Syringic acid inhibits apoptosis pathways via downregulation of p38MAPK and JNK signaling pathways in H9c2 cardiomyocytes following hypoxia/reoxygenation injury

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Abstract. Syringic acid (SA), a naturally occurring O-methylated trihydroxybenzoic acid monomer extracted from *Dendrobium nobile* Lindl., has been demonstrated to attenuate renal ischemia-reperfusion (I/R) injury. However, the role of SA in myocardial I/R injury is unclear. The present study aimed to clarify the cardioprotective effect of SA in myocardial I/R injury *in vitro* and explore the potential molecular mechanisms. In the present study, it was revealed that pretreatment with SA increased the viability and inhibited oxidant stress in H9c2 cardiomyocytes that had suffered hypoxia/reoxygenation (H/R). SA also markedly downregulated B-cell lymphoma 2 (Bcl-2) expression and inhibited the expression of Bcl-2-like protein 4 (Bax) and cleaved caspase-3 in H9c2 cardiomyocytes induced by H/R. In addition, SA significantly alleviated H/R-induced the phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK) in H9c2 cardiomyocytes. In conclusion, the present study demonstrated that SA inhibits the apoptosis of H9c2 cardiomyocytes following H/R injury via reduced activation of the p38MAPK and JNK signaling pathways. The results support the therapeutic usage of SA in the treatment of myocardial infarction.

Introduction

Myocardial infarction (MI) is a major cause of mortality and disability worldwide (1). Although there have been recent advances in the treatment of MI, the general prognosis is unsatisfactory (2-4). Restoration of blood supply, termed reperfusion, has been used to treat ischemic myocardium and prevent further tissue damage. However, reperfusion following a period of prolonged ischemia can often cause myocardial ischemia-reperfusion (I/R) injury, leading to damage of cardiac tissues (5). The underlying mechanisms behind myocardial I/R injury are associated with a number of factors, including substantial free radical production, intracellular calcium overload, increased inflammation, myocardial necrosis and apoptosis (6). Thus, inhibition of oxidative stress and myocardial apoptosis is beneficial in the treatment of myocardial I/R injury.

Syringic acid (SA), a naturally occurring O-methylated trihydroxybenzoic acid monomer extracted from *Dendrobium nobile* Lindl., exhibits a variety of biological actions including anti-inflammatory, anti-tumor and anti-oxidant properties (7-9). A recent study demonstrated that SA prevented oxidative stress in l-arginine-induced acute pancreatitis (9). In addition, SA was revealed to protect against I/R injury. Tokmak *et al* (10) reported that SA pretreatment in spinal cord I/R reduced oxidative stress and neuronal degeneration. SA also attenuated renal I/R injury (11). However, the role of SA in myocardial I/R injury remains to be elucidated. The present study aimed to clarify the cardioprotective effect of SA from myocardial I/R injury *in vitro*, and the potential molecular mechanisms were also explored. The results demonstrated that SA inhibited apoptosis signaling in H9c2 cardiomyocytes via downregulation of p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK) signaling pathways following hypoxia/reoxygenation (H/R) injury.

Materials and methods

Cell culture and treatment. H9c2 rat cardiomyocyte cell line was obtained from the American Type Culture Collection (Manassas, VA, USA.). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C in a humidified incubator with 5% CO_2.

For treatment, H9c2 cells at a density of $1x10^4$ cells/well were pretreated with various concentrations of SA (0.1, 1 and

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10 μM; Sigma-Aldrich; Merck KGaA) for 24 h. Then, the cultures were introduced into a humidified N_2 hypoxic chamber (2% O_2) at 37˚C for 6 h and then reoxygenated 5 h at 37˚C in 5% CO_2 (95% O_2). Normoxic control cells were incubated at 37˚C in 5% CO_2.

Cell viability assay. Cell viability was detected using a Cell Counting kit-8 (CCK-8) assay. In brief, following treatment, the medium was removed and replaced with fresh DMEM (100 μl/well). Then, 10 μl CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well, and the microplates were incubated at 37˚C for 2 h. The absorbance was measured at 490 nm using a microplate reader (Benchmark; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cytotoxicity assay. Cell cytotoxicity was measured by a lactate dehydrogenase (LDH) assay using the Cytotoxicity Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Briefly, H9c2 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well. Following treatment, the medium was collected to measure the LDH activity according to the manufacturer's protocol. The colorimetric compound was measured at 530 nm using a microplate reader (Benchmark; Bio-Rad Laboratories, Inc.).

Measurement of cellular levels of superoxide dismutase (SOD) and malondialdehyde (MDA). H9c2 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well. Following treatment, the activity of SOD in media was analyzed using a SOD kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The MDA level was measured with an MDA kit (Beyotime Institute of Biotechnology, Jiangsu, China). For colorimetric analysis, the absorbance at 532 nm was recorded using a microplate reader (Spectra Max 190; Molecular Devices, LLC, Sunnyvale, CA, USA).

Western blot analysis. The proteins were extracted from H9c2 cells using radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was determined by a BCA protein assay kit (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 30 μg protein was separated by 10% SDS-PAGE electrophoresis followed by electroblotting onto a nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, UK). Following blocking with 5% nonfat dry milk in PBS for 4 h, the membrane was incubated overnight at 4˚C with primary rabbit anti-mouse antibodies (dilution, 1:1,000) targeting B-cell lymphoma 2 (Bcl-2; cat. no. sc-783), Bcl-2-like protein 4 (Bax; cat. no. sc-6236), cleaved caspase-3 (cat. no. sc-98785), phospho (p)-p38MAPK (cat. no. sc-101759), total p38MAPK (cat. no. sc-535), p-JNK (cat. no. sc-135642), total JNK (cat. no. sc-572) and GAPDH (cat. no. sc-25778; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Subsequently, the membrane was washed and incubated with goat anti-rabbit peroxidase-conjugated immunoglobulin G (cat. no. sc-516087; Santa Cruz Biotechnology, Inc.) diluted 1:3,000 in the blocking buffer for 1 h. Then the blot was washed in TBST buffer (TBS containing 0.1% Tween-20) and positive bands were visualized using enhanced chemiluminescence reagents (Bio-Rad Laboratories, Inc.). Densitometry was performed using Gel-Pro Analyzer software version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Statistical analysis was carried out using a one-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SA protects H/R-induced H9c2 cell injury. The protective effects of SA on H9c2 cell cytotoxicity caused by H/R were investigated. The results of the CCK-8 assay demonstrated that, compared with the normoxia group, cell viability was significantly reduced following H/R treatment. Pretreatment with SA for 24 h before H9c2 cells suffered with 6/5 h H/R injury markedly inhibited H9c2 cell injury, compared with the H/R group (Fig. 1A).

It was further analyzed whether SA pretreatment influences the cell death of H9c2 cells. As demonstrated in Fig. 1B, H9c2 cells subjected to 6/5 h H/R injury caused an marked increase of LDH release. By contrast, pretreatment of cells with SA for 24 h downregulated LDH release compared with the H/R group.

SA inhibits oxidant stress in H/R induced H9c2 cells. Oxidative stress is widely reported to serve a critical role in mediating myocardial I/R injury and thus the effect of SA on oxidant stress in H9c2 cells in response to H/R injury was investigated. As demonstrated in Fig. 2A, H/R treatment significantly decreased SOD level in H9c2 cells compared with the normoxia group and this parameter was significantly increased by SA pretreatment. By contrast, pretreatment with SA significantly reduced MDA levels induced by H/R in H9c2 cells (Fig. 2B).

SA attenuates H/R induced H9c2 cell apoptosis. Oxidative stress is one of the major stimuli involved in the myocardial apoptosis observed during I/R. Thus, the effect of SA on H9c2 cell apoptosis in response to H/R injury was investigated. As demonstrated in Fig. 3, H9c2 cells exposed to 6/5 h H/R injury caused a marked increase of Bax expression. By contrast, pretreatment of cells with SA for 24 h markedly decreased the H/R-induced Bax expression. SA pretreatment significantly increased the expression of Bcl-2 in H9c2 cells and significantly decreased the level of cleaved caspase-3 following H/R.

SA inhibits the activation of p38MAPK and JNK signaling pathways in H9c2 cells. Since p38MAPK and JNK are involved in myocardial injury caused by I/R, western blot analysis was performed to investigate whether SA inhibited the activation of p38MAPK and JNK in H9c2 cells. As demonstrated in Fig. 4, H/R treatment significantly induced the phosphorylation of p38MAPK and JNK, compared with the normoxia group. However, SA significantly alleviated H/R-induced phosphorylation of p38MAPK and JNK in H9c2 cells.

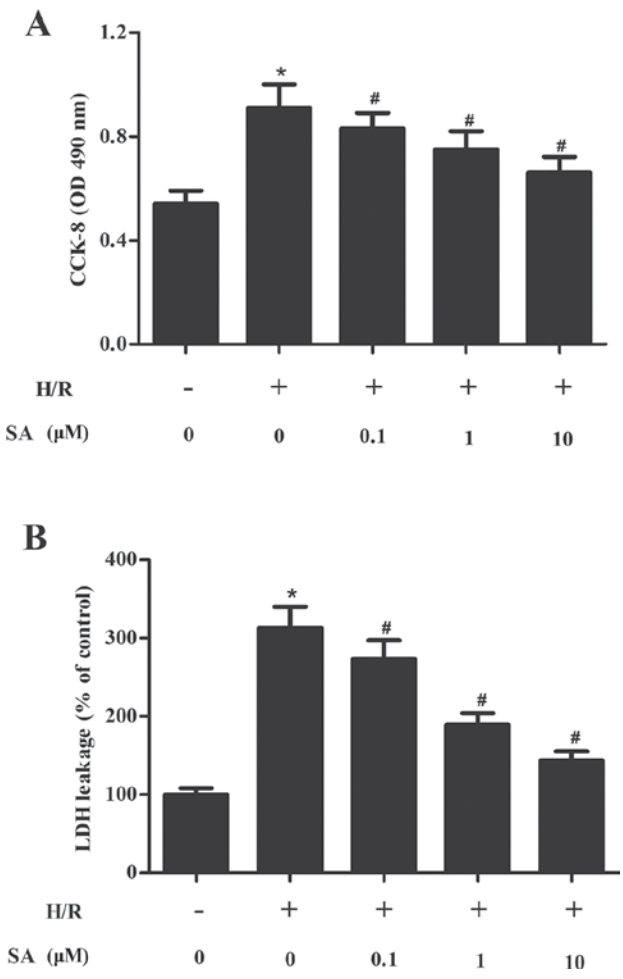

Figure 1. SA protects against H/R-induced H9c2 cell injury. H9c2 cells were pretreated with various concentrations of SA (0.1, 1 and 10 μM) for 24 h and underwent H/R injury for 6/5 h. (A) Cell viability was detected by the CCK-8 assay. (B) Cell cytotoxicity was assessed by the LDH assay. The data represent the mean ± standard deviation of three independent experiments. *P<0.05 vs. the normoxia group and #P<0.05 vs. the H/R group. CCK-8, Cell Counting kit-8; H/R, hypoxia/reoxygenation; SA, syringic acid; LDH, lactate dehydrogenase.

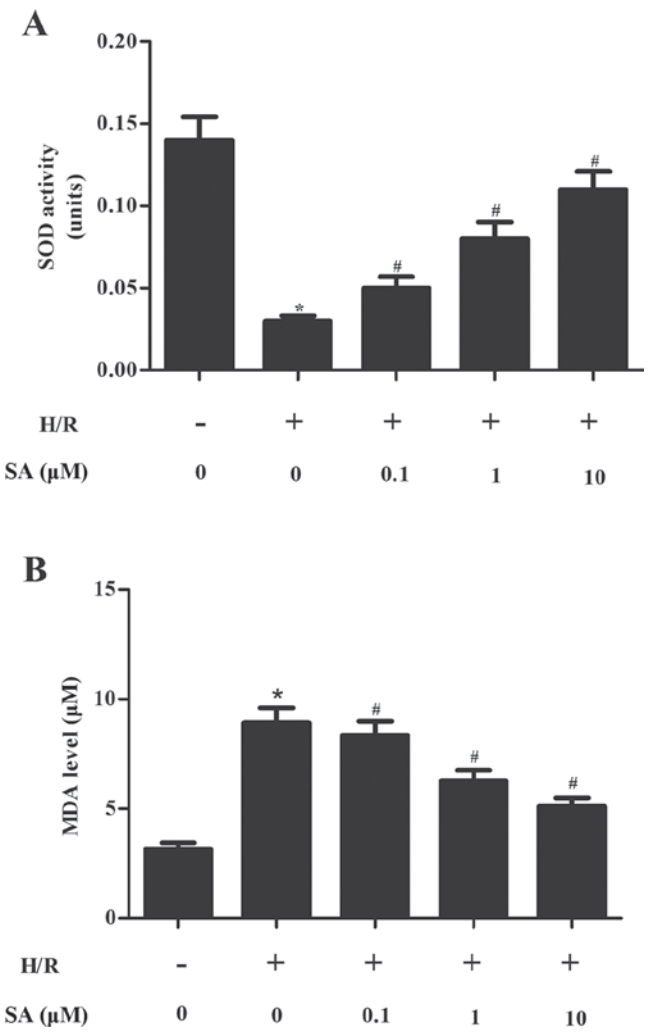

Figure 2. SA inhibits oxidant stress in H/R-induced H9c2 cells. H9c2 cells were pretreated with various concentrations of SA (0.1, 1 and 10 μM) for 24 h and underwent hypoxia/reoxygenation (H/R) injury for 6/5 h. (A) the activity of SOD in media was analyzed. (B) MDA level was measured by a method based on a reaction with thiobarbituric acid. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the normoxia group and #P<0.05 vs. the H/R group. SOD, superoxide dismutase; H/R, hypoxia/reoxygenation; SA, syringic acid; MDA, malondialdehyde.

Discussion

The present study demonstrated that pretreatment with SA increased the viability and inhibited oxidant stress in H9c2 cardiomyocytes following H/R. SA also markedly upregulated Bcl-2 expression and inhibited increases in Bax and cleaved caspase-3 in H9c2 cardiomyocytes induced by H/R. In addition, SA significantly alleviated the H/R-induced phosphorylation of p38MAPK and JNK in H9c2 cardiomyocytes.

A growing body of evidence indicates that oxidative stress contributes to the development of myocardial I/R injury (12-14). The endogenous defense system, primarily the antioxidant enzyme system (involving SOD and glutathione peroxidase), is critical for attenuating the injury induced by I/R (15). It has been reported that the activity of SOD is considerably reduced following myocardial I/R injury. MDA is the end-product of lipid peroxidization and is increased in myocardial tissue following myocardial I/R injury (16). The present study observed that SA inhibited oxidant stress in H9c2 cardiomyocytes following H/R. Thus, the antioxidant activity of SA may contribute towards a beneficial effect against myocardial I/R injury.

Apoptosis is an active gene-directed cell death process which serves a key role in myocardial reperfusion injury (17,18). Bcl-2 forms a heterodimer with Bax, thereby preventing Bax homodimerization and the activation of caspase-3 (19). Caspase-3, a cysteine protease, also serves a critical role in apoptosis (20). H/R has been demonstrated to elicit cardiomyocyte apoptosis in conjunction with the activation of caspases and an imbalance of pro-/anti-apoptotic proteins (21). Consistent with the previous studies, the present study noted that H/R markedly upregulated expression of Bax and downregulated expression of Bcl-2 in H9c2 cells. However, pretreatment of cells with SA for 24 h markedly decreased Bax expression and increased Bcl-2 expression in H9c2 cells induced by H/R. SA pretreatment significantly decreased the level of cleaved caspase-3 induced by H/R. Thus, the anti-apoptosis activity of SA may contribute towards a beneficial influence against myocardial I/R injury.

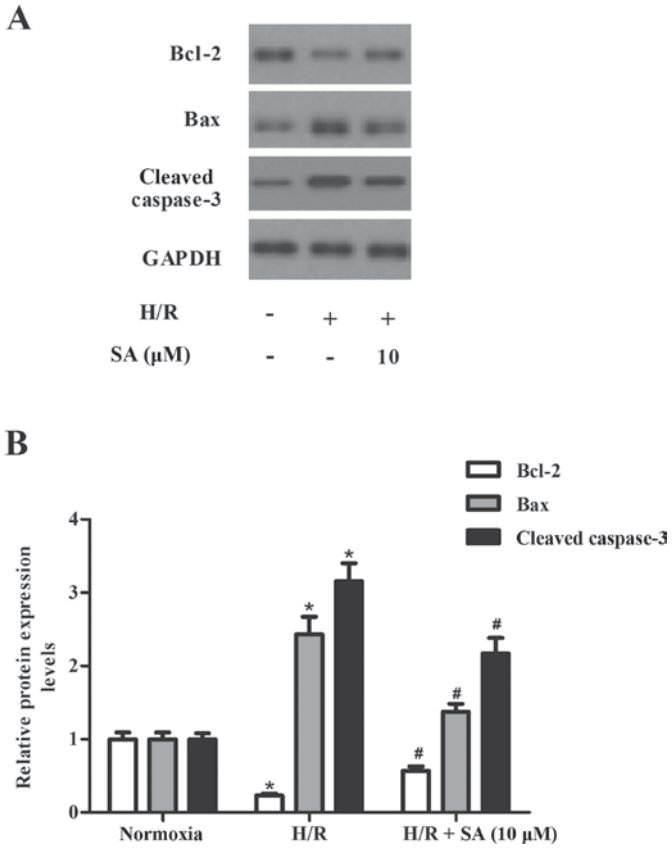

Figure 3. SA attenuates H/R-induced H9c2 cell apoptosis. H9c2 cells were pretreated with 10 μM SA for 24 h and underwent H/R injury for 6/5 h. (A) The expression levels of Bcl-2, Bax and cleaved-caspase-3 were detected using western blotting. (B) Band density was measured and normalized to that of GAPDH. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the normoxia group and #P<0.05 vs. the H/R group. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-like protein 4; H/R, hypoxia/reoxygenation; SA, syringic acid.

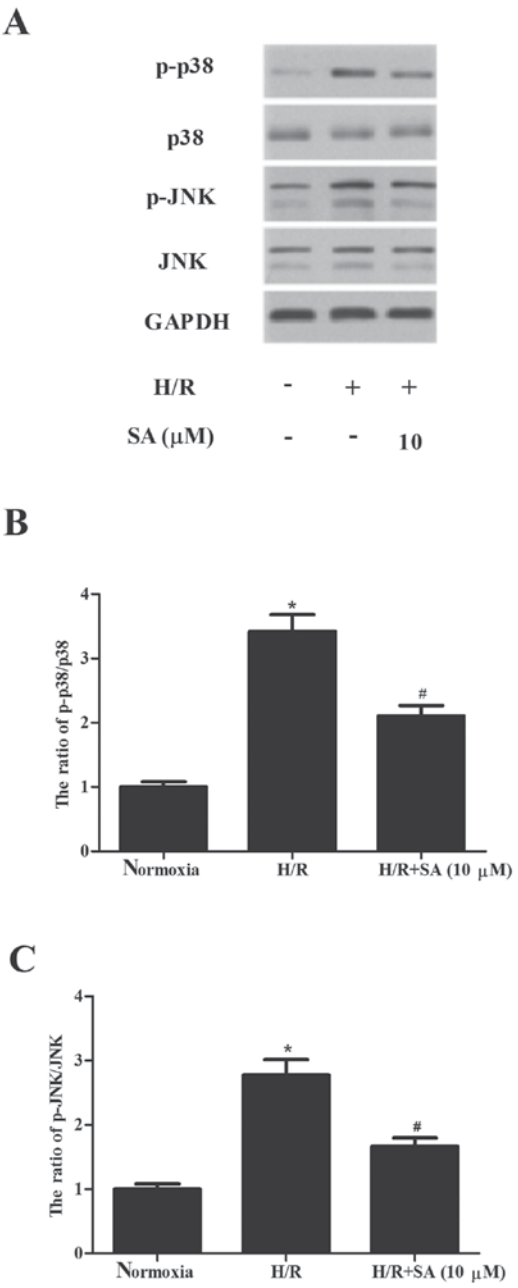

Figure 4. SA inhibits the activation of p38MAPK and JNK signaling pathways in H9c2 cells. H9c2 cells were pretreated with 10 μM SA for 24 h and underwent H/R injury for 6/5 h. (A) The expression levels of p-p38, p38, p-JNK and p-JNK were detected using western blotting. Band density was measured and normalized to that of GAPDH for (B) p-p38/p38 and (C) p-JNK/JNK. The data represent the mean ± standard deviation of three independent experiments. *P<0.05 vs. the normoxia group and #P<0.05 vs. the H/R group. p-, phospho-; p38, p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; H/R, hypoxia/reoxygenation; SA, syringic acid.

Previous studies have demonstrated that myocardial I/R is associated with MAPK activation (22-24). Cook *et al* (25) reported enhanced activation of JNK and p38MAPK in heart tissue from patients with heart failure caused by ischemic disease. Studies have demonstrated that the targeted inhibition of p38MAPK and JNK reduced cardiomyocyte apoptosis and improved cardiac performance following I/R injury (26-28). Engelbrecht *et al* (29) reported that pre-treatment with SB203580, a p38 inhibitor, produced a significant increase in cell viability and attenuation of the apoptotic index in neonatal cardiomyocytes during simulated I/R, while SP600125, a specific JNK inhibitor, caused a significant increase in caspase-3 activation and apoptotic index. Another study confirmed that treatment with the novel JNK inhibitor AS601245 during myocardial ischemia and reperfusion significantly reduced myocardial apoptosis in anaesthetized rats (23). The present study demonstrated that H/R treatment significantly induced the phosphorylation of p38MAPK and JNK. However, SA significantly alleviated H/R-induced phosphorylation of p38MAPK and JNK in H9c2 cells. These results imply that SA can induce cardio-protection via inhibition of p38MAPK and JNK signaling pathways in H9c2 cells.

In conclusion, the present study demonstrated that SA inhibited apoptosis via downregulation of p38MAPK and JNK signaling pathways in H9c2 cardiomyocytes following H/R injury. These results support the therapeutic use of SA in the treatment of MI.

Acknowledgements

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