ESTROGEN RECEPTOR ALPHA MEDIATES ACUTE MYOCARDIAL PROTECTION IN FEMALES

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ABSTRACT

Sex differences in myocardial recovery have been reported following acute ischemia and reperfusion injury. Estrogen and the estrogen receptors are critical determinants of cardiovascular gender differences. However, the mechanistic pathways responsible for these differences remain unknown. We hypothesized that estrogen receptor alpha is an important modulator of: 1) myocardial functional recovery following ischemia; and 2) inflammatory signaling via mitogen activated protein kinases (MAPK). To study this, adult male and female wildtype (WT) and estrogen receptor alpha knockout (ER1KO) mouse hearts were isolated, perfused via Langendorff model, and subjected to 20 minutes ischemia, 60 minutes reperfusion. Myocardial contractile function (LVDP, +dP/dT, -dP/dT) was continuously recorded. After ischemia/reperfusion, hearts were assessed for expression of inflammatory cytokines (ELISA), and activation of mitogen activated protein kinases and caspase-3 (Western blot). Data were analyzed with two-way ANOVA or student’s t-test, p<0.05 statistically significant. ER1KO females exhibited significantly less functional recovery than WT females and were similar to WT males. Activated ERK was increased in female WT hearts compared to female ER1KO. Activated JNK was decreased in female WT hearts compared to female ER1KO. No significant differences were found between male WT, female WT, male ER1KO, and female ER1KO in activated p38 MAPK, proinflammatory cytokine expression, and pro-apoptotic signaling. Estrogen receptor alpha plays a role in the protection observed in the female heart. Differential activation of MAPK may mediate this protection. Further studies are necessary to delineate these mechanistic pathways.

KEY WORDS
cardiac ischemia, sex hormones, inflammation, MAPK
INTRODUCTION

Myocardial infarction is the leading cause of mortality in the United States for men and women. However, reperfusion of blood flow to ischemic myocardium is accompanied by the deleterious phenomenon, ischemia/reperfusion (I/R) injury (28, 29). Gender differences have recently been noted in I/R injury. Investigations in both humans (1, 17, 18, 41) and animals (8, 42) have found less tissue injury, improved survival, and diminished inflammatory response in females when compared to males after cardiac injury. Estrogen has widely been implicated in the cardioprotection found in females (3, 4, 10, 25, 31, 40, 48). Indeed, our lab recently demonstrated that exogenous 17-beta-estradiol decreased inflammatory signaling and cardiac dysfunction following acute ischemia (43). Traditionally, estrogen mediates its physiologic effects by binding to an intracellular estrogen receptor (ER) that functions as a ligand-modulated nuclear transcription factor (27). No study has clearly elucidated the mechanistic contribution of the ER in myocardial I/R injury.

Two estrogen receptor molecules have been identified: the original ER-alpha, and the more recently discovered ER-beta (23). Expression of ER-alpha and ER-beta varies in different tissues and species (11). In murine as well as human myocardial tissue, the presence of ER-alpha is well established (16, 19, 31). In contrast, Mendelsohn et al (30) and others (13, 22, 45) have questioned whether murine hearts express ER-beta. This uncertainty regarding the expression of the estrogen receptor in myocardium highlights the need to delineate the role of ER-alpha and ER-beta in myocardial I/R injury.

Myocardial inflammation also plays a critical role in I/R injury and is characterized by the expression of inflammatory cytokines (7, 29) and the activation of the mitogen-activated protein kinase (MAPK) family, p38 MAPK, c-jun N-terminal kinase (JNK), and extracellular signal-regulated protein kinase p42/p44 (ERK) (20). However, differences in the I/R response have been noted between these kinases; whereas p38 MAPK and JNK activity were related to
myocardial dysfunction (26), ERK activation was observed to improve cardiac functional recovery (20). Gender differences have also been observed in MAPK signaling (2, 21, 42), and estrogen has been associated with decreased p38 MAPK signaling (42, 43). No investigation has determined the role of the ER in MAPK signaling.

Therefore, we hypothesize that in female murine hearts, ER-alpha confers cardioprotection following I/R, and that ER-alpha regulates cytokine expression, inhibits p38 MAPK and JNK signaling, activates ERK, and regulates apoptotic signaling in female myocardium subjected to I/R. The purpose of this study was to determine the effect of ER-alpha on postischemic myocardial function, inflammatory signaling, and apoptotic signaling using mice with a targeted mutation of ER-alpha.

MATERIALS AND METHODS

**Animals**

A total of 20 strain C57BL/6J mice [10 with targeted mutation of ER-alpha (ER1KO), transgenic mouse strain Tg(cre/Esr1)5Amc, and 10 wild type (WT) (The Jackson Laboratory, Bar Harbor, ME)] were fed a standard diet and acclimated in a quiet quarantine room for one 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).

All isolated mouse hearts were subjected to the same I/R protocol: 15 minute equilibration period, 20 minutes global ischemia (37°C), and 60 minutes total reperfusion. Mouse hearts were divided into four experimental groups: normal females (n=5), ER1KO females (n=5), normal males (n=5), and ER1KO males (n=5).

**Isolated heart preparation (Langendorff)**
Mice were anesthetized (sodium pentobarbital, 60 mg/kg i.p.) and heparinized (500 U i.p.), and hearts were rapidly excised via median sternotomy and placed in 4°C Krebs-Henseleit (KH) solution. The aorta was cannulated and the heart was retrograde perfused in the isolated, iso-volumetric Langendorff mode (70 mm Hg) with KH solution (in mM: 11 dextrose, 110 NaCl, 1.2 CaCl₂, 4.7 KCl, 20.8 NaHCO₃, 1.18 KH₂PO₄, 1.17 MgSO₄) at 37°C. The KH solution was bubbled with 95% O₂ / 5% CO₂ (Medipure) to achieve a PO₂ of 450 to 460 mmHg, PCO₂ 39 to 41 mmHg, and pH 7.39 to 7.41. Total ischemic time was less than 45 s. The perfusion buffer was continuously filtered through a 0.45 micron filter to remove particulates. A pulmonary arteriotomy and left atrial resection were performed before insertion of a water-filled latex balloon through the left atrium into the left ventricle. The pre-load volume (balloon volume) was held constant during the entire experiment to allow continuous recording of the left ventricular developed pressure. The balloon was adjusted to a mean left ventricular end-diastolic pressure of 8 mmHg (range 6-10 mmHg) during the initial equilibration. Pacing wires were fixed to the right atrium and left ventricle and hearts were paced at 6 Hz, 3 V, 2 ms (approximately 350 beats / min) 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 was 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 maximal positive and negative values of the first derivative of pressure (+dP/dt and -dP/dt) were calculated using PowerLab software.

**Coronary Effluent LDH activity**

Coronary effluent (1 ml) was collected at 10, 20, 30, and 40 min into reperfusion, and then frozen at -70°C until assay. The assay was performed with an LDH Cytotoxicity Detection
Kit (Roche Diagnostics, Indianapolis, IN). The assay was performed according to the manufacturer’s instructions. All samples were measured in duplicate.

**Myocardial Proinflammatory Cytokine Expression**

Heart tissue and coronary effluent from various time points were homogenized separately 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 μg/ml Leupeptin, 1 mM PMSF, and centrifuged at 12000 rpm for 5 minutes. Myocardial tumor necrosis factor alpha (TNF), interleukin (IL)-1beta, and IL-6 in the cardiac tissue were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA kit (R&D Systems Inc., Minneapolis, MN). ELISA was performed according to the manufacturer’s instructions. All samples and standards were measured in duplicate.

**Western blotting**

Western blot analysis was performed to measure MAPK and apoptosis-related 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 μg/ml Leupeptin, 1 mM PMSF, and centrifuged at 12000 rpm for 5 minutes. The protein extracts (30 μg/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: p38 MAPK antibody, phosphor-p38 MAPK (Thr180/Tyr182) antibody, JNK antibody, phosphor-JNK (Thr183/Tyr185) antibody, ERK antibody, phosphor-ERK (Thr202/Tyr204) antibody (Cell Signaling Technology, Beverly, MA), caspase-3 (H-277) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Bcl-2 (Ab-4) antibody, and GAPDH antibody (Oncogene Research Products, San Diego, CA). 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) and band density was analyzed using ImageJ software (NIH).

**Presentation of data and statistical analysis**

All reported values are mean ± SEM. Data was compared using two-way analysis of variance (ANOVA) with post-hoc Bonferroni test or Student’s t-test (Female WT vs Female ER1KO and Male WT vs Male ER1KO). A two-tailed probability value of less than 0.05 was considered statistically significant. Representative gels are shown with all lanes/samples from the same gel for each respective figure.

**RESULTS**

**Myocardial function**

Maximum positive and negative dP/dt were impaired at the start of reperfusion. Female ER1KO hearts demonstrated more depression of +dP/dt and elevation of -dP/dt compared to female WT hearts (Figure 1). Male WT also demonstrated more depression of +dP/dt and elevation of -dP/dt compared to female WT hearts. Male WT, male ER1KO, and female ER1KO hearts exhibited similar impairments of contractility and compliance.

**Myocardial necrosis**

LDH in the coronary effluent from hearts used in these studies was undetectable, possibly due to the small amount of tissue, the dilution of a non-recirculating model, and the early time points measured.

**Myocardial MAPK signaling pathway following ischemia/reperfusion**

The myocardial activation of phosphorylated p38 (active), nonphosphorylated p38 (total) MAPK, phosphorylated JNK, nonphosphorylated JNK, phosphorylated ERK and nonphosphorylated ERK were assessed by Western blot (Figure 2,3,4). The phosphorylated
forms of ERK were increased in female WT hearts compared to female ER1KO. The phosphorylated forms of JNK were decreased in female WT hearts compared to female ER1KO. Total p38 MAPK, activated p38 MAPK, total JNK, and total ERK were equivalent in female WT, female ER1KO, male WT, and male ER1KO following I/R.

**Myocardial caspase cascades following ischemia/reperfusion**

The expression of apoptosis-related and inflammation-related caspases in I/R-injured myocardium was assessed by Western blot. Caspase-3 cleavage/activation products were not significantly different in female WT and male WT compared to female ER1KO and male ER1KO as shown in Figure 5.

**Myocardial inflammatory response to ischemia/reperfusion**

Myocardial production of TNF, IL-1beta and IL-6 in heart tissue as well as coronary effluent at various time points were measured via ELISA. Compared to female WT and male WT hearts, female ER1KO and male ER1KO had equivalent myocardial TNF, IL-1beta and IL-6 levels following I/R injury as shown in Figure 6.

**DISCUSSION**

The results of this study demonstrate that in animals with a higher baseline of endogenous estrogen (females but not males), ER-alpha (1) mediates acute myocardial functional protection following I/R; and (2) increases protective ERK ½ activation and decreases pro-apoptotic JNK activation during myocardial ischemia.

ER-alpha may play an important role in modulating the contractile dysfunction induced by I/R injury for several reasons. Previously we demonstrated that 17-beta-estradiol, a non-selective ER ligand, increased functional recovery and decreased inflammation and apoptosis following acute ischemia in males as well as ovariectomized females (43). Recently, Booth et al found that a selective ER-alpha agonist, 4,4',4''-[4-propyl-(1H)-pyrazole-1,3,5-triy]tris-phenol, reduced infarct size, release of troponin-I, and deposit of the membrane attack complex after I/R.
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in rabbit hearts (6). ICI 182,780 and ZM-182780, both potent ER antagonists, also have been found to reverse the cardioprotection conferred by 17-beta-estradiol (35) and selective ER-alpha agonists (6, 37). A study limited to male mice (with presumably lower estrogen levels) also found significantly lower coronary flow rate in ER-alpha knockouts (47). Indeed, the results of this study corroborate the latter indirect evidence that ER-alpha mediates cardioprotection in females; female ER1KO hearts demonstrated a statistically significant decrease in functional recovery of contractility (+dP/dT) and compliance (-dP/dT) in comparison with female WT hearts. Recently, both estradiol (36) as well as 16alpha-LE2 (37), an ER-alpha selective ligand, significantly reduced cardiac hypertrophy, and increased cardiac output. Thus, it is possible that ER-alpha mediates improved contractility and compliance in females via a reduction in cardiac hypertrophy and infarct reduction.

MAPKs are critically involved in regulatory signaling pathways which ultimately lead to inflammation and cardiac hypertrophy (9). Activation of p38 MAPK and JNK is a critical step in the generation of deleterious myocardial inflammation after I/R injury whereas ERK activation has been found to improve cardiac functional recovery (20). Similarly, transfection of cardiomyocytes with adenoviral vectors expressing upstream activators for p38 MAPK and JNK induces transcriptional and morphological changes associated with the hypertrophy (44, 46); activation of ERK has not been associated with myocardial hypertrophy (38, 39). Gender differences have been noted in MAPK signaling (2, 21, 42). In particular, estrogen has been found to modulate activation of the MAPK signal cascade (42, 43). Previously, we determined that estrogen decreased MAPK inflammatory signaling, inflammatory cytokine expression, and apoptotic signaling following acute ischemia in males as well as ovariectomized females (43). However, Migliaccio et al determined that the activation of MAPK pathway by estrogen requires the ligand occupancy of the estrogen receptor (32). Indeed, in this study, ER-alpha was found to increase protective ERK ½ activation and decrease pro-apoptotic JNK activation during
myocardial ischemia in females; no significant differences were found in p38 MAPK activation in female WT and female ER1KO hearts. This differential activation confirms previous findings that estradiol induced a rapid activation of ERK and JNK, but had only a marginal effect on p38 MAPK activation (33). Therefore, these findings suggest that ER-alpha may mediate reduced cardiac hypertrophy and inflammation via differential activation of the MAPK family.

We found no significant difference in the myocardial production of TNF, IL-1, IL-6, and caspase-3 between female WT and female ER1KO as well as between male WT and male ER1KO. Previously, exogenous estrogen decreased myocardial inflammatory cytokine production and increased post ischemic cardiac function, suggesting that estrogen exerts a protective effect on myocardium via decreased myocardial inflammation (43). This discrepancy in myocardial inflammatory cytokine production between estrogen exposure and ER-alpha exposure may reflect an estrogen receptor independent mechanism. It is widely recognized that estrogen binds to intracellular receptors and modulates transcription and protein synthesis, triggering genomic events responsible for physiologic effects (5). However, accumulating data suggest that some metabolites of estradiol are biologically active and mediate multiple effects on the cardiovascular systems that are largely independent of estrogen receptors; catecholestradiols and methoxyestradiols are implicated in this process (12). In other systems, investigators have found that the protective effect of estrogen on inflammatory cytokine production was not dependent on ER-alpha signaling (15, 24). Thus, this discrepancy in cytokine production between estrogen exposure and ER-alpha exposure intimates that estrogen may exert its protective effects on myocardium via different receptor dependent and independent pathways.

This study did not address the role of ER-beta in myocardial I/R injury in murine animals. Several investigators (13, 22, 45) have questioned whether murine hearts express ER-beta. Oliveira et al found that ICI 182,780, an ER receptor antagonist has no effect on ER-beta in rats (34). Although Gabel et al suggests that ER-beta in rats plays a role in gender differences in
I/R injury under hypercontractile conditions, no differences were discovered under normal contractile conditions (14). This study demonstrates under normal contractile conditions that ER1KO hearts exhibited significantly less functional recovery than WT females and were similar to WT males. Whether similar findings can be applied to ER-beta remains to be determined, and can be a potential aspect to address in future studies.

There still remains a great deal of controversy over the role of gender and injury. This study confirms the protective effects of estrogen receptor alpha in females. This may also lend insight into the mechanistic pathways behind the variation in clinical outcomes between males and females after MI. Perhaps modification of estrogen receptor alpha dependent mechanisms associated with I/R will alter the myocardial response to ischemia in menopausal females and potentially males.

DISCLOSURES

None.
REFERENCES


9. Clerk A, Michael A, and Sugden PH. Stimulation of the p38 mitogen-activated protein kinase pathway in neonatal rat ventricular myocytes by the G protein-coupled receptor agonists,


FIGURE LEGENDS

**Figure 1.** Changes in myocardial function following I/R in male wildtype hearts (M WT), female wildtype hearts (F WT), male estrogen receptor alpha knock outs (M ER1KO), and female estrogen receptor alpha knock outs (F ER1KO). A. +dP/dt maximum (% of Equilibration); B. -dP/dt maximum (% of Equilibration); C. Recovery of +dP/dt after I/R at end reperfusion (% of Equilibration); D. Recovery of +dP/dt after I/R at end reperfusion (% of Equilibration). Results are mean±SEM, *p<0.05 at the corresponding time points.

**Figure 2.** I/R induced myocardial phosphorylation of p44/42 (ERK). The expression of activated ERK after I/R injury in male wildtype hearts (M WT), female wildtype hearts (F WT), male estrogen receptor alpha knock outs (M ER1KO), and female estrogen receptor alpha knock outs (F ER1KO). A. Shown are representative immunoblots. Row 1.Phosphorylated (activated) ERK; Row 2. Total ERK. All samples were on the same membrane. B. Densitometry data of phosphorylated ERK (% of total ERK). Results are mean±SEM, *p<0.05 F WT vs M WT, #p<0.05 F WT vs F ER1KO.

**Figure 3.** I/R induced myocardial phosphorylation of JNK. The expression of activated JNK after I/R injury in male wildtype hearts (M WT), female wildtype hearts (F WT), male estrogen receptor alpha knock outs (M ER1KO), and female estrogen receptor alpha knock outs (F ER1KO). A. Shown are representative immunoblots. Row 1. Phosphorylated (activated) JNK; Row 2. Total JNK. All samples were on the same membrane. B. Densitometry data of phosphorylated JNK (% of total JNK). Results are mean±SEM, *p<0.05 versus M WT, #p<0.05 F WT vs F ER1KO.
Figure 4. I/R induced myocardial phosphorylation of p38 MAPK. The expression of activated ERK after I/R injury in male wildtype hearts (M WT), female wildtype hearts (F WT), male estrogen receptor alpha knock outs (M ER1KO), and female estrogen receptor alpha knock outs (F ER1KO). A. Shown are representative immunoblots. Row 1. Phosphorylated (activated) p38 MAPK; Row 2. Total p38 MAPK. All samples were on the same membrane. B. Densitometry data of phosphorylated p38 MAPK (% of total p38 MAPK). Results are mean±SEM.

Figure 5. Myocardial expression of proapoptotic enzymes in male wildtype hearts (M WT), female wildtype hearts (F WT), male estrogen receptor alpha knock outs (M ER1KO), and female estrogen receptor alpha knock outs (F ER1KO). A. Shown are representative immunoblots. All samples were on the same membrane. B. Densitometry data of caspase-3 (% of GAPDH). Results are mean±SEM.

Figure 6. Effect on myocardial TNF, IL-1β and IL-6 production after I/R in male wildtype hearts (M WT), female wildtype hearts (F WT), male estrogen receptor alpha knock outs (M ER1KO), and female estrogen receptor alpha knock outs (F ER1KO). Results are mean±SEM.
Figure 1.

A.

B.

C.

D.
Figure 2.

A.

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B.
Figure 3.

A.

M WT       F WT        F ER1KO       M ER1KO

p-JNK (active)

JNK (total)

B.
Figure 4.

A.

M WT  F WT  F ER1KO  M ER1KO

p-p38 (active)

B.
Figure 5.

A.

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B.

Cleavage caspase-3/GAPDH (%)

- M WT: 90%
- F WT: 80%
- F ER1KO: 70%
- M ER1KO: 60%

p=0.058
p=0.13
Figure 6.

A.

B.