Astragaloside IV regulates the PI3K/Akt/HO-1 signaling pathway and inhibits H9c2 cardiomyocyte injury induced by hypoxia-reoxygenation

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Abstract

Astragaloside IV (AS-IV) is one of the main pharmacologically active compounds found in Astragalus membranaceus. AS-IV has protective effects against ischemia-reperfusion injury (IRI), but its mechanism of action has not yet been determined. This study aims to investigate the effect of AS-IV on IRI and its effect on the PI3K/Akt/HO-1 signaling pathway through in vitro experiments. Firstly, a cell culture model of myocyte hypoxia-reoxygenation (H/R) injury was replicated. After AS-IV treatment, cell viability, reactive oxygen species (ROS) levels, as well as the content or activity of the cellular factors lactate dehydrogenase (LDH), superoxide dismutase (SOD), malondialdehyde (MDA), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), were measured to evaluate the effect of treatment with AS-IV. The effect of AS-IV on HO-1 protein expression and nuclear Nrf2 and Bach1 protein expression was determined by Western blotting. Finally, a reversal of the effect of AS-IV treatment was observed following co-incubation with a PI3K inhibitor. Our results show that AS-IV has good protective effect on H/R injury and has anti-oxidative stress and anti-inflammatory effects. It can regulate the expression of Nrf2 and Bach1 proteins in the nucleus and promote the expression of HO-1 protein, while a PI3K inhibitor can partially reverse the above effects. This study suggests that the PI3K/Akt/HO-1 signaling pathway may be a key signaling pathway for the anti-IRI effect of AS-IV.

Keywords Astragaloside IV; H9c2 cardiomyocyte; hypoxia-reoxygenation injury; Heme oxygenase (HO-1); Phosphatidylinositol 3-kinase/Akt pathway
Introduction

The traditional Chinese medicine consisting of the root of *Astragalus membranaceus Bunge* has been widely used in the treatment of various diseases for several thousands of years. Astragaloside-IV (AS-IV) is one of the main pharmacologically active substances of *A. membranaceus Bunge*. Studies have confirmed that AS-IV has protective effect against cardiovascular disease, ischemic encephalopathy, diabetes, liver fibrosis, and other diseases. Its mechanism of action involves anti-inflammatory, anti-oxidative, anti-fibrotic, and immunoregulatory actions. In recent years, *in vitro* and *in vivo* studies have shown that AS-IV is protective against myocardial ischemia-reperfusion injury (IRI) and its mechanism of action is mainly related to anti-oxidative stress and anti-inflammatory pathways. However, the specific target molecules and signaling pathways involved still await further study. Heme oxygenase (HO-1) is recognized as an important anti-oxidative stress and tissue protective enzyme that produces CO and biliverdin, and can also induce multiple anti-oxidative and anti-inflammatory signaling pathways, which significantly reduce cellular damage and protect organ function. As a novel protective factor in myocardioocytes, HO-1 plays an important role in mitigating damage due to myocardial IRI through the anti-inflammatory, anti-oxidative stress, anti-apoptotic, and anti-arrhythmic effects of its products. In this study, we further studied the mechanism through which AS-IV induces HO-1 expression and its related signaling pathways on an *in vitro* model of myocardial IRI.

1. Materials and methods

1.1 Main materials

The H9c2 rat cardiomyocyte cell line was purchased from The Institute of Life Sciences cell bank(Shanghai, China). AS-IV (Lot No.H-013-170614), purity 99.06%, was purchased from Herbpurify Co., Ltd(Chengdu,China). and its molecular structure is shown in Figure 1. AS-IV (5g) was stored at the Institute of Biochemistry and Molecular Biology of Ningbo University to permit future reference and verification.

Dulbecco’s Modified Eagle Medium (DMEM) was purchased from Gibco Co(Grand Island, NY, USA.). Fetal bovine serum (FBS) was purchased from Hyclone Co.,( Logan, Utah, USA). Cell Counting Kit-8 (CCK-8) was purchased from Lianke Biology Technology Co., Ltd(Hangzhou,China). Reactive oxygen species (ROS),lactate dehydrogenase (LDH), superoxide dismutase (SOD), and malondialdehyde (MDA) kits were purchased from Institute of Bioengineering(Nanjing,China). Tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) ELISA kits were purchased from USCN Co., Ltd (Wuhan,China). Cell lysis buffer for Western and nuclear protein extraction kit, BCA protein quantification kit, and ECL luminescence kit for Western blotting were purchased from Beyotime Biotechnology Co. (Shanghai,China). PI3K, p-Akt, Akt, HO-1, Nrf2, Bach1, GAPDH, and
Histone H3 rabbit anti-rat polyclonal antibodies were purchased from Proteintech Co (Shanghai, China). Cobalt protoporphyrin (CoPP, HO-1 agonist) and zinc(II) protoporphyrin IX complex (ZnPP, HO-1 inhibitor) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). PI3K inhibitor LY294002 was purchased from Selleck Co. (Houston, Texas, USA).

1.2 Cell culture and hypoxia-reoxygenation (H/R) model replication

H9c2 cardiomyocytes were seeded in DMEM containing 10% FBS, placed in a constant temperature incubator at 37 °C under 5% CO2. Cells were subcultured based on their density and logarithmic growth phase (approximately 80% confluent) cells were used for experiments. After optimizing experimental conditions, the H9c2 H/R model was determined: the cells were cultured for 24 hours, then cultured for 12 hours under hypoxia and hypoglycemia, followed by reoxygenation in media containing optimal glucose for 8 hours.

1.3 Toxicity of ASIV on normal H9c2 cells

H9c2 cells in logarithmic growth phase were divided into the control group and four AS-IV treatment groups (0.1μM, 1μM, 10μM, 100μM). After incubating for 20h, cell viability was measured using CCK-8. Cell viability (%) = (test well OD - blank well OD) / (control well OD - blank well OD) x 100.

1.4 Determination of AS-IV effective concentration

H9c2 cells in logarithmic growth phase were divided into the control group, H/R model group, and four AS-IV treatment groups (0.1μM, 1μM, 10μM, 100μM). During H/R model establishment, the cells were incubated in the presence of AS-IV for 20h, cell viability was measured using CCK-8, and the LDH content of the cell supernatant was measured using the LDH kit.

1.5 Cell grouping and treatment methods

Logarithmic growth phase cells were randomly divided into the following groups: control group (cultured under normal conditions for 20h), H/R model group (hypoxic for 12h followed by reoxygenation for 8h), H/R+AS-IV group (100μM AS-IV administered for 20h during H/R model establishment), H/R+AS-IV+ZnPP group (100 μM AS-IV and ZnPP administered for 20 h during H/R model establishment), and H/R+CoPP group (positive control group, CoPP treatment for 20h during H/R model establishment). After treatment, the cell supernatant was collected and the concentrations or activities of SOD, MDA, IL-6, and TNF-α were measured. Three replicate wells were set up for each group and each experiment was performed in triplicate.
1.6 Determination of SOD and MDA activities and IL-6 and TNF-α content

SOD activity was measured using the -hydroxylamine method, while the MDA activity was measured using the thiobarbituric acid method. The contents of IL-6 and TNF-α were measured by ELISA. All measurements were performed according to kit instructions.

1.7 Determination of intracellular ROS

ROS levels were determined using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. Cells were seeded in 6-well plates, treated with AS-IV for 20h, and were stained with DCFH-DA(10 μ M) in a staining buffer for 30min at 37℃. Then, random cells were photographed under an inverted fluorescence microscope and five images were selected for each group. The intensity of fluorescence was determined and the mean fluorescence intensity (MFI) was determined.

1.8 Treatment with PI3K inhibitor LY294002

Logarithmic growth phase cells were randomly divided into the following groups: control group (culture under normal conditions for 20h), H/R model group, H/R+ASIV group, and H/R+ASIV+ LY294002 group (20μM LY294002 pre-treatment for 2h, followed by 100μM ASIV for 20h). At the end of the treatment, cells were collected for measurement of PI3K/HO-1 signaling pathway protein expression. Three replicate wells were set up for each group and the experiment was repeated in triplicate.

1.9 Western blot

Cells were collected and total protein (to assay PI3K,p-Akt,Akt and HO-1 content) as well as nuclear protein (to assay Nrf2 and Bach1 content) were extracted. Protein concentration was determined using the BCA method. Thirty micrograms of protein were loaded from each well and separated via gel electrophoresis on 12% polyacrylamide gel. The separated proteins were transferred onto PVDF membranes. A blocking solution containing 5% skim milk was incubated with the membranes on a shaker at room temperature for 1 h, followed by three TBST washes. Rabbit anti-rat PI3K, p-Akt, Akt, HO-1, Nrf2, and Bach1 polyclonal antibodies were added to the respective reaction and allowed to incubate overnight at 4℃. The membrane was washed three times with TBST on the following day, then goat anti-rabbit IgG secondary antibody was added and incubated at room temperature for 1 h. The membrane was washed 3 times with TBST. The ECL kit instructions were followed to expose the proteins of interest and the image was analyzed using Image J. The relative expression of each protein of interest was normalized using either GAPDH (PI3K, Akt, HO-1) or HistoneH3(Nrf2, Bach1).
1.10 Statistical analysis

The data were analyzed and processed using SPSS19.0. Data were expressed as mean ± standard deviation (\( \bar{x} \pm s \)). One-way ANOVA was used for comparison between groups. The LSD method was used for further comparison between the two groups. P<0.05 indicates statistically significant differences.

2 Results

2.1 AS-IV reduces cytotoxicity to H9c2 cells induced by H/R injury

The CCK-8 assay demonstrates cell viability. The range of concentrations of AS-IV used in this experiment (0.1μM– 100μM) have no significant effect on the viability of normal cultured H9c2 cells (P>0.05) (Figure 2A). Compared to the control group, the viability of the H/R group decreased significantly (P<0.01). Compared to the H/R group, the AS-IV 10μM and 100μM treatment groups showed significantly increased cell viability (P<0.01), while the AS-IV 0.1μM and 1μM treatment groups showed no significant effect (Figure 2B). The LDH assay indicates that LDH activity in the H/R group was significantly higher than that in the control group (P<0.01). Compared with the H/R group, treatment with 10 μM and 100 μM AS-IV significantly decreased the LDH activity level (P<0.01). However, AS-IV administered at 0.1 μM and 1 μM had no significant effect on LDH activity (P>0.05) (Figure 2C). These results indicate that the viability of H/R model cells is significantly decreased, and that H/R injury-induced cytotoxicity is significantly increased. AS-IV treatment at concentrations of 10μM and 100μM significantly increases cell viability and reduces cytotoxicity. As results using 100μM was superior to those obtained with 10μM AS-IV, subsequent experiments were performed with AS-IV100 μM.

2.2 AS-IV alleviates oxidative damage of H9c2 cells induced by H/R injury

Experimental results show that when compared to the control group, ROS level and MDA activity increased significantly in the H/R group, while SOD activity decreased significantly (P<0.01). Compared with the H/R group, ROS level and MDA activity decreased significantly in the AS-IV and CoPP treatment groups, while SOD activity increased significantly (P<0.01). Compared with the AS-IV group, ROS level and MDA activity increased significantly in the ASIV+ZnPP treatment group, while SOD activity decreased significantly (P<0.01) (Fig. 3AB). These findings indicate that AS-IV and CoPP have significant anti-oxidative stress effects, and ZnPP can partially reverse the anti-oxidative stress effects of AS-IV.

2.3 AS-IV reduces the inflammatory response of H9c2 cells induced by H/R injury

Experimental results show that compared with the control group, the levels of IL-6 and
TNF-α secreted by cells in the H/R group were significantly increased ($P<0.01$). Compared to the H/R group, the cells in the AS-IV and CoPP treatment groups secreted significantly less IL-6 and TNF-α ($P<0.01$). Compared with the AS-IV group, IL-6 and TNF-α secretion increased significantly in the AS-IV+ZnPP treatment group ($P<0.01$) (Figure 4AB). This indicates that AS-IV and CoPP have significant anti-inflammatory effects and that ZnPP can partially reverse the anti-inflammatory effects of AS-IV.

2.4. Effect of AS-IV on activation of PI3K/Akt /HO-1 signaling pathway

Western blotting showed that the expression of PI3K and p-Akt in the H/R group was significantly lower than that in the control group ($P<0.01$). Compared with the H/R group, the expression of PI3K and p-Akt in the AS-IV group increased significantly ($P<0.01$). Compared with the AS-IV group, PI3K and p-Akt expression in the PI3K-specific inhibitor LY294002 group decreased significantly ($P<0.01$) (Fig. 5A D G). Compared with the control group, the expression of HO-1 in the H/R group increased significantly increased ($P<0.01$). Compared with the H/R group, the expression of HO-1 in the AS-IV group increased significantly ($P<0.01$). In comparison, the expression of HO-1 in the LY294002 group decreased significantly ($P<0.01$) (Figs. 5B and G). The results suggest that AS-IV promotes HO-1 protein expression, and PI3K-specific inhibitors partially reverse this effect. In addition, ASIV had no influence to the basal expression of PI3K, p-Akt, HO-1, Nrf2 and Bach1 ($P>0.05$). (Figure S1 available online)

Western blotting also showed that the expression of Nrf2 in the H/R group was significantly higher than that in the control group ($P<0.01$). Compared with the H/R group, the expression of Nrf2 in the AS-IV group increased significantly ($P<0.01$). Compared with the AS-I-V group, the expression of Nrf2 in the LY294002 group decreased significantly ($P<0.01$), (Fig. 5E and H). Compared with the control group, the expression of Bach1 increased significantly in the H/R group ($P<0.01$). Compared to the H/R group, the expression of Bach1 decreased significantly in the AS-IV group ($P<0.01$). Compared with the AS-IV group, the expression of Bach1 increased significantly in the LY294002 group ($P<0.01$) (Fig. 5F and H). The results suggest that AS-IV can significantly increase nuclear Nrf2 expression, while decreasing nuclear Bach1 expression, and PI3K specific inhibitors can partially reverse this effect.

3 Discussion

Reducing myocardial IRI has been a primary focus of researchers and clinicians. 9) It is generally believed that the occurrence and development of IRI is due to a cascade of multi-factor interactions, which are mutually causal and mutually influential, and ultimately lead to the apoptosis and death of cardiomyocytes. Free radical damage and inflammatory
responses are believed to play key roles in myocardial IRI. In recent years, the role of HO-1 in myocardial IRI has received a great deal of attention. Numerous studies have shown that HO-1 and its metabolites are widely involved in anti-oxidative stress, anti-inflammatory damage, anti-cell proliferation, anti-apoptosis, inhibition of platelet aggregation, regulation of vascular tone, and intracellular signaling and play a very important role in cardiovascular diseases. HO-1 appears to be particularly closely related to myocardial IRI, as the expression of HO-1 is significantly increased in the early stage of myocardial IRI. Over-expression of the HO-1 gene in cardiomyocytes or increased myocardial HO-1 expression via gene therapy or drug therapy can significantly reduce the infarct surface area due to ischemia during IRI and significantly improve myocardial function. Mice deficient in the HO-1 gene showed more severe IRI. In this study, by replicating the myocardial H/R cell culture model, we were able to separately administer HO-1 agonists and inhibitors. The study also confirmed that HO-1 can significantly inhibit the expression of inflammatory factors and increase the level of anti-oxidants, indicating that HO-1 is protective against myocardial H/R injury via protection against oxidative stress and inhibition of inflammation.

The above results suggest that by highly expressing HO-1 levels, myocardial IRI can be attenuated to some extent. Both in vitro and in vivo experiments have confirmed that AS-IV has significant protective effects against myocardial IRI, but its mechanism of action needs to be further explored. In this study, the mechanism of action of AS-IV was further studied using an in vitro myocardial H/R model. The results show that after treatment with AS-IV, the expression of HO-1 is significantly increased, the secretion of cytokines is significantly reduced, and the anti-oxidative stress capacity of cells is enhanced. A HO-1 inhibitor partially reversed the above effects, suggesting that induction of HO-1 was the source of the above changes. The expression of HO-1 is one of the mechanisms of action by which AS-IV protects against myocardial H/R injury. But what is the signaling pathway through which AS-IV induces HO-1?

Studies have shown that the regulation of HO-1 gene expression occurs mainly at the transcriptional level. Nuclear factor E2-related factor 2 (Nrf2) is an important transcriptional regulator of anti-oxidative stress. When cardiomyocytes are under oxidative stress, Nrf2 is rapidly phosphorylated, dissociates, activates, and translocates to the nucleus, where it binds to the anti-oxidative stress response element (ARE) to promote the expression of anti-oxidant proteins such as HO-1 and SOD to combat oxidative stress caused by ischemia and hypoxia. Tanaka et al. found that deletion of the Nrf2 gene results in a significant decrease in the expression of various baseline and inducible anti-oxidant genes, including HO-1, and an overall increase in oxidative damage. On the other hand, gene regulation of HO-1 is also negatively regulated by some Nrf2 repressors, such as BTB-CNC allogeneic 1 (BTB and CNC homology 1, Bach1), and deletion of the Bach1 gene can lead to increased expression.
of HO-1. The mechanism by which Bach1 and Nrf2 antagonize the expression of anti-oxidant genes may be related to the competitive binding of ARE sequences in the nucleus. Oxidative stress phosphorylates Bach1 tyrosine 486, allowing for rapid nuclear exit and for Nrf2 to bind to available AREs, resulting in the production of anti-oxidant enzymes.

The above experimental results suggest that the dynamic equilibrium relationship between Nrf2 and Bach1 in the nucleus affects the transcription of antioxidant enzymes such as HO-1. Phosphatidylinositol 3-kinase (PI3K) is an intracellular phosphatidylinositol kinase that is a second messenger located on the plasma membrane. Akt is a key downstream gene in the PI3K/Akt pathway. Akt induces phosphorylation of serine and threonine residues and activates or inhibits downstream target gene expression. The PI3K/Akt signaling pathway is essential for heart and vascular health. As an important part of the body’s protective mechanism, the PI3K/Akt pathway plays a major role in controlling cell growth, survival, proliferation, and migration, and improves overall function in IRI through activating Akt. PI3K may affect the expression of HO-1 by regulating the balance of Nrf2 and Bach1. Sun et al. showed that inhibition of the PI3K/Akt pathway significantly reduces HO-1 protein expression, thereby attenuating the protective effects of HO-1 on cardiomyocytes. Joung et al. stimulated HepG2 cells with capsaicin to activate the PI3K/Akt pathway, increasing the nuclear translocation of Nrf2 and the transcriptional activity of AREs, and thereby increasing the expression of HO-1 in cells. Other studies have shown that chlorophyllin induces nuclear translocation of Nrf2 via activation of the PI3K pathway, up-regulating HO-1 expression in human umbilical vein endothelial cells in a time- and dose-dependent manner. These findings strongly confirm that PI3K/Akt upregulates the Nrf2-ARE pathway and mediates HO-1 expression. There are fewer reports on whether PI3K-mediated HO-1 expression is simultaneously achieved by down-regulating Bach1 activity. In an experiment on Jurkat tumor cells, the PI3K signaling pathway was shown to activate Nrf2 and inhibit Bach1 activity, thereby regulating HO-1 expression.

However, there are only a few studies on whether PI3K/Akt signaling pathway regulates HO-1 in myocardial IRI. A recent study showed that a Chinese herbal medicine extract (TFCC) protects against myocardial injury and enhances cellular antioxidant defense capacity by inducing the phosphorylation of Akt, which subsequently activates the Nrf2/HO-1 signaling pathway. In addition, two studies showed that AS-IV has protective effects on the cardiovascular system by regulating the PI3K/Akt signaling pathway. However, whether AS-IV further induces HO-1 expression by regulating the PI3K/Akt signaling pathway and its downstream effectors remains unclear. The results of this study show that the effect of AS-IV on myocardial H/R injury can significantly increase the expression of PI3K and p-Akt in cells, thereby increasing the nuclear expression of Nrf2 and decreasing the nuclear expression of Bach1, and further increased the expression of HO-1 protein. A PI3K-specific inhibitor can partially reverse the above effects. These results...
suggest that AS-IV may affect the nuclear translocation of Nrf2 and Bach1 by Activating the PI3K/Akt signaling pathway thereby regulating the expression of HO-1. Therefore, this study confirms that the PI3K/Akt/HO-1 signaling pathway is a key signaling pathway for the anti-myocardial hypoxia-reoxygenation injury of AS-IV.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.
References


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Figure 1. Chemical structure of AS-IV.
Molecular formula, C41H68O14, molecular weight, 784.9702 g/mol.
Figure 2. AS-IV attenuates H9c2 cytotoxicity induced by H/R injury.
A. H9c2 cells were treated with AS-IV for 20h, and cell viability was measured using CCK-8.
B. Pre-I/R injury for 8 hours and incubation with AS-IV for 12 hours, the cell viability was measured using CCK-8.
C. Pre-I/R injury for 8 hours and incubation with AS-IV for 12 hours, the content or activity of the cellular LDH was measured.

All experiments were repeated at least three times. Data are presented as mean±S.D. **P<0.01 vs. control group, ***P<0.05 vs. H/R group. n=3
Figure 3. AS-IV attenuates oxidative damage in H9c2 cells induced by H/R injury.

A,B, ROS levels in H9c2 cells were determined using DCFH-DA staining. CSOD activity was measured in the lysed cell supernatant; D. MDA activity was measured in the lysed cell supernatant. Data are presented as mean±S.D. All experiments were repeated at least three times. ##P<0.01 vs. control group, ** P<0.05 vs. H/R group, △△ P<0.05 vs. H/R+ASIV group. n=3

(Color figure can be accessed in the online version.)
Figure 4. ASIV attenuates H/R injury-induced inflammatory response in H9c2 cells. 
A. ELISA of secreted IL-6 in lysed cell supernatant; B, ELISA of secreted TNF-α in lysed cell supernatant. Data are presented as mean±S.D. All experiments were repeated at least three times. ## P<0.01 vs. control group, ** P<0.05 vs. H/R group, △△P<0.05 vs. H/R+ASIV group. n=3
Figure 5. Effect of AS-IV on activation of PI3K/Akt/HO-1 signaling pathway.
A, B, and G show the expression of PI3K and HO-1 protein in the total protein extract and their semi-quantitative analysis when normalized to GAPDH. C, D and G show the expression of Akt protein and its level of phosphorylation. E, F, and H show the expression of Nrf2 and Bach1 and their semi-quantitative expression analysis when normalized to histone. LY294002, a specific inhibitor of PI3K, was administered concurrently with AS-IV for 20 h at a concentration of 20 μM. All experiments were repeated at least three times. Data are presented as mean±S.D. ### P<0.01 vs. control group, ** P<0.05 vs. H/R group, △△P<0.05 vs. H/R+ASIV group. n=3