Astragaloside IV Alleviates the Myocardial Damage Induced by Lipopolysaccharide via the Toll-Like Receptor 4 (TLR4)/Nuclear Factor kappa B (NF-κB)/Proliferator-Activated Receptor α (PPARα) Signaling Pathway

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Background: We previously reported that astragaloside IV (As-IV) can alleviate myocardial damage induced by lipopolysaccharide (LPS). However, the anti-inflammatory effects of As-IV following LPS stimulation in mice and H9C2 cardiomyocytes remain unclear. The present study was designed to explore the mechanism of action of As-IV.

Material/Methods: In vivo, C57BL/6J mice were randomly divided into 5 groups: the control group, the LPS group (10 mg/kg), and 3 LPS groups receiving different doses of As-IV (20, 40, and 80 mg/kg). The protective effect of As-IV on LPS-stimulated H9C2 cardiomyocytes was evaluated in vitro. Cardiac function was detected by echocardiography, and H&E staining was used to evaluate morphologic changes. Cardiomyocyte viability was detected by MTT assay. ELISA was used to detect free fatty acid (FFA), interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor alpha (TNF-α) levels in mouse serum and in cell supernatant. Adenosine triphosphate (ATP) and adenosine monophosphate (AMP) contents in myocardial tissues and cells were detected by high-performance liquid chromatography. ATP5D and TLR4/NF-κB/PPARα signaling pathway proteins (TLR4, NF-κB, p65, and PPARα) were detected by Western blotting.

Results: As-IV significantly improved cardiac function, myocardial cell viability, and pathological changes and reduced FFA, IL-1β, IL-6, and TNF-α levels. The ATP/AMP ratio in the cardiac tissues of mice and in H9C2 cardiomyocytes was increased compared to that in the LPS group. In addition, As-IV enhanced ATP synthase and PPARα protein expression. In H9C2 cardiomyocytes, the p65-specific inhibitor BAY11-7082 exerted similar effects as As-IV.

Conclusions: As-IV alleviates LPS-induced myocardial damage by modulating TLR4/NF-κB/PPARα signaling-mediated energy biosynthesis.

MeSH Keywords: Lipopolysaccharides • NF-kappa B • PPAR alpha • Toll-Like Receptor 4

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Background

Inflammatory reactions are common pathological processes leading to many diseases. In contrast to the low-grade inflammation caused by metabolic diseases, lipopolysaccharide (LPS) can promote excessive activation of the body’s immune system, infiltration of inflammatory cells into myocardial tissue, and overexpression of inflammatory factors in a short period of time, resulting in acute inflammation. An excessive or persistent inflammatory response may further lead to pathological damage in the body, such as myocardial damage, and may even cause septic shock [1, 2].

LPS is the main component of the outermost layer of the cell wall of gram-negative bacteria and is one of the most effective stimulants in the immune system [3]. LPS is the main cause of acute inflammation leading to cardiac dysfunction [4], which is accompanied by changes in energy metabolism-related factors during LPS-induced cardiomyopathy. According to reports, TLR4 is the major LPS receptor and a key mediator of proinflammatory responses. TLR4 activates downstream effectors via adaptor proteins (including MyD88), including mitogen-activated protein kinase (MAPK), NF-κB and phosphatidylinositol 3-kinase (PI3K). These pathways regulate expression of proinflammatory cytokines [5, 6] and control gene expression related to cell survival and apoptosis [7].

In many heart failure models, the key transcription factors involved in fatty acid oxidation metabolism mainly include PPARs and PGC-1α [8]. PPARs regulate the lipid and glucose metabolism of ligand-dependent transcription factors. Three kinds of PPAR subtypes exist: PPARα, PPARβ/δ and PPARγ [9]. PPARα mainly affects the metabolism of fatty acids [10], and its activation reduces lipid levels. PPARα activation has been shown to improve myocardial function and energy metabolism during stress overload in heart failure. PPARα activation has also been suggested as a potential treatment strategy for heart failure. PPARα is expressed in the heart and regulates the oxidation of fatty acids. TLR4 is stimulated by LPS to activate the downstream effector NF-κB, which then exhibits high expression and may lead to lower expression of PPAR, thus affecting fatty acid metabolism and the use of cardiac muscle energy [11]. Therefore, the TLR4/NF-κB/PPARα signaling pathway plays a vital role in energy metabolism in myocardial injury.

Traditional Chinese medicine has been widely used worldwide for centuries. Astragaloside IV (As-IV) is a major small molecule and active substance extracted from the traditional Chinese medicine Astragalus membranaceus [12]. Existing research has proven that As-IV has a very broad range of pharmacological effects, for example, anti-inflammatory effects [13], anti-apoptotic effects [14–17], regulatory effects on energy metabolism [18] and anti-inflammatory effects [19]. As-IV has potential therapeutic effects in cardiovascular disease because of its beneficial pharmacological and biochemical activities. As-IV has been demonstrated to have a protective effect on LPS-induced myocardial injury, but the specific mechanism is still unclear [20]. Septic myocardial injury models were prepared both in vivo and in vitro to observe the effect of As-IV on cardiomyopathy after inhibiting energy metabolism and the TLR4/NF-κB/PPARα signaling pathway.

Material and Methods

Reagents

As-IV (purity: 98%) was provided by Jingzhu Biotechnology Co., Ltd. (Nanjing, China). LPS, BAY, adenosine triphosphate (ATP) and adenosine monophosphate (AMP) standards were provided by Sigma-Aldrich (St. Louis, MO, USA). ELISA kits for determination of free fatty acid (FFA), tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) levels were provided by R&D Systems (Minneapolis, MN, USA). TLR4, NF-κB, p65, PPARα, ATP5D and GAPDH antibodies were purchased from Abcam (Cambridge, MA, USA). All other reagents are analytically pure reagents.

Animal model

The C57BL/6j mice (20±2 g, 4 weeks old) used in all experiments were purchased from the Experimental Animal Center of Jinzhou Medical University (Jinzhou, China). All the experimental procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The C57BL/6j mice were randomly divided into 5 groups, with 10 mice in each group: the (1) control group, (2) LPS group, (3) LPS+As-IV-20, (4) LPS+As-IV-40, and (5) LPS+As-IV-80. The doses of As-IV were selected based on previous literature [13]. As-IV was dissolved in 1% CMC Na solution. As-IV was administered intragastrically for 7 consecutive days. The control group and LPS group were intragastrically treated with the same volume of autoclaved PBS. One hour after the last administration, the mice in the LPS groups (2, 3, 4, and 5) were intraperitoneally injected with 10 mg/kg LPS (Sigma-Aldrich). LPS was injected intraperitoneally (i.p.) to evoke inflammation in the C57BL/6j mice as previously described [21]. The control mice were intraperitoneally injected with autoclaved PBS. After LPS treatment for 8 h, the animals underwent cardiac function measurements. After the measurements, blood samples were collected through the eyelids for further analysis. Then, heart tissue was harvested; a portion of tissue was placed in 4% paraformaldehyde, and another portion was stored in liquid nitrogen.

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ANIMAL STUDY

Cell culture and treatment

The rat cardiomyocyte H9C2 cell line, which was obtained from Wuhan Boster Bioengineering Company, Ltd. (HuBei, China), was cultured in DMEM containing 10% fetal bovine serum (HyClone, South America) at 37°C and 5% CO₂ in 100 IU/ml streptomycin and 100 IU/ml penicillin (Amresco, OH, USA). The H9C2 cells were passaged periodically and subcultured to 90% confluence. The cells were randomly divided into 4 groups: the (1) control group, (2) LPS group, (3) LPS+As-IV group (As-IV, 80 µM), and (4) LPS+BAY (10 µM). We conducted a sufficient number of preliminary experiments before the formal test and determined that an As-IV concentration of 80 µM is the optimal experimental concentration. As-IV was dissolved in DMSO, and the final concentration in the medium was not higher than 0.1%. The NF-κB inhibitor BAY was dissolved in high-glucose DMEM medium. The H9C2 cells in the treatment groups were treated with LPS (1 µM) for 24 hours, and the H9C2 cells in the LPS+As-IV group and the LPS+BAY group were treated with As-IV (80 µM) and BAY (10 µM), respectively, for 1 hour before LPS administration (1 µM).

Determination of cardiac function

Eight hours after LPS injection into the mice, sevoflurane was used for anesthesia, and the skin was prepared. The EF, FS, LVIDd, and LVIDs were determined by echocardiography.

Histological analysis

Myocardial tissue was fixed with formaldehyde for 24 hours and then embedded with paraffin and cut into 5-µm sections. Hematoxylin and eosin staining was performed. LAS software (V4.3) was used for analysis.

MTT assay

H9C2 myocardial cells were inoculated in 96-well plates for 24 h (5×10⁴/well). Then, As-IV and BAY were added to the wells. One hour after treatment, the cells were treated with LPS (1 µM) for 24 h. Next, 50% MTT solution (5 mg/ml) (Sigma) was added to each well, followed by incubation at 37°C for 4 h. The solution in each well was subsequently replaced with 150 µL of DMSO, and MTT-formazan crystals were dissolved. After 10 minutes of breeding in a cradle, the absorbance was measured at 490 nm with a microplate reader (Semmerfeld Instrument Co., USA). * P<0.05 and ** P<0.01 indicate statistically significant differences.

Cytokines in the serum and cellular supernatant

The levels of FFA, IL-1β, TNF-α and IL-6 in mouse serum and cell supernatant were determined using commercial ELISA kits (R&D, Minneapolis, MN, USA). All measurements were performed in triplicate according to the manufacturer’s instructions.

Immunofluorescence

H9C2 cardiomyocytes were seeded into 96-well plates. The cells were fixed in 4% paraformaldehyde in PBS for 20 min, 0.5% TritonX-100 was used to permeate the cells in PBS for 20 min, and 5% BSA was used to block the cells at room temperature for 30 min. Then, the cells were diluted with NF-xb and p65 (1:100) antibodies and incubated at 4°C overnight. The next day, fluorescence staining was measured for 1 hour, and DAPI was added for 5 minutes. After washing the specimens with PBST, images were captured at 200× magnification under fluorescence microscopes.

Western blotting

Using a commercially available Nuclear and Cytoplasm Extraction Kit (Active Motif), nuclear proteins were isolated from the heart tissues of mice or from H9C2 cells. The proteins (20 µg) were separated by 10% SDS-PAGE, transferred to hydrophobic polyvinylidene (PVDF) membranes and blocked with 1% BSA. The membranes were incubated with primary antibodies for TLR4, p65, ATP5D, PPARα, GAPDH and Lamin B at 4°C overnight. Detection was performed with enhanced chemiluminescence reagents. The results were analyzed with Quantity One software (Bio-Rad Laboratories, Hercules).

High-performance liquid chromatography

ATP and AMP levels in myocardial tissues and cells were measured by high-performance liquid chromatography according to the machine test requirements of the samples.

Statistical analysis

The results are displayed as the mean ± standard deviation (SD). Data were processed using one-way analysis of variance (ANOVA), and differences between treatments were calculated, followed by a t test (Prism 5 for Windows, GraphPad Software Inc., USA). * P<0.05 and ** P<0.01 indicate statistically significant differences.

Results

Effects of As-IV on cardiac function

The EF, FS, LVIDd and LVIDs in the LPS group were significantly lower than those in the control group, which decreased by 47.42%, 55.93%, 30.62% and 36.45%, respectively. These results show that LPS administration can induce heart dysfunction. In contrast, these changes were considerably enhanced.
with As-IV (20, 40, and 80 mg/kg) treatment. Our data suggest that As-IV can attenuate cardiac dysfunction in LPS-induced myocardial injury. The results are shown in Figure 1.

**Effects of As-IV on cardiac tissue morphology and cell viability**

Myocardial tissue showed infiltration by inflammatory cells, abnormal deformation of myocardial fibers and vacuolation of some myocardial cells in the LPS group compared with myocardial tissue in the control group. As the pretreatment concentration of As-IV increased, inflammatory cell infiltration gradually decreased, and the myocardial fiber structure gradually returned to normal. In the cell experiments, the cell survival rate in the LPS group obviously decreased compared with that in the control group. The activity of H9C2 cardiomyocytes in the As-IV and p65 inhibitor BAY groups was notably increased compared to that in the LPS group (Figure 2).

**Effects of As-IV on the MTT assay results**

The cell survival rate in the LPS group obviously decreased compared with that in the control group. The activity of H9C2 cardiomyocytes in the As-IV and p65 inhibitor BAY groups was markedly increased compared to that in the LPS group (Figure 3).

**Effects of As-IV on inflammatory cytokines in mouse serum and cellular supernatant**

We assessed the inflammatory cytokines generated in cell supernatants and mouse serum. The levels of cytokines IL-6, IL-1β and TNF-α in serum and cell supernatants after LPS administration were significantly increased. In contrast, administration of As-IV (20, 40, and 80 mg/kg) appeared to downregulate IL-6, IL-1β and TNF-α levels in a dose-dependent manner, with BAY and As-IV exhibiting similar effects (Figure 4).

**Effects of As-IV on cardiac energy biosynthesis and ATP5D expression**

The results showed that the ATP/AMP ratio in heart tissues and H9C2 cells decreased, the FFA content increased, and ATP5D protein expression decreased in the LPS groups compared with those in the control group. However, As-IV treatment significantly increased the ATP/AMP ratio in mouse tissues and H9C2 cells, decreased the FFA content, and increased the protein expression of ATP5D compared with the corresponding results in the groups that received LPS treatment alone. BAY and As-IV had similar effects (Figure 5).
Effects of As-IV and BAY on nuclear expression of NF-κB according to immunofluorescence

The expression of p65 was obviously increased in the LPS group compared with that in the control group (P<0.01). Compared with the LPS group, the As-IV group and BAY group exhibited decreased p65 expression in the nucleus (P<0.01). As-IV and NF-κB inhibitors have been suggested to not only prevent the transcription of p65 in the nucleus but also activate downstream factors related to p65. These inhibitors had certain protective effects against myocardial damage similar to As-IV (Figure 6).

Effects of As-IV on the expression of TLR4

As an upstream signaling molecule of NF-κB, TLR4 contributes to the activation of inflammatory cells and thus plays an indispensable role in the pathogenesis of myocardial injury caused by inflammation and cardiac dysfunction. In this experiment, the protein expression of TLR4 was elevated in both cardiac tissues and H9C2 cells compared to that in the control group. However, As-IV treatment obviously reduced the expression of TLR4 protein compared to LPS treatment. These results suggest that As-IV can increase expression of the LPS-4 receptor (Figure 7).

Effects of As-IV and BAY on expression of NF-κB and PPARα

An inseparable association exists between the NF-κB/PPARα signaling pathway and ATP5D, which affects the biosynthesis of energy. The results showed that protein expression of the NF-κB subunit p65 was decreased in the cytoplasm but that...
p65 protein expression was increased in the nuclear fraction; the protein expression of PPARα was decreased in cardiac tissues and H9C2 cells compared with that in the control group. However, compared with LPS treatment, As-IV treatment significantly reduced expression of p65 in the nuclear fraction but simultaneously increased that of PPARα. The inhibitor of p65, BAY, had an effect on PPARα expression similar to that of As-IV (Figure 8).

**Discussion**

LPS is the main component of the outermost layer of the cell wall of gram-negative bacteria and is a key ligand for TLR4. LPS is widely used as an inducer of endotoxins in research [22] and causes a systemic inflammatory response through infection, which leads to the production and release of various inflammatory factors. Inflammatory processes result in various cardiac insufficiencies, including acute heart injury [23], ischemic shock [24] and ischemia-reperfusion injury [25].

As-IV can improve cardiac function and reduce ventricular remodeling by stimulating fatty acid β-oxidation. We previously...
confirmed the efficacy of As-IV based on its protective effect against cardiac hypertrophy in Iso-induced mice, which has a certain degree of association with inhibition of the TLR4/NF-κB signaling pathway \[20,26,27\]. However, very few studies have investigated the role of PPARα in myocardial injury, which may represent a new treatment direction.

In our research, we evaluated the protective effects of As-IV on LPS-induced myocardial injury. The EF, FS, LVIDd and LVIDs were measured as the mean ±SD. n=3. ** P<0.01 vs. the control group, * P<0.05, ## P<0.01 vs. the LPS model group.

Figure 5. Effects of As-IV on cardiac energy biosynthesis and the ATP/AMP ratio in heart tissues and H9C2 cells (A, B). The levels of FFA in serum and cellular supernatant (C, D). The protein expression of ATP5D in heart tissues and H9C2 cells (E, F). Data were measured as the mean ±SD. n=3. ** P<0.01 vs. the control group, * P<0.05, ## P<0.01 vs. the LPS model group.

significantly improved with As-IV treatment compared with LPS treatment. The protective effect of As-IV was also confirmed by observing changes in cardiac tissue morphology. In this study, As-IV significantly reduced expression of IL-6, IL-1 β, TNF-α and FFAs in mouse serum and cell supernatant and increased the ATP/AMP ratio and ATP5D expression in H9C2 cells and heart tissues. Western blotting demonstrated that As-IV inhibited expression of p65 nucleoprotein and TLR4 protein and upregulated the level of p65 in the cytosol and protein expression of PPARα in H9C2 cells and heart tissues. Furthermore, we examined the effects of the NF-κB p65 inhibitor BAY11-7082 on H9C2 cells. These data suggest that the protective effects of As-IV on myocardial injury may be associated with the TLR4/NF-κB/PPARα signaling pathway.

Figure 6. Effects of As-IV and BAY on NF-κB nuclear expression according to immunofluorescence. Data were measured as the mean ±SD. n=7. ** P<0.01 vs. the control group, ** P<0.01 vs. the control group, ## P<0.01 vs. the LPS model group.

Figure 7. Western blotting was used to detect the effect of As-IV on TLR4 protein expression. TLR4 expression in cardiac tissue and H9C2 cells (A, B). Data were measured as the mean ±SD. n=3. ** P<0.01 vs. the control group, * P<0.05, ** P<0.01 vs. the LPS model group.
**Figure 8.** Western blot analysis of the effect of As-IV on PPARα and p65 protein expression. Expression of p65 in the cytosol and nuclear fractions of cardiac tissue (A, C). Expression of p65 in the cytosol and nuclear fractions of H9C2 cells (B, D). Expression of PPARα in cardiac tissue and H9C2 cells (E, F). Data were measured as the mean ±SD. n=3. **P<0.01 vs. the control group, # P<0.05, ## P<0.01 vs. the LPS model group.
At the local and systemic levels, IL-1β, as a major proinflammatory cytokine, can activate inflammatory responses [28]. IL-6 is a multifunctional cytokine and is a type of interleukin that is involved in the inflammatory response in vivo [29]. When the levels of these proinflammatory cytokines exceed a certain limit, tissue damage and even severe septic shock can occur. Therefore, inflammatory diseases must be treated with a method that inhibits the release of proinflammatory cytokines [30].

Many studies have confirmed that TLR4 can induce activation of the NF-κB pathway if its expression is stimulated by LPS [31,32]. NF-κB is a pleiotropic transcription inducible factor involved in the regulation of a variety of biological phenomena. NF-κB activation is involved in apoptosis in cardiomyocytes and the release of cytokines during inflammation [33]. NF-κB can be activated by a variety of stimuli, thus causing phosphorylation of IKK as well as its subsequent ubiquitination and degradation, followed by translocation of NF-κB subunits to the nucleus and binding to the target gene promoter region, thereby regulating expression of inflammatory factors such as IL-1β, IL-6 and TNF-α [34].

The expression levels of IL-1β, IL-6 and TNF-α inflammatory factors in the LPS group increased obviously compared with those in the control group, and p65 in the nucleus of the LPS group was also obviously increased. In the nucleus, the expression of p65 was significantly higher according to the immunofluorescence experiments, indicating that NF-κB is involved in myocardial injury. The expression levels of IL-1β, IL-6 and TNF-α inflammatory factors and the upstream signaling molecule TLR4 were significantly lower in the As-IV group than those in the LPS group, and the expression in the high-dose group was obviously lower than that in the low-dose group. These results show that As-IV has an inhibitory effect on the release of downstream inflammatory factors such as TNF-α, IL-6, and IL-1β. The mechanism may be related to reducing the expression of the membrane receptor protein TLR4, and the cardioprotective effects of As-IV may be related to its anti-inflammatory effects. Simultaneously, As-IV promoted nuclear translocation of p65, increased expression of p65 in the cytoplasm, and reduced the corresponding nuclear expression of p65. Thus, As-IV improves the state of myocardial injury. No significant difference in protein expression was observed in the H9C2 cardiomyocytes between the As-IV group and the BAY group, and no significant difference in inflammatory cytokine levels were found in the H9C2 cells. Therefore, we believe that the protective effects of As-IV in myocardial injury may be related to inhibition of NF-κB.

Studies have shown that the biological energy of the myocardium depends on effective synthesis of ATP and the ATP synthase subunit. ATPSDL is one of the major enzymes in mitochondrial ATP synthesis. Cardiac dysfunction is usually associated with mitochondrial dysfunction, which leads to a decrease in the ATP/AMP ratio and an insufficient energy supply for cardiac function. The ultimate results of acute inflammation are myocardial dysfunction and energy deficits. The transfer of energy substrates from fatty acids to glucose is a sign of metabolic remodeling during the progression of heart failure [35]. Regulating energy metabolism may become a therapeutic target for heart failure in the future [36]. The results show that FFAs in the As-IV group decreased significantly and that this decrease was related to the concentration of As-IV. As-IV can alleviate damage to the myocardial membrane caused by FFAs. At the same time, As-IV apparently increased the ratio of ATP to AMP and increased the energy supply in myocardial tissues and H9C2 cells. After alleviating the low energy supply in injured myocardial tissue due to acute inflammation, myocardial function gradually returned to normal.

ATP is mainly derived from the oxidation of fatty acids inside mitochondria [37,38], and PPARα can promote the oxidation of fatty acids. Therefore, the role of PPARα in the heart cannot be ignored [39]. The NF-κB signaling pathway has been shown to be one of the most important signaling transduction pathways in myocardial inflammatory injury, and this pathway inhibits the activity of PPARα, thereby affecting the oxidation of fatty acids in myocardial injury. Activation of the NF-κB pathway has been determined to promote the oxidation of fatty acids.

According to reports, the agonist of PPARα, fenofibrate, regulates inflammation by regulating TLR4-mediated signaling pathways and reduces serum proinflammatory mediator levels in patients with atherosclerosis [40]. In the process of inflammation, PPAR participates in regulation of the TLR4 signaling pathway [30].

PPARα has become an attractive target for improving metabolic remodeling. Since myocardial function is closely related to energy metabolism, metabolic pathways are potential therapeutic targets for the treatment of cardiac insufficiency [41,42], and activation of PPARα has been proposed as a therapeutic method. In this experiment, we added the inhibitor BAY as a positive control in the in vitro experiment, and the above experimental results confirmed the protective effect of As-IV on myocardial injury. However, in the in vivo experiment, we did not add BAY to observe the final result. This is a weakness of this experiment. We will continue to study the effect of the BAY in future experiments.

Conclusions

As-IV can alleviate the myocardial damage induced by LPS through the TLR4/NF-κB/PPARα signaling pathway.
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