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17β Estradiol as a Receptor Mediated Cardioprotective Agent

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Running Title: 17β Estradiol and Cardioprotection

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Abstract

Cardiac tissue that undergoes an ischemic episode exhibits irreversible alterations that become more extensive upon reperfusion. Estrogen treatment has been reported to protect against reperfusion injury, but the mechanism remains unknown. The cardioprotective effects of 17β□estradiol, a biologically active form of the hormone, and 17α□estradiol were assessed in an in vivo occlusion-reperfusion model. Anesthetized, ovariectomized rabbits were administered 17β□estradiol (20μg), 17α□estradiol (1mg), or vehicle intravenously 30 min before a 30 min occlusion of the left anterior descending (LAD) coronary artery followed by 4 hr of reperfusion. Infarct size as a percent of area at risk decreased in the 17β□estradiol treated group (18.8 $\pm$ 1.7) as compared to 17α□estradiol (41.9 $\pm$ 4.8; $p < 0.01$) or vehicle groups (48 $\pm$ 5.5; $p < 0.001$). Similar results were obtained when infarct size was expressed as a percent of total left ventricle. The second arm of the study was to assess ICI 182,780, an estrogen receptor antagonist, for its effects on infarct size in ovariectomized female rabbits treated with 17β□estradiol. ICI 182,780 was administered intravenously one hr before the administration of 17β□estradiol (20μg) or vehicle. The hearts were subjected to 30 min LAD coronary artery occlusion and 4 hr reperfusion. Pretreatment with ICI 182,780 significantly limited the infarct size sparing effect of 17β□estradiol when expressed as a percent of the risk region (53.0 $\pm$ 5.0). The results indicate that 17β□estradiol protects the heart against ischemia-reperfusion injury and that the observed cardioprotection is mediated by the estrogen receptor.

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Estrogen has a wide variety of biological effects upon target tissues involving actions mediated via a classical, nuclear pathway or an alternative pathway as well as receptor independent antioxidant properties (Bhavnani et al. 2001). In the classical model of estrogen action, binding of the steroid agonist to the receptor initiates receptor dimerization, nuclear translocation and gene transcription. The alternative pathway involves the activation of receptors at either membrane or cytosolic locations as well as interaction with ligand or voltage gated ion channels (Nadal et al. 2000; Nadal et al. 2001). Activation of both membrane and cytosolic targets can lead to the activation of kinase cascades thought to be responsible for the rapid time course (from seconds to minutes) of the primary effect that is too fast to involve either RNA synthesis or protein translocation.

In addition to their role in female reproductive function, estrogens have beneficial actions on unrelated tissues. Estrogen receptors are present in cardiac myocytes, arterial smooth-muscle cells, and endothelial cells. Previous studies indicate that estrogens exert beneficial effects on the cardiovascular system, in part through their actions on hepatic lipid metabolism (Rosano GMC and Panina G 1999). However, several studies concluded that hormone replacement therapy (HRT) does not confer a benefit towards preventing heart disease or stroke and should not be used in postmenopausal women for the sole purpose of heart disease prevention (Nelson et al. 2002; Humphrey et al. 2002; Writing Group 2002).

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Recent experimental evidence suggests that the biologically active 17β-estradiol reduces both the extent of irreversible myocardial injury and the incidence and duration of reperfusion-induced ventricular tachycardia and ventricular fibrillation (Tsai et al. 2002). Myocardial infarct size resulting from coronary artery occlusion and reperfusion was reduced in male rabbits after acute treatment with 17β-estradiol whereas 17α-estradiol had no beneficial effect (Hale et al. 1996). Estradiol is reported to induce vasorelaxation (Gilligan et al. 1994; Vehkavaara et al. 2000) and to act as a free radial scavenger (Kuhl 1993; Telci et al. 2002). Estrogens have antioxidant activity and may inhibit the oxidation of LDL (Sack et al. 1994) by affecting superoxide dismutase, each of these effects, acting alone or in concert, may contribute to estradiol's non-genomic mediated cardioprotective effects. The exact mechanism of estrogen's protection is currently unknown and the elucidation of the mechanism may aid in the development of more specific pharmacological interventions such as selective estrogen receptor modulators (SERMs) for the treatment and/or prevention of postmenopausal cardiovascular disease.

The aim of the present study was to assess the cardioprotective effects of 17β-estradiol and 17α-estradiol in ovariectomized, (female) New Zealand White rabbits subjected to myocardial ischemia and reperfusion. Protocols were designed to evaluate whether protection afforded by estradiol was mediated by a receptor dependent action or a steroidal, nonestrogenic effect.

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Methods

Guidelines for Animal Research. The procedures used in this study were 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 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 Publ. N. (NIH) 86-23.

Surgical Preparation. Ovariectomized, (female), New Zealand White rabbits (2.6-3.2 kg) were anesthetized with a combination of xylazine (3.0 mg/kg) and ketamine (35 mg/kg) administered intramuscularly, followed by an intravenous injection of sodium pentobarbital (15 mg/kg). After insertion of a cuffed endotracheal tube, the animals were placed on positive-pressure ventilation with 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 micro-tip pressure transducer (Millar Inst. Inc, Huston, Tx, U.S.A) 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; Deknatel, Fall River, Ma, U.S.A) was passed under the artery and passed through a short length of polyethylene tubing. Simultaneous downward displacement of the polyethylene tubing while applying

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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 minutes 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 (Figure 1). The animals were allowed to stabilize for 15 minutes before beginning the protocol that involved two experimental groups. Group 1 consisted of 21 rabbits randomized equally among three treatment regimens consisting of: 17α-estradiol (n=7), 17β-estradiol (n=7), or vehicle (20% DMSO 80% PEG; n=7) administered 30 minutes before occlusion of the left anterior descending coronary artery. Rabbits in Group 2 were treated with the estrogen receptor antagonist ICI 182,780 dissolved in PEG one hour before acute treatment with 17β-estradiol (n=5), or vehicle (n=5).

Determination of Infarct Size. At the completion of the 4 hour reperfusion period, the hearts were removed, the aorta was cannulated, and the coronary vascular bed was perfused on a Langendorff apparatus with Krebs-Henseleit Buffer at a constant flow of 22-24 ml/min. The hearts were perfused with buffer for 10 minutes to clear the vascular compartment of plasma and blood cellular elements. Forty-five milliliters of a 1% solution of triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4, 37°C) was

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perfused through the heart. TTC demarcates the non-infarcted myocardium within the area at risk with a brick-red color, indicating the presence of a formazan precipitate resulting from 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. Upon 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 2 ml of a 0.25% solution of Evans Blue was injected slowly through a side-arm port connected to the aortic cannula. The dye was passed through the heart for 10 sec 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 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 photocopied and enlarged. The photocopies were scanned and downloaded into Adobe PhotoShop™ (Adobe Systems Inc. Seattle, WA). The areas of the normal left ventricle non-risk 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 as well as percentage of the total left ventricle.

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Evaluation of neutrophil accumulation. Tissue samples to be assayed for myeloperoxidase (MPO) activity were obtained from a separate set of experiments to avoid interference with the enzymatic assay. Samples of area at risk (AAR) were obtained from myocardium immediately distal to the site of vessel occlusion. Samples of were weighed and immediately frozen in liquid nitrogen until assayed. Tissue samples obtained from AAR were, placed in buffer (50 mm sodium phosphate, pH 6.0) and homogenized with a polytron homogenizer (Tekmar Co., Cincinnati, OH, U.S.A.). The homogenates were centrifuged for 30 min (3,000g, 4°C), and the supernatants removed. MPO activity was determined by measuring the change in absorbance at 460 nm resulting from the conversion of H_2O_2 in the presence of O-dianisidine (Sigma, St. Louis, MO, U.S.A.), as described previously (Bradley PP 1982). The MPO activity was normalized to the weight of the sample.

Morphological Changes. Tissue samples used for electronmicroscopy were obtained from a separate group of experiments. Upon completion of the designated protocol, hearts were perfused for 3 min with 2.5% glutaraldehyde and 1%$LaCl_3$ in 0.1M sodium cacodylate buffer (pH 7.44). The electron-dense $LaCl_3$ serves as an indicator of blood vessel integrity (Haack et al. 1981). Tissue samples from the left ventricular myocardium immediately below the site of occlusion were cut into segments measuring approximately 1 mm on a side. The samples were fixed for an additional 2 hrs at room temperature and then overnight at 4°C. After washing with 0.1M sodium cacodylate buffer, the samples were dehydrated in an ethanol series and embedded in EM bed-812 (Electron Microscopy Sciences). Tissue blocks were sectioned with a Reichert ultramicrotome, placed onto

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formvar coated copper grids, and then stained with 4% uranyl acetate. Sections were observed with the use a Phillips CM-10 electron microscope.

Materials. Unless otherwise specified, 17β-estradiol and 17α-estradiol as well as all materials used to prepare buffer solutions were purchased from Sigma Chemical Co (St. Louis, MO U.S.A). The estrogen receptor antagonist, ICI 182,780, was provided by Wyeth Pharmaceuticals (Princeton, NJ, U.S.A).

Statistical Analysis. The data are expressed as the mean ± S.E.M. Differences between control and experimental groups were determined using a one-way analysis of variance (ANOVA) for multiple groups or repeated measures as appropriate. Post-test differences between groups were determined using Bonferoni post test. A value of $P < 0.05$ was considered to be significant.

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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 decrease in arterial blood pressure followed by a return to the baseline value was observed after intravenous administration of estradiol or the vehicle control (data not shown). 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 **Figure 2A** the RPP decreased in each of three groups (17α-estradiol, 17β-estradiol, and vehicle) from equilibration to thirty minutes after treatment and then remained stable throughout the duration of the protocol. As seen in **Figure 2B**, RPP was reduced similarly in all groups with no significant difference among groups. The RPP remained stable throughout the duration of the protocol with no significant difference among groups.

Electrophysiologic data did not demonstrate any changes on administration of estradiol. All animals exhibited ST-segment elevation during the induction of regional myocardial ischemia. The ST-segment changes resolved towards baseline upon removal of the occlusive ligature. In all groups, premature ventricular complexes were present immediately after reperfusion. No deaths, from either cardiac arrhythmias or cardiac failure were noted in any of the groups.

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Effects of Estrogen Receptor Agonists on Myocardial Infarct Size. Each of the three treatment groups consisted of seven animals in which 17β-estradiol (20μg), 17α-estradiol (1mg) or the control vehicle was administered intravenously 30 minutes before the induction of regional myocardial ischemia followed by four hours of reperfusion. The size of the area at risk expressed as a percent of the total left ventricle was similar in each of the three groups (**Figure 3 A**). Rabbits treated with 17β-estradiol developed significantly smaller infarcts expressed as a percent of the area at risk compared to rabbits treated with vehicle or 17α-estradiol (**Figure 3B**). A significant reduction in myocardial infarct size was also observed in the 17β-estradiol treated group when the data were expressed as a percent of the total left ventricle (**Figure 3C**).

Effects of 17β-estradiol, 17α-estradiol and vehicle on morphological changes after ischemia and reperfusion. The tissue samples for electronmicroscopy were obtained from the left ventricular free wall of hearts that underwent 30 minutes of ischemia followed by 4 hr of reperfusion. In both the vehicle and 17α-estradiol treated hearts sarcomere structural features were altered and contracture band necrosis was present. The mitochondria were markedly swollen with disrupted cristae and osmophillic inclusion bodies. In the 17β-estradiol treated hearts, the sarcomere structure was relatively normal and the mitochondria appeared intact with only minimal swelling. The virtual absence of contraction bands in 17β-estradiol treated hearts was in marked contrast with changes observed in the heart tissue specimens obtained from both the vehicle treated and 17α-estradiol treated animals (**Figure 4**). The ability of 17β-estradiol to prevent contraction band necrosis is consistent with the observation that hearts

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pretreated with 17β-estradiol protected myocardial tissue from irreversible injury as determined by the TTC histochemical method for demarcation of viable and non-viable tissue.

Effects of ICI 182,780 on Attenuation of Myocardial Infarct Size by 17β-Estradiol. Each of three treatment groups consisted of five animals - a vehicle treated group, a group treated with ICI 182,780 (1mg/rabbit) plus vehicle and a group treated with ICI 182,780 plus 17β-estradiol. The selective estrogen receptor antagonist ICI 182,780 was administered intravenously one hour before the animals were treated with 17β-estradiol (20μg/rabbit) or vehicle. Either vehicle or 17β-estradiol was administered thirty minutes before induction of regional myocardial ischemia (30 min) after which reperfusion was maintained for a period of four hours. The size of the area at risk or ischemic region expressed as a percent of the total left ventricle was similar in each treatment group (**Figure 5A**).

Rabbits treated with ICI 182,780 and 17β-estradiol developed similar infarcts expressed as a percent of the area at risk compared to the control rabbits treated with vehicle (**Figure 5B**). Similarly, a reduction in myocardial infarct size was not observed when the data were expressed as a percent of the total left ventricle (**Figure 5C**). The findings support the concept that the cardioprotective effect of 17β-estradiol is mediated via activation of the estrogen receptor and the beneficial cardioprotective actions of 17β-estradiol can be attenuated by the pure antiestrogen ICI 182,780.

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Effects of ICI 182,780 on morphological changes after ischemia and reperfusion. The tissue samples for electronmicroscopy were obtained from the left ventricular free wall of hearts that underwent 30 minutes of ischemia followed by 4 hr of reperfusion. In all of the treatment groups: vehicle, ICI 182,780 plus vehicle, and ICI 182,780 plus 17β-estradiol sarcomere structural features were altered and contraction bands were present. The mitochondria were swollen with disrupted cristae and osmophillic inclusion bodies (**Figure 6**). The inability of 17β-estradiol to prevent contraction band necrosis following pretreatment with ICI 182,780 is consistent with the observation that the cardioprotective effect of 17β-estradiol is mediated via the estrogen receptor.

Effects of Estrogen Receptor Agonists and ICI 182,780 on Neutrophil Accumulation. To assess the degree of neutrophil infiltration, a separate set of myocardial tissue samples for each treatment group was obtained for the determination of MPO content (**Figure 7A and 7B**). The AAR from each treatment group was assayed for MPO activity. The MPO activity in the risk regions of control and treated groups was found to correlate with the degree of infarct size. The administration of 17β-estradiol resulted in a decrease in MPO activity in the AAR of animals receiving drug compared with 17α-estradiol and control. The data are indicative of decreased neutrophil accumulation within the AAR of animals treated with 17β-estradiol. The inhibition of neutrophil accumulation in the AAR of animals treated with 17β-estradiol was attenuated by pretreatment with the estrogen receptor antagonist ICI 182,780.

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Discussion

An earlier study in rabbits demonstrated cardioprotection associated with the acute administration of 17β-estradiol, but not with 17α-estradiol (Hale et al. 1996). In the present report, we demonstrated a similar outcome in that acute pretreatment with 17β-estradiol, but not 17α-estradiol, results in a decrease in myocardial infarct size in an *in vivo* rabbit model of regional myocardial ischemia/reperfusion. In addition, we demonstrate that the cardioprotection afforded by 17β-estradiol occurs via a receptor dependent mechanism and that the observed reduction in myocardial injury is abrogated by pretreatment with the estrogen receptor antagonist ICI 187,280.

The question of whether or not hormone replacement therapy (HRT) is associated with cardioprotective properties in postmenopausal women has been a subject of heightened clinical interest for many years. Publication of the Heart and Estrogen/Progestin Replacement Study (HERS II) (Grady et al. 2002) and the early termination of the Women's Health Initiative (WHI) (Writing Group 2002) plus two meta-analyses (Nelson et al. 2002; Humphrey et al. 2002) all lead to the same conclusion: HRT does not confer a benefit towards preventing heart disease or stroke and should not be used in postmenopausal women for the sole purpose of heart disease prevention a position endorsed by the American Heart Association (Mosca et al. 2001). Although these studies did not show a benefit of HRT, estrogen deficiency seems to be involved in many pathological processes including arteriosclerosis (Henderson et al., 1986), osteoporosis (Turner et al., 1994), and degenerative processes in the CNS (Fillit et al., 1986). The exact mechanism of action of estrogens in these various hormone responsive tissues are

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not fully defined. The present study indicates a receptor mediated protective effect on infarct size with acute treatment of 17β-estradiol. In addition to its antioxidant properties, estrogen has demonstrated anti-inflammatory properties such as a reduction in the expression of adhesion molecules in the brain (Galea E et al. 2002). Estradiol has also been shown to protect isolated human pancreatic cell islets from cytokine mediated cell death in a receptor dependent manner (Contreras JL et al. 2002). These effects may contribute to the protective effects of estrogen demonstrated in this study, however, the effects of HRT are systemic and this study alone cannot suggest that the opinion concerning HRT is incorrect. The elucidation of the protective effects of estradiol in certain tissues may lead to the development of improved tissue-selective estrogens and SERMs thereby eliminating the detrimental effects of prolonged HRT treatment including the alterations in the atherogenic process that lead to myocardial infarction.

Estrogens elicit a variety of cellular responses by acting upon intracellular or plasma membrane receptors. A number of transcriptional effects of estrogens are the result of events involving classic estrogen nuclear receptors ERα and ERβ (Beato and Klug 2000). It generally is accepted that estrogens act at the genomic level by binding to intracellular estrogen receptors. The molecular mechanism used by environmental chemicals to exert their hormone-like actions is only partially resolved. It is believed that xenoestrogens act at the genomic level by binding to intracellular estrogen receptors. There is a compelling body of data to suggest that estrogens trigger nongenomic effects in pancreatic beta cells. Both xenoestrogens and the circulating hormone, 17β-estradiol, bind with high affinity to a common membrane binding site unrelated to the intracellular estrogen receptors ERα

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and ERβ. This binding site is shared by dopamine, epinephrine, and norepinephrine and has the pharmacological profile of the γ adrenergic receptor (Nadal et al. 1998; Nadal et al. 2000; Ropero et al. 1999). At physiological concentrations, 17β-estradiol, closes K(ATP) channels in a rapid and reversible manner occurring through a receptor located at the plasma membrane, distinct from the classic cytosolic estrogen receptor. The acute administration of conjugated equine estrogen or 17β-estradiol significantly attenuates the severity and incidence of ventricular arrhythmias during ischemia and reperfusion (McHugh et al. 1995; Node et al. 1997b). The results from several studies suggest that estrogen is cardioprotective against reperfusion injury and reperfusion arrhythmias by acting via different ATP-sensitive potassium channels and that this protection is independent of an estrogen receptor mechanism. The degree of involvement of the two types of ATP-sensitive potassium channels in estradiol-induced infarct size and antiarrhythmic activity during ischemia and reperfusion remains uncertain. In the present study we did not focus on the antiarrhythmic or antifibrillatory actions of 17β-estradiol. However, there is no doubt that more must be done to determine if 17β-estradiol has a beneficial effect in terms of preventing ventricular arrhythmias/fibrillation in the ischemic and post-ischemic heart.

The action of estrogen on the vascular wall include increased production of nitric oxide (Fraser et al. 2000; Zhai et al. 2000; Node et al. 1997a), which occurs within minutes, and induction of inducible nitric oxide synthase and increased production of prostacyclin (PGI2), which develop more slowly. Each of these changes promote vasodilation. Thus, the ability of estrogens to reduce the injury associated with myocardial ischemia and

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reperfusion (Delyani et al. 1996; Hale et al. 1996; Squadrito et al. 1997) may involve one or more of the proposed mechanisms resulting from a combination of genomic and non-genomic effects. Myocardial infarct size resulting from coronary artery occlusion and reperfusion was reported to be reduced by 17β-estradiol in a dose-dependent fashion, irrespective of gender differences. The infarct size-limiting effect of estrogen as well as that of ischemic preconditioning was abolished by 5-hydroxydecanoate, suggesting that the cardioprotective effect of estrogen may result from activation of myocardial mitochondrial K(ATP) channels (Lee et al. 2000).

In the present study, administration of the estrogen-receptor antagonist ICI 182,780 (fulvestrant, Faslodex™) alone to the ovariectomized rabbit did not influence myocardial infarct size. ICI 182,780 is an ER antagonist that is reported to down regulate cellular levels of the ER and has no known agonist effects (Wakeling et al. 1994; Howell et al. 2000). Pretreatment with ICI 182,780 significantly impaired the ability of 17β-estradiol to protect the rabbit heart against irreversible myocardial injury in response to regional ischemia and reperfusion. The rapid onset of the cardioprotective effect of 17β-estradiol as well as the attenuation of the protection by ICI 182,780 would support the concept that 17β-estradiol is acting *via* a membrane or cytosolic receptor and not by way of affecting a nuclear receptor. The present findings however, do not rule out the possibility that chronic administration of 17β-estradiol would not confer cardioprotection by more than one mechanism involving both membrane (non-genomic) and nuclear receptors (genomic) or by way of an action on a diversity of plasma membrane estrogen binding sites. When estrogen binds to a receptor, both rapid non-genomic and long-term genomic

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effects may become operative. The genomic effects should be related to the distribution of estrogen receptors, while some of the non-genomic actions might be independent of ERs. Thus, estrogen mediated effects may be the result of antioxidant properties, altered enzymatic activity or ion channel interactions that are independent of specific receptor activation (Bracamonte and Miller 2001).

In conclusion, 17β-estradiol provides a protective effect on ischemic myocardium as evidenced from its ability to reduce the extent of myocardial injury associated with regional ischemia and reperfusion. Whereas these findings are in agreement with that of previous authors the current study extends upon the knowledge base by demonstrating that the observed cardioprotective effect is inhibited by ICI 182,780, a pure anitestrogen. The latter observation provides compelling support for the concept that the cardioprotection exhibited by 17β-estradiol is achieved, in part, by a non-genomic, receptor mediated mechanism. To our knowledge this is the first demonstration indicating that the acute cardioprotective actions of 17β-estradiol are mediated via a non-genomic receptor mechanism. Future studies will be directed towards determining if selective estrogen receptor modifiers (SERMs) can confer similar cardioprotective benefits.

There is compelling evidence that estrogen does not act only at the transcriptional level, but has several novel routes of action including activation of receptors on the plasma membrane as well as in the cytoplasm. The alternative, or non-genomic, pathways may be initiated at either membrane or cytosolic locations and result in either direct local

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effects (modulation of channel activity, release of nitric oxide) or effects such as the regulation of gene transcription secondary to the activation of signaling cascades. Thus, multiple potential mechanisms may contribute to the decreased infarct size in animals treated with 17β-estradiol during the acute phase response. Although the evidence presented in the present study suggests a receptor mediated non-genomic mechanism due to the timeline of activation, caution should be exercised against over-interpretation of the data. For example, it is thought that the membrane and cytosolic receptors are structurally similar to the classic nuclear estrogen receptor as demonstrated by the ability of several antibodies raised against different epitopes of the classic ER to recognize these receptors, however, we can not be completely certain that the pure antiestrogen ICI 182,780 interacts with these receptors. Further studies must be done to resolve these important issues.

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Figure Legends:

Figure 1

Experimental Protocol for acute treatment with 17α-estradiol, 17β-estradiol, or vehicle (Group 1) or ICI 182,780 one hour before acute administration of 17b-estradiol or vehicle (Group 2).

Figure 2

Rate-pressure product for 17α-estradiol, 17β-estradiol, and vehicle (control) treated animals (A) and ICI 182780 plus vehicle, ICI 182780 plus 17 β-estradiol or vehicle treated animals (B). Rate-pressure product was calculated with the formula beats/min x mm Hg/100. No significant differences were noted between groups at any time point or within the same group throughout the reperfusion period. Arrow indicates the onset of reperfusion.

Figure 3

Effects of 17α-estradiol and 17β-estradiol on myocardial infarct size after 30 min of left circumflex coronary artery occlusion and 4 hr reperfusion as compared with vehicle. 17α-estradiol was administered at a doses of 1mg/rabbit and 17β-estradiol was administered at a dose of 20μg/rabbit. The risk regions were similar between groups, which indicates that the degree of injury was similar (A). Infarct size after ischemia/reperfusion (B) is expressed as a percent of the AAR and (C) total left ventricle.

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The infarct region is significantly decreased in the group treated with 17β-estradiol as compared with vehicle. Animals treated with 17α-estradiol did not demonstrate a reduction in infarct size. Results are expressed as the mean + S.E.M. ** $P < 0.01$ versus control, *** $P < 0.001$ versus control.

Figure 4

Representative electron micrographs of hearts from control animals and animals treated with 17α-estradiol and 17β-estradiol after 30 min ischemia and 4 hr reperfusion. In the vehicle and 17α-estradiol treated hearts sarcomere structural features are obliterated and contracture bands are present. The mitochondria are markedly swollen with disrupted crystal and osmophilic inclusion bodies. In the 17β-estradiol-treated hearts, the sarcomere structure is relatively normal and the mitochondria appear intact with only minimal swelling. The virtual absence of contraction bands stands in marked contrast with those observed in the control hearts. Magnification:19000x

Figure 5

Infarct size, expressed as the percent of the left ventricle or area at risk (AAR), was used to assess the ability of the 17β-estradiol to protect the myocardium in the presence of ICI 182,780 after ischemia and reperfusion. The AAR when expressed as a percent of left ventricle indicated that the tissue amount subject to the ischemia episode was similar in all groups (A). Rabbits pretreated with ICI 182,780 and then treated with 17β-estradiol developed similar infarcts expressed as a percent of the AAR compared to rabbits treated with vehicle (B). No reduction in myocardial infarct size was observed when the data

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were expressed as a percent of the total left ventricle (C). Pretreatment with ICI 182,780 attenuates cardioprotection afforded by 17β-estradiol indicating that 17β-estradiol protects the myocardium via a receptor-mediated mechanism.

Figure 6

Representative electron micrographs of hearts from animals treated with the vehicle, estrogen receptor antagonist ICI 182,780 alone, or ICI 182,780 and 17β-estradiol. In all groups sarcomere structural features are altered and contracture bands are present. The markedly swollen mitochondria with disrupted cristae and osmophillic inclusion bodies indicate irreversible damage. The inability of 17β-estradiol to prevent contraction band necrosis following pretreatment with ICI 182,780 is consistent with the observation that the cardioprotective effect of 17β-estradiol is mediated via the estrogen receptor.

Magnification: 19000x

Figure 7

Neutrophil accumulation in the area at risk (AAR) as determined by myeloperoxidase (MPO) activity in tissues from animals treated with 17α-estradiol, 17β-estradiol, or vehicle (A) and animals treated with ICI 182,780 and vehicle, ICI182,780 and 17β-estradiol or vehicle (B). The MPO activity in the AAR from 17β-estradiol treated animals was decreased as compared with vehicle-treated animals. The decrease in MPO activity seen in the 17β-estradiol treated animals was eliminated with the pretreatment of the estrogen receptor antagonist ICI 182,780. Values are normalized to the tissue wet weight and represent the mean + SEM ** $P < 0.01$ versus control.

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Figure 1

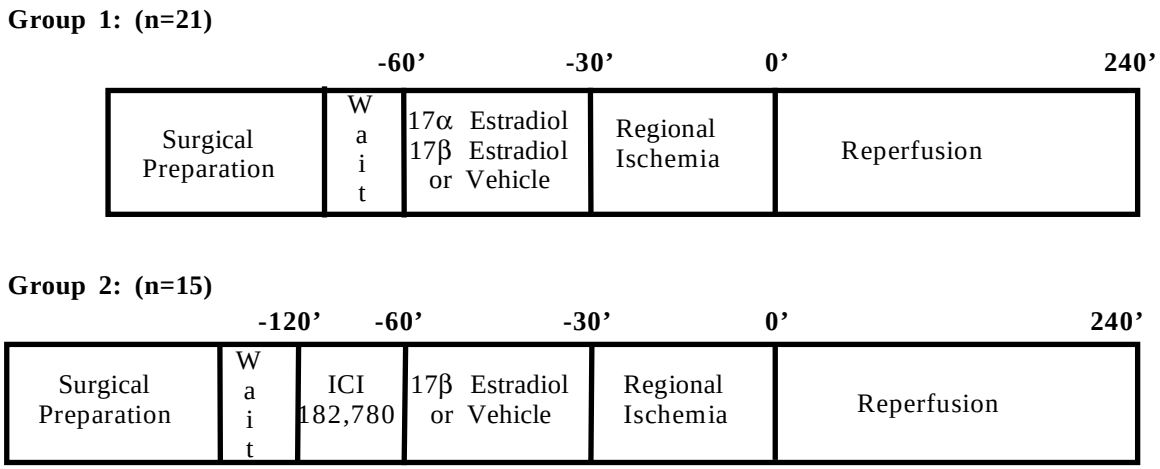

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Figure 2

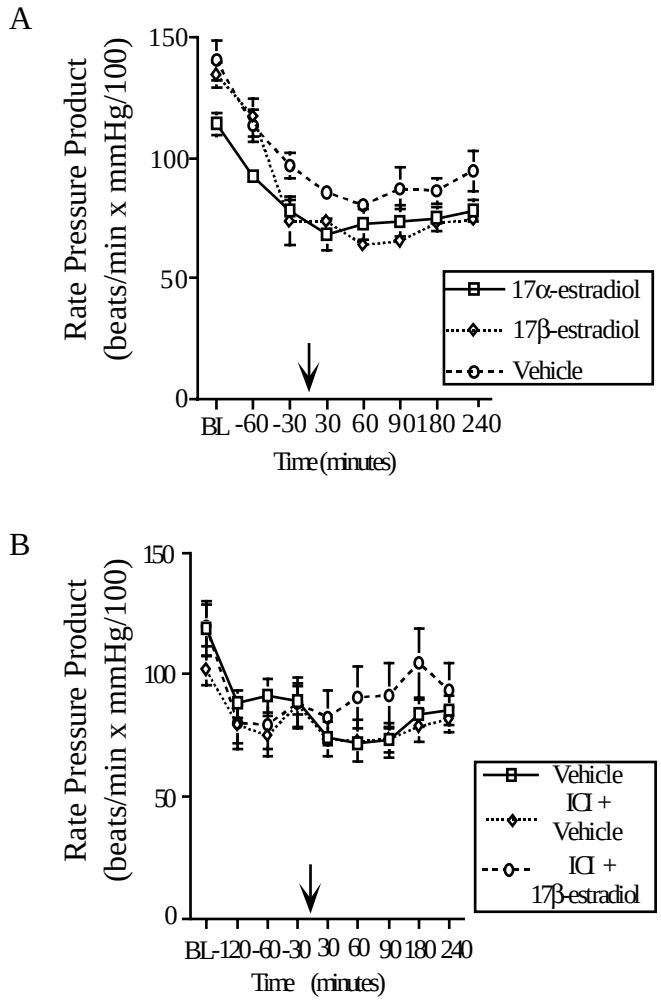

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Figure 3

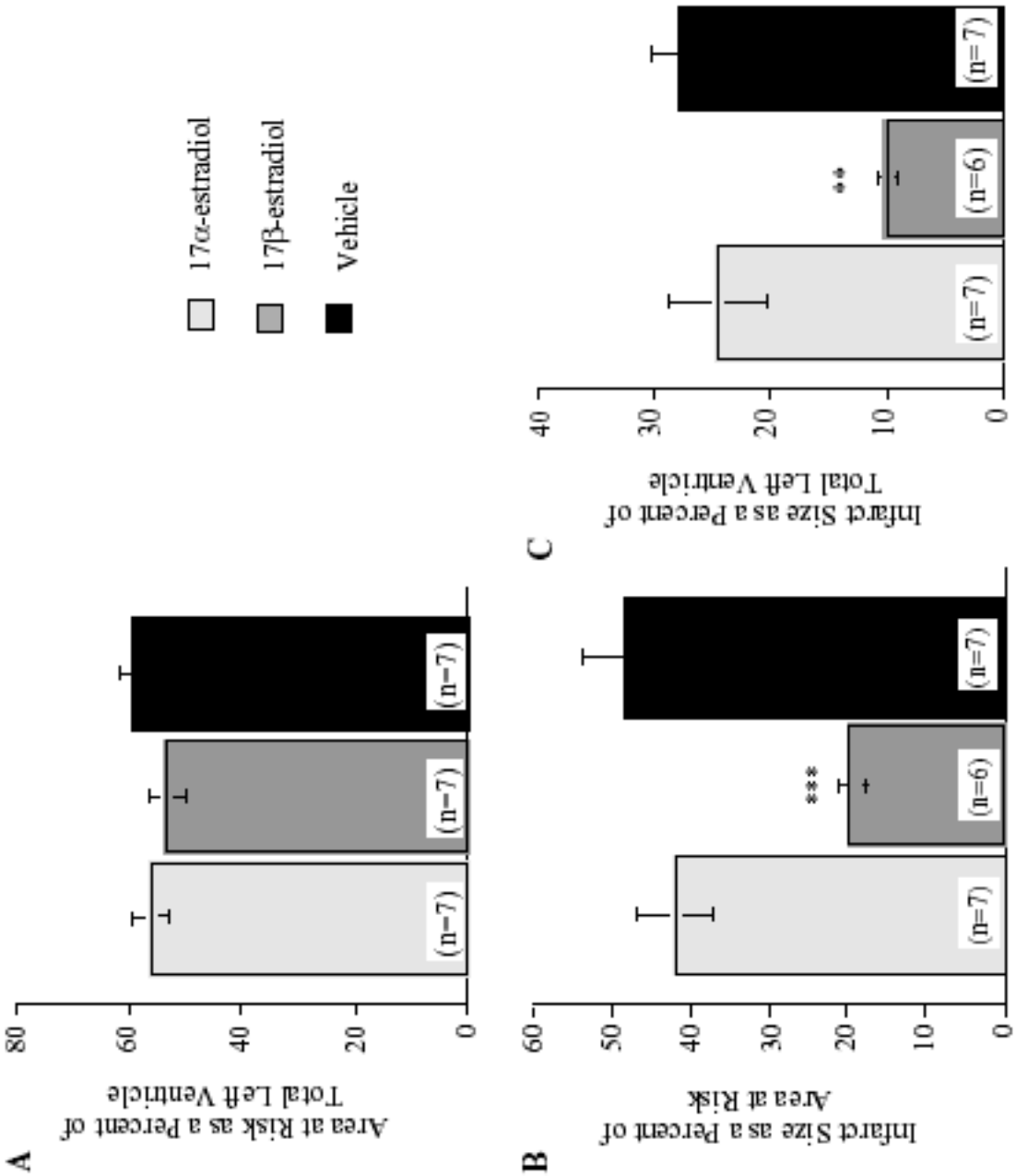

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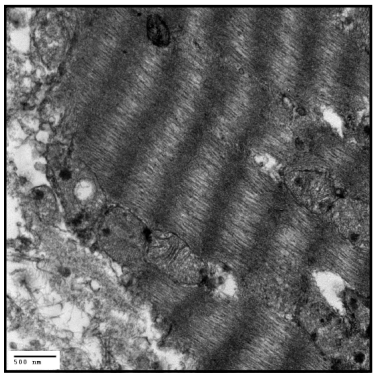
Figure 4



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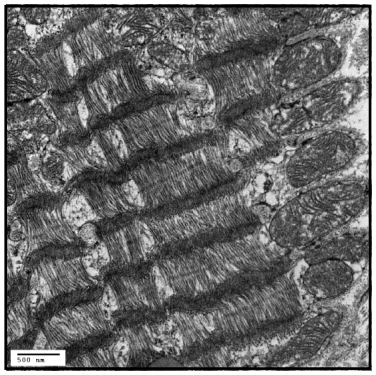

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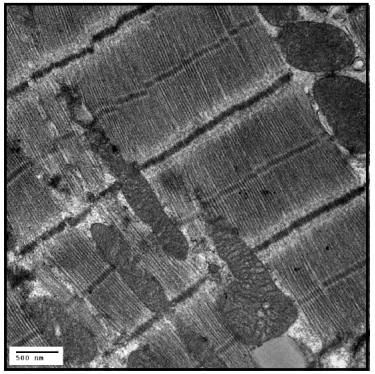

17β-estradiol

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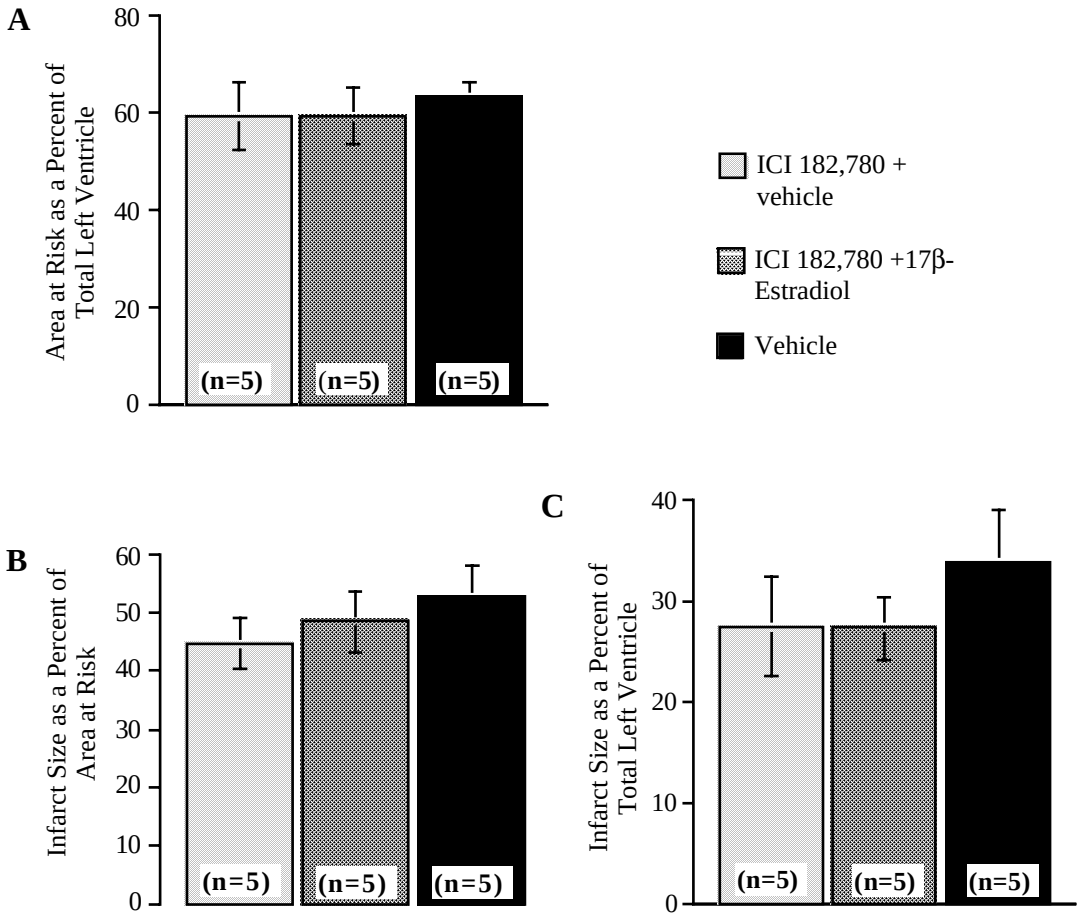
Figure 5



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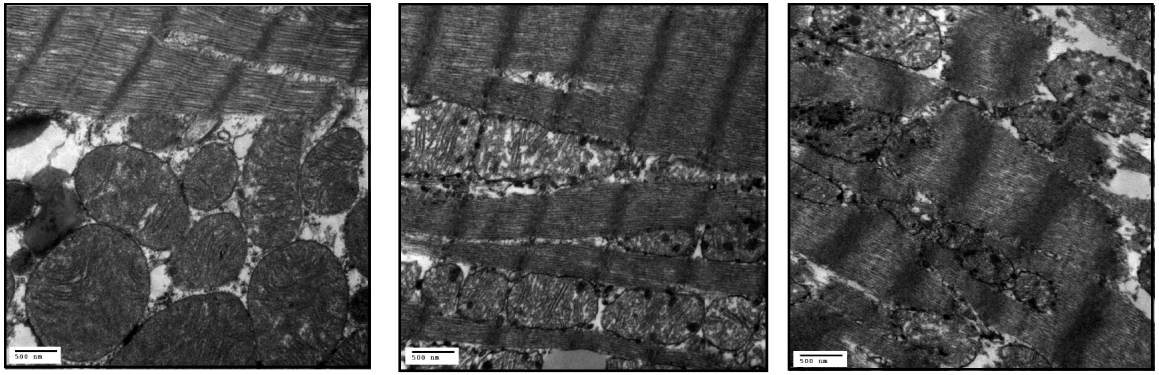

Vehicle | ICI 182,780 + Vehicle | ICI 182,780 + 17β-estradiol

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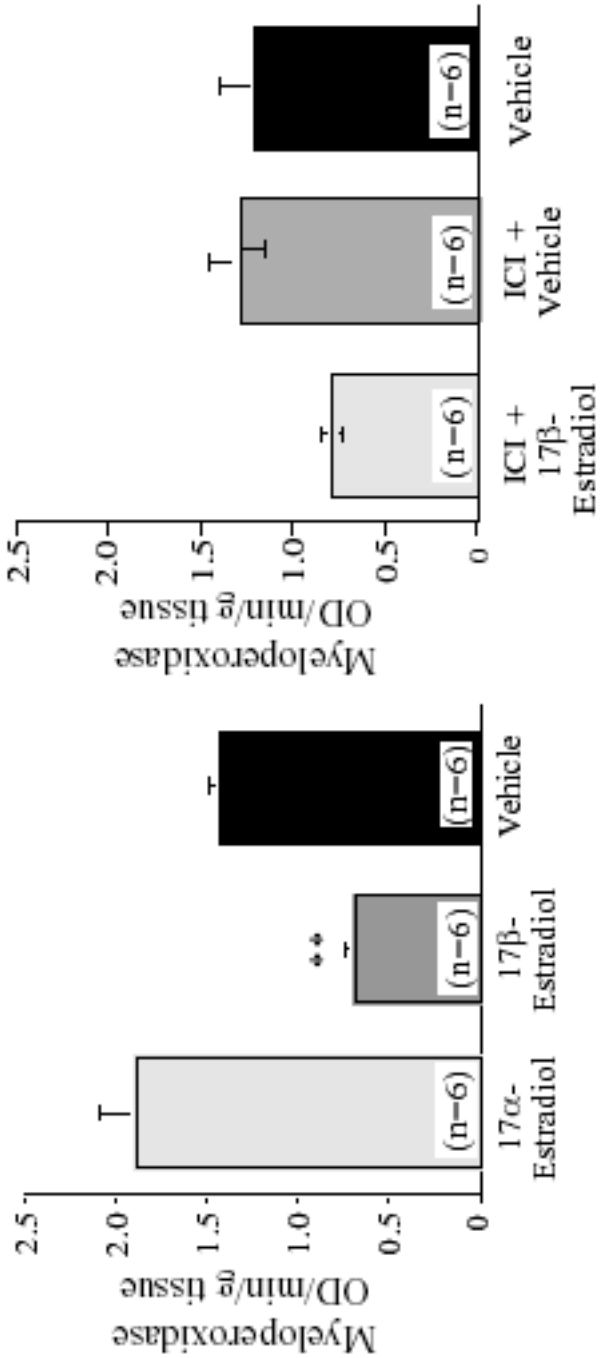
Figure 7



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