Sex differences in the myocardial inflammatory response to ischemia-reperfusion injury

Meijing Wang,1,2 Lauren Baker,1,2 Ben M. Tsai,1 Kirstan K. Meldrum,3 and Daniel R. Meldrum1,2,4

Departments of 1Cellular and Integrative Physiology, 2Surgery, and 3Urology, and the 4Indiana Center for Vascular Biology and Medicine, Indiana University School of Medicine, Indianapolis, Indiana

Submitted 23 June 2004; accepted in final form 10 September 2004

Wang, Meijing, Lauren Baker, Ben M. Tsai, Kirstan K. Meldrum, and Daniel R. Meldrum. Sex differences in the myocardial inflammatory response to ischemia-reperfusion injury. Am J Physiol Endocrinol Metab 288: E321–E326, 2005. First published September 14, 2004; doi:10.1152/ajpendo.00278.2004.—The myocardium generates inflammatory mediators during ischemia-reperfusion (I/R), and these mediators contribute to cardiac functional depression and apoptosis. The great majority of these data have been derived from male animals and humans. Sex has a profound effect over many inflammatory responses; however, it is unknown whether sex affects the cardiac inflammatory response to acute myocardial I/R. We hypothesized the existence of inherent sex differences in myocardial function, expression of inflammatory cytokines, and activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway after I/R. Isolated rat hearts from age-matched adult males and females were perfused (Langendorff), and myocardial contractile function was continuously recorded. After I/R, myocardium was assessed for expression of TNF-α, IL-1β, and IL-6 (RT-PCR, ELISA); IL-1α and IL-10 mRNA (RT-PCR); and activation of p38 MAPK (Western blot). All indexes of postischemic myocardial function [left ventricular developed pressure, left ventricular end-diastolic pressure, and maximal positive (+dP/dt) and negative (−dP/dt) values of the first derivative of pressure] were significantly improved in females compared with males. Compared with males, females had decreased myocardial TNF-α, IL-1β, and IL-6 (mRNA, protein) and decreased activation of p38 MAPK pathway. These data demonstrate that hearts from age-matched adult females are relatively protected against I/R injury, possibly due to a diminished inflammatory response.

MYOCARDIAL ISCHEMIA is the leading cause of death in both men and women. Although a great deal of data exists regarding the influence of age and sex (and sex steroids) on the development of coronary artery disease, very little information exists concerning the influence of sex on the response of the myocardium itself to an acute insult such as ischemia-reperfusion (I/R). Cannon and Dinarello (3) and Lynch et al. (20) first discussed the effect of sex on inflammation. Recently, sex differences have been demonstrated in trauma and sepsis injury models, and sex hormones have been implicated in the mechanistic differences between males and females (14). However, it remains unknown whether sex has any effect on the response of otherwise normal myocardium to acute injury. This may be important, since the myocardium itself intensely generates inflammatory mediators, such as TNF-α, IL-1β, and IL-6, in response to I/R injury (12, 21–26, 33). These inflammatory mediators contribute to myocardial functional depression and cardiomyocyte apoptosis. Observations of decreased mortality from sepsis in females have been correlated with decreased inflammatory cytokines (2, 17, 28). Furthermore, Horton et al. (14) have demonstrated improved myocardial function in females after burn injury.

A critical component of the signal transduction pathway leading to myocardial inflammation is the activation of p38 mitogen-activated protein kinase (MAPK) (4, 30, 32, 35). Increased activation of myocardial p38 MAPK occurs after I/R injury (9, 19). In this regard, inhibition of p38 MAPK attenuates I/R-induced myocardial injury (29). In other systems, it appears that sex differences exist in the p38 MAPK signaling pathway. Angele et al. (1) demonstrated increased activated p38 MAPK in splenic and peritoneal macrophages in males after trauma-hemorrhage injury, whereas activated p38 MAPK was decreased in injured females compared with uninjured control groups.

We hypothesized that sex may have an important role in the acute inflammatory response of otherwise normal hearts. Therefore, the purposes of this study were to investigate potential sex differences in 1) functional recovery of otherwise normal myocardium, 2) proinflammatory cytokine (IL-1, IL-6, TNF-α) production, and 3) p38 MAPK activation after I/R.

METHODS

Animals. Age-matched adult male and female Sprague-Dawley rats (weight 280–300 g; Harlan, Indianapolis, IN) were fed a standard diet and acclimated in a quiet quarantine room for 2 wk 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).

Experimental groups. Rats were divided into four experimental groups: male stability control (n = 6), female stability control (n = 5), male I/R (n = 14), and female I/R (n = 13). Each I/R experiment lasted a total of 82 min, beginning with a mandatory equilibration period (15 min), followed by a standard I/R insult (27 min of global ischemia at 37°C and 40 min of reperfusion). Stability control hearts underwent 82 min of oxygenated perfusion without any periods of ischemia to ensure preparation stability.

I/R protocols. Rats were anesthetized and heparinized with an intraperitoneal injection of pentobarbital sodium (Nembutal, 60 mg/kg) and heparin sodium (500 U; SoloPak Laboratories, Elk Grove Village, IL). After sternotomy, hearts were rapidly excised into 4°C Krebs-Henseleit solution. The aorta was cannulated, and the heart was perfused (37°C) with oxygenated buffer within 45 s. Hearts were perfused in the isolated, isovolumetric Langendorff mode (70 mmHg).
with modified Krebs-Henseleit solution (in mM: 5.5 glucose, 119 NaCl, 1.2 CaCl₂, 4.7 KCl, 25 NaHCO₃, 1.18 KH₂PO₄, 1.17 MgSO₄) and saturated with 95% O₂-5% CO₂ to achieve a PO₂ of 440–460 mmHg, a PCO₂ of 39–41 mmHg, and a pH of 7.39–7.41 (ABL-4 blood gas analyzer; Radiometer, Copenhagen, Denmark). The perfusion buffer was continuously filtered through a 0.45-μm microfilter to remove particular contaminants. 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 preload volume (balloon volume) was held constant during the entire experiment to allow continuous recording of the left ventricular developed pressure (LVDP). The balloon was adjusted to a mean left ventricular end-diastolic pressure (LVEDP) of 8 mmHg (range 6–10 mmHg = both the peak and the plateau of the LVEDP-LVDP curve) during the initial equilibration. Pacing wires were fixed to the right atrium, and pulmonary outflow tract and hearts were paced at 6 Hz, 3 V, and 2 ms (350 beats/min) throughout perfusion. Data were continuously recorded using a computerized MacLab 8 preamplifier/digitizer (AD Instruments, Milford, MA) and an Apple Quadra 800 computer (Apple Computers, Cupertino, CA). 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. The maximal positive and negative values of the first derivative of pressure (+dP/dt and −dP/dt, respectively) were calculated using PowerLab software.

**RT-PCR.** Total RNA was extracted from each heart by use of RNA STAT-60 (Tel-Test, Friendswood, TX). A quantity of 0.5 g of total RNA was subjected to cDNA synthesis using a cloned AMV first-strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA). cDNA from each sample was used for multiple PCR for TNF-α, IL-1β, IL-1α, and IL-6, using message screen rat inflammatory cytokine multiplex PCR kits (Biosource, Camarillo, CA), and for IL-10 using rat dual-PCR kit (Maxim Biotech, South San Francisco, CA), respectively. One negative control used deionized distilled water (ddH₂O) instead of the RNA sample, and a second negative control used ddH₂O instead of reverse transcriptase to exclude the presence of genomic contaminants. Positive controls were included in the kit to verify appropriate expression of respective markers. PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide. Representative gel photographs are shown, and densitometry was performed to assess relative quantity and represented as a ratio to GAPDH.

**Myocardial TNF-α, IL-1, and IL-6 in the coronary effluent.** Coronary effluent was collected every 10 min during reperfusion. Myocardial TNF-α, IL-1β, and IL-6 in the coronary effluent were determined by ELISA (R&D Systems, Minneapolis, MN). ELISA was performed by adding 50 μl of each sample (tested in duplicate) to wells in a 96-well plate of a commercially available ELISA kit according to the manufacturer’s instructions.

**Western blotting.** Western blot analysis was performed on rat hearts, followed by I/R to measure the p38 MAP kinase pathway. 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

---

**Fig. 1.** Changes in myocardial function after ischemia and reperfusion in age-matched adult male (n = 14), female (n = 13), male stability control (n = 6), and female stability control (n = 5) rat hearts perfused with modified Krebs-Henseleit solution. A: left ventricular (LV) developed pressure. B: LV end-diastolic pressure. C: positive value of the first derivative of pressure (+dP/dt). D: negative value of the first derivative of pressure (−dP/dt). E: coronary flow in 15 min of equilibration (Eq) and 80 min of experiment. Results are means ± SE. *P < 0.001 vs. male ischemia-reperfusion (I/R) at the corresponding time points in A–D. *P < 0.05 vs. male I/R at 15 min of equilibration.
mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM PMSF and centrifuged at 12,000 rpm for 5 min. The protein extracts (30 μg/lane) were eletrophoresed on a 12% Tris-HCl gel (Bio-Rad, Hercules, CA) 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 h and then incubated with the following primary antibodies: p38 MAP kinase antibody and phospho-p38 MAP kinase (Thr180/Tyr182) antibody (Cell Signaling Technology, Beverly, MA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody and detection using SuperSignal West Pico Stable Peroxide Solution (Pierce, Rockford, IL).

Presentation of data and statistical analysis. All reported values are means ± SE. Data were compared at the corresponding time points between groups with the use of two-way ANOVA with post hoc Bonferroni test or Student’s t-test. A two-tailed probability value of <0.05 was considered statistically significant.

RESULTS

Myocardial function. After I/R there was a significant (P < 0.001) decrease in LVDP in both groups. (Fig. 1A). Recovery of LVDP in the postischemic period was significantly higher in females than in males (74.6 ± 12.1 vs. 43.1 ± 4.0%, P < 0.001). LVEDP was elevated in response to I/R, as shown in Fig. 1B. Females demonstrated lower LVEDP at each time point after ischemia than males (P < 0.001). However, there was no significant change of LVDP and LVEDP in male and female stability controls after 82 min of perfusion. Both +dP/dt and −dP/dt were impaired after ischemia (+dP/dt, male 1,313 ± 118.13 vs. female 2,081.1 ± 169.27, P < 0.001; −dP/dt, male −760.55 ± 61.41 vs. female −1,169.3 ± 107.6, P < 0.001) (Fig. 1, C and D). There were no significant differences in +dP/dt and −dP/dt between 15 and 80 min of perfusion in stability control (male, +dP/dt 3,128 ± 132.0 vs. 3,151 ± 122.2 mmHg/s, −dP/dt −1,768 ± 95.28 vs. −1,622 ± 79.03 mmHg/s; female, +dP/dt 3,109 ± 142.8 vs. 3,078 ± 145.4 mmHg/s, −dP/dt −1,864 ± 117.7 vs. −1,651 ± 125.5 mmHg/s). Coronary flow was also significantly (P < 0.05) decreased in males. However, there was no marked difference in postischemic coronary flow compared with equilibration females (Fig. 1E). The female hearts were the same size as the male hearts.

Sex differences in inflammatory response to I/R injury. Myocardial and coronary effluents were assessed for mRNA and protein levels of TNF-α, IL-1β, IL-1α, IL-6, and IL-10 to investigate whether sex differences exist in response of inflammation to I/R injury. I/R resulted in TNF-α, IL-1β, IL-1α, and IL-6 mRNA expression (baseline: TNF-α, under detection; IL-1β, 0.183 ± 0.084; IL-1α, 0.063 ± 0.024; IL-6, 0.178 ± 0.093) (Fig. 2) and induced TNF-α, IL-1β, and IL-6 protein production (Fig. 3). However, females had significantly less myocardial TNF-α, IL-1α, IL-1β, and IL-6 (Figs. 2B and 3). I/R upregulated myocardial IL-10, which acts as a negative regulator of inflammatory cytokine synthesis. IL-10 mRNA expression (Fig. 4) was increased in both males and females after I/R injury. However, there were no differences between males and females in I/R-induced myocardial IL-10 mRNA levels.

Effect of sex on p38 MAPK signaling pathway after I/R injury. Activation of the p38 MAPK pathway was determined by assessment (Western blot) of the phosphorylated proteins of p38 MAPK in male and female hearts after I/R injury (Fig. 5). Total p38 MAPK was equivalent between sexes after I/R. However, the activated forms of p38 MAPK were increased significantly in males compared with females.

DISCUSSION

This study represents the initial demonstration of sex-related differences in myocardial function and inflammation after acute I/R injury. Specifically, females demonstrated 1) better functional recovery, 2) decreased inflammatory cytokine production, and 3) less activation of the p38 MAPK signaling pathway.

Acute injury in the form of ischemia, endotoxemia, or burn trauma results in myocardial functional suppression, in part via the local production of inflammatory mediators such as TNF-α, IL-1β, and IL-6. I/R injury induces the local production of TNF-α, IL-1β, and IL-6 (13, 21, 23, 33). Finkcl et al. (10) reported that TNF-α or IL-1 induces depression of myocardial function in an ex vivo crystalloid-superfused papillary muscle preparation. Indeed, a locally produced inflammatory mediator such as TNF-α, IL-1, or IL-6 may be an important contributor to postischemic myocardial dysfunction, apoptosis, and/or hypertrophy. Others have reported the existence of significant sex differences in immunological response after trauma-hemorrhage or other acute injury (1, 2, 15, 16). In our study, female hearts, which had better functional recovery after acute ische-
mia, expressed less myocardial TNF-α, IL-1β, and IL-6 protein levels than male hearts subjected to the same I/R insult. 

I/R induced an increase in myocardial IL-10 mRNA in both males and females, but there were no sex differences in myocardial IL-10 mRNA. IL-10 is a cytokine synthesis inhibitory factor that inhibits synthesis of TNF-α, IL-1, and IL-6 (7, 8, 11, 27). Myocardial TNF-α and IL-6 may induce the expression of IL-10, which may have a counterregulatory role in proinflammatory cytokine production after I/R. However, it does not appear that IL-10 is responsible for the...
decreased synthesis of proinflammatory cytokines observed in females.

Activation of myocardial p38 MAPK after I/R injury in the heart has been observed in animal and human studies (9, 19, 32). Inhibition of p38 MAPK activation results in improved myocardial function after I/R injury (18, 29). p38 MAPK may mediate myocardial dysfunction through the activation of immune cells and apoptosis. Indeed, activation of p38 MAPK is involved in mediating proinflammatory cytokine production (32, 35). Activation of p38 MAPK is required for TNF-α and IL-1 production in cardiomyocytes (30, 32). Furthermore, p38 MAPK inhibition reduces IL-6 production in cultured myocytes and vascular smooth muscle cells (4). Our observation of decreased p38 MAPK activation in females after I/R correlates with their decreased myocardial cytokine production.

Population-based studies on sex differences in mortality after acute myocardial infarction have yielded conflicting results. Some studies reported increased mortality in younger women compared with men (5, 31, 34), whereas other studies demonstrated increased mortality in older women compared with age-matched men (6). These effects are likely multifactorial, with involvement of comorbidities such as diabetes mellitus affecting overall mortality. Whereas these clinical studies examined patients with coronary artery disease, the current study examines acute myocardial injury in healthy cardiac tissue. This allows us to investigate the effect of estrogen on myocardial injury without confounding factors such as preexisting coronary artery disease, age, and associated comorbidities.

GRANTS

This research was supported in part by National Institute of General Medical Sciences Grant R01-GM-070628, the Clarian Values Fund, the Showalter Trust, and the Cryptic Masons Medical Research Foundation (all D. R. Meldrum).

REFERENCES


