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ORIGINAL ARTICLE

S-Propargyl-cysteine (SPRC) attenuated lipopolysaccharide-induced inflammatory response in H9c2 cells involved in a hydrogen sulfide-dependent mechanism

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Abstract The present study attempts to investigate the effects of *S*-propargyl-cysteine (SPRC), a sulfur-containing amino acid, on lipopolysaccharide (LPS)-induced inflammatory response in H9c2 cardiac myocytes. We found that SPRC prevented nuclear factor-κB (NF-κB) activation assessed by NF-κB p65 phosphorylation and IκBα degradation, suppressed LPS-induced extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation and intracellular reactive oxygen species (ROS) production. Furthermore, incubation of H9c2 cells with SPRC induced phosphorylation of Akt in a time- and concentration-dependent manner. In addition, SPRC attenuated LPS-induced mRNA and protein expression of tumor necrosis factor-α (TNF-α), and mRNA expression of intercellular adhesion molecule-1 (ICAM-1) and inducible nitric oxide synthase (iNOS). The effects of SPRC were abolished by cystathionine γ-lyase [CSE-an enzyme that synthesizes hydrogen sulfide (H_2S)] inhibitor, DL-propargylglycine (PAG), SPRC-induced Akt phosphorylation and TNF-α release was also abolished by the phosphoinositide 3-kinase (PI3K) inhibitor LY294002. Furthermore, SPRC also increased LPS-induced down-regulation expression of CSE and H_2S level in H9c2 cells. PAG abolished SPRC-induced up-regulation of H_2S level. Therefore, we concluded that SPRC produced an anti-inflammatory effect in LPS-stimulated H9c2 cells partly through the CSE/H_2S pathway by impairing IκBα/NF-κB signaling and by activating PI3K/Akt signaling pathway.

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Introduction

Sepsis causes cardiac contractile dysfunction, which is a major cause of morbidity and mortality in septic patients (Parrillo et al. 1985). The production of pro-inflammatory cytokines induced by bacterial endotoxin lipopolysaccharide (LPS) contributes to myocardial dysfunction during sepsis (Hickson-Bick et al. 2008). However, the underlying mechanisms remain not fully understood; increasing evidences suggest that LPS-induced myocardial dysfunction is mediated by multiple pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α) (Ceylan-Isik et al. 2010; Zhu et al. 2010a, b), intercellular adhesion molecule-1 (ICAM-1) (Davani et al. 2006; Boyd et al. 2008), inducible nitric oxide synthase (iNOS) (Ceylan-Isik et al. 2010), etc. It had been shown that reducing systemic inflammatory response could improve myocardial function (Takeuchi et al. 2000).

Activation of multiple stresses signaling processes such as oxidative stress and mitogen-activated protein kinases (MAPKs) play pivotal roles in the pathogenesis of septic cardiac dysfunction (Yuan et al. 2009). Reactive oxygen species (ROS) are now recognized as important signaling molecules and able to modulate various genes' transcription via activation of MAPKs (Yuan et al. 2009; Zhao et al. 2009; Ceylan-Isik et al. 2010) and nuclear factor-κB (NF-κB) (Peng et al. 2005). NF-κB activities are inhibited by inhibitory proteins IκBs and present as an inactive IκBs/NF-κB complex in the cytoplasm; upon stimulation, this complex is broken down and results in activation of

NF-κB, which translocates into the nucleus and mediates target genes expression (Carayol et al. 2006). Various reports have shown that activation of PI3K-Akt signaling negatively regulates LPS-induced inflammatory response (Guha and Mackman 2002; Schabbauer et al. 2004). In the present study, we investigate this pathway further.

Dietary garlic (*Allium sativum*) has been recognized for its beneficial health effects on cardiovascular diseases mediated by its hydrogen sulfide (H_2S) releasing compounds, including *S*-allylcysteine (SAC) (Benavides et al. 2007; Chuah et al. 2007; Rose et al. 2005). Likewise, we had demonstrated that *S*-propargyl-cysteine (SPRC), a new sulfur-containing amino acid and derivative from SAC, exerts cardioprotective and anti-inflammatory effects through modulating endogenous H_2S level (Gong et al. 2011b; Wang et al. 2009, 2010). H_2S has long been considered as an environmental pollutant but now is regarded as the third endogenous signaling gasotransmitter (Wang 2002). H_2S production attributes to three key enzymes in the biosynthesis pathway, cystathionine γ-lyase (CSE) (Zhao et al. 2001) and cystathionine β-synthetase (Li et al. 2009a, b) and the newest one 3-mercaptopyruvate sulfurtransferase (Shibuya et al. 2009), while CSE is the main H_2S-forming enzyme in cardiovascular system (Zhao et al. 2001; Wang 2003). It exerts multiple physiological and pathophysiological roles in the cardiovascular diseases (Pearson et al. 2006; Sun et al. 2008; Wang 2002, 2009; Zhao et al. 2001). Recent studies reported that H_2S is indeed an important molecule in the host's defense against oxidative stress in cardiovascular system (Geng et al. 2004; Yan et al. 2006). More recently, H_2S has also been recognized to exhibit important anti-inflammatory functions (Zhu et al. 2010a, b; Szabó 2007). However, some other reports demonstrated that H_2S aggravates inflammatory response (Bhatia et al. 2005). The role of H_2S in inflammation is still controversial or may be double-edged. Therefore, the purpose of the present study was to determine a potential role of SPRC–H_2S in preventing LPS-induced inflammatory processes in H9c2 cells and its possible mechanisms.

Materials and methods

Materials

2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Molecular probes (Eugene, OR, USA). Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco (Gibco-BRL, Paisley, UK). Lipopolysaccharide (LPS, *E. coli* 0127:B8), 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002, a specific PI3K inhibitor), and DL-propargylglycine (PAG, a CSE inhibitor) were purchased from Sigma-Aldrich. SPRC (also named it as ZYZ-802) was synthesized from the reaction of L-cysteine with propargyl bromide and purified by re-crystallization from an ethanol–water mixture (96.1%) as described previously (Wang et al. 2009). The final product was verified by ^{1}H nuclear magnetic resonance spectroscopy, purity was over 99% determined by high performance liquid chromatography.

Cell culture studies

H9c2 rat embryonic ventricular myocardial cells (ATCC, Manassas, VA) were maintained in DMEM containing 1,500 mg/L $NaHCO_3$, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

For experiments, cells were grown to confluence in six-well plates, 60 mm dishes, or 100 mm dishes (Costar, Cambridge, MA). Cells were serum starved for 3 h. To assess the effect of LPS or SPRC on Akt or ERK1/2 phosphorylation, cells were incubated with 10 μg/mL LPS or 1 μmol/L SPRC for different periods (15, 30, 45, 60, and 120 min). To assess the effects of LPS on inflammatory genes expression, cells were incubated with LPS (10 μg/mL) for different periods (1, 2, 4, 6, and 8 h). In some experiments, cells were preincubated with or without PAG (2 mmol/L) or LY294002 (10 μmol/L) for 30 min. Subsequently, incubated 1–0.01 μmol/L SPRC for another 30 min before co-incubated with LPS (10 μg/mL) for various periods: 6 h for measurement of TNF-α, H_2S production, and CSE expression; 2 h for measurement of mRNA levels and intracellular ROS generation (1 μmol/L SPRC); and 30 min for measurement of Akt, ERK1/2 and NF-κB p65 phosphorylation, and IκBα protein.

TNF-α level analysis by enzyme-linked immunosorbent assay (ELISA)

The level of TNF-α in the supernatants was measured using a commercially available kit (R&D systems, Minneapolis, MN) according to the manufacture's instructions. Final results were expressed as pg of cytokine/mL media.

Detection of mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from H9c2 cells with TRIzol Reagent (TakaRa, TaKaRa Biotechnology, Dalian, China) following the manufacturer's instructions. Total RNA (2 μg) of each sample was reverse-transcribed into first-strand cDNA and amplified using a PrimeScriptP^{TM} 1st Strand cDNA Synthesis Kit (TakaRa) according to the

manufacturer's directions. PCR conditions include denaturation (94°C for 30 s); annealing (56°C for 45 s); and extension (72°C for 30 s); number of cycles (30). PCR products were separated by 1.2% TAE-agarose gel electrophoresis and densitometry analysis was performed using Alpha Imager (Alpha Innotech Corp, San Leandro, CA). The primers used in this experiment are indicated in Table 1.

Assay for ROS

Intracellular ROS was determined spectrofluorometrically by the oxidation of the specific probe H_2DCFDA to 2′,7′-7-dichlorofluorescein (DCF) as described previously (Wang and Joseph 1999). Briefly, cells were plated on dishes or 24-well plates were pretreated with SPRC, then incubated with H_2DCFDA (10 μmol/L) for 30 min at 37°C. Then stimulated with LPS for 2 h, and the fluorescence intensity was measured at an excitation and emission wavelength of 485 and 530 nm, respectively, using a fluorescence microscope (Carl Zeiss) or a fluorescence spectrophotometer (M1000, TECAN, Austria GmbH, Austria).

Western blot analysis

Cells were washed twice with ice-cold PBS and lysed in lysis buffer (Tris–HCl 10 mmol/L, NP-40 0.5%, NaCl 0.15 mol/L, Na_3VO_4 1 mmol/L, NaF 10 mmol/L, phenylmethylsulfonyl fluoride 1 mmol/L, EDTA 1 mmol/L, aprotinin 10 μg/mL, leupeptin 10 μg/mL, and pepstatin 1 μg/mL) for 30 min at 4°C. Protein concentration was determined with a BCA protein assay kit (Beyotime, Haimen, China). Equal amounts (30 μg) of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membrane (Millipore). After being blocked with 5% nonfat dry milk, membranes were incubated overnight at 4°C with: polyclonal rabbit anti-Akt, polyclonal rabbit anti- phosphorylated (p)-Akt (Ser^{473}), polyclonal rabbit anti-ERK1/2, polyclonal rabbit anti-p-ERK1/2 (Thr^{202}/Tyr^{204}), polyclonal rabbit anti-p65, polyclonal rabbit anti-p-p65 (Ser^{536}), polyclonal rabbit anti-IκBα (Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit anti-GAPDH, or polyclonal rabbit anti- CSE (Santa Cruz Biotechnology) antibodies. HRP-conjugated goat anti-rabbit IgG (ICL Lab, Newberg, OR, USA) was used as a secondary antibody. The reactions were developed with SuperSignal West Duro chemiluminescent substrate (Pierce, Inc) and signal intensity was detected by Alpha Imager (Alpha Innotech Corp, San Leandro, CA) and quantified by Quantity One software (Bio-Rad, Hercules, CA, USA).

Table 1 Primer sequences used in the present study

Genes	Sequences
GAPDH	
Sense	5′-TCAACGGCACAGTCAAGG-3′
Antisense	5′-AGCATCAAAGGTGGAAGAAT-3′
TNF-α	
Sense	5′-CCGATTTGCCATTTCATACC-3′
Antisense	5′-AAGTAGACCTGCCCGGACTC-3′
ICAM-1	
Sense	5′-AAACGGGAGATGAATGGT-3′
Antisense	5′-CTGAGCCTTCTGTAACTTGTAT-3′
iNOS	
Sense	5′-AGGGAGTGTTGTTCCAGGTG-3′
Antisense	5′-TCCTCAACCTGCTCCTCACT-3′

H_2S concentration assay

The concentration of H_2S in supernatant was determined as described previously (Wang et al. 2009). Briefly, 500 μL of media was added into a 1.5 mL microtube containing 0.25 mL of 1% zinc acetate to trap H_2S. Next, *N*,*N*-dimethyl-p-phenylenediamine dihydrochloride (20 μmol/L, 133 μL) in 7.2 M HCl and $FeCl_3$ (30 μmol/L, 133 μL) in 1.2 M HCl were added. Following, trichloroacetic acid (10% W/V, 250 μL) was added to precipitate the proteins present and centrifuged at 24,000*g* for 5 min. Absorbance (670 nm) of aliquots from the supernatant was determined using a 96-well microplate reader (TTECAN, Austria GmbH, AustriaT).

Statistical analysis

Data are presented as the mean ± SEM obtained from triplicate experiments. Differences between mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test performed using SPSS 12.0. Statistical significance was considered at P less than 0.05.

Results

SPRC inhibited LPS-induced TNF-α mRNA and protein expression

Incubation of H9c2 cells with LPS induced inflammatory genes expression in a time-dependent manner, as shown in Fig. 1a. Interestingly, LPS elicited peak levels of mRNA of inflammatory factors almost observed after 2 h of incubation (Fig. 1a). Based on this time course response to LPS, 2 h was chosen for subsequent experiments. The increase TNF-α mRNA level response to LPS was attenuated by

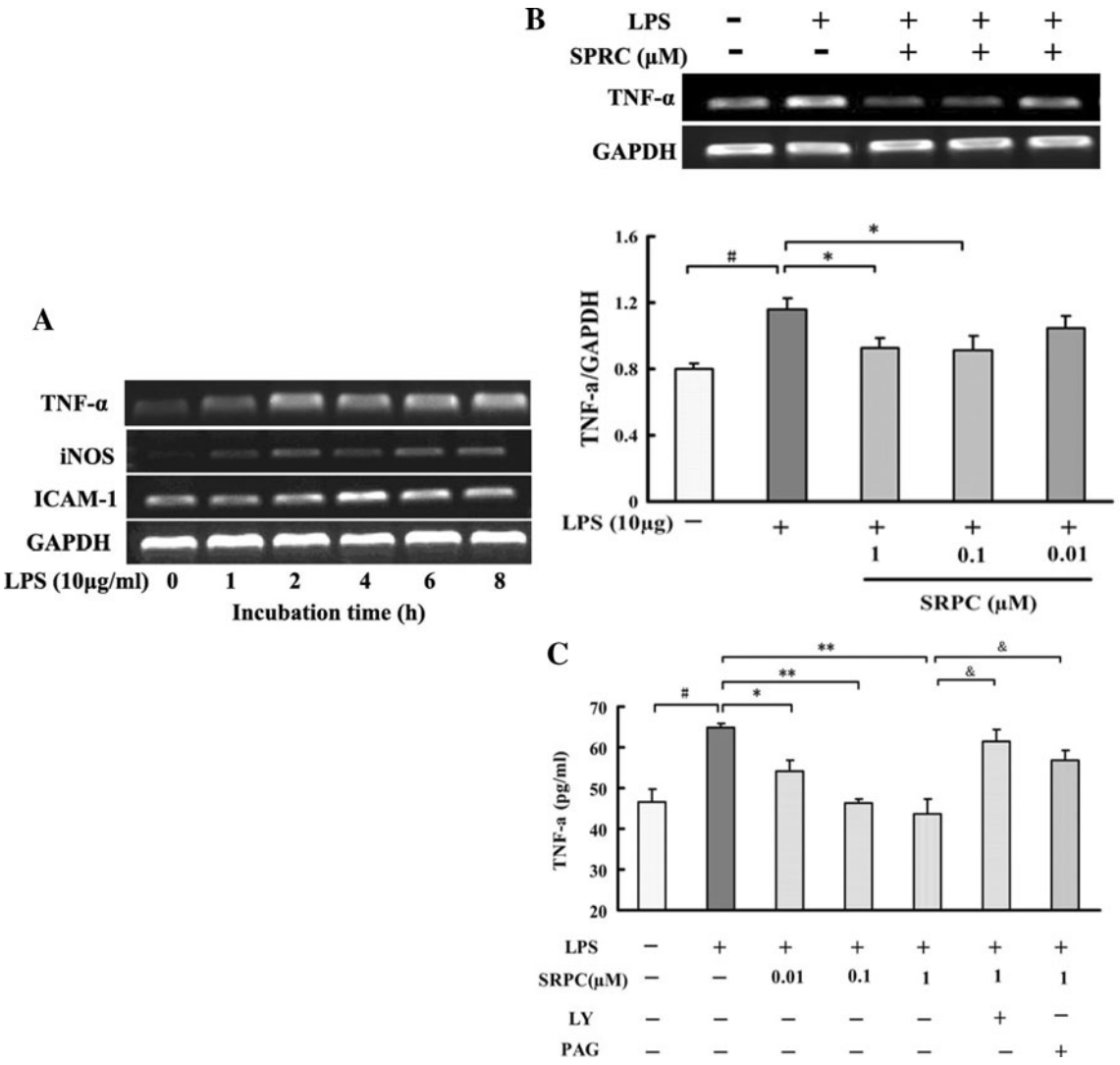

Fig. 1 SPRC attenuated LPS-induced mRNA and protein expression of TNF-α. **a** Cells were incubated with 10 μg/mL LPS for indicated periods, inflammation-related genes mRNA was measured by RT-PCR. **b** TNF-α mRNA was analyzed by RT-PCR, GAPDH as the internal standard control. Data represent mean ± SEM of three independent experiments. $^{\#}P < 0.05$ compared with non-treated control; $^{*}P < 0.05$ compared with LPS-treated control. **c** Cells were incubated with 10 μmol/L LY294002 (LY) or 2 mmol/L DL-propargylglycine (PAG) for 30 min before adding SPRC, then stimulated with LPS for 6 h, the TNF-α was measured by ELISA. Data represent mean ± SEM of three independent experiments. $^{\#}P < 0.05$ compared with non-treated control TT; $^{*}P < 0.05$ compared with LPS-treated control; $^{\&}P < 0.05$ compared with LPS plus LY or PAG

SPRC (Fig. 1b). Consistent with the effect of SPRC on mRNA expression of TNF-α, level of TNF-α was elevated in culture media at 6 h after LPS-stimulated cells compared with untreated control cells. SPRC significantly attenuated LPS-induced TNF-α production in a concentration-dependent manner (Fig. 1c). But the effect of SPRC was blocked by the LY294002 or PAG.

SPRC inhibited LPS-induced inflammatory genes expression in H9c2 cells

As shown in Fig. 2, after 2 h stimulation by LPS, ICAM-1 and iNOS mRNA levels were significantly greater than in control. SPRC (1 μmol/L) treatment also significantly inhibited LPS-induced ICAM-1 and iNOS mRNA expression ($P < 0.05$) (Fig. 2a, b).

SPRC activated PI3K/Akt signaling pathway in H9c2 cells

We investigated whether SPRC could activate the PI3K/Akt pathway and affect LPS-induced Akt phosphorylation. Incubation of H9c2 cells with 1 μmol/L SPRC induced phosphorylation of Akt in a time-dependent manner. Interestingly, SPRC elicited a biphasic response, with peak level of p-Akt observed after 30 min of incubation

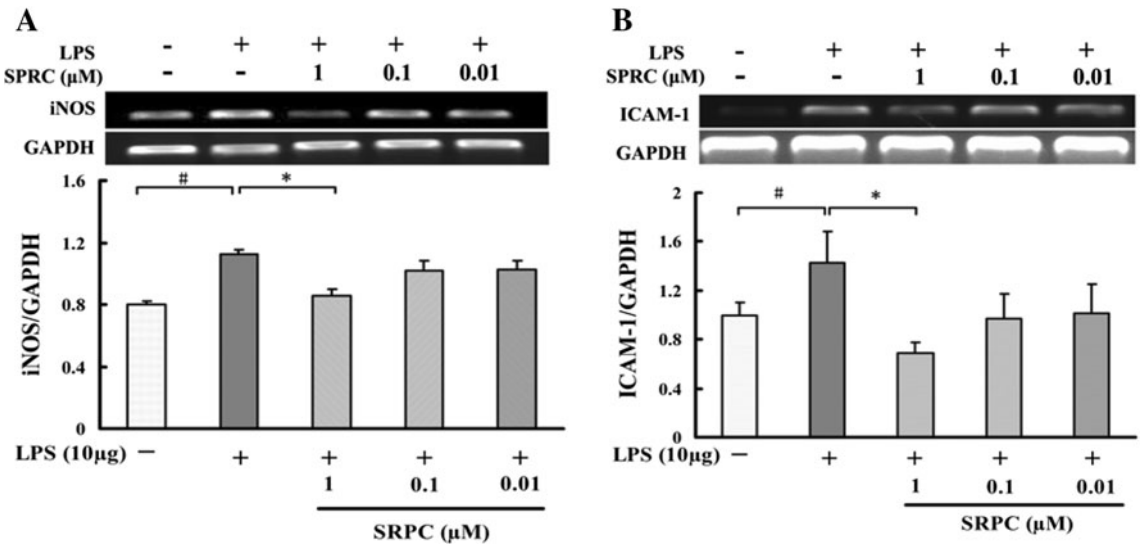

Fig. 2 SPRC inhibited LPS-induced iNOS and ICAM-1 mRNA production in H9c2 cells. Cells were incubated with indicated concentrations of SPRC for 30 min, then stimulated with 10 μg/mL LPS for 2 h, iNOS (**a**) and ICAM-1 (**b**) mRNA were analyzed by RT-PCR, GAPDH as the internal standard control. Data represent mean ± SEM of three independent experiments. $^{\#}P < 0.05$ compared with non-treated control; $^{*}P < 0.05$ compared with LPS-treated control

($P < 0.05$), followed by a drop at 45 min and then a steady increase up to 120 min ($P < 0.05$) (Fig. 3a).

Incubation with LPS elicited a peak level of p-Akt at 30 min, followed by dropping to normal level (Fig. 3b). But pretreatment with SPRC for 30 min before LPS stimulation, Akt phosphorylation was elevated initially and further increased steadily during the 30 min (Fig. 3b). The level of p-Akt was always significantly higher in the SPRC-treated cells, compared with LPS-treated only ($P < 0.05$). Pre-incubation with LY294002 abolished the phosphorylation of Akt independent of the presence of SPRC (Fig. 3b). Furthermore, SPRC (1 μmol/L) significantly induced the phosphorylation of Akt compared with LPS-treated control cells ($P < 0.05$). Importantly, SPRC-induced Akt phosphorylation was blocked by the PI3 K inhibitor LY294002 or CSE inhibitor PAG (Fig. 3c). These data strongly suggested that SPRC caused Akt phosphorylation involvement of activation of the PI3 K pathway and H_2S.

SPRC inhibited LPS-induced ERK1/2 phosphorylation in H9c2 cells

It is interesting to know whether SPRC could affect LPS-induced ERK1/2 phosphorylation. We found that LPS increased ERK1/2 phosphorylation in a time-dependant manner, with peak levels of p-ERK1/2 observed after 45 min of incubation ($P < 0.05$), and followed by a drop at 60–120 min (Fig. 4a). Hence, subsequent experiment was followed after LPS-stimulation for 30 min. Consistent with this hypothesis, pretreatment with SPRC for 30 min before LPS stimulation, SPRC inhibited LPS-induced ERK1/2 phosphorylation in a concentration-dependent manner (Fig. 4b). Furthermore, the inhibitory action of SPRC on ERK1/2 phosphorylation was blocked using a CSE inhibitor PAG (Fig. 4b). These results strongly suggested that SPRC inhibited LPS-induced ERK1/2 phosphorylation.

SPRC inhibited LPS-induced NF-κB p65 phosphorylation and IκBα degradation in H9c2 cells

Previous studies had shown that LPS activated the nuclear translocation of NF-κB in cardiac myocytes and other cell types via phosphorylation at p65 Ser^{536} (Madrid et al. 2001; Hall et al. 2005). To determine whether SPRC affects LPS-induced NF-κB p65 phosphorylation and IκBα degradation, immunoblotting studies were performed. Results showed that stimulation of the cells with LPS caused rapid degradation of IκBα. Pretreatment with SPRC concentration-dependently inhibited IκBα degradation (Fig. 4c). Using an antibody specific for Ser^{536}-phosphorylated p65 of NF-κB, the phosphorylation of p65 was evident after LPS exposure for 30 min. Consistent with the effects of SPRC on LPS-induced IκBα degradation, SPRC also inhibited LPS-induced phosphorylation of NF-κB p65 in a concentration-dependent manner (Fig. 4d). However, the effect of SPRC was abolished by pretreatment with PAG (Fig. 4c, d). Our data, therefore, strongly suggested that SPRC inhibited both LPS-induced phosphorylation of p65 and degradation of IκBα in H9c2 cells, thereby preventing NF-κB activation and NF-κB-mediated inflammatory genes expression.

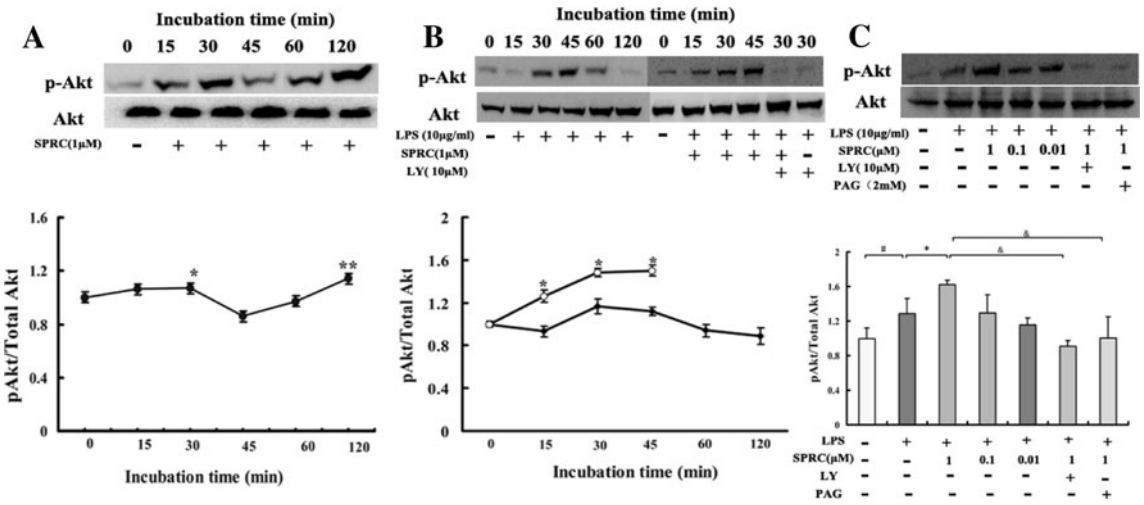

Fig. 3 SPRC induced Akt phosphorylation in LPS-stimulated H9c2 cells. **a** Cells were incubated with 1 μmol/L SPRC for indicated times. The densitometric analysis gave the related ratio of p-Akt/Akt. Data represent mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.05$ compared with non-treated control. **b** Cells were incubated with and without 1 μmol/L SPRC (*open circles*) before addition of 10 μg LPS (*filled circles*) for a period of indicated times. The densitometric analysis gave the related ratio of p-Akt/Akt. Data represent mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared with LPS-treated control. **c** Cells were incubated with 10 μmol/L LY294002 or 2 mmol/L PAG for 30 min before adding indicated concentrations of SPRC for 30 min; subsequently, stimulated with LPS for another 30 min. The densitometric analysis gave the related ratio of p-Akt/Akt. Data represent mean ± SEM of three independent experiments. $^{\#}P < 0.05$ compared with non-treated control; $^{*}P < 0.05$ compared with LPS-treated control; $^{\&}P < 0.05$ compared with LPS plus PAG or LY

SPRC decreased LPS-induced intracellular ROS production

The level of intracellular ROS was significantly increased in cells stimulated with LPS as compared to control, indicated by the increase on DCF fluorescence. However, the increase in DCF fluorescence was significantly decreased by SPRC pretreatment (1 μmol/L) (Fig. 5). These results suggested that SPRC was capable of reducing intracellular ROS accumulation following LPS stimulation. It was likely that the ability of SPRC to inhibit intracellular ROS production contributed to its effective protection of cardiomyocytes against LPS-induced inflammatory response.

SPRC attenuated LPS-induced down-regulation CSE expression and H_2S production

To verify whether SPRC modulates CSE expression and H_2S level in H9c2 cells induced by LPS. The results of western blot analysis showed that stimulation of H9c2 cells with LPS resulted in markedly reducing CSE expression. Interestingly, we observed that SPRC ameliorated LPS-induced down-regulation of CSE expression (Fig. 6a). Meanwhile, SPRC also up-regulated H_2S level in culture media induced by LPS (Fig. 6b). But the effects of SPRC were blocked by PAG.

Discussion

In this study, we determined a mechanism by which a new sulfur-containing amino acid SPRC derived from SAC attenuated LPS-induced inflammatory events in H9c2 cells, in part, by modulating the production of endogenous H_2S, an important mediator in the pathophysiology of cardiovascular diseases (Wang 2002). The results are consistent with previous study that SPRC attenuated TNF-α release and NF-κB activation in LPS- or beta-amyloid-treated rats (Gong et al. 2011a, b). The cytoprotective effects offered by SPRC may be mediated by alleviation of ROS accumulation, ERK1/2 signaling cascade, and NF-κB activation. The beneficial myocardial effect of SPRC was also associated with activation of PI3 K/Akt signaling pathway.

On the basis of previous literature and the current results, it is apparent that the inflammatory responses induced by LPS in cardiomyocytes include the initial induction of ROS, which leads to the activation of intracellular signaling pathways (e.g., MAPKs) and transcription factors (e.g., NF-κB) and to the induction of inflammatory mediators, including TNF-α, ICAM-1, and iNOS. All of these mediators that may be involved in the depression of cardiac function (Haudek et al. 2001; Davani et al. 2004; Ceylan-Isik et al. 2010). We examined the release of TNF-α from H9c2 cells after LPS stimulation; furthermore, we provided additional data showing that LPS stimulated the mRNA

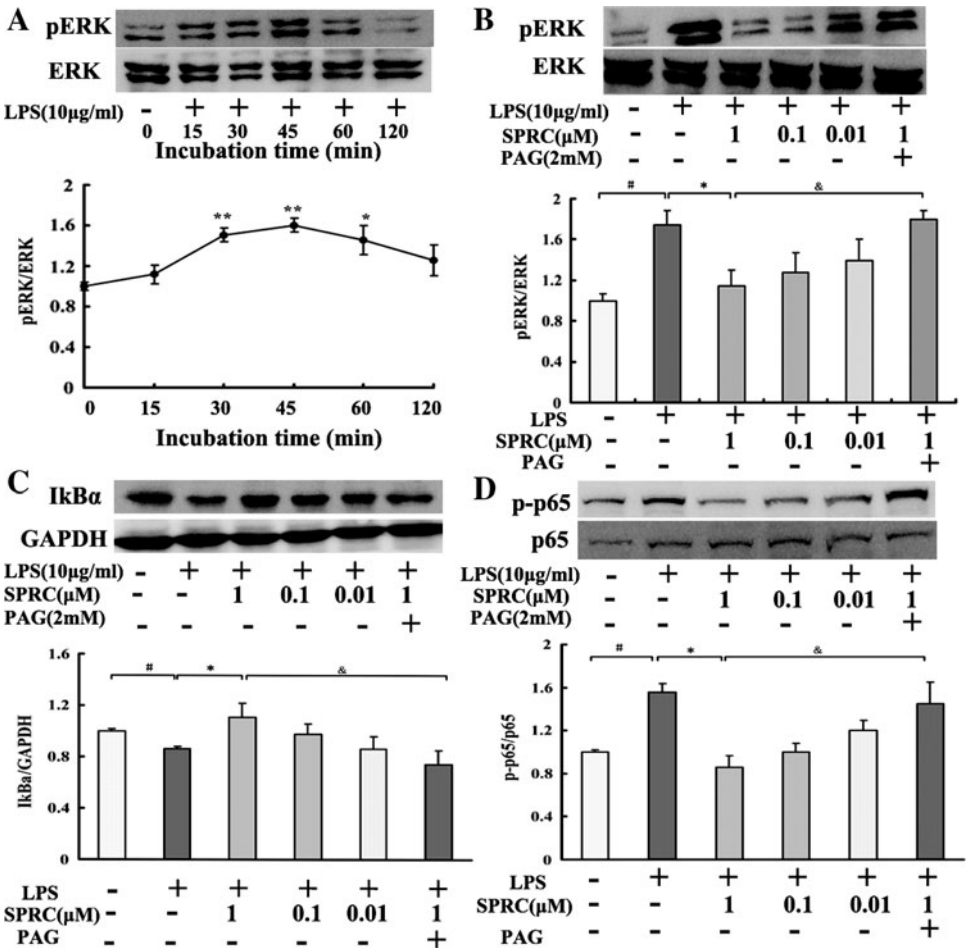

Fig. 4 SPRC inhibited LPS-evoked ERK1/2 phosphorylation, IκBα degradation and p65 phosphorylation in H9c2 cells. **a** Cells were incubated with 10 μg/mL LPS for indicated times. The total and phosphorylation levels of ERK1/2 were measured by western blot. Each assay was done in triplicate. **b** Cells incubated without or with 2 mmol/L PAG for 30 min followed by indicated concentration of SPRC for 30 min. Thereafter, cells were then incubated for 30 min with 10 μg/mL LPS. The densitometric analysis gave the related ratio of p-ERK1/2/ERK1/2. **c** Cells were incubated without or with 2 mmol/L PAG for 30 min followed by indicated concentration of SPRC for 30 min before incubation with 10 μg/mL LPS for 30 min. IκBα level was analyzed by western blot. **d** Cells were incubated without or with 2 mmol/L PAG for 30 min followed by indicated concentration of SPRC for 30 min before incubated with 10 μg/mL LPS for 30 min. The densitometric analysis gave the related ratio of p-p65/p65. Data represent mean ± SEM of three independent experiments. $^{\#}P < 0.05$ compared with non-treated control; $^{*}P < 0.05$ compared with LPS-treated control; $^{\&}P < 0.05$ compared with LPS plus PAG

expression of TNF-α, ICAM-1 and iNOS in H9c2 cells. Several studies suggest a beneficial effect for cardiac dysfunction through inhibition of cardiac inflammatory processes in sepsis (Ceylan-Isik et al. 2010). On the contrary, SPRC treatment attenuated the LPS-induced release of TNF-α and mRNA expression of TNF-α, iNOS, and ICAM-1 in H9c2 cells. These findings led us to postulate that the effects of SPRC may be partly mediated by an H_2S-dependent pathway. To further support the hypothesis, our results demonstrated that LPS resulted in markedly reducing CSE expression and H_2S level. The results are consistent with the report that a decreased CSE expression and H_2S level were observed under inflammatory condition (Chen et al. 2009; Szabó 2007). However, SPRC significantly elevated the CSE and H_2S level, which were abolished by PAG (Wang et al. 2009, 2010), a potent, active site-directed, irreversible inhibitor of CSE (Thompson et al. 1982). Our results were consistent with the report that sulfur-containing amino acids can modulate the endogenous H_2S production and ameliorated myocardial injury and oxidative stress (Chang et al. 2008). Although there are some earlier reports indicating that LPS may up-regulate CSE expression in inflammation (Li et al. 2009a, b; Zhu et al. 2010a, b), our findings suggested that LPS down-regulated the CSE expression and H_2S level in H9c2 cells. The inconsistency between the present study and earlier study may be a result of different cell lines used. In the present study, SPRC increases level of H_2S significantly

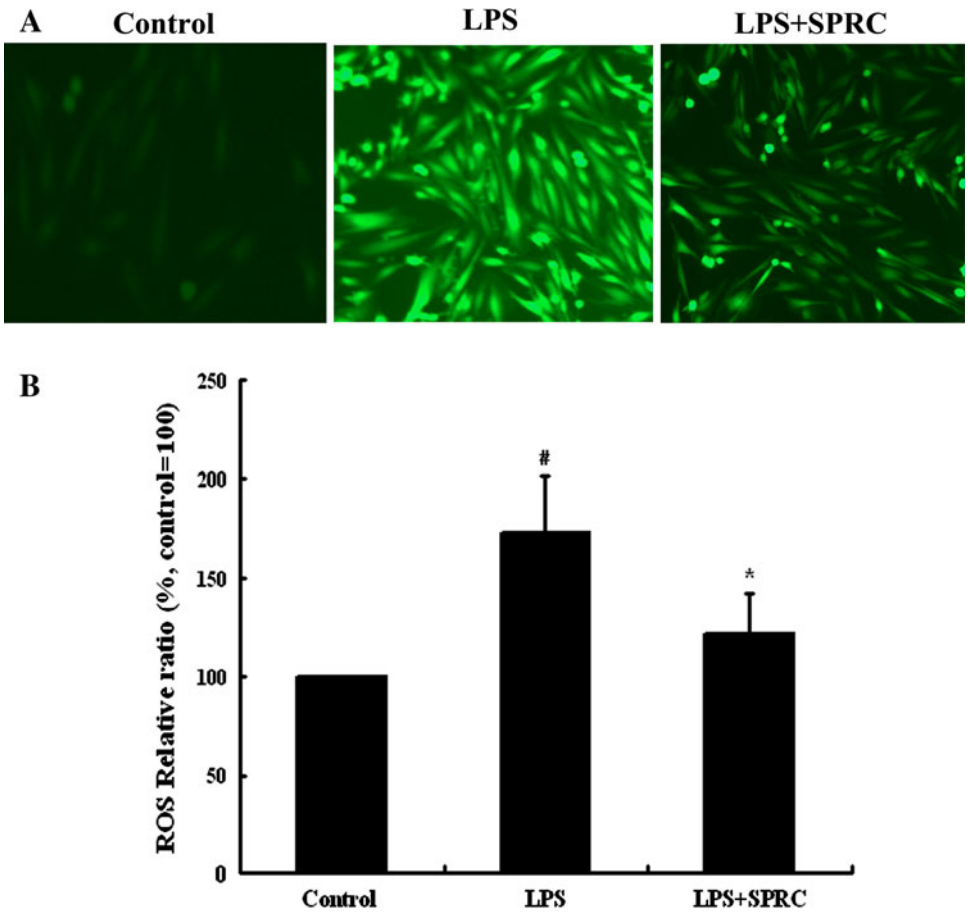

Fig. 5 SPRC attenuated intracellular ROS production in LPS-stimulated H9c2 cells. **a** Cells were incubated with 1 μmol/L SPRC for 30 min, then stimulated with 10 μg/mL LPS for 2 h, intracellular ROS was detected by a fluorescent microscope. Each assay was done in triplicate and had similar results. **b** Cells were incubated with 1 μmol/L SPRC for 30 min, then stimulated with 10 μg/mL LPS for 2 h, intracellular ROS was detected by a fluorescence spectrophotometer. Data represent mean ± SEM of three independent experiments. $^{\#}P < 0.05$ compared with non-treated control; $^{*}P < 0.05$ compared with LPS-treated control

and exerts obvious anti-inflammatory effects (Gong et al. 2011b). A similar circumstance has been reported that H_2S produced an anti-inflammatory effect in LPS-stimulated microglia (Hu et al. 2007) and macrophages (Zhu et al. 2010a, b; Whiteman et al. 2010), although in the earlier studies H_2S may play a role in inflammatory disease (Li et al. 2009a, b; Bhatia et al. 2005). The role of H_2S in systematic inflammation may have a pro- or anti-inflammatory effect under different conditions.

The mechanism underlying the cytoprotection of SPRC may be mediated by inhibition of NF-κB activation (Gong et al. 2011b), which controls the expression of inflammatory mediators (Valen et al. 2001). A crucial step in the activation of NF-κB is the degradation of IκBα (Schmitz et al. 2004). LPS stimulates the canonical NF-κB activation pathway through degradation of IκBα and phosphorylation of NF-κB p65 subunit (Wright et al. 2002; Hall et al. 2005), NF-κB p65 subsequently translocates from the cytoplasm to the nucleus, in turn, triggers a large amount of genes encoded for inflammatory mediators (Hall et al. 2005). Accordingly, prevention of NF-κB activation by overexpression of IκBα or inhibition of TNF-α-induced IκBα degradation inhibits TNF-α production (Zhang and Frei 2001), which consequently improves myocardial function (Haudek et al. 2001). In this study, SPRC treatment inhibited LPS-induced inflammatory mediators expression in association with inhibition of IκBα degradation and NF-κB activation. LPS-induced oxidative stress is a major source of NF-κB activation (Peng et al. 2005), and SPRC has been shown to reduce myocardial infarction-triggered oxidative stress in rats (Wang et al. 2009, 2010). Therefore, attenuation of oxidative stress by SPRC may contribute to suppress NF-κB activation. In addition, modulating the endogenous production of H_2S by SPRC (Wang et al. 2009, 2010), which has wide-ranging cellular effects including regulation of NF-κB activity (Gong et al. 2010, 2011a; Whiteman et al. 2010). Further studies addressing this aspect are therefore needed.

The interplay between Akt and MAPKs in LPS-induced inflammation is diverse. In response to LPS, ROS generation is markedly elevated and involved in the mechanisms of TNF-α expression in LPS-induced myocardial myocytes response (Shang et al. 2006). Evidence also indicates that ROS contribute to LPS-stimulated MAPKs (ERK1/2, p38, and JNK1/2) signaling pathways in myocardial cells (Ceylan-Isik et al. 2010). These findings are in line with the crucial role of MAPKs signaling and oxidative stress in sepsis-induced myocardial contractile dysfunction (Haudek

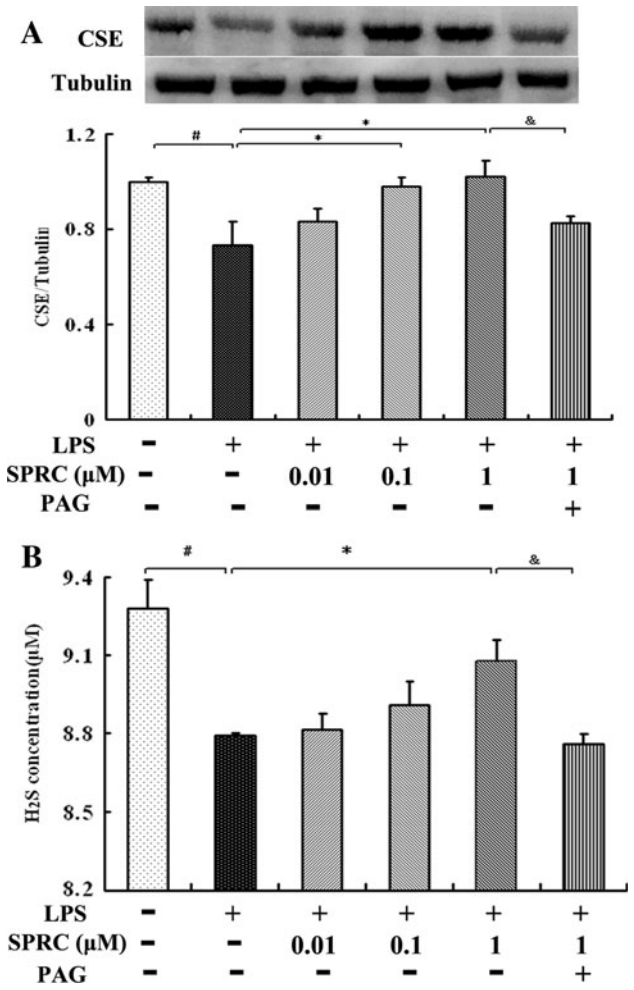

Fig. 6 SPRC attenuated LPS-induced downregulation of CSE and H_2S level in H9c2 cells. Cells were pre-incubated without or with PAG (2 mmol/L) for 30 min, then cells were incubated with indicated concentrations of SPRC for another 30 min. Thereafter, cells were then stimulated for 6 h with 10 μg/mL LPS. **a** The densitometric analysis gave the related ratio of CSE/Tubulin. **b** The culture media was collected and measured the H_2S level. Data represent mean ± SEM of three independent experiments. $^{\#}P < 0.05$ compared with non-treated control; $^{*}P < 0.05$ compared with LPS-treated control; $^{\&}P < 0.05$ compared with LPS plus PAG

et al. 2001; Davani et al. 2004; Ceylan-Isik et al. 2010). Studies using other model systems suggest that intracellular ROS production results in the activation of specific signaling cascades, including ERK1/2 (Thakur et al. 2006). Our results demonstrated that SPRC attenuated LPS-elicited activation of ERK1/2, but not p38 and JNK1/2, consistent with the findings of enhanced ROS accumulation and inflammatory mediators expression. Furthermore, LPS can induce iNOS expression in cardiomyocytes, and iNOS, in turn, triggers intracellular ROS accumulation. Pharmacological inhibition of iNOS also suppressed ROS production (Yuan et al. 2009). Importantly, SPRC alleviated LPS-induced iNOS expression, which may be associated with suppression of intracellular ROS generation and signaling cascades activation. Our current observation of the beneficial effect of SPRC against LPS-induced intracellular ROS accumulation is somewhat consistent with H_2S protective role against oxidative stress partly via suppression expression of iNOS and therefore NO production (Oh et al. 2006; Yan et al. 2006). It has been reported that PI3K/Akt pathway acts as a negative regulator of pro-inflammatory cytokines production (Guha and Mackman 2002; Schabbauer et al. 2004). Inhibition of PI3K/Akt enhances LPS-induced inflammatory factors expression (Schabbauer et al. 2004). Conversely, activation of the PI3K/Akt pathway inhibits inflammatory response and increases survival in endotoxic model. The PI3K-Akt pathway has been shown to regulate negatively many kinases including ERK1/2 pathway, which mediate induction of inflammatory genes (Guha and Mackman 2002). In this study, we found that SPRC induced Akt phosphorylation and inhibited TNF-α production. The effect of SPRC was abolished by PI3K inhibitor LY294002. Although the mode of action behind SPRC remained unclear, our results showed that SPRC activated the PI3K/Akt pathway and, hence, attenuated LPS-induced inflammatory response and ERK activation in H9c2 cells.

Taken together, our results clearly indicated that SPRC attenuated LPS-induced H9c2 cells activation. The effects of SPRC implied that it would be useful for either the prevention or the treatment of cardiovascular inflammatory diseases.

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