Influence of androgen on myocardial apoptosis and expression of myocardial IR and IRS-1 in chronic heart failure rat models

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Abstract. The present study aimed to investigate the effect of androgens on chronic heart failure (CHF) in a rat model. A total of 120 Sprague Dawley male rats were randomly divided into the following groups: (A) sham operation group, (B) castrated group, (C) heart failure (HF) group, (D) castrated + HF group, and (E) castrated + HF + testosterone (T) replacement therapy group. There were 20 rats in group A, and 25 rats in the other groups. Surgical castration was performed on groups B, D and E, and T replacement therapy was administered to group E. Groups C, D and E were treated with doxorubicin hydrochloride to prepare the CHF animal model. The insulin sensitivity index (ISI) was calculated from fasting blood glucose and fasting insulin levels. Echocardiography was performed. Venous blood was collected for plasma T level test. Myocardial tissue was used for apoptosis index analysis. The expression levels of myocardial insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) were measured by reverse transcription semi-quantitative polymerase chain reaction. Compared with group A, the T level and ISI decreased, whereas the expression level of IR and IRS-1 were increased in the CHF group (P<0.05). Following castration, the T level and ISI were significantly decreased, and the expression of IR and IRS-1 were increased compared with the uncastrated CHF rats (P<0.01). Following androgen administration, the ISI increased, expression of IR and IRS-1 decreased, and the myocardial apoptosis index decreased (P<0.05). Taken together, these results demonstrated that androgen supplementation could improve insulin resistance and affect the expression of IR and IRS-1 in CHF, thereby reducing myocardial apoptosis and improving cardiac function.

Introduction

Heart failure (HF) results from impaired ventricular filling and/or ejection function caused by a variety of cardiac structural or functional diseases (1). As cardiac output does not meet the requirements of body tissues for metabolism, HF behaves similarly to a series of syndromes that are characterized by having a clinical manifestation of pulmonary circulation deficiency and/or congestion of systemic circulation, and organ-tissue blood perfusion insufficiency (2). The main symptoms of HF include difficulty in breathing which limits physical activity and fluid retention. Previously, chronic HF (CHF) has been considered to be a clinical syndrome characterized by chronic inflammation combined with a neuroendocrine disorder (3). It is well documented that the systemic insulin resistance phenomenon exhibited in patients with CHF is caused by a variety of factors (4).

The mechanism of insulin resistance in HF is rather complex. The current hypothesis is that activation of the renin-angiotensin aldosterone system, sympathetic nervous system activation, cytokine and nerve endocrine hormone alterations (e.g., interleukin-6), inflammatory factor release caused by reduced insulin secretion, and insulin sensitivity decreases are the main causes of insulin resistance (5). Additionally, there may be other functional mechanisms (4,6,7).

It has been reported that androgen functions in vivo and androgen receptors occur in cardiac tissue (8,9). Marsh et al (10) demonstrated the presence of a functional androgen receptor, which regulates gene expression in myocardial cells, indicating that the cardiovascular system is a target of androgens. Sex hormones and insulin are known to interact in the body. Testosterone (T) and estradiol (E2) are considered to be useful in maintaining normal insulin sensitivity in the physiological concentration range; however, outside this range, these hormones are able to promote insulin resistance (11). It has been demonstrated that there is a gender difference in terms of the effect of sex hormones on insulin sensitivity. For instance, T can lead to insulin resistance in females, whereas T significantly improves insulin sensitivity in peripheral tissues.
in males (12). T therapy may improve insulin sensitivity in both normal and diseased populations, including in obese men or diabetics (13). However, the level of androgens in HF, and whether androgen supplementation could improve insulin resistance in HF, remains to be elucidated.

The aim of the present study was to analyze the effect of androgens on myocardial apoptosis and the expression of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) in a CHF rat model. In addition, the theoretical foundation of the pathophysiological factors influencing the prognosis and outcome of CHF was investigated.

Materials and methods

Animal modeling. A total of 120 Sprague Dawley male rats (7 weeks old) weighing 230±20 g were enrolled in the present study. The rats were randomly divided into five groups, the sham group contained 20 rats whereas the other four groups contained 25 rats. The five groups were named as follows: (A) the sham operation group (20 rats), (B) castrated group, (C) HF group, (D) castrated + HF group, and (E) castrated + HF + T replacement therapy group. The rats were kept at 25˚C, 0.03% CO₂ and 12/12 h light/dark cycle with free access to food and water.

Animal models were prepared as follows. The rats were fed normally for 1 week, and testectomy was performed on rats in groups B, D and E. First, the rats were anesthetized with ether and fixed in dorsal recumbency. Lidocaine was then injected locally at the midpoint of the line connecting the two knees in front of the genitals. A 1-1.5 cm incision was made using ophthalmic scissors, and forceps were inserted between the skin and muscles for elevation of the skin to expose the muscles of the abdominal wall. The incision was extended through the body wall to penetrate the abdominal cavity and enlarge the surgical window. In the surgical field, the bladder was located. Fat tissue is present on the two sides of the bladder along with the testicular artery and vein converging at the head of the testicle. The fat tissue was elevated and the testicles and epididymal artery and vein were ligated using a suture and an incision was made at the head of the testicle. The fat tissue was then injected subcutaneously once every 2 days to group E for a total of 30 days. The rats in groups C, D and E received intraperitoneal injections of doxorubicin hydrochloride at a dose of 2.5 mg/kg once every 5 days, 6 times in total. The cumulative dose was 15 mg/kg. Following the last administration, food and water intake, mobility, shedding of hair, and mortality of the rats were observed for 3 weeks. For groups A and B, an equal volume of 0.9% sodium chloride was injected intraperitoneally once every 5 days, 6 times in total. After 1 week of normal diet, and the second day after castration in groups B, D, and E, group A was given subcutaneous injection of peanut oil until the end of the study. The current study was approved by the Medical Ethics Committee of Yan’an Hospital Affiliated to Kunming Medical University.

Echocardiography. Echocardiography was performed at the beginning and the end of the experiment, using a Mindray M7 portable ultrasound machine. The following indicators were detected: Left ventricular diastolic diameter, left ventricular end systolic diameter, the thickness of the interventricular septum and posterior wall, left ventricular end diastolic volume and left ventricular end systolic volume. Ejection fraction (EF), left ventricular fraction shortening (FS) and left ventricular mass (LVM) were calculated using the Devereux formula (14).

Sample collection and storage. At the end of experiment, venous blood was sampled and the rats were sacrificed. The hearts were harvested and the left ventricles were preserved at -80°C. Fasting plasma glucose (FPG), fasting insulin (FIS) and T were measured at the beginning and the end of the experiment. Blood samples were collected from the tail vein. FPG was detected by the glucose oxidase method, with the OneTouch® UltraVue™ blood glucose monitoring system and glucose strips (Johnson & Johnson, New Brunswick, NJ, USA). FIS was measured using Human Insulin ELISA kit (catalog no. ab200011; Abcam, Cambridge, UK). The insulin sensitivity index (ISI) was calculated as follows: ISI=1/(FPGxFIS).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA of left ventricular myocytes in rats was extracted with TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) from the left ventricular myocardial tissue of rats, followed by 1.5% agarose gel electrophoresis. The samples with clear 28S and 18S rRNA and no obvious degradation were subjected to RT-PCR using an All-in-One™ First-Strand cDNA Synthesis kit (GeneCopoeia, Inc., Rockville, MD, USA). Using the RT-PCR products as templates, semiquantitative PCR amplification was performed according to the manufacturer’s protocol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primers of IR (the PCR product was 154 bp) were: Forward, 5'-GTTCGTGAGATCTGCTCC-3' and reverse, 5'-GCTGAGGATTGTGTGCTG-3'. The primers of IRS-1 (the PCR product was 160 bp) were: Forward, 5'-GTTGCAGCATGGTGAGTCT-3' and reverse, 5'-TCCGGTGGAGGTTTCTGAC-3'. The primers of β-actin were: Forward, 5'-TGGATTTAGAAGCTAAGCA-3' and reverse, 5'-TGTCGAGCTAC-3'. The primer of β-actin was used as the internal control and the primers of β-actin were forward, 5'-TGGGTATGGGAAATCTCTGGCA-3' and reverse, 5'-TGTTGCCATAGAGGTTCTTACG-3'. The cycling program was as follows: 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 5 sec, and annealing at 56°C for 30 sec and extension at 68°C for 30 sec. The products were subjected to 1.0% agarose gel electrophoresis and visualized with ethidium bromide. Intensity of the bands was visualized on the Tanon-5200 Chemiluminescent Imaging system (Tanon Science and Technology Co., Ltd., Shanghai, China).
and the relative PCR amounts of IR and IRS-1 were recorded as I/\(I(\beta\)-actin).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) detection. TUNEL method was used to detect cell apoptosis using a commercial kit according to manufacturer’s protocol (Roche Applied Science, Penzberg, Germany). Briefly, sections were de-waxed in xylene (5 min), rehydrated in graded ethanol (2 min) and then rinsed in PBS for 5 min three times. Sections were digested with Proteinase K (20 µg/ml; Sigma Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 15 min and then soaked in PBS for 5 min. Endogenous peroxidase was quenched with 3% \(H_2O_2\) in PBS for 5 min. Enzymatic reaction was stopped, the slides were rinsed with PBS, and the samples were incubated in peroxidase substrate for 30 min at RT in an humidified chamber. Then, sections were counterstained with 5% methyl green solution for 10 min and mounted with permanent mounting media DPX (Panreac SA, Barcelona, Spain). Images were captured under a BX53TR research microscope (Olympus, Tokyo, Japan). The cells with brown granules were considered to be apoptotic cells. A total of six rats in each group were taken and five images were captured for each specimen. The total number of cardiac cells and apoptotic cells were recorded and the mean values were calculated. The formula for calculating the apoptotic index was as follows: Apoptosis index (AI)=number of apoptotic cells/total number of myocardial cells x100%.

Statistical analysis. All statistical analyses were performed using SPSS for Windows software (version 17.0; SPSS, Inc., Chicago, IL, USA). The data were expressed as the mean ± standard deviation. Multiple comparisons were performed using one-way analysis of variance and Student-Newman-Keuls test was used to examine the differences between the two groups when a significant result (P<0.05) was identified. The experiments were repeated ≥ three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Model suitability. To assess the suitability of the animal model, the number of rats that survived in each group was recorded. As demonstrated in Table I, the survival rate of groups A and B were 100 and 96%, respectively. The survival rate of the rats in group D was 72%, which was the lowest out of the five groups, whereas the survival rate of group C (80%) was lower compared with group E. The results demonstrated that the animal model was successfully prepared.

Results of the serological indices. To investigate the effect of androgen on insulin resistance in rats with CHF, the FPG, FIS and ISI were measured in all groups. The results are illustrated
in Fig. 1 and Table II. Multiple comparison tests demonstrated that there were no differences in the five groups in FPG, FIS, and ISI prior to animal modeling. Following modeling, however, ISI of the group B was decreased compared with group A, and FPG and FIS in group D was increased compared with group E (P<0.01; Fig. 1A and B). However, the ISI in group D was significantly lower than that in group E (P<0.01; Fig. 1C). These results demonstrated that androgens could improve insulin resistance in peripheral blood of rats with HF.

Results of heart Doppler ultrasound. To characterize the effect of androgen on cardiac structure and function, color Doppler ultrasound was performed and echocardiographic indices (EF, FS and LVM) prior to and following modeling were observed. As demonstrated in Fig. 2 and Table III, EF and FS in group B were decreased compared with group A. EF and FS in group D were significantly decreased compared with group E, whereas the LVM in group D was significantly increased compared with group E (P<0.01). In conclusion, the results of the present study demonstrated that androgen supplementation could improve cardiac function and ventricular hypertrophy in rats with HF.

Comparison of T levels. In order to analyze the alterations in androgen levels in rats with CHF, the T level in rats of the different groups was measured and compared, and these results are shown in Fig. 3. The T level in CHF rats (group C) was decreased compared with group A and increased compared with group B, whereas the T level of group D was significantly decreased compared with group A (P<0.01; Table IV). These observations demonstrated that androgen levels are decreased in CHF rats.

Table II. Comparison of FPG, FIS and ISI in the 5 groups of Sprague Dawley rats.

<table>
<thead>
<tr>
<th>Variable</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPG, mmol/l</td>
<td>4.30±0.24</td>
<td>4.51±0.44</td>
<td>4.48±0.37</td>
<td>5.33±0.41</td>
<td>4.26±0.58</td>
</tr>
<tr>
<td>FIS, ng/ml</td>
<td>2.17±0.84</td>
<td>3.2±1.664</td>
<td>4.546±1.74</td>
<td>7.39±1.55</td>
<td>4.276±1.17</td>
</tr>
<tr>
<td>ISI</td>
<td>0.107±0.018</td>
<td>0.069±0.017</td>
<td>0.049±0.017</td>
<td>0.025±0.015</td>
<td>0.055±0.011</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation of the mean. *P<0.01 vs. group A; †P<0.01 vs. group B; ‡P<0.01 vs. group C; •P<0.01 vs. group D; ‣P<0.05 vs. group A; ††P<0.05 vs. group B; †‡P<0.05 vs. group C. FPG, fasting plasma glucose; FIS, fasting insulin; ISI, insulin sensitivity index.
Expression of IR and IRS-1. To verify the underlying mechanism of androgen on insulin resistance in rats with CHF, the expression levels of IR and IRS-1 in different groups were tested by RT-PCR (Fig. 4A). The results are exhibited in Fig. 4B and C. Following statistical analysis, myocardial IR expression levels in groups B, C, D and E were significantly increased compared with group A (P<0.01; Table V). Compared with group B, myocardial IR expression level in group D (P<0.01) increased, whereas no significant differences were demonstrated in groups C and E (P>0.05). Compared with group C, myocardial IR expression level in group D was significantly increased (P<0.01), whereas no statistically significant difference was demonstrated compared with group E (P>0.05). Compared with group D, myocardial IR expression level in group E decreased significantly (P<0.01). These results indicated that the expression levels of the IR and IRS-1 in cardiac muscle cells of CHF rats with androgen deficiency increased, and insulin resistance occurred.

Apoptosis of rat myocardial cells. In order to investigate the underlying effect of androgens on the apoptosis of cardiac cells in rats, a TUNEL assay was performed. The results are demonstrated in Fig. 5. The apoptosis of myocardial cells in CHF rats (group C) was significantly increased compared with groups A and B (Table VI). In rats with androgen deficiency and HF (group D), myocardial apoptosis was significantly increased compared with group E (P<0.01; Fig. 6). These observations suggested that apoptosis of myocardial cells in rats with androgen deficiency and HF occurs, and that apoptosis is improved in rats with CHF following T supplementation.

Discussion

In the present study, compared with the sham operation group (group A), the biochemical indicators and cardiac indices demonstrated that, when CHF occurred, the androgen levels were reduced, the insulin sensitivity decreased, and insulin resistance developed in the body. Additionally, the results of the present study demonstrated that ventricular remodeling occurred, myocardial IR expression increased, and cardiac insulin resistance developed. Doehner et al. (15) demonstrated that dehydroepiandrosterone (DHEA) levels in 53 cases of male patients with CHF were significantly decreased compared with healthy controls. Kontoleon et al. (16) demonstrated that T levels in patients with CHF was significantly decreased, and the T level is positively correlated with heart index. Zhou et al. (17) argued that, in CHF male castrated rats, the decreased T level aggravated cardiac insufficiency, whereas treatment with

Table III. Comparison of heart color Doppler echocardiography indexes (EF, FS, LVM) in the 5 groups of Sprague Dawley rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF, %</td>
<td>83.78±0.674</td>
<td>69.53±0.903a</td>
<td>55.42±0.905ab</td>
<td>35.23±1.66ac</td>
<td>64.55±0.88c,d,e</td>
</tr>
<tr>
<td>FS, %</td>
<td>50.29±0.35</td>
<td>39.87±0.59c</td>
<td>20.86±0.26c,b</td>
<td>15.5±0.43c</td>
<td>35.98±0.25c,e</td>
</tr>
<tr>
<td>LVM, g</td>
<td>0.74±0.122</td>
<td>0.78±0.125</td>
<td>0.85±0.130l</td>
<td>0.94±0.129b</td>
<td>0.80±0.129c</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation of the mean. a P<0.01 vs. group A; b P<0.01 vs. group B; c P<0.01 vs. group C; d P<0.05 vs. group B; e P<0.01 vs. group D; f P<0.05 vs. group A. EF, ejection fraction; FS, left ventricular fraction shortening; LVM, left ventricular mass.

Table IV. Comparison of T levels in the 5 groups of Sprague Dawley rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Variable</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T, ng/ml</td>
<td>1.62±0.08</td>
<td>1.10±0.09a</td>
<td>1.40±0.13ab</td>
<td>0.79±0.18c</td>
<td>1.29±0.12b,cd</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation of the mean. a P<0.01 vs. group A; b P<0.01 vs. group B; c P<0.01 vs. group C; d P<0.01 vs. group D. T, testosterone.
physiological T was able to protect the contractile function of the heart. A previous study demonstrated that insulin resistance exists in patients with HF, resulting in normal glucose levels and insulin hyperlipidemia during fasting or glucose load (18). These results all are consistent with the results of the present study.

When insulin resistance occurs in the body, the heart muscle also exhibits insulin resistance. According to a previous study, when insulin resistance occurs, the myocardial glucose uptake is reduced and fatty acid uptake increases, which leads to an increase in myocardial lipotoxicity (4). Simultaneously, the free fatty acid level increases, which in turn causes insulin-mediated glucose utilization barriers, aggravating insulin resistance (19). In the present study, it was demonstrated that IRs in group C were increased compared with group A, but decreased compared with group D. These results indicated that during CHF, the expression of IRs increased and insulin resistance occurred in the cardiac myocytes. The IR may also be associated with an increased intake of fatty acids and a decreased uptake of glucose by myocytes. In the present study, however, no significant alterations in the expression of IRS-1 in myocardial cells of group C was demonstrated compared with group A. The possible reasons may be that, when CHF occurs, insulin resistance may arise in the receptor itself, or the number of insulin receptors in cardiac cells is reduced, or a mutation on the receptor gene will occur. These underlying mechanisms remain to be confirmed.

Myocardial cell programmed death is alternatively referred to as myocyte apoptosis, which not only may affect heart development, but also serves an important pathophysiological role in primary hypertension, HF, arrhythmia, and other cardiovascular diseases. In the present study it was demonstrated that, compared with group A, the expression of myocardial IR increased in groups C and D. The myocardial cell apoptosis index in group C increased compared with group A, whereas
group D was increased compared with group C. These results indicated that myocardial insulin resistance during CHF could induce the apoptosis of myocardial cells. Therefore, it was hypothesized that improvement in insulin resistance in patients with CHF could increase the protective effect of insulin on the myocardium and reduce apoptosis of the myocardial cells, which, in turn, could delay the progression of HF. With the recent advances in the understanding of the mechanisms of signal transduction, it has been demonstrated that insulin as a mitogenic compound is able to promote proliferation and differentiation. When combined with the IR, insulin activates the tyrosine kinase in the β-subunit of the IR, thereby causing phosphorylation of IRS-1 (20). Additionally, insulin is able to connect with downstream proteins containing SH2 domains and thereby generate signaling cascades in the phosphoinositide 3-kinase/protein kinase B signaling pathway, which initiates insulin-resistant cell apoptosis (21).

In the present study, it was demonstrated that myocardial IR and IRS-1 expression, and myocardial cell apoptosis indexes were decreased in group E compared with group D. This indicates that androgen treatment is able to improve the insulin-resistant state during CHF by reducing myocardial cell IR and IRS-1 expression, which leads to a decrease in myocardial cell apoptosis, thereby leading to an improvement in cardiac function. Androgens are mainly produced in the testes of male animals, although a small amount of androgen is secreted by the adrenal glands (22). Androgens serve their physiological role via their receptors. Previous studies have demonstrated that androgens serve a wide range of functions.
in vivo, and androgen receptors have been demonstrated in cardiac tissue (8,9). This indicates that androgens are likely to produce cardiovascular benefits by acting on receptors in the myocardium.

In light of the results of the present study a CHF rat model was created, and data were obtained to aid the elucidation of the underlying mechanism of androgens in CHF therapy. It may be hypothesized that androgen levels and insulin sensitivity decreased in CHF, whereas the expression of the IR and IRS-1 in cardiac muscle cells increased, and insulin resistance occurred. Supplementation with androgens could improve insulin resistance, reduce the expression of the IR and the IRS-1 in cardiac muscle cells, and thereby reduce the apoptosis of myocardial cells in CHF. Additionally, androgen may reduce the apoptosis of cardiac muscle cells in HF by improving insulin resistance. However, there are several limitations associated with the present study. The study sample size was small, and a larger sample size would be required for further study. In addition, the present study is based on an animal model, and further studies are required in a clinical setting to confirm these conclusions.

Acknowledgements

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References