Andrographolide Attenuates LPS-Induced Cardiac Malfunctions Through Inhibition of IκB Phosphorylation and Apoptosis in Mice

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Key Words
Cardiac malfunction • LPS • Andrographolide • Apoptosis

Abstract
Background/Aims: Cardiac malfunction is a common complication in sepsis and significantly increases the mortality of patients in septic shock. However, no studies have examined whether andrographolide (And) reduces LPS-induced myocardial malfunction. Methods: Left ventricular systolic and diastolic functions were examined using echocardiography. TNF-α and IL-1β protein levels were detected by an enzyme-linked immunosorbent assay (ELISA). NO oxidation products were determined using Griess reagent. Protein expression levels of inhibitors of NF-κBα (IκB) and phospho-IκB were determined via Western blot. Oxidative injury was determined by measuring myocardial lipid peroxidation and superoxide dismutase activity. Cardiac apoptosis was examined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) and cardiac caspase 3/7 activity. Results: And blunted LPS-induced myocardial malfunctions in mice. LPS induced TNF-α, IL-1β, and NO production as well as I-κB phosphorylation. Cardiac apoptosis was attenuated via incubation with And, but the extent of oxidative injury remained unaffected. Conclusion: And prevents LPS-induced cardiac malfunctions in mice by inhibiting TNF-α, IL-1β, and NO production, IκB phosphorylation, and cardiac apoptosis, indicating that And may be a potential agent for preventing myocardial malfunction during sepsis.

Introduction
The systemic inflammation of sepsis is initiated by pathogen-associated molecules, such as lipopolysaccharide (LPS), and is a leading cause of death in the intensive care unit [1, 2]. Recent progress has been made in the management of sepsis, but the mortality of sepsis is still high [2]. Myocardial malfunction is a common complication of sepsis inpatients...
or in animal models of LPS-induced endotoxemia and 40% of patients with sepsis develop cardiac dysfunction characterized by ejection fraction reductions, biventricular dilatations, and reduced responses to fluid administration [3, 4]. Emerging evidence has demonstrated that LPS-induced cardiac dysfunction is mediated by cardiac apoptosis as well as nuclear factor-Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB)-modulated overexpression of multiple proinflammatory mediators, including IL-1β, TNF-α, and NO [4-8]. Cardiac abnormalities have been found to be reversible in surviving patients, but not in non-survivors [9], suggesting that myocardial dysfunction significantly contributes to the mortality of patients in septic shock. In addition, in septic patients, the occurrence of myocardial dysfunction significantly increases mortality from 20% to 70% ~ 90% [10]. Therefore, the development of effective approaches for attenuating cardiac dysfunction during sepsis is imperative.

Andrographis Paniculata has been widely used as folk medicine in China for the treatment of viral infection, diarrhea, dysentery, and fever for hundreds of years. Additional hepatoprotective, antihypertensive, anti-inflammatory, and antithrombotic effects have also been reported, and Andrographis Paniculata has been used in antitoxins for snake bites. Moreover, andrographolide (And), extracted and purified from Andrographis Paniculata, is currently prescribed for treatment of inflammation-related diseases, such as laryngitis, upper respiratory tract infection, rheumatoid arthritis, and cancer [11-13]. Recent studies have revealed that this herb also has cardiovascular effects [14-16], and And has been shown to protect hearts against ischemia/reperfusion [17]. These findings indicate that And may be a favorable alternative to cardiovascular therapy. And protects against LPS-induced injury by inactivating NF-κB [18-25]. NF-κB plays a pivotal role in the pathogenesis of inflammation, prompting drug design for the treatment of human inflammatory diseases to focus on inhibiting NF-κB activation [26].

However, thus far, no reported data has suggested that And prevents LPS-induced cardiac dysfunction in vivo.

**Materials and Methods**

**Animals and experimental design**

All procedures for animal use were approved by the Committee on the Ethics of Animal Experiments of the First People’s Hospital of Yancheng. Male BALB/c mice (6-8 weeks old, 22-25 g) were supplied by the Yangzhou experimental animal center. The mice were kept in 26°C temperature controlled room and given free access to food and water. Animals underwent an acclimatization period of at least 7 days before use in our study. The mice were then randomly divided into four groups: control, LPS, And + LPS, and And alone. Distilled water (0.1 mL/10 g body weight) or And (10 mg/kg; Sigma) was administered intragastrically once a day for 7 days. One hour after intragastrical treatment on day 7, normal saline (0.2 mL/10 g bodyweight) or LPS (Escherichia coli, 055:B5; Sigma; 20 mg/kg, 0.2 mL/10 g body weight) was injected intraperitoneally.

**Echocardiographic examinations**

After LPS administration, echocardiographic evaluation was performed using a commercially available echocardiograph (Vevo2100, VisualSonics, Canada). M-mode and Doppler echocardiography were performed according to previously described methods [27]. Briefly, each mouse was carefully maintained under light anesthesia by inhalation of a mixture of 2% isoflurane and oxygen via a nose cone. Left ventricular systolic function was assessed by fractional shortening (FS) and ejection fraction (EF) parameters. Peak early-diastolic transmitral velocities (E wave) and peak late-diastolic transmitral velocities (A wave) across mitral valve inflow were examined on Doppler flow tracings and were used to calculate E/A ratios, a commonly used parameter of left ventricular diastolic function. All echocardiography procedures were performed by the same qualified technician, and the data were averaged from at least three cardiac cycles.

**Measurements of cardiac TNF-α, IL-1β, and NO**

The mice were sacrificed under euthanasia at 1 and 4 h after injection of LPS or normal saline. Hearts were harvested and homogenized thoroughly on ice in a lysis buffer. Heart tissue homogenates
were centrifuged at 3,600 g for 10 min, and the supernatant was obtained and stored at -80°C until analysis. TNF-α and IL-1β protein levels were detected by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer’s instructions (R&D Systems, Minneapolis, USA). Supernatant protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime, Nantong, China); TNF-α and IL-1β levels are expressed as picograms per milligram of protein. NO production was estimated from the determination of NO oxidation products (nitrate and nitrite), referred to as NOx. NOx levels were determined using Griess reagent.

Western blot analysis of NF-κBα inhibition (IκB) and phosphorylated IκB

The mouse hearts were removed 0.5 h after LPS or normal saline injection and washed once with cold normal saline, then homogenized thoroughly on ice in a split buffer RIPA (Beyotime, Nantong, China) that included phosphatase inhibitor, protease inhibitor, and phenyl methanesulfonyl fluoride. The homogenate was centrifuged for 30 min at 12,000 rpm and 4°C. Protein concentrations of the homogenates were determined using a bicinchoninic acid protein assay kit (Beyotime, Nantong, China). The lysates (100 mg of protein) were separated via 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was incubated in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) supplemented with 5% bovine serum albumin for 1 h to block nonspecific protein binding at room temperature. The membrane was cut according to the marker and then incubated overnight at 4°C in a 1:2,000 dilution of IκB antibodies or a 1:600 dilution of the phosphorylated IκB (phospho-IκB) antibodies (Abcam, Cambridge, UK). After washing the membrane three times with 0.1% TBST, the membrane was incubated in a 1:800 dilution of horseradish peroxidase-conjugated secondary monoclonal antibodies (R&D Systems, Minneapolis, USA) for 1 h at room temperature, washed three times with 0.1% TBST, and then developed using an enhanced chemiluminescence detection reagent (Pierce Biotechnology, Rockford, USA). The results were quantified by scanning densitometry.

Measurements of myocardial lipid peroxidation and superoxide dismutase activity

Heart homogenates were prepared at 12 h after injection of LPS or saline. An assay of thiobarbituric acid - reactive substances (TBARSs) was performed to assess the level of myocardial lipid peroxidation, a marker of oxidative injury. Myocardial superoxide dismutase (SOD) activity was measured using a xanthine oxidase kit (Jiancheng, Nanjing, China). Enzyme activity was presented as the amount of activity units per milligram of protein.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay

Myocardial apoptosis was assessed at 12 h after LPS administration by quantifying the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay using apoptosis detection kit in situ (Roche, New Jersey, USA). Mouse hearts were fixed in 10% phosphate-buffered formaldehyde and were paraffin embedded. Sections were incubated with proteinase K for 30 min at room temperature and then washed with PBS. Sections were incubated with the TUNEL reaction mixture and two negative controls for 60 min at 37°C in a humidified atmosphere in the dark, after which 50 uL of converter-POD was added to the sample for 30 min in a humidified chamber. Visualization of the reaction was achieved using a DAB substrate system. Apoptotic cells were counted in five randomly selected fields per section, corresponding to at least 70 cells examined at 40 x magnification. The apoptotic index was expressed as the percentage of the total number of myocardial cells.

Caspase 3/7 activity determination

Cardiac caspase 3/7 activity was monitored by measuring the relative cleavage of Asp-Glu-Val-Asp (DEVD) (Promega, WI, USA). Mouse hearts were harvested at 2 h after LPS or normal saline injection and thoroughly homogenized with 1 mL normal saline on ice. The homogenate was centrifuged at 3,600 g at 4°C for 10 min. Then, Apo-ONE caspase 3/7 reagent (100 µL) was added to each well in a 96-well plate containing 100µL of the sample and incubated at 37°C for 90 min. The resulting fluorescence was determined at 499 nm using a spectrofluorometer.

Statistical analysis

All data are expressed as the means ± SEMs. Experimental groups were compared using one-way ANOVA followed by the Student-Newman-Keuls test or an unpaired Student’s t test. The difference between means was considered statistically significant when P was less than 0.05.
Results

And prevents LPS-induced myocardial malfunction in vivo

Left ventricular function was examined by echocardiography 12 h after vehicle or LPS injection. As shown in Fig. 1 and 2, LPS injection caused significant reduction of left ventricular systolic function in mice. In contrast, And significantly prevented this LPS-induced decrease in the left ventricular EF and FS, indicating that And attenuates LPS-induced myocardial systolic malfunction. In addition, administration of And alone did not alter left ventricular EF and FS compared with control.

To assess the left ventricular diastolic function, E/A ratios were calculated from Doppler-derived mitral inflow measurements. LPS injection reduced E/A ratio values, an effect that was reversed by treatment with And (Fig. 3). These results indicate that And also inhibits LPS-induced diastolic malfunction. No significant differences in E/A ratios were observed between the control and And alone group.

LPS-induced cardiac TNF-α, IL-1β and NO productions are inhibited by And

Enhanced production of TNF-α, IL-1β and NO has implicated them as the major mediators of myocardial malfunction during sepsis [4-8]. Therefore, we have investigated the effects of And on LPS-induced cardiac TNF-α, IL-1β and NO production to examine the anti-inflammatory properties of And. As shown in Fig. 4, TNF-α and IL-1β concentrations in heart tissue at 4 h after LPS injection was significantly lower in the And+ LPS group than in the LPS group, indicating that And inhibited LPS-induced IL-1β and TNF-α over-expression in these hearts. Cardiac TNF-α and IL-1β were not detectable in the control and And alone groups. In addition, LPS increased cardiac NO levels at 12 h after LPS injection, an effect that was reversed by And (Fig. 4).

Fig. 1. Representative M-mode echocardiograms obtained from mice 12 h after injection. (A-D) represent control, LPS, And + LPS, and And alone groups, respectively.
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Oxidative damage plays a vital role in endotoxemia, which enhances the production of reactive oxygen species (ROS) and lipid peroxidation (such as TBARS) in hearts, and antioxidants decrease cardiac TBARS levels and improve rats' survival after LPS injection [28]. In addition, And was found to affect ROS production [29]. Therefore, we further observed the effects of And on cardiac TBARS content and SOD activity in mice. As shown in Tables 1 and 2, LPS did not induce an increase in TBARS content and SOD activity in hearts, and And did not alter TBARS content and SOD activity in hearts at 12 h after LPS or saline injection.

And inhibits IκB phosphorylation induced by LPS in the mouse heart

IκB phosphorylation is a vital transcription factor for inflammatory gene expression. Moreover, cardiac IκB over-expression not only inhibits inflammatory gene production but also prevents cardiac malfunction after LPS injection [30].
collected at 0.5 h after injection, and expressions of total IκB and phospho-IκB were assessed by Western blotting. Our results suggested that And affects cardiac IκB phosphorylation in mice, indicating that And inhibited the activation of IκB. As shown in Fig. 5A, LPS treatment in vivo induced an increase in cardiac IκB phosphorylation. Meanwhile, And significantly reversed LPS-induced phosphorylation of IκB in hearts.
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Fig. 5. Effects of And on LPS-induced IκB phosphorylation in the mouse heart. The upper row shows representative Western blot analysis of total and phospho-IκB in the mouse heart. The lower row represents image analysis of total and phospho-IκB, n = 5. *P < 0.05 compared with the control group; #P < 0.05 compared with LPS group.

Fig. 6. And inhibited LPS-induced cardiomyocyte apoptosis and cardiac caspase 3/7 activation. (A-D) represent photomicrographs of TUNEL-stained sections from the control, LPS, And + LPS, and And alone groups, respectively. (E) Quantification of TUNEL-positive cardiomyocytes at 12 h after normal saline or LPS injection. (F) Cardiac caspase 3/7 activity examined at 2 h after injection, n = 10. *P < 0.05 compared with the control group; #P < 0.05 compared with the LPS group.

And inhibits LPS-induced cardiac apoptosis and cardiac caspase 3/7 activation

LPS induced a rapid activation of apoptosis to affect myocardial malfunction [31]. To examine whether the cardio protective effects of And are related to the prevention of LPS-induced cardiac apoptosis, cardiac sections were examined by TUNEL staining for apoptosis. Fewer apoptotic cells were found in the control group (Fig. 6A), compared with the LPS
group at 12 h after LPS injection (Fig. 6B). In contrast, And significantly suppressed LPS-induced myocardial apoptosis (Fig. 6C). Moreover, caspase 3/7 activities in hearts from the LPS group were significantly elevated at 2 h after LPS injection compared with the control group, and caspase 3/7 activities in hearts at 2 h after LPS injection was lower in the And + LPS group than in the LPS group (Fig. 6D).

**Discussion**

The results of the present study have demonstrated that And reversed LPS-induced cardiac malfunction in mice by inhibiting cardiac apoptosis, cardiomyocyte IκB phosphorylation, and TNF-α, IL-1β, and NO production. Our study found that And significantly reversed LPS-induced reduction of left ventricular EF, FS, and E/A ratio values in vivo, suggesting that And attenuates cardiac systolic and diastolic malfunction in endotoxin-induced sepsis.

The mechanisms of LPS-induced cardiac malfunction remain controversial. Various inflammatory mediators, such as TNF-α and IL-1β, support a vital role in the development of myocardial malfunction in sepsis [4-6]. LPS, TNF-α and IL-1β have direct negative inotropic effects on cardiomyocytes via induction of NO synthase. And over-production of NO causes contractile malfunction via cyclic guanosine 3', 5'-monophosphate in sepsis [8, 32, 33]. Moreover, phosphorylation of IκB has been shown to lead to the expression of NF-κB-controlled genes for cytokines that are associated with cardiac malfunction in the adult mammalian heart [34].

In the study reported herein, LPS administration led to phosphorylation of IκB and over-production of TNF-α, IL-1β, and NO in hearts. And inhibited IκB phosphorylation and the production of TNF-α, IL-1β and NO in mice hearts after LPS injection. These observations indicated that And suppressed IκB phosphorylation as well as the production of TNF-α, IL-1β and NO in vivo, resulting in the reversal of cardiac malfunction in LPS-induced mice.

On the other hand, some studies have suggested that the generation of ROS, such as superoxide and peroxynitrite, plays a vital role in the induction of myocardial malfunction caused by LPS [35]. We further examined myocardial SOD activity and TBARS content in mice and found that LPS did not increase myocardial SOD activity and TBARS content at 12 h after injection, which is consistent with a previous study [36]. Moreover, And did not affect myocardial TBARS levels and SOD activities in LPS-induced mice. These results indicate that the protections of And against LPS-induced cardiac malfunction do not prevent oxidative stress. However, although myocardial SOD activity and TBARS content did not increase at 12 after LPS treatment, the possibility that oxidative stress could play a role in LPS-induced cardiac malfunction cannot be ruled out. For instance, different results could be obtained for measurements made earlier or later than 12 h after LPS injection [37].

Because it is well known that LPS-induced activation of apoptotic pathways results in cardiac malfunction [7, 38], we further evaluated whether And prevented LPS-induced cardiac apoptosis and found that And significantly inhibited cardiac caspase 3/7 activation and apoptosis induced by LPS. Further studies will be needed to determine the potential mechanisms for inhibition of LPS-induced cardiomyocyte apoptosis by And; such studies should consider the opening of the mitochondrial permeability transition pore [39], stimulation of calcium-sensing receptors [40], and activation of cannabinoid type-2 receptors as possible mechanisms[41].

In summary, our study is the first to demonstrate that And prevents LPS-induced cardiac malfunction in mice by inhibiting cardiac myocardial apoptosis and IκB phosphorylation. These findings indicate that And may be a potential agent for confronting septic cardiac malfunction.

**Disclosure Statement**

We declare that there are no conflicts of interest in this study.
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