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

Meijing Wang², Yue Wang², Brent Weil², Aaron Abarbanell², Jeremy Herrmann², Jiangning Tan², Megan Kelly², and Daniel R. Meldrum¹, ², ³, ⁴
Clarian Cardiovascular Surgery¹ and the Departments of Surgery², Cellular and Integrative Physiology³, and the Center for Immunobiology⁴, Indiana University School of Medicine, Indianapolis, Indiana

Running Head: ERβ protects ischemia-damaged female heart via PI3K/Akt

Correspondence:
Meijing Wang, M.D.
635 Barnhill Drive, MS 2031F
Indianapolis, Indiana 46202
meiwang@iupui.edu
Phone: 317-274-0827
Fax: 317-274-6896
ABSTRACT

Background: Females have a lower incidence of heart failure and improved survival after myocardial ischemia/reperfusion (I/R) versus males. Although estrogen-suppressed cardiomyocyte apoptosis may be mediated through the PI3K/Akt, it is unclear whether this action is mediated via estrogen receptor beta (ERb). Therefore, we hypothesized that ERb mediates estrogen-induced cardioprotection through PI3K/Akt and anti-apoptotic signaling in females, but not males. Methods and Results: Isolated male and female hearts from ERb knockout (ERbKO) and wild type (WT) mice (n=5/group) were subjected to 20-minute ischemia followed by 60-minute reperfusion (Langendorff). Ablation of ERb significantly decreased post-ischemic recovery of LVDP in female hearts, but not males. Reduced activation of PI3K and Akt was noted in female ERbKO hearts, which was associated with increased expression of caspase-3 and -8, as well as decreased Bcl-2 levels compared to WT. However, ERbKO did not change myocardial STAT3, SOCS3, VEGF, TNFR1 and TNFR2 in either gender following I/R. Furthermore, deficiency of ERb increased myocardial JNK activation in females, but increased ERK1/2 activity in males during acute I/R. Conclusions: ERb mediates myocardial protection via up-regulation of PI3K/Akt activation, decreased caspase-3, -8, and increased Bcl-2 in female hearts following I/R. These findings provide evidence of ERb-mediated PI3K/Akt and anti-apoptotic signaling in the myocardium, and may lend insight into the mechanistic pathways behind the observed variation in clinical outcomes between males and females after MI.

Keywords: ischemia/reperfusion, apoptotic signaling, cardioprotection, estrogen receptor
INTRODUCTION

Myocardial ischemia/reperfusion (I/R) injury occurs during cardiac surgery and leads to production of inflammatory cytokines, as well as cardiomyocyte apoptosis and necrosis, all of which exacerbate post-ischemic myocardial dysfunction. Although there have been many studies focused on minimizing the adverse effects of I/R injury, few have resulted in significant clinical benefits. Sex differences have been noted in the myocardial response to ischemic injury with female exhibiting improved cardiac function, diminished inflammatory response and reduced apoptotic signaling (17, 19). Studies from our group and others have further demonstrated that estrogen mediates cardioprotection in females following acute I/R (10, 25). However, this notion has been challenged recently by controversial results from clinical trials which did not demonstrate a cardioprotective effect of hormone replacement therapy on post-menopausal females (16). This led to a recognition that estrogen-mediated cardioprotection appears more complicated than originally thought and requires more research.

The effects of estrogen are mediated mostly through estrogen receptor alpha (ERα) and/or estrogen receptor beta (ERβ), both of which are expressed in the heart and have been involved in regulating cardioprotection (9, 22). In fact, deficiency of the ERβ gene worsens cardiac dysfunction following myocardial ischemic injury (2, 6, 13). In addition, a selective ERβ agonist, 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), protects the myocardium from acute ischemia in ovariectomized (OVX) females (11). Furthermore, 17-beta-estradiol (E2) has been shown to suppress cardiomyocyte apoptosis via activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway during myocardial infarction (12, 15). However, it is unknown whether this action is mediated through ERβ. On the other hand, our group has previously demonstrated TNFR1 signaling resistance as well as up-regulated signal transduction and activator of transcription 3 (STAT3)/suppressor of cytokine signaling 3 (SOCS3)/VEGF
cascade in female hearts following I/R (23, 24). It is important to elucidate whether ERβ plays a role in these pathways.

Therefore, with the use of mice containing a targeted deletion of ERβ, this study aims to determine: 1) whether ERβ mediates protection of cardiac function through PI3K/Akt and anti-apoptotic signaling in females, but not males following acute I/R injury; and 2) whether ERβ may this facilitate cardioprotection via TNFR/STAT3/SOCS3/VEGF-mediated cascades.

**MATERIALS AND METHODS**

*Animals*

A total of 20 male and female C57BL/6J mice (16±4 weeks) with and without ERβ deficiency (ERβKO) (Taconic Farms, Inc., Hudson, NY) were fed a standard diet and acclimated in a quiet quarantine room for more than one month 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).

All isolated mouse hearts were subjected to the same I/R protocol as described previously (23, 24). Mouse hearts were divided into four 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 Inc., Milford, MA) and an Apple G4 PowerPC computer (Apple Computer Inc., Cupertino, CA).
Western blotting

Western blot analysis was performed to measure PI3K/Akt signaling, STAT3 activation, SOCS3 expression, apoptotic proteins (caspase-3, -8 and Bcl-2), and mitogen-activated protein kinases (MAPKs) (p38 MAPK, c-jun N-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, Saint Louis, MO) and was centrifuged at 12000 rpm for 10 minutes. 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 hour and then incubated with the following primary antibodies: PI3K, phosphor-PI3K, Akt, phosphor-Akt, STAT3, phosphor-STAT3 (Tyr705), p38 MAPK, phosphor-p38 MAPK, JNK, phosphor-JNK (Thr183/Tyr185), ERK1/2, phosphor-ERK1/2 (Thr180/Tyr182) (1:1000 dilution, Cell Signaling Technology, Beverly, MA), caspase-3, -8 and SOCS3 (1:200 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Bcl-2 (Oncogene Research Products, San Diego, CA) and GAPDH (1:5000 dilution, Biodesign International, Saco, Maine). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (Pierce, Rockford, IL) and detection 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).

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 (Duo Set ELISA Development System, R&D Systems Inc., 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 mean ± SEM. Data was compared using one-way analysis of variance (ANOVA) with post-hoc Tukey 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 probability value of less than 0.05 was considered statistically significant.

RESULTS

Myocardial function

LVDP at the end of reperfusion was exhibited as % of equilibration. I/R injury decreased myocardial LVDP in both WT and ERβKO mice (figure 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 to 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 to male WT. However, ablation of the ERβ gene neutralized this difference following I/R.

Effects of ERβ on myocardial PI3K/Akt signaling during acute ischemia/reperfusion

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% (% of p-PI3K/T-PI3K) in WT to 14.6% (figure 2A), and Akt from 190% (% of p-Akt/T-Akt) to 91.3% in female hearts (figure 2B). Although deficiency of ERβ decreased PI3K activation, the KO did not affect myocardial Akt activation in males (figure 2).

Effects of ERβ on myocardial apoptotic proteins after ischemia/reperfusion injury

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

Effects of ERβ on myocardial TNFR/STAT3/SOCS3/VEGF cascade after ischemia/reperfusion injury

Although a trend of decreased STAT3 activation was observed in female ERβKO hearts, deficiency of ERβ did not significantly change myocardial STAT3 activation, expression of TNF receptors (1 and 2), SOCS3 levels, or VEGF production in either gender after acute I/R (figure 4).

Effects of ERβ on myocardial activation of MAPKs after ischemia/reperfusion 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 (figure 5). Interestingly, enhanced JNK activation was observed in female ERβKO myocardium in response to I/R, while activation of p38 MAPK and ERK1/2 was not changed by deficiency of ERβ in females (figure 5). This 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 hypercontractile 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 when compared to males), but not in males (figure 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 non-selective ER ligand, improved functional recovery, reduced myocardial inflammation, and diminished pro-apoptotic signaling following acute ischemia in males as well as OVX females (25). Recently, deficiency of ERβ has been shown to worsen post-ischemic myocardial dysfunction in female hearts with hypercontractile condition (2). In addition, DPN, a selective ERβ agonist, restores 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 ERβKO hearts when compared to female WT after acute I/R, whereas ablation of ERβ did not affect post-ischemic 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 non-genomic events trigged 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 non-genomic actions of E2 in various types of cells (18). Up-regulation 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 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 non-genomic effect of PI3K/Akt activation. In this study, we found significantly reduced activation of the PI3K/Akt pathway in female ERβKO hearts, but not 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 pro-apoptotic proteins caspase-3 and -8, while reducing anti-apoptotic protein Bcl-2 levels in female hearts in response to acute I/R.

E2-regualted Akt activation may also 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, while 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 has also 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 through 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.
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); administration of E2 up-regulates STAT3 activation, which partially mediates non-genomic effects of E2 (1). Therefore, the question arises whether ERβ is involved in regulating myocardial STAT3/SOCS3/VEGF cascades in females. In this study, we found that ablation of ERβ did not affect myocardial expression of TNFR1 or TNFR2. Additionally, ERβKO did not change myocardial STAT3/SOCS3/VEGF signaling following I/R. However, further investigation is required to determine whether the myocardial TNFR/STAT3/SOCS3/VEGF cascade is related to E2-mediated cardiac protection, and if so, whether ERα plays a role in this action.

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 and 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 up-regulate activation of the protective ERK ½, decrease the pro-apoptotic JNK activation, and improve myocardial function in females during acute ischemia (22). Herein, we further found significantly increased JNK activation in female ERβKO hearts, while 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.

**ACKNOWLEDGEMENTS**

This work was supported in part by NIH R01GM070628, NIH R01HL085595 and NIH K99/R00 HL0876077.

**DISCLOSURES**

None.
REFERENCES


FIGURE LEGENDS

Figure 1. Myocardial left ventricular developed pressure (LVDP) at the end of ischemia/reperfusion (I/R) (represented as % of equilibration) in wildtype (WT) and estrogen receptor beta knock out (ERβKO) of male and female. Deficiency of the ERβ gene decreased post-ischemic myocardial LVDP in female hearts, but not males. Results are mean ± SEM, n=5/group, *p<0.05 vs. MWT, #p<0.05 vs. F WT.

Figure 2. Activation of myocardial PI3K/Akt signaling in male and female wildtype (WT) and estrogen receptor beta knock out (ERβKO) hearts following I/R. A: Ablation of ERβ reduced PI3K activation in both genders, but with relatively greater effect in female hearts. B: ERβKO decreased myocardial Akt activity only in female hearts, but not males. Representative immunoblots (2 lanes/group) are shown on the left panel. Densitometry data (% of phosphor-/total-) are on the right. Results are mean ± SEM, *p<0.05, **p<0.01 vs. the corresponding WT. Experiments were repeated in two different sessions.

Figure 3. The expression of myocardial caspase-3, caspase-8 and Bcl-2 in ERβKO and wildtype hearts of both genders after I/R. A: Increased caspase-3 activity was noted in female ERβKO hearts, but not males. B: Deficiency of the ERβ gene increased caspase-8 activity in female hearts, but decreased its activity in males following I/R. C: ERβKO decreased anti-apoptotic protein Bcl-2 in female hearts. All shown are representative immunoblots (2 lanes/group) on the left and densitometry bar graph (% of GAPDH) on the right. Mean ± SEM, *p<0.05, **p<0.01 vs. M WT, ##p<0.01 vs. F WT. Experiments were repeated in two different sessions.

Figure 4. Activation of myocardial STAT3 (A), expression of SOCS3 (B), TNFR1 (C), TNFR2 (C) and VEGF (D) in ERβKO and wildtype hearts of both genders after I/R. ERβKO did not
affect myocardial TNFR/STAT3/SOCS3/VEGF cascades following I/R. Representative immunoblots (2 lanes/group) are shown on the left of A, B and C. Densitometry bar graph is on the right. D shows ELISA data of VEGF. Results are mean ± SEM.

**Figure 5.** Activation of myocardial p38 MAPK (A), JNK (B), ERK1/2 (C) (represented as % of phosphor-/total-) in ERβKO and wildtype hearts of both genders after I/R. Ablation of ERβ did not affect myocardial p38 MAPK activation in either gender (A). However, ERβKO significantly increased JNK activation in female hearts (B) and elevated ERK1/2 activity in males following I/R (C). All shown are representative immunoblots (2 lanes/group) on the left and densitometry bar graph on the right. Mean ± SEM, *p<0.05 vs. the corresponding WT. Experiments were repeated in two different sessions.

**Figure 6.** Simplified schematic illustrates how the ERβ mediates activation of PI3K/Akt and apoptotic pathway, and thus, protects the female myocardium in response to ischemia/reperfusion. ERβ-regulated JNK and ERK1/2 signaling is also shown here.
Figure 1

[Bar chart showing comparison of Post-ischemic LVDP (% of Eq) between different groups (M WT, M ErbKO, F WT, F ErbKO).]
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-PI3K</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-PI3K</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Akt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-Akt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**p-PI3K/T-PI3K (%)**

**p-Akt/T-Akt (%)**
Figure 3

A

![Western Blot Images]

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>GAPDH</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

B

![Western Blot Images]

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-8</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>GAPDH</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

C

![Western Blot Images]

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>GAPDH</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
Figure 4

A

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-STAT3</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>T-STAT3</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

![Graph](graph1.png)

B

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS3</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>GAPDH</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
</tr>
</tbody>
</table>

![Graph](graph2.png)

C

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFR1</td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
</tr>
<tr>
<td>TNFR2</td>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>GAPDH</td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
</tr>
</tbody>
</table>

![Graph](graph3.png)
D

![Graph showing VEGF levels for different genotypes](image)
Figure 5

A

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-p38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-p38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-JNK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-JNK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>MWT</th>
<th>M ERβKO</th>
<th>FWT</th>
<th>F ERβKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ERK1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-ERK1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p=0.08 vs. MWT
Figure 6

Ischemia/reperfusion

↑ PI3K/Akt

Cell death

Apoptosis

Dysfunction

Cardiomyocyte

Caspase-8, -3

Bcl-2

E2

Member associated ERβ

PI3K/Akt

JNK

ERK1/2

Apoptosis

Dysfunction