Estrogen receptor β mediates increased activation of PI3K/Akt signaling and improved myocardial function in female hearts following acute ischemia

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Wang M, Wang Y, Weil B, Abarbanell A, Herrmann J, Tan J, Kelly M, Meldrum DR. Estrogen receptor β mediates increased activation of PI3K/Akt signaling and improved myocardial function in female hearts following acute ischemia. Am J Physiol Regul Integr Comp Physiol 296: R972–R978, 2009. First published February 11, 2009; doi:10.1152/ajpregu.00045.2009.—Females have a lower incidence of heart failure and improved survival after myocardial ischemia-reperfusion (I/R) compared with males. Although estrogen-suppressed cardiomyocyte apoptosis may be mediated through the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway, it is unclear whether this action is mediated via estrogen receptor β (ERβ). Therefore, we hypothesized that ERβ mediates estrogen-induced cardioprotection through PI3K/Akt and antiapoptotic signaling in females but not in males. Isolated male and female hearts from ERβ knockout (ERβKO) and wild-type (WT) mice (n = 5 mice/group) were subjected to 20-min ischemia followed by 60-min reperfusion (Langendorff). Ablation of ERβ significantly decreased postischemic recovery of left ventricular developed pressure in female, but not male, hearts. Reduced activation of PI3K and Akt was noted in female ERβKO hearts, which was associated with increased expression of caspase-3 and -8, as well as decreased Bcl-2 levels compared with WT. However, myocardial STAT3, SOCS3 (suppressor of cytokine signaling 3), VEGF, and TNF receptors 1 and 2 levels did not change in ERβKO of either sex following I/R. Furthermore, deficiency of ERβ increased myocardial JNK activation in females but increased ERK1/2 activity in males during acute I/R. We conclude that ERβ mediates myocardial protection via upregulation of PI3K/Akt activation, decreased caspase-3 and -8, and increased Bcl-2 in female hearts following I/R. These findings provide evidence of ERβ-mediated PI3K/Akt and antiapoptotic signaling in the myocardium and may lend insight into the mechanistic pathways behind the observed variation in clinical outcomes between males and females after myocardial infarction.

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MATERIALS AND METHODS

Animals. A total of 20 male and female C57BL/6J mice (16 ± 4 wk) with and without ERβ deficiency (ERβKO; Taconic Farms, Hudson, NY) were fed a standard diet and acclimated in a quiet quarantine room for more than 1 mo 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” [DHENV Publication No. (NIH) 85-23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

All isolated mouse hearts were subjected to the same I/R protocol as described previously (23, 24). Mouse hearts were divided into four groups.

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experimental groups: normal males (n = 5), ERβKO males (n = 5), normal females (n = 5), and ERβKO females (n = 5).

Isolated heart preparation (Langendorff). Experiments were performed with the use of a Langendorff apparatus as described previously for mouse hearts (24). The left ventricular developed pressure (LVDP) was continuously recorded using a PowerLab 8 preamplifier/digitizer (AD Instruments, Milford, MA) and an Apple G4 PowerPC computer (Apple Computer, Cupertino, CA).

**Western blotting.** Western blot analysis was performed to measure PI3K/Akt signaling, STAT3 activation, SOCS3 expression, apoptotic proteins (caspase-3 and -8 and Bcl-2), and mitogen-activated protein kinases (MAPKs: p38 MAPK, c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated protein kinase p42/p44 (ERK1/2)). Heart tissue was homogenized in cold radioimmunoprecipitation assay (RIPA) buffer (product no. R 0278; Sigma, St. Louis, MO) and centrifuged at 12,000 rpm for 10 min. The protein extracts (15 µg/lane) were subjected to electrophoresis on a 4–12% Bis-Tris protein gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. The membranes were incubated in 5% dry milk for 1 h and then incubated with the following primary antibodies: PI3K, p-PI3K, Akt, p-Akt, STAT3, p-STAT3 (Tyr705), p38 MAPK, p-p38 MAPK, c-Jun, p-c-Jun (Thr180/Tyr182) (1:1,000 dilution; Cell Signaling Technology, Beverly, MA); caspase-3 and -8 and SOCS3 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); Bcl-2 (Oncogene Research Products, San Diego, CA); and GAPDH (1:5,000 dilution, Biosdesign International, Saco, ME). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (Pierce, Rockford, IL), and detection was performed using SuperSignal West Pico stable peroxide solution (Pierce). Films were scanned using an Epson Perfection 3200 scanner (Epson America, Long Beach, CA), and band density was analyzed using ImageJ software (NIH, Bethesda, MD).

**Enzyme-linked immunosorbent assay.** Myocardial VEGF levels in the cardiac tissue were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA set (Duoset ELISA development system; R&D Systems, Minneapolis, MN). ELISA was performed according to the manufacturer’s instructions. All samples and standards were measured in duplicate.

**Presentation of data and statistical analysis.** All reported values are means ± SE. Data were compared using one-way ANOVA with post hoc Tukey’s test or Student’s t-test (male WT vs. female WT, male WT vs. male ERβKO, and female WT vs. female ERβKO). A two-tailed P value of <0.05 was considered statistically significant.

**RESULTS**

**Myocardial function.** LVDP at the end of reperfusion was exhibited as a percentage of equilibration. I/R injury decreased myocardial LVDP in both WT and ERβKO mice (Fig. 1).

Deficiency of the ERβ gene did not significantly affect myocardial recovery of LVDP in male hearts following I/R (35.9 ± 8 vs. WT 45.2 ± 4.7%), whereas ERβKO markedly decreased LVDP recovery in female hearts (43 ± 6.3%) compared with female WT (62.9 ± 2.9%, P < 0.05). In addition, sex difference existed in I/R-depressed myocardial function with improved LVDP in female WT hearts compared with male WT. However, ablation of the ERβ gene neutralized this difference following I/R.

**Effects of ERβ on myocardial PI3K/Akt signaling during acute I/R.** Myocardial ischemia increases activation of the PI3K/Akt pathway, which in turn mediates cardiomyocyte survival in response to I/R injury. Ablation of the ERβ gene significantly decreased myocardial PI3K/Akt activation in females after I/R. ERβKO reduced activation of PI3K from 47.6% (percentage of p-PI3K/total PI3K) in WT to 14.6% (Fig. 2A) and Akt from 190% (percentage of p-Akt/total Akt) to 91.3% in female hearts (Fig. 2B). Although deficiency of ERβ decreased PI3K activation, the KO did not affect myocardial Akt activation in males (Fig. 2).

**Effects of ERβ on myocardial apoptotic proteins after I/R injury.** Given that the experimental period in this study is too brief (20-min ischemia followed by 60-min reperfusion) to detect significant apoptosis, we measured proapoptotic signaling by examining caspase-3 and -8 and antiapoptotic protein Bcl-2 expression instead. ERβKO significantly increased myocardial expression of caspase-3 and -8 and reduced Bcl-2 levels in females following I/R (Fig. 3). This suggests that ERβ may mediate antiapoptotic signaling and improve cardiomyocyte survival in female hearts. However, ablation of ERβ appeared to have an opposite effect in males, since a trend of decreased caspase-3 and -8 and enhanced Bcl-2 levels were noted in male ERβKO hearts (Fig. 3). It is possible that sex differences exist in ERβ-mediated apoptotic signaling following acute I/R.

**Effects of ERβ on myocardial PI3K/Akt pathway after I/R injury.** MAPK pathways (p38 MAPK, JNK, and ERK1/2) play important roles in myocardial inflammation, cardiac function, and cardiomyocyte death following I/R. In males, ablation of ERβ did not affect myocardial activation of p38 MAPK and JNK, whereas increased ERK1/2 activation was noted in the KO hearts (Fig. 5). Interestingly, enhanced JNK activation was observed in female ERβKO myocardium in response to I/R, whereas activation of p38 MAPK and ERK1/2 was not changed by deficiency of ERβ in females (Fig. 5). This finding suggests that specific sex differences exist in ERβ-mediated MAPK signaling.

**DISCUSSION**

Accumulating evidence has demonstrated that ERβ is involved in mediating E2-induced cardioprotection in females following I/R combined with isoproterenol-induced hypercon-
tractile condition (2, 11). In the present study, by utilizing mice lacking ERβ, we found that ERβ mediates acute myocardial functional protection likely through activation of PI3K/Akt pathway and decreased apoptotic signaling following I/R in females (presumably having a higher baseline levels of endogenous estrogen compared with males) but not in males (Fig. 6).

ERβ is a member of the nuclear receptor gene family of transcription factors and may play an important role in protecting against myocardial dysfunction caused by I/R injury. Previously, we indicated that E2, a nonselective ER ligand, improved functional recovery, reduced myocardial inflammation, and diminished proapoptotic signaling following acute ischemia in males as well as OVX females (25). Recently, deficiency of ERβ was shown to worsen postischemic myocardial dysfunction in female hearts with hypercontractile condition (2). In addition, DPN, a selective ERβ agonist, restored...
E2-mediated cardiac protection in OVX females under conditions of enhanced contractility following I/R injury (11). In our study, significantly decreased recovery of LVDP was noted in female ERβ/H9252 KO hearts compared with female WT after acute I/R, whereas ablation of ERβ did not affect postischemic myocardial functional recovery in males. This result further confirms that ERβ mediates protection of myocardial function not only in female hearts associated with isoproterenol-enhanced contractility (2, 11) but also in normal females during acute I/R.

ERβ has been shown to mediate E2-induced cardioprotection through genomic mechanisms including regulation of metabolic gene expression (2, 11) and modulation of ion channel expression and calcium-handling protein (6, 13). However, it is also possible that ERβ may be located in the plasma membrane and mediates nongenomic events triggered by E2 (18). Akt and its upstream signal, PI3K, play critical roles in mediating cell survival and apoptosis (12). It is evident that the E2-activated PI3K/Akt pathway functions as one of the acute nongenomic actions of E2 in various types of cells (18). Upregulation of PI3K/Akt by administration of E2 results in endothelial nitric oxide synthase activation via a transcription-independent mechanism (3). In addition, activation of the PI3K/Akt pathway is required for E2-suppressed apoptosis and metabolic gene expression (2, 11).
E2-protected myocardial function in the heart following ischemia (12). Although most studies on the interaction of estrogen receptor with the PI3K/Akt pathway have indicated a crucial role for ERα as a membrane-associated receptor engaged in this action (18), little information exists regarding the importance of ERβ in the E2-mediated nongenomic effect of PI3K/Akt activation. In this study, we found significantly reduced activation of the PI3K/Akt pathway in female ERβ KO hearts, but not in males, following acute I/R. This provides evidence that ERβ is also involved in PI3K/Akt-regulated cardioprotection in females. In fact, ERβ has been shown to mediate E2-induced antiapoptotic effects through the PI3K/Akt pathway in skeletal muscle cells (21). In addition, administration of E2 protects cardiac H9c2 cells from oxidative stress-induced apoptosis through ERβ-activated Akt signaling (20). Consistent with these observations, we have demonstrated that deficiency of the ERβ gene significantly increases levels of proapoptotic proteins caspase-3 and -8 while reducing antiapoptotic protein Bcl-2 levels in female hearts in response to acute I/R.

E2-regulated Akt activation also may mediate myocardial protection via additional mechanisms. Previous studies have demonstrated, for example, that OVX-reduced Akt activation occurs with decreased myocyte contractile function and impaired intracellular calcium handling, whereas E2-upregulated Akt is associated with restored cardiac contractility and intracellular calcium homeostasis (15). This suggests that Akt signaling may play a role in E2-mediated protection of myocardial function in addition to reduction of apoptosis. Indeed, activation of the Akt pathway also has been linked to a lower incidence of arrhythmias after myocardial infarction (14). In addition, delivery of a constitutively active Akt mutant gene markedly improves myocardial function in vivo following acute I/R and protects hypoxia-induced cardiomyocyte dysfunction in vitro, likely by preventing hypoxia-induced abnormalities in calcium transients and shortening (7). Therefore, it is possible that decreased activation of Akt may lead to reduced myocardial recovery of LVDP in female ERβKO hearts following acute I/R in our study.

![Diagram](https://example.com/diagram.png)
TNFR1 signaling has been shown to mediate detrimental effects of TNF on the myocardium (8, 24), whereas the TNFR2 pathway conducts protective effects (4, 23). Evidence suggests that the balance of TNFR1 and TNFR2 signaling shifts in favor of TNFR2 and its beneficial effects in female hearts during I/R (23, 24). However, it is unclear whether ERβ is engaged in the regulatory balance of the TNFR1 and TNFR2 pathways. In addition, TNFR2-induced cardioprotection appears to be mediated through STAT3, SOCS3, and VEGF in female hearts. STAT3 has been shown to be a direct target gene for estradiol (1). Therefore, the question arises whether ERα partially mediates nongenomic effects of E2 (1). Therefore, the regulatory balance of the TNFR1 and TNFR2 pathways. In both ERα/H9251 KO mice, the myocardial TNFR1 signaling has been shown to mediate detrimental effects of TNF on the myocardium (8, 24), whereas the TNFR2 signaling shifts in favor of TNFR2 and its beneficial effects in female hearts during I/R (23, 24). However, it is unclear whether ERβ is engaged in the regulatory balance of the TNFR1 and TNFR2 pathways. In addition, TNFR2-induced cardioprotection appears to be mediated through STAT3, SOCS3, and VEGF in female hearts. STAT3 has been shown to be a direct target gene for estradiol (1). Therefore, the regulatory balance of the TNFR1 and TNFR2 pathways. In both ERα/H9251 KO mice, the myocardial TNFR1 signaling has been shown to mediate detrimental effects of TNF on the myocardium (8, 24), whereas the TNFR2 signaling shifts in favor of TNFR2 and its beneficial effects in female hearts during I/R (23, 24). However, it is unclear whether ERβ is engaged in the regulatory balance of the TNFR1 and TNFR2 pathways. In addition, TNFR2-induced cardioprotection appears to be mediated through STAT3, SOCS3, and VEGF in female hearts. STAT3 has been shown to be a direct target gene for estradiol (1). Therefore, the regulatory balance of the TNFR1 and TNFR2 pathways.

MAPKs (p38 MAPK, JNK, and ERK1/2) have been shown to mediate myocardial responses during injury. Activity of p38 MAPK and JNK is related to myocardial dysfunction (5), whereas ERK1/2 activation improves cardiac functional recovery following I/R (22). In addition, E2 has been reported to block hypoxia-induced activation of p38 MAPK and JNK, thus protecting the myocardium (5, 25). Conversely, E2-increased activation of ERK1/2 has been shown to inhibit cardiomyocyte apoptosis during myocardial ischemia (22). It is evident that both ERα and ERβ are involved in regulating myocardial MAPKs (5, 22). In fact, ERα has been shown to upregulate activation of the protect ERK1/2, decrease the proapoptotic JNK activation, and improve myocardial function in females during acute ischemia (22). In the present study, we further found significantly increased JNK activation in female ERβKO hearts but elevated ERK1/2 activation in male ERβKO following I/R. This suggests that sex differences exist in ERβ-mediated MAPK signaling in the hearts in response to acute ischemic injury.

Perspectives and Significance

This study provides the direct evidence of ERβ-mediated cardioprotection in female hearts following acute I/R. Improved myocardial function is associated with ERβ-activated PI3K/Akt pathway and subsequently decreased apoptotic signaling in female hearts in response to I/R. Further investigation is required to elucidate the detailed mechanisms on the specific function of ER subtypes in the heart. Understanding effects of estrogen and estrogen receptors may help in advancing therapeutic manipulations in menopausal females and, potentially, males.

GRANTS

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