Original Article
Shensongyangxin protects cardiomyocytes against LPS-induced injury through the NFκB pathway

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Abstract: Shensongyangxin (SSYX) is a Chinese medicine compound used to treat cardiac tachyarrhythmias. We have previously demonstrated that SSYX can protect cardiomyocytes against pressure overload-induced cardiac hypertrophy. However, whether SSYX exerts an effect on lipopolysaccharide (LPS)-induced myocardial injury remains unknown. H9c2 rat cardiomyocytes were subjected to LPS in the presence or absence of different SSYX concentrations (0.1, 10, 50, and 100 μg/mL). By CCK-8 testing, TUNEL staining, Polymerase chain reaction (PCR) and Western blot analyses showed that 0.1, 10, 50, or 100 μg/mL SSYX did not affect the cell viability. However, LPS increased the release of TNF-α, IL-1β, and IL-6 from cardiomyocytes, which were attenuated by 10 and 50 μg/mL SSYX pretreatment. Moreover, SSYX protected cardiomyocytes against mitochondrial apoptosis by inhibiting the elevation of Bax and c-caspase3 as well as the decrease of Bcl-2. LPS-induced NFκB activation was abolished by SSYX, as well as by the NFκB inhibitor, Bay117082, which also mitigated the pro-inflammatory and pro-apoptotic effects of LPS. Results indicate that the activation of NFκB may be responsible for LPS-induced cardiac injury. Therefore, SSYX conferred a protecting effect on cardiomyocytes exposed to LPS possibly by inhibiting the NFκB pathway.

Keywords: Shensongyangxin, cardiomyocytes, lipopolysaccharide, inflammation, apoptosis, NFκB

Introduction
Myocardial dysfunction is a common complication that considerably contributes to sepsis mortality in patients [1]. During sepsis, lipopolysaccharide (LPS) is recognized as an important pathogen-associated molecular pattern that is responsible for exaggerated inflammatory response [2] and apoptosis [3]. Recent, studies have focused on cardiomyocytes to address the mechanisms underlying sepsis-induced myocardial dysfunction. In cardiomyocytes, LPS stimulates the production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, which contribute to myocardial depression during sepsis [4, 5]. In addition, the activation or upregulation of multiple stress signaling cascades, such as mitogen-activated protein kinase (MAPK) and NFκB, has been speculated to perform a pivotal function in the pathogenesis of sepsis-associated cardiac contractile dysfunction [6, 7]. However, a unique and efficacious clinical management for sepsis-associated myocardial dysfunction and heart failure has been lacking. Drugs constraining LPS-induced inflammation and apoptosis have yet to be discovered.

Shensongyangxin (SSYX), which consists of 12 ingredients namely, ginseng, Radix, dogwood, Salvia, semen (fried), mistletoe, red peony, Eupolyphaga, nard, berberine, Kadsura, and keel, is a Chinese medicine compound that is conventionally used to treat cardiac tachyarrhythmias, such as tachyarrhythmias, bradycardia, paroxysmal atrial fibrillation (AF), and premature ventricular [8-10]. Our previous study revealed that SSYX can attenuate pressure overload-induced cardiac hypertrophy and fibrosis, which are involved in inhibiting the Akt and TGFβ/Smad pathways [11]. SSYX also elim-
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Materials and methods

Chemicals and reagents

LPS was purchased from Sigma (Munich, Germany). TUNEL Cell Death Detection kit (Roche Diagnostics, USA) was used to analyze cell apoptosis. Primary antibodies: Bax, Bcl-2, c-caspase-3, total (T) NFκB and IκBα, and phosphorylated (p-) NFκB and IκBα were obtained from Cell Signaling Technology (Boston, USA). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was obtained from Santa Cruz (Texas, USA). Secondary antibodies: goat anti-Rabbit IRdye@800 CW IgG and goat anti-Mouse IRdye@800 CW were obtained from LI-COR (Lincoln, USA). The Transcriptor First Strand cDNA Synthesis Kit was purchased from Roche (Basel, Switzerland). The NFκB inhibitor, Bay117082, was obtained from Merck (Darmstadt, Germany).

Cell culture

H9c2 rat cardiomyocytes were obtained from the Cell Bank of Academy of Science (Shanghai, China). Cells were cultured in standard Dulbecco’s modified Eagle’s medium, which was supplemented with 10% fetal bovine serum, 1% penicillin (100 U/ml) and streptomycin (100 mg/ml), in a CO₂ incubator with 5% CO₂ at 37°C. Cells were treated with different concentrations of SSYX and 10 µg/ml LPS for 12 h.

Cell viability assay

Cell viability was evaluated using CCK-8 assay, in accordance with the manufacturer’s instructions. Briefly, 10 µl of CCK-8 solution was added to each well of a 96-well plate, and the absorbance was measured at 450 nm by using an ELISA reader (Synergy HT, Bio-tek, Vermont, USA) after 4 h incubation. The effect of SSYX on cell viability was expressed as the percentage cell viability compared with that of the vehicle group, which was set at 100%.

TUNEL staining

Cells were grown on cover slips in a 24-well plate, fixed in 4% paraformaldehyde and then permeabilized in 0.1% Triton X-100 after treatment. Cells were incubated in TUNEL reaction mixture for 1 h at 37°C. Nuclei were labeled with 4’, 6 diamidino-2-phenylindole (DAPI) and DNA fragmentation was quantified under high-power magnification (×200). The percentages of TUNEL-positive cells relative to DAPI-positive cells were calculated by an investigator in a blinded manner.

Western blot

Cells in six-well plates were harvested and lysed in RIPA lysis buffer. Protein concentration was measured using a BCA protein assay kit by an ELISA reader (Synergy HT, Bio-tek, Vermont, USA). Extracted protein (50 µg) from each sample was separated on 8% to 12% SDS-PAGE gels, and the proteins were then transferred onto polyvinylidene difluoride membranes. These membranes were blocked with 5% non-fat milk powder and then incubated with primary antibodies overnight at 4°C. The membranes were subsequently incubated with secondary antibodies. Western blots were scanned and analyzed using a two-color infrared imaging system (Odyssey, LI-COR, USA). Specific protein

Figure 1. Effect of SSYX on cell viability, obtained using a Cell Counting kit-8 assay. Treatment with the indicated concentrations of SSYX (0.1, 10, 50, and 100 µg/ml) with LPS (10 µg/ml) for 12 h. *P<0.05 compared with the PBS group, #P<0.05 compared with the LPS group. Data are expressed as mean ± standard error of the mean. LPS, lipopolysaccharide; SSYX, Shensongyangxin.

inates angiotensin II-induced myocardial hypertrophy. These findings indicate that SSYX can protect the cardiovascular system. However, little is known about the effect of SSYX on LPS-induced cardiomyocyte injury. In current study, H9c2 rat cardiomyocytes were subjected to LPS in the presence or absence of SSYX. The results showed SSYX can attenuate the release of inflammatory cytokines and reduce apoptosis. The protective effect of SSYX on LPS-induced cardiomyocyte injury is associated with the inhibition of the NFκB pathway.
expression levels were normalized against the expression of GAPDH.

**Quantitative real-time RT-PCR**

Cells in six-well plates were harvested, and total RNA was extracted using TRIZol. Yields and purities were spectrophotometrically estimated with A260/A280 and A230/A260 ratios by using a SmartSpec Plus spectrophotometer (Bio-Rad, California, USA). RNA (2 mg of each sample) was reverse-transcribed into cDNA by using oligo (DT) primers and the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). PCR amplifications were quantified by a LightCycler 480 SYBR Green 1 Master Mix (Roche, Switzerland). The results were normalized against GAPDH gene expression.

**Statistical analysis**

Data were presented as mean ± standard error on the mean (SEM). Statistical calculations were performed in SPSS13.0 software package. Analysis of Variance (ANOVA) was used. *P* values of less than 0.05 were considered significant.

**Results**

**Effect of SSYX on LPS-induced H9c2 cardiomyocyte cytotoxicity**

The effects of SSYX on the LPS-induced cytotoxicity in H9c2 cells are shown in Figure 1. LPS markedly decreased the cell viability. Pretreatment with 0.1 and 10 μg/mL SSYX can not prevent cell injury induced by LPS, whereas pretreatment with 50 and 100 μg/mL SSYX significantly inhibited LPS-induced cytotoxicity in H9c2 cells. Thus, 50 and 100 μg/mL SSYX were used to further study the effects of SSYX on LPS-induced cardiac injury.

**Effects of SSYX on LPS-induced release of TNF-α, IL-1β, and IL-6 from H9c2 cardiomyocytes**

The effects of SSYX on the induction of TNF-α, IL-1β and IL-6 in response to LPS were measured by RT-PCR. LPS significantly stimulated the release of TNF-α, IL-1β and IL-6. As shown in Figure 2, SSYX treatment significantly attenuated this increase in a concentration-dependent manner.

**Effects of SSYX on apoptosis among LPS-stimulated cardiomyocytes**

TUNEL staining was used to identify apoptotic nuclei. Only 2.68% ± 0.27% TUNEL-positive nuclei were detected in control cells at the end of the experiment, and LPS significantly increased the percentage of apoptotic cells to 13.11% ± 0.35%. SSYX treatment at 50 and 100 μg/mL reduced the percentage of TUNEL-positive cells to 3.47%, and 3.42%, respectively (Figure 3A). In addition, both 50 and 100 μg/mL SSYX decreased the protein expression of Bax and c-caspase-3, but increased that of Bcl-2 in H9c2 cells after LPS stimulation (Figure 3B).

**SSYX reduced NF-κB activation in response to LPS**

The mechanisms underlying the anti-inflammatory and anti-apoptotic effects of SSYX on LPS-treated H9c2 cells were investigated via Western blot analysis. The results showed that LPS increased the phosphorylation and degradation of IκB and subsequently increased the
phosphorylation level and nuclear translocation of NFkB-p65. In comparison, 50 and 100 μg/mL SSYX decreased the degradation of IκB and subsequently decreased the phosphorylation level and nuclear translocation of NFkB-p65, as confirmed by immunofluorescence staining (Figure 4A, 4B).

The NFkB inhibitor Bay117082 (10 μM) was used to further confirm that NFkB was the specific signal transduction pathway involved in the anti-inflammatory and anti-apoptotic roles of SSYX. Cardiomyocytes incubated with LPS were then treated with SSYX (100 μg/mL) in the presence of Bay117082 (10 μM). LPS-induced an increase in apoptosis, whereas the elevated mRNA expression levels of TNF-α, IL-1β and IL-6 were nearly abolished by Bay117082. However, combination of SSYX and Bay117082 cannot further decrease the TUNEL-positive cells, as well as the release of TNF-α, IL-1β, and IL-6 (Figure 4C, 4D). These data suggest that SSYX suppressed LPS-induced inflammation and apoptosis depending on the NFkB pathway.
Cardiac dysfunction is common in patients with severe sepsis, and act as an important predictor of mortality. However, effective drugs to cure the dysfunction in this condition are lacking [12, 13]. SSYX is a widely used traditional Chinese medicine, and has been widely administered...
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istered in patients with cardiovascular diseases [14, 15]. However, the effects of SSYX on LPS-induced myocardial dysfunction have yet to be fully defined. To our knowledge, our study is the first to observe that SSYX significantly attenuated LPS-induced inflammatory and apoptotic responses in H9c2 cardiomyocytes. Furthermore, the cardioprotective effects of SSYX were closely associated with apoptosis and NFκB signaling pathways.

LPS is a major structural component of Gram-negative bacteria and a key mediator of the body's response to infection, which is responsible for multi-organ dysfunction characterizing septic shock, and of which myocardial dysfunction is a recognized manifestation [3, 6, 13]. In the present study, we first investigated the protective effects of SSYX against LPS-induced cardiotoxicity by CCK8 assay. The results showed that cells treated with LPS demonstrated loss of cell viability. However, pretreatment with a certain concentration of SSYX markedly decreased the loss of cell viability. These findings suggest that SSYX significantly protected H9c2 cells against LPS-induced cytotoxicity.

Endotoxin-induced inflammatory response and cardiomyocyte apoptosis perform a considerable function in the pathogenesis of multiple cardiovascular diseases, thereby significantly increasing the mortality rate in patients with sepsis [16, 17]. LPS stimulation has been widely demonstrated to result in myocardial inflammation injury. Experimental evidence indicates that LPS can induce inflammatory factors, such as TNF-α, IL-6, and IL-1β, which further cause cardiomyocyte apoptosis and heart failure [18-20]. In our study, we confirmed that LPS decreases cardiac function and increases the release of TNF-α, IL-6, and IL-1β in H9c2 cells. Moreover, our results demonstrate that SSYX inhibited the LPS-induced release of TNF-α, IL-6, and IL-1β in H9c2 cardiomyocytes, suggesting that the cardioprotective effects of SSYX are associated with anti-inflammatory properties.

Apoptosis has been shown to perform an important function in the pathogenesis of cardiac dysfunction, and cardiac injury, as well as in the pathological changes in cardiovascular diseases [21, 22]. Consistent with previous reports [22, 23], the current experimental findings indicate that LPS significantly elevated apoptosis in H9c2 cells. To examine whether SSYX affected myocardial apoptosis induced by LPS stimulation, we investigated the expression of apoptosis-related proteins and detected the apoptosis-positive cells. Caspase-3 is a key mediator of apoptosis, and its activation leads to DNA injury and apoptotic cell death. The expression of Bax, which functions as a pro-apoptotic protein, increased in response to LPS stimulation, whereas the expression of the anti-apoptotic protein Bcl-2 decreased [22-24]. As expected, SSYX markedly decreased the number of apoptotic cells in response to LPS stimulation in H9c2 cells. Furthermore, the attenuation of myocardial apoptosis by pretreatment with SSYX was associated with the increased expression of Bcl-2 and the decreased expression of Bax and caspase-3. These findings suggest that SSYX attenuated the development of LPS-induced cardiomyocyte apoptosis. Therefore, SSYX may be beneficial to patients with certain heart diseases.

To date, the possible mechanisms by which SSYX attenuated the LPS-induced inflammatory response and apoptosis in H9c2 cells remain unclear. As has been extensively documented, the transcriptional activation of NFκB-responsive genes in the LPS-induced signaling cascade is considered to be a crucial step [25]. The inactive form of NFκB is sequestered in the cytoplasm, where the protein is bound by the IκB family proteins, including IκBα. Once NFκB is activated by a stimulus, IκBα is phosphorylated by IKK and then degraded, resulting in the translocation of NFκB subunits from the cytoplasm to the nucleus [26, 27]. Clinically, the NFκB binding activity increased in patients with acute inflammation and septic cardiac dysfunction and is correlated with clinical severity and mortality [28]. Thus, we examined the NFκB and IκBα activities, as well as the nuclear translocation of NFκB. LPS increased both NFκB phosphorylation and nuclear translocation, but these processes were significantly attenuated by pretreatment with SSYX. These findings suggest that SSYX protected H9c2 cells against LPS stimulation by inhibiting the NFκB signaling pathway. However, further experiments are needed to determine the molecular mechanisms by which SSYX regulates the NFκB signaling pathway.

In conclusion, this investigation is the first to define a function of SSYX for LPS-induced
inflammation and apoptosis in H9c2 cardiomyocytes. Moreover, the mechanism underlying the protective role of SSYX in H9c2 cardiomyocytes from LPS stimulation appears to be related to the apoptosis and NFκB signaling pathways. Overall, these findings provide further understanding about the pharmacological effects of SSYX and the pathways imparting its protective effects. SSYX may hold future clinical promise in the development of novel therapeutic strategies to treat inflammatory injury and apoptosis in cardiovascular diseases.

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Disclosure of conflict of interest

None.

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