Protection of Astragaloside Derivate on Oxidative Stress and Hypertrophy in Cardiomyocytes

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Abstract: **Objective** The astragaloside IV (ASI) has been proved to play an important role in protecting against cell death on cardiovascular diseases. This study aims to investigate the effect of the astragaloside derivate (ASId) on confronting oxidative stress and hypertrophy in myocardial cells. **Methods** Following exposure embryonic rat cardiac H9c2 cells to hydrogen peroxide (H_2O_2) and angiotensin II for developing oxidative stress and hypertrophy, ASId at final concentrations (0.1, 1, and 10 μmol/L) was added to study its role in protecting cardiomyocytes by biochemical detection and cell size measurement. In addition, the mitochondrial permeability transition pore (mPTP) opener atractyloside (20 μmol/L) and inhibitor cyclosporin A (CSA) (1 μmol/L) were employed to investigate the possible mechanisms for anti-oxidation. **Results** ASId at 1 and 10 μmol/L in cultures suppressed oxidative stress at different degrees, which induced the decrease in LDH activity and MDA content, and also the increase in SOD activity in comparable with the model group; The mPTP opener atractyloside and inhibitor CSA weakened and strengthened the role of ASId, respectively. ASId at 10 μmol/L inhibited cell hypertrophy, and the cell diameter, surface area, and protein content were all decreased in comparable of those cells in model group. **Conclusion** ASId is involved in the cytoprotective effects on oxidative stress through a pathway mediated by mPTP, and also has a protective effect against hypertrophy.

Key words: astragaloside derivate; cardiomyocytes; H9c2 cells; hypertrophy; oxidative stress

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Introduction

Congestive heart failure (CHF), with the increasing prevalence, is the final common sequelae of severe heart disease. The pathogenesis is complicated, which is relevant to neuroendocrine hyperexcitability, excitation contraction coupling dysfunction, cardiac myocyte hypertrophy, oxidative damage and apoptosis, and so on. Recently, following the therapeutical advance of CHF, new theory target point such as oxidative stress, myocardial remodeling, and apoptosis were emphasized and supplied as new ways for treatment (Frey *et al*, 2004; Das, 2007).

As we all know, reactive oxygen species (ROS) are derived from mitochondrial and cellular oxidases that generate superoxide ($O_2^{\cdot-}$) (Sawyer *et al*, 2002). $O_2^{\cdot-}$ is quickly converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2), and in turn converted to hydroxyl radical via the Fenton reaction (Jung and Reszka, 2001). These highly toxic ROS reactions with cellular molecules, including nucleic acids, proteins, and lipids, caused cellular damage, including membrane lipid peroxidation, the functional lose of protease and DNA, and so on. It has been reported to modulate cellular and functional responses in the heart, suggesting a role for ROS in short-term cardiac regulation (Zhang *et al*, 2005). Moreover, the mitochondrial permeability transition pore (mPTP), a non-specificity passage between the exterior and interior membrane of chondrosome, participates in myocardial preservation against injury (Halestrap *et al*, 2004).

Cardiac hypertrophy is a response of the heart to a wide range of extrinsic stimuli, such as arterial hypernsion, valvular heart disease, myocardial infarction, and hypertrophic cardiomyopathy. Although this process is initially compensatory for the workload increases, its prolongation frequently results in CHF, arrhythmia, and

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sudden death (Lorell and Carabello, 2000). On the cellular level, the hypertrophic phenotype is characterized by an increase in cell size and myofibrillar assembly, reactivation of fetal genes (e.g. atrial natriuretic factor, ANF), and expression increase of contractile elements (e.g. myosin light chain-2, MLC-2) (Sugden and Clerk, 1998).

In summary, finding out the drugs which can reverse oxidative stress and hypertrophy may provide a therapeutic method against the heart failure. Recently, more and more researches have focused on astragaloside IV (ASI), one of the main active components of *Astragali Radix*. ASI can mitigate oxidative stress-induced cell death and hypertrophy in myocardial cells (Guan *et al*, 2007; Xu *et al*, 2007; Wu, 2005; Zhang *et al*, 2010), which may be the therapy for viral myocarditis and CHF. Astragaloside derivate (ASId) was derived from ASI by reforming structures, but with higher water solubility. And ASId may have more druggability than ASI because water has lower toxicity as a solvent (Wang *et al*, 2010). However, little is known about the role of ASId in the cardiomyocytes. Hence H9c2 cells were applied to studying the effects of ASId on H_2O_2-induced oxidative stress and angiotensin II (AngII)-induced hypertrophy. The H9c2 cell line is a clonal cardiomyocyte derived from embryonic rat ventricles, and these cells maintain many molecular markers of cardiomyocytes and show morphological characteristics of immature embryonic cardiomyocytes, although these cells have dedifferentiated to an extent (Hescheler *et al*, 1991). H9c2 cell line is usually used as an *in vitro* model for studying the cellular mechanisms of oxidative stress and commonly as an experimental model of hypertrophy. Therefore, these cells were chosen for the present study.

Methods

Cells and reagents

H9c2 cell line was purchased from Shanghai Institutes for Biological Science. ASId (Batch No. 20070929) was provided by Tianjin Institute of Pharmaceutical Research. Trypsin-EDTA was from GIBCO (America); DMEM HIGH was from Tianrun Puda (Beijing, China); Fetal bovine serum (FBS) was from Hyclone (Thermo centific, USA); AngII (No. CAS038K5106), Cyclosporin A (No. CAS59865-13-3), and atractyloside potassium salt (No. CAS102130-43-8) were from Sigma (St. Louis, MO, USA) and Losartan was from Beijing Maijin Medicine Technology Company (No. 20050806); Bicinchoninic acid (BCA) assay kit was from Gaining Jinnuo (Beijing, China); SOD, malonaldehyde (MDA), and lactate dehydrogenase (LDH) assay kits were from Jiancheng (Nanjing, China).

Cell culture

H9c2 cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS containing 100 U/mL Penicilin and 100 mg/mL Streptomycin at 37 ℃ in a 5% CO_2 incubator. Medium was changed every 3 d. For preparation of the test sample, H9c2 cells were plated at a density of 2×10^5 cells/mL and kept for 12 h in serum-free medium.

Experimental procedure

Oxidative stress was induced by H_2O_2 following the procedure: cells were randomly divided into seven groups: (1) normal group; (2) solvent group: cells were treated with solvent containing 25% ethanol and 25% propylene glycol; (3) model group: cells were treated with 500 μmol/L H_2O_2; (4) ASId group: cells were pretreated with ASId (0.1, 1, and 10 μmol/L) for 1 h before H_2O_2 incubation; (5) astragalus group: cells were pretreated with *Astragalus* injection 150 g/L for 1 h before H_2O_2 incubation; (6) atractyloside group: cells were pretreated with atractyloside (20 μmol/L) for 20 min before incubation of ASId (10 μmol/L) and H_2O_2; (7) cyclosporin A (CSA) group: cells were pretreated with CSA (1 μmol/L) for 20 min before the incubation of ASId (10 μmol/L) and H_2O_2. After culturing for 24 h, the supernatant of cells was collected and prepared for the LDH, SOD, and MDA detection.

Hypertrophy was induced by AngII following the procedure: cells were randomly divided into six groups: (1) normal group; (2) solvent group: cells were treated with solvent containing 25% ethanol and 25% propylene glycol; (3) model group: cells were treated with 1 μmol/L AngII; (4) ASId group: cells were pretreated with ASId (0.1, 1, and 10 μmol/L) for 30 min before AngII incubation; (5) Losartan group: cells were treated with 1 μmol/L AngII antagonist Losartan for 30 min before AngII incubation; (6) astragalus group: cells were pretreated with *Astragalus* injection 150 g/L for 30 min before AngII incubation. After 6 d duration, cells were treated for the foreword experiments.

LDH activity, MDA content, and SOD activity assays

After treatment, the supernatant was collected for testing the LDH activity, MDA content, and SOD activity using the assay kits according to the manufacturer's instructions. LDH activity and MDA content in the medium released from the cytoplasm of cells were considered to be indicators of oxidative stress, and SOD activity was an index of the body ability to clean the free radical. The absorbances were 440, 532, and 550 nm, respectively.

Determination of cell volume

Cells were digested via 0.25% trypsilin, and images of rounded cells were acquired by Nikon invert microscope (ECLIPE, Japan) with × 20 lens of attached digital camera. Measurements of each cell diameter and surface area were made using pathology image analysis analytical system software. One hundred cells per experimental group were determined randomly.

Protein assay

To evaluate whether or not the increase in cell volume was caused by elevated cellular components or merely cell swelling, protein assay was performed. Protein concentrations were measured by BCA method using BCA protein content assay kit according to the manufacturer's instruction with a Sunrise Eliasa (Tecan, Austria) at the 562 nm absorbance. The whole protein content was normalized relative to the total cell numbers.

Statistical analysis.

All results were expressed as $\bar{x} \pm s$. Comparison between multiple groups was performed by One-way analysis of variance (ANOVA) with software Origin 7.5. Difference was statistically significant ($P < 0.05$).

Results

Effect of ASId on LDH activity, MDA content, and SOD activity in H_2O_2-treated H9c2 cells

In model group, LDH activity and MDA content increased by 17.7% and 54.2%, and SOD activity decreased by 10.3% ($P < 0.05$) compared with solvent group, which indicated that H9c2 cells were damaged by lipid peroxidation irritation with H_2O_2. The protection was observed at different degrees by ASId pretreatment (1 and 10 μmol/L) compared with model group; LDH activity decreased by 10.5% ($P > 0.05$) and 19.7% ($P < 0.05$); MDA content decreased by 53.8% ($P < 0.05$) and 38.9% ($P < 0.05$); SOD activity increased by 8.3% ($P < 0.05$) and 8.3% ($P < 0.05$), respectively (Table 1). But there were no statistic differences at concentration of 0.1 μmol/L.

Table 1 Effects of ASId on activity of LDH and SOD, and MDA content in H9c2 cells treated by H_2O_2 ($\bar{x} \pm s$, $n = 5$)

Group	Dose	LDH / ($U \cdot L^{-1}$)	SOD / ($U \cdot mL^{-1}$)	MDA / ($nmol \cdot mL^{-1}$)
control	—	1513 ± 110	1.77 ± 0.15	4.73 ± 1.09
solvent	—	1493 ± 165	1.74 ± 0.14	4.67 ± 1.05
model	—	1758 ± 175$^{\Delta}$	1.56 ± 0.09$^{\Delta}$	7.20 ± 2.09$^{\Delta}$
ASId	10^{-7} $mol \cdot L^{-1}$	1858 ± 260	1.72 ± 0.23	4.93 ± 0.72
	10^{-6} $mol \cdot L^{-1}$	1574 ± 105	1.69 ± 0.08*	3.33 ± 2.03*
	10^{-5} $mol \cdot L^{-1}$	1412 ± 181*	1.69 ± 0.07*	4.40 ± 1.19*
astragalus	150 $mg \cdot mL^{-1}$	1400 ± 256*	1.73 ± 0.10*	4.27 ± 1.19*

$^{\Delta}P < 0.05$ *vs* solvent group; $^{*}P < 0.05$ *vs* model group

Effect of atractyloside and CSA on the role of ASId against oxidative stress in H_2O_2-treated H9c2 cells

The leakage of LDH in the cells pretreated with mPTP opener atractyloside was higher than that in the cells pretreated with ASId (10 μmol/L) only ($P < 0.05$), which indicated that atractyloside could weaken the role of ASId on antiperoxidation, while the mPTP inhibitor CSA could strengthen the antiperoxidation of ASId, with higher SOD activity ($P < 0.05$). The results suggested that the mPTP opener atractyloside and inhibitor CSA could weaken and strengthen the protection of ASId on H9c2 cells, respectively, so there might be a certain relationship of the protection between ASId against oxidative stress and mPTP in myocardial cells (Table 2).

Effect of ASId on AngII-induced cardiomyocyte hypertrophy

A significant enlargement of the cell size and protein synthesis was visualized (Fig. 1). The diameter and surface

Table 2 Effects of atractyloside and CSA on ASId against oxidative stress H9c2 cells treated by H_2O_2 ($\bar{X} \pm s$, $n = 5$)

Group	Dose / (mol·L^{-1})	LDH / (U·L^{-1})	SOD / (U·mL^{-1})	MDA / (nmol·mL^{-1})
model	—	1758 ± 175	1.56 ± 0.09	7.20 ± 2.09
TY6052	1×10^{-5}	1412 ± 181*	1.69 ± 0.07*	4.40 ± 1.19*
atractyloside + TY6052	$2 \times 10^{-5} + 1 \times 10^{-5}$	1678 ± 165^{+}	1.68 ± 0.22	4.13 ± 1.86
CSA + TY6052	$1 \times 10^{-6} + 1 \times 10^{-5}$	1429 ± 323	1.84 ± 0.12^{+}	3.47 ± 1.10

$^{*}P < 0.05$ *vs* model group; $^{+}P < 0.05$ *vs* ASId (10 μmol·L^{-1}) group

area of H9c2 cells were increased by 6.7% ($P < 0.05$) and 15.9% ($P < 0.05$) and protein content increased by 27.9% ($P < 0.05$) in the AngII-treated cells relative to solvent controls. After incubation of ASId in the cultures, the cell diameter, surface area, and protein content were decreased by 6.9%, 14.1%, and 20.4% at concentration of 10 μmol/L compared with those cells in model group ($P < 0.05$), but there were no statistic differences at concen- trations of 0.1 and 1 μmol/L. (Tables 3, 4, and Fig. 1).

Discussion

ASI, one of the main active constituents of *Astragali Radix*, has been indicated to mitigate myocardial damage caused by peroxidation, inhibit cardiac myocyte apoptosis, and lessen myocardial fibrosis, which may be the therapy for viral myocarditis and CHF. ASId was derived from ASI by reforming structures, but with higher water solubility and efficacy. In present study, the role of ASId on heart failure was studied in rats, as far as we know, it was the first time to observe the cytoprotection against oxidative stress and hypertrophy on cultured cardiomyocyte.

Table 3 Effect of ASId on diameter and surface area of AngII-induced hypertrophy in H9c2 cells ($\bar{X} \pm s$, $n = 5$)

Group	Dose	Diameter / μm	Surface area / μm^2
control	—	18.87 ± 1.01	263.84 ± 30.69
solvent	—	18.90 ± 0.98	262.10 ± 32.16
model	—	20.16 ± 0.73$^{\triangle}$	303.74 ± 20.26$^{\triangle}$
ASId	0.1 μmol·L^{-1}	20.23 ± 1.67	306.27 ± 52.67
	1.0 μmol·L^{-1}	19.55 ± 1.14	284.05 ± 35.32
	10.0 μmol·L^{-1}	18.75 ± 1.12*	261.00 ± 29.57*
Losartan	1.0 μmol·L^{-1}	18.84 ± 0.81*	263.99 ± 23.09*
astragalus	150.0 g·L^{-1}	18.24 ± 0.73**	249.2 ± 22.09**

$^{\triangle}P < 0.05$ *vs* solvent group; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model group

Table 4 Effect of ASId on protein of AngII-induced hypertrophy in H9c2 cells ($\bar{X} \pm s$, $n = 5$)

Group	Dose	Protein content / (μg·10^{-5} cells)
control	—	635.0 ± 92.0
solvent	—	609.0 ± 181.0
model	—	779.2 ± 98.1$^{\triangle}$
ASId	0.1 μmol·L^{-1}	724.6 ± 154.0
	1.0 μmol·L^{-1}	666.5 ± 88.1
	10.0 μmol·L^{-1}	620.0 ± 89.7*
Losartan	1.0 μmol·L^{-1}	658.2 ± 60.9*
astragalus	150.0 g·L^{-1}	652.4 ± 65.1*

$^{\triangle}P < 0.05$ *vs* solvent group; $^{*}P < 0.05$ *vs* model group

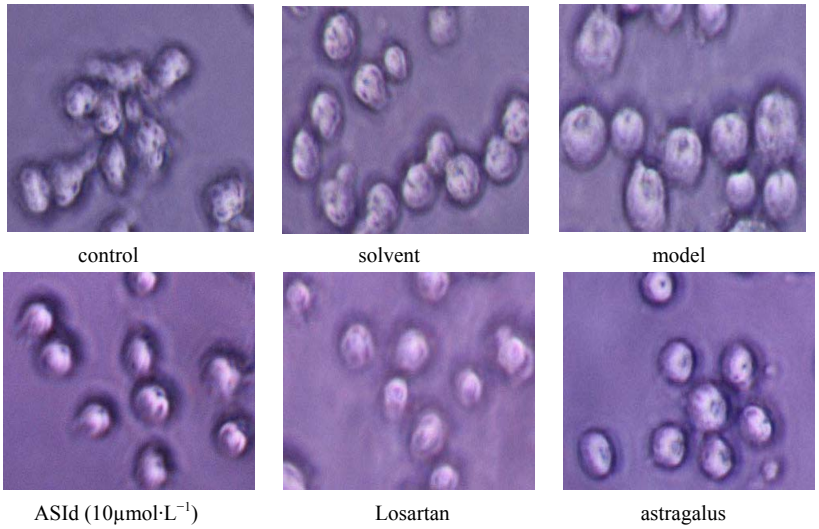

Fig. 1 Effects of pretreatment with ASId in AngII-induced hypertrophy in H9c2 cells

Representative images of diameter and surface area in each group were observed following 6 h treatment

Oxidative stress and cardiac hypertrophy are believed to be the significant pathophysiology basis of CHF (Finkel and Holbrook, 2000) (Fig. 2). Oxidative stress evoked the alteration of mitochondrial permeability, resulting in leakage of intramembrane protein-like cytochrome C, and eventually induced the apoptosis and whereafter CHF (Fig. 2) (Hitomi *et al*, 2004). Thus, it is of great significance to search for the effective method of anti-oxidation. In this research, H9c2 cells were pretreated with or without three concentrations of ASId before H_2O_2 incubation for inducing oxidative stress, the results of increasing LDH activity and MDA content and decreasing SOD activity were observed, indicating that cells were damaged by lipid per-oxidation. After incubation of ASId (0.1, 1, and 10 μmol/L), there was a dose-effect relationship in decreasing LDH activity and MDA content and increasing SOD activity in 1 and 10 μmol/L groups compared with the model group. ASId (10 μmol/L) decreased LDH activity by 19.7% ($P < 0.05$), MDA content by 38.9% ($P < 0.05$), and SOD activity by 8.3% ($P < 0.05$). These findings indicate that ASId can protect cardiomyocytes from oxidative stress. The mechanisms underlying these effects remain incompletely defined. It is thought that the open of mPTP is the common damage or death passage of several organs and cells, such as myocardial ischemia/reperfusion, cerebral ischemia, and renal ischemia (Zheng, Li, and Zhang, 2001). The open of mPTP is relevant to apoptosis factor, oxidative damage, mitochondrial swelling, and so on. Accordingly, the mPTP opener atractyloside and inhibitor CSA were also added in our experiments. Atractyloside and CSA could weaken and strengthen the protection of ASId in H9c2 cells, respectively. Results suggested that ASId was involved in the cytoprotective effects on the oxidative stress induced by H_2O_2, and might be through a pathway mediated by mPTP.

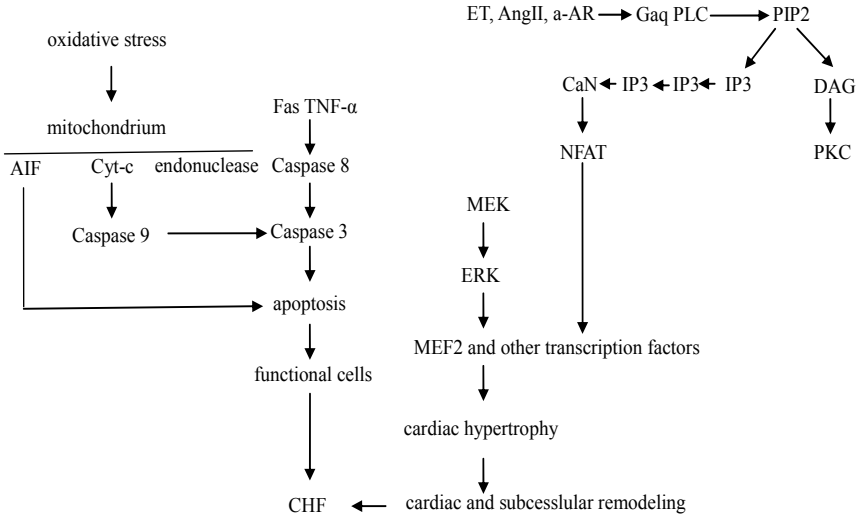

Fig. 2 Mechanism of oxidative stress and hypertrophy in heart failure

Cardiac hypertrophy is an adaptive response to hemodynamic overload or neurohormonal activation to normalize wall tension and maintain systolic function, which is also an important precipitating factor of CHF, inducing heart remodeling and the descent of heart function, resulting in CHF at last through complicated signal transduction (Fig. 2). Thus, pharmacological targeting of key mediators of hypertrophy may prevent the development of cardiac failure. In this study H9c2 cells were pretreated with AngII, and a significant enlargement of the cell size and protein synthesis was detected, and also the success of hypertrophy model was verified. Our experiments found that, ASId (10 μmol/L) decreased cell diameter, surface area, and protein content by 6.9%, 14.1%, and 20.4% compared with those cells in the model group, which should prove the protection of ASId on hypertrophy. These rusults were in consistent with the reports that ASId could inhibit compensatory hypertrophy and left ventricular (LV) remodelling. After iv injection of ASId (1 mg/kg) in rats for two weeks, LV internal diameters at end-diastole and end-systole (LVIDd and LVIDs)

decreased by 6.3% and 13.6%, and LV volum at end-diastole and end-systole (EDV and ESV) decreased by 15.2% and 34.0%, in addition, LV systolic wall stress and cardiac hypertrophy were decreased by 35.5% and 9.5% (Wang *et al*, 2010), which all confirmed the protection of ASId against hypertrophy.

These results suggested that the therapeutical effect of ASId on heart failure could be finished by resisting oxidation mediated by mPTP and hypertrophy, so that a therapeutic drug against the heart failure may be provided. However, our results just only provide a basis insight for investigating the effect of ASId on CHF, and more mechanisms such as neurohumor factors and inflammation, which could induce CHF, should be further studied.

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