Astragaloside IV prevents lipopolysaccharide-induced injury in H9C2 cardiomyocytes

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[ABSTRACT] This study aimed to investigate the protective effects of astragaloside IV (AS IV) on lipopolysaccharide (LPS)-induced injury in H9C2 cardiomyocytes. H9C2 Cardiomyocytes were cultured with LPS (10 µg·mL⁻¹) for 4 h and treated with AS IV at 50, 100, and 150 µmol·L⁻¹ for various durations. Cell viability was determined by MTT. The content of released TNF-α and IL-6 from cardiomyocytes were evaluated by enzyme-linked immunosorbent assay (ELISA). The levels of superoxidase dismutase (SOD), malondialdehyde (MDA), lactate dehydrogenase (LDH), and creatine phosphate kinase (CK) were measured by using commercial available kits. The mRNA and protein expression levels of NF-κB p65 were measured by RT-PCR and Western blotting, respectively. And the NF-κB p65 activity was measured by ELISA. Our results demonstrated that AS IV at 50, 100, and 150 µmol·L⁻¹ markedly inhibited the release of TNF-α and IL-6 and decreased NF-κB expression, compared with the model group. Moreover, the improved SOD activity and decreased MDA, LDH and CK levels were detected after AS IV treatment. In summary, AS IV could increase the activities of antioxidant enzymes, inhibite lipid peroxidation, and down-regulate the inflammatory mediators involved in the inflammatory responses. These results demonstrated that AS IV could prevent LPS-induced injury in cardiomyocytes.

[KEY WORDS] Astragaloside IV; Lipopolysaccharide; H9C2 Cardiomyocytes


Introduction

Sepsis is a common complication in neonatal intensive care units. The incidence of neonatal sepsis is 1–5 per 1000 live births, and its mortality rate is 5%–20% [1]. The incidence of sepsis and sepsis-related deaths is increasing by 1.5 % per year [2]. One of the major causes of death among the affected sepsis patients is severe hypotension associated with a decrease in cardiac output [3]. Currently, accumulating evidence indicates that myocardial depression is a common feature of sepsis in both neonates and experimental models of lipopolysaccharide (LPS)-induced endotoxicemia [4-5].

Astragaloside IV (AS IV, Fig. 1) is one type of saponin purified from Astragalus membranaceus, which has been used in Traditional Chinese Medicine as a major component of many polyherbal formulations for tissue and organ repaiment and regeneration [6]. Nowadays, AS IV is regarded as the quality standard for Astragalus membranaceus injection in the Pharmacopoeia of the People's Republic of China [7]. Previous studies have shown that AS
IV has a broad range of pharmacological properties, including the promotion of axonal maturation, antiviral and anti-inflammatory activities, and the ability to reduce infarct size as well as to improve post-ischemic heart function [8-9]. In addition, AS IV may enhance immune cell proliferation and antibody production, relax cardiovascular smooth muscle, and protect the ischemic-hypoxic myocardium and brain in patients with cardiovascular and cerebrovascular diseases [7]. The present study was designed to investigate the protective effects of AS IV on LPS-induced injury in cardiomyocytes.

Materials and Methods

Reagents

AS IV (purity: ≥ 99%) were purchased from National Institutes for Food and Drug Control (Beijing, China). LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA). The kits for superoxide dismutase (SOD), malondialdehyde (MDA), lactate dehydrogenase (LDH), and creatine phosphate kinase (CK) were purchased from the Institute of Jiancheng Bioengineering (Nanjing, China). The enzyme-linked immunosorbent assay (ELISA) kits for determination of IL-6 and TNF-α were produced by Nanjing KeyGEN Bio-tech Co., Ltd. (Nanjing, China). The NF-κB p65 activity kit was produced by Dakewe Biotech Co., Ltd. (Beijing, China).

Cell culture, LPS-induced injury, and drug treatment

H9C2 cardiomyocytes were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and were cultured in high glucose Dulbecco’s Modified Eagle's Medium (DMEM) of Sciences (Shanghai, China), and were cultured in high relative humidity in a CO2 incubator containing 5% CO2 at 37 °C. H9C2 cardiomyocytes were seeded at 1 × 10⁴ cells/mL. H9C2 cardiomyocytes were seeded at 1 × 10⁴ cells/mL. The medium was discarded and 100 µL of dimethyl sulfoxide (DMSO) was added to each cell, and the plate was kept on a shaker for 15 min to dissolve the formazan crystals formed in intact cells. Micro plate reader (Bio-Rad, Hercules, CA, USA) was used to measure the absorbance at 595 nm. The results were expressed as the percentages of reduced MTT, assuming the absorbance of control cells as 100%.

Measurement of LDH and CK content

The concentrations of LDH and CK were detected using LDH and CK kits according to manufacturer’s instruction. The NF-κB and IL-6 measurement

The concentrations of TNF-α and IL-6 in the culture media were detected using ELISA kits. The medium was collected and cytokine levels were quantified with the kits. The lower detection limits of the TNF-α and IL-6 ELISA kits were 15 and 7.8 pg·mL⁻¹, respectively.

Measurement of MDA level and SOD activity

The levels of MDA, SOD activity were measured by commercial kits according to manufacturer’s instruction. The mRNA expression levels by RT-PCR and real-time PCR

The SYBR green (Invitrogen, Carlsbad, CA, USA) by Step One system (Applied Biosystems, Foster City, CA, USA) PCR Master Mix was used according to the manufacturer’s instructions. The amplified products were electrophoresed with 1.5% agarose gel and visualized by GoldView™ (SBS Genetech, Beijing, China) and UV irradiation.

The SYBR green (Invitrogen, Carlsbad, CA, USA) was used to normalize cDNA levels.
NF-κB p65 protein expression by Western blotting

The cells were homogenized, washed with PBS, and incubated in lysis buffer in addition to a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) to obtain extracts of proteins. The samples were loaded onto 10% SDS-PAGE gels and were electrophoresed onto nitrocellulose membrane. The blots were incubated with the appropriate concentration of anti-p65 antibody (Santa Cruz Biotechnology, CA, USA). After washing, the blots were incubated with horseradish peroxidase conjugated second antibody. The membranes were stripped and re-blotted with anti-GAPDH antibody (Santa Cruz Biotechnology, CA, USA) to verify the equal loading of protein in each lane. Quantification of protein expression was normalized to GAPDH using imaging system (NIH Image J, Bethesda, MD, USA).

Statistical analysis

The results were expressed as Mean ± SD and analyzed by one-way ANOVA, with the Statistical Product and Service Solutions (SPSS 12.0, USA). Differences were considered significant if $P < 0.05$.

Results

Cell viability

The effects of AS IV on the cell viability of the H9C2 cardiomyocytes are shown in Fig. 2 (A) and Table 1. Compared with control, the cell viability of the model group markedly decreased; compared with model group, the cell viability of the treatment markedly increased.

Effects of AS IV on LDH and CK

The effects of AS IV on LDH and CK in H9C2 cardiomyocytes can be seen in Figs. 2B–2C and Table 1. The levels of the LDH and CK were dramatically increased in the model group; AS IV decreased the LDH and CK levels, compared with model group.

Effects of AS IV on LPS-induced release of IL-6 and TNF-α

The levels of the cytokines IL-6, TNF-α (Table 2) from H9C2 cardiomyocytes were dramatically increased in the model group; AS IV decreased the IL-6 and TNF-α levels, compared with model group.

Effects of AS IV on LPS-induced release of SOD and MDA

Compared with control group, the level of SOD in the model group decreased and the MDA content increased; compared with model, the level of SOD in the treatment group increased and the MDA content decreased. The result can be seen in Table 3.

![Fig. 2 Astragaloside IV prevented against LPS-induced injury of H9c2 cardiomyocytes. (A) Cell viability was assessed by MTT assay. (B, C) Cell death was measured by LDH and CK release](image)

Values are expressed as Mean ± SD. *$P < 0.05$, **$P < 0.01$ vs control; $P < 0.05$, ***$P < 0.01$ vs model

<table>
<thead>
<tr>
<th>Table 1 Astragaloside IV prevents H9c2 cardiomyocytes against LPS-induced injury (Means ± SD, $n = 9)$</th>
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<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>LPS (10 mg·L$^{-1}$)</td>
</tr>
<tr>
<td>AS IV (50 µmol·L$^{-1}$)</td>
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<td>AS IV (100 µmol·L$^{-1}$)</td>
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<td>AS IV (150 µmol·L$^{-1}$)</td>
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*$P < 0.05$, **$P < 0.01$ vs control; $P < 0.05$, ***$P < 0.01$ vs model
Table 2  Effects of astragaloside IV on SOD and MDA
(Means ± SD, n = 9)

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U·L⁻¹)</th>
<th>MDA (µmol·g⁻¹)</th>
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<tr>
<td>Control</td>
<td>32.56 ± 2.24</td>
<td>11.22 ± 2.16</td>
</tr>
<tr>
<td>LPS (10 µmol·L⁻¹)</td>
<td>9.53 ± 1.48</td>
<td>45.77 ± 3.26</td>
</tr>
<tr>
<td>AS IV (50 µmol·L⁻¹)</td>
<td>15.42 ± 1.69</td>
<td>36.42 ± 2.56</td>
</tr>
<tr>
<td>AS IV (100 µmol·L⁻¹)</td>
<td>19.48 ± 1.85</td>
<td>29.55 ± 1.97</td>
</tr>
<tr>
<td>AS IV (150 µmol·L⁻¹)</td>
<td>24.83 ± 2.12</td>
<td>22.67 ± 2.09</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 vs control; *P < 0.05, **P < 0.01 vs model

Effect of AS IV on LPS-induced NF-κB p65 mRNA expression

The mRNA expression of NF-κB p65 was increased after LPS treatment in H9C2 cardiomyocytes (Fig. 3 and 4) (P < 0.05). In the AS IV groups, the mRNA level of NF-κB p65 was significantly decreased, compared with the model group (P < 0.05).

Fig. 3  Effects of astragaloside IV on mRNA of NF-κB p65 by RT-PCR. A (control); B (LPS); C [AS IV (50 µmol·L⁻¹)]; D [AS IV (100 µmol·L⁻¹)]; E [AS IV (150 µmol·L⁻¹)]

Values are expressed as Mean ± SD. *P < 0.05, **P < 0.01 vs control; *P < 0.05, **P < 0.01 vs model

Fig. 4  Effects of astragaloside IV on NF-κB p65 mRNA expression by real-time PCR

Values are expressed as Mean ± SD. *P < 0.05, **P < 0.01 vs control; *P < 0.05, **P < 0.01 vs model

Effect of AS IV on LPS-induced NF-κB p65 protein expression

The expression of NF-κB p65 protein was increased by LPS in H9C2 cardiomyocytes (Fig. 5). In the AS IV groups, the protein levels of NF-κB p65 were significantly decreased, compared with the model group (P < 0.05).

Fig. 5  Effects of astragaloside IV on NF-κB p65 protein expression A (control); B (LPS); C [AS IV (50 µmol·L⁻¹)]; D [AS IV (100 µmol·L⁻¹)]; E [AS IV (150 µmol·L⁻¹)]

Values are expressed as Mean ± SD. *P < 0.05, **P < 0.01 vs control; *P < 0.05, **P < 0.01 vs model

Effect of AS IV on LPS-induced NF-κB p65 activity

Compared with control, the NF-κB p65 activity in the model group was increased, which was reversed by the treatment with AS IV (Fig. 6).

Fig. 6  Effects of Astragaloside IV on NF-κB p65 activity in H9C2 cells

Values are expressed as Mean ± SD. *P < 0.05, **P < 0.01 vs control; *P < 0.05, **P < 0.01 vs model

Discussion

Huangqi (Radix Astragali Mongolici) is used for a wide range of treatment effects in cardiovascular disorders, hepatitis, kidney disease, and skin diseases, which constituents include saponins, polysaccharides and flavonoids [11]. More than 40 constituents in Astragalus saponins have been identified from the astragalus root, of which AS IV is the main
bacteria and a key mediator of the body’s response to infection [16-17], which is responsible for the multiorgan dysfunctions induced by lipopolysaccharide (LPS) [24]. The level of MDA is often used as an indicator of oxidative damage and as a marker for free radicals [100 and 150 μmol·L⁻¹]. We confirmed that LPS resulted in decreased cell viability, which were evidenced by elevated LDH and CK activities in the culture medium. The treatment with different concentrations (100 and 150 μmol·L⁻¹) of AS IV greatly reversed the loss of cell viability and reduced LDH and CK activities in a dose-dependent manner. The results strongly suggested that AS IV exerted a protective effect against the LPS-induced cardiotoxicity. However, the role of AS IV in LPS-induced myocardial dysfunction has yet not been fully defined.

LPS induces expression of pro-inflammatory cytokines such as TNF-α and IL-6 from cardiomyocytes, and the cytokines in turn triggers apoptosis in cardiomyocytes [19], which are early predictors of organ dysfunction [20]. TNF-α and IL-6 have been demonstrated to impair cardiac contractile function in intact animals, isolated hearts, and cardiomyocytes [21-22]. Therefore, plasma concentrations of TNF-α and IL-6 are valuable predictors of the prognosis of these conditions. In the present study, we demonstrated that AS IV inhibited LPS-induced release of IL-6 and TNF-α from H9C2 cardiomyocytes.

Oxidative stress and its consequent lipid peroxidation aggravate free radicals chain reactions, and activate inflammatory mediators [23]. Antioxidant enzymes are decreased in the cardiomyocytes, depressing defense mechanisms against oxidative stress [24]. The level of MDA is often used as an indication of oxidative damage and as a marker for free radicals-induced lipid peroxidation [25]. SOD, a primary defense factor, reduces the oxidative stress and the activation of inflammatory mediators [25]. The two markers are therefore a direct measure of oxidative damage [23]. The MDA contents are increased and SOD levels are decreased both in human and experimental animal studies with sepsis [26-27]. In the present study, the MDA content was significantly higher while SOD activities were significantly lower in the model group, compared with control group. AS IV reduced MDA and increased SOD activities, indicating that the cardioprotective property of AS IV was at least in part associated with its antioxidant activity.

NF-κB comprises a family of transcription factors that exist in the cytoplasm in an inactive form by virtue of its association with IkBs [28]. Once activated, NF-κB is translocated to the nucleus from the cytoplasm, then activates the consensus sequence related genes, including TNF-α, IL-6, and IL-2, which were involved in immune and inflammatory responses [29-30]. It is well known that NF-κB p65 is key signaling pathways accounting for the expressions of proinflammatory cytokines induced by LPS. Hence, we investigated the possibility that AS IV inhibited the production of TNF-α and IL-6 by interfering with the activation of NF-κB. In the present study, LPS caused a significant increase in NF-κB p65 expression compared with the control group, which was reversed by AS IV, showing downregulation of NF-κB p65 at both mRNA and protein expression levels. Moreover, we observed a significant reduction in LPS-induced NF-κB activity in response to the treatment with AS IV. We therefore speculate that the prevention of LPS-induced NF-κB activation is an important mechanism by which AS IV protects against sepsis.

In conclusion, the present study demonstrated that AS IV attenuated LPS-induced injury in H9C2 cardiomyocytes, by increasing the activities of antioxidant enzymes and inhibiting lipid peroxidation. AS IV could down-regulate the inflammatory mediators involved in the inflammatory responses, including TNF-α, IL-6, and NF-κB p65. These findings should be further investigated in preclinical and clinical settings in the future.

References


