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Original Article

Role of endogenous testosterone in TNF-induced myocardial injury in males

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Abstract: Background: Gender-specific disparities have been observed in myocardium exposed to tumor necrosis factor-α (TNF). Male myocardium demonstrates greater loss in cardiac function in the presence of a given TNF level compared to female. In addition, we have previously demonstrated that estrogen has little influence on reducing TNF-caused myocardial dysfunction in female hearts, suggesting that male hormone – testosterone may be responsible for gender differences in TNF-mediated myocardial damage. Therefore, in this study, we hypothesize that endogenous testosterone plays a detrimental role in TNF-induced myocardial injury in male hearts. Methods: Isolated mouse hearts from age-matched adult males, females, castrated males and males treated with androgen receptor blocker-flutamide were subjected to 45 minutes of TNF infusion via a Langendorff model. Left ventricular developed pressure (LVDP) and heart rate were continuously recorded. After TNF infusion, heart tissue was analyzed for myocardial levels of caspase-8 and caspase-3 by Western blot assay. Results: TNF infusion significantly depressed LVDP, but not heart rate in males. Myocardial rate pressure product (RPP, LVDP*heart rate) was markedly decreased in male hearts compared to females in exposure to TNF, which was associated with higher levels of TNF-induced caspase-8 and caspase -3. Importantly, depletion of endogenous testosterone by castration or blockade of androgen receptor by flutamide treatment abolished TNF-decreased RPP in male hearts. However, castration or flutamide treatment did not affect TNF production and myocardial expression of TNFR1 and TNFR2. Conclusion: Our study shows that testosterone is critical to the gender difference in TNF-induced detrimental effects on myocardium. Relative low threshold for TNF-caused myocardial damage in males is likely due to the interaction of testosterone with downstream signals of TNFR1 and/or TNFR2.

Keywords: Gender differences, TNF, myocardial function, testosterone, estrogen

Introduction

Myocardial inflammation follows cardiac ischemia and plays a critical role in cardiomyocyte death and myocardial dysfunction. Tumor necrosis factor-α (TNF), an important inflammatory mediator, is increased following myocardial ischemia [1-3]. TNF has been reported to depress cardiac function, induce inflammatory signaling and increase myocyte apoptosis [1-3]. Our previous studies have indicated that direct infusion of TNF into isolated rodent hearts significantly decreases cardiac contractile function [4, 5]. Ablation of TNF receptor (TNFR) 1 protects myocardial functional recovery and promotes cardiomyocyte survival following acute ischemia/reperfusion (I/R) injury [6]. In addition, TNF has been shown to damage ventricular function by causing sarcoplasmic reticulum (SR) calcium leak [7]. However, little information exists regarding the role of TNF in heart rate and in the energy demand of the heart.

On the other hand, gender-specific disparities have been observed in the myocardium exposed to exogenous TNF. Females exhibit relative resistance to TNF-induced depressive effects on myocardial function [5, 8]. Deficiency of TNFR1 neutralizes gender differences in TNF-depressed myocardial function [6]. It is well documented that the advantage of female gender in cardiovascular system is mainly attributable to the beneficial effects of estrogen [9-12]. Studies from our group and others have demonstrated that either acute or chronic administration of estrogen provides cardioprotection following myocardial I/R injury [12-15]. Therefore, it is postulated that estrogen may play a role in

alleviating TNF-mediated deleterious effects on female hearts. However, our previous evidence has shown that there are no differences in myocardial function between metestrous and proestrus females following TNF infusion [5], suggesting that endogenous testosterone may play a role in TNF-induce myocardial dysfunction.

Indeed, the heart can accumulate testosterone at higher concentrations than other androgen target organs [16], and functional androgen receptors are present in isolated cardiac myocytes [17]. In addition, we have demonstrated that endogenous testosterone had a deleterious effect on myocardial function, and proinflammatory and proapoptotic signaling following I/R [18]. However, it remains unclear whether testosterone is responsible for TNF-mediated detrimental effects on male hearts. Therefore, in the current study, we hypothesized that gender differences in TNF-depressed cardiac function would be likely due to the deleterious effect of testosterone. To investigate this important unknown, we utilized castration to deplete endogenous testosterone or flutamide treatment to block androgen receptor in male mouse hearts.

Material and methods

Animals

Age matched male and female C57BL mice (9-10 weeks, Jackson Laboratories, Bar Harbor, ME) were fed a standard diet and acclimated in a quiet quarantine room for 1 week before experiments. Ten male mice (5-6 week old) underwent bilateral castration followed by a 4-week recovery and were purchased as surgical modified animals from the Jackson lab. Subcutaneous implantation of 21-day release pellets containing 5 mg of flutamide (androgen receptor blocker) was performed in 10 male mice during 8-week old. Those animals were utilized after 21 days. The animal protocol was reviewed and approved by the Institutional 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 Pub. No. 85-23, revised 1996).

Isolated heart preparation (Langendorff)

Mice were anesthetized using isoflurane and heparinized (500 U ip). The mouse hearts were rapidly excised via a median sternotomy and placed in a 4ºC Krebs-Henseleit solution. The aorta was cannulated under a dissection microscope, and the heart was perfused in the isovolumetric mode (70 mmHg) with oxygenated (95% O_2 and 5% CO_2) Krebs-Henseleit solution (37ºC). A water filled latex balloon was passed into the left ventricle. End diastolic pressure (EDP) was adjusted to a level between 8-15 mmHg. A 5 ml syringe was filled with 200 ng/ml of TNF dissolved in perfusate (Krebs-Henseleit solution) and secured in the infusion pump. The syringe and tubing were connected to a two-way stop-cock above the aortic root. During equilibration, pulmonary artery effluent was collected to determine the coronary flow rate. Based on the coronary flow rate, the TNF infusion rate and volume were calculated and preset to ensure that 500 pg/ml/min or 2500 pg/ml/min of TNF was infused into isolated mouse hearts. The TNF doses were chosen based on our previous literature [5, 6]. Data were continuously recorded with a PowerLab 8 preamplifier/digitizer (AD instruments, Milford, MA). The left ventricular developed pressure (LVDP) and heart rate (BPM) were measured. Immediately at the end of TNF infusion, hearts were snap frozen with liquid nitrogen and stored at -80°C.

Experimental groups

A total of 50 mice were divided into the following groups (n=4-6/group): 1) male vehicle control; 2) male TNF (500pg/ml/min or 2500pg/ml/min); 3) female vehicle control; 4) female TNF (500pg/ml/min or 2500pg/ml/min); 5) castrated male (castrated M) vehicle control; 6) castrated M TNF (2500pg/ml/min); 7) Male flutamide (M Flut) vehicle control; 8) M Flut TNF (2500pg/ml/min). The isolated mouse hearts were subjected to the same infusion protocol: a 15-minute equilibration period and a 45-minute TNF infusion period. Controls were infused with a perfusate vehicle during the experimental period.

Western blotting

Heart tissue was homogenized in cold RIPA buffer (Sigma, Saint Louis, MO) and centrifuged at 12000 rpm for 10 minutes. The protein extracts (30 μg/lane) were subjected to electrophoresis on a 4-12% Bis-Tris protein gel (Invitrogen, Carlsbad, CA). The membranes were incubated with the primary antibodies: caspase-

3, caspase-8, TNFR1, TNFR2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and GAPDH (Biodesign International, Saco, Maine), and then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody. Detection was performed using supersignal west pico stable peroxide solution (Pierce, Rockford, IL).

TNF ELISA

Blood (~0.8ml) was collected during sternotomy. After clotting, these samples were centrifuged at 5000rpm for 10 minutes to get serum, which were stored at -80°C. Serum and myocardial TNF were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA set (R&D Systems Inc., Minneapolis, MN and BD Biosciences, San Diego, CA). ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate.

Statistical analysis

All reported values are mean ± SEM. Data were compared with a two-way ANOVA with Bonferroni post-test or unpaired Student's *t* test when appropriate (GraphPad Software, San Diego, CA). A probability value <0.05 was considered statistically significant.

Results

Effect of TNF on heart rate and myocardial contractile function

Our previous study has indicated that infusion of TNF at a dose of 500pg/ml/min was able to significantly damage myocardial contractility and compliance in male mouse hearts [6]. In addition, myocardial dysfunction was directly dependent on the dose of TNF infused into isolated rat hearts [5]. However, in those studies, we preset heartbeats to the same rate before and during TNF infusion in all isolated hearts. Therefore, it could miss the evidence of that TNF infusion might affect heart rate. Herein, we did not utilize pacing wire and let heart have natural rhythm beating. Although a trend of decreased heart rate was observed in response to TNF infusion, neither 500pg/ml/min nor 2500pg/ml/min of TNF infusion significantly changed heart rate (**Figure 1A**). In addition, markedly depressed LVDP and +/- dP/dt were noticed in isolated male hearts, which was not dependent on the dose of infused TNF (**Figure 1B, 1C and 1D**).

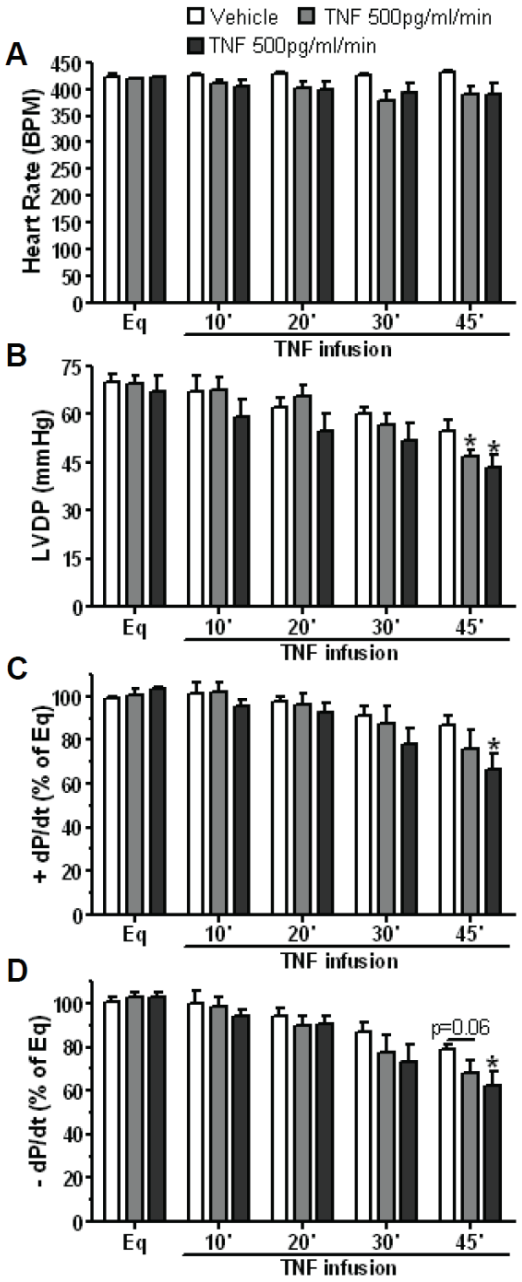

Figure 1. The effect of TNF on heart rate (A), left ventricular developed pressure (B), the maximal positive value of the first derivative of pressure (+ dP/dt, C) and the – dP/dt (D) in male hearts. TNF infusion duration was 45 minutes and was initiated after a 15-minute equilibration (Eq) period. All results are mean ± SEM of raw data. N= 4-6/group, *p<0.05 vs. according Eq and vehicle at corresponding time.

To better understand the role of TNF in the workload of heart, we utilized rate pressure product (RPP), calculated as the product of heart rate and LVDP. RPP is able to directly indicate the energy demand of the heart. Here, we found that both 500pg/ml/min and 2500pg/ml/min of TNF significantly decreased RPP to the similar degree in male hearts (**Figure 2C**). However, 2500pg/ml/min of TNF infusion took effect on damaging the myocardium 10 minutes earlier than 500pg/ml/min of TNF did following TNF infusion (**Figure 2A** and **1B**).

Female hearts were resistant to TNF-induced injury

Our previous observation has shown that female myocardium was resistant to TNF-depressed cardiac function [5, 6]. In this study, our data further demonstrated that TNF did not significantly decrease RPP in female hearts, even for the dosage of 2500pg/ml/min (**Figure 3A** and **3B**). Additionally, in agreement with our previous findings, gender difference also existed in myocardial RPP following TNF infusion with worse RPP noticed in male hearts compared to females (**Figure 3C**).

Not surprisingly, TNF infusion significantly increased active levels of pro-apoptotic proteins: caspase-8 and caspase-3 in male hearts (**Figure 4**). On the contrary, TNF used here did not augment cleaved levels of caspase-8 and caspase-3 in female myocardium (**Figure 4**), which was in correspondence to the results of RPP.

Male hormone – testosterone in TNF-induced myocardial damage

To determine the role of endogenous testosterone in TNF-induced myocardial injury, we utilized castration to deplete endogenous testosterone or flutamide treatment to block androgen receptor. During exposure to 2500 pg/ml/min of TNF, castration or flutamide treatment neutralized TNF-depressed myocardial RPP in male hearts as shown in **Figure 5A** and **5B**. In addition, TNF infusion did not increase active levels of caspase-8 and caspase-3 in castrated or flutamide treated male hearts (**Figure 6**).

Testosterone in TNF production and TNFR expression

To elucidate how testosterone interacts with

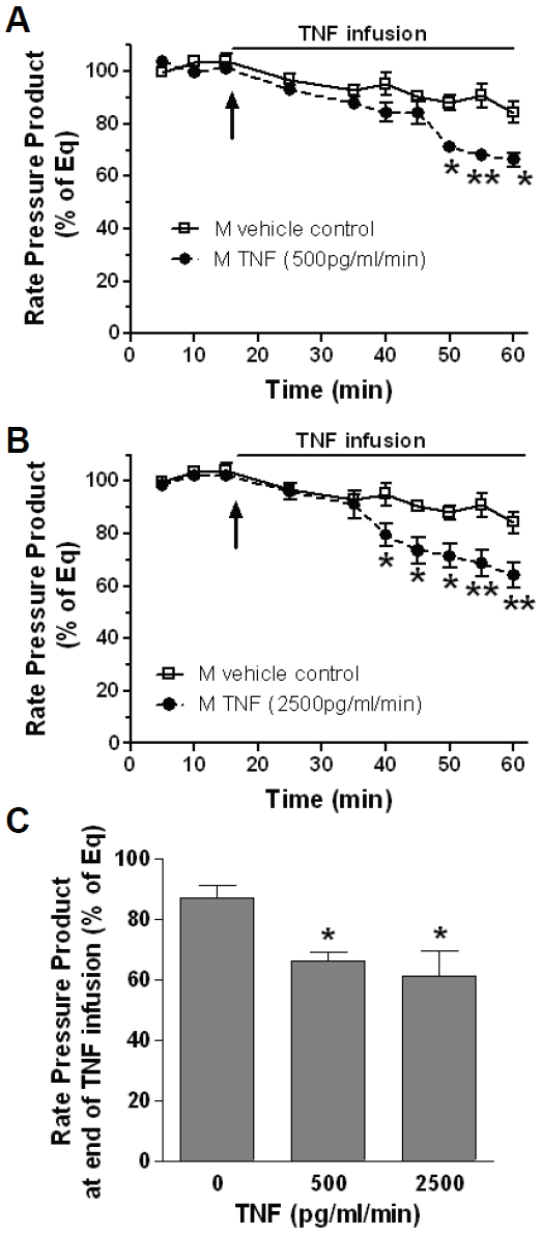

Figure 2. Changes of rate pressure product (RPP, LVDP*heart rate) in male hearts following 45-minute infusion of TNF, represented as % of equilibration (Eq). A, TNF infusion at 500pg/ml/min; B, TNF infusion at 2500pg/ml/min; C, Myocardial RPP at the end of TNF infusion (after 45-min infusion of TNF). Mean ± SEM, n= 4-6/group, *p<0.05, **p<0.01 vs. vehicle.

TNF-caused cardiac injury, we determined whether testosterone plays a role in regulating TNF production or mediating myocardial TNFR expression. We observed similar serum levels of

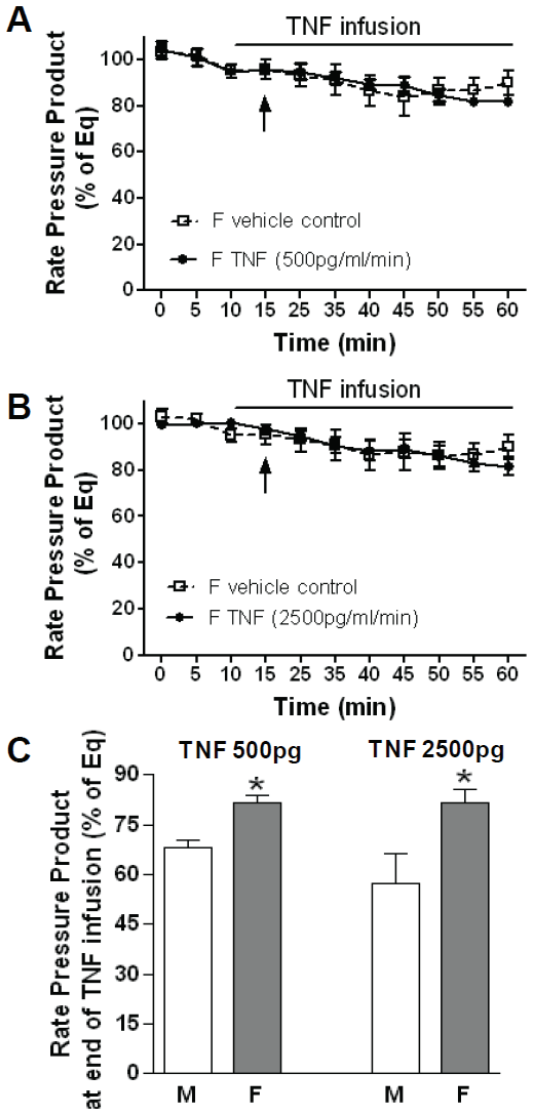

Figure 3. Myocardial rate pressure product (RPP) in females following 45-minute TNF infusion. A, 500pg/ml/min of TNF; B, 2500pg/ml/min of TNF; C, Myocardial RPP at the end of TNF infusion (after 45-min infusion of TNF) between males and females. All results are represented as the percent of equilibration (eq). Mean ± SEM, n= 4-6/group, *p<0.05 vs. male.

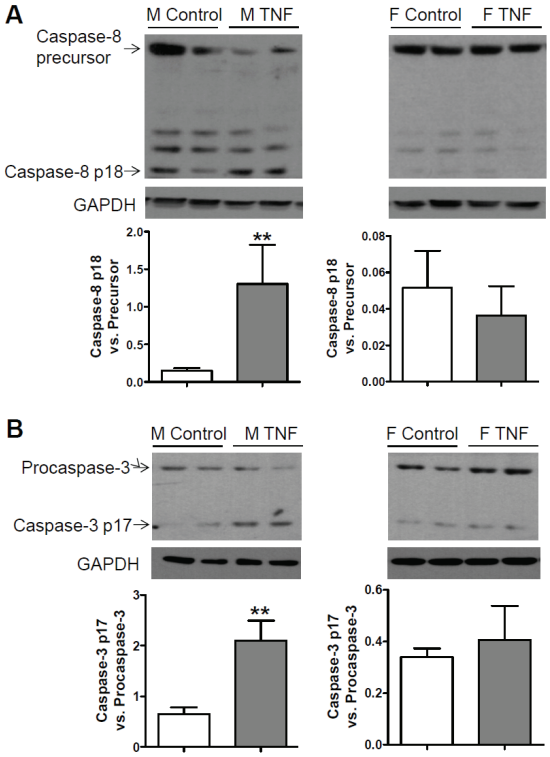

Figure 4. Myocardial apoptotic signaling between male and female hearts after 45-minute TNF infusion (2500pg/ml/min). A, Active/cleavage caspase-8 expression (p18) is shown by representative blots (2 lanes/group) and bar graph shows densitometry data vs. caspase-8 precursor); B, Western blot data exhibit active/cleavage caspase-3 expression (p17) (2 lanes/group) with densitometry results (vs. procaspase-3). N= 3-5/group, Mean ± SEM, **p<0.01 vs. male control.

TNF between normal male and castrated male (**Figure 7A**). In addition, there was no significant difference in myocardial TNF expression among normal male, castrated male and flutamide treated male (**Figure 7B**). Furthermore, depletion of endogenous testosterone by castration or blockade of androgen receptor by using flutamide neither affected myocardial TNFR1 expression nor changed TNFR2 levels in male hearts (**Figure 7C** and **7D**).

Discussion

There are gender differences in the myocardial response to pro-inflammatory cytokine - TNF exposure [5, 6]. In addition, previous studies have shown that TNF is able to disrupt excitation-contraction coupling and affect SR Ca2+ leak [7, 19]. In the current study, we expanded these findings to effects of TNF on the energy demand of the heart (exhibited as RPP), and the role of endogenous testosterone in TNF-stressed myocardial RPP. We found that direct coronary infusion of TNF did not significantly affect heart rate, but decreased RPP in isolated male mouse hearts. Additionally, female hearts

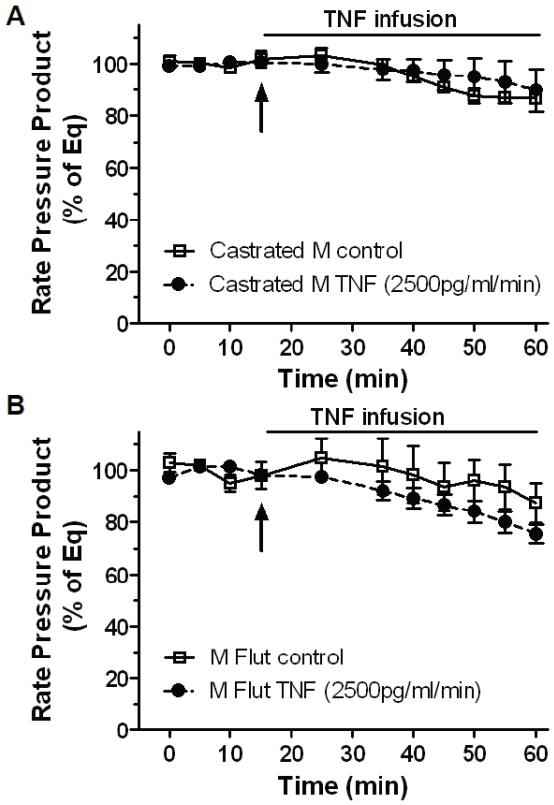

Figure 5. The role of endogenous testosterone in TNF -caused depression of myocardial function. A, Myocardial RPP in castrated male hearts following 45-minute TNF infusion (2500pg/ml/min); B, Changes of RPP in flutamide-treated male hearts exposed to TNF (2500pg/ml/min). Results are represented as % of equilibration (Eq). Mean ± SEM, n= 4-6/group.

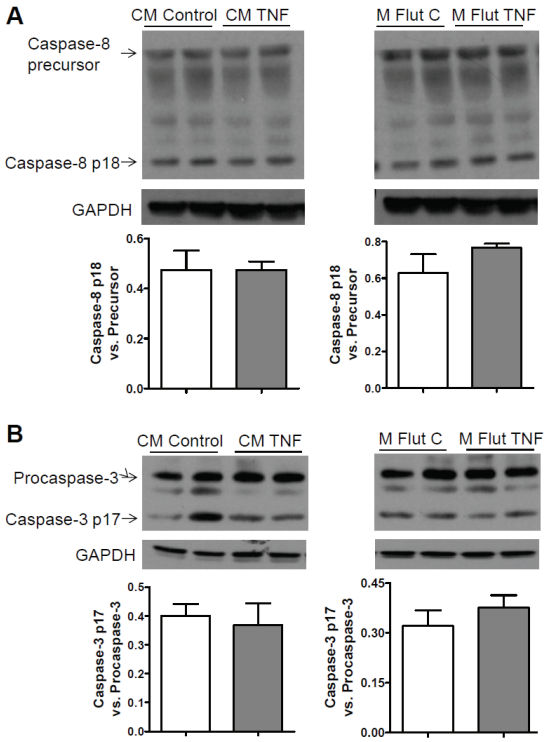

Figure 6. Myocardial levels of cleaved caspase-8 and caspase-3 in castrated males (CM) and flutamide-treated males without (C) or with 45-minute TNF infusion (2500pg/ml/min). A, Active/cleavage caspase-8 expression with densitometry data. B, Active/cleavage caspase-3 expression with quantitative results. Shown are representative immunoblots (2 lanes/group). N= 3-5/group, mean ± SEM.

were resistant to TNF-depressed RPP. These disparities in myocardial RPP were associated with differences in TNF-induced caspase-8 and caspase-3 between male and female. Most importantly, depletion of endogenous testosterone by castration or blockade of androgen receptor by flutamide treatment abolished TNF-decreased RPP in male hearts. However, testosterone did not affect serum and myocardial TNF production, as well as expression of cardiac TNFR1 and TNFR2.

It is established that gender differences exist in the myocardial response to acute injury, including burn injury [20], sepsis [21, 22], trauma-hemorrhage [23] and I/R [6, 10]. These disparities may be partly due to gender differences in the levels of inflammatory cytokine produced [10], or different threshold for injury-initiated myocardial response. In fact, our previous evidence has indicated a higher threshold for endotoxin-induced cardioprotection in female hearts [22]. In addition, gender-specific differences have also been observed in TNF-depressed myocardial function and female hearts exhibit a higher threshold for TNF-induced myocardial injury [5, 6]. The current study also confirms that differences exist in the magnitude of TNF-burdened myocardial RPP between male and female, with the relative lower threshold in male hearts compared to females.

Substantial amount of evidence has indicated that greater cardioprotection in female hearts is primarily attributed to the beneficial effects of estrogen [8, 11, 12, 24-27]. However, our previous study has demonstrated that there was no significant difference in the TNF-induced func-

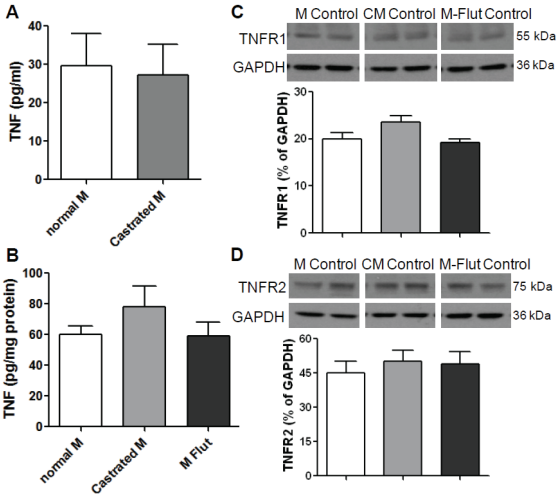

Figure 7. The role of endogenous testosterone in TNF production and expression of TNF receptors (TNFR) in males. A, ELISA data reveals serum TNF levels between normal male and castrated male (n=7/group). B, myocardial levels of TNF among normal male, castrated male and male treated with flutamide (ELISA). C, myocardial TNFR1 expression with quantitative data among normal male, castrated male and male treated with flutamide (Western blot). D, cardiac TNFR2 expression with densitometry results. N=4-5/group, mean ± SEM.

tional depression between the proestrus female hearts (presumably with the higher levels of circulating estrogen when compared to metestrous/diestrus) and metestrus/diestrus female hearts [5], suggesting that fluctuations in estrogen seem to have little influence on TNF -caused myocardial dysfunction, and that another sex hormone, testosterone, may play a role in mediating TNF-induce myocardial injury in male hearts.

In fact, accumulated evidence has shown that testosterone exerts a negative effect on cardioprotection. Testosterone has been reported to worsen myocardial dysfunction following major trauma [28] and to decrease antioxidants, leading to the impairment of cardiac function during myocardial infarction [29]. Exogenous androgen supplementation has been indicated to increase apoptosis in adult rat ventricular myocytes [30]. Additional evidence has also demonstrated that testosterone may exert adverse effects on lipoproteins, thrombosis, and cardiac hypertrophy [28, 31-33]. Furthermore, studies from our group have reported that testosterone exacerbates I/R-induced myocardial damage in male hearts [18, 34]. Depletion of endogenous testosterone by castration or blockade of androgen receptor by flutamide treatment preserved myocardial function following I/R, reduced I/R-induced myocardial inflammatory cytokine production, and decreased activation of p38 MAPK and expression of apoptotic-related proteins [18]. In contrast, acute administration of exogenous testosterone worsened myocardial functional recovery associated with upregulated myocardial inflammatory signaling [34]. In this study, we observed that disruption of action of endogenous testosterone by castration or flutamide treatment abolished TNF-induced myocardial damage in male hearts, indicating that testosterone plays a detrimental role in mediating myocardial dysfunction in response to TNF.

Now, the question becomes how testosterone interacts with TNF signaling, and thus, worsens the effect of TNF on myocardium. With respect to the role of testosterone in reduction of circulating TNF production [35, 36], we postulated that castration might increase basal levels of TNF, leading to myocardium resistant to exogenous TNF-induced injury. However, our results demonstrated that castration did not affect serum TNF levels in males. In addition, castration or flutamide treatment did not significantly change myocardial TNF expression either. Therefore, it is unlikely that testosterone deteriorated TNF-depressed cardiac function via the effect of testosterone on TNF production. On the other hand, TNF initiates its biological effects through binding to TNFR1 and TNFR2, both of which are expressed in the heart. Studies from our group have demonstrated that female hearts were resistant to TNFR1-mediated deleterious effects and exhibited enhanced TNFR2-induced beneficial action compared to male hearts following acute I/R injury [6, 8]. Of note, myocardial expression of TNFR1 and TNFR2 was not different between male and female, suggesting that gender-specific differences in TNF-depressed myocardial RPP are not attributable to the magnitude of expression of TNFR1 and/or TNFR2. However, it is unknown whether testosterone plays a role in mediating myocardial expression of TNFR1 and TNFR2. In this study, depletion of endogenous testosterone or blockade of androgen receptor did not significantly affect expression of TNFR1 and TNFR2 in male hearts, implying that testosterone likely interacts with the downstream signals

of TNFR1 and/or TNFR2 to convey its adverse effects on the heart during stress condition.

In summary, our findings represent that testosterone plays an important role in gender differences in the deleterious effects imposed by TNF on the myocardium, and males prone to TNF-induced myocardial damage following acute injury are likely due to the adverse effects of testosterone. However, other studies have suggested that testosterone may have a beneficial effect on myocardial function. For example, testosterone reduced myocardial ischemia in patients with coronary artery disease [37], and low plasma testosterone levels have been correlated with several risk factors for myocardial infarction [38, 39]. Therefore, further investigations are required to determine the detailed mechanisms behind testosterone-implicated TNF-induced myocardial injury. Understanding these mechanisms may allow for the development of novel interventions to attenuate the detrimental effects of TNF after myocardial ischemia.

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