Astragaloside IV protects heart from ischemia and reperfusion injury via energy regulation mechanism

Lei Tu\textsuperscript{a,b}, PhD; Chun-Shui Pan\textsuperscript{a,c,d}, PhD; Xiao-Hong Wei\textsuperscript{a}, MD; Li Yan\textsuperscript{a,c,d}, MD; Yu-Ying Liu\textsuperscript{a,c,d}, MD; Jing-Yu Fan\textsuperscript{c}, PhD; Hong-Na Mu\textsuperscript{a,b}, MD; Quan Li\textsuperscript{a,c,d}, PhD; Lin Li\textsuperscript{a}, MD; Yu Zhang\textsuperscript{a,b}, MD; Ke He\textsuperscript{a,b}, PhD; Xiao-Wei Mao\textsuperscript{a,b}, MD; Kai Sun\textsuperscript{a,c,d}, MD; Chuan-She Wang\textsuperscript{a,b,c,d}, MD; and Jing-Yan Han\textsuperscript{a,b,c,d}, MD, PhD

\textsuperscript{a}Tasly Microcirculation Research Center, Peking University Health Science Center, Beijing 100091, China
\textsuperscript{b}Department of Integration of Traditional Chinese and Western Medicine, School of Basic Medical Sciences, Peking University, Beijing 100091, China
Running title: Astragaloside IV and reperfusion injury

Abstract

Objective: This study was designed to investigate the protective potential of AS-IV against ischemia and I/R induced myocardial damage, with focusing on possible involvement of energy metabolism modulation in its action and the time phase in which it takes effect.
Methods: SD rats were subjected to 30 min LADCA occlusion, followed by reperfusion. MBF, myocardial infarct size, and cardiac function were evaluated. Myocardial structure and myocardial apoptosis were assessed by double immunofluorescence staining of F-actin and TUNEL. Content of ATP, ADP, and AMP in myocardium, cTnI level, expression of ATP5D, P-MLC2, and apoptosis related molecules were determined.

Results: Pretreatment with AS-IV suppressed MBF decrease, myocardial cell apoptosis, and myocardial infarction induced by I/R. Moreover, ischemia and I/R both caused cardiac malfunction, decrease in the ratio of ATP/ADP and ATP/AMP, accompanying with reduction of ATP 5D protein and mRNA, and increase in P-MLC2 and serum cTnI, all of which were significantly alleviated by pretreatment with AS-IV, even early in ischemia phase for the insults that were implicated in energy metabolism.

Conclusions: AS-IV prevents I/R induced cardiac malfunction, maintains the integrity of myocardial structure through regulating energy metabolism. The beneficial effect of AS-IV on energy metabolism initiates during the phase of ischemia.

Key Words: energy metabolism, cardiac function, myocardial structure, ATP 5D

Abbreviations used in this article: AS-IV, Astragaloside IV; AAR, Area at risk; cTnI, Cardiac troponin I; HR, Heart rate; I/R, Ischemia/reperfusion; LV, Left ventricle; LVSP, Left ventricular systolic pressure; LVDP, Left ventricular diastolic pressure; LVEDP, Left ventricular end diastolic pressure; +dp/dtmax, Left ventricular maximum upstroke velocity;

This article is protected by copyright. All rights reserved.
dp/dtmax, Left ventricular maximum descent velocity; LADCA, Left anterior descending coronary artery; MBF, Myocardial blood flow; PCI, Percutaneous coronary intervention; P-MLC2, Phosphorylated myosin light chain 2; TTC, Triphenyltetrazolium chloride; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

Introduction

Ischemic heart disease is among the top causes of death in the world [12]. PCI has currently been applied widely to deal with acute coronary syndrome, myocardial infarction and stable angina. Although PCI can restore the blood flow in myocardium rapidly, it does not reduce the risk of serious heart events because of reperfusion injury [1, 3]. Thus, strategy to prevent reperfusion injury and improve PCI outcome is currently appealing in clinic.

Ischemia/reperfusion (I/R) injury occurs in two phases, ischemia and reperfusion [7]. During ischemia phase, ischemic hypoxia uncouples oxidative phosphorylation from the respiratory chain, resulting in the cessation of ATP synthesis and depletion of ATP [19], which is thought to play a key role in ischemic myocardial injury. ATP deficiency causes depolymerization of F-actin [10], disarranging thin filament of cardiac myocytes. Yet, cardiac myocyte contracture initiates when the cellular ATP content decreases [8], which contributes to the cardiac malfunction. In the phase of reperfusion, restoration of blood flow and oxygen supply provokes hypoxanthine oxidation and massive oxygen free radical production, leading to reperfusion injury. In light of the critical importance, strategies
directing to intervene in energy metabolism disorder are a tempting alternative for protection of I/R induced myocardium injury.

AS-IV (molecular structure is shown in Fig. 1 [9]) is one of the components derived from a traditional herbal medicine, *Radix Astragalus*. In traditional Chinese Medicine, *Radix Astragalus* has been used to deal with cardiovascular diseases for years, and thought to be able to improve energy metabolism of the heart. AS-IV was detected in the plasma of rat after given Chinese Medicine which contains *Radix Astragalus* [18]. Recent studies showed that AS-IV could prevent I/R injury by inhibiting the oxidation stress and interfering with nuclear factor kappa B pathway [6, 21]. However, whether AS-IV could improve the energy metabolism is not clear. The present study was designed to investigate the effect of AS-IV on I/R induced myocardial damage, with particularly focusing on the possible involvement of energy metabolism modulation in its action and the time phase over I/R challenge in which it takes effect.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats, weighing 240 to 260 g, were purchased from the Animal Center of Peking University (Certificate no. SCXK (Jing) 2006-0008). The rats were housed in cages at temperature 22 ± 2 °C, humidity 40 ± 5%, under a 12-hour light/dark cycle, and received standard diet and water *ad libitum*. The rats were fasted for 12 hours before
experiment but allowed to access water freely. The investigations conformed to Guide of Peking University Animal Research Committee. Experiment protocols were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2010-001).

**Drug and reagents**

AS-IV was obtained from Feng Shan Jian Medicine Research Co. Ltd. (Kunming, China). It was dissolved in saline to make a solution of concentration of 1 mg/ml and 10 mg/ml for different doses before experiment.

Pentobarbital sodium was purchased from Beijing Chemical Agent Ltd (Beijing, China). ELISA Kits for ATP, ADP, AMP and Cardiac troponin I (cTnI) were from Beijing Huanya Biomedicine Technology CO. Ltd (Beijing, China). The antibodies against phosphorylated myosin light chain 2 (P-MLC2), cTnI, Bax and Bcl-2 were bought from Cell Signaling Technology (CST, Boston, Massachusetts, USA), the antibody against ATP 5D was from Santa Cruz Biotechnology (Santa Cruz, California, USA).

**Myocardial I/R model and animal grouping**

Animals were anaesthetized with 2% pentobarbital sodium (60 mg/kg) by intraperitoneal injection, and placed in a supine position. A tracheal cannula was inserted via mouth, with one end being connected with an animal breathing apparatus (ALC-V8, Shanghai ALCOTT BIOTECH CO., China), which was set at the breathing ratio 1:1, the
frequency 75/min, and tidal volume 12 ml/kg. A thoracotomy was performed to exposure the heart, and the LADCA was ligated with a 5/0 silk. The suture silk was released after 30 min, allowing reperfusion for 90 min. The animals in Sham and AS-IV groups underwent the same procedure but without ligation of suture silk. Ninety minutes before ischemia, the animals in AS-IV pretreatment groups were administrated through gavage with AS-IV in saline at a dose of either 1 mg/kg or 10 mg/kg. The animals in Sham group and I/R group received saline in the same way at 1 ml/kg. Three or six animals were enrolled in each group for determination of each parameter (See Table 1 for detail).

**Myocardial blood flow**

After left thoracotomy, MBF was determined at baseline, 30 min after ischemia, and 90 min after reperfusion by using Laser-Doppler Perfusion Imager (PeriScan PIM3 System; PERIMED, Stockholm, Sweden), as previously described [22]. Briefly, heart was exposed and a computer-controlled optical scanner directed a low-powered He-Ne laser beam over the exposed heart. The scanner head was positioned in parallel to the surface of heart at a distance of 18 cm. At each measuring site, the beam illuminated the tissue to a depth of 0.5 mm. A color-coded image denoting specific relative perfusion level was displayed on a video monitor, and all images were evaluated with the software LDPIwin 3.1 (PeriScan PIM3 System; PERIMED, Stockholm, Sweden). The magnitude of MBF was represented by different colors, with blue to red denoting low to high. Results were expressed as percentages of the baseline MBF [22].

This article is protected by copyright. All rights reserved.
Heart function test

A cannulation was inserted into left ventricle through right carotid artery, which was connected to a bio-function experiment system BL-420F (Chengdu Taimen technology Ltd, Chengdu, China). LVSP, LVDP, LVEDP, +dp/dtmax, dp/dtmax, were evaluated at baseline, 30 min after ischemia, and 90 min after reperfusion with a BL-420F equipment [11].

Myocardial infarct size

At 30 min after ischemia and 90 min after reperfusion, LADCA was ligated, and 2 ml of 0.35% Evans Blue (Sigma, St. Louis, MO, USA) was administrated through femoral vein. Hearts were rapidly excised and sliced into 5 sections (1 mm thick), parallel to the atrioventricular groove, from the apex cordis to the ligation site. Slices were incubated for 15 min at 37 °C in a 0.375% solution of triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA), and then photographed with a stereoscope connected with Digital sight (DS-5M-U, NIKON, Nanjing, China). In so treated slices, infarction zone was stained white, AAR was pink, while non-infarction zone was blue. The myocardial area of infarct, AAR and LV was analyzed on each slice, respectively, by Image-Pro Plus 6.0 (Media Cybernetic, Bethesda, MD, USA) (n=6). The ratios of AAR/LV (%) and infarct area/AAR (%) were calculated, and the values from 5 slices were averaged and used to score the degree of myocardial infarction [17].

This article is protected by copyright. All rights reserved.
Double staining of F-actin and TUNEL

At 30 min after ischemia and 90 min after reperfusion, heart was perfused with saline, and then removed and fixed in 4% paraformaldehyde solution for 48 h, processed for paraffin section (5 μm). Sections were subjected to double staining of F-actin and TUNEL. F-actin was labeled with rhodamine phalloidine (R415, Invitrogen, Carlsbad, California, USA), and TUNEL staining was undertaken by a cell death detection kit (Roche, Basel, Switzerland), according to the manufacture’s instruction. Then DyLight™ 549 and DyLight™ 488-labeled secondary antibodies were applied (KPL, Gaithersburg, Maryland, USA), and the nuclei were labeled with Hoechst33342. Five fields were selected from the surrounding infarction areas of the left ventricle for each section at ×40 magnification of objective, and observed with a Laser Scanning Confocal Microscope (TCS SP5, Leica, Mannheim, Germany). The number of the TUNEL-positive cells in the five fields were counted, and the average was calculated and expressed as cell number per field.

cTnI content in serum

Blood was collected and serum prepared using heparin as an anticoagulant at 30 min after ischemia and 90 min after reperfusion, and then samples were centrifuged for 15 minutes at 1000g at 4°C. The supernatant was harvested, and the content of cTnI was detected using a rat cTnI ELISA Kit by microplate reader (MULTISKAN MK3, Thermo, San Jose, CA, USA) [20].

This article is protected by copyright. All rights reserved.
Assessment of energy metabolism

At 30 min after ischemia and 90 min after reperfusion, rats were perfused with saline under anesthesia, and the hearts were removed (n=6). The tissue from left ventricle was sampled at about 2 mm under ligature, quickly frozen in liquid nitrogen, and stored at -80 °C for a maximum of 1 week before use. The whole protein of the tissues was extracted with a protein extraction kit (Applygen Technologies, Beijing, China), according to manufacturer’s instruction. Briefly, eighty to one hundred mg of tissue was cut into pieces, mixed with 1 ml of RIPA containing 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, and 5 mM PMSF. The mixture was homogenized, incubated on ice for 30 min, and centrifuged at 19357 g, 4°C, for 10 min. The myocardial content of ATP, ADP and AMP was assessed with ELISA by microplate reader (MULTISKAN MK3, Thermo, San Jose, CA, USA), according to manufacturer’s instruction.

Western blotting assay and real-time PCR

Rats were sacrificed 30 min after ischemia and 90 min after reperfusion, and 200 mg of myocardial tissue was sampled from the surrounding of infarct area of left ventricle, and stored at -80 °C (n=3). The whole protein was extracted as described above. The concentration of whole protein was determined with a BCA protein assay kit (Applygen Technologies, Beijing, China), according to the manufacture’s instruction. For each sample, the assessment was undertaken twice, taking the average as the concentration.
The whole protein was mixed with 2× electrophoresis sample buffer. After separated on 12% SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membrane. After 1 h blocking with 5% nonfat dry milk or 5% BSA, rinsing with TBS-Tween for 3 times, 5 min each, the membrane with target proteins was cut and incubated overnight at 4°C with antibodies, respectively, against P-MLC2 (1:1000), ATP5D (1:200), Bcl-2 (1:1000), Bax (1:1000), and cTnI (1:2000). And then the membranes were rinsed 3 times, 5 min each, incubated with secondary antibody for 1 h at room temperature, followed by rinsing with TBS-Tween 3 times, 10 min each time. The protein was quantified by scanning densitometry in the X-film using a bio-image analysis system (Image-Pro plus 6.0). The result of each group was expressed as a relative optical density to that from Sham group.

In addition, real-time quantitative PCR was performed to detect the mRNA level of ATP 5D from each sample in accordance to the manufacturer’s protocol. RNA was extracted using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer protocol. RNA was applied for reverse transcription using a Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lifesciences, UK) to generate the first strand cDNA mix. Real-time PCR was performed utilizing the ABI PRISM sequence detection system 7500 (Perkin-Elmer Applied Biosystems). Primer sequences (all Rattus): ATP5D—forward, 5’-CACTGTGAATGCGGACTCCT-3’; reverse, 5’-GGATTTGGATCTCAGCCCGT-3’; GAPDH—forward, 5’-AGTTCAACGGCACAGTCAAG-3’; reverse, 5’-TACTCAGCACCCAGCATCACC-3’.

The PCR reaction mixture (25 μl) included 2×Maxima SYBR Green/ROX qPCR Master Mix,
reverse transcription product cDNA, forward and reverse primers, nuclease-free water. The reactions took place in a 96-well plate at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 1 min and plate read. All tests were performed in triplicate.

Statistical analysis

All data were expressed as mean ± SE. Statistical analysis was carried out with SAS 9.3 statistical software, and one-way analysis of variance was used, and then for post hoc testing, Fisher’s least-significant-difference test was used for multiple comparisons between groups. For repeated measurement data, the linear mixed effects models were analyzed, and least squares means were calculated between the groups of different time points. P < 0.05 was considered as statistically significant.

Results

Effect of AS-IV on myocardial infarct size

The effect of AS-IV on myocardial infarct size at different doses was determined by Evans blue-TTC staining. As showing in the Figure 2A, the pink area represents ischemic myocardial tissue, the white area represents the infarction region, and the blue area represents normal myocardial tissue. We found that AS-IV was effective at the dose of 10 mg/kg, and thus chose this dose for all the experiments below. The representative heart slices in I-30 min and I/R-90 min groups are shown in Figure 3A and Figure 3D, respectively. Obviously, myocardial tissue slices from I-30 min group exhibited ischemia but no infarct. By contrast,
noticeable ischemia and infarct areas were observed in myocardial tissue slices in I/R-90 min group. As compared to I/R-90 min group, pretreatment with AS-IV significantly decreased the I/R-90 min induced myocardial infarct size, but retained a similar area of ischemic region. As shown in Fig.3.B-C, E-F, quantitative analysis of AAR/ LV and infarct area/AAR confirmed the above results.

**Effect of AS-IV on MBF**

Figure 4A shows the color images acquired by the Laser Scanning Doppler in the four groups at different time point. A prominent decrease in MBF occurred from 30 min after ischemia, and persisted till 90 min after reperfusion in I/R-group. AS-IV pretreatment prevented MBF from decrease at 30 min ischemia, and this protective role remained by I/R-90 min. Figure 4B is the quantitative evaluation of MBF changes in the four groups, which confirmed the impression from Figure 4A.

**Effect of AS-IV on heart function**

Heart function was assessed in different conditions to evaluate the role of AS-IV in protecting heart against ischemia and reperfusion injury. As noticed in Fig.5, in comparison with Sham group, ischemia 30 min caused a significant decline in $+\frac{dp}{dt_{max}}$, and an apparent elevation in LVDP, LVEDP and $-\frac{dp}{dt_{max}}$, indicating an impairment on heart function. Reperfusion for 90 min led to a further decline in $+\frac{dp}{dt_{max}}$ as well as a significant decrease in LVSP, and a sustained increase in $-\frac{dp}{dt_{max}}$, but did not deteriorate LVDP and LVEDP. The protective role of AS-IV pretreatment for LVDP and LVEDP exhibited already.
at 30 min ischemia, but only at 90 min reperfusion for other parameters examined. No significant change was observed in heart rate in any group over the observation, nor among the groups at any time point (Fig.5A).

**Effect of AS-IV on cTnI in myocardium and serum**

As a marker of myocardial damage [2], cTnI level in myocardial tissue and serum was assessed by Western Blotting and ELISA, respectively. The expression of cTnI in myocardial tissue decreased significantly 30 min after ischemia (Fig.6A and C) and 90 min after reperfusion (Fig.6B and D), as compared with Sham group. In contrast, the level of cTnI in serum was very low in Sham group, but increased evidently 30 min after ischemia (Fig.6E), and 90 min after reperfusion (Fig.6F). Noticeably, the change in cTnI level in both myocardial tissue and serum after ischemia and I/R was significantly attenuated by pretreatment with AS-IV (Fig. 6C).

**Effect of AS-IV on energy metabolism**

To address the energy metabolism in different conditions, the ratio of ATP/ADP and ATP/AMP in cardiac tissue was explored. As shown in Fig.7 A-D, AS-IV alone had no effect on either ATP/ADP or ATP/AMP compared with Sham group. Notably, ATP/ADP and ATP/AMP decreased dramatically at 30 min after ischemia, and remained at low level by I/R-90 min, indicating a more catabolism of ATP. However, pretreatment with AS-IV significantly prevented ATP/ADP and ATP/AMP from reduction both at 30 min after ischemia and 90 min after reperfusion.

This article is protected by copyright. All rights reserved.
We next determined the expression of ATP 5D and P-MLC2 in myocardial tissue. As a subunit of ATP synthase, the expression of protein and mRNA of ATP 5D had an obvious reduction in response to I-30 min and I/R-90min (Fig.8.A2-A3, B2-B3). The level of P-MLC2 increased prominently in response to 30 min ischemia (Fig.8.A4) and I/R-90min challenge (Fig.8. B4). Of notice, pretreatment with AS-IV prevented all the alterations evoked by ischemia and I/R.

**Effect of AS-IV on cardiac structure and myocardial cell apoptosis**

To gain insight into the effect of AS-IV on the alteration in myocardium structure and apoptosis, double staining of F-actin and TUNEL was carried out for the surrounding infarction areas of the left ventricle myocardial tissue from various groups. The representative images are displayed in Fig.9 A and B, wherein nuclei were stained blue, F-actin red, and TUNEL-positive cells green. At 30 min after ischemia, myocardial tissue became injury with disrupted myocardial fibers and few apoptotic cells, while this injury was protected against by pretreatment with AS-IV. At 90 min after reperfusion, the myocardial tissue displayed more distinct alterations compared with Sham group, exhibiting rupture of myocardial fibers, degradation of F-actin, and numerous TUNEL-positive cells. These changes were all alleviated by pretreatment with AS-IV.

**Effect of AS-IV on the expression of apoptosis-related proteins**

Bcl-2 and Bax have been well accepted as apoptosis regulated proteins, with Bcl-2 acting as an anti-apoptosis factor, and Bax as a pro-apoptosis molecule [13]. Therefore, we
investigated the expression of Bcl-2 and Bax by Western blotting, and calculated the ratio of Bax/Bcl-2. As shown in Fig.10, the expression of Bcl-2 and Bax had no obvious change among the groups at 30 min after ischemia, while at 90 min after reperfusion the ratio of Bax/Bcl-2 significantly increased as compared to Sham group, and such upregulation was suppressed by pretreatment with AS-IV.

**Discussion**

The present study showed that I/R induced myocardium injury manifested differently in ischemia and reperfusion phase. In ischemia phase the injury presented as a decrease in MBF, ATP/ADP, ATP/AMP, the expression of ATP 5D and the content of cTnI in myocardium, and an increase in phosphorylation of MLC2 and the content of cTnI in serum, accompanying with disrupted F-actin; while in reperfusion phase, MBF remained deceased compared to baseline implying a no-reflow due to the impairment of microvasculature, and apoptosis and infarction occurred in addition to the insults observed in ischemia phase. Interestingly, pretreatment with AS-IV attenuated I/R elicited insults in myocardium in both ischemia and reperfusion phase, suggesting it as a potential option for protecting heart from I/R injury.

As expected, most of ischemia evoked insults are associated with disorder of energy metabolism. Energy metabolism disorder in ischemia phase was documented in the present
case by the reduction in ATP/ADP and ATP/AMP. F-actin integrity in myocardium was found disrupted in this phase, an outcome that is known to depend on ATP availability. LVEDP and LVDP elevated significantly at 30 min ischemia, indicative of a so called ischemic contracture, which is related to ATP depletion [15], since a too low ATP availability leads to crossbridges remaining trapped in a rigor state [14]. Ischemia led to an increase in phosphorylation of MLC2 in present study. MLC2 phosphorylation are mediated by myosin light chain kinase as well as by protein kinase C, both of which are Ca^{2+} dependent, and ATP depletion caused cytoplasmic Ca^{2+} overload may be expected to increase MLC2 phosphorylation [16]. Of notice, ATP 5D expression decreased in ischemia phase. As one of the subunits of ATP synthase, ATP 5D plays an important part in ATP synthesis [4]. The result of the present study implies that reduced ATP 5D expression along with hypoxia contributes to the observed ATP depletion. Importantly, AS-IV pretreatment protected against energy metabolism disorder and the resultant insults, suggesting the beneficial role of AS-IV initiated early in the ischemia phase. Moreover, the results that AS-IV attenuated MBF and ATP 5D expression suggested that the protective role of AS-IV in energy metabolism is attributable to increased oxygen supply as well as to enhanced ATP synthesis capacity. Nonetheless, the detailed mechanism responsible for the beneficial role of AS-IV in energy metabolism needs further study.

AS-IV is widely used to cope with various diseases, including acute kidney injury [6], Parkinson’s disease [21], and diabetic nephropathy [5]. Several studies reported that AS-IV exerted its multiple action through inhibiting oxidative stress, interfering in NF-kappa B
mediated inflammatory process, and Bax-mediated apoptosis pathways [5, 21]. In a myocardial I/R injury model in the present study, the anti-apoptosis potential of AS-IV was further documented, as shown by the reduction of TUNEL-positive cells after AS-IV pretreatment. Pretreatment with AS-IV significantly prevented the increase in Bax/Bcl-2 ratio after I/R, suggesting that the anti-apoptosis effect of AS-IV was related to the suppression of Bax-mediated pathway. AS-IV protection of cardiac myocytes from apoptosis ultimately led to the attenuation of myocardial injury, as evidenced by the reduction in myocardial infarct size. Of notice, I/R induced cardiac myocyte apoptosis and myocardium infarction emerged at 90 min after reperfusion but not at 30 min after ischemia, showing that these impairments are reperfusion injury in nature. On the other hand, an increase in serum cTnI level and a decrease of cTnI content in heart tissue were observed already at 30 min after ischemia, indicating that cardiac myocyte injury took place in this phase in a form of non-apoptosis. AS-IV prevented ischemia induced increase in serum cTnI level, implying that AS-IV protects cardiac myocyte from ischemia injury via a mechanism other than depressing apoptosis. This mechanism is most likely a modulation of energy metabolism.

In conclusion, AS-IV pretreatment protected against myocardium injury and cardiac malfunction after I/R, which may be related to its potential to restore the energy metabolism disorder occurred in ischemia phase. The results provide support for AS-IV as a novel therapeutic approach to protect against I/R-induced myocardial injury. In addition, the results of present study opened an avenue for development of new drug to cope with cardiac I/R injury by targeting energy metabolism.

This article is protected by copyright. All rights reserved.
Perspectives:

Most studies of I/R mainly concern oxidative stress and apoptosis. In present study, we aimed at energy metabolism, and found that AS-IV may prevent I/R injury via energy metabolism modulation. It may open a new way to deal with cardiac I/R injury.

Acknowledgment

This work was supported by the National Natural Science Foundation of China [81273637] for Jing-Yan Han.

References:


5. Gui D, Huang J, Guo Y, Chen J, Chen Y, Xiao W, Liu X and Wang N. Astragaloside iv ameliorates renal injury in streptozotocin-induced diabetic rats through...


This article is protected by copyright. All rights reserved.


Table

Table1. Number of animals for different experimental groups and various parameters.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>AS-IV</th>
<th>I-30</th>
<th>AS-IV + I-30</th>
<th>Sham</th>
<th>AS-IV</th>
<th>I/R-90</th>
<th>AS-IV + I/R-90</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (I)</td>
<td>AS-IV (I)</td>
<td>I-30 (I)</td>
<td>AS-IV + I-30 (I)</td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This article is protected by copyright. All rights reserved.
The same animals were used for detection of myocardial blood flow and myocardial infarct size, and the same was true for detection of hemodynamics and ATP/ADP/AMP content assay. Sham (I): Sham group at 30 min after ischemia; AS-IV (I): AS-IV plus Sham group at 30 min after ischemia; I-30min: Ischemia 30 min group; AS-IV + I-30 min: treatment with AS-IV plus I-30 min group; Sham (I/R): Sham group at 90 min after reperfusion; AS-IV (I/R): AS-IV plus Sham group at 90 min after reperfusion; AS-IV + I/R-90 min: treatment with AS-IV plus I/R-90 min group.

**Figure Captions**

**Fig.1.** Structure of AS-IV.

**Fig.2.** The effect of AS-IV at different doses on myocardial infarct size of rats subjected to 30 min ischemia followed by 90 min reperfusion. A: Representative slices of left ventricle
stained by Evans blue-TTC at 90 min after reperfusion. B and C: Quantitative measurement of AAR/LV and infarct area/AAR area at 90 min after reperfusion. *$P<0.05$ vs. Sham group. $P<0.05$ vs. I/R-90 group. Data are mean ± SE (n=6).

**Fig.3.** The effect of AS-IV on myocardial infarct size of rats subjected to either 30 min ischemia or 30 min ischemia followed by 90 min reperfusion. A and D: Representative slices of left ventricle stained by Evans blue-TTC at 30 min after ischemia and 90 min after reperfusion following 30 min ischemia, respectively. B and C: Quantitative measurement of AAR/LV and infarct area/AAR area at 30 min after ischemia. *$P<0.05$ vs. Sham (I) group. E and F: Quantitative analysis of AAR/LV and infarct area/AAR area at 90 min after reperfusion following 30 min ischemia. * $P<0.05$ vs. Sham (I/R) group, $P<0.05$ vs. I/R-90 group. Data are mean ± SE (n=6).

**Fig.4.** The effect of AS-IV on MBF of rats. A: Representative images of MBF acquired by Laser Scanning Doppler Perfusion Imager in Sham (I/R) group, AS-IV (I/R) group, I/R-90 group and AS-IV + I/R-90 group at baseline, 30 min after ischemia, and 90 min after reperfusion. B: Time course of MBF in various groups. The linear mixed effects models were analyzed for repeated measurement data, and least squares means were calculated between the groups of different time points. *$P<0.05$ vs. Sham (I/R) group, $P<0.05$ vs. I/R-90 min group. Values are means ± SE (n=6).

**Fig.5.** The effect of AS-IV on rat cardiac function. Presented are the time courses of HR (A), LVSP (B), $+dp/dt_{max}$ (C), LVEDP (D), LVDP (E) and $-dp/dt_{max}$ (F) in Sham (I/R)
group, AS-IV (I/R) group, I/R-90 group and AS-IV + I/R-90 group, respectively. The linear mixed effects models were analyzed for repeated measurement data, and least squares means were calculated between the groups of different time points. *$P<0.05$ vs. Sham (I/R) group, #$P<0.05$ vs. I/R-90 group. Values are means ± SE (n=6).

**Fig.6.** The effect of AS-IV on the level of cTnI in heart and serum. A, B, C, and D: The representative Western blotting bands and semi-quantitative analysis of cTnI in heart at 30 min after ischemia (A, C) and 90 min after reperfusion following 30 min ischemia (B, D), respectively. E and F: The semi-quantitative analysis of cTnI level in serum at 30 min after ischemia (E) and 90 min after reperfusion (F), respectively. In C and E: *$P<0.05$ vs. Sham (I) group, #$P<0.05$ vs. I-30 group. In D and F: *$P<0.05$ vs. Sham (I/R) group, #$P<0.05$ vs. I/R-90 group. Values are means ± SE (n=3).

**Fig.7.** The effect of AS-IV on the energy metabolism in the myocardium of rats. A and C: The effects of AS-IV on the ratio of ATP/ADP, and ATP/AMP in myocardium at 30 min after ischemia. *$P<0.05$ vs. Sham (I) group, #$P<0.05$ vs. I-30 group. B and D: The effects of AS-IV on the ratio of ATP/ADP, and ATP/AMP in myocardium at 90 min after reperfusion. *$P<0.05$ vs. Sham (I/R) group, #$P<0.05$ vs. I/R-90 group. Values are mean ± SE. (n=3).

**Fig.8.** The effect of AS-IV on the expression of ATP 5D and P-MLC2 in myocardium of rats. A: The expression of ATP 5D and P-MLC2 at 30 min after ischemia in various groups. Shown are representative Western blotting bands (A1), semi-quantitative analysis of ATP 5D (A2) and P-MLC2 (A4) protein, and the mRNA level of ATP 5D (A3), respectively. *$P<0.05$
vs. Sham (I) group, *P<0.05 vs. I-30 group. B: The expression of ATP 5D and P-MLC2 at 90 min after reperfusion in various groups. Shown are the the representative Western blotting bands (B1), semi-quantitative analysis of ATP 5D (B2) and P-MLC2 (B4) protein, and mRNA level of ATP 5D (B3), respectively. *P<0.05 vs. Sham (I/R) group, #P<0.05 vs. I/R-90 group. Values are means ± SE (n=3).

**Fig.9.** The effect of AS-IV on myocardial apoptosis and F-actin. A and B: Presented are the representative photographs of double staining of F-actin and TUNEL. Nuclei are stained with blue, F-actin red, and TUNEL-positive cells green (arrow). Bar = 25 μm. C and D: Quantitative analysis of apoptosis cells among the various groups. Ordinates are cell number per field. *P<0.05 vs. Sham (I/R) group, #P<0.05 vs. I/R-90 group. Values are means ± SE (n=3).

**Fig.10.** The effect of AS-IV on the expression of Bcl-2 and Bax in myocardium of rats. A and B: The representative Western blotting bands of Bcl-2 and Bax in myocardium at 30 min after ischemia (A) and 90 min after reperfusion (B), respectively. C and D: The semi-quantitative analysis of Bax/Bcl-2 at 30 min after ischemia (C) and 90 min after reperfusion (D), respectively. *P<0.05 vs. Sham (I/R) group, #P<0.05 vs. I/R-90 group. Values are means ± SE (n=3).
Figure 2

Figure 3
Figure 4

A

Baseline
I-30
I/R-90

Sham AS-IV I/R AS-IV+I/R

B

Myocardial blood flow (%)

0 30 60 90 120

Baseline I-30 I/R-90

I R

Figure 5

A

Heart Rate (bpm)

0 100 200 300 400

Baseline I-30 I/R-90

B

LVSP (mmHg)

0 50 100 150

Baseline I-30 I/R-90

C

LVDP (mmHg)

0 5 10 15

Baseline I-30 I/R-90

D

Baseline I-30 I/R-90

E

Baseline I-30 I/R-90

F

Baseline I-30 I/R-90