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POST-ISCHEMIC INFUSION OF 17- β -ESTRADIOL PROTECTS MYOCARDIAL FUNCTION AND VIABILITY

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Abstract

Background—Females demonstrate improved cardiac recovery after ischemia-reperfusion (I/R) injury compared to males. Attenuation of myocardial dysfunction with pre-ischemic estradiol suggests that estrogen may be an important mediator of this cardioprotection. However, it remains unclear whether post injury estradiol may have clinical potential in the treatment of acute myocardial infarction. We hypothesize that post-ischemic administration of 17 β -estradiol will decrease myocardial I/R injury, and improve left ventricular cardiac function.

Materials and Methods—Adult male Sprague-Dawley rat hearts (n=20) were isolated, perfused with Krebs-Henseleit solution via Langendorff model, and subjected to 15 min of equilibration, 25 min of warm ischemia, and 40 min reperfusion. Experimental hearts received post-ischemic 17 β -estradiol infusion, 1nM (n=4), 10 nM (n=4), 25 nM (n=4), or 50 nM (n=4), throughout reperfusion. Control hearts (n=4) were infused with perfusate vehicle.

Results—Post-ischemic recovery of left ventricular developed pressure was significantly greater with 1nM (51.6 \pm 7.4%) and 10 nM estradiol (47.7 \pm 8.6%) than with vehicle (37.8 \pm 9.7%) at end reperfusion. There was also greater recovery of the end diastolic pressure with 1 nM (47.8 \pm 4.0 mmHg) and 10 nM estradiol (54.0 \pm 4.0) compared to vehicle (75.3 \pm 7.5). Further, 1nM and 10 nM estrogen preserved coronary flow after ischemia and decreased coronary effluent LDH compared to controls. Estrogen at 25 nM and 50 nM did not provide additional benefit in terms of functional recovery. Estrogen at all concentrations increased ERK phosphorylation.

Conclusions—Post-ischemic infusion of 17 β -estradiol protects myocardial function and viability. The attractive potential for the clinical application of post-ischemic estrogen therapy warrants further study to elucidate the mechanistic pathways and differences between males and females.

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Keywords

sex hormones; estrogen; myocardial infarction; recovery

INTRODUCTION

Cardiovascular disease is the leading cause of mortality in the United States for men and women. Reperfusion therapy, the treatment of choice for myocardial infarction, is accompanied by the deleterious effects of ischemia / reperfusion (I/R) injury (1,2). Gender specific differences have been noted in I/R injury. Clinically, females seem to have relative cardiac protection from acute infarctions compared to males (3). Similarly, in animal studies, proestrus females demonstrate increased myocardial protection from I/R injury (4,5), and improved systolic and diastolic function after ischemia (6) compared to males. Female animals also exhibit decreased left ventricular remodeling after ischemic injury (7,8), possibly due to decreased levels of inflammatory cytokines such as tumor necrosis factor α (TNF), interleukin (IL)-6, and IL-1 β (9-12).

Estrogen is believed to play an important role in female cardioprotection from I/R injury. Chronic administration of estrogen provides protection from I/R injury in isolated hearts undergoing global ischemia and in hearts undergoing *in vivo* left anterior descending coronary artery (LAD) obstruction (13). Acute administration of estrogen before LAD occlusion also decreases cardiac myocyte injury and necrosis (14,15), infarct size (16), and the incidence of ventricular arrhythmia (17). Estrogen may mediate these effects through estrogen receptor-alpha which increases extracellular signal-regulated protein kinase 1/2 (ERK) and decreases c-Jun NH(2)-terminal kinase (JNK) signaling (18). Estrogen receptor-alpha also appears important in decreasing calcium accumulation and increasing nitrite production, thereby minimizing ATP depletion and increasing myocardial perfusion (19). Intracellular estrogen signaling, by way of estrogen receptor alpha, is critical to female resistance to I/R injury.

Post-insult administration of estrogen appears to be cardioprotective in trauma-hemorrhage and sepsis models. Administration of 17 β -estradiol fifteen minutes before the end of resuscitation following trauma-hemorrhage improves left ventricular and hepatocellular functioning (20,21). Similarly, administration of estrogen following induction of sepsis decreases oxidative organ damage, improves liver function, and decreases pro-inflammatory cytokines (22,23). However, it remains unknown whether post ischemic administration of estrogen confers myocardial protection in the isolated heart.

The administration of post-ischemic estrogen to confer cardioprotection has theoretical clinical appeal. We therefore hypothesize that post-ischemic administration of 17 β -estradiol will decrease myocardial I/R damage, and improve left ventricular cardiac function in the isolated heart. The purposes of this study are to 1) assess myocardial function as measured by left ventricular developed pressure (LVDP), end diastolic pressure (EDP), the maximal positive and negative values of the first derivative of pressure (+dP/dt and -dP/dt), and coronary flow in male rats subjected to I/R and treated with 17 β -estradiol; and 2) establish the most effective post-ischemic dose of 17 β -estradiol via a dose-response curve.

MATERIALS AND METHODS

Animals

Age-matched (250–300 g, 9- to 10-week) normal male Sprague–Dawley rats (Harlan, Indianapolis, IN) were fed a standard diet and acclimated in a quiet quarantine room for 1 week before the experiments. The animal protocol was reviewed and approved by the Indiana Animal

Care and Use Committee of Indiana University. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1985).

Experimental groups

All isolated rat hearts were subjected to the same I/R protocol: 15-min equilibration period, 25 min of global index ischemia (37°C), and 40 min of total reperfusion. Male rats were divided into five experimental groups: 1) vehicle infusion (n = 4); 2) 17 β -estradiol infusion, 1 nM (n = 4); 3) 17 β -estradiol infusion, 10 nM (n = 4); 4) 17 β -estradiol infusion, 25 nM (n = 4); and 5) 17 β -estradiol infusion, 50 nM (n = 4). As a reference, the circulating level of estrogen in a proestrus female rat is 0.2 nM. The 17 β -estradiol solution was prepared with water soluble β -Estradiol (Sigma-Aldrich, St. Louis, MO) and Krebs-Henseleit solution. Vehicle and estradiol solutions were infused through a port above the aortic root throughout reperfusion (40 min). The rate of the 17 β -estradiol infusion was titrated to the coronary flow in order to maintain the desired experimental concentration at all times.

Isolated heart preparation (Langendorff)

Rats were anesthetized (sodium pentobarbital, 60 mg/kg intraperitoneal) and heparinized (500 U intraperitoneal), and hearts were rapidly excised via median sternotomy and placed in 4°C Krebs–Henseleit solution. The aorta was cannulated and the heart was perfused (70 mmHg) with oxygenated (95% O₂/5% CO₂) Krebs–Henseleit solution (37 °C). A left atrial resection was performed prior to insertion of a water-filled latex balloon through the left atrium into the left ventricle. The preload volume (balloon volume) was held constant during the entire experiment to allow continuous recording of the LVDP and EDP. The balloon was adjusted to a mean EDP of 5 mmHg (range 4–8 mmHg) during the initial equilibration. Pacing wires were fixed to the right atrium and hearts were paced at approximately 6 Hz, 3 V, 2 ms (350 bpm) throughout perfusion. A three-way stopcock above the aortic root was used to create global ischemia, during which the heart was placed in a 37°C degassed organ bath. Coronary flow was measured by collecting pulmonary artery effluent. Data were continuously recorded using a PowerLab 8 preamplifier/digitizer (AD Instruments Inc., Milford, MA) and an Apple G4 PowerPC computer (Apple Computer Inc., Cupertino, CA). The +dP/dt and -dP/dt were calculated using PowerLab software. After reperfusion, the heart was removed from the apparatus, immediately sectioned, and snap frozen in liquid nitrogen.

Measurement of Myocardial Necrosis

Coronary effluent from various time points was collected. Lactate dehydrogenase (LDH) was determined using a commercially available cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN).

Western blotting

Western blot analysis was performed to measure ERK proteins. Heart tissue was homogenized in cold buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -Glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml Leupeptin, 1 mM PMSF, and centrifuged at 12000 rpm for 5 minutes. The protein extracts (30 μ g/lane) were subjected to electrophoresis on a 12% tris-HCl gel from Bio-Rad and transferred to a nitrocellulose membrane, which was stained by Naphthol Blue-Black to confirm equal protein loading. The membranes were incubated in 5% dry milk for 1 hour and then incubated with the following primary antibodies: ERK antibody, phosphor-ERK (Thr202/Tyr204) antibody (Cell Signaling Technology, Beverly, MA). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody. Detection was performed using supersignal west pico stable peroxide

solution (Pierce, Rockford, IL). Films were scanned using an Epson Perfection 3200 Scanner (Epson America, Long Beach, CA).

Presentation of data and statistical analysis

All reported values are mean \pm S.E.M. Data were compared using two-way analysis of variance (ANOVA) with post-hoc Bonferroni test or Student's *t*-test. A two-tailed probability value of less than 0.05 was considered statistically significant.

RESULTS

Postischemic recovery of LVDP (expressed as percentage of preischemic function) was significantly greater ($p < .05$) in the 1 nM group ($51.6 \pm 7.4\%$) and 10 nM group ($47.7 \pm 8.6\%$) than with vehicle ($37.8 \pm 9.7\%$) at end reperfusion (Fig 1A,B). However, there was no significant difference in postischemic recovery of LVDP in the 25 nM ($37.5 \pm 7.9\%$, Fig. 1C), or 50 nM group ($40.7 \pm 5.2\%$, Fig. 1D) compared to vehicle ($37.8 \pm 9.7\%$). Using the above data, a dose-response curve was generated (Fig. 2).

Left ventricular end diastolic pressure (mmHg) was elevated in response to I/R. However, male rats in the 1 nM (47.8 ± 4.0 mmHg) and 10 nM (54.0 ± 4.0 mmHg) group had significantly decreased EDP after forty minutes of reperfusion compared to those who received vehicle (75.3 ± 7.5 , $p < .001$, Fig. 3A,B). The end reperfusion EDP of rats in the 25 nM and 50 nM groups was 72.8 ± 4.5 (Fig. 3C) and 70.0 ± 1.9 (Fig. 3D) respectively. Estrogen administration at these levels did not result in significantly different EDPs as compared to control. Maximum positive and negative dP/dt were impaired at the beginning of reperfusion. Significant differences were not noted between experimental groups and vehicle. While post-ischemic infusion of estrogen markedly reduces the EDP after I/R injury, it may not significantly improve contractility or compliance.

Coronary flow was markedly decreased in the control (9.5 ± 1.0 to 7.5 ± 0.9 ml/min), 25 nM and 50 nM groups after ischemia, but remained stable in the 1 nM (9.6 ± 0.6 to 10.3 ± 1.0 ml/min) and 10 nM (9.8 ± 0.3 to 9.8 ± 1.1 ml/min) group (Fig. 5B). Therefore, post-ischemic estrogen infusion at a concentration of 1 nM or 10 nM may help to maintain post-ischemic coronary flow, thereby promoting increased functional recovery.

Coronary effluent LDH was markedly increased after ischemia in all groups (Fig. 6A). However, LDH in 10 nM (1.9 ± 0.1 μ U/ml) and 25 nM (2.4 ± 0.4 μ U/ml) groups during early reperfusion were significantly lower than LDH in controls (Fig. 6B).

The myocardial activation of phosphorylated ERK and nonphosphorylated ERK were assessed by Western blot (Figure 7). The phosphorylated forms of ERK were increased in estradiol treated hearts compared to vehicle control hearts. However, no difference was found in the phosphorylated forms of ERK among different estradiol concentrations following I/R.

DISCUSSION

The results of this study indicate that post-ischemic infusion of estrogen at a concentration of 1 nM or 10 nM (1) decreases myocardial injury and necrosis; (2) lowers EDP throughout reperfusion; (3) increases end reperfusion LVDP; and (4) preserves coronary flow. Post ischemic estradiol at all concentrations also increases phosphorylation of ERK. This study demonstrates that post-ischemic administration of estrogen may improve left ventricular functional recovery and viability.

Post ischemic functional recovery was marked by an improvement in the EDP, LVDP, and coronary flow in male rats. Elevations in end diastolic pressure, a marker of diastolic cardiac function, are typically seen with increasing severity of cardiac injury. This elevation is typically viewed as a reduction in the unstressed volume of the ventricle, which may be due to myocardial contracture or edema (24). In this study, EDP was elevated above equilibrium in all groups, indicating cardiac injury with the induced ischemia. However, estrogen at a dose of 1 nM or 10 nM significantly decreased the EDP during reperfusion as compared to control and other estrogen doses, indicating that this dose of estrogen protected hearts from ischemic injury. The protective effects of estrogen administration were also seen in other measures of systolic function. LVDP was improved at 40 minutes of reperfusion, although a significant difference was not observed at other time points during reperfusion. Interestingly, estrogen at all doses did not affect $+dP/dT$ or $-dP/dT$ between experimental groups. This discrepancy in measures of myocardial function suggests that estrogen may mediate its acute positive effects not by increasing contractility, but instead by increasing the end diastolic fluid volume of the heart.

Furthermore, coronary flow (25-27) was substantially decreased with ischemia in other experimental groups, but was maintained in the 1 nM and 10 nM group. This may be due to inhibition of voltage gated or receptor operated calcium channels. Estrogen has also been shown to promote coronary vasodilation (28). It is possible that a low dose of estrogen induces increased coronary vasodilation, improved cardiac perfusion, and overall increased functional recovery. However, it is important to note that other Langendorff models may differ in baseline coronary flow. Several reasons may exist which may account for differences in coronary flow such as Langendorff model (ADI vs others), perfusion pressure (70 vs 95 mmHg), and mode of perfusion (constant flow vs constant pressure). Thus, estradiol's protective ability to preserve coronary flow may be greater or alternatively less depending on the magnitude of baseline coronary flow.

The acute cardioprotective effects of estrogen may occur through several mechanisms. Reactive oxygen species (ROS) are important mediators of myocardial dysfunction from I/R injury (29). Estrogen has been shown to decrease the production of oxygen radicals during I/R injury (30). Recent evidence shows that estrogen activates PI3K/Akt signaling. Activation of this pathway effectively decreases ROS generation, decreases pro-apoptotic p38 MAPK activation, and increases anti-apoptotic p38 β MAPK signaling (31,32). Estrogen also exerts an antioxidant effect by increasing reduced glutathione, a free radical scavenger in the myocardium (33,34). Thus, one mechanism that estrogen may utilize in conferring cardioprotection from I/R injury is the reduction of oxidative damage.

In addition to decreasing oxidative damage, the post-ischemic protective effects of estrogen may be mediated through decreased proinflammatory cytokine signaling. This may be mediated in part by decreased TNF signaling through TNF receptor resistance (6,13). Furthermore estrogen has previously been shown to upregulate the cardioprotective ERK MAPK pathway, while down-regulating JNK signaling. These pathways are also likely affected with post-ischemic administration of estrogen (18). Indeed, in this study, post ischemic estradiol infusion was associated with increased ERK phosphorylation compared to vehicle controls. Therefore, by minimizing proinflammatory cytokine signaling, estrogen effectively decreases cardiac dysfunction after I/R.

Estrogen also provides protection from I/R injury by decreasing apoptosis. Estrogen prevents the loss of *cytochrome c* from myocardial mitochondria, and decreases its accumulation in the cytosol. It reduces caspase-3 activation and decreases DNA strand breaks (35,36). Estrogen also reduces cardiac apoptosis through a reduction in pro-apoptotic nuclear factor- κ B (NF- κ B), and also through increased PI3K/Akt signaling (37,38). Interestingly, post ischemic estrogen compared to vehicle in this study also reduced myocardial necrosis as measured by

coronary effluent LDH. By reducing cardiac cell death, estrogen increases the amount of viable myocardium after I/R injury, thereby facilitating increased cardiac recovery after I/R injury.

Our experiment, performed with leukocyte-free, buffer-perfused hearts demonstrates that post-ischemic administration of estrogen provides effective cardioprotection. Investigations have shown that estrogen modulates immune cells by decreasing systemic IL-6 release, MAPK activation, and Kupffer cell cytokine production (21,39) after trauma-hemorrhage. Thus, in the future, *in vitro* studies on the heart may be helpful to test the possibility of additional cardioprotection with post-ischemic estrogen administration mediated through immune cell modulation.

The results of this study show that a post-ischemic infusion of 17 β -estradiol at a concentration of 1 nM or 10 nM decreases myocardial dysfunction in male rats after I/R injury. It adds support to the use of estrogen as a therapeutic intervention for acute myocardial ischemia. Indeed, post-ischemic administration is even more clinically relevant and has attractive therapeutic potential. Further study in this area is imperative to further delineate the mechanisms of estrogen protection and to study the gender differences associated with post-ischemic estrogen administration.

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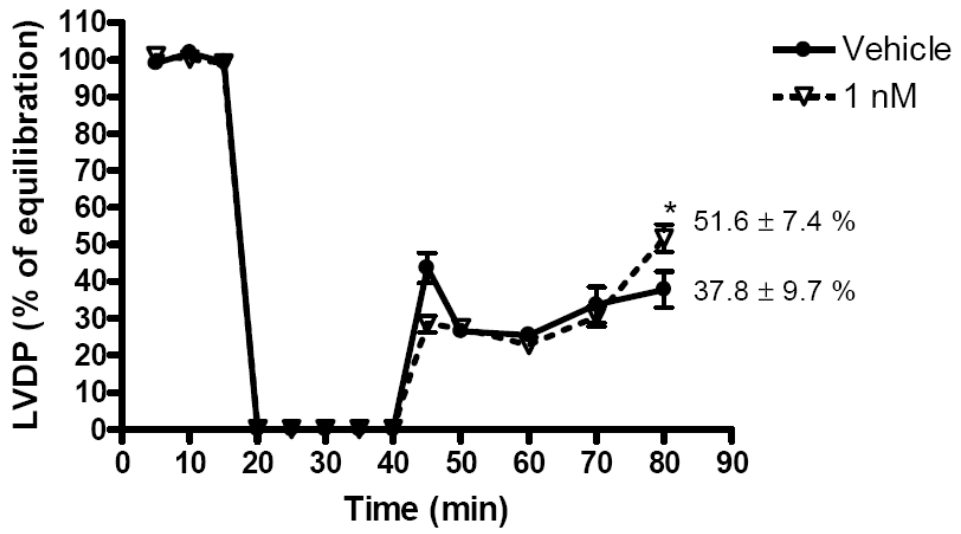
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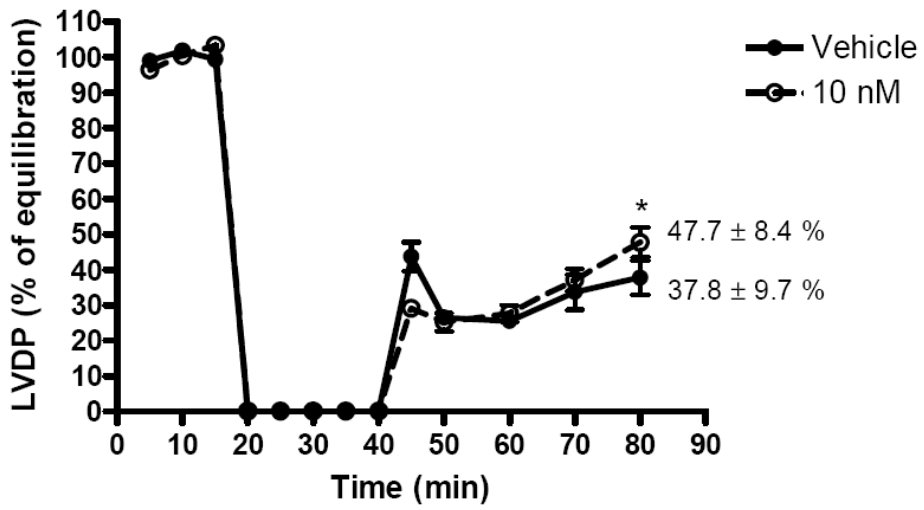
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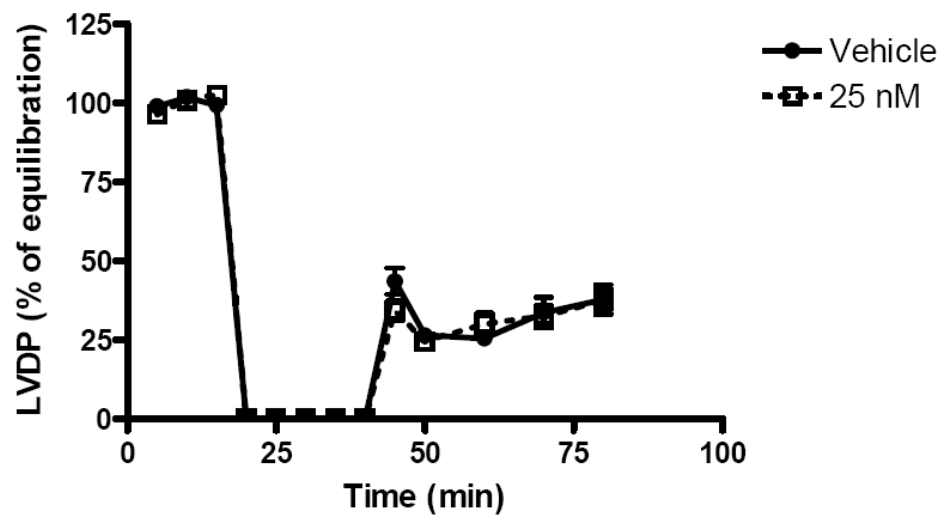
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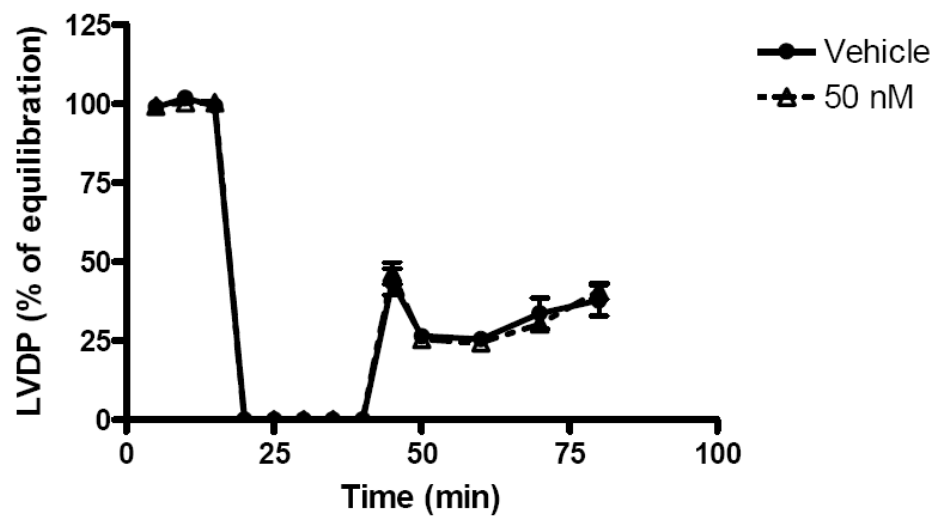
B.



C.



D.

**FIG. 1.**

Changes in left ventricular developed pressure (LVDP % of equilibration) after I/R. (A) 1 nM compared to vehicle. (B) 10 nM compared to vehicle. (C) 25 nM compared to vehicle. (D) 50 nM compared to vehicle. Results are mean \pm SEM, * p <0.05 vs vehicle.

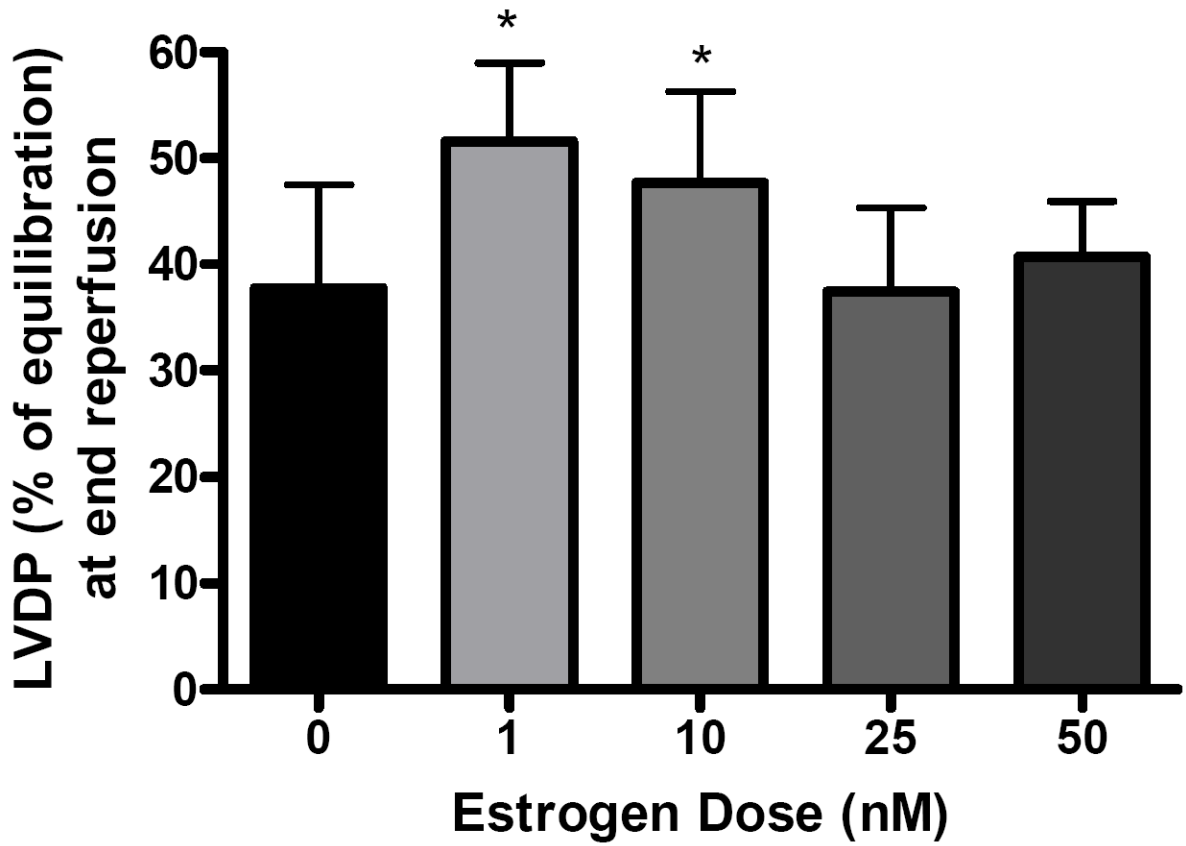
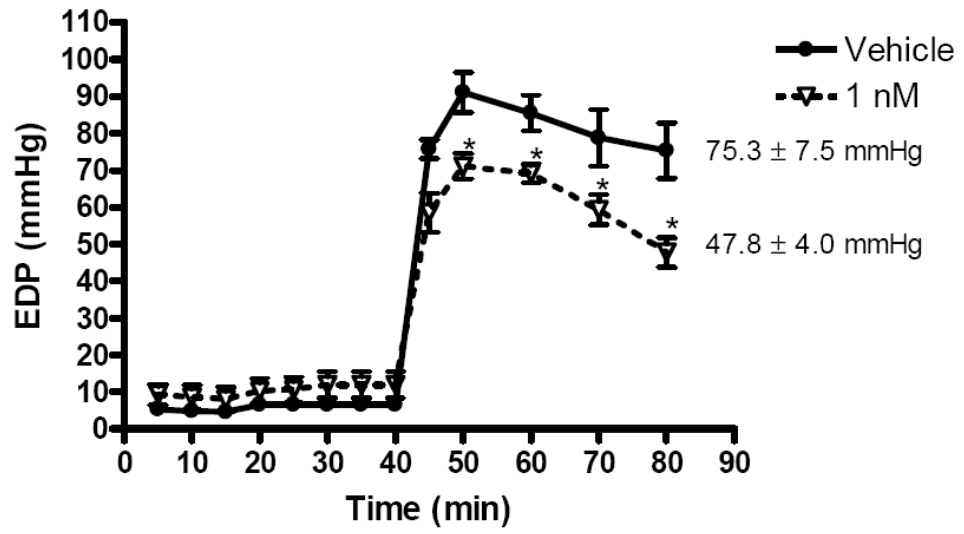
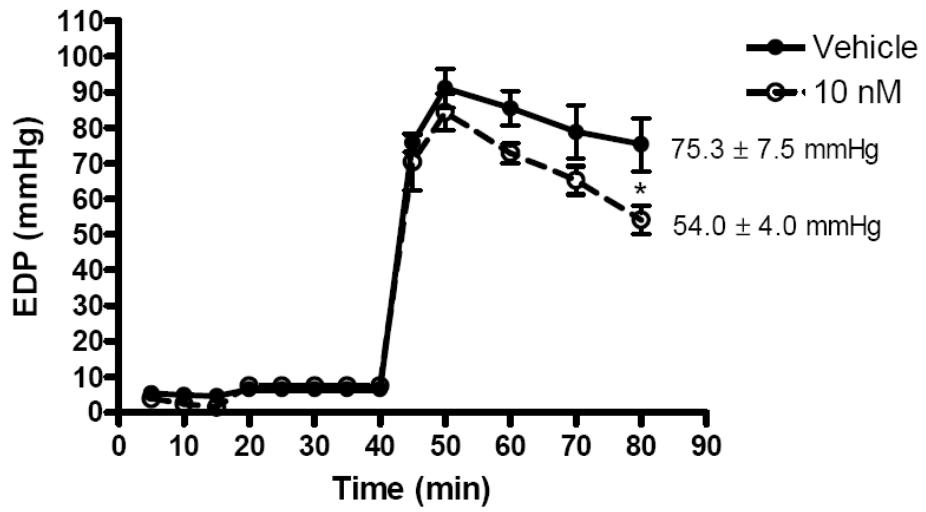


FIG. 2. End reperfusion left ventricular developed pressure (LVDP % of equilibration) versus dose of estrogen infusion at 0 (vehicle), 1, 10, 25, and 50 nM. Results are mean \pm SEM, * p <0.05 vs vehicle.

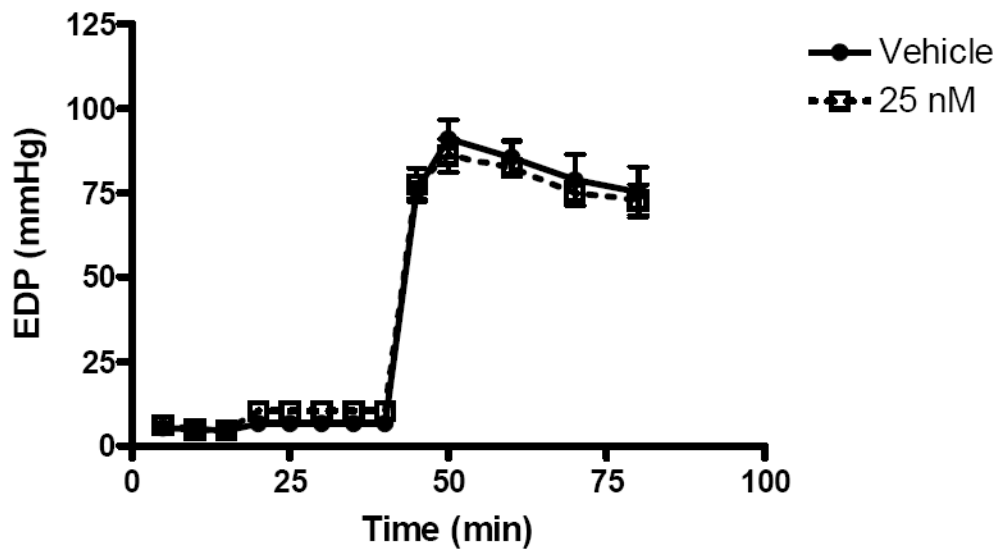
A.



B.



C.



D.

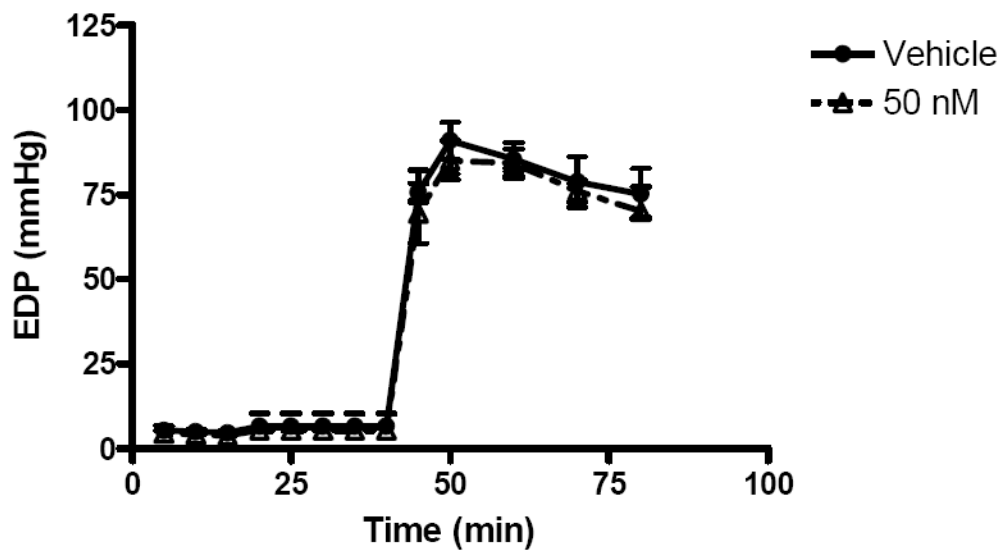


FIG. 3.

Changes in left ventricular end diastolic pressure (EDP) after I/R. (A) 1 nM compared to vehicle. (B) 10 nM compared to vehicle. (C) 25 nM compared to vehicle. (D) 50 nM compared to vehicle. Results are mean \pm SEM, * p <0.05 vs vehicle.

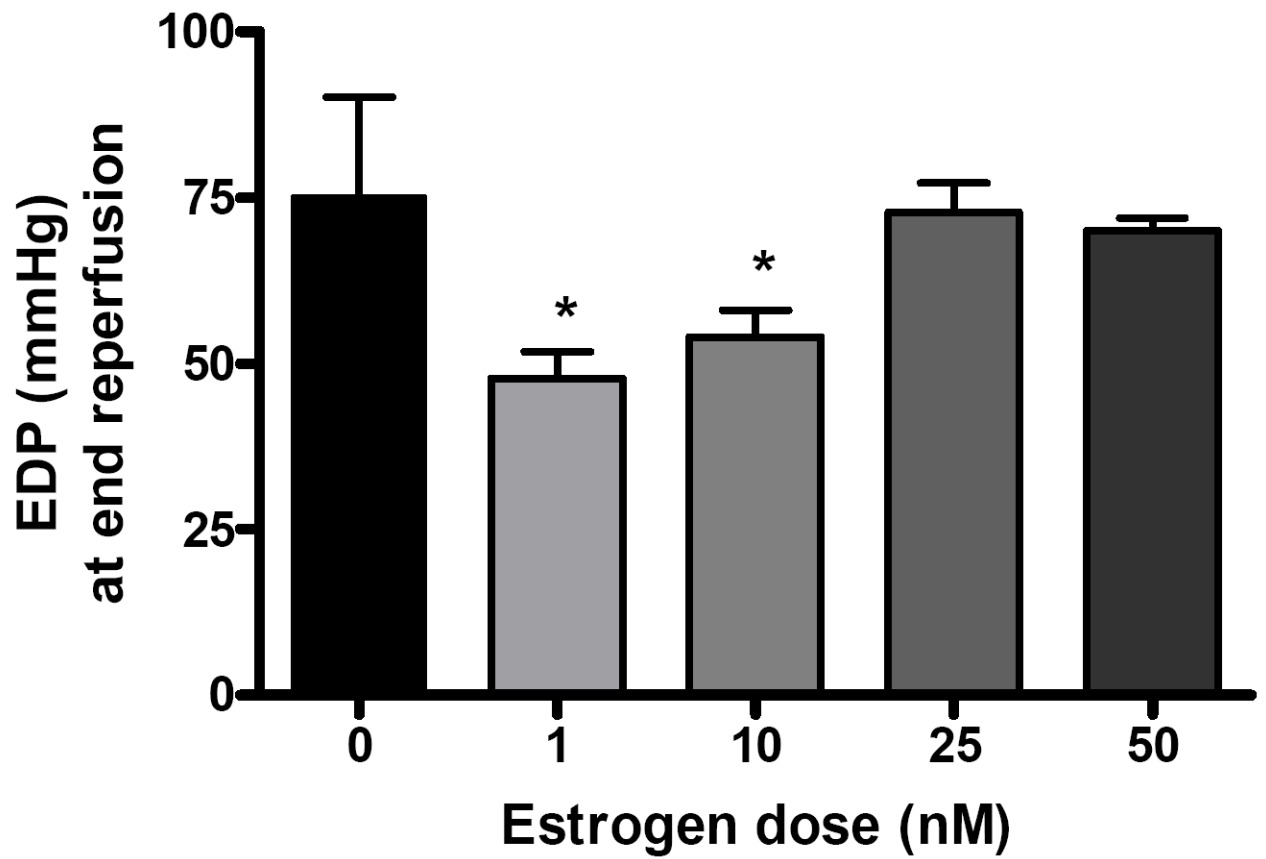
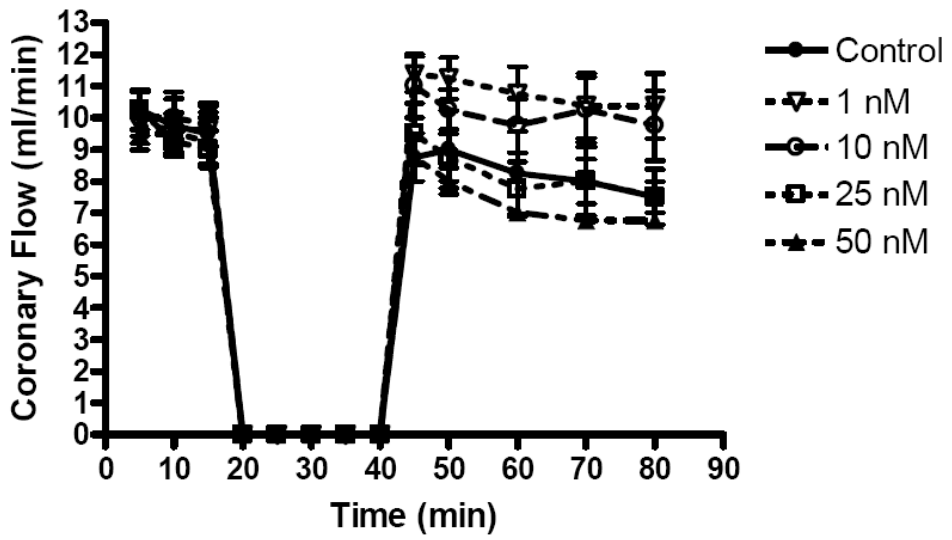


FIG. 4. End reperfusion end diastolic pressure (EDP mmHg) versus dose of estrogen infusion at 0 (vehicle), 1, 10, 25, and 50 nM. Results are mean \pm SEM, * p <0.05 vs vehicle.

A.



B.

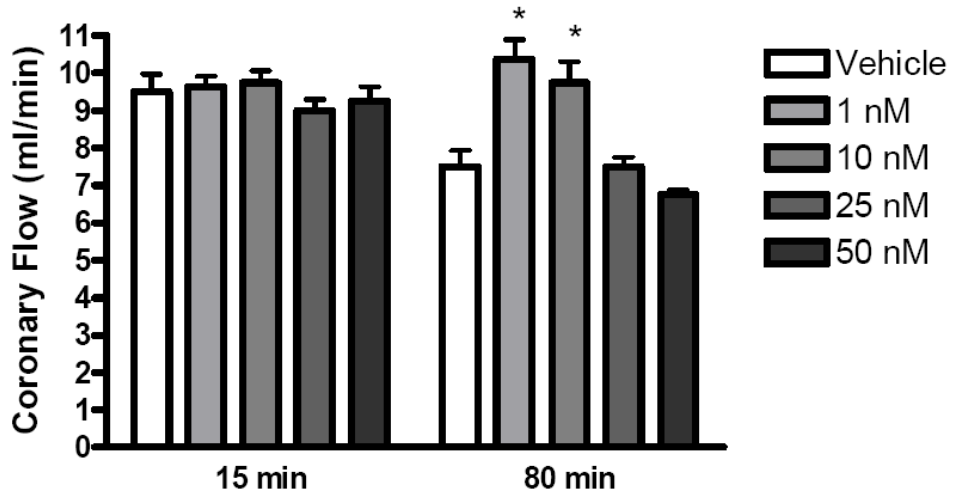
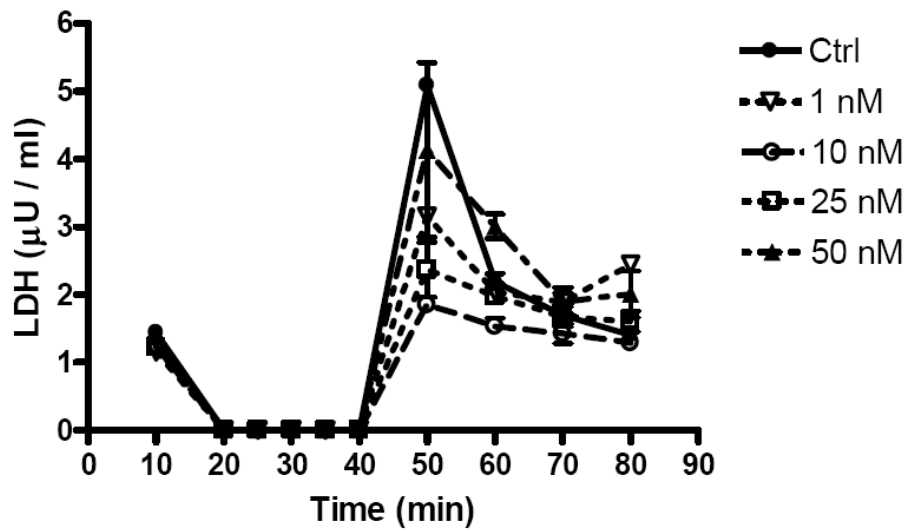


FIG. 5. (A) Changes in coronary flow after I/R. (B) Coronary flow at 15 minutes (after equilibration and before ischemia) and 80 minutes (at the end of reperfusion). 1, 10, 25, and 50 nM groups compared to vehicle. Results are mean \pm SEM, * $p < 0.05$ vs vehicle.

A.



B.

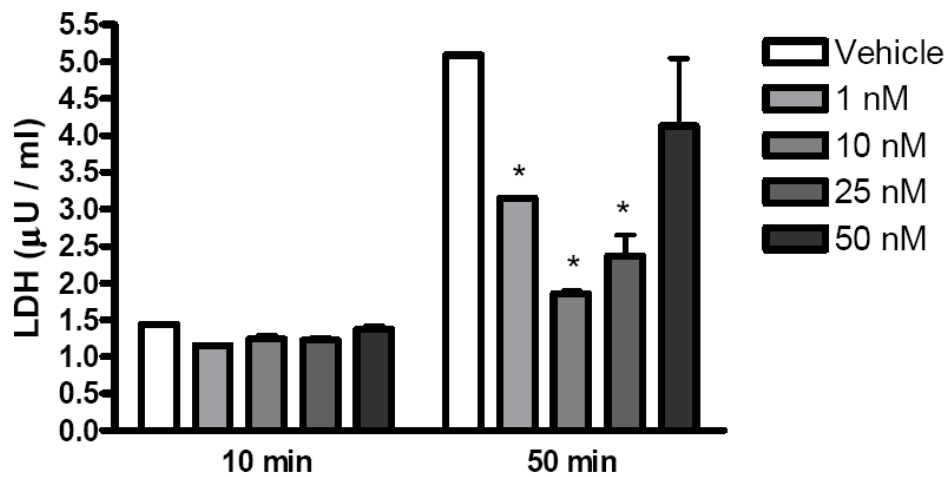


FIG. 6. (A) Changes in coronary effluent LDH after I/R. (B) Coronary effluent LDH at 10 minutes (during equilibration, before ischemia) and 50 minutes (early reperfusion). 1, 10, 25, and 50 nM groups compared to vehicle. Results are mean ± SEM, **p*<0.05 vs vehicle.

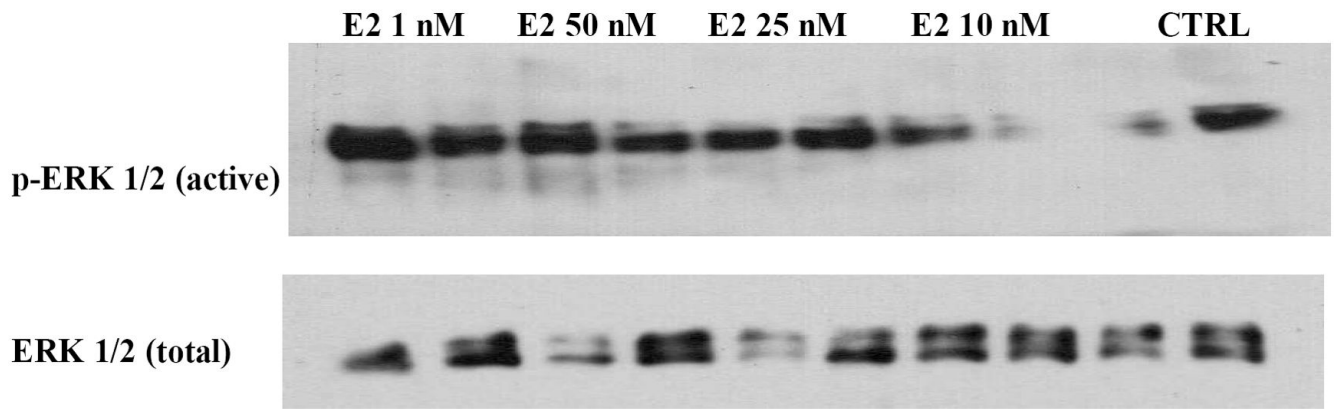


FIG 7. I/R induced myocardial phosphorylation of p44/42 (ERK). The expression of activated ERK after I/R injury in control hearts and hearts exposed to estradiol at various concentrations. Shown are representative immunoblots. Row 1. Phosphorylated (activated) ERK; Row 2. Total ERK. All samples were on the same membrane.