Astragaloside IV protects cardiomyocytes from anoxia/reoxygenation injury by upregulating the expression of Hes1 protein

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<td>Huang, Huang; The First Affiliated Hospital of Nanchang University, Department of Cardiac Surgery</td>
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<td>Lai, Songqing; The First Affiliated Hospital of Nanchang University, Department of Cardiac Surgery</td>
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<td>Qi, Wanghong; The First Affiliated Hospital of Nanchang University, Department of Cardiac Surgery</td>
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<td>Liu, Jichun; The First Affiliated Hospital of Nanchang University, Department of Cardiac Surgery</td>
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Astragaloside IV protectes cardiomyocytes from anoxia/reoxygenation injury by upregulating the expression of Hes1 protein

Huang Huang 1, Songqing Lai 1, Qing Wan 2, Wanghong Qi 1, Jichun Liu 1,*

1 Department of Cardiac Surgery, The First Affiliated Hospital of Nanchang University, No. 17, Yong Wai Zheng Street, Nanchang, Jiangxi 330006, P.R. China

2 Department of Pharmacy, The First Affiliated Hospital of Nanchang University, No. 17, Yong Wai Zheng Street, Nanchang, Jiangxi 330006, P.R. China

*Corresponding author: Jichun Liu, Department of Cardiac Surgery, The First Affiliated Hospital of Nanchang University, No. 17, Yong Wai Zheng Street, Nanchang, Jiangxi 330006, P.R. China, Tel/Fax: 86-791-88692707, China.

E-mail: liujichun999@163.com
Abstract

Astragaloside IV (ASI), a traditional Chinese medicine, is a main active ingredient of astragalus membranaceus. Many clinical studies have found that ASI protects cardiomyocytes in cardiovascular diseases, but the underlying mechanisms remain obscure. The aim of this study was to investigate the molecular mechanisms responsible for the protective effects of ASI in cardiomyocytes from anoxia/reoxygenation (A/R) injury. According to the previous studies, we hypothesized that the cardioprotective effects of ASI against A/R injury might be associated with Notch 1-Hes1 signaling pathway. In this study, neonatal rat primary cardiomyocytes were preconditioned with ASI prior to A/R injury. Our results showed that ASI effectively increased the cell viability, decreased the content of MDA, decreased the activities of CPK and LDH, increased the activities of GSH-Px and SOD, reduced the reactive oxygen species (ROS) generation and the loss of mitochondrial membrane potential ($\Delta\psi_m$). ASI inhibited the mitochondrial permeability transition pore (mPTP) opening and activation of caspase-3, and finally decreased the cell apoptosis in cardiomyocytes. Furthermore, ASI upregulated Hes1 protein expression. However, pretreatment with DAPT, a Notch1 inhibitor, effectively attenuated the cardioprotective effects of ASI against A/R injury, except MDA, SOD, GSH-Px and the ROS generation. Taken together, we demonstrated that ASI could protect against A/R injury via the Notch1-Hes1 signaling pathway.
Key words—Astragaloside IV; Notch1 signaling pathway; Hes1; cardioprotection; anoxia/reoxygenation; precondition; neonatal rat primary cardiomyocytes
1. Introduction

Ischemia/reperfusion (I/R) injury is a major cause of death and disability worldwide (Hausenloy and Yellon 2013). The calcium overload and the generation of ROS could result in opening of mPTP, rupture of the plasma membrane and resultant cell death (Minamino 2012). Astragaloside IV (ASI), extracted from the leguminous plants astragalus membranaceus, is a main biological activity ingredient of the astragaloside and whose molecular formula is C$_{41}$H$_{68}$O$_{14}$. As a traditional Chinese medicine, ASI has been suggested to exert protective effects in multiple human disorders, including cardiovascular diseases, hepatitis, kidney disease, and skin diseases (Ren et al. 2013). Among which, cardioprotective effect of ASI was investigate extensively and the potential mechanisms might be associated with regulation of energy metabolism, change of Ca$^{2+}$-ATPase activity, dysregulations of Toll-like receptor 4/Nuclear factor-κB (TLR4/NF-κB), PI3K/Akt and HIF-1α signaling pathways (Jia et al. 2014; Lu et al. 2015; Si et al. 2014; Tu et al. 2013; Xu et al. 2007). However, the detailed molecular mechanisms underlying cardioprotective effects of ASI remain largely unclear.

The Notch signaling pathway consists of transmembrane receptors Notch 1-4 that are activated by interacting with the ligands Delta 1-4 and jagged1-2 (Kopan and Ilagan 2009). Notch interacts with its ligands, and then there are serial cleavage events of the Notch receptor by ADAM family metalloproteases and γ-secretase, resulting in translocation of Notch intracellular domain to the nucleus and interacts with the
DNA-binding protein recombinant signal-binding protein for RBP-Jκ region, and acts on other downstream targets, such as Hes1 (Schroeter et al. 1998). The Notch1 signaling pathway can regulate multiple cellular processes, such as cell development, cell differentiation, cell proliferation, cell apoptosis, and cell regeneration (Gude et al. 2008; Schwanbeck et al. 2011; Tremblay et al. 2013). Hes1, which is a downstream target gene of Notch1 signaling pathway, has been demonstrated to protect the cardiomyocytes via activating the PI3K/Akt signaling pathway and modulating Notch1/Hes1-PTEN/Akt signaling (Yu et al., 2015; Zhou et al. 2014). And the functions of some Chinese medicines, such as Xiaotan Sanjie, salidroside, xinfeng capsule and indirubin-3'-monoxime, have been found to be relative to Notch1/Hes1 signaling pathway (Lee et al. 2008; Wan et al. 2012; Yan et al. 2014; Zhao et al. 2014).

Thus, we hypothesized that the ASI protects cardiomyocytes from A/R injury might be associated with Notch1-Hes1 signaling pathway. We used the neonate SD rat primary cardiomyocytes to establish A/R injury model in vitro. We assessed cell viability, certain biochemical parameters, the expression of Hes1 protein, ROS, ∆ψm, mPTP opening, and cell apoptosis to evaluate the cardioprotective effects of ASI.
2. Materials and methods

2.1. Reagents

ASI (purity: > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). DAPT (γ-secretase inhibitor, inhibits the Notch1 signaling pathway and Hes1 expression) was purchased from Selleckchem (S2215, USA).

2.2. Cell culture

Cardiomyocytes were isolated from 2-3 days old Sprague-Dawley (SD) rats (Medical College of Nanchang University, China) as previously described (Watkins et al. 2011). Briefly, the hearts of the neonatal SD rats were eviscerated in D-Hank's solution and the ventricles were digested with 0.1% trypsin. Cells were collected by centrifugation at 60 g for 5 minutes, resuspended with 15% FCS in 60mm culture dishes, incubated in a 95% O₂ and 5% CO₂ incubator at 37 °C for 2 hours to remove other cells. The supernatant was collected and plated on culture dishes with a density of 1×10⁶ cells/dish. Cardiomyocytes were washed 24 hours later and then incubated for another 3 days prior to following assays. The animal experiments were approved by the Ethics Committee of Nanchang University and conducted according to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Experimental groups and treatments
The primary neonatal rat cardiomyocytes were randomly divided into different experimental groups: (1) Control group: cardiomyocytes were cultured with medium in the incubator of 5% CO\textsubscript{2} for 5 hours; (2) A/R group: cardiomyocytes were subject to anoxia condition by adding anoxia solution and incubated in the chamber with 95% N\textsubscript{2} and 5% CO\textsubscript{2} for 3 hours. Following 3 hours of anoxia, cells were replaced with reoxygenation solution and then incubated in a chamber with 95% O\textsubscript{2} and 5% CO\textsubscript{2} for 2 hours (Guo et al. 2015); (3) The concentration-effect ASI+A/R group: cardiomyocytes were pretreated with ASI (10, 20, 40, 80 µM) 24 hours before A/R treatment; (4) ASI+A/R group: cardiomyocytes were pretreated with 40µM ASI 24 hours before A/R treatment; (5) ASI+DAPT+A/R group: cardiomyocytes were pretreated with 40 µM ASI and 10 µM DAPT (Liu et al. 2014) 24 hours before A/R treatment; (6) DAPT+A/R group: only DAPT was added to the cardiomyocytes 24 hours before A/R treatment.

2.4. MTS assay

A colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS) assay was used to evaluate the cell viability of cardiomyocytes. Primary cardiomyocytes were cultured in 96-well plates with a density of $1 \times 10^4$ cells per well. Cardiomyocytes were incubated with 20 µl MTS (Promega, USA) in 100 µl medium at 37 °C for 2 hours following the manufacturer’s instructions. After 2 hours, a microplate reader (Bio-Rad 680, USA) was used to determine the absorbance value of each well at a wavelength of
490 nm. The percentage of viable cells was calculated with the control cells set as 100%.

2.5. Western blot analysis

Cells were harvested and incubated with the lysis buffer (Vazyme, China) and vibrated at 4°C for 15 minutes. Proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 10% nonfat milk in TBST buffer for 2 hours. Membranes were incubated with antibodies (1:500 dilution) for Hes1 (Abcam, USA) and β-actin (Affinity, USA) overnight at 4°C, followed by incubation with HRP-labeled IgG secondary antibodies (1:2000 dilution) (ZSGB-BIO, China) at room temperature for 2 hours. The enhanced chemiluminescence method was used to detect the immune complexes. The result images were analyzed by Quantity One software (Bio-Rad, USA).

2.6. The determination of biochemical parameters

The culture media or cell lysate supernatants were collected. The content of malondialdehyde (MDA), and the activities of lactate dehydrogenase (LDH), creatine phosphate kinase (CPK), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were detected by using certain assay kits (Jiancheng, China) following the instructions of manufacturer.

2.7. Detection of intracellular ROS

2,7-dichlorofluorescein diacetate (DCFH-DA) was used to detect the accumulation
of intracellular ROS. DCFH-DA can be converted into DCFH$_2$ by intracellular esterases, DCFH$_2$ can be oxidized into highly fluorescent DCF by ROS. The cells were collected and incubated in DMEM culture medium with 10 µM DCFH-DA (Invitrogen, USA) at 37 °C for 20 minutes, the fluorescence was measured by BD FACSCalibur excited at 488 nm and emitted at 525 nm.

2.8. Determination of ∆ψm

∆ψm was evaluated by the method of JC-1 (Invitrogen, USA) staining according to the instructions of manufacturer. Carbonyl cyanide $m$-chlorophenyl hydrazone (CCCP)-treated cells had their membrane potential disrupted. Briefly, the cells were harvested and incubated with 200 µM JC-1 for 20 minutes at 37 °C. The fluorescence was determined by using by BD FACSCalibur excited at 530 nm and emitted at 580 nm for red fluorescence, then excited at 485 nm and emitted at 530 nm for green fluorescence. The level of ∆ψm was expressed as the ratio of the red to green intensity.

2.9. Opening of the mPTP

The mitochondrias were isolated from cells with a mitochondrial/cytosolic fractionation kit (QIAGEN, Germany). The opening of the mPTP causes mitochondrial swelling after adding 200 µM CaCl$_2$. The absorbance at 520 nm was continuously recorded to detect the extent of mPTP opening and the reduction in the absorbance at 520 nm/min (∆ODmin$^{-1}$) were used to analyse the degree of mPTP opening.

2.10. Caspase-3 activity assay
A total of $2 \times 10^7$ cells were collected and resuspended in the lysis buffer on ice in 100 µl culture medium for 15 minutes, centrifuged at 16,000 g for 15 minutes at 4 °C and transferred the supernatant to a cold tube, then added the detection buffer and acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), finally incubated 2 hours at 37 °C. The caspase-3 enzyme activity was determined with caspase-3 activity assay kit (Beyotime, China) by detecting the absorbance at 405 nm.

2.11. Apoptosis assay

Apoptosis assay was measured with Annexin V/PI Apoptosis Detection Kit (BD Biosciences, USA). For each tube, a total of $2 \times 10^6$ cells were collected and resuspended in 500 µl binding buffer, 10 µl of Annexin V-FITC and 5 µl of PI were added and vortexed gently, then incubated in the dark for 15 minutes at room temperature and analyzed by BD FACSCalibur. The percentage of positively stained cells was determined.

2.12. Statistical analysis

All data were expressed as mean ± SEM. Examined data were assessed by using ANOVA. $P$ values <0.05 were considered statistically significant.

3. Results

3.1. ASI increased the viability of cardiomyocytes suffered A/R

The effect of ASI on the viability of neonatal rat primary cardiomyocytes was examined by MTS assay. As shown in Fig 1.A, cardiomyocytes undergoing A/R
decreased viability significantly ($p<0.01$, vs. control group). ASI+A/R groups treated with different concentrations of ASI could significantly increase the cell viability in a dose-dependent manner relative to A/R group ($p<0.01$), and 40 µM ASI+A/R group exhibited the highest cell viability ($p<0.01$). However, the cell viability decreased when ASI+A/R groups treated with a higher concentration of 80 µM, which demonstrated the higher concentration might have the doses toxic injury. The optimal concentration and precondition time was 40 µM ASI for 24 hours before A/R treatment, which was used in our subsequent experiments. In Fig. 1.B, our data also suggested that DAPT could inhibit this effect significantly ($p<0.01$). Therefore, we hypothesized that the cardioprotective effects of ASI were associated with Notch1-Hes1 signaling pathway.

3.2. ASI upregulated Hes1 expression in cardiomyocytes subjected to A/R

To investigate whether Hes1 was involved in the process of ASI-induced cardioprotection and the optimal concentration of ASI, the expression of Hes1 protein was evaluated by western blot analysis. Cardiomyocytes were pretreated with ASI with different concentrations (10, 20, 40, 80 µM) for 24 hours prior to the A/R treatment, respectively. As shown in Fig.2A, the expression of Hes1 in all of the ASI+A/R groups with different concentrations were higher than control and A/R group ($p<0.01$), and 40 µM ASI+A/R group presented with the highest expression of Hes1 ($p<0.01$). The optimal concentration and precondition time was similar to the results of MTS. Furthermore, the expression of Hes1 protein was significantly decreased in ASI+DAPT+A/R and
DAPT+A/R when compared with control, A/R and ASI+A/R group (p<0.01) (Fig.2.B). These results suggested that ASI protected cardiomyocytes from A/R-induced injury associated with up-regulation of Hes1.

3.3. ASI decreased the activities of LDH and CPK of cardiomyocytes undergoing A/R

Following A/R treatment, the activities of CPK and LDH in culture mediums were analyzed to evaluate the damages of cell membranes during A/R. There was a significant increase of the activities of LDH and CPK in A/R group compared with control group (p<0.01), yet decreased significantly in ASI+A/R group relative to A/R group (p<0.01). Interestingly, DAPT treatment significantly increased the activities of LDH and CPK compared with ASI+A/R group (p<0.01) (Fig.3).

3.4. ASI decreased the ROS generation and oxidative stress damage induced by A/R

DCFH-DA fluorescence probe was used to analyze intracellular ROS level in cardiomyocytes. There was a striking increase of ROS in A/R group (p<0.01, vs. control group), while a significant decrease of ROS in ASI+A/R and DAPT+A/R group (p<0.01, vs. A/R group). Notably, DAPT significantly decreased the ROS level in ASI+DAPT+A/R group (p<0.01, vs. ASI+A/R group and DAPT+A/R group), indicating that DAPT decreased the ROS generation and the effect of ASI to decrease oxidative stress might be independent with Notch1/Hes-1 signaling pathway (Fig. 4). Moreover, MDA was increased in A/R group (p<0.01, vs. control group), while decreased
significantly in ASI+A/R group ($p<0.01$, vs. A/R group). Meanwhile, the activities of GSH-Px and SOD, two major antioxidases in cells, were reduced in A/R group ($p<0.01$, vs. control group), but increased in ASI+A/R group ($p<0.01$, vs. A/R group). The results in ASI+DAPT+A/R group showed that the content of MDA was decreased, while the activities of GSH-Px and SOD were increased ($p<0.01$, vs. AST+A/R group), which indicated that DAPT decreased oxidative stress damage and the antioxidative effect of ASI might be independent with Notch1/Hes1 signaling pathway (Table 1).

### 3.5. ASI prevented $\Delta\psi_m$ loss in cardiomyocytes after A/R injury

JC-1 fluorescent probe was used to determine the $\Delta\psi_m$ in cardiomyocytes subjected to A/R injury. The ratio of fluorescence in upper right quadrant and lower right quadrant was used to evaluate the level of $\Delta\psi_m$. In the A/R group, there was a significant decrease of $\Delta\psi_m$ relative to control group ($p<0.01$) (Fig. 5). However, ASI+A/R group significantly prevented the loss of $\Delta\psi_m$ ($p<0.01$, vs. A/R group). A decrease of $\Delta\psi_m$ was observed in both the ASI+DAPT+A/R and DAPT+A/R groups ($p<0.01$, vs. ASI+A/R group), indicating that DAPT could abolish the effect of ASI on $\Delta\psi_m$.

### 3.6. ASI inhibited the opening of mPTP undergoing A/R

In Fig. 6, the extent of mPTP opening was analysed by the change of absorbance at 520 nm/min ($\Delta OD_{520} \text{min}^{-1}$). $\Delta A_{520}$ was significantly increased in the A/R group ($p<0.01$, vs. control group), while it was attenuated in ASI+A/R group ($p<0.01$, vs. A/R group). A significant decrease in $\Delta A_{520}$ was observed in ASI+DAPT+A/R group ($p<0.01$, vs. A/R group).
ASI+A/R group), indicating that the effect of ASI in preventing mPTP opening was abolished by DAPT.

### 3.7. ASI reduced the activity of caspase-3 induced by A/R

As shown in Fig. 7, the activity of caspase-3 significantly increased in A/R group ($p<0.01$, vs. control group), while ASI+A/R group had a significantly decreased activity of caspase-3 ($p<0.01$, vs. A/R group). Moreover, the activity of caspase-3 increased significantly in ASI+DAPT+A/R group ($p<0.01$, vs. ASI+A/R group)

### 3.8. ASI inhibited the cardiomyocyte apoptosis suffered A/R

In order to test the protective effect of ASI against A/R-induced apoptosis in cardiomyocytes, cells were collected and stained with Annexin V and PI, then analyzed by flow cytometer (Fig. 8). Our results showed that the proportion of apoptotic cells was increased in A/R group ($p<0.01$, vs. control group) and reduced significantly in the ASI+A/R group ($p<0.01$, vs. A/R group). However, the apoptotic rates of cardiomyocyte in ASI+DAPT+A/R group and DAPT+A/R group were higher when compared with ASI+A/R group ($p<0.01$).

### 4. Discussion

Astragaloside IV, the best biological activity ingredient in the Astragaloside and extracted from the leguminous plants astragalus membranaceus, has been used as a drug in traditional Chinese medicine for the treatment of cardiovascular disorders, hepatitis,
kidney disease, and skin diseases (Ren et al. 2013). ASI plays an important role in the treatment of cardiovascular diseases by regulating the myocardial energy metabolism, calcium homeostasis, anti-oxidation and anti-apoptosis effect (Liu et al. 2014; Tu et al. 2013; Yin et al. 2014). ASI could prevent LPS-induced injury in cardiomyocytes by increasing the activities of antioxidant enzymes, inhibiting lipid peroxidation, and down-regulating the inflammatory mediators involved in the inflammatory responses (Wang et al. 2015). Moreover, we found ASI preconditioning significantly increased the viability of cardiomyocytes and decreased the activities of CPK and LDH, which were in accordance with previous studies (Wang et al. 2015; Zheng et al. 2012). In the present study, ASI was shown to increase the cell viability significantly in a dose-dependent manner. The cell viability was the highest at a final concentration of 40 µM, suggesting that ASI protected cardiomyocytes against A/R and the optimum concentration and preconditioning time was 40 µM 24 hours prior to A/R. However, the cell viability decreased when ASI+A/R groups treated with a higher concentration of 80 µM, which demonstrated the higher concentration might have the doses toxic injury.

ASI has been demonstrated the cardioprotective effects via regulating energy metabolism, Ca\(^{2+}\)-ATPase activity, TLR4/NF-κB, PI3K/Akt and HIF-1α signaling pathway (Jia et al. 2014; Lu et al. 2015; Si et al. 2014; Tu et al. 2013; Xu et al. 2007). However, the mechanism of ASI protection against I/R injury is not completely clear. Notch1 signaling pathway is involved in a wide range of physiological processes,
including mitogenesis, cell survival under stressful conditions, metastasis and transcriptional regulation (Croquelois et al. 2008; Schwanbeck et al. 2011; Tremblay et al. 2013). Moreover, Notch1-Hes1 signaling pathway has been demonstrated to protect the cardiomyocytes against I/R injury (Chen et al. 2013; Pei et al. 2015; Pei et al. 2013; Yu and Song 2014). Our results also showed that the expression of Hes1 protein was highest in 40 μM ASI 24 hours prior to A/R group. To investigate whether the cardioprotective effects of ASI was Hes1-dependent, we used the DAPT (γ-secretase inhibitor, which can inhibit the Notch1 signaling pathway) to abolish these effects. However, these effects were abolished when DAPT was used, which suggested that the cardioprotective effects of ASI were related to the Notch1-Hes1 signaling pathway. So Hes1 may be involved in the protection of ASI against A/R.

Curcumin had been proved a protective effect on the HUVECs against H$_2$O$_2$ by inhibiting Notch signaling pathway to increase the cell viability, adhesive ability and migratory ability and decrease the apoptotic index, ROS generation and several biochemical parameters (Yang et al. 2013). Moreover, activated the Notch1 signaling could increased myocardial cell viability, prevented cardiomyocyte apoptosis, and reduced loss of the Δψm against I/R injury in H9C2 cells (Zhou et al. 2013a; Zhou et al. 2013b). ROS activated Notch signaling in SK-N-MC cells and led to cell apoptosis, EUK134 prevented H$_2$O$_2$/menadione-induced SK-N-MC cells death by modulating Notch signaling pathway (Kamarehei and Yazdanparast 2014). Thus, we hypothesized that the
cardioprotective effects of ASI against I/R injury were associated with Notch1-Hes1 signaling pathway.

During A/R, the damage of the electron transport chain caused the mitochondrial ROS generation and oxidative stress injury (Li et al. 2010). The overload of mitochondrial Ca\(^{2+}\) and increase of ROS generation results in the mPTP opening, which further compromises cellular energetic signal, the resultant low ATP and altered ion homeostasis, finally causes the plasma membrane rupture and cell death (Griffiths 2012; Zorov et al. 2014). In this study, we determined that ASI preconditioning decreased the lipid peroxidative product MDA, as well as increasing the activities of GSH-Px and SOD, two major antioxidases in cells, which indicates that ASI treatment could reduce the oxidative stress injury induced by A/R injury, which was in accordance with previous study (Shao et al. 2014). And these effects of ASI to the oxidative stress injury might be independent with Notch1/Hes-1 signaling pathway. ASI had the neuroprotective effect against dopaminergic neurotoxicity induced by 1-methyl-4-phenylpyridinium ion in SH-SY5Y cells by increasing the cell viability, reducing the generation of ROS, decreasing the Bax/Bcl-2 ratio and the activity of caspase-3 (Zhang et al. 2012). ASI could reduce ROS generation in renal proximal tubular cells to attenuate epithelial-to-mesenchymal transition induced by glycated albumin (Qi et al. 2014). Our study also suggested that the cardioprotective effect of ASI may be related to mechanisms including ROS production. In our results, ASI preconditioning significantly reduced ROS
generation caused by A/R injury. DAPT has been found the effect of decreasing the ROS generation in N2a cells (Sheng et al. 2009) and reducing the oxidative stress injury in HUVECs (Yang et al. 2013). These results indicated that reducing the generation of ROS and oxidative stress injury might be one of the mechanisms underlying the cardioprotective effects of ASI. The mechanisms of ASI protection against A/R injury are complicated, which involved in multiple factors and signaling pathways. However, the mechanisms of ASI to decreased ROS generation and oxidative stress damage maybe independent with Notch1/Hes-1 signaling pathway, which may involved in the other factors or signaling pathways. And the mechanisms of DAPT to decrease ROS generation and oxidative stress damage are still not fully elucidated.

The mitochondrial Ca\(^{2+}\) channel open probability depends on the cytosolic Ca\(^{2+}\) concentration and ∆ψm (Williams et al. 2015). The loss of ∆ψm is mediated by the mPTP opening in the inner mitochondrial membrane, resulting in matrix swelling, the rupture of outer membrane, and the release of apoptotic signaling molecules, such as cytochrome C from the inter membrane space (Tang et al. 2014). Reducing the cardiocyte injury induced by A/R via inhibiting cell apoptosis is a rational strategy to protect cardiomyocytes against A/R injury. Mitochondrial dysfunction also leads to the apoptotic signal cascade (Su et al. 2014). Caspase-3, one of the key effectors in cell apoptosis, is initiated and played an important role in the mitochondria-mediated apoptosis (Lee et al. 2010). ASI prevented H\(_2\)O\(_2\)-induced loss of ∆ψm, the opening of mPTP and reperfusion
injury by inhibiting GSK-3β through the NO/cGMP/PKG signaling pathway in H9c2 cell (He et al. 2012). ASI had been proved to promote the cardiocytes survival, attenuate the LDH release, the ROS production and cell apoptosis, stabilize the ∆ψm and reduce the intracellular calcium overload, which were induced by H2O2 in human umbilical vein endothelial cell (Guan et al. 2015). ASI could protect against amyloid beta1-42 neurotoxicity by preventing the loss of ∆ψm, inhibiting the opening of mPTP, enhancing ATP generation, increasing the activity of cytochrome c oxidase and reducing cytochrome c release from mitochondria (Sun et al. 2014). In this study, we showed that ASI precondition could prevent the loss of ∆ψm, inhibit the opening of the mPTP, reduce the activity of caspase-3 and consequently reduce the apoptosis induced by A/R. Consistent with the above results, all of these effects were abolished in the presence of DAPT.

In summary, we demonstrated that ASI can protect cardiomyocytes from A/R injury by increasing the cell viability, decreasing ROS generation, preventing ∆ψm loss, inhibiting the opening of mPTP, reducing the activity of caspase-3 and inhibiting the final cell apoptosis. Moreover, most of these cardioprotective effects were abolished by DAPT, but ROS and oxidative stress damage generation was significantly decreased by DAPT, except MDA, SOD, GSH-Px and the ROS generation. However, the mechanisms of ASI to decreased ROS generation and oxidative stress damage maybe independent with Notch1/Hes-1 signaling pathway. And the mechanisms of DAPT to decrease ROS generation and oxidative stress damage are still not fully elucidated. These findings
suggest that ASI precondition can upregulate the expression of Hes1 protein, indicating that the cardioprotective effects of ASI against A/R injury involves the Notch1-Hes1 signaling pathway.

Acknowledgements

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increases mitochondrial activity and leads to reduced vulnerability to apoptosis:
1362-1375.

activation by post-ischemia treatment with astragaloside IV attenuates myocardial

proteins are involved in the signal crosstalk between endoplasmic reticulum stress
2014: 234370.

astragaloside IV against amyloid beta1-42 neurotoxicity by inhibiting the

puerarin in cardiomyocytes from anoxia/reoxygenation injury are mediated by


Table 1. The content of MDA, the activities of SOD and GSH-Px induced by A/R.

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<th>GSH-Px (µM)</th>
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<tr>
<td>C</td>
<td>1.25 ± 0.22</td>
<td>145.26 ± 13.08</td>
<td>30.28 ± 4.35</td>
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<tr>
<td>A/R</td>
<td>3.41 ± 0.62**</td>
<td>92.31 ± 8.13**</td>
<td>12.25 ± 1.78**</td>
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<td>ASI + A/R</td>
<td>1.57 ± 0.36▲▲</td>
<td>138.44 ± 12.87▲▲</td>
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<td>DAPT + A/R</td>
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<tr>
<td>ASI + DAPT + A/R</td>
<td>1.46 ± 0.45##</td>
<td>146.27 ± 12.92##</td>
<td>27.89 ± 2.15##</td>
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</table>

Values were mean ± SEM for five individual experiments. **p<0.01, vs. control group;▲▲p<0.01, vs. A/R group;##p<0.01, vs. ASI + A/R group.
Figure captions

Figure 1. Effects of ASI on cell viability in cardiomyocytes subjected to A/R injury.

The results of MTS assay showed that A/R group decreased the cell viability of cardiomyocytes (\( **p<0.01, \text{ vs. control group} \)), the ASI+A/R group could significantly increase the cell viability (\( \▲\▲p<0.01, \text{ vs. A/R group} \)), and 40 µM ASI+A/R group was the highest (\( ###p<0.01, \text{ vs. 10, 20, 80 µM ASI+A/R group} \)). (B) A/R group could decrease the cell viability of cardiomyocytes (\( **p<0.01, \text{ vs. control group} \)), ASI+A/R groups increased the cell viability (\( \▲\▲p<0.01, \text{ vs. A/R group} \)), while Notch1 inhibitor DAPT abolished the effects of ASI on cell viability (\( ###p<0.01, \text{ vs. ASI+A/R group} \)). Data were expressed as the mean ± SEM, \( n=5 \).

Figure 2. The effect of ASI on the expression of Hes1 in cardiomyocytes suffered A/R injury. Hes1 expression was evaluated by western blot, β-actin was used as an internal control. (A) In the concentration-effect ASI+A/R group, the expression of Hes1 protein in the ASI+A/R groups were higher than A/R group (\( \▲\▲p<0.01, \text{ vs. A/R group} \)), and 40 µM ASI+A/R group was the highest (\( ###p<0.01, \text{ vs. 10, 20, 80 µM ASI+A/R group} \)). (B) The expression of Hes1 protein significantly increased in ASI +AR group (\( \▲\▲p< 0.01, \text{ vs. A/R group} \)) and decreased in both the ASI+DAPT+A/R and DAPT+A/R groups (\( ###p<0.01, \text{ vs. ASI+A/R group} \)). Data were expressed as the mean ± SEM, \( n=3 \).
Figure 3. Effects of ASI on the activities of CPK and LDH in cardiomyocytes subjected to A/R injury. The activities of CPK and LDH of A/R group were increased than control group (**p<0.01, vs. control group), while the activities decreased significantly in ASI+A/R group (▲▲p<0.01, vs. A/R group). DAPT significantly increased the activities of CPK and LDH (##p<0.01, vs. ASI+A/R group). Data were expressed as the mean ± SEM, n=5.

Figure 4. ASI pretreatment decreased the ROS generation of cardiomyocytes induced by A/R, and the effect was independent with Notch1-Hes1 signaling pathway. (A) Flow cytometric analysis the cell fluorescence for DCF. (B) Column bar graph of cell fluorescence for DCF. Data were expressed as the mean ± SEM, n=3. **p<0.01, vs. control group; ▲▲p<0.01, vs. A/R group; ##p<0.01, vs. ASI + A/R group and DAPT+A/R group.

Figure 5. ASI pretreatment alleviated the loss of ∆ψm in cardiomyocytes suffered A/R, while DAPT abrogated the effect. (A) Representative dot plots of flow cytometry. (B) ∆ψm was calculated with ratio of red/green fluorescence obtained from flow cytometry. The ratio of fluorescence in upper right quadrant and lower right quadrant was used to evaluate the level of ∆ψm. Data were expressed as the mean ± SEM, n=3. **p<0.01, vs. control group; ▲▲p<0.01, vs. A/R group; ##p<0.01, vs. ASI + A/R group.
Figure 6. ASI preconditioning suppressed mPTP opening of A/R treated-cardiomyocytes, while DAPT attenuated the effect. (A) After the addition of 200 µM CaCl$_2$, the absorbance value at A520 was monitored over 20 minutes to reflect the opening of mPTP. (B) Changes in absorbance value at 520 nm/min (\(\Delta\text{ODmin}^{-1}\)) were used to express the extent of mPTP opening (\(\Delta\text{OD} = \text{A}520_{0\text{min}} - \text{A}520_{20\text{min}}\)). Data were expressed as the mean ± SEM, \(n=3\). **\(p<0.01\), vs. control group; ▲▲\(p<0.01\), vs. A/R group; ##\(p<0.01\), vs. ASI + A/R group.

Figure 7. ASI pretreatment influenced the activity of caspase-3 induced by A/R injury, while DAPT abrogated the effect. The column bar graph represented the activity of caspase-3 in different groups. Data were expressed as the mean ± SEM, \(n=5\). **\(p<0.01\), vs. control group; ▲▲\(p<0.01\), vs. A/R group; #\(p<0.01\), vs. ASI + A/R group.

Figure 8. ASI pretreatment inhibited the apoptosis of cardiomyocytes suffered by A/R, while DAPT abrogated the effect. (A) Representative dot plots of flow cytometry (x-axis and y-axis represented Annexin V and PI staining, respectively). (B) The evaluation of apoptotic cell populations. Data were expressed as the mean ± SEM, \(n=3\). **\(p<0.01\), vs. control group; ▲▲\(p<0.01\), vs. A/R group; #\(p<0.01\), vs. ASI + A/R group.
Figure 1
94x119mm (300 x 300 DPI)
Figure 2.
125x210mm (300 x 300 DPI)
Figure 3.
47x30mm (300 x 300 DPI)
Figure 4.
137x121mm (300 x 300 DPI)
Figure 5.
148x140mm (300 x 300 DPI)
Figure 6.
76x77mm (300 x 300 DPI)
Figure 7.
52x36mm (300 x 300 DPI)
Figure 8.
152x149mm (300 x 300 DPI)