Astragalus Improved Cardiac Function of Adriamycin-Injured Rat Hearts by Upregulation of SERCA2a Expression

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Abstract: The traditional Chinese medical herb Astragalus, the dried root of Astragalus membranaceus (Fisch.) Bge., has been widely applied to treat patients with cardiovascular disease in China and has profound cardioprotective effects. This study investigated the effect of Astragalus on hemodynamic changes in adriamycin (ADR)-injured rat hearts and its underlying molecular mechanism. Sprague-Dawley rats were divided into four groups: control, ADR only, ADR + low dose of Astragalus and ADR + high dose of Astragalus. Rats were injected intraperitoneally with 6 equal doses of ADR (cumulative dose, 12 mg/kg) over a period of 2 weeks. Treatment of Astragalus began 1 day before the onset of ADR injection and was given orally once a day for 50 days (3.3 or 10 g/kg/day). Five weeks after the final injection of ADR, rats treated with ADR only showed a significant inhibition of cardiac diastolic function accompanied by the presence of ascites, a remarkable reduction in body weight and heart weight as well as survival rate compared to the controls. Moreover, SERCA2a mRNA and protein expressions in hearts were obviously downregulated by ADR. However, this impaired cardiac function was significantly improved in both doses of Astragalus feeding groups. The amount of ascites was also reduced in a similar extent in these 2 groups. Only the high dose treatment of Astragalus significantly attenuated the changes of SERCA2a expression in injured hearts and improved survival. These results indicated that Astragalus could improve cardiac function of ADR-injured rat hearts, which was partly mediated by upregulation of SERCA2a expression.

Keywords: Astragalus; Adriamycin; Cardiac Function; SERCA2a; Rat.

Introduction

Adriamycin (ADR), an anthracycline antibiotic, is one of the most effective antineoplastic agents for the treatment of a variety of malignancies, including lymphoma, leukemia, and...
solid tumors. However, the clinical use of ADR has been seriously limited by undesirable side effects, especially dose-dependent myocardial injury leading to potentially lethal congestive heart failure (Singal et al., 2000). Several mechanisms by which anthracyclines cause myocardial injury have been suggested, such as free-radical formation, myocyte apoptosis, lipid peroxidation, mitochondrial impairment, alterations in calcium handling, and direct suppression of muscle-specific gene expression (Minotti et al., 2004). A number of studies have indicated that the cardiotoxicity of ADR is independent of its antineoplastic effect and it may be reduced without diminishing its therapeutic effect (Myers et al., 1977; Siveski-Iliškovic et al., 1995; Sacco et al., 2001).

Astragalus, a Chinese traditional medicine named Huang-qi, is derived from the root of Astragalus membranaceus (Fisch.) Bge. and contains medicinally active compounds such as astragalosides, polysaccharides, flavones and trace essential minerals. Astragalus has been routinely used in the form of injection and oral dosages for the treatment of cardiovascular diseases in China, especially for heart failure, and its therapeutic efficacy has been confirmed by clinical studies (Miller, 1998). Pharmacological research showed that Astragalus had potent cardioprotective effects including an improvement in cardiac function, an inhibition of calcium overloading and a reduction of cardiac hypertrophy in failed heart (Su et al., 2005). Both in vivo and in vitro studies have provided evidence that the antioxidant effect may be one of the underlying mechanisms by which Astragalus protects myocardium (Hong et al., 1994; Chen et al., 1995; Ma et al., 1999). Moreover, Astragalus was found to possess immune stimulating activity and may be effective in inhibiting malignant tumors (Rios and Waterman, 1997). Therefore, we speculate that Astragalus is an exciting and potentially promising medicine to protect ADR-injured hearts. Although it has been reported that Astragalus could alleviate the ADR-induced pathological changes of myocardium in rats (Yu et al., 2005), there is no direct experimental evidence to support that Astragalus may improve cardiac function injured by ADR and the possible mechanisms have not been worked out.

Herewith, we are the first to assess the effects of Astragalus on cardiac function of ADR-injured rat hearts by studying the hemodynamic changes. In order to investigate the possible molecular mechanism underlying the improvement of cardiac performance, we compared the mRNA and protein expressions of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a) in the injured hearts of rats treated with or without Astragalus.

**Materials and Methods**

*Drugs and Reagents*

Adriamycin hydrochloride for injection (10 mg) was obtained from Hualian Pharmaceutical Co., Ltd., Shanghai, China. Astragalus injections were purchased from Fuda Pharmaceutical Co., Ltd, Shanghai, China, and contained 2 g crude material per milliliter, in which Astragaoside IV was no less than 0.08 mg/ml. All reagents for RT-PCR were obtained from Invitrogen Corporation (Carlsbad, CA, USA). The synthesis of PCR primers were performed by SBS Genetotechnology Co., Ltd., Beijing, China. Protease inhibitor cocktail was procured from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The enhanced ECL-plus
kit was purchased from Amersham Biosciences Co., Ltd. (Piscataway, NJ, USA). The antibodies used in the methods described below have been specified in detail in the respective references.

Animals

Five-week-old male Sprague-Dawley rats (140 to 160 g) were obtained from the Experimental Animal Center, Second Military Medical University, Shanghai, China. They were housed under standard laboratory conditions and given standard rodent chow and free access to water. Investigations using experimental animals were conducted in accordance with internationally accepted principles for laboratory animal use and care. The experimental protocol was approved by the Ethical Committee for Medical and Biological Research in Tongji University.

Experimental Protocols

After 3 days of acclimatization, rats were randomly assigned into 4 groups: control (n = 8), Adrimycin only (ADR, n = 15), ADR plus low dose of Astragalus (ADR + As-L, n = 15) and ADR plus high dose of Astragalus (ADR + As-H, n = 15). The myocardial injury was induced as previously described (Matsui et al., 1999). Briefly, rats were injected intraperitoneally with 6 equal doses of ADR (2 mg/kg each time, with a cumulative dose of 12 mg/kg) or equivalent volume of normal saline over a period of 2 weeks. Astragalus was given orally from the day before ADR treatment at a dose of 3.3 or 10 g/kg/day in ADR plus As-L or ADR plus As-H groups, respectively, and the treatment lasted for 50 days. Animals in control and ADR groups received an equivalent volume of vehicle p.o. during the experimental period. The dosages of Astragalus were selected on the bases of our previous pharmacological studies for Astragalus (Su et al., 2008). When the 2 drugs were given on the same day, Astragalus was administered 3 hours before ADR injection. Intraperitoneal injection of ADR was suspended after 2 weeks, while Astragalus administration was continued for 5 weeks more. Throughout the study, the rats were weighed twice a week and the mortalities were observed until the termination of the treatment. At the 24th hour after the last administration of Astragalus or vehicles, hemodynamic parameter were measured, ascitics were collected from the peritoneal cavity, and hearts were removed rapidly, weighed and rinsed in ice-cold saline. Left ventricles (LV) were rapidly frozen in liquid nitrogen and preserved at -80°C for further analysis.

Hemodynamic Studies

Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). A polyethylene catheter was cannulated in the right carotid artery and advanced carefully into the left ventricle. The catheter filled with normal saline solution was connected to a ADI MLT1050/D high-fidelity transducer, which was in turn connected to a ADI ML110 pressure processor amplifier. After an equilibration period of 10 min, the hemodynamic parameters including the left ventricular
systolic pressure (LVSP), the left ventricular end-diastolic pressure (LVEDP), the maximal rate of rise in LVP (+LVdP/dt\textsubscript{max}), the maximal rate of decrease in LVP (-LVdP/dt\textsubscript{max}) and the heart rate (HR) were monitored (Thomas et al., 2004). The pressure signals were simultaneously recorded by ADI ML740 PowerLab/4SP physiological data acquisition and analysis system.

**RT-PCR Analysis**

Total RNA was extracted using TRIZOL reagent. For reverse transcription, 4 µg of total RNA in each sample was performed in 25 µl reaction volumes containing 200 U of M-MLV reverse transcriptase, 30 U of RNase inhibitor, 10 mM dNTPs, 5 × RT buffer and 0.5 µg random primer for 60 min at 37°C. For the polymerase chain reaction, each sample containing 10 pmol upstream and downstream primers (GAPDH-1, 5′-GCC ATC AAC GAC CCC TTC ATTG-3′ and GAPDH-2, 5′-TGC CAG TGA GCT TCC CGT TC -3′, 597 bp; SERCA2a-1, 5′-ATG AGA TCA CAG CTA TGA CTG GTG -3′ and SERCA2a-2, 5′-GCA TTC ATC ATC TCT ATG GTG ACT AG -3′, 653 bp), 200 µM dNTPs, 10 × PCR buffer and 1 U Taq DNA polymerase in a final volume of 20 µl, was amplified for 22 (SERCA2a) or 23 (GAPDH) cycles. The amplification profile involved denaturation at 94°C for 30 sec (SERCA2a) or 15 sec (GAPDH), primer annealing at 60°C for 30 sec (SERCA2a) or 62°C for 15 sec (GAPDH) and primer extension at 72°C for 90 sec (SERCA2a) or 45 sec (GAPDH). After the last cycle, samples were incubated at 72°C for 8 min to extend incomplete products. The PCR products were electrophoretically detected on a 1.5% agarose gel stained with ethidium bromide. The density of each band was measured by Pharmacia Biotech ImageMaster VDS analysis system.

**Western Blot Analysis**

Proteins were extracted from freshly frozen LV, myocardial tissue was homogenized in a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM PMSF, 5 mM DTT, 1% Triton X-100, 10% glycerol, 0.1% SDS and 1% deoxycholic acid with protease inhibitors. Total protein concentration of each sample was measured by the method of (Bradford, 1976). Samples (100 µg and 10 µg total proteins per lane) were electrophoretically separated on 8% and 12% SDS-PAGE for the determination of SERCA2a and GAPDH, respectively. Then each SDS-PAGE was transferred to a PVDF membrane using Bio-Rad Mini Trans-Blot system. Membranes were blocked by incubation with 5% non-fat dried milk in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween20, pH 7.5) for 2 hours at room temperature. The blots were then probed with primary antibodies to SERCA2a (ABR Inc., Golden, CO, USA, 1:1,000) or GAPDH (Abcam Inc., Cambridge, MA, USA, 1: 20,000) as an internal control overnight at 4°C. After washing, the blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Chemicon International Inc., Temecula, CA, USA, 1: 5,000) for 2 hours at room temperature. The targeted bands were visualized by using enhanced ECL-plus kit exposed to X-ray film. Bands were analyzed by FURI-982 Bio-Image density scan and analysis system.
Statistical Analysis

Data were expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey’s method, and chi-square test was performed to measure the significance of the mortality result, p < 0.05 was considered a significant difference in groups.

Results

Body Weight, Heart Weight, Ascites and Mortality

The average initial body weight of rats was 174 ± 2.3 g. At the end of the experimental period, rats in ADR group presented a significant amount of ascites (p < 0.001) and a remarkable 25% reduction in both body weight and heart weight (p < 0.001) compared to the controls. As a consequence, the heart-to-body weight ratio was not changed. Both body weight and heart weight were gained more in ADR plus As-H group than ADR group during the experimental period, but not significantly. In both doses of Astragalus administered groups, the amount of peritoneal fluid in rats was about one third that seen in ADR group (p < 0.001) (Table 1).

There were no deaths in control group (0% mortality). In ADR group, mortality was elevated to 40% (6 rats died out of 15 died). The treatment with low and high doses of Astragalus reduced mortality to 33% (5 rats out of 15 died) and 13% (only 2 rats out of 15 died), respectively. Thus the survival rate was improved significantly by the higher dose of Astragalus treatment (p < 0.01).

Cardiac Function in vivo

In ADR only treated rats, the major effect of ADR was a marked alteration of cardiac diastolic function: 180% increase in LVEDP (p < 0.001) and 21% decrease in -LVdp/dtmax (p < 0.05) compared with controls. LVSP was increased slightly in ADR group but no obvious difference from that in control group. In ADR + As-L group, LVEDP was decreased significantly by 41% (p < 0.05) compared to the ADR group. While in ADR + As-H group,

| Table 1. Effects of Astragalus on ADR-Induced Changes in Body Weight, Heart Weight and Ascites in Rats |
|-------------------------------------------------|----------|----------|----------|----------|
| Groups          | N  | BW (g)  | HW (g)   | HW/BW   | Ascites (ml) |
| CON              | 8  | 422.2 ± 11.7### | 1.20 ± 0.04### | 2.85 ± 0.04 | 0### |
| ADR              | 9  | 318.3 ± 9.1***   | 0.90 ± 0.03***  | 2.85 ± 0.05 | 9.42 ± 0.99*** |
| ADR + As-L       | 10 | 330.0 ± 19.6***  | 0.95 ± 0.05***  | 2.92 ± 0.05 | 3.16 ± 0.57### |
| ADR + As-H       | 13 | 362.5 ± 15.0*    | 1.01 ± 0.04**   | 2.72 ± 0.13 | 3.23 ± 0.65### |

BW, body weight; HW, heart weight; HW/BW, the ratio of heart weight to body weight; CON, control; ADR, Adriamycin; As-L, Astragalus, 3.3 g/kg/day; As-H, Astragalus, 10 g/kg/day. Values are expressed as mean ± SEM (n = 8–13). *p < 0.05, **p < 0.01, ***p < 0.001 vs. CON group. ###p < 0.001 vs. ADR group.
Table 2. Effects of Astragalus on ADR-Induced Changes in Cardiac Function in Rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVSP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>+LVdP/dtmax (mmHg/s)</th>
<th>-LVdP/dtmax (mmHg/s)</th>
<th>HR (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>127.5 ± 5.9</td>
<td>4.31 ± 1.64***</td>
<td>6284 ± 394</td>
<td>5286 ± 278#</td>
<td>379 ± 13</td>
</tr>
<tr>
<td>ADR</td>
<td>136.1 ± 7.0</td>
<td>12.06 ± 1.08***</td>
<td>6555 ± 512</td>
<td>4167 ± 192*</td>
<td>342 ± 14</td>
</tr>
<tr>
<td>ADR + As-L</td>
<td>145.8 ± 8.1</td>
<td>7.06 ± 1.24#</td>
<td>5943 ± 228</td>
<td>4671 ± 248</td>
<td>368 ± 5</td>
</tr>
<tr>
<td>ADR + As-H</td>
<td>132.1 ± 4.1</td>
<td>5.13 ± 1.05***</td>
<td>5395 ± 242</td>
<td>5177 ± 251#</td>
<td>373 ± 10</td>
</tr>
</tbody>
</table>

LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; ±LVdP/dtmax, maximal rate of increase and decrease of left ventricle pressure development; HR, heart rate; CON, control; ADR, Adriamycin; As-L, Astragalus, 3.3 g/kg/day; As-H, Astragalus, 10 g/kg/day. Values are expressed as mean ± SEM (n = 8). *p < 0.05, **p < 0.001 vs. CON group. #p < 0.05, ##p < 0.01, ###p < 0.001 vs. ADR group.

LVEDP was decreased by 57% (p < 0.01) and -LVdp/dtmax was clearly increased by 24% (p < 0.05) compared to the ADR group. However, there were no distinct changes in heart rate among the four groups (Table 2).

Expressions of SERCA2a mRNA and Protein

To study the molecular mechanisms underlying the beneficial effects of Astragalus on cardiac function of ADR-injured rat hearts, alterations in expressions of SERCA2a mRNA and protein in left ventricular tissue were measured. As compared to the controls, the level of SERCA2a mRNA expression in myocardium was reduced markedly by 29% (p < 0.05) in ADR group. In ADR + As-H group, the SERCA2a mRNA expression was increased obviously by 36% (p < 0.05) compared to the ADR group (Fig. 1). The changes in protein levels were paralleled to the changes in mRNA levels as was evidenced by a nearly 38% (p < 0.01) downregulation of SERCA2a protein expression in ADR group compared to the controls. While SERCA2a protein expression was upregulated significantly by 57% (p < 0.01) in ADR + As-H group compared to the ADR group (Fig. 2).

Discussion

The rat model is considered suitable for the research of ADR-induced cardiac injury because it emulates the structural as well as functional changes observed in patients and is highly reproducible (Seifert et al., 1994). Therefore, this animal model was employed in the present study, and the total dose of ADR to induce myocardial injury was set at 12 mg/kg as previously described (Xu et al., 2001).

In the present research, rats with ADR injection manifested general toxicity characterized by a significant reduction in body weight compared to the controls irrespective of Astragalus treatment. The decline of body weight might result from a decrease of food intake during ADR treatment, or from toxic effects of ADR on bone marrow and on the gastro-intestinal system, or more generally from non-specific modifications of protein synthesis induced by ADR. However, the real delay in body weight gain in ADR-treated rats was probably underestimated, owing to the ascites accumulation observed at the end of the experiment.
Figure 1. Effects of Astragalus on SERCA2a mRNA expression in ADR-injured rat hearts (A) RT-PCR results for SERCA2a and GAPDH in left ventricle obtained from CON (lane 2), ADR (lane 3), ADR + As-L (lane 4) and ADR + As-H (lane 5) groups, respectively; lane 1 shows molecular weight markers. (B) Summarized data for the effect of Astragalus on SERCA2a mRNA level (normalized by GAPDH). CON, control; ADR, Adriamycin; As-L, Astragalus, 3.3 g/kg/day; As-H, Astragalus, 10 g/kg/day. Values are expressed as mean ± SEM (n = 8). *p < 0.05 vs. CON group. #p < 0.05 vs. ADR group.

Similarly, all rats in ADR, ADR + As-L and ADR + As-H groups had significantly decreased heart weights compared to the controls. This reflected the toxic effects of ADR on hearts, however these effects were not minimized by Astragalus treatment. In earlier studies, some scholars evaluated the degree of cardiac failure based on the increase or decrease of ascites in rats with ADR-induced cardiomyopathy (Siveski-Iliškovic et al., 1995). Therefore, the presence of ascites in ADR-treated rats observed in our study was believed to be mainly associated with cardiac dysfunction, although the involvement of drug-induced peritonitis cannot be completely ruled out. Both doses of Astragalus treatment reduced the amount of ascites to a similar extent indicating that Astragalus was likely to have beneficial effects on cardiac function.

Hemodynamic study showed that ADR injection caused a significant increase in LVEDP, a distinct decrease in -LVdp/dt_max and a slight increase in LVSP. These results were similar to those observed in the previous report (Thomas et al., 2004). Since the hemodynamic analysis was only performed at the 5th week after suspension of ADR, few of the rats exhibited
Figure 2. Effects of Astragalus on SERCA2a protein expression in ADR-injured rat hearts (A) Representative immunoblots for SERCA2a and GAPDH in left ventricle obtained from CON (lane 1), ADR (lane 2), ADR + As-L (lane 3) and ADR + As-H (lane 4) groups, respectively. (B) Summarized data for the effect of Astragalus on SERCA2a protein expression (normalized by GAPDH). CON, control; ADR, Adriamycin; As-L, Astragalus, 3.3 g/kg/day; As-H, Astragalus, 10 g/kg/day. Values are expressed as mean ± SEM (n = 8). **p < 0.01 vs. CON group. ##p < 0.01 vs. ADR group.

signs of heart failure associated with a reduction of cardiac contractility. At this stage, the cardiac systolic function was nearly preserved, owing to an activation of the sympathetic nervous system which maintained LVSP. The earlier study also suggested that the depression of cardiac contractility induced by ADR only occurred in the decompensated phases (Gorodetskaya et al., 1990). While the main reason for the LVEDP increase and -dP/dt max decrease was a marked reduction of the left ventricular diastolic function. In our study, the impaired diastolic function by ADR was improved significantly by both doses of Astragalus treatment. Especially in 10 g/kg/day Astragalus feeding group, the improvement of cardiac function was evidenced by a marked alteration of both LVEDP and -LVdP/dt max. It has been reported that Astragaloside IV could improve cardiac function in rats with isoproterenol-induced myocardial injury (Xu et al., 2007). Therefore, the improvement of left ventricular diastolic function observed in our research was thought to be due to Astragaloside IV, the main pharmacologically active ingredient in Astragalus (Li and Cao, 2002; Zhang et al., 2006). Furthermore, the survival benefits of 10 g/kg/day Astragalus treatment might be partly attributed to the prominent improvement in cardiac function.
The sarcoplasmic reticulum plays a pivotal role in regulating the intracellular concentration of Ca$^{2+}$. One of the sarcoplasmic reticulum Ca$^{2+}$-cycling proteins, the sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA2a), is responsible for 75–92% reuptake of intracellular calcium resulting in cardiac relaxation (Giannini et al., 2005). Depression of SERCA2a mRNA level and protein content has been reported in different types of failing hearts (Temsah et al., 2000; Satoh et al., 2003; Su et al., 2008). Thus, it has been proposed that sarcoplasmic reticulum remodeling may be an important mechanism for the sarcoplasmic reticulum dysfunction in the pathophysiology of cardiac injury (Frank et al., 2002). Some authors have suggested the genetic regulation of SERCA2a to be important in anthracycline cardiomyopathy (Arai et al., 1998; Arai et al., 2000). In our study, the treatment with 10 g/kg/day Astragalus significantly attenuated SERCA2a down-regulation in ADR-injured rat hearts. Furthermore, it has been shown in our previous studies that Astragulus could upregulate SERCA2a expressions in left ventricular tissues in rats with pressure overload-induced heart failure (Su et al., 2008). These results indicated that Astragulus might improve cardiac function in different models through the regulation of SERCA2a expression.

In conclusion, the present study showed that Astragalus had beneficial effect on diastolic function of ADR-injured rat hearts, and the underlying molecular mechanisms was associated with the upregulation of SERCA2a expression in myocardium. However, it would be important to elucidate the effect of Astragalus on the antioxidant status of the heart and myocyte apoptosis in this special animal model. Although it is difficult to extrapolate rat data into human, the results offer an important substantiation for using Astragalus to protect hearts against ADR-induced cardiac dysfunction and provide a survival benefit in patients with ADR chemotherapy.

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References


