Estrogen Reduces Cardiac Injury and Expression of $\beta_1$-Adrenoceptor upon Ischemic Insult in the Rat Heart

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ABSTRACT

To test the hypothesis that estrogen confers cardioprotection by suppressing the expression of $\beta_1$-adrenoceptor ($\beta_1$-AR), we first correlated the infarct size in response to ischemic insult and $\beta_1$-AR stimulation with the expression of $\beta_1$-AR in sham, ovariectomized (Ovx) and estrogen replaced (Ovx + E$_2$) rats. When $\beta_1$-AR is being activated during ischemia, the infarct size was significantly greater in Ovx than in the sham and Ovx + E$_2$ rats. There is a negative correlation between the infarct size and the expression level of $\beta_1$-AR as revealed by Western blotting and supported by binding analysis. Incubation of ventricular myocytes from Ovx rats with estrogen at $10^{-8}$ M for 24 and 48 h, but not 12 h, significantly reduced lactate dehydrogenase release when the myocytes are subjected to simulated ischemia. The cardioprotective effect of 24 h estrogen incubation was accompanied by a reduction in the protein expression level of $\beta_1$-AR, which is estrogen receptor-dependent, whereas the lack of protection of 12-h estrogen incubation was not accompanied by any alterations in the expression level of $\beta_1$-AR. Together, the result from present study suggested that it is most likely that the cardioprotective effect of long-term estrogen replacement is due to suppressing the enhanced expression of cardiac $\beta_1$-AR in the Ovx rats, which in turn reduces cardiac injury when $\beta_1$-AR is activated by sympathetic hyperactivity during ischemia. Therefore, suppression of the enhanced expression of cardiac $\beta_1$-AR in Ovx rats represents a novel cardioprotective mechanism of estrogen replacement therapy.

The sympathetic nervous system is one of the most important extrinsic mechanisms regulating the cardiac function through $\beta_1$-adrenergic receptor ($\beta_1$-AR) stimulation. The $\beta_1$-AR subtype 1 ($\beta_1$-AR) is the predominant subtype in the heart known to cause an increase in heart rate and contractility when stimulated (Sperelakis and Wahler, 1988; Schomig and Richardt, 1990). Stimulation of cardiac $\beta_1$-AR activates adenylyl cyclase via Gs protein leading to an increase in cAMP accumulation, which in turn increases Ca$^{2+}$ influx via the L-type Ca$^{2+}$ channel (Sperelakis and Wahler, 1988; Schomig and Richardt, 1990). The resultant cardiac responses are increase in heart rate and contractility. Sympathetic hyperactivity during acute myocardial ischemia may lead to abnormal cardiac functions such as malignant arrhythmias and infarction (Dart et al., 1984; Tsien et al., 1986; Catelli et al., 2003).

It is well established that women have a lower incidence of ischemic heart diseases before menopause but lose this advantage with the onset of menopause, indicating that the female sex hormone, particularly estrogen, plays a pivotal role in reducing the risk for ischemic heart diseases (Sullivan et al., 1998; Grodstein and Stampfer, 1995). Besides affecting systemic factors such as altering the ratio of low-density to high density lipoprotein and improving glucose metabolism (Furman et al., 1958; Oliver and Boyd, 1959), estrogen has been shown to reduce both myocardial infarct size and ventricular arrhythmias induced by ischemia/reperfusion without changing hemodynamic parameters, suggesting that estrogen might directly act on the heart (Hale et al., 1997). A recent study showed that the expression of $\beta_1$-AR is up-regulated in the heart of ovariectomized rat, an effect rectified by estrogen replacement (Thawornkaiwong et al., 2003). The finding implies that estrogen may affect cardiac responses to sympathetic stimulation by altering the expression of the $\beta_1$-AR in the heart. In support of this, acute administration of 17$\beta$-estradiol at physiological concentration of $10^{-9}$ M, which itself had no effect, inhibits the enhanced Ca$^{2+}$ influx via the L-type Ca$^{2+}$ channel by $\beta_1$-AR stimulation in the heart (Meyer et al., 1998; Li et al., 2000). This result indicates that estrogen inhibits the action of $\beta_1$-AR stimulation. We therefore hypothesize that estrogen at physiological concentration may confer cardioprotection against ischemic insult by inhibiting the effect of $\beta_1$-AR stimulation.

ABBREVIATIONS: $\beta_1$-AR, $\beta_1$-adrenoceptor; Ovx, ovariectomy; Ovx + E$_2$, ovariectomy with estrogen replacement; LDH, lactate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction.
during ischemia. In the present study, we first correlated the \( \beta_1 \)-AR expression and effects of isoproterenol stimulation on cardiac injury and cellular cAMP content (in one experiment) during ischemia in the isolated heart preparation from female, ovariectomized, and estrogen-replaced rats. We further determined the effect of 17\( \beta \)-estradiol incubation for different periods on \( \beta_1 \)-AR expression in ventricular myocytes from ovariectomized rats and cardiac injury in response to \( \beta \)-AR stimulation during ischemia. Results suggested that estrogen confers cardioprotection via, at least partly, suppressing the enhanced expression of \( \beta_1 \)-AR in the ovariectomized rat heart. The action of the female sex hormone is at the myocardiwm and estrogen receptor-mediated.

**Materials and Methods**

**Experimental Animals.** The study was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Female Sprague-Dawley rats weighting 190 to 210 g were purchased from Charles River Breeding Laboratories (Wilmington, MA) and randomly divided in two groups. One group served as normal control. The other group underwent bilateral ovariectomy and divided into two subgroups. One week after ovariectomy (Ovx), one subgroup was implanted with subcutaneous 60-day release pellets containing 1.5 mg of 17\( \beta \)-estradiol (Innovative Research of America, Toledo, OH) (Ovx + E\(_2\)). The sustained releasing pellets were designed to maintain estrogen concentration within physiological range. Ovariectomy and implantation of pellets was performed under intraperitoneal anesthesia using sodium pentobarbitol (Abbott Diagnostics, Abbott Park, IL).

**Experimental Protocol.** Three series of experiments were performed. The first (Fig. 1) series of experiments was designed to study the impact of different estrogenic status on infarct size when the heart was subjected to \( \beta \)-AR stimulation during ischemia. Three types of rats were used: sham-operated female, Ovx, and Ovx + E\(_2\). The rats were killed, and hearts were removed and mounted to the Langendorff apparatus. The hearts were subjected to regional myocardial ischemia for 30 min. One group was perfused with Krebs-Ringer solution throughout the whole experiment (Fig. 1A). In another group, the hearts were perfused with 10\(^{-5}\) M isoproterenol, a selective \( \beta \)-AR agonist during the ischemic period (Fig. 1B). Then, the hearts were reperfused with normal Krebs-Ringer solution for another 120 min. We also determined the effect of acute estrogen treatment on infarct size when the hearts of female and Ovx rats were subjected to \( \beta \)-AR stimulation during ischemia. The isolated perfused rat hearts were prepared as described above. The hearts were perfused with Krebs-Ringer solution containing 10\(^{-9}\) M 17\( \beta \)-estradiol for 20 min and isoproterenol (10\(^{-7}\) M) was added to the same solution 10 min before the onset of ischemia. During ischemia, the hearts were perfused with Krebs-Ringer solution containing both 17\( \beta \)-estradiol and isoproterenol. Then, the hearts were reperfused with normal Krebs-Ringer solution for another 120 min. In the second series of experiments, both the mRNA and protein expression level of \( \beta_1 \)-AR as well as cellular cAMP content was determined. The cAMP level was determined to confirm cardiac response to \( \beta \)-AR stimulation. The third series of experiment was first to determine whether estrogen acted directly on the heart, second to determine the duration of estrogen exposure required for cardioprotection, and third to correlate the expression of \( \beta_1 \)-AR with myocardial injury. Isolated myocytes from Ovx rats were exposed to 17\( \beta \)-estradiol at 10\(^{-9}\) M for 12, 24, and 48 h. At the end of experiment, lactate dehydrogenase (LDH) level in the culture medium and protein expression of \( \beta_1 \)-AR were determined. The effect of estrogen was also determined with the presence of an estrogen receptor antagonist \( \alpha \)-9(4,4,5,5,5-pentafluoropentylsulfinylmethyl)loestra-1,3,5(10)-triene-3,17\( \beta \)-diol (ICI182,780).

**Langendorff-Perfused Isolated Rat Heart Preparation.** Five weeks after ovariectomy, rats were decapitated with a guillotine. The hearts were removed immediately and mounted on a Langendorff apparatus. The hearts were perfused retrogradely with Krebs-Ringer solution equilibrated with 95% O\(_2\) + 5% CO\(_2\) at a constant pressure of 80 cm of H\(_2\)O and a flow rate of 10 ml min\(^{-1}\). The temperature of the perfusing solution was maintained at 37\(^\circ\)C.

**Measurement of Ischemic Risk Zone and Infarct Size.** The ischemic risk zone and the infarct size were determined as described previously (Wang et al., 2001). Briefly, a fine silk thread was passed below the left anterior descending coronary artery. The ends of the thread were passed through a propylene tube to form a snare. Pulling of the ends of the thread produced ischemia, whereas release of the occlusion produced reperfusion. The former was confirmed by pericardial cyanosis, whereas the latter by pericardial hyperemia. The hearts were allowed to stabilize for 10 min. Any hearts exhibited arrhythmias during this period were discarded.

**Cardiac Membrane Preparation.** Cardiac membrane was prepared from the left ventricle of the heart as described previously (Yu et al., 2001). The pellet was dispersed with a Polytron in 400 μl of the original buffer. The resuspended pellets were stored at –80\(^\circ\)C until use. Protein concentration was determined as described previously (Lowry et al., 1951).

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**Fig. 1.** The effect of hormonal environment on infarct size in response to \( \beta \)-AR stimulation during ischemia in female, ovariectomized, and estrogen-replaced rats. Infarct size is expressed as percentage of ischemic risk area. A, without isoproterenol stimulation. B, with isoproterenol stimulation. Data are represented as mean ± S.E.M of eight hearts in each group. *, \( P < 0.05 \) versus female. #, \( P < 0.05 \) versus Ovx.
Western Blotting Analysis. Western blotting analysis was carried out as described previously (Thawornkaiwong et al., 2003). Briefly, 60 μg of protein was diluted in loading buffer and denatured for 5 min. Protein samples were separated by 10% polyacrylamide gel electrophoresis in the presence of SDS-polyacrylamide gel electrophoresis and then electrochemically transferred onto polyvinylidene difluoride membrane at 100 V for 1.5 h. The membrane was then incubated with polyclonal antibodies against β1-AR (diluted 1:500) overnight at 4°C. Then, there were three 10-min washes in Tris-buffered saline/Tween 20 solution. The horseradish peroxidase-linked anti-rabbit IgG with a dilution of 1:1000 was used as secondary antibody and incubated for 1 h at room temperature followed by three 10-min washes in Tris-buffered saline/Tween 20 solution. Protein bands were detected using enhanced chemiluminescence detection system and visualized by autoradiography. Densitometric analysis was conducted using Syngene CCD BIO acquisition and analysis software (Hitachi Genetics System, Alameda, CA).

β-AR Binding Assay. The binding assay for the β-AR was performed as described previously (Cervoni et al., 1981). One hundred micrograms of ventricular sarcolemmal membrane protein in a total volume of 400 μl of binding buffer containing 50 mM HEPES, pH 8.0, 4 mM MgCl2 was incubated with various concentrations of [3H]dihydroalprenolol and enhanced chemiluminescence detection kit (TRK 432; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Nonspecific binding was evaluated in parallel set of experiments with the addition of 100 μM alprenolol. The binding parameters, including receptor density (Bmax) and dissociation constant (Kd), were determined by nonlinear regression using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

RT-PCR Analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 50 mg of left ventricular tissue was homogenized in 1 ml of TRIzol reagent using a Polytron homogenizer. The resultant RNA pellet was dried at room temperature for 15 min and resuspended in 50 μl of diethyl pyrocarbonate-treated water.

Two micrograms of total RNA was converted into cDNA using Superscript II RNase−H− reverse transcriptase and oligo(dT)12−18 primer (Invitrogen). Then, 2.5% of the first-strand reaction cDNA was subsequently amplified by PCR in 25 μl of 1× PCR buffer containing 200 μM dNTPs, 1.5 mM MgCl2, and 1 μM primer specific for the β1-AR (forward primer, CTG CTA CAA CGA CCC CAA GT; reverse primer, CCC AGC CAG TTG AAG AAC AG) and 1 U of AmpliTaq Gold polymerase. Conditions for amplification were as follows: 95°C for 7 min (heat activating the Taq polymerase) followed by 25 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 60 s. A final extension at 72°C for 8 min was included in all instances. Control amplifications were performed with GAPDH to confirm the efficiency of PCR amplification in each reaction and to allow semiquantification of the PCR products. Parallel samples with no reverse transcriptase served as negative control. Amplification products were electrophoresed on 1% (w/v) agarose gels and visualized by ethidium bromide staining. PCR products were quantified using Syngene CCD BIO acquisition and analysis software (Hitachi Genetics System).

Isolation and Culture of Rat Ventricular Myocytes. Cardiac ventricular myocytes were isolated from the hearts of Ovx rats, using a collagenase perfusion method as described previously (Yu et al., 1999). After isolation, they were allowed to stabilize for at least 30 min before experiments. The cells were preplated on 100-mm culture dishes for 30 min. The suspending cells were then resuspended in 5 ml of minimal essential medium containing 1.25 mM Ca2+, 5% fetal bovine serum, 5 μg/ml insulin, 5 μg/ml apo-transferrin, 100 U/ml penicillin G, and 100 μg/ml streptomycin and seated at a density of 3 × 105 cells/well on standard six-well plates and incubated with/without water-soluble 17β-estradiol for 12, 24, and 48 h. Then, cardiac membrane was prepared and Western blotting analysis was carried out to determine the expression of β1-AR. Because it took 24 h for the estrogen to suppress the expression of β1-AR (see Results), the cellular injury in response to β1-AR stimulation during ischemia was correlated with β1-AR expression in myocytes incubated with estrogen for 24 h. To induce ischemia, the culture medium was removed and replaced with a modified glucose-free Krebs-Ringer solution supplemented with 16 mM K+, 10 mM 2-deoxy-D-glucose, and 0.75 mM sodium hydrosulfite. The pH of the ischemic solution was adjusted to 6.5. The cells were then incubated for 2 h in the ischemic solution with or without 10−7 M of isoproterenol at 37°C in CO2 incubators and then transferred back to normal culture medium for another 2 h.

LDH Assay. The LDH activity in the culture medium was determined at the end of ischemia/reperfusion as an indication of cellular injury. After completion of ischemia/reperfusion, the ischemic buffer in each well of the culture dish was assayed for LDH as described previously (Zhou et al., 2001). Total LDH was considered as the sum of the LDH released into the media during ischemia/reperfusion plus the residual LDH present in the cells. The LDH release was presented as the amount released into the media over the total LDH (released plus cellular content). All samples were assayed in triplicate.

cAMP Assay. The method for determination of cAMP content was performed as described previously (Bian et al., 2000). Briefly, 3 × 105 myocytes isolated were placed in each well. Then, 10−7 M isoproterenol was added to the cells and incubated for 10 min at 37°C. At the end of the treatment, cAMP was extracted and stored at −20°C for subsequent determination of cAMP. The protein content of the samples was determined as described previously (Lowry et al., 1951). The intracellular cAMP content was measured with a cAMP assay kit (TRK 432; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK).

Serum Estrogen Level. Rat serum E2 levels were measured using solid phase 125I radioimmunoassay technique (Diagnostic Research Laboratory) according to the manufacturer’s instructions. Blood samples were collected from the rats after decapitation. In addition to the serum estrogen level, body weight was also monitored as a noninvasive physiological measure of estrogen depletion after ovariectomy.

Drugs and Chemicals. Alprenolol, 17β-estradiol, water-soluble 17β-estradiol, isoproterenol, and type-1 collagenase were purchased from Sigma-Aldrich (St. Louis, MO). ICI182,780 was purchased from Toscris Cookson Inc. (Ballwin, MO). Specific β1-adrenergic antibody and horseradish peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [3H]Dihydroalprenolol and enhanced chemiluminescence detection kit was purchased from Amersham Biosciences UK, Ltd. TRIzol reagent was purchased from Invitrogen. Superscript II RNase−H− reverse transcriptase was purchased from Invitrogen. All drugs were dissolved in double distilled H2O or Krebs solution except 17β-estradiol, which was dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was ≤0.01%, which itself had no effect on the heart.

Isoproterenol (10−7 M) was chosen based on a previous study (Yu et al., 1999) that showed that at this concentration, isoproterenol significantly increased the heart rate and contractility and the effects were abolished by 10−6 M propranolol, a selective β-AR antagonist.

Statistical Analysis. Data were expressed as mean ± S.E.M. Two-way analysis of variance with post hoc test was used to determine the differences among multiple groups. For analysis of drug
effects, the nonparametric Kruskal-Wallis test was used. $P < 0.05$ was considered statistically significant.

**Results**

General Features of Experimental Animals. Five weeks after ovariectomy, the female rats exhibit a significant reduction in serum estrogen level accompanied by a significant increase in body weight. The heart/body weight was also significantly reduced in Ovx rats. These ovariectomy-induced alterations were reversed in estrogen-treated animals (Table 1).

**Effect of β-AR Stimulation and Regional Ischemia on Infarct Size in Isolated Rat Hearts.** To mimic the in vivo situation when there is sympathetic overactivity during ischemia, $10^{-7}$ M isoproterenol was administered to the isolated perfused rat heart subjected to regional ischemia. Left coronary artery occlusion produced an ischemic risk zone of $48 \pm 4$, $49 \pm 5$, and $46 \pm 3$% of the ventricular volume in sham, Ovx, and Ovx + E$_2$ rats, respectively. There was no significant difference in infarct size among the three groups of rats (Fig. 1A). When isoproterenol was administered during the ischemic period, the infarct size increased significantly in all three groups of rats (Fig. 1B). The infarct size was significantly greater in Ovx rats than in female rats ($57.3 \pm 3.2$ versus $42.5 \pm 3.5$) and physiological estrogen replacement after ovariectomy restored the infarct size to a level comparable with the female counterparts ($45.2 \pm 2.6$ versus $42.5 \pm 3.5$). These results suggested that the estrogen level of the animal is an important factor affecting the cardiac injury in response to β-AR stimulation during myocardial ischemia.

Effect of Acute Administration of 17β-Estradiol on Infarct Size Induced by β-AR Stimulation and Regional Ischemia in Isolated Perfused Hearts of Sham and Ovx Rats. Because acute estrogen treatment has been shown to inhibit β-AR stimulation in the heart, we determined whether this would lead to reduction in infarct size in sham and Ovx rats. As shown in Fig. 2, pretreatment of the hearts with $10^{-9}$ M 17β-estradiol before the addition of isoproterenol did not reduce the deleterious effect of β-AR activation during ischemia. There is no significant difference in infarct size between groups with or without estrogen pretreatment in both female ($45.3 \pm 6.5$ versus $42.3 \pm 3.5$) and Ovx rats ($52.2 \pm 4.2$ versus $57.3 \pm 3.2$) (Fig. 2). These results indicated that acute estrogen treatment did not affect the exaggerated cardiac injury in response to β-AR stimulation during ischemia.

**Effect of β-AR Stimulation on cAMP Content in Left Ventricular Myocytes of Female, Ovx, and Ovx + E$_2$ Rats.** Because β-AR activation stimulate adenylyl cyclase, the cAMP content in response to isoproterenol stimulation was studied. In all three groups of rats, isoproterenol significantly increased cellular cAMP content (Fig. 3). However, the isoproterenol-induced change in cAMP content was significantly greater in the Ovx group compared with the female group. In Ovx + E$_2$ rats, the cAMP content induced by isoproterenol was significantly less than that in the ovariectomized groups. The result indicated that estrogenic level of the animal affect the cardiac response to β-AR stimulation in terms of cAMP accumulation.

**Effect of Ovariectomy and Estrogen Replacement on β$_1$-AR Expression.** To determine the β$_1$-AR expression, we measured the mRNA and protein contents of β$_1$-AR by Western blotting and performed direct binding assay in the left ventricular tissue of the three groups of rats. As shown in
Fig. 4. The mRNA (Fig. 4A) and the protein (Fig. 4B) levels of β1-AR were up-regulated in Ovx rats and suppressed in Ovx + E2 rats to a level comparable with that of normal female rats. In agreement with the Western blotting result, the $B_{\text{max}}$ of β1-AR was significantly higher in the Ovx than that of the sham and Ovx + E2 rats (Table 2). There was no difference in $K_d$ values among three groups of rats (Table 2). The results showed that estrogen deficiency induced a significant change in the density, but not the affinity, of the β1-AR in the heart. The result suggested a suppressive action of estrogen on β1-AR expression in the heart as revealed by Western blotting analysis. This observation was further supported by a reduction in the β1-AR density as shown by radioligand binding study.

To further determine whether the increase in β1-AR expression in Ovx rat hearts was a result of direct effect of estrogen on the myocardium, ventricular myocytes from Ovx rats were incubated with estrogen (10^{-9} M)-supplemented medium for 12, 24, and 48 h. Incubation of ventricular myocytes with 10^{-9} M 17β-estradiol for 24 and 48 h, but not 12 h, significantly reduced the protein expression of β1-AR (Fig. 5). The effect of estrogen was abolished by coincubation with 10^{-8} M ICI 182,780, a high-affinity estrogen receptor antagonist. These data indicate that the action of estrogen takes place at the myocardium and is estrogen receptor-mediated.

TABLE 2

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<tr>
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<th>$K_d$</th>
<th>$B_{\text{max}}$</th>
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<tr>
<td></td>
<td>nM</td>
<td>fmol/mg protein</td>
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<tr>
<td>Female</td>
<td>5.2 ± 1.30</td>
<td>171.6 ± 10.1*</td>
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<tr>
<td>Ovx</td>
<td>5.6 ± 0.98</td>
<td>128 ± 7.4</td>
</tr>
<tr>
<td>Ovx + E2</td>
<td>4.8 ± 1.14</td>
<td>128 ± 7.4</td>
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*P < 0.05 vs. female.
Effect of β-AR Stimulation during Simulated Ischemia in Isolated Ventricular Myocytes Incubated with 17β-Estradiol. To examine whether estrogen confers cardioprotection through modulation of the expression level of β1-AR in the heart, we compared the injuries of isolated ventricular myocytes from Ovx rat incubated with vehicle or 10⁻³ M 17β-estradiol for 12 and 24 h in response to β-AR stimulation during simulated ischemia. As shown in Fig. 6A, addition of 10⁻⁷ M isoproterenol in the lethal simulated ischemia solution significantly increased the LDH release in all groups compared with corresponding controls without isoproterenol, indicating an exaggerated cellular injury in response to β-AR stimulation during ischemia. The effect of isoproterenol on cellular injury was antagonized by 10⁻⁶ M propranolol, indicating that the effect is β-AR-dependent. More importantly, as shown in Fig. 6B, the percentage of increase in LDH release was significantly lower in cells treated with estrogen for 24 h when the β₁-AR expression was significantly reduced (Fig. 5), an effect antagonized by ICI182,780. Furthermore, the increased release of LDH in response to β-AR stimulation was similar between the vehicle and the group incubated with 10⁻⁹ M 17β-estradiol for 12 h (Fig. 6B) where there was no change in the β₁-AR expression (Fig. 5). The observation suggested that the protective effect of estrogen is most likely a result of its suppressive action on β₁-AR expression.

Discussion

The present study has provided evidence that the estrogenic environment, not acute administration of estrogen, is important for cardioprotection. First, acute administration of 10⁻⁹ M 17β-estradiol to isolated hearts of female and Ovx rats did not confer cardioprotection. Second, female rats with intact ovaries and Ovx rats with estrogen replacement had significantly smaller infarct size than Ovx rats in response to β-AR stimulation during ischemia in the isolated perfused heart preparation. Third, incubation of ventricular myocytes from Ovx rats with estrogen for 24 h, but not 12 h, reduced cardiac injury induced by ischemia and β-AR stimulation. Furthermore, the effect of estrogen is mediated by the estrogen receptor at the level of myocardium as a selective estrogen receptor antagonist abolished its effect in isolated ventricular myocytes.

A more interesting finding of the present study is that the cardioprotective effect of female sex hormone is most likely due, at least partly, to its direct suppressive effect on the enhanced β₁-AR expression in the heart of Ovx rats, which in turn reduces the cardiac responses to β-AR stimulation during ischemia. This is based on the following observations. First, myocardial infarct was negatively correlated with β₁-AR expression in female, Ovx, and Ovx + E₂ rats. Second and more importantly, incubation of ventricular myocytes from Ovx rats with 10⁻⁹ M 17β-estradiol for 24 h, which suppressed the β₁-AR expression, was accompanied by cardioprotection against injury induced by β-AR stimulation during ischemia, whereas incubation of ventricular myocytes with 10⁻⁹ M 17β-estradiol for 12 h neither suppressed the β₁-AR expression nor did it confer cardioprotection. However, there exists the possibility that estrogen might produce cardioprotection through mechanisms unrelated to β-AR after the 24-h incubation period (Jovanovic et al., 2000).

Isoproterenol at 10⁻⁴ M or higher has been shown to produce cardiotoxicity in cardiomyocytes by generating free radicals, which is independent of β-AR (Ramos et al., 1983; Ramos and Acosta, 1983). However, our result indicated that propranolol, a specific β-AR antagonist, blocks the cardiotoxicity effect of isoproterenol, indicating that at 10⁻⁷ M the deleterious effect of isoproterenol on LDH release is β-AR-dependent. In the present study, we focused on β₁-AR because its role in the regulation of cardiac function has been well established. However, there is evidence that in humans, β₂-AR also mediates contractile response to β-AR agonists, suggesting it may also play an important role in the regulation of cardiac function (Zerkowski et al., 1986). Further studies are needed to delineate the role of this receptor subtype in cardioprotection of estrogen.

In the present study, we found that there was no significant difference in infarct size among female, Ovx, and Ovx +
E$_2$ rats without $\beta$-AR stimulation during ischemia. This is in agreement with a recent study by McNulty et al. (2000), who showed that estrogen withdrawal by ovariectomy for a 6- to 8-week period did not increase the myocardial infarct size of the isolated rat heart compared with normal and estrogen-replaced rats. On the other hand, the present study has shown that myocardial infarct size of female rats was smaller in response to $\beta$-AR stimulation during ischemia than that in Ovx rats and estrogen replacement restored the ability of the Ovx rats to resist ischemic injury. The observation indicates the importance of $\beta$-AR in the pathogenesis of myocardial infarct induced by ischemia/reperfusion.

Interestingly, acute administration of 17$\beta$-estradiol at 10$^{-9}$ M did not alter the infarct size induced by $\beta$-AR stimulation during ischemia in both female and Ovx rats, indicating that a sudden increase in estrogen level is not responsible for the cardioprotective effect. In two previous studies, it was shown that acute administration of 17$\beta$-estradiol at 10$^{-9}$ M inhibited the enhanced Ca$^{2+}$ influx across the L-type Ca$^{2+}$ channel by $\beta$-AR stimulation (Meyer et al., 1998; Li et al., 2000). In spite of this, our previous study showed that acute administration of 17$\beta$-estradiol at 10$^{-9}$ M did not alter the arrhythmic action of $\beta$-AR stimulation during myocardial ischemia (Li et al., 2000). Because arrhythmia and infarction involve a multitude of factors, the effect of acute administration of estrogen at physiological concentrations alone may not be sufficient to reduce arrhythmia or infarction induced by ischemia/reperfusion and $\beta$-AR stimulation.

A previous study showed that both the protein expression and receptor binding sites of the $\beta$-AR were up-regulated in the heart of ovariectomized rats (Thaworkinwong et al., 2003). In the present study, we showed that in addition to protein level and binding sites, the mRNA level was also increased in the heart of ovariectomized rats. Similarly, in the female rat cortex, estradiol treatment for 48 h reduces both the number of binding sites and mRNA level of the $\alpha_2$-AR (Karkanis et al., 1997). Therefore, one common mechanism for estradiol regulation of adrenergic receptor expression is to affect its mRNA production. The mechanism by which estrogen reduces the synthesis of $\beta$-AR is not fully understood. Although there is no evidence that estrogen response elements are located upstream of the $\beta$-AR promoter sequence, these elements can influence gene transcription from distant upstream sites (Seyfred and Gorski, 1990; Gronehmyer, 1991). There are both positive and negative transcriptional regulatory sites in the promoter region of the rat $\beta_1$-AR gene to which both positive- and negative-acting nuclear factors can bind and thereby influence $\beta_1$-AR mRNA transcription (Kirigiti et al., 2000). These nuclear factors are therefore potential targets of estradiol action. Moreover, it has been shown that estrogen and estrogen receptors interacted with other proteins known as coactivators and corepressors to modify gene transcription in cell specific manner (Halachmi et al., 1994). It is possible that estrogen through interaction with other proteins affects the stability of $\beta_1$-AR mRNA in the heart. Further studies are needed.

Estradiol has been shown to suppress the responsiveness to $\beta$-AR stimulation in the hypothalamus of rats (Ungar et al., 1993; Ansonoff and Etgen, 2001) and myometrium of rats (Ruzyczky and DeLoia, 1997; Engstrom et al., 2001). In the hypothalamus, estrogen uncouples the receptor and the G protein (Ungar et al., 1993; Ansonoff and Etgen, 2001), whereas in the myometrium, estrogen increases the $\beta$-AR-inactivating kinase (Ruzyczky and DeLoia, 1997). In addition to decreased $\beta$-AR expression, these may also be mechanisms responsible for decreased responsiveness to $\beta$-AR stimulation.

In conclusion, this study has provided evidence that estrogenic environment is important for cardiac response to ischemia and $\beta$-AR stimulation. Deprivation of estrogen in ovariectomized rat up-regulates $\beta_1$-AR and increases injury in the heart during ischemia when $\beta$-AR is activated by sympathetic nervous system. It is most likely that at physiological concentration estrogen exerts a tonic inhibition on the expression of $\beta_1$-AR of the heart, thus reducing the cardiac responses to $\beta$-AR stimulation during ischemia. So suppression of the enhanced $\beta$-AR expression in Ovx hearts represents a novel cardioprotective mechanism of estrogen replacement therapy.

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