Tumor Necrosis Factor Receptor 1 Signaling Resistance in the Female Myocardium During Ischemia

Meijing Wang, MD, MS; Ben M. Tsai, MD; Paul R. Crisostomo, MD; Daniel R. Meldrum, MD

Background—Tumor necrosis factor-α (TNF) is increased in myocardial tissue after ischemia and reperfusion (I/R). TNF contributes to postischemic myocardial dysfunction and induces proinflammatory signaling, which may be mediated by the 55-kDa TNF receptor (TNFR1). In humans, there is a direct correlation between functional capacity, survival, and circulating TNF levels. Although decreasing the TNF level in animals was beneficial after myocardial ischemia, simply decreasing the bioavailability of TNF in humans with heart failure was not beneficial. This led to the important appreciation that TNF may have beneficial or deleterious effects in the heart, depending on which of its receptors is activated. Females have a lower incidence of heart failure and a higher heart failure survival than males. We hypothesized that TNFR1 signaling resistance occurs in the female myocardium during ischemia.

Methods and Results—Hearts from male and female TNFR1-knockout and wild-type (WT) mice were subjected to I/R. Female WT mice had better postischemic recovery than did male WT mice, an effect that appeared to be due to TNFR1 signaling resistance in females. Female WT mice had less myocardial depression after TNF infusion despite equivalent TNFR1 expression. Interestingly, TNFR1 ablation improved postischemic myocardial function, decreased activation of p38 mitogen-activated protein kinase, and reduced expression of interleukins-1β and -6 in males but not in females. Furthermore, WT females expressed more of the suppressor of cytokine signaling protein 3 after I/R, which may in part explain TNFR1 signaling resistance in the female myocardium.

Conclusions—This study demonstrates that sex differences exist in myocardial TNF signaling by TNFR1 after I/R. (Circulation. 2006;114[suppl I]:I-282–I-289.)

Key Words: ischemia ■ receptors ■ sex

Sex-specific differences have been noted in cardiovascular disease. Females have a lower incidence of heart failure and higher heart failure survival than do males. Myocardial ischemia and reperfusion (I/R) is a leading cause of heart failure and death in civilized countries. Tumor necrosis factor-α (TNF) may play an important role in the development of heart failure. In humans, there is a direct correlation between functional capacity, survival, and circulating TNF levels. Cardiac myocytes themselves and heart mast cells produce substantial amounts of TNF in response to ischemia. TNF is upregulated systemically and in the myocardium during myocardial ischemia. Indeed, locally produced TNF may be an important contributor to postischemic myocardial dysfunction and cardiomyocyte apoptosis, as well as play an important role as an upstream cytokine inducer. Animal studies performed by many different investigators clearly showed that decreasing the bioavailability of TNF by various means has beneficial effects after acute I/R. However, clinical studies have indicated that simply decreasing the bioavailability of TNF in heart failure patients appears to lack benefit. This led to the important appreciation that TNF itself may have beneficial or detrimental effects, depending on which of its receptors is activated.

It is now recognized that TNF acts by binding to a 55-kDa receptor (TNFR1) and/or a 75-kDa receptor (TNFR2). Although these 2 receptors induce both distinct and overlapping responses, TNF-induced myocardial contractile dysfunction and TNF-induced cardiac myocyte apoptosis are initiated by binding to TNFR1. Cardiac dysfunction and myocardial infarct size have been reduced by using anti-TNF antibody to neutralize local TNF release from cardiac myocytes and by using soluble TNFR1 (sTNFR1) to block the binding of TNF to TNFR1 after myocardial infarction. Recent evidence demonstrates significantly improved myocardial function in TNFR1-knockout mice (TNFR1KO) compared with wild-type (WT) mice after myocardial infarction. In addition, females show better survival and reduced myocardial infarct size than do males in normal WT mice after myocardial infarction, but there were no sex-related differences in the expression of TNFR1 protein and TNF in the myocardium. This suggests that the female survival advantage may be associated with their resistance to myocardial TNFR1 signaling. Therefore, we hypothesized that TNFR1 signaling resistance exists in female myocardium during I/R.
Methods

Animals
C57BL/6j mice (mean±SD weight, 24.8±0.81 g at 16±2 weeks of age; 20 with targeted deletion of TNFR1 [TNFR1KO] and 16 WT) of both sexes from Jackson Laboratories, Bar Harbor, Me) 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 (NIH publication No. 85-23, revised 1985).

Isolated Heart Preparation (Langendorff)
Mice were anesthetized (sodium pentobarbital, 60 mg/kg IP) and heparinized (500 U IP), and hearts were rapidly excised via 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 with a PowerLab/digitizer (AD Instruments Inc, Milford, Mass) and an Macintosh G4 computer (Apple Computer, Cupertino, Calif).

Isolated WT (n=3/sex) and TNFR1KO (n=4/sex) mouse hearts were subjected to TNF infusion (500 pg/mL per min) for 30 minutes after equilibration. Others were divided into 4 groups (male WT n=5, male TNFR1KO n=6, female WT n=5, and female TNFR1KO n=6) and subjected to the same I/R protocol: a 15-minute equilibration period, 20 minutes of global ischemia (37°C), and 60 minutes of reperfusion.

Reverse Transcription–Polymerase Chain Reaction
Total RNA was extracted from the left ventricle with RNA STAT-60 (Tel-Test, Friendswood, Tex): 0.1 µg of total RNA was subjected to cDNA synthesis with a cloned AMV first-strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, Calif). The cDNA from each sample was used for polymerase chain reaction (PCR) for TNF, interleukin (IL)-1β, and IL-6 with the use of dual quantitative PCR kits (Maxim Biotech, South San Francisco, Calif). PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Densitometry was performed to assess relative quantity and represented as a ratio to the housekeeping gene.

Myocardial TNF, TNFR1, and TNFR2
Myocardial TNF, TNFR1, and TNFR2 in cardiac tissues were determined by ELISA with a commercially available ELISA set (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 phenylmethylsulfonyl fluoride, and centrifuged at 12,000 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. The membranes were blocked in 5% dry milk for 1 hour and incubated with the following primary antibodies: p38 mitogen-activate protein kinase (MAPK), phosphor-p38 MAPK (Thr180/Tyr182; 1:1000), c-Jun NH2-terminal kinase (JNK; 1:1000), phosphor-JNK (1:1000), extracellular signal-regulated kinase (ERK1/2; 1:1000), phosphor-ERK1/2 (1:1000) (all from Cell Signaling Technology, Beverly, Mass), TNFR1 (1:100), and suppressor of cytokine signaling protein-3 (SOCS-3; 1:100) (the last 2 from Santa Cruz Biotechnology, Santa Cruz, Calif), followed by incubation with horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse IgG secondary antibody and detection with supersignal west pico stable peroxide solution (Pierce, Rockford, Ill). Films were scanned and band density was analyzed with NIH Imagej software.

Results

Sex-Related Differences Existed in Myocardial Function After I/R
I/R resulted in markedly impaired left ventricular developed pressure (LVDP), +dP/dt, and −dP/dt in male and female WT (n=5/sex) and TNFR1KO (n=6/sex) mouse hearts. A, LVDP; B, +dP/dt; C, −dP/dt. Results are mean±SEM and are represented as the percentage of equilibration (eq). *P<0.05 vs male I/R.

Figure 1. Myocardial function after I/R in male (n=5) and female (n=5) mouse hearts. A, LVDP; B, +dP/dt; C, −dP/dt. Results are mean±SEM and are represented as the percentage of equilibration (eq). *P<0.05 vs male I/R.
WT mouse hearts. LVDP, +dP/dt, and −dP/dt were significantly different between male and female WT mice after I/R (Figure 1). Recovery of LVDP, +dP/dt and −dP/dt in the postischemic period was significantly higher in females than in males.

Expression of Myocardial TNF and TNFR1 After I/R
ELISA results showed no difference in the level of myocardial TNF protein among male and female WT or TNFR1KO mice (Figure 2A). In addition, the expression of myocardial TNF1 protein demonstrated no sex-specific differences in WT mice by Western blotting and ELISA (Figure 2B and 2C). Not surprisingly, the level of TNF1 was not detectable in TNFR1KO mice by ELISA (Figure 2C). Furthermore, no sex differences existed in myocardial TNFR2 after I/R (Figure 2D).

TNF Decreased Myocardial Function Through TNFR1
TNF infusion resulted in a progressive decrease in myocardial function in WT mice. During the 10-minute equilibration period, hearts maintained a stable LVDP, +dP/dt, and −dP/dt, which were significantly depressed by 30 minutes of TNF infusion in both sexes (Figure 3A through 3C). However, exposure to equivalent amounts of TNF resulted in increased myocardial function in female WT hearts compared with males. Interestingly, sex differences disappeared in myocardial function in response to TNF infusion in TNFR1KO mice (Figure 3 E through 3G). TNFR1KO mice had improved myocardial function when exposed to TNF compared with WT in males.

Effect of TNFR1 on Myocardial Function After I/R
Sex-specific differences in myocardial function were diminished in the TNFR1KO in response to I/R (Figure 4). TNFR1 deficiency resulted in improved postischemic recovery of myocardial function compared with that in WT males but not in females after I/R.

Myocardial MAPK Signaling Pathway After I/R
The phosphorylated/active form of p38 MAPK was markedly decreased in female WT compared with male WT mice after I/R (Figure 5). TNFR1 deficiency resulted in significantly lower activation of p38 in males but not in females. In addition, there were no sex-specific differences in activation of JNK and ERK1/2 in both WT and TNFR1KO mice after I/R.

Effect of TNFR1 on Expression of Myocardial Proinflammatory Cytokines After I/R
Myocardial tissue TNF and IL-1β mRNA levels were significantly lower in females compared with males (Figure 6).
TNFR1 did not change TNF gene expression in either sex. However, TNFR1 deficiency decreased the expression of IL-1β and IL-6 mRNAs in males but not in females after I/R injury.

Expression of SOCS-3 Protein in Mouse Hearts After I/R
After I/R, sex-specific differences were found in SOCS-3 protein level in WT mouse hearts (Figure 7). The SOCS-3 protein level was significantly increased in male TNFR1KO compared with male WT mice after I/R. However, TNFR1 deficiency did not affect myocardial SOCS-3 expression in female hearts after I/R.

**Discussion**
Cardiac myocytes themselves produce substantial amounts of TNF in response to I/R. Locally produced myocardial TNF has a direct effect on myocardial dysfunction. Improved myocardial function and reduced infarct size have been observed in mice lacking TNF during I/R in an in vivo model. In the present study, we demonstrated that sex-related differences exist in postischemic recovery of myocardial contractile function in WT mice. However, there were no sex differences in the levels of TNFR1 or the expression of TNF after I/R. In addition, females had less myocardial contractile dysfunction when exposed to equivalent doses of TNF compared with males. These results suggest that females are resistant to myocardial TNF signaling after I/R. This is the first study to demonstrate that sex-related differences exist in myocardial TNF signaling after I/R.

It is now recognized that TNFR1 and/or TNFR2 engagement is essential for many of the biological responses of TNF. Both receptors exist in most cell types, including cardiac myocytes. However, the majority of TNF effects, including TNF-induced myocardial contractile dysfunction and TNF-induced cardiac myocyte apoptosis, are initiated by binding to TNFR1. Moreover, ablation of TNFR1 improved myocardial function and survival in transgenic mice with overexpression of myocardial TNF. Myocardial function in TNFR1KO mice was significantly improved compared with WT mice after myocardial infarction. In addition, using sTNFR1 improved cardiac function and decreased myocardial infarct size by blocking the binding site of TNF in the cell membrane. Our data in male mice were consistent with those previous studies. However, we also demonstrated that a TNFR1 deficiency exerted disparate effects in males compared with females after I/R. In contrast to males, TNFR1 deficiency had no effect on postischemic myocardial recov-
ery in females. Furthermore, TNFR1 and TNF levels were not significantly different in males and females. This suggested that the improved postischemic myocardial functional recovery in females may be attributed to their resistance to myocardial TNF signaling initiated by TNFR1. A potential explanation for this sex-related disparity is that increased binding of sTNFR1 to TNF decreases the effect of I/R-induced TNF on myocardial dysfunction in females, because higher levels of sTNFR1 have been demonstrated in females compared with males.15

The question then becomes which downstream signals are targeted by TNF through TNFR1. MAPKs are important mediators in multiple physiological and pathophysiological processes. They transduce signals from the cell surface to the intracellular plasm and the nucleus. Three MAPKs have been identified in mammals: p38 MAPK, JNK, and ERK. Previously, we have demonstrated that myocardial I/R injury resulted in elevated expression of TNF with activation of myocardial p38 MAPK, whereas p38 MAPK inhibition led to improved myocardial function after I/R injury. Recent evidence suggests that TNF-mediated activation of p38 MAPK and JNK occurs via both TNFR1 and TNFR2 in airway smooth muscle.16 However, TNF does not cause activation of ERK.16 In contrast, other studies have shown that TNF-stimulated activation of p38 MAPK is mediated by TNFR1 alone and that JNK activation by TNFR1 is more transient than that of TNFR2 in cell culture.17 Taken together, the ability of TNF to activate p38 MAPK through TNFR1 is clear. In parallel, the present study demonstrates that TNFR1 deficiency decreases activation of p38 MAPK after I/R in males. We also note that sex-related differences exist in myocardial p38 MAPK activation by TNFR1 after I/R. In line with previous studies, it is not surprising that TNFR1 deficiency does not affect activation of myocardial JNK or ERK after I/R in either sex.

TNF is an upstream cytokine that initiates the reperfusion-dependent cytokine release in experimental canine myocardial I/R,4 and it may induce other inflammatory cytokines via p38 MAPK. Activation of p38 MAPK is required for TNF and IL-1β production in cardiomyocytes,18 and p38 MAPK-regulated production of IL-1β and IL-6 is induced by TNF in murine embryo fibroblasts.19 Moreover, TNF-induced IL-6 production is mediated by p38 MAPK, and this process occurs at the transcriptional level.20 However, it is still unclear which receptor(s) is involved

Figure 4. Myocardial function was recorded after I/R in male WT (n = 5), male TNFR1KO (n = 6), female WT (n = 5), and female TNFR1KO (n = 6) mouse hearts. Results are represented as percentage of equilibration (eq). A and D, LVDP; B and E, +dP/dt; C and F, −dP/dt; G–I, LVDP, +dP/d, and −dP/dt at the end of reperfusion. Results are mean ± SEM. *P < 0.05 vs male WT I/R.
in TNF-induced inflammatory cytokine production in the I/R-injured myocardium. In this study, we show that TNFR1 deficiency decreases myocardial IL-1β and IL-6 mRNA after I/R, and this decrease is correlated with lower p38 MAPK activation and improvement in postischemic myocardial function in males. This suggests that I/R-induced TNF binding to TNFR1 results in activation of p38 MAPK, which subsequently regulates elevated expression of myocardial IL-1β and IL-6 mRNA in part.

Acute injury such as I/R results in myocardial functional suppression in part via the activation of an inflammatory response. We have shown that TNFR1 deficiency decreases myocardial IL-1β and IL-6 mRNA after I/R, and this decrease is correlated with lower p38 MAPK activation and improvement in postischemic myocardial function. These findings support the role of TNFR1 in regulating myocardial inflammatory cytokine production and highlight the importance of TNFR1 in mediating the inflammatory response during myocardial injury.
cascade. Therefore, elucidating the regulatory mechanisms of the cytokine cascade may help identify therapeutic targets for myocardial protection. Emerging evidence suggests that SOCS proteins are major inhibitors of cytokine signaling via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, and SOCS may be induced by an initial stimulus to inhibit the cells’ response to subsequent stimuli.21 Here, we explored the expression of SOCS-3 in the heart after I/R. We found that sex-specific differences exist in myocardial expression of SOCS-3 in response to I/R. Specifically, WT females had higher levels of SOCS-3 compared with WT males and that TNFR1 deficiency increased myocardial SOCS-3 expression in males but not in females. The significance of STAT-3 in SOCS-3 expression and TNF signaling is as follows: (1) STAT-3 deficient cells lose their ability to induce SOCS-3 in response to IL-6,22 which suggests that SOCS-3 may depend on STAT-3; (2) hypoxia has been observed to strongly induce activation of STAT-3, which also results in elevated expression of SOCS-3; (3) liver fibrosis formation is mediated by STAT-3 via TNFR124; and (4) intracellular delivery of SOCS-3 into mouse macrophage resulted in decreased production of TNF and IL-6 in response to lipopolysaccharide.25 Altogether, we speculate that I/R-induced TNF binding to TNFR1 may result in STAT-3 phosphorylation, which increases myocardial SOCS-3 expression. SOCS-3, in turn, has an inhibitory effect on inflammatory cytokine signaling and myocardial dysfunction.

Sources of Funding
This work was supported in part by National Institutes of Health grant GM070628 (D.R.M.) and American Heart Association award 0526008Z (M.W.).

Disclosures
None.

References


Tumor Necrosis Factor Receptor 1 Signaling Resistance in the Female Myocardium During Ischemia
Meijing Wang, Ben M. Tsai, Paul R. Crisostomo and Daniel R. Meldrum

Circulation. 2006;114:I-282-I-289
doi: 10.1161/CIRCULATIONAHA.105.001164
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/114/1_suppl/I-282

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/