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BOTH ENDOGENOUS AND EXOGENOUS TESTOSTERONE DECREASE MYOCARDIAL STAT3 ACTIVATION AND SOCS3 EXPRESSION FOLLOWING ACUTE ISCHEMIA AND REPERFUSION

Meijing Wang, M.D.², Yue Wang, Ph.D.², Aaron Abarbanell, M.D.², Jiangjing Tan, M.D., Ph.D.², Brent Weil, M.D.², Jeremy Herrmann, M.D.², and Daniel R. Meldrum, M.D.^{1,2,3,4}

¹ Clarian Cardiovascular Surgery, Indiana University School of Medicine, Indianapolis, Indiana

² Department of Surgery, Indiana University School of Medicine, Indianapolis, Indiana

³ Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana

⁴ Center for Immunobiology, Indiana University School of Medicine, Indianapolis, Indiana

Abstract

Background—Signal transducer and activator of transduction 3 (STAT3) pathway has been shown to be cardioprotective. We observed decreased STAT3/suppressor of cytokine signaling 3 (SOCS3) in male hearts, which was associated with worse post-ischemic myocardial function compared to females. However, it is unclear whether this down-regulation of myocardial STAT3/SOCS3 is due to testosterone in males. We hypothesized that following ischemia/reperfusion (I/R): 1) endogenous testosterone decreases myocardial STAT3 and SOCS3 in males; 2) administration of exogenous testosterone reduces myocardial STAT3/SOCS3 in female and castrated male hearts.

Methods—To study this, hearts from I/R injury (Langendorff) were homogenized and assessed for phosphorylated-STAT3 (p-STAT3), total-STAT3 (T-STAT3), SOCS3 and GAPDH by western blot. Groups: age-matched adult males, females, castrated males, males with androgen receptor blocker-flutamide implantation, females and castrated males with chronic (3-week) 5alpha-dihydrotestosterone (DHT) release pellet implantation or acute (5-minute) testosterone infusion (ATI) prior to ischemia (n=5–9/group).

Results—Castration or flutamide treatment significantly increased SOCS3 expression in male hearts after I/R. However, only castration increased myocardial STAT3 activation. Notably, DHT replacement or ATI markedly decreased myocardial STAT3/SOCS3 in castrated males and females subjected to I/R.

Conclusion—These results suggest that endogenous and exogenous testosterone decrease myocardial STAT3 activation and SOCS3 expression following I/R. This represents the initial demonstration of testosterone-downregulated STAT3/SOCS3 signaling in myocardium.

Correspondence: Daniel R. Meldrum, MD, 635 Barnhill Drive MS 2017, Indianapolis, Indiana 46202, dmeldrum@iupui.edu, Phone: 317-313-5217, Fax: 317-274-6896.

DISCLOSURES

None.

Keywords

Ischemia/reperfusion; STAT3; SOCS3; myocardial function; testosterone

INTRODUCTION

Myocardial ischemia/reperfusion (I/R) injury causes cardiomyocyte death, myocardial inflammation, cardiac dysfunction and myocardial infarction. Much research has been devoted to the development of methods to protect the myocardium from I/R injury^{1, 2}. Understanding the detailed cellular and molecular events that modulate the severity of myocardial ischemia will potentially allow us to develop novel techniques for the treatment of myocardial infarction. We have reported gender-specific differences in hearts subjected to acute I/R^{3, 4}. Specifically, male hearts exhibit decreased functional recovery, elevated myocardial inflammation and increased cardiomyocyte apoptosis in response to I/R^{3, 4}. Although the majority of studies have indicated that the sex hormone-estrogen is cardioprotective, the cardiac effects of testosterone remain controversial. Some studies have demonstrated that testosterone plays a cardioprotective role in the hearts following I/R⁵. However, we and others have clearly shown that depletion of endogenous testosterone or blockade of testosterone receptor in males improved cardiac functional recovery in response to acute I/R⁶. In addition, administration of exogenous testosterone worsened cardiac function in castrated males and normal females following I/R⁷. Furthermore, synthetic derivatives of testosterone (anabolic steroids) have been reported to induce ventricular myocyte apoptosis and reduce cardiac contractility⁸. Therefore, this controversy highlights the need to further investigate the effects of testosterone on the myocardium following I/R and the mechanism testosterone affects the heart.

Gender differences also exist in myocardial signal transducer and activator of transcription 3 (STAT3) activation and suppressor of cytokine signaling 3 (SOCS3) expression during I/R, with lower levels of active STAT3 and SOCS3 level in male hearts, which in turn, is associated with worse functional recovery compared to females^{9, 10}. STAT3 is known to play an important role in cell survival/apoptosis, proliferation and inflammatory responses^{11, 12}. Recent studies have indicated that I/R injury is able to activate STAT3, which provides protective effects in the ischemic heart^{13, 14}. Indeed, blockade of the STAT3 pathway tends to worsen myocardial injury after myocardial infarction¹⁵. Further evidence supporting the benefits of STAT3 comes from cardiomyocyte-restricted deficiency of STAT3. These cells demonstrated increased apoptosis and reduced local growth factor production in response to I/R¹³. With respect to SOCS3, we have previously demonstrated that STAT3-induced cardioprotection is likely mediated through SOCS3¹⁶. However, it is unknown what role testosterone plays in modulating myocardial STAT3/SOCS3 signaling in response to acute I/R.

Therefore, in this study, we hypothesized that: 1) endogenous testosterone will decrease myocardial STAT3 activation and SOCS3 expression in male hearts following I/R; 2) administration of exogenous testosterone will reduce myocardial STAT3/SOCS3 in female and castrated male hearts in response to acute I/R.

MATERIALS AND METHODS

Animals

Normal male and female (9–10 weeks) Sprague-Dawley rats (Harlan, Indianapolis, IN) were fed a standard diet and acclimated in a quiet quarantine room for two weeks before the experiments. Eight male rats (100–125g, 5–6 weeks) received bilateral castration and were allowed at least 4 weeks recovery. Subcutaneous implantation of 21-day release pellets

containing 10 mg of flutamide (testosterone receptor blocker) was performed in normal male rats and 100 mg of 5 α -dihydrotestosterone (DHT) release pellets were subcutaneously implanted in castrated male and normal female rats (animals were utilized at the end of the 21 day blockade). 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).

Rats were divided into eight experimental groups: normal females (n=9), normal male (n=8), castrated males (n=8), males treated with flutamide (M+flutamide n=9), castrated males or females with chronic DHT administration (n=8 for each), and castrated males (n=6) or females (n=5) with acute testosterone infusion (ATI) prior to ischemia. Acute testosterone infusion (17 β -hydroxy-4-androstenone) was administered at physiologic levels, 10 ng/ml/min $\times$ 5 min prior to ischemia.

All isolated rat hearts were subjected to the same I/R protocol as previously described^{3, 6}: 15 minute of equilibration period, 25 minutes of global ischemia (37°C), and 40 minutes of reperfusion.

Isolated heart perfusion (Langendorff)

Rats were anesthetized (sodium pentobarbital, 60 mg/kg i.p.) and heparinized (500 U i.p.), 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 (70 mm Hg) with oxygenated (95% O₂/5% CO₂) Krebs-Henseleit solution (37°C). 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. Data 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). After I/R experiment, heart tissue was rapidly frozen in liquid nitrogen and kept in the -80°C freezer until western blot assay.

Western blotting

Western blot analysis was performed to measure phosphorylated-STAT3 (p-STAT3), total-STAT3 (T-STAT3) and SOCS3 protein expression in all groups. Hearts were homogenized in cold radio-immunoprecipitation assay (RIPA) buffer (Sigma, Saint Louis, MO) and was centrifuged at 12000 rpm for 10 minutes. The protein extracts (15 μ g/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: T-STAT3, p-STAT3 (Tyr-) (Cell Signaling Technology, Beverly, MA), SOCS3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and GAPDH (Biosdesign International, Saco, Maine), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody. Protein bands were detected 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).

Presentation of data and statistical analysis

All reported values are mean $\pm$ SEM. Data was compared using the Student's t test. A two-tailed probability value of less than 0.05 was considered statistically significant.

RESULTS

Negative effects of testosterone on myocardial STAT3 activation following I/R

Gender differences were evident in myocardial STAT3 activation after I/R, with relative lower levels seen in male hearts (figure 1). However, depletion of endogenous testosterone by castration significantly increased myocardial activation of STAT3 by two times in male hearts subjected to acute I/R. Blockade of androgen receptor by using flutamide did not affect myocardial levels of active STAT3 in males (figure 1).

In addition, chronic administration of DHT markedly reduced myocardial activation of STAT3 in castrated males to the level seen in normal males (figure 2A and B). Similarly, treatment with DHT also significantly decreased active STAT3 in female hearts following I/R (figure 2C and D).

Furthermore, 60% lower levels of myocardial STAT3 activation were noted in castrated males with acute testosterone infusion prior to ischemia (figure 3A and B). Additionally, ATI significantly reduced active STAT3 to 30% in female hearts (figure 3C and D).

Negative effects of testosterone on myocardial SOCS3 expression in response to I/R

There were gender differences in myocardial SOCS3 expression following I/R. Males demonstrated significantly lower levels of SOCS3 compared to female hearts (figure 4). In line with STAT3 activation, depletion of endogenous testosterone by castration markedly increased SOCS3 expression by five times in males (figure 4) and “adding back” testosterone by chronic implantation of DHT significantly reduced myocardial levels of SOCS3 in castrated males by more than 50% (figure 5). Unlike the observation of STAT3 activation in flutamide treated hearts, inhibition of the testosterone receptor significantly increased SOCS3 levels in male hearts by four times (figure 4). In addition, a trend toward lower levels of SOCS3 (↓ 40%) was noted in castrated male hearts with ATI (figure 6). Interestingly, administration of exogenous testosterone by chronic DHT implantation or ATI did not significantly affect myocardial expression of SOCS3 in response to I/R (data not shown).

DISCUSSION

To our knowledge, this is the first study showing the effects of testosterone on myocardial STAT3/SOCS3 in response to I/R. Here, we observed that: 1) gender differences existed in myocardial STAT3 activation and SOCS3 expression following I/R; 2) depletion of endogenous testosterone significantly increased activation of myocardial STAT3 and SOCS3 in male hearts, whereas blockade of testosterone receptor only increased SOCS3 levels in males subjected to I/R; 3) Administration of exogenous testosterone to castrated male and female hearts decreased their STAT3 activity and SOCS3 level. Taking all together, the present study demonstrates that testosterone plays a negative role in myocardial STAT3 activation and SOCS3 expression following I/R.

Myocardial ischemia is a leading cause of heart failure and death in both men and women. Accumulating evidence has demonstrated that gender appears to play an important role in the myocardial response to ischemia. Clinically, men have higher overall incidence of heart failure, more progression to heart failure, worse age-matched cardiac contractility than women, and worse preservation of myocardial mass as they age¹⁷. Experimentally, animal studies have indicated that male hearts are more sensitive to acute I/R injury as exhibited by worse myocardial functional recovery, higher proinflammatory cytokine production and increased apoptotic signaling following I/R^{3, 4}. Although overwhelming data have established the protective effects of estrogen in females, testosterone is also an important sex hormone, which may lead to deleterious effects seen in the male heart subjected to acute injury. In fact, Dr.

Chaudry's group has already found that testosterone plays a negative role in myocardial function following trauma-hemorrhage¹⁸. In addition, some studies have demonstrated that testosterone increases myocyte apoptosis and worsens cardiac contractile function⁸. Recently, evidence from our group and others has further shown the adverse effects of testosterone on myocardial response to I/R injury^{6, 7, 19}.

Depletion of endogenous testosterone by castration or blockade of androgen receptor by flutamide increases post-ischemic functional recovery in male hearts⁶, whereas acute testosterone infusion prior to ischemia results in further depression of myocardial function in castrated male or female hearts following I/R when compared to their counterparts⁷. These data demonstrated that both endogenous and exogenous testosterone exert negative effects on myocardial function following acute I/R injury. However, it is unclear by what mechanisms testosterone worsens cardiac function in response to I/R.

Based on our results, STAT3/SOCS3 is implicated in the effects of gender on the heart²⁰. I/R has been shown to activate STAT3 and thereby, to protect the myocardium from ischemic injury via up-regulation of anti-apoptotic signals^{21, 22}, inhibition of anti-angiogenic factors, and promotion of myocardial capillary formation¹³. In addition, SOCS3 plays an important role in controlling inflammatory mediators, and thus, exerts beneficial effects in response to injury^{16, 23}. It is known that STAT3 deficient cells lose their ability to induce SOCS3²⁴, which suggests that STAT3-provided cardioprotection may partly be mediated through SOCS3. In this study, we found decreased myocardial levels of STAT3/SOCS3 in male hearts. In addition, castration or flutamide treatment increased active STAT3 and SOCS3 expression. Moreover, testosterone "adding back" by chronic implantation or acute infusion reduced STAT3 activation and SOCS3 levels in castrated male and female hearts when compared to their untreated counterparts. Therefore, it is possible testosterone-worsened myocardial function through decreased STAT3/SOCS3 signaling in response to acute I/R injury.

STAT3 has been shown to play an anti-apoptotic role in response to myocardial ischemia. Conditioned knockout of STAT3 in cardiomyocytes increases apoptotic signaling after myocardial infarction¹³. Blockade of active STAT3 increases caspase-3 activity and increases cardiomyocyte apoptosis following myocardial I/R injury¹⁵. On the other hand, increased pro-apoptotic proteins caspase-3, -8 and decreased anti-apoptotic protein Bcl-2 have been noted in males after I/R compared to females³. Conversely, castration or flutamide treatment significantly decreases myocardial caspase-3 and increases Bcl-2 levels in male hearts following I/R⁶, whereas ATI prior to ischemia leads to elevated caspase-3 and decreased Bcl-2 in castrated male and female hearts subjected to I/R⁷. Taken together, therefore, it can be postulated that testosterone reduces myocardial STAT3 activation, which results in increased apoptotic signaling and partly worsens myocardial dysfunction in male hearts following I/R. STAT3 also up-regulates SOCS-3 expression, which is involved in inhibiting cytokine signaling. Interestingly, higher levels of myocardial proinflammatory cytokines (TNF- α , IL-1 β and IL-6) have been observed in male hearts following I/R³. However, depletion of endogenous testosterone by or blockade of androgen receptor significantly decreases myocardial production of TNF- α , IL-1 β and IL-6 in males⁶. Therefore, it is possible that testosterone decreases STAT3 activity in male hearts, which further down-regulates SOCS3, increasing myocardial inflammatory cytokine production, and depressing post-ischemic functional recovery.

Now the question arises as to how exactly testosterone mediates myocardial STAT3/SOCS3 following I/R. In other words, is it via genomic or nongenomic pathway? In this study, depletion of endogenous testosterone significantly increased myocardial STAT3 activation after I/R, but not flutamide treatment. However, both castration and flutamide administration increased SOCS3 expression. It suggests that testosterone mediates STAT3 and SOCS3 likely through

different mechanisms. It may down-regulate STAT3 by non-genomic effects, which may not require the involvement of androgen receptors. However, inhibition of SOCS3 is likely through genomic pathways involving the action of testosterone receptor so that administration of androgen receptor blocker-flutamide only affected myocardial SOCS3 expression, not STAT3 activation in males following I/R. In addition, both DHT and ATI did not significantly change SOCS3 expression in female hearts, suggesting that testosterone may interact with estrogen together to mediate SOCS3 expression in female hearts. These interesting unknowns meet further investigation to determine the detailed mechanisms involved in deleterious effects of testosterone seen in I/R injury.

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A

B

Figure 1.

The measurement of p-STAT3 and T-STAT3 protein expression in female, male, castrated male and flutamide treated male hearts after I/R injury by Western blot. A. Gel blots of p-STAT3 and T-STAT3 in female (n=3 of individual heart samples), male (n=3), castrated male (n=4) and flutamide treated male (n=4) hearts. B. Densitometry bar graph represents the % of p-STAT3/T-STAT3. Results are mean $\pm$ SEM, *p<0.05 vs. female and # p<0.05 vs. male. Experiments were repeated at least twice.

Figure 2.

Effects of chronic DHT implantation on myocardial STAT3 activation in castrated male and female hearts following I/R. Gel blots of p-STAT3 and T-STAT3 in males (n=3), castrated males with (n=4) or without DHT treatment (n=3) (A), or in females with (n=3) or without DHT treatment (n=4) (C). Relative levels of active STAT3 represented as % of p-STAT3/T-STAT3 are shown in B and D. Results are mean $\pm$ SEM, *p<0.05 vs. male or female, # p<0.05 vs. castrated male. Experiments were repeated at least twice.

Figure 3.

Effects of acute testosterone infusion (ATI) on expression of activated STAT3 and total STAT3 in the heart after I/R. Immunoblots of p-STAT3 and T-STAT3 in males, castrated males with or without ATI (n=3/group) are shown in A, while females with or without ATI (n=3/group) are shown in C. B and D. Densitometry bar graph represents the % of p-STAT3/T-STAT3. Results are mean $\pm$ SEM, *p<0.05 vs. male, ***p<0.001 vs. female and #p<0.05 vs. castrated male. Experiments were repeated at least twice.

A

B

Figure 4. Effects of endogenous testosterone on myocardial SOCS3 expression in the heart following I/R in female, male, castrated male and flutamide treated male hearts (n=3/group). A. Immunoblots of SOCS3 and GAPDH; B. Bar graph of % of SOCS3/GAPDH. Results are mean $\pm$ SEM, *p<0.05 vs. female and # p<0.05 vs. male. Experiments were repeated at least twice.

Figure 5.

Expression of myocardial SOCS3 in the hearts subjected to I/R with or without chronic DHT administration. A. Immunoblots of SOCS3 and GAPDH in males (n=3), castrated males with (n=4) or without DHT treatment (n=3). B. Relative levels of SOCS3 as % of SOCS3/GAPDH. Results are mean $\pm$ SEM, *p<0.05 vs. male, # p<0.05 vs. castrated male. Experiments were repeated at least twice.

Figure 6.

Effects of acute testosterone infusion (ATI) prior to ischemia on myocardial SOCS3 levels in castrated male hearts following I/R (n=3/group). A. Immunoblots of SOCS3 and GAPDH; B. Bar graph of % of SOCS3/GAPDH. Results are mean $\pm$ SEM, * p <0.05 vs. male. Experiments were repeated at least twice.