A value of *P* < 0.05 was considered to be significant. Statistical analysis was performed using Graph Pad Prism (GraphPad Software, San Diego, CA).

## RESULTS

### Hemodynamic Effects

Hemodynamic variables were obtained to determine the effects of estradiol in mediating alterations in arterial blood pressure and heart rate. An immediate insignificant decrease in arterial blood pressure (5–8 mmHg) followed by an immediate return to the baseline values was observed after intravenous administration of the ER-specific agonists PPT and DPN as well as E2 and the vehicle control. The rate-pressure product (RPP), defined as mean arterial blood pressure multiplied by the heart rate divided by 100, was used as an indicator of myocardial oxygen consumption. As depicted in Fig. 2A, the RPP decreased in each of the groups (PPT, DPN, E2, and vehicle) from equilibration to 30 min after treatment and then remained stable throughout the duration of the protocol. As seen in Fig. 2, B and C, RPP was reduced similarly in animals

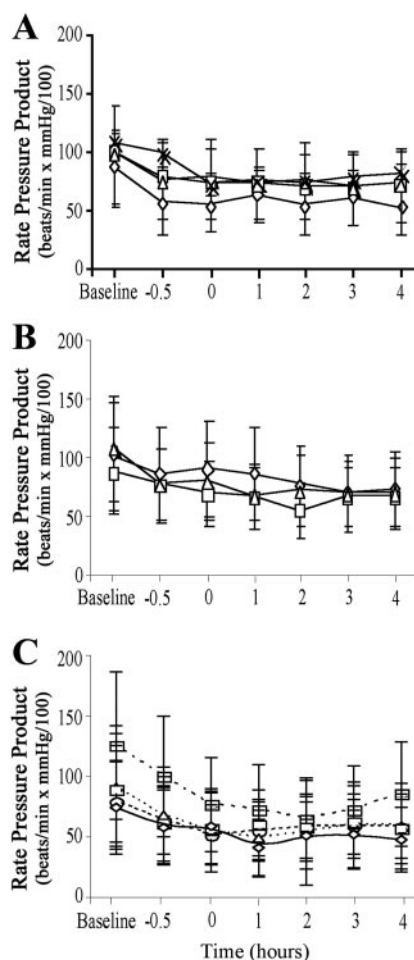


Fig. 2. Rate-pressure product for PPT ( $\Delta$ ), DPN ( $\times$ ), E2 ( $\square$ ), and vehicle ( $\diamond$ )-treated intact female rabbits (A); ICI-182,780 plus PPT ( $\Delta$ ), ICI-182,780 plus E2 ( $\square$ ), or vehicle ( $\diamond$ )-treated intact female rabbits (B); and PPT (3 mg/kg,  $\Delta$ ; 10 mg/kg,  $\circ$ ), DPN ( $\boxplus$ ), E2 ( $\square$ ), and vehicle ( $\diamond$ )-treated ovariectomized female rabbits (C). Rate-pressure product was calculated with the formula (beats/min  $\times$  mmHg)/100. No significant differences were noted between groups at any time point or within the same group throughout the reperfusion period. Time 0 indicates the onset of reperfusion.

treated with ICI-182,780 and in ovariectomized rabbits administered PPT, DPN, E2, or vehicle. The RPP remained stable throughout the duration of both protocols with no significant difference among groups.

Electrophysiological data did not demonstrate any changes on administration of PPT, DPN, E2, or vehicle. All animals exhibited ST segment elevation during the induction of regional myocardial ischemia. The ST segment changes resolved toward baseline on removal of the occlusive ligature. In all groups, premature ventricular complexes were present immediately after reperfusion. A significant increase in reperfusion arrhythmia was observed in intact rabbits administered 3 mg/kg DPN but not in the other experimental groups. The increase in arrhythmogenic activity did not occur in ovariectomized females administered the same dose of DPN (Table 1).

### Infarct Size

*PPT and E2, but not DPN, reduced infarct size in intact female rabbits.* In group 1, 34 intact female rabbits were randomized to the following treatment groups: PPT (ER $\alpha$



agonist; 3 mg/kg), DPN (ER $\beta$  agonist; 3 mg/kg) E2 (20  $\mu$ g/rabbit), or vehicle. Treatment was administered intravenously 30 min before the induction of regional myocardial ischemia followed by 4 h of reperfusion. Rabbits treated with either E2 or PPT developed significantly smaller infarcts expressed as a percentage of the area at risk compared with vehicle-treated rabbits. Infarct expressed as a percentage of area at risk did not differ between vehicle- and DPN-treated animals (Fig. 3A). The size of the area at risk expressed as a percentage of the total left ventricle was similar in each of the treatment groups (Fig. 3B).

*The nonspecific ER antagonist ICI-182,780 attenuated myocardial protection afforded by PPT and E2 treatment.* Each of three treatment groups consisted of five animals: a vehicle-treated group, a group treated with ICI-182,780 (1 mg/rabbit) plus PPT (3 mg/kg), and one treated with ICI-182,780 (1 mg/rabbit) plus E2 (20  $\mu$ g/rabbit). The ER antagonist ICI-182,780 was administered intravenously in combination with either PPT or E2. Combination treatment was administered 30 min before induction of regional myocardial ischemia (30 min), after which reperfusion was maintained for a period of 4 h. Rabbits treated with ICI-182,780 and PPT or E2 developed similar-sized infarcts expressed as a percentage of the area at risk compared with the control rabbits treated with vehicle (Fig. 4A). The findings suggest that the cardioprotective effect of E2 is mediated by the ER and support the concept that the cardioprotective actions of PPT are a result of ER $\alpha$  activation. The size of the area at risk or ischemic region expressed as a percentage of the total left ventricle was similar in each treatment group (Fig. 4B).

*PPT dose dependently reduced infarct size in ovariectomized female rabbits.* As seen in intact female rabbits, PPT and E2 reduced infarct size in ovariectomized rabbits. PPT dose dependently reduced infarct size and required a higher dose to reduce infarct size to the levels of estrogen than required in intact female rabbits (Fig. 5A). The size of the area at risk as a percentage of the total left ventricle was similar in each group (Fig. 5B).

Table 1. Incidence of VF during reperfusion and death due to VF in female New Zealand White rabbits

Treatment		Incidence of VF	Mortality Due to VF
<i>Group 1: Intact females</i>			
Vehicle (n = 8)		0/8 (0%)	0%
E2 (n = 8)		0/8 (0%)	0%
PPT (3mg/kg) (n = 8)		0/8 (0%)	0%
DPN (3mg/kg) (n = 10)		4/10 (40%)	20%
<i>Group 2: Intact females</i>			
Vehicle (n = 5)		0/5 (0%)	0%
ICI+E2 (n = 5)		0/5 (0%)	0%
ICI+PPT (n = 5)		0/5 (0%)	0%
<i>Group 3: Ovariectomized females</i>			
Vehicle (n = 5)		0/5 (0%)	0%
E2 (n = 5)		0/5 (0%)	0%
PPT (3mg/kg) (n = 5)		0/5 (0%)	0%
PPT (10mg/kg) (n = 5)		0/5 (0%)	0%
DPN (3mg/kg) (n = 5)		0/5 (0%)	0%

E2, 17 $\beta$ -estradiol; PPT, 4,4',4''-(4-propyl-(1H)-pyrazole-1,3,5-triyl) trisphenol; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; ICI, ICI-182,780; VF, ventricular fibrillation.

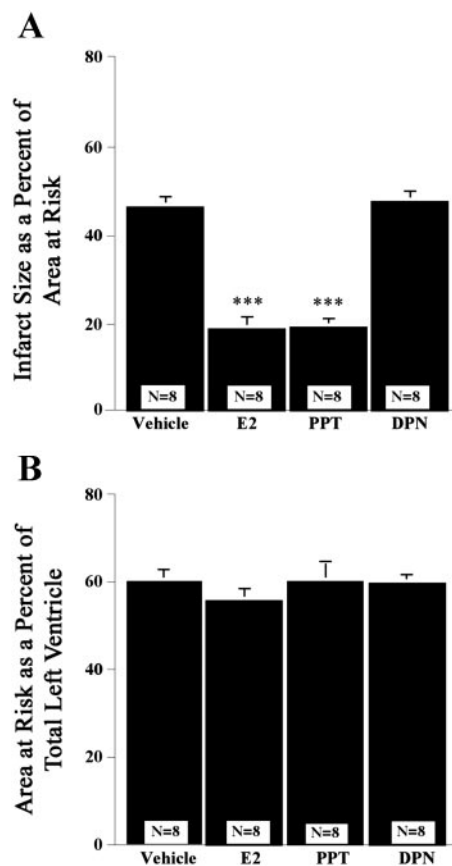


Fig. 3. Effects of PPT [estrogen receptor (ER)- $\alpha$ ], DPN (ER $\beta$ ), and E2 on myocardial infarct size after 30 min of coronary artery occlusion and 4 h reperfusion compared with vehicle. Infarct size after ischemia-reperfusion is expressed as a percentage of the area at risk (AAR; A). The infarct region is significantly decreased in the group treated with PPT and E2 compared with vehicle. Animals treated with DPN did not demonstrate a reduction in infarct size. The risk regions were similar between groups, which indicates that the degree of injury was similar (B). Results are expressed as means  $\pm$  SE. \*\*\* $P < 0.001$

#### Biochemical Markers of Irreversible Myocardial Injury

*Plasma concentrations of biochemical markers of cardiac damage are reduced after PPT and E2 treatment.* Plasma concentrations of cTnI were similar in all groups at baseline. PPT- and E2-treated rabbits exhibited significantly lower values of cTnI at 2 and 4 h after the onset of reperfusion compared with plasma from vehicle-treated animals. Plasma concentrations of cTnI from DPN-treated animals did not differ from vehicle (Fig. 6A).

*Markers of irreversible myocardial injury are similar to vehicle after administration of ICI-182,780.* Plasma concentrations of cTnI were similar in all groups at baseline. Combined treatment with ICI-182,780 and PPT or E2 abolished the reduction in plasma cTnI levels at 2 and 4 h after the onset of reperfusion. (Fig. 6B).

#### Immunofluorescence

*Immunofluorescence is reduced in hearts from PPT- and E2- but not DPN-treated animals.* Left ventricular tissue sections used for immunofluorescence were taken from hearts that had been subjected to 30 min of regional ischemia followed by 4 h

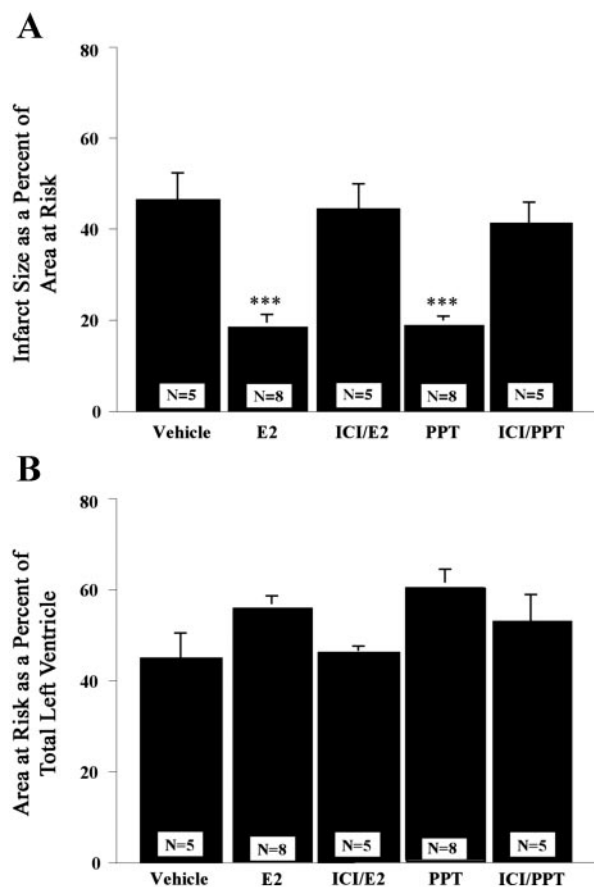


Fig. 4. Infarct size, expressed as the percentage of AAR (A), was used to assess the ability of E2 and the ER $\alpha$  agonist PPT to protect the myocardium in the presence of the ER antagonist ICI-182,780 (ICI). Rabbits treated with a combination of ICI-182,780 and PPT or E2 developed similar infarcts expressed as a percentage of the AAR compared with rabbits treated with vehicle. The risk regions were similar between groups (B). Results are expressed as means  $\pm$  SE. \*\*\* $P < 0.001$ .

of reperfusion. Hearts from vehicle-treated animals demonstrated bright fluorescence with both chicken anti-rabbit MAC and chicken anti-rabbit CRP antibodies, indicating the deposition of both proteins in the area of infarction. Conversely, hearts treated with PPT or E2 exhibited reduced fluorescence and, therefore, a reduction in deposition of CRP and MAC. Heart tissue from DPN-treated rabbits exhibited fluorescence similar to that in heart tissue samples from vehicle-treated rabbits (Fig. 7A). The mean intensity of fluorescence in heart sections obtained after treatment with E2 was significantly lower in tissue sections stained for either MAC or CRP compared with hearts from vehicle-treated animals (Fig. 7, B and C). Heart sections from animals treated with PPT demonstrated a decrease in mean fluorescence; however, this was not significantly different from vehicle ( $P < 0.08$ ). The mean intensity of fluorescence in heart sections from DPN-treated animals was similar to that of vehicle.

*Increased MAC and CRP deposition is seen in animals treated with a combination of ICI-182,780 and PPT or E2.* Hearts from all groups treated with ICI-182,780 (except for sham treated) demonstrated bright fluorescence with both chicken anti-rabbit MAC and chicken anti-rabbit CRP antibodies, indicating the deposition of both proteins in the area of

infarction. Administration of the ER antagonist eliminated the protective effects previously seen with PPT and E2 administration (data not shown).

#### Ventricular Arrhythmia

*DPN treatment increases reperfusion arrhythmia in intact female rabbits.* An increase in ventricular fibrillation during reperfusion was observed in intact female rabbits pretreated with DPN (3 mg/kg). In addition, there was an increase in mortality due to ventricular fibrillation during reperfusion (Table 1). Arrhythmias were not observed in the PPT-, E2-, or vehicle-treated female rabbits nor were they observed in ovariectomized rabbits treated with the same dose of DPN.

#### DISCUSSION

The administration of estrogen before the induction of regional myocardial ischemia and reperfusion is reported to reduce the extent of irreversible myocardial injury by a mech-

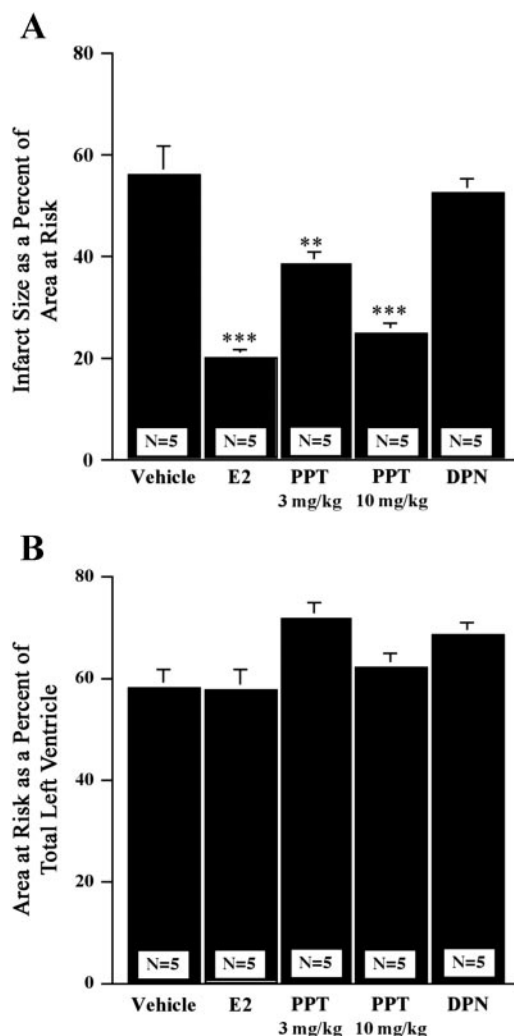


Fig. 5. Effects of PPT, DPN, and E2 on myocardial infarct size in ovariectomized female rabbits. The infarct as a percentage of the AAR is dose dependently decreased with PPT administration compared with vehicle. Animals treated with E2, but not DPN, showed a significant reduction in infarct size (A). The risk regions were similar between groups (B). Results are expressed as means  $\pm$  SE. \*\* $P < 0.01$ , \*\*\* $P < 0.001$

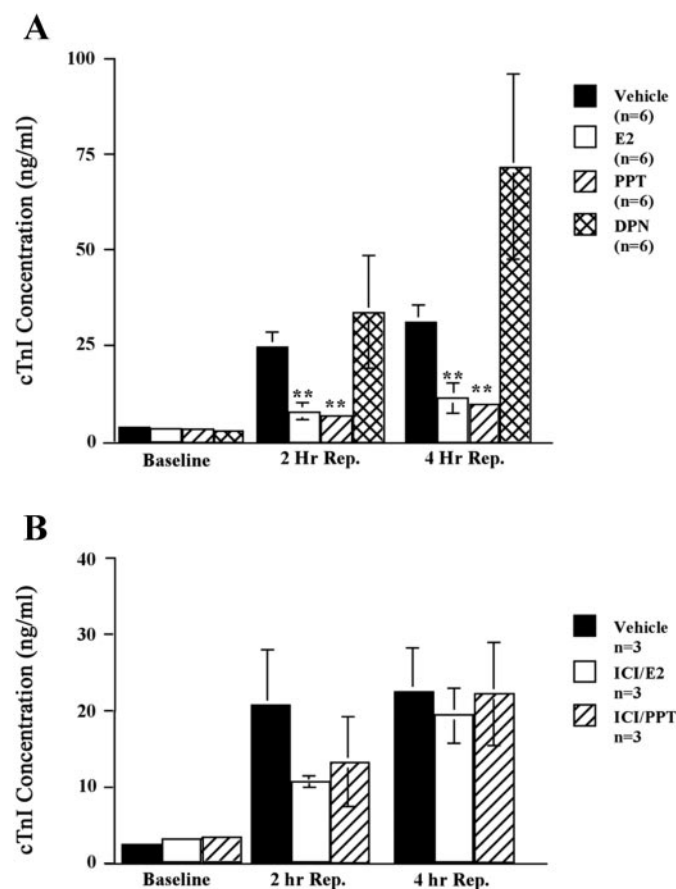


Fig. 6. Plasma levels of cardiac troponin-I (cTnI) as determined by ELISA assay in samples from intact females treated with PPT, DPN, E2, or vehicle (A) and with ICI-182,780 and PPT, ICI-182,780 and E2, or vehicle (B). Administration of PPT and E2, but not DPN, significantly reduced plasma concentrations of the cardiac-specific marker of damage cTnI at 2 and 4 h after reperfusion (Rep) compared with vehicle control. Coadministration of ICI-182,780 with E2 or PPT eliminated the reduction in plasma concentrations of cTnI. Values are presented as means  $\pm$  SE.  $^{***}P < 0.01$ .

anism involving activation of the ER (6). The goal of our study was to determine which ER is involved in the observed cardioprotection. There are two ERs, ER $\alpha$  and ER $\beta$ , which are differentially expressed throughout body tissues (9), including cardiac myocytes and endothelial cells (33). Activation of ER $\alpha$  and ER $\beta$  can cause homo- and heterodimer formation, suggesting that different compositions of the dimer differentially regulate gene expression (21, 29). Data also suggest that ER $\beta$  can antagonize the actions of ER $\alpha$  (28). The above observations have led to the concept that selective ER modulators (SERMs) might retain the protective effects of estrogen, such as activation of receptors in the heart, without the adverse effects of stimulating breast or ovarian proliferation. The development of cardioprotective SERMs is limited by an incomplete understanding of the mechanism of activation of ER $\alpha$  and ER $\beta$  on the cardiovascular system.

To date, studies with knockout mice have yielded conflicting results. Both ER $\alpha$  and ER $\beta$  have been shown to contribute to myocardial protection after ischemia and reperfusion (13, 47). Recent knockout studies have suggested that ER $\beta$  mediates gender differences in ischemia-reperfusion injury under conditions of enhanced contractility (13), the development of

pressure overload hypertrophy (37) and the aggravation of heart failure (34). Despite these convincing studies, there is still no consensus on whether ER $\beta$  is present in the myocardium. Some reports show that ER $\beta$  cannot be detected in the adult heart (24), whereas in other studies ER $\beta$  is abundant in the nucleus (41, 44), and one report shows ER $\beta$  exclusively in the mitochondria (45). Because of these conflicting data, the effects of estrogen on the myocardium have been suggested to be indirect (12). It is possible that, as seen in neonatal cardiomyocytes, ER $\alpha$  and ER $\beta$  are expressed only when estradiol is added to the culture medium (19). Furthermore, some of the data are from male ER knockout animals without the addition of exogenous estrogen to activate the ER. These situations are not physiologically relevant as they demonstrate the effects of the lack of the ER but not the physiological response to activation of the expressed receptor subtype. These problems, as well as the possibility of residual ER proteins in the knockout animals, make the interpretation of knockout data difficult.

Our study utilized the ER-specific agonists PPT (ER $\alpha$ ) and DPN (ER $\beta$ ) to determine the effects of ER-specific activation on the myocardium after ischemia and reperfusion. We demonstrated that the reduction in myocardial damage after ischemia and reperfusion was mediated by activation of ER $\alpha$ . Administration of the ER $\alpha$ -specific agonist PPT resulted in a reduction in infarct size similar to that seen with E2 treatment. The infarct-sparing effect was not observed with the administration of the ER $\beta$ -selective agonist DPN. On the other hand, the administration of DPN to intact female rabbits resulted in an increase in reperfusion arrhythmias and development of lethal ventricular fibrillation. The protective effects of both PPT and E2 were reduced significantly by the combined treatment with the nonspecific ER antagonist ICI-182,780, suggesting that the protection by PPT is related to activation of the receptor and not due to a nonspecific action of the agonist. Previous studies have shown that ICI-182,780 alone did not affect infarct size (6). In addition, consistent with the observed protection in intact females, PPT administration was accompanied by a dose-dependent reduction in reperfusion injury in ovariectomized females, which lack endogenous estrogens. The low dose of PPT (3 mg/kg) did not confer the same degree of protection in ovariectomized animals compared with intact females. Basal activation of the ER or incomplete discrimination of the ER subtype in intact females may be the cause of both the dose-dependent difference in myocardial rescue seen with PPT administration as well as the increase in ventricular fibrillation that occurred in intact females treated with DPN.

As another method of quantifying cardiac injury after ischemia-reperfusion, serum concentrations of biochemical markers of tissue injury were measured. cTnI is a component of the contractile machinery within myocytes. During irreversible injury and cell lysis, the protein is released into the blood and can be measured using a specific immunoassay. As expected, based on the infarct size data, acute treatment with PPT and E2 significantly decreased the plasma concentration of cTnI compared with vehicle. Treatment with the ER $\beta$  agonist DPN had no effect on cTnI release compared with vehicle. Coadministration of ICI-182,780 with PPT or E2 abolished the reduction in cTnI release.

An important aspect of complement-mediated myocardial injury that has been the focus of recent research is the role of



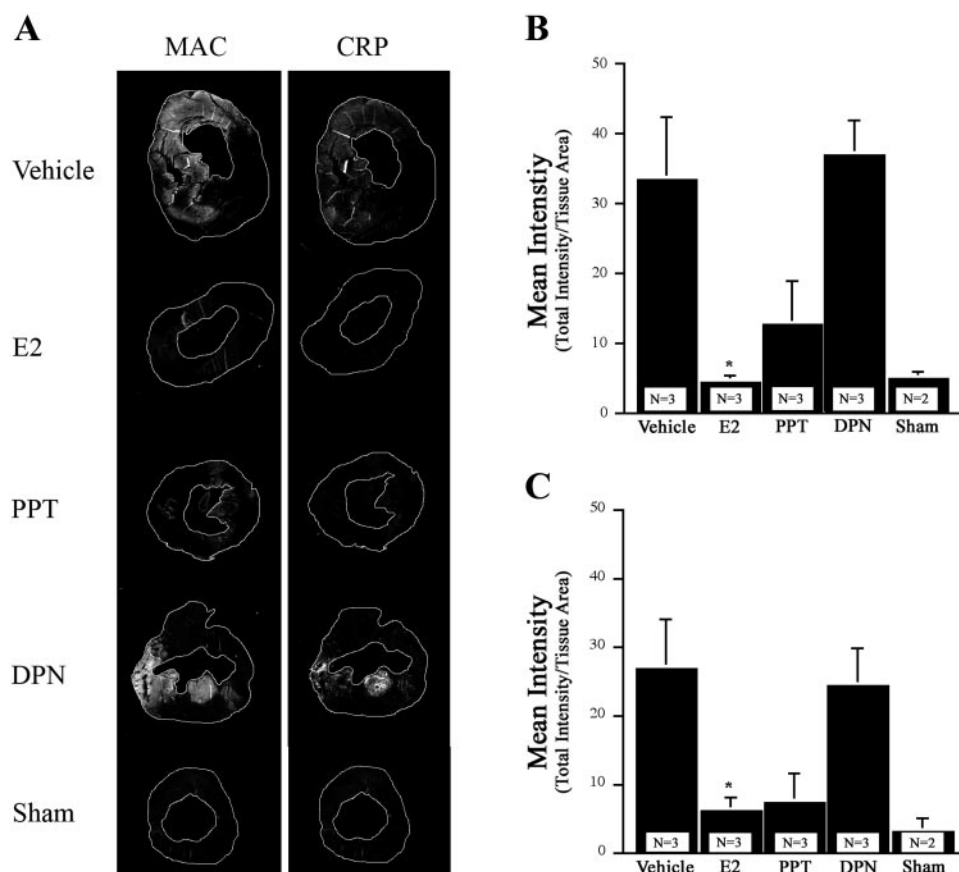


Fig. 7. Representative immunofluorescent pictures of hearts from animals treated with PPT, DPN, E2, or vehicle (A). Areas of bright deposits indicate sites of membrane attack complex (MAC) formation and C-reactive protein (CRP) deposition. Note that the most intense staining is localized to the risk region of the vehicle- and DPN-treated hearts. Hearts from animals treated with E2 showed a significant reduction in mean intensity of staining for MAC (B) and CRP (C). Results are expressed as means  $\pm$  SE. \* $P < 0.05$ .

CRP in the activation of the complement system. Once thought of as only a nonspecific indicator of systemic inflammation, recent epidemiological studies indicate that CRP may be directly involved in the pathogenesis of ischemic heart disease through the activation of the complement system (2, 4, 11). Moreover, increased CRP concentrations are reported to be associated with increased mortality due to cardiovascular events (10, 46). Therefore, CRP may be an indicator of myocardial injury, as well as being involved in the pathogenesis of irreversible myocyte injury (10, 35, 46). Systemic administration of CRP as well as elevations in endogenous CRP are reported to increase the extent of myocardial necrosis, through complement-dependent mechanisms (3, 16). CRP activates the classical complement pathway, which provides a possible mechanism linking CRP to mortality due to myocardial infarction (25, 32). An immunofluorescent method was used to determine the presence of tissue-bound CRP and MAC. Both PPT and estrogen administered 30 min before the induction of regional myocardial ischemia reduced the deposition of both CRP and MAC localized within the area of infarct. The inhibition of immune complex deposition was attenuated by the coadministration of the ER antagonist ICI-182,780.

This study is the first to demonstrate that specific activation of ER $\alpha$  by PPT protects the myocardium subjected to ischemia and reperfusion. The results suggest that activation of ER $\alpha$  is associated with a reduction in myocardial injury and immune complex deposition in a rabbit model of regional ischemia and reperfusion. Although the exact mechanism by which PPT and E2 protect the myocardium remains to be determined, we

provide evidence that activation of ER $\alpha$  leads to inhibition of the complement system, possibly through inhibition of CRP, thereby leading to an attenuation of myocardial ischemia-reperfusion injury. The presence of the MAC in infarcted myocardium and the ability of inhibitors of the complement cascade to protect the ischemic myocardium underscore the importance of estrogen's effects on the immune response during reperfusion (15, 27, 40).

ERs have been demonstrated in cultured myocytes, fibroblasts, vascular cells, and the immune system, where they can exert redundant, nonredundant, or opposing biological effects (21, 28, 29). Although we have shown that estrogen protects the myocardium from ischemia-reperfusion injury through activation of ER $\alpha$ , the compound was administered systemically; therefore, we do not know the direct effect of ER $\alpha$  activation on the cardiovascular system. Additional studies need to be performed utilizing isolated organ systems or conditional knockouts to determine the specific effects of ER $\alpha$  activation on the heart and vasculature. Furthermore, because estrogen acts equally on both ER $\alpha$  and ER $\beta$ , the involvement of ER $\alpha$  in acute myocardial protection does not completely rule out a role for ER $\beta$  in the chronic setting. It is necessary to perform long-term estrogen replacement studies to determine the effects of selective ER $\alpha$  activation in ischemia-reperfusion injury.

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