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Acute Post-Ischemic Treatment with Estrogen Receptor-α Agonist or Estrogen Receptor-β Agonist Improves Myocardial Recovery

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Abstract

Background—Female hearts following ischemia/reperfusion (I/R) injury demonstrate improved functional recovery compared to male, which suggests a protective role for estrogen. Acute post-ischemic treatment with 17-β-estradiol (E2) attenuates myocardial dysfunction. However, it is unknown by which estrogen receptor (ER) E2 mediates this acute cardioprotection during I/R. Therefore, we hypothesize that post-ischemic infusion of the selective ER-α agonist (PPT) or the selective ER-β agonist (DPN) will improve myocardial function following I/R.

Methods—Isolated, perfused hearts (Langendorff) from adult male rats were subjected to 25-minute ischemia followed by 40-minute reperfusion. Hearts (n=4–6/group) were randomly infused with either perfusate, PPT or DPN at 1 nM, 10 nM, or 100 nM throughout reperfusion. After I/R, heart tissue was analyzed for TNF-α, IL-1β, VEGF and LDH.

Results—Post-ischemic treatment with 10 nM of PPT significantly improved myocardial function. Additionally, 10 or 100 nM of DPN significantly increased myocardial functional recovery following I/R, with maximum benefit at the 10 nM dose. A trend towards lower levels of LDH was noted in DPN and PPT treated groups following I/R. Neither PPT nor DPN affected myocardial production of TNF-α or IL-1β. However, higher levels of myocardial VEGF were noted in the PPT treated group compared to control.

Conclusions—Both ER-α and ER-β are involved in mediating E2-induced rapid cardioprotection following I/R. Advancing our understanding of both ER subtypes may be useful for the development of novel strategies that may benefit both males and females in response to myocardial ischemia.

Keywords

estrogen receptor; myocardial ischemia; cardiac function; sex hormones

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DISCLOSURES
None.

Introduction

Gender disparities in cardiovascular disease suggest a protective role for estrogen in myocardial ischemia/infarction [1, 2]. Estrogen has been reported to protect the myocardium from ischemia/reperfusion injury (I/R) in animal models through anti-apoptotic, anti-inflammatory signaling, as well as intracellular calcium regulatory mechanisms [1, 3]. Following I/R injury, chronic estrogen administration has been shown to provide cardioprotection in *in vitro* and *in vivo* animal experiments [4]. In addition, acute estrogen treatment before left anterior descending coronary artery ligation results in less cardiac myocyte necrosis and reduced infarct size [5]. However, with respect to therapeutic potential in the treatment of acute myocardial infarction, post-insult administration of estrogen has more clinical appeal. Recently, our group demonstrated that post-ischemic infusion of 17β-estradiol (E2) throughout the 40 minute reperfusion period improved left ventricular (LV) function compared to control [6].

Estrogen exerts its effect on the target cell by diffusing through the cell membrane and binding to the receptors, estrogen receptor (ER)-α and/or ER-β [7–9]. A selective ER-α agonist, 4,4′,4″-[4-propyl-(1H)-pyrazole-1,3,5-triyl]tris-phenol (PPT), has been shown to reduce infarct size after I/R in rabbit hearts [10]. ICI 182,780 and ZM-182780, both potent ER antagonists, have also been found to abolish E2 [11] or ER-α [10, 12] induced cardioprotection. In addition, our group has demonstrated that both ER-α and ER-β are involved in mediating cardiac protection following I/R injury by using ER-α knockout (KO) and ER-β KO mouse hearts [9, 13]. However, it remains unknown by what mechanisms acute administration of E2 after ischemia protects the myocardium. Therefore, based on our previous findings, the purpose of this study was to determine which receptor (ER-α and/or ER-β) mediates acute cardiac protection by using selective ER agonists following I/R injury. We hypothesized that: 1) post-ischemic administration of the selective ER-α agonist (PPT) or the selective ER-β agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) will improve left ventricular (LV) function; and 2) PPT- or DPN-induced cardioprotection will be associated with less myocardial damage (LDH release), reduced pro-inflammatory mediator production and increased VEGF production.

Materials and Methods

Animals

Normal male (250–300g, 8–10 weeks) Sprague-Dawley rats (Harlan, Indianapolis, IN) were fed a standard diet and acclimated in a quiet quarantine room for two weeks 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 1996).

Experimental Groups

Rat hearts were randomly divided into 7 experimental groups and all hearts in each group were subjected to the same I/R protocol: 15 minute equilibration period (any drop of LVDP will be < 10% during this time), 25 minutes of global ischemia (37°C), and 40 minutes of reperfusion (figure 1). ER-α selective agonist PPT (1, 10, 100 nM/min, respectively, n=4–6/group), or ER-β selective agonist DPN (1, 10, 100 nM/min, respectively, n=4/group) was infused after ischemia and throughout the entire reperfusion period. Control hearts (n=6) were infused with Krebs-Henseleit (KH) solution. The proper agonist concentration was adjusted based on the coronary flow (CF) rate.

Isolated Heart Preparation (Langendorff)

Rats were anesthetized (sodium pentobarbital, 60 mg/kg, intraperitoneal (ip) and heparinized (500 units, ip). Hearts where rapidly excised via median sternotomy and placed in 4°C KH solution. The aorta was cannulated and coronary arteries were retrograde perfused with oxygenated (95% O_2 + 5% CO_2), 37°C KH solution. Cardiac decompression was obtained by pulmonary arteriotomy. The left atrial appendage was excised and a water filled latex balloon was passed into the left ventricle. Pacing wires where affixed to the right atrium and left ventricle. The heart was paced at 350 BPM (6 Hz, 3 V, 2 ms). End diastolic pressure (EDP) was adjusted to a level between 6–10 mmHg and held constant throughout the entire experiment. Hearts unable to generate a left ventricular developed pressure (LVDP) >80 mmHg were excluded. Hearts were maintained at the same settings for 15 consecutive minutes of equilibration before proceeding to ischemia. A 3-way stopcock located above the aortic cannula was utilized to induce global ischemia. At the point of reperfusion, retrograde flow was slowly re-established (75 mmHg) and maintained for 40 minutes. Coronary flow rate was measured at regular intervals. Data (LVDP, EDP, HR, +dP/dt, and −dP/dt) were continuously recorded using a PowerLab 8 preamplifier/digitizer (AD Instruments Inc., Milford, MA) and a Mac mini computer (Apple Computer Inc., Cupertino, Ca). Immediately at the end of reperfusion, hearts were snap frozen with liquid nitrogen and stored at −70°C.

Lactate Dehydrogenase Activity (LDH)

Hearts (control n=6, 10 nM DPN n=4, 10 nM PPT n=6) were homogenized as previously described by Wang et al. [9]. LDH assay was performed in supernatants and coronary effluents according to manufacturer instructions, in duplicate, using a commercially available Cytotoxicity Detection Kit (Roche Applied Science, Indianapolis, IN).

ELISA

Supernatants collected from the myocardial homogenate were analyzed for IL-1β, TNF-α, and VEGF concentration using commercially available ELISA kits (R & D Systems, Inc., Minneapolis, MN) (5). Cardiac tissue IL-1β, TNF-α and VEGF ELISAs were performed according to the manufacturer instructions. All standards and samples were measured in duplicate.

Presentation of Data and Statistical Analysis

All data are reported as mean ± SEM. Data were analyzed by 2-way ANOVA with post-hoc Bonferroni or a two-tailed Student's *t*-test. A probability value of <0.05 was considered statistically significant.

Results

Post-ischemic infusion of ER-α agonist or ER-β agonist improved myocardial functional recovery

Ischemia and reperfusion injury resulted in markedly depressed myocardial function as demonstrated by a decrease in myocardial LVDP, +dP/dt and an elevation of EDP, −dP/dt (figure 2–5). Although a trend of increased myocardial LVDP recovery was noted in post-ischemic treatment with 1, 10 and 100 nM of the ER-α agonist (PPT), only 10 nM of PPT significantly improved myocardial function following I/R (figure 2A and 2C). In addition, both 10 and 100 nM doses of ER-β agonist (DPN) significantly restored myocardial LVDP after I/R, with the maximum effect at 10 nM concentration (figure 2B and 2C).

End diastolic pressure, at a constant preload during equilibration, indicated severity of myocardial injury by progressively increasing following ischemia and the beginning of

reperfusion. The value of EDP was reduced after 15 minutes of reperfusion showing a recovery of myocardium from I/R injury. Similarly with LVDP, both PPT (10 nM) and DPN (10 and 100 nM) treatment after ischemia significantly improved recovery of EDP when compared to control (figure 3).

Other indices of myocardial function, +dP/dt (a measure of myocardial contractility) and −dP/dt (a measure of myocardial compliance), were also measured in this study. Not surprisingly, post-ischemic infusion of PPT and DPN significantly protected myocardial +dP/dt and −dP/dt during I/R injury. The maximum beneficial effects of PPT and DPN were induced at the 10 nM dose (figure 4 and 5).

Effects of ER-α agonist or ER-β agonist on myocardial damage following I/R

Lactate dehydrogenase (LDH) was measured in this study to demonstrate cardiac damage. Elevated LDH release usually indicates the damage of cell membrane integrity and is one of the most widely used indicators for cell viability and tissue injury. Theoretically, worse myocardial damage from I/R injury will result in higher levels of LDH in the coronary effluent and the less LDH will remain in cardiac tissue. Due to the under-detectable levels of LDH in coronary effluent, we measured the value of LDH in cardiac tissue. A trend of higher levels of LDH was observed in PPT (10 nM) and DPN (10 nM) -treated myocardium after I/R compared to the control group (figure 6). This suggests that post-ischemic treatment with 10 nM PPT or DPN might reduce LDH release into coronary effluent. In other words, infusion of ER-α agonist or ER-β agonist possibly decreased cardiac damage following I/R injury.

Effects of ER-α agonist or ER-β agonist on myocardial cytokine production

Treatment with 10 nM PPT or DPN appeared to reduce myocardial levels of IL-1β (figure 7A), whereas there was no change in TNF levels (figure 7C). IL-1β levels were 23% lower in the DPN group and 8% lower in the PPT group versus control. Interestingly, the PPT 10 nM group demonstrated significantly greater production of VEGF compared to control (185 ±11 pg/mg protein vs. 138±16 mg/mg protein), whereas DPN showed no effect (figure 7D).

Discussion

Females demonstrate improved myocardial function following I/R injury when compared to males [3]. Administration of E2 attenuates cardiac dysfunction, suggesting that E2 may be involved in mediating this cardioprotection in females [4]. Additionally, post-injury treatment with E2, demonstrates protection of myocardial function against I/R and may have clinical potential in the treatment of acute myocardial infarction [6]. In this study, we further determined by what mechanisms E2 conveys this acute cardiac protection following I/R. Herein, by utilizing specific agonists of ER-α or ER-β, we found that both ER-α and ER-β are involved in mediating E2-induced acute cardioprotection in response to I/R injury. Post-ischemic infusion of ER-α agonist-PPT at a concentration of 10 nM or ER-β agonist-DPN at a concentration of 10 nM or 100 nM reduces myocardial injury. In this study, post-injury treatment with either ER-α agonist or ER-β agonist improved myocardial systolic (LVDP) and diastolic function (EDP), as well as cardiac compliance (+/− dP/dt), suggesting that ER-α and ER-β may mediate E2-induced acute positive effects on myocardial function not only by increasing contractility, but by increasing the end diastolic fluid volume of the heart.

Estrogen has been shown to block voltage gated or receptor operated calcium channels and to promote coronary vasodilation, thereby resulting in improved cardiac perfusion, and overall protection of myocardial function [14]. In addition, reactive oxygen species (ROS) are important mediators of myocardial dysfunction caused by I/R injury. E2 has been observed to decrease the production of oxygen radicals in I/R-injured myocardium [15]. E2 demonstrates antioxidant

effects by increasing I/R-reduced glutathione, a free radical scavenger in the heart [16]. Therefore, it is possible that E2-reduced oxidative damage may mediate the protection of cardiac function in response to I/R injury.

Although the majority of effects of E2 are completed through genomic mechanism, the acute cardioprotective effects of E2 may occur via binding to membrane associated receptors. In fact, there is evidence that ER-α or ER-β is located in the membrane and mediates non-genomic events triggered by E2 [17, 18]. In addition, these receptors can be altered with post-translational modification such as phosphorylation, S-nitrosylation, and O-GlcNAcylation [1], thereby, initiating the effects of E2 through rapid, non-genomic pathways (e.g. MAPK, AKT, PI3K/AKT, PKA/PKC) [2]. Estrogen-inhibited increase in ROS production has been shown to be mediated through ER-α [19]. Conversely, ER-β is likely involved in regulating estrogen-increased NOS activation, NO production and E2–modulated intracellular calcium [20]. Therefore, it is possible that an ER-α agonist or ER-β agonist may improve myocardial function through different mechanisms in this study. However, this requires further investigation to determine the detailed mechanisms by which ER-α or ER-β mediates cardiac protection following I/R injury.

In addition to decreasing oxidative damage, regulating intracellular calcium, and increasing myocardial NOS activation and NO production, estrogen may protect the myocardium from I/R injury by decreasing myocyte death [4, 21]. Estrogen prevents the release of cytochrome *c* from myocardial mitochondria, and decreases its accumulation in the cytosol [21]. Indeed, it is evident that ER-α and ER-β are localized in the mitochondrial membrane, and are responsible for reducing injury/stress-induced apoptosis [17]. In addition, estrogen has been reported to suppress myocyte apoptosis via upregulation of the PI3K/Akt signaling pathway [22]. In this study, post-ischemic infusion of either PPT (ER-α agonist) or DPN (ER-β agonist) demonstrates a trend toward reduction of myocardial necrosis as measured by cardiac tissue LDH, suggesting that ERα and ER-β likely reduce cardiac cell death and decrease myocardial damage, thereby leading to increased myocardial recovery following I/R.

Finally, the protective effects of E2 may also be mediated through reduced proinflammatory cytokine production. Estrogen has been shown to act as an immunomodulator of inflammation in chronic heart disease [23, 24]. In addition, ER agonists have been demonstrated to have anti-inflammatory properties in some disease models [4]. However, there is no significant difference in myocardial production of TNF-α and IL-1β between ER agonist treated group and untreated group. Further study is required to determine whether the decreased inflammation associated with E2 is mediated through rapid, non-genomic pathway during myocardial ischemia. Interestingly, in this study, we found that post-ischemic administration of ER-α agonist-PPT significantly increases myocardial VEGF production. Considering the protective effects of VEGF on the heart, it can be postulated that ER-α agonist may mediate cardiac protection through increased VEGF.

In this study, the Langendorff model was employed to test our hypothesis. This isolated heart perfusion system avoids the potential confounding effects of systemic actions as occurs when the heart is subjected to I/R in vivo. In addition, this model has direct clinical implications as global myocardial ischemia is routinely employed by cardiac surgeons during operation. However, this is an in vitro model which is not able to provide the evidence for the chronic effects of ER agonists on myocardial infarction. Therefore, further investigations using an in vivo myocardial infarction model are required for completely understanding the chronic actions of post-infarction treatment with ER agonists.

In summary, acute post-ischemic administration of ER-α agonist-PPT or ER-β agonist-DPN significantly attenuates myocardial dysfunction following I/R injury, suggesting that both ER-

α and ER-β may mediate protection against myocardial I/R. Although a trend exists toward decreased myocardial damage and reduced pro-inflammatory cytokine production, as well as increased pro-survival cytokine (VEGF) has been noted in PPT or DPN treated group, further study is required to investigate the detailed mechanisms on the specific function of ER subtypes in the heart. Elucidation of knowledge regarding both ER subtypes will improve our understanding of gender and estrogen effects and may help in the development of novel strategies with benefit for both man and woman in response to acute myocardial injury.

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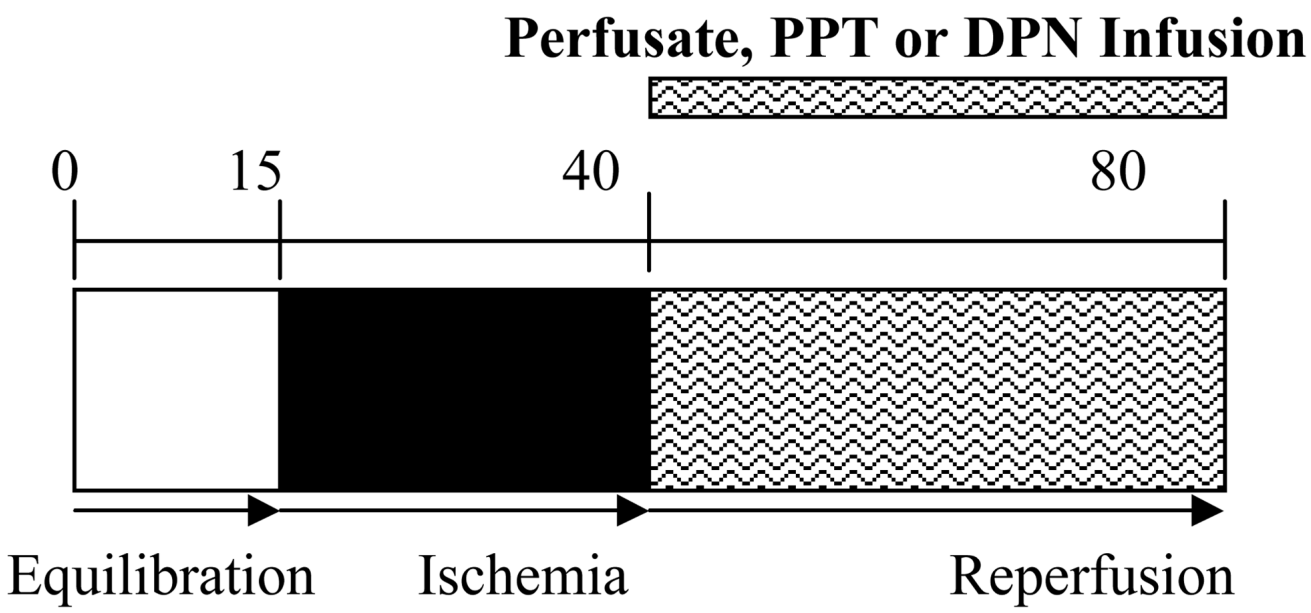

Figure 1.
Simplified schematic illustrates experimental protocol. Isolated rat hearts were subjected to 15 minutes of equilibration, followed by 25 minutes of warm ischemia (37°C) and then 40 minutes of reperfusion. During reperfusion period, either perfusate, PPT (1 nM, 10 nM, or 100 nM), or DPN (1 nM, 10 nM, or 100 nM) is infused.

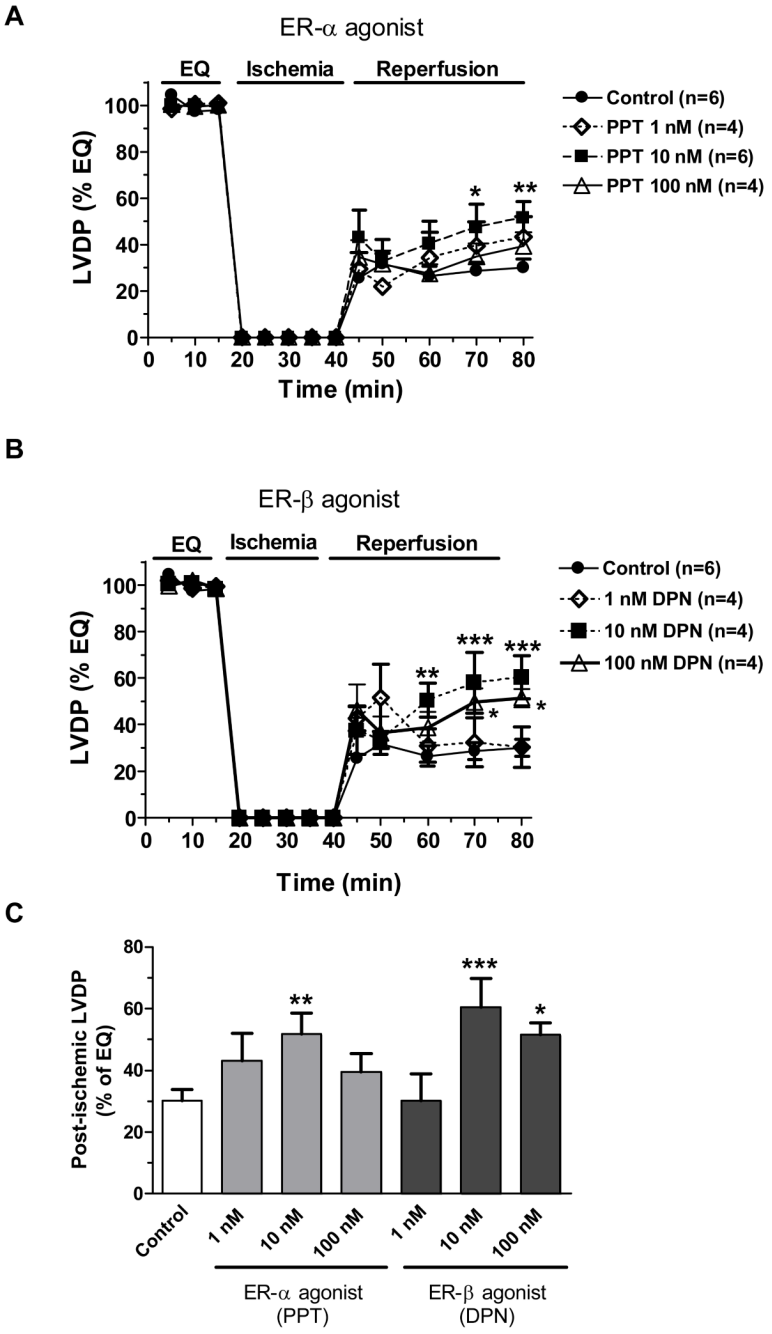

Figure 2.

Changes in LVDP (% of baseline) measured during equilibration, ischemia and reperfusion. (A) Post-ischemic infusion of PPT at concentration of 1 nM, 10 nM or 100 nM compared to control; (B) Post-ischemic administration of DPN at concentration of 1 nM, 10 nM or 100 nM compared to control; (C) LVDP recovery (% of baseline) at the end of reperfusion in all groups. Results are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 vs. corresponding control.

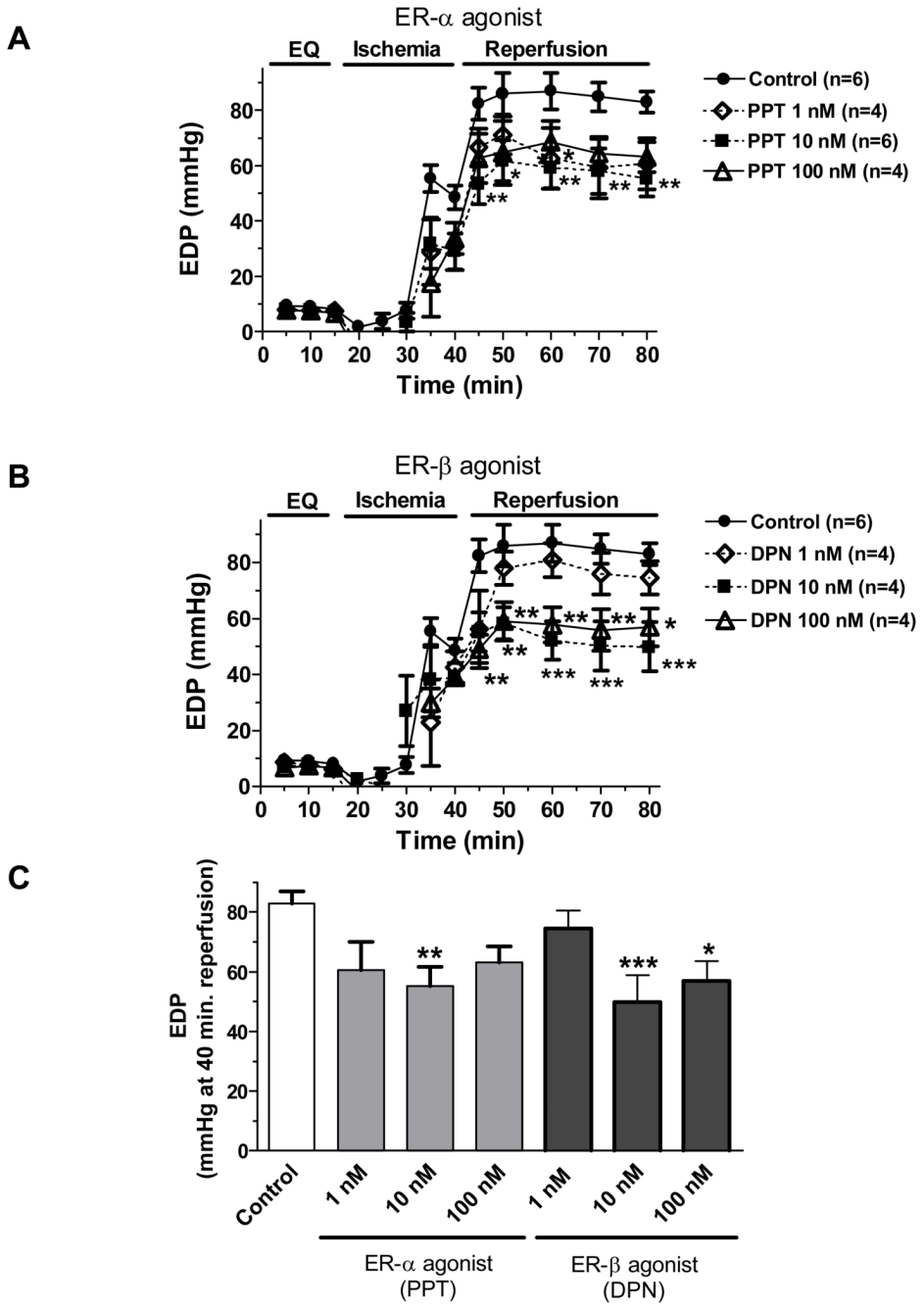

Figure 3.

Changes in EDP (mmHg) throughout equilibration and ischemia/reperfusion in (A) post-ischemic treatment with PPT (1 nM, 10 nM or 100 nM) compared to control; (B) post-ischemic infusion of DPN (1 nM, 10 nM or 100 nM) compared to control; (C) comparison of EDP at the 40-minute reperfusion in all groups. Results are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 vs. corresponding control.

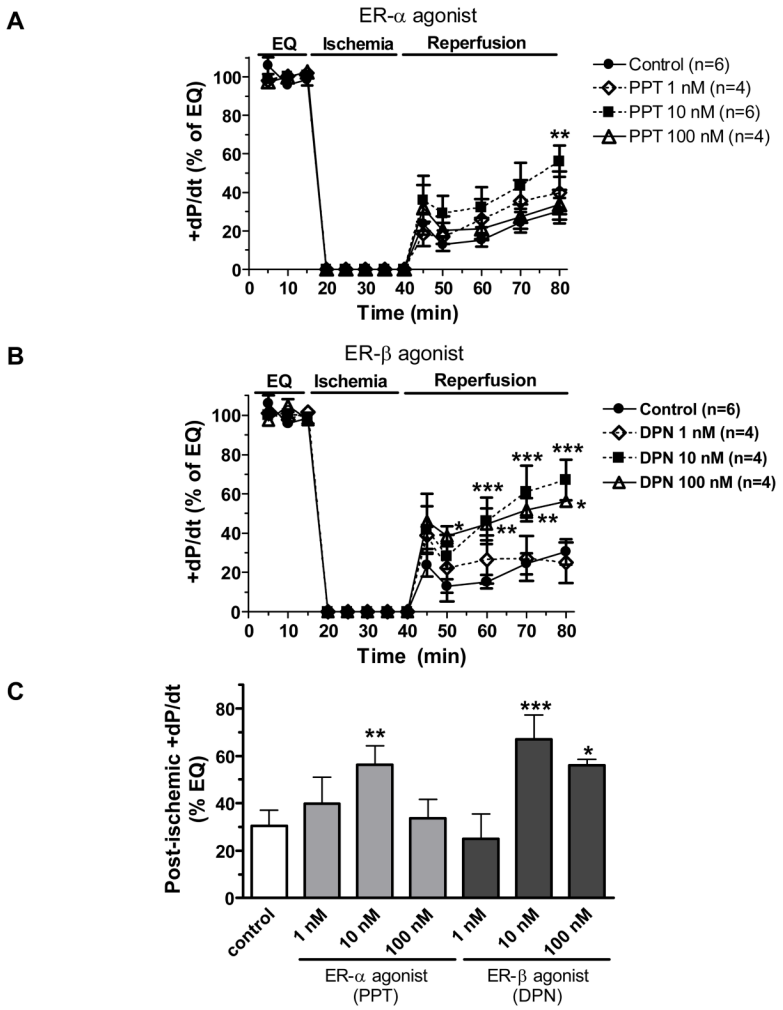

Figure 4.

Changes in the maximum positive value of the first derivative of pressure (+dP/dt) throughout equilibration and I/R (% of baseline). (A) Post-ischemic infusion of PPT (1 nM, 10 nM or 100 nM) compared with control; (B) Post-ischemic treatment with DPN (1 nM, 10 nM or 100 nM) compared to control; (C) Comparison of +dP/dt at the end of reperfusion in all groups. Results are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 vs. corresponding control.

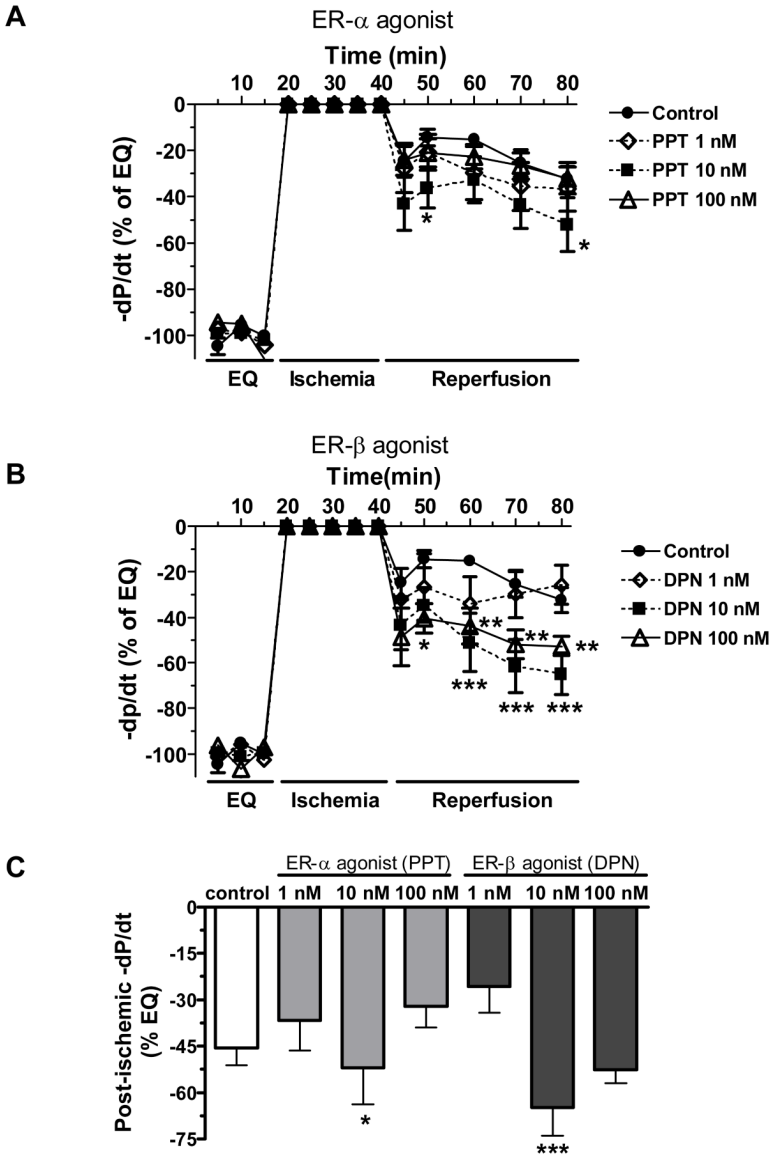

Figure 5.

Changes in the maximum negative value of the first derivative of pressure (−dP/dt) throughout equilibration and I/R (% of baseline). (A) Post-ischemic administration of PPT (1 nM, 10 nM or 100 nM) compared with control; (B) Post-ischemic infusion of DPN (1 nM, 10 nM or 100 nM) compared to control; (C) comparison of −dP/dt at the end of reperfusion in all groups. Results are mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 vs. corresponding control.

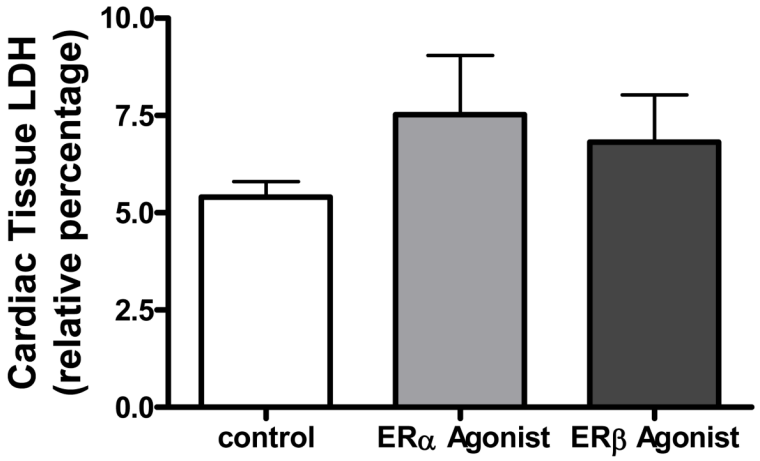

Figure 6.
Relative level of cardiac tissue LDH in Control (n=6), PPT 10 nM (n=6), and DPN 10 nM (n=4) after I/R injury.

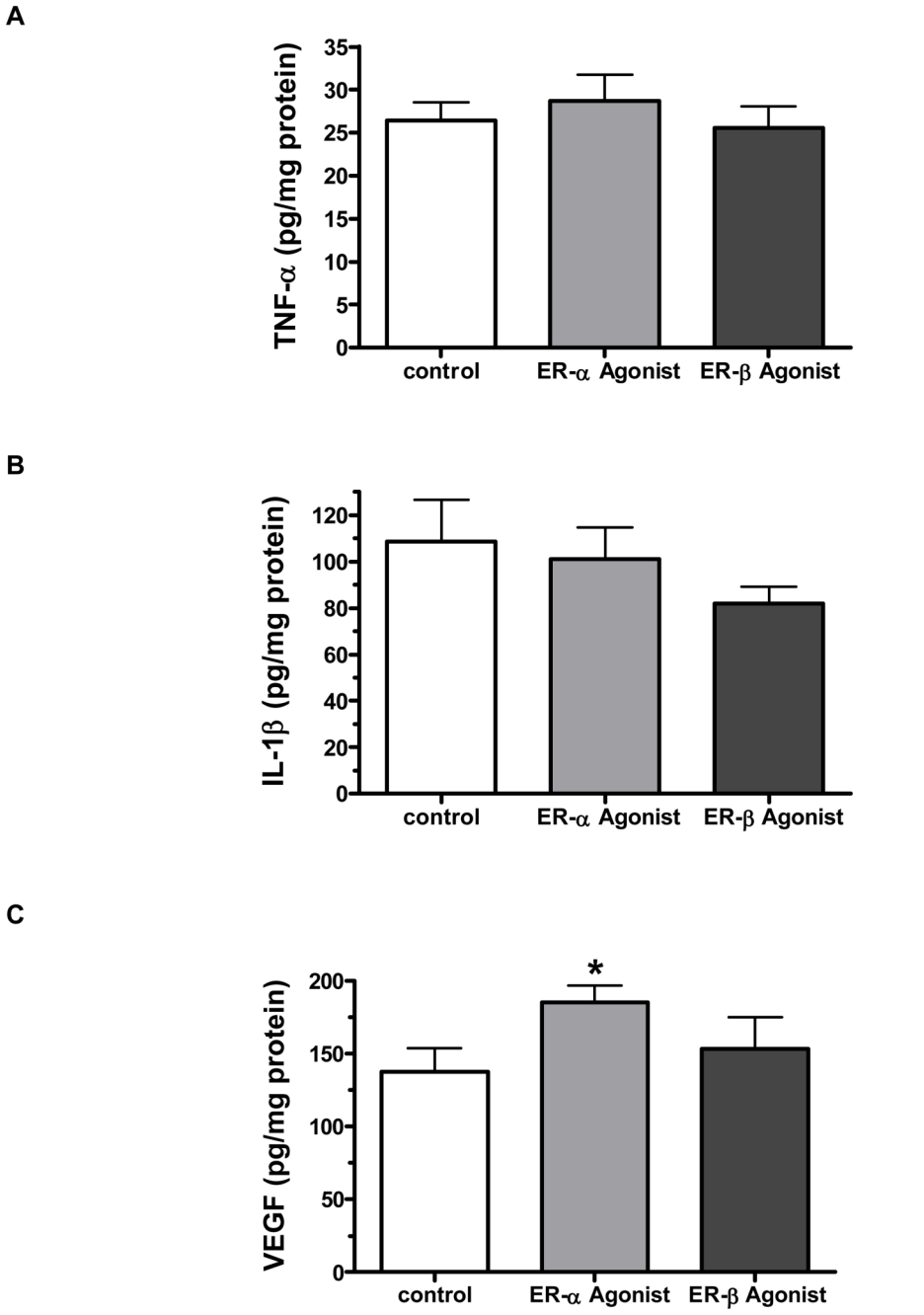

Figure 7.

Myocardial production of TNF-α, IL-1 and VEGF after I/R injury. Neither PPT (ER-α agonist) nor DPN (ER-β agonist) post-ischemic administration significantly affected myocardial levels of TNF-α (A) and IL-1β (B). However, post-ischemic infusion of ER-α agonist – PPT, but not DPN throughout reperfusion significantly increased myocardial VEGF production (C). Results are mean ± SEM, *$p<0.05$ vs. control.