Mechanisms of Sex Differences in TNFR2-Mediated Cardioprotection

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**Background**—TNFR1/TNFR2 signaling may mediate different cellular and molecular responses (injury versus protection) and the balance may be affected by sex hormones. Previous studies have shown that females have improved myocardial functional recovery, TNFR1 signaling resistance, and increased SOCS3 expression after acute ischemia/reperfusion when compared with males. However, it is unknown whether the TNFR2 pathway protects the myocardium from ischemia/reperfusion injury, and if so, whether sex differences exist in TNFR2-mediated cardioprotection. Therefore, we hypothesized that (1) TNFR2 mediates myocardial protection from ischemia/reperfusion through STAT3, SOCS3, and vascular endothelial growth factor in both sexes; and (2) TNFR2 elicits greater protective signaling in females compared with males.

**Methods and Results**—Isolated male and female mouse hearts from TNFR2 knockout, TNFR1/2 knockout, and wild-type (C57BL/6J or B6129SF2/J; n = 5 to 6/group) were subjected to 20 minutes ischemia followed by 60 minutes reperfusion. TNFR2 deficiency decreased postischemic myocardial recovery in both sexes but had a greater effect on females. The deleterious effects of TNFR2 ablation were associated with a decrease in mRNA and protein levels of SOCS3, STAT3, and vascular endothelial growth factor as well as an increase in myocardial interleukin-1-beta production in female hearts. However, a significant increase in JNK activation and interleukin-1-beta protein levels was noted in male TNFR2KO hearts after ischemia/reperfusion. Additionally, TNFR1/2 knockout decreased myocardial function in female hearts but not males. This observation was associated with a decrease in mRNA levels of SOCS3, STAT3, and vascular endothelial growth factor and an increase in myocardial p38 mitogen-activated protein kinase activation in females.

**Conclusions**—Sex differences in the mechanisms of TNFR2-mediated cardioprotection occur by increasing STAT3, SOCS3, and vascular endothelial growth factor in females and by decreasing JNK in males. (Circulation. 2008;118[Suppl 1]:S38–S45.)

**Key Words:** ischemia ■ sex ■ tumor necrosis factor receptor

Proinflammatory signaling has been implicated in the pathophysiology of a number of conditions related to cardiothoracic surgery.1–4 Interestingly, sex differences have been observed in myocardial proinflammatory signaling after ischemia and reperfusion injury (I/R). Cardiothoracic surgeons recognize that men and women respond differently to cardiothoracic surgical procedures. Indeed, clinical differences in sex-related outcomes appear to be multifactorial and complex. Clinically, women have a lower overall incidence of heart failure, slower heart failure progression, better age-matched cardiac contractility, and better preservation of myocardial mass as they age compared with men.5 Indeed, our previous animal studies have demonstrated that females have better myocardial functional recovery, decreased proinflammatory cytokine production, and reduced apoptotic signaling after I/R.6,7

Ischemia and reperfusion injury results in substantial amounts of myocardial proinflammatory cytokine production such as tumor necrosis factor α-α (TNF). Numerous studies have demonstrated that TNF contributes to alterations in calcium homeostasis, stimulation of cardiomyocyte apoptosis as well as induction of other cytokines associated with myocardial dysfunction after acute ischemia.8 Interestingly, although animal studies performed by several different investigators have shown that decreasing the bioavailability of TNF has beneficial effects after I/R,9,10 others have suggested that TNF may actually be protective.11 Indeed, clinical studies have indicated that simply decreasing the bioavailability of TNF in patients with heart failure appears to lack benefit.12,13 These observations have led to the important appreciation that TNF may mediate different cellular and molecular responses (injury versus protection) depending on which of its receptors are activated.

TNF initiates its biological actions by binding to a 55-KDa receptor (TNFR1) and/or a 75-KDa receptor (TNFR2), both of which are present on cardiac myocytes.14 The majority of...
TNF-induced myocardial dysfunction and myocyte apoptosis is initiated by the activation of TNFR1. Ablation of TNFR1 has been reported to improve myocardial function and survival in mice after myocardial infarction, whereas ablation of TNFR2 has been associated with increased heart failure rates and a reduction in survival after infarction. A previously published study from our laboratory has also shown that improved myocardial functional recovery in females was associated with TNFR1 signaling resistance after I/R. However, it remains unknown whether TNFR2 plays a role in the sex differences observed in the myocardial response to I/R. Therefore, we hypothesized that TNFR2 signaling plays a cardioprotective role in the myocardial response to I/R through STAT3, SOCS3 and vascular endothelial growth factor (VEGF) in both sexes but with a relative greater benefit in females.

Materials and Methods

Statement of Responsibility

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Animals

C57BL/6J mice with and without targeted deletion of TNFR2 (TNFR2 knock out [KO] and wild-type [WT]) and B6129SF2/J mice with and without targeted deletion of TNFR1/2 (TNFR1/2 KO and WT) of both sexes (Jackson Laboratories, Bar Harbor, Maine) were fed a standard diet and acclimated in a quiet quarantine room for 2 months 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 (National Institutes of Health publication No 85-23, revised 1985).

Isolated mouse hearts were divided into: (1) male WT; (2) male TNFR2 KO; (3) male WT/H11032; (4) male TNFR1/2 KO; (5) female WT; (6) female TNFR2 KO; (7) female WT/H11032; and (8) female TNFR1/2 KO and subjected to 15-minute equilibration period, 20 minutes of global ischemia (37°C), and 60 minutes of reperfusion (n=5 to 6 animals/group).

Isolated Heart Preparation (Langendorff)

Experiments were performed with the use of a Langendorff apparatus as described previously for use in mouse heart. Briefly, mice were anesthetized (sodium pentobarbital, 60 mg/kg intraperitoneally) and heparinized (500 U intraperitoneally), and hearts were rapidly excised through median sternotomy and placed in 4°C Krebs-Henseleit solution. The aorta was cannulated and the heart was perfused (90 mm Hg) with oxygenated (95% O2/5% CO2) Krebs-Henseleit solution (37°C). Data were continuously recorded using a PowerLab 8 preamplifier/digitizer (AD Instruments Inc, Milford, Mass) and an Apple G4 PowerPC computer (Apple Computer Inc, Cupertino, Calif).

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from each heart’s left ventricle using RNA STAT-60 (TEL-TEST, Friendswood, Texas). A total of 0.5 μg of total RNA was subjected to cDNA synthesis using a cloned AMV first-strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, Calif.).
bad, Calif). cDNA from each sample was analyzed for 18S (assay ID# Hs99999901_s1), TNF (assay ID# Mm00443258_m1), interleukin-6 (IL-6; assay ID# Mm00446190_m1), VEGFa (assay ID# Mm00437304_m1), SOCS3 (assay ID# Mm00545913_s1), and STAT3 (assay ID# Mm00456961_m1) by using TaqMan gene expression assay (real-time polymerase chain reaction; Applied Biosystems, Foster City, Calif). Densitometry was performed to assess relative quantity and represented as a ratio to male WT control (without I/R).

Enzyme-Linked Immunosorbent Assay
Myocardial TNF, IL-6, IL-1β, and VEGF in the cardiac tissue were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA set (BD Opt EIA ELISA set; BD Biosciences Pharmingen, San Diego, Calif, and Duo set ELISA Development System; R&D Systems Inc, Minneapolis, Minn). ELISA was performed according to the manufacturer’s instructions. All samples and standards were measured in duplicate.

Western Blotting
Heart tissue was homogenized in cold buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin, and 1 mmol/L PMSF and centrifuged at 12 000 rpm for 5 minutes. The protein extracts (20 μg/lane) were subjected to electrophoresis on a 4% to 12% tris-Hepes gel from Pierce and transferred to a nitrocellulose membrane. The membrane was incubated in 5% dry milk for 1 hour and then incubated with the following primary antibodies: STAT3, phosphor-STAT3 (Tyr705), p38 mitogen-activated protein kinase (MAPK), phosphor-p38 MAPK (Thr180/Tyr182), JNK, phosphor-JNK (Thr183/Tyr185), ERK1/2 and phosphor-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology, Beverly, Mass), caspase-3, -8, and SOCS-3 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), and GAPDH antibody (Biosdesign International, Saco, Maine) followed by incubation with horseradish peroxidase–conjugated goat antirabbit or antimouse IgG secondary antibody and detection using Supersignal West Pico Stable Peroxide solution (Pierce, Rockford, Ill). Films were scanned using an Epson Perfection 3200 Scanner (Epson America, Long Beach, Calif) and band density was analyzed using ImageJ software (National Institutes of Health).

Presentation of Data and Statistical Analysis
All reported values are mean±SEM. Data were compared using 2-way analysis of variance with post hoc Bonferroni test or Student t test. A 2-tailed probability value of <0.05 was considered statistically significant.

Results
Effects of TNFR2 and TNFRI/2 on Myocardial Function After Ischemia–Reperfusion
No differences in baseline absolute ±dP/dt were observed during equilibration among all groups. TNFRI/2 deficiency further impaired myocardial function after I/R, which was exhibited by depressed +dP/dt and −dP/dt in both male and female mouse hearts. Additionally, TNFRI/2 KO neutralized sex differences in myocardial functional recovery (Figure 1). Interestingly, ablation of both TNFR1 and TNFRI/2 genes decreased recovery of postischemic myocardial function in females, but not in males (Figure 2), which resulted in a lower recovery of +dP/dt and −dP/dt in female double KOs compared with males after I/R.
Effects of Tumor Necrosis Factor Receptors on Myocardial Tumor Necrosis Factor, Interleukin-6 and Interleukin-1β after Ischemia and Reperfusion

Ischemia-induced production of myocardial TNF, IL-6, and IL-1β has been observed in our previous studies as well as the present one (data not shown). Ablation of TNFR2 or TNFR1/2 did not significantly affect mRNA and protein levels of myocardial TNF and IL-6 in male and female hearts after I/R (data not shown). However, deficiency of TNFR2 increased myocardial IL-1β protein levels by 2-fold in males and by 3-fold in females (Figure 3A), whereas TNFR1/2 KO hearts did not show any significant difference in IL-1β levels (Figure 3B).

Ablation of TNFR2 or TNFR1/2 significantly decreased VEGFa mRNA by 43% or 56% in female hearts, but not in males (Figure 4A–B). Additionally, markedly reduced VEGF protein levels were noted in female TNFR2 KO compared with female WT and male TNFR2 KO (Figure 4C). Furthermore, a trend of lower protein levels of VEGF was observed in female TNFR1/2 KO hearts after I/R (Figure 4D). However, this observation was not seen in males (Figure 4C–D).

Effects of Tumor Necrosis Factor Receptors on Myocardial SOCS3 and STAT3 After Ischemia and Reperfusion

Our results indicated that TNFR2 and TNFR1/2 ablation significantly decreased mRNA levels of SOCS3 and STAT3 in females, but not in males (Figure 5A–B, E–F). Female WT (C57BL/6J) had higher protein levels of SOCS3 and p-STAT3 compared with male WT, which was neutralized by deficiency of TNFR2 in female hearts, whereas ablation of TNFR2 did not significantly reduce SOCS3 levels and STAT3 activation in males (Figure 5C, G). In contrast, TNFR1/2 KO increased SOCS3 protein levels and had no effect on myocardial STAT3 activation in both sexes (Figure 5D, H).

Effects of Tumor Necrosis Factor Receptors on Mitogen-Activated Protein Kinases After Ischemia and Reperfusion

Ablation of the TNFR2 gene did not change activation of p38 MAPK in the myocardium subjected to I/R in either sex (Figure 6A). However, double knockout of TNFR1 and TNFR2 increased myocardial p38 MAPK activation in female hearts, but not in males (Figure 6B). Interestingly,
significantly augmented myocardial JNK activation was noted in male TNFR2 KO hearts, but not in male TNFR1/2 KO (Figure 6C–D). In addition, neither TNFR2 nor TNFR1/2 deficiency affected myocardial JNK activation in females after I/R. Furthermore, ablation of TNFR2 or TNFR1/2 had no effects on myocardial ERK activation in both sexes (Figure 6E–F).

**Effects of Tumor Necrosis Factor Receptors on Proapoptotic Signaling After Ischemia and Reperfusion**

To study the role of TNF receptors in the myocardial response to I/R, it was necessary to determine the degree of apoptosis given that the TNF receptor is usually recognized as a death receptor. However, our experimental period was too brief to detect significant apoptosis in the myocardium. Therefore, we examined myocardial expression of caspase-8 and caspase-3 by Western blot. Deficiency of TNFR2 and TNFR1/2 KO had no significant effects on caspase-8 and caspase-3 expression in males after I/R (Figure 7A–D). Conversely, TNFR2 and TNFR1/2 ablation appeared to increase myocardial caspase-3 levels in females (Figure 7C–D).

**Discussion**

Sex differences in cardiothoracic surgical outcomes and heart failure progression is widely accepted as a clinical given.5 To investigate the potential signaling mechanisms involved, we conducted the present studies and the results indicated the...
following: (1) ablation of the TNFR2 gene decreased myocardial functional recovery in both males and females; (2) double KO of TNFR1 and TNFR2 reduced postischemic functional recovery only in female hearts; and (3) sex differences in the mechanisms of TNFR2-mediated cardioprotection after ischemia occur by increasing STAT3, SOCS3, and VEGF in females and by decreasing JNK in males.

Cardiac myocytes and macrophages produce substantial amounts of TNF in response to I/R. The biological function of TNF is regulated through 2 structurally distinct receptors: TNFR1 and TNFR2, both expressed in cardiac myocytes. The majority of the deleterious effect of TNF on the myocardium is clearly mediated by TNFR1, including short-term negative inotropic effects and long-term TNF-induced apoptosis.8 Conversely, TNFRII signaling appears to conduct protective effects.15,16 The results of this study as well as that of previous literature would suggest that the balance of TNFR1 and TNFR2 signaling in female hearts may be shifted to favor salutary effects of TNF conducted by TNFR2 during I/R. Our data suggest that enhanced TNFR2 signaling may exist in female hearts subjected to acute I/R. However, it is unclear as to which downstream signals TNFR2 targets to improve myocardial function.

Inflammatory cytokines undoubtedly play a critical role in mediating postischemic myocardial dysfunction.17 In this regard, TNF is also important in initiating the reperfusion-dependent cytokine release seen in myocardial I/R.18 Thus, it

**Figure 6.** The expression of activated myocardial p38 MAPK, JNK, and ERK1/2 and total p38 MAPK, JNK, and ERK1/2 after I/R in TNFR2 KO, TNFR1/2 KO, and WT of both sexes. Representative immunoblots (2 lanes/group) and densitometry data of phosphorlated p38 MAPK (percent of total p38 MAPK) indicate p38 MAPK activation in TNFR2 KO (A) and TNFR1/2KO hearts (B) after I/R. Phosphorylated JNK (activation) and nonphosphorylated (total) JNK are shown in C (TNFR2 KO) and D (TNFR1/2 KO). Representative immunoblots (2 lanes/group) and densitometry bar graph of phosphorylated ERK (percent of total ERK) demonstrate active myocardial ERK in TNFR2 KO (E) and TNFR1/2 KO (F). Mean±SEM, n=4 to 6/group. *P<0.05 versus male WT, **P<0.01 versus female WT, #P<0.05 versus male WT and @P<0.05 versus male TNFR2 KO.
can be postulated that TNFR2 may mediate myocardial protection through downregulation of other proinflammatory cytokines or the upregulation of beneficial growth factors. Indeed, there is evidence that ablation of TNFR2 exaggerated myocardial dysfunction accompanied by its upregulation of IL-6 and IL-1β in noninfarced myocardium compared with WT in a murine myocardial infarction model. In addition, impaired posts ischemic recovery has been associated with a decrease in both mRNA and protein levels of VEGF in TNFR2 KO muscle tissue. Here, we found that deficiency of the TNFR2 gene increased myocardial IL-1β protein levels in both male (by approximately 2-fold) and female (by approximately 3-fold) hearts after I/R. However, there were no significant differences in expression of TNF and IL-6 between TNFR2 KO and WT mice (data not shown). Interestingly, ablation of TNFR2 reduced myocardial VEGF expression in females, but not in males. Therefore, it is possible that the combination of upregulated IL-1β and decreased VEGF production in females caused a greater degree of myocardial dysfunction in female hearts compared with WT. However, TNFR1/2 KO did not show similar effects on myocardial IL-1β and VEGF production (except VEGFα mRNA in female hearts) after I/R.

Emerging evidence suggests that suppressor of cytokine signaling protein 3 (SOCS3) may be induced by various stimuli and may participate in the important process of controlling proinflammatory signals. Our previous study has demonstrated that a deficiency of TNFR1 increased myocardial SOCS3 protein levels in males but not in females after I/R. We further found that ablation of TNFR2 significantly reduced SOCS3 protein in females, whereas double KO of TNFR1 and TNFR2 augmented myocardial SOCS3 levels in both sexes. These data suggest that TNFR1 signaling may downregulate SOCS3 expression in both sexes, whereas TNFR2 signaling may represent a compensatory mechanism to upregulate SOCS3 expression in females.

On the other hand, the signal transducer and activator of transcription 3 (STAT3) pathway exerts cardioprotective signaling in the ischemic heart, most likely through the induction of growth factors, the suppression of apoptosis, and the upregulation of SOCS3. In this study, females exhibited higher levels of myocardial SOCS3 and STAT3 activation compared with male hearts (C57BL/6J) in response to I/R. In addition, ablation of TNFR2 significantly decreased SOCS3 and STAT3 (mRNA and protein) in female hearts after I/R. These results suggest that improved posts ischemic myocardial function in females is likely associated with TNFR2 upregulated STAT3 activation and SOCS3 expression in females. Furthermore, overexpression or activation of STAT3 in cardiomyocytes has been shown to increase myocardial VEGF production. Therefore, it is possible that TNFR2 ablation decreased the STAT3 pathway and thereby reduced myocardial VEGF production (both mRNA and protein) in females. These data also result in an appreciation that TNF receptors might regulate VEGF expression differently between male and female hearts after I/R.

Recent evidence also suggests that TNF-mediated activation of p38 MAPK and JNK occurs through both TNFR1 and TNFR2 in bronchial smooth muscle. Additionally, our previous work has indicated that deficiency of TNFR1 decreased myocardial p38 activation in males. We further demonstrated that ablation of TNFR2 did not change p38 activation after I/R. Taken together, these data would suggest that I/R-induced p38 MAPK activation is more likely mediated through TNFR1 in both sexes. However, noting that females have an inherent resistance to TNFR1 signaling after
I/R, it is not surprising that TNFR1/2 ablation increased myocardial p38 MAPK activation in females, but not males. This result is consistent with impaired myocardial function in female TNFR1/2 KO hearts after I/R. On the other hand, TNFR2 KO significantly increased myocardial JNK activation, whereas TNFR1/2 ablation appeared to reduce activation of JNK in male hearts. These data suggest that TNFR1 may be responsible for activation of JNK or TNFR2 may downregulate myocardial JNK activation in males after I/R. Moreover, we found that neither TNFR2 ablation nor TNFR1/2 ablation affected myocardial ERK activation in either sex after I/R.

TNF binding to TNFR1 results in recruitment and activation of procaspase-8. Caspase-8 then activates downstream caspase-3 and induces the classic extrinsic death pathway. In this study, our data suggest that TNFR2 ablation and TNFR1/2 KO had no significant effects on caspase-8 and caspase-3 expression in males after I/R. Those results are inconsistent with previous studies in that TNFR1 is required for caspase-8-mediated extrinsic proapoptotic signaling. This discrepancy likely resulted from a limitation of our experimental model, a short experimental time period.

**Sources of Funding**

This work was supported in part by National Institutes of Health (NIH) R01GM070628 (D.R.M.), NIH R01HL085595 (D.R.M.), NIH K99/R00 HL087607 (M.W.), American Heart Association Grant-in-aid (D.R.M.), and American Heart Association Postdoctoral Fellowship 0725663Z (P.R.C.).

**Disclosures**

None.

**References**

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Circulation. 2008;118:S38-S45
doi: 10.1161/CIRCULATIONAHA.107.756890
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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