Attenuation of the Na/K-ATPase/Src/ROS amplification signaling pathway by astaxanthin ameliorates myocardial cell oxidative stress injury

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Abstract. The 3S, 3'S-ASTaxanthin (3S, 3'S-AST) isomer has strong antioxidant activity; however, its protective roles and potential mechanisms against oxidative stress damage in cardiomyocytes have not been investigated. Na^+/K^+-ATPase (NKA)/Src signal activation has an important role in increasing reactive oxygen species (ROS) production. The aim of the present study was to investigate the protective effects and mechanism of 3S, 3'S-AST on hydrogen peroxide (H_2O_2)-induced oxidative stress injury in H9c2 myocardial cells. The protective effects of 3S, 3'S-AST on H_2O_2-induced H9c2 cell injury was observed by measuring lactate dehydrogenase and creatine kinase myocardial band content, cell viability and nuclear morphology. The antioxidant effect was investigated by analyzing ROS accumulation and malondialdehyde, glutathione (GSH) peroxidase, GSH and glutathione reductase activity levels. The protein expression levels of Bax, Bcl-2, caspase-3 and cleaved caspase-3 were analyzed using western blotting to determine cardiomyocyte apoptosis. Western blot analysis of the phosphorylation levels of Src and Erk1/2 were also performed to elucidate the molecular mechanism involved. The results showed that 3S, 3'S-AST reduced the release of LDH and promoted cell viability, and attenuated ROS accumulation and cell apoptosis induced by H_2O_2. Furthermore, 3S, 3'S-AST also restored apoptosis-related Bax and Bcl-2 protein expression levels in H_2O_2-treated H9c2 cells. The phosphorylation levels of Src and Erk1/2 were significantly higher in the H_2O_2 treatment group, whereas 3S, 3'S-AST pretreatment significantly decreased the levels of phosphorylated (p)-Src and p-ERK1/2. The results provided evidence that 3S, 3'S-AST exhibited a cardioprotective effect against oxidative stress injury by attenuating NKA/Src/Erk1/2-modulated ROS amplification.

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Introduction

Acute myocardial infarction (MI) is a common cause of death worldwide. The annual mortality and morbidity rates following MI are 7 and 22%, respectively (1). Reperfusion therapies, including primary percutaneous coronary intervention and thrombolytic therapy, are commonly used to attenuate MI damage (2). However, reperfusion therapies may cause myocardial ischemia reperfusion (I/R) injury when the blood flow returns to the myocardial tissue (2). The potential molecular mechanisms of I/R injury include oxidative stress, inflammation, calcium overload and cytokine release (3). A large number of reactive oxygen species (ROS) are produced within minutes of reperfusion, which could oxidize target proteins to trigger oxidative stress injury, as a physiological second messenger signaling molecule (4). Abundant natural biologically active substances from diverse sources (e.g. luteolin, coptisine, curcumin) can potentially decrease I/R injury through an antioxidant effect (5-7). Therefore, natural biologically active substances can be used to control the development of oxidative stress injury in order to improve the health of patients with I/R.

Astaxanthin (AST) belongs to a natural xanthophyll carotenoid that is well-known for its antioxidant, anti-inflammatory, anti-apoptosis and anticancer abilities (8-13). *Haematococcus pluvialis (H. pluvialis)* is the best natural AST resource as it primarily contains the 3S, 3'S-AST isomer. The 3S, 3'S-AST molecule is regarded as the most effective biological antioxidant to eliminate free radicals (14). AST has an α-hydroxyketone structure, which is responsible for its strong antioxidant activity by capturing singlet oxygen and reacting with free radicals (15). The antioxidant activity of AST is 500 times and 10 times higher compared with that in vitamin E and β-carotene, respectively (14,16). In recent years, AST has been reported to protect against cardiovascular disease. In rats with isoproterenol-induced MI, AST treatment decreased the weight of the heart, inflammatory cell

infiltration and myocardial fibrosis by improving antioxidant enzyme activity (9). Furthermore, AST also attenuated cardiac dysfunction and fibrosis in a mouse MI model (17). However, the protective roles of 3S, 3'S-AST against ROS-induced damage of cardiomyocytes and the potential mechanisms involved have not been elucidated.

During I/R injury, cardiac Na^+/K^+-ATPase (NKA) function was found to be altered, and elucidation of the mechanism involved would be important to develop novel approaches for therapeutic intervention (18). NKA is a transmembrane enzyme responsible for transporting Na^+ and K^+ ions across the cytomembrane, which establishes and maintains the ion concentration gradient across the cell plasma membrane (19). In addition to its Na pump function, NKA was also discovered to function as a signal transduction protein. The NKA/Src/Erk1/2 signaling pathway was found to be used as a feed-forward amplifier for ROS signaling that aggravates atherosclerosis, dyslipidemia, obesity and diabetes (20,21). Wang *et al* (22) demonstrated that ROS could active the NKA/Src/Erk1/2 signaling pathway to further trigger ROS production. Emerging studies have demonstrated the NKA/Src/ROS signal pathway is an underlying target for protecting the heart from I/R injury (23). Therefore, the present study was designed to investigate whether 3S, 3'S-AST exerts protective effects against myocardial oxidative stress damage by attenuating NKA/Src/Erk1/2 signaling-modulated ROS amplification.

Materials and methods

Materials. The 3S, 3'S-AST compound was obtained from Sigma-Aldrich (cat. no. SML0982; Merck KGaA), with ≥97% purity. Antibodies against Bax (cat. no. 2772S), Bcl-2 (cat. no. 3498S), caspase-3 (cat. no. 9665S), cleaved-caspase-3 (cat. no. 9664S), Erk1/2 (cat. no. 4695S) and phosphorylated (p)-Erk1/2 (Thr202/Tyr204; cat. no. 9101S) were purchased from Cell Signaling Technology, Inc. NKA (cat. no. cs-58629) and anti-c-Src antibodies (cat. no. cs8056) were obtained from Santa Cruz Biotechnology, Inc., while anti-p-Src pY418 antibody (cat. no. 44-660G) was obtained from Thermo Fisher Scientific, Inc. Goat anti-rabbit IgG (cat. no. 925-68021) and goat anti-mouse IgG (cat. no. 925-68020) secondary antibodies were purchased from LI-COR Biosciences. Nitrocellulose (NC) membranes were purchased from EMD Millipore, while RIPA lysis buffer was purchased from Boster Biological Technology. FBS was obtained from Biological Industries and the MTT kit (cat. no. M2128) and DAPI fluorescent dye (cat. no. D9542) were purchased from Sigma-Aldrich (Merck KGaA).

Cell culture and treatment. H9c2 cells (rat embryonic cardiomyocytes) were obtained from the Cell Bank of Shanghai Academy of Sciences and cultured in DMEM (cat. no. C11995500BT; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin at 37˚C in a humidified incubator with 5% CO_2. The H9c2 cells were passaged regularly and cultured to 70-80% confluence prior experimentation. Control group was treated with DMEM without FBS; DMSO group was treated with DMEM and DMSO without FBS. To generate a ROS-induced myocardial cell injury model, H9c2 cells were exposed to 100 μM H_2O_2 for 1 or 24 h and analyzed for further analysis. A total of 50 mg 3S, 3'S-AST powder was dissolved in 5 ml DMSO and prepared into a stock solution of 16.8 mM. Cells were pretreated with 3S, 3'S-AST (5, 10 and 20 μM) for 1 h prior to H_2O_2 administration.

Cell viability assay. Cell viability was detected using an MTT kit according to the manufacturer's instructions. H9c2 cells were plated into 96-well plates ($1x10^3$ cells/well), cultured with DMEM and treated with 3S, 3'S-AST (5, 10, 20 μM) and H_2O_2 (50, 100, 200, 400 μM) for 24 h. A total of 15 μl MTT solution was added into each well and co-incubated for 4 h, at 37˚C. After removing the medium, 150 μl DMSO was added to each well. The absorbance was measured at a wavelength of 490 nm using a microplate absorbance reader (Multiskan; Thermo Fisher Scientific, Inc.) and analyzed to assess cell viability.

Cell apoptosis assay. To detect cell apoptosis, H9c2 cells were stained using a DAPI fluorescent dye to observe nuclear morphology. H9c2 cells were plated into six-well plates ($1x10^5$ cells/well), washed with PBS three times and fixed in 4% paraformaldehyde (Beyotime Institute of Biotechnology) at room temperature for 10 min. The cells were then washed three times with PBS and stained using 3 ml DAPI staining solution (1 μg/ml) for 10 min at 37˚C. The nuclear morphology was observed using fluorescence microscopy (Olympus Corporation; magnification, x200). Healthy living cells presented with uniform staining of the nuclei. Apoptotic cells presented with pyknosis and fragmentation of the nuclei.

Lactate dehydrogenase (LDH) release analysis. Cell damage was determined using an LDH assay kit (cat. no. A020; Nanjing Jiancheng Bioengineering Institute) by detecting LDH release of myocardial cells. H9c2 cells were cultured at a density of $1x10^5$ cells/well in six-well plates. A total of 20 μl cell culture medium was added into another 96-well plate, with 300 μl reaction mixture from the kit and the mixture was incubated for 30 min at 37˚C. LDH activity was measured using a microplate reader at a wavelength of 450 nm.

Creatine kinase-myocardial band (CK-MB) activity analysis. CK-MB activity levels were measured using a CK-MB assay kit (cat. no. E006; Nanjing Jiancheng Bioengineering Institute) according to manufacturer's protocol. Briefly, H9c2 cells were lysed with PBS buffer, and freeze/thawed three times on ice for 30 min, followed by centrifugation at 12,000 x g for 15 min at 4˚C to isolate the cell debris. The protein content of samples was examined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). CK-MB activity levels were detected at a wavelength of 340 nm using a microplate absorbance reader and expressed as U/g protein.

Malondialdehyde (MDA) and glutathione peroxidase (GSH-px) level detection. The levels of MDA and GSH-px in H9c2 cells were detected using MDA (cat. no. A003) and GSH-px assay kits (cat. no. A005) (both from Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. Briefly, H9c2 cells were lysed with PBS buffer and freeze/thawed 3 times on ice for 30 min. The

supernatant was isolated by centrifugation at 12,000 x g for 15 min at 4˚C. The MDA content was measured at a wavelength of 532 nm and expressed as nmol/mg protein. The GSH-px activities of the cell lysate was detected at a wavelength of 412 nm according to the produced enzyme-catalyzed reaction product.

GSH and glutathione reductase (GR) level detection. The levels of GSH and GR in H9c2 cells were detected using GSH (cat. no. A006) and GR assay kits (cat. no. A062) (both from Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. Briefly, the H9c2 cells were lysed with PBS buffer and freeze/thawed three times on ice for 30 min then. The mixture was then centrifuged at 12,000 x g for 15 min at 4˚C to isolate the cell debris. GSH activity levels were detected at a wavelength of 405 nm and expressed as μmol/g protein, while GR activity levels were detected at a wavelength of 340 nm using a spectrophotometer and expressed as U/g protein.

Intracellular ROS measurements. A ROS assay kit (cat. no. S0033; Beyotime Institute of Biotechnology) was used to detect intracellular ROS levels according to the manufacturer's instructions. 2'-7'-dichlorofluorescin diacetate (DCFH-DA) was diluted with serum-free medium (1:1,000). Cells were seeded into a six-well plate ($1x10^5$ cells/well) and treated with H_2O_2 and 3S, 3'S-AST for 24 h. The culture medium was removed and the cells were incubated with DCFH-DA (10 μM) for 20 min at 37˚C. Subsequently, the cells were washed with serum-free medium (three times for 5 min each time) and images were obtained using a fluorescence microscope (Olympus Corporation; magnification x100). ROS fluorescence intensity was analyzed using Image-Pro Plus (version 5.0; MediaCybernetics, Inc.).

Western blot analysis. Following treatment, H9c2 cells were scraped using a scraper and the protein lysates were extracted using a solubilization buffer (containing RIPA and 1% protease inhibitor). The debris was removed by centrifugation at 12,000 x g for 15 min at 4˚C. The protein concentration was determined using a BCA assay kit. The protein samples (80 μg) were added to 5X loading buffer (Beyotime Institute of Biotechnology) and heated at 95˚C for 5 min using metal heating block. Protein samples were separated using 10% SDS-PAGE and transferred onto a NC membrane, at a constant current of 300 mA for 1 h under wet transfer conditions. The NC membranes were blocked with 5% skimmed milk at room temperature, and the proteins were incubated with the following antibodies overnight at 4˚C: NKA (1:200), c-Src (1:200), p-Src (1:1,000), Erk1/2 (1:1,000), p-Erk1/2 (1:1,000), Bax (1:500), Bcl-2 (1:500), caspase-3 (1:500), cleaved-caspase-3 (1:500) and GAPDH (1:1,000). The membranes were then incubated with the following fluorescent secondary antibodies: Goat anti-rabbit (1:10,000) and goat anti-mouse (1:10,000). The bands were captured and quantified using an Odyssey Imaging System (LI-COR Biosciences).

Statistical analysis. Statistical analysis was performed using SPSS software (version 22.0; IBM Corp.). The results are represented as the mean ± SEM from at least three independent experiments. Analysis between two groups were performed using Student's t-test. Analysis between multiple groups were performed using one-way ANOVA analysis followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

3S, 3'S-AST attenuates ROS-induced cell injury in rat cardiomyocytes. To investigate the protective effects of 3S, 3'S-AST in cardiomyocytes under oxidative stress damage *in vitro*, the rat H9c2 embryonic heart-derived cell line was treated with H_2O_2 to simulate oxidative stress injury. The cell viability was evaluated using an MTT assay. As shown in Fig. 1A and B, H_2O_2 significantly decreased cell viability in H9c2 cells in dose-dependent manner, and pretreatment with 3S, 3'S-AST significantly inhibited the decrease of cell viability caused by H_2O_2. Furthermore, with the increase in the dose of 3S, 3'S-AST, the effect of inhibition was more significant. Because the 100 μM H_2O_2 decreased cell viability to ~60%, this concentration was used for subsequent experiments. LDH and CK-MB are well-known indicators of cell injury (24); Therefore, they were measured in the present study. The LDH content in the culture medium of H_2O_2-treated cells was significantly higher compared with the control group, and treatment with H_2O_2 significantly increased CK-MB content in H9c2 cells. However, pretreatment with 3S, 3'S-AST significantly reduced this increase (Fig. 1C and D). No significant difference was observed in the 5 μM 3S, 3'S-AST-treated group compared with the H_2O_2-treated group.

3S, 3'S-AST inhibits ROS-induced cardiomyocyte apoptosis. DAPI DNA fluorescent staining was utilized to investigate the effect of 3S, 3'S-AST on cell apoptosis. The morphology of the nucleus in the H9c2 cells showed uniform staining in the control and DMSO groups. By contrast, H_2O_2-treated cells showed nuclear pyknosis and fragmentation. Compared with the H_2O_2-treated group, the 3S, 3'S-AST-treated group showed markedly attenuated cell apoptosis, but no significant difference was observed in the 5 μM 3S, 3'S-AST-treated group ($P>0.05$) (Fig. 2A). Western blot analysis was performed to evaluate the effect of 3S, 3'S-AST on the expression levels of pro-apoptosis proteins (Bax, caspase-3 and cleaved-caspase-3) and anti-apoptosis protein (Bcl-2) in each group. The protein expression levels of caspase-3, cleaved-caspase-3 and Bax were significantly downregulated, while the expression level of Bcl-2 was significantly upregulated in the 3S, 3'S-AST-treated groups compared with the H_2O_2-treated group. These effects were attenuated treatment with 10 and 20 μM 3S, 3'S-AST (Fig. 2B-F).

Effects of 3S, 3'S-AST on ROS accumulation and antioxidant activity. DCFH-DA staining was used to determine ROS accumulation. Cells treated with 3S, 3'S-AST significantly attenuated intracellular ROS level induced by H_2O_2 (Fig. 3A and B). It was reported that the NKA/Src signaling pathway, which regulates ROS amplification, plays a key role in oxidative stress damage (23). In the present study, the results indicated that 3S, 3'S-AST may act as an antioxidant to reduce the ROS level and inhibit the NKA/Src/ROS amplification loop.

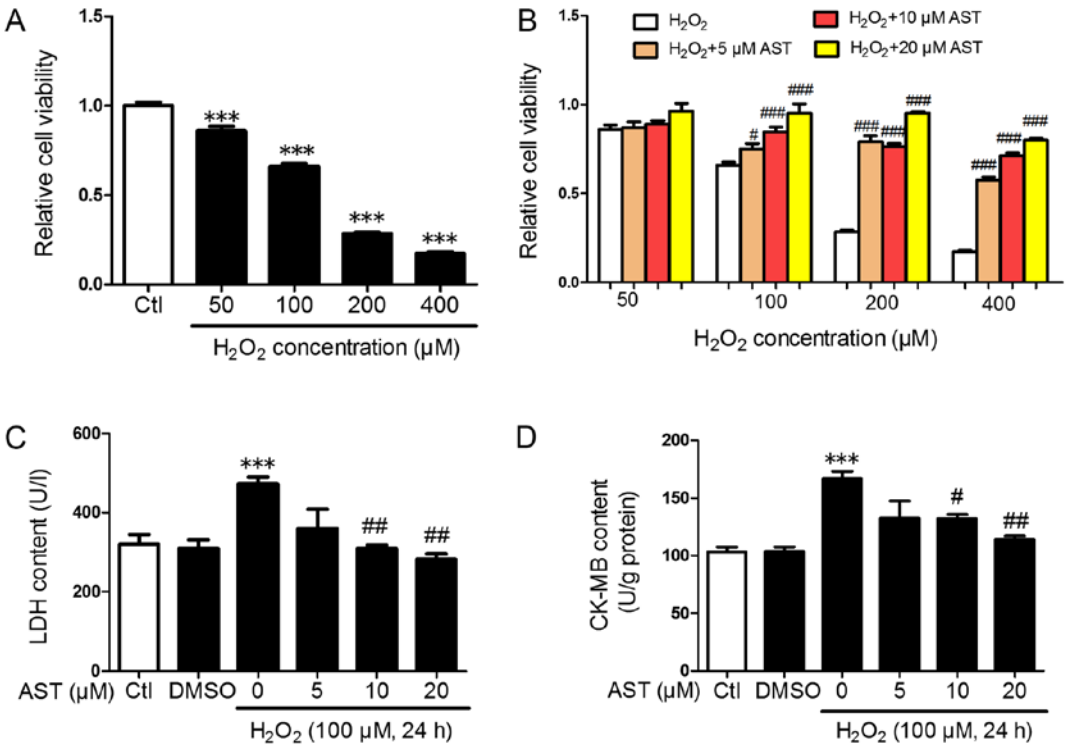

Figure 1. 3S, 3'S-AST attenuates reactive oxygen species-induced injury in H9c2 cells. (A) Cell viability in H9c2 cells treated with different concentrations of H_2O_2 (50, 100, 200, and 400 μM) for 24 h. n=8. ***P<0.001 vs. Ctl. (B) Cell viability in cells pretreated with various concentrations of 3S, 3'S-AST (5, 10 and 20 μM) for 1 h, and then treated with H_2O_2. n=8. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. H_2O_2. (C) H_2O_2 (100 μM, 24 h) upregulated the LDH content in the culture supernatant of H9c2 cells, while 3S, 3'S-AST pretreatment decreased LDH content. n=4. ***P<0.001 vs. Ctl; $^{\#\#}$P<0.01 vs. H_2O_2. (D) H_2O_2 (100 μM, 24 h) upregulated CK-MB content in H9c2 cells while 3S, 3'S-AST pretreatment decreased CK-MB content. n=3. ***P<0.001 vs. Ctl; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs, H_2O_2. Data are expressed as the mean ± standard error of the mean. AST, 3S, 3'S-ASTaxanthin; LDH, lactate dehydrogenase; CK-MB, creatine kinase-myocardial band; Ctl, control.

Furthermore, the effects of 3S, 3'S-AST on MDA content and GSH-px, GSH and GR activity levels were also measured. The results revealed that treatment with H_2O_2 significantly increased MDA content and significantly decreased GSH-px, GSH and GR activity levels in H9c2 cells. The effects of 10 and 20 μM 3S, 3'S-AST were more effective compared with cells treated with 5 μM 3S, 3'S-AST (Fig. 3C-F).

Effects of 3S, 3'S-AST on the NKA/Src signaling pathway. ROS is a specific ligand of NKA and could induce activation of Src and Erk1/2 and activated NKA/Src/Erk1/2 signaling further promoted ROS production (22). Therefore, the effects of 3S, 3'S-AST on the phosphorylation of Src and its downstream regulator Erk1/2 in H_2O_2-treated H9c2 cells was determined. As shown in Figs. 4 and 5, the phosphorylation of Src and Erk1/2 was significantly upregulated in H_2O_2-treated cells compared with controls. However, 3S, 3'S-AST inhibited the activation of Src and Erk1/2 and did not alter the protein expression levels of NKA in cells with ROS-induced damage. These results indicated that 3S, 3'S-AST attenuated the activities of the NKA/Src/Erk1/2 signaling pathway, which acted as one upstream pathway of ROS production and apoptosis.

Discussion

Restoring blood flow has been confirmed to be an efficient approach to recover ischemic myocardium (25). However, myocardial reperfusion will elicit an increase in ROS production in cardiomyocytes (26). Some therapeutic drugs, such as rosuvastatin (27), have been available to prevent oxidative stress injury from I/R; however, the therapeutic effects for I/R injury remain unsatisfactory (28). Therefore, it is essential to discover safe and effective therapeutic agents. The present study demonstrated the function of 3S, 3'S-AST in protecting cardiomyocytes from ROS-induced injury *in vitro*. The results indicated that 3S, 3'S-AST treatment inhibited cardiomyocyte apoptosis and improved antioxidant activity. It was further indicated that 3S, 3'S-AST treatment prevented intracellular ROS generation by reducing the activity of the NKA/Src/Erk1/2/ROS amplification signal transduction pathway.

AST exists in three stereoisomeric forms (3S, 3'S; 3R, 3'R and 3R, 3'S)(29). *H. pluvialis*-derived AST primarily contains the 3S, 3'S-form, and synthetic AST is a mixture of the three stereoisomers [(3S, 3'S):(3R, 3'S):(3R, 3'R), 1:2:1 ratio] (29). It was reported that synthetic AST could reduce MI size in I/R rabbits (30); however, humans are unable to use synthetic AST (31), while *H. pluvialis*-derived AST is the only AST permitted for human consumption (31). Synthetic AST has 20 times lower antioxidant capacity than natural AST and to date has not been approved for human consumption. The United States Food and Drug Administration and the European Food Safety Authority has approved the use of AST from *H. pluvialis*, as a food ingredient in the food production process and it can be used as an additive at dosages below 12-24 mg/day within 30 days (32). Initial preclinical studies also support the antioxidant ability of AST at a particular dose. For example,

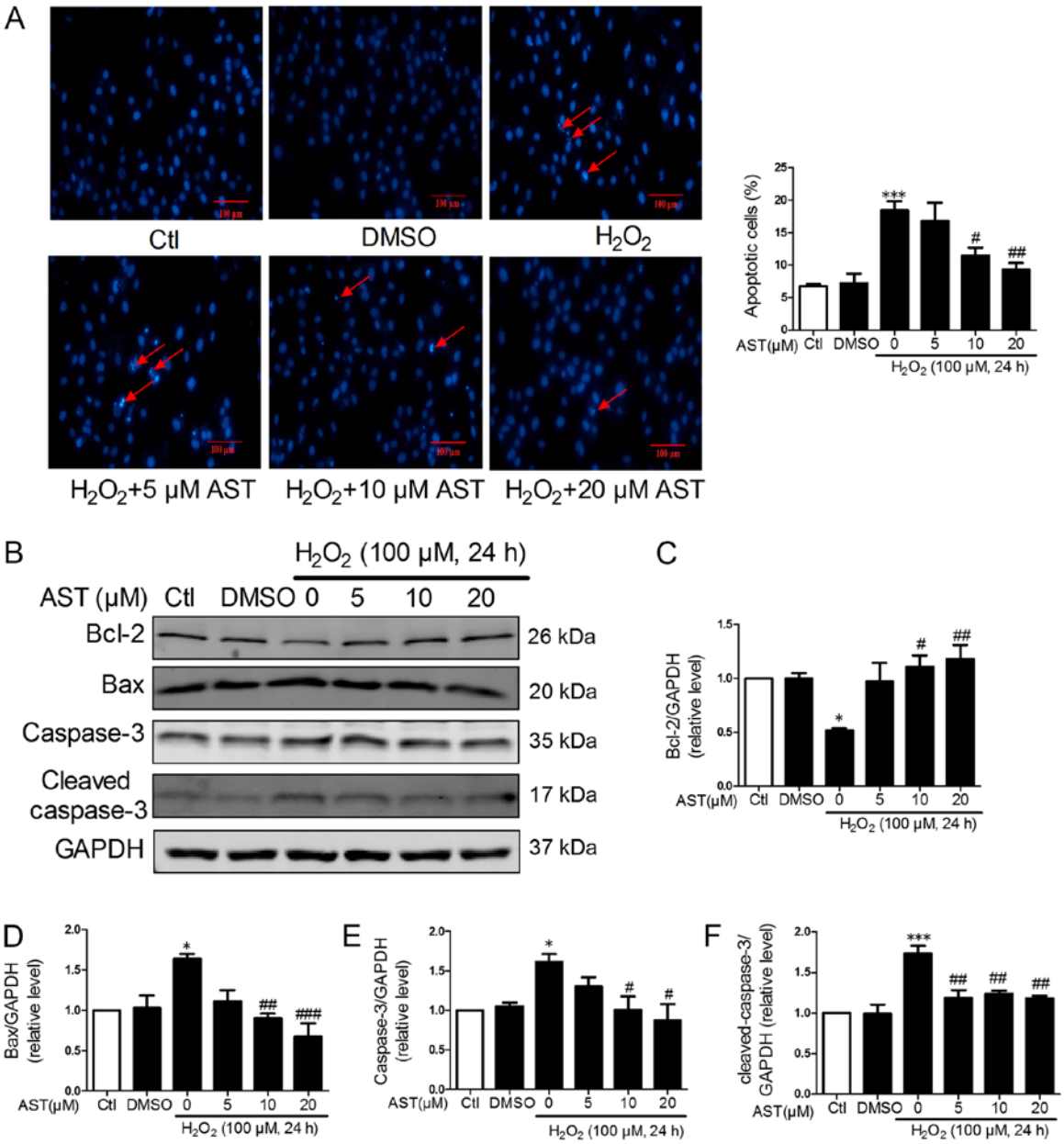

Figure 2. 3S, 3'S-AST attenuates reactive oxygen species-induced cell apoptosis. (A) Pretreatment with 3S, 3'S-AST decreased the number of apoptotic cells caused by treatment with H_2O_2 (100 μM, 24 h). The red arrows indicate apoptotic cells. Scale bar, 100 μM. (B) Expression levels of Bcl-2, Bax, caspase-3 and cleaved-caspase-3 were determined by western blot analysis. Densitometric analysis of (C) Bcl-2, (D) Bax, (E) caspase-3 and (F) cleaved-caspase-3. Data are expressed as the mean ± standard error of the mean. n=3. *P<0.05 and ***P<0.001 vs. Ctl; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H_2O_2. AST, 3S, 3'S-AST. AST, 3S, 3'S-ASTaxanthin; Ctl, control.

Iwamoto *et al* (33) found that the resistance of volunteers to low-density lipoprotein oxidation was enhanced when AST was administered at doses of 1.8-21.6 mg/day for 14 days. Yoshida *et al* (34) showed that triglyceride and high-density lipoprotein-cholesterol levels were ameliorated in patients administered with AST at doses of 12-18 mg/day for 12 weeks. Furthermore, in a human clinical study, the beneficial action of dietary AST was identified at doses of 2 and 8 mg/day for 8 days and was found to regulate the immune response, oxidative damage and inflammation (35). However, a previous study investigating the anti-fibrotic effects of different concentrations of AST (10, 20 and 40 μM) on LX-2 cells revealed that AST could modulate proliferation and apoptosis of LX-2 cells at the cellular level (36). H9c2 cells pre-treated with 0.5-8 μM AST for 6 h and co-incubated with homocysteine for 72 h revealed that AST alleviated homocysteine induced cytotoxicity (37). In the present study, different concentrations of 3S, 3'S-AST (5, 10 and 20 μM) was used to treat H9c2 cells to investigate the effects and mechanism of 3S, 3'S-AST on oxidative stress. It was found that 10 and 20 μM 3S, 3'S-AST was more effective compared with that for 5 μM, and almost no significant difference was observed in cells treated with 5 μM 3S, 3'S-AST compared with H_2O_2-treated cells.

The bioavailability of AST is affected a number of factors, including host-related conditions, such as sex, age, obesity, smoking and alcohol consumption, as well as the structure of AST or its dispersion medium (14). Some trials have investigated the bioavailability of AST from *H. pluvialis* under

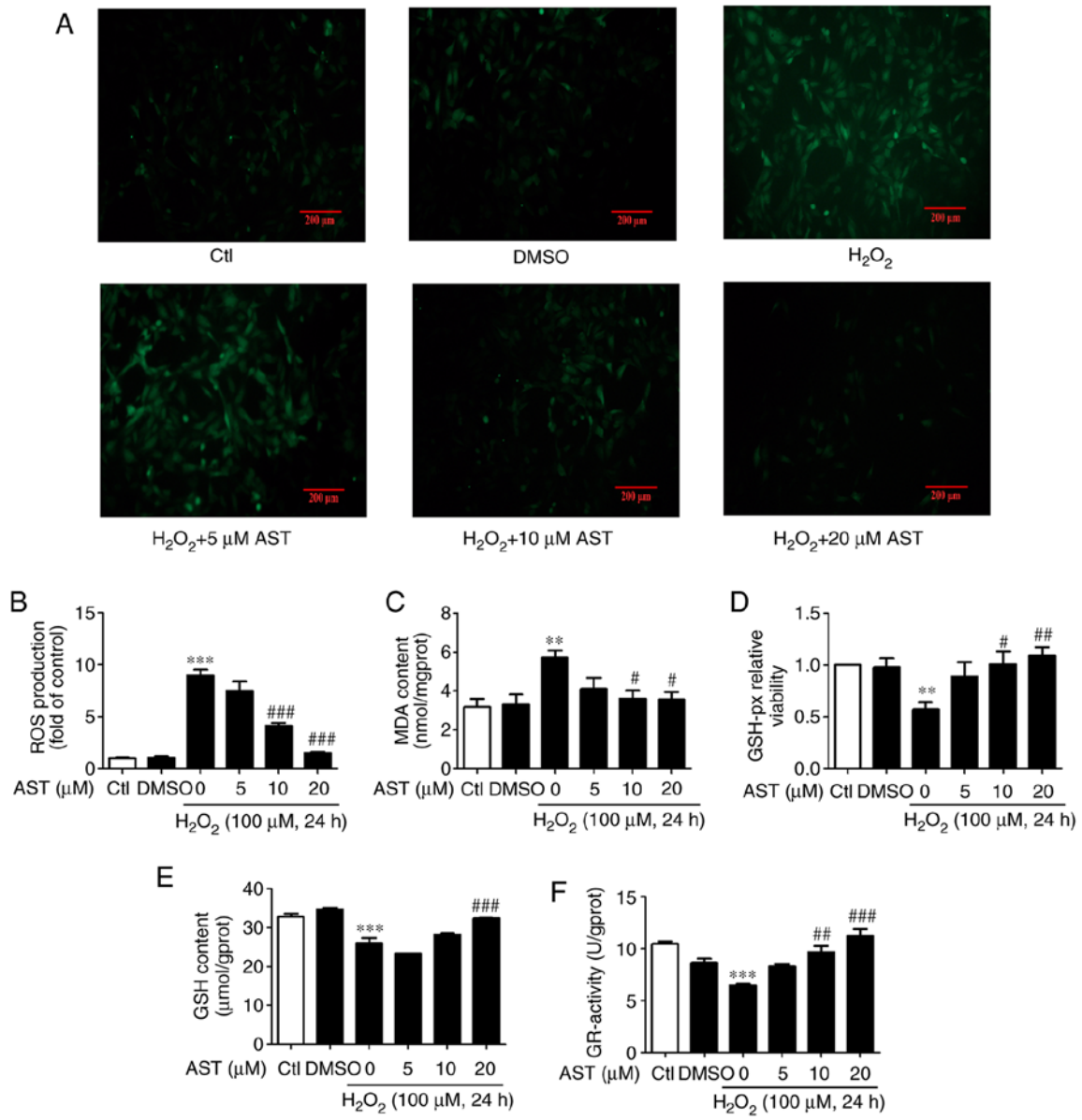

Figure 3. 3S, 3'S-AST inhibits ROS-induced ROS accumulation and increases antioxidation activity. (A) Representative ROS staining in H9c2 cells treated with H_2O_2 (100 μM, 24 h) and pretreated with 3S, 3'S-AST. Scale bar, 200 μM. (B) Quantitative analysis of ROS fluorescence intensity. n=3. (C) Pretreatment with 3S, 3'S-AST decreased MDA content in H9c2 cells treated with H_2O_2 (100 μM, 24 h). n=4. (D) Pretreatment with 3S, 3'S-AST increased GSH-px activity in H9c2 cells treated with H_2O_2 (100 μM, 24 h). n=3. (E) Pretreatment with 3S, 3'S-AST increased GSH content in H9c2 cells treated with H_2O_2 (100 μM, 24 h). n=3. (F) Pretreatment with 3S, 3'S-AST increased GR activity in H9c2 cells treated with H_2O_2 (100 μM, 24 h). n=3. Data are expressed as the mean ± standard error of the mean. **P<0.01, ***P<0.001 vs. Ctl; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H_2O_2. AST, 3S, 3'S-ASTaxanthin; Ctl, control; ROS<, reactive oxygen species; MDA, malondialdehyde; GSH-px, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione.

different factors. Okada *et al* (38) showed that the metabolism of AST in a group of smokers was faster compared with the non-smokers group, which may be attributed to the antioxidant effect of AST. A previous study found that dietary oils may enhance the absorption of AST bioavailability in humans (39). In addition, Mercke *et al* (40) determined the bioavailability of AST from *H. pluvialis* dispersed in synthetic hydrophilic surfactant polysorbate 80 (B), which is a lipid-based formulation, was 1.7-3.7 times higher compared with the commercial formulation. Furthermore, studies have shown that the antioxidant ability of AST from *H. pluvialis* was improved in rat plasma and liver tissues after being dissolved in olive oil (41,42). Satoh *et al* (43) administered participants with an oil-based natural AST-rich product for 4 weeks at 20 mg daily, and there were no safety concerns reported by evaluating toxicity and efficacy levels. As aforementioned, further research should focus on improving host-related conditions and identifying new biomaterials to act as astaxanthin vectors to improve its bioavailability.

Baburina *et al* (44) revealed that AST could inhibit opening of a large-conductance channel (mitochondrial permeability transition pore) in the rat heart following mitochondrial calcium overload or oxidative stress by reducing the serine/threonine protein kinase B/cAMP-responsive element-binding protein signaling pathways in the mitochondria. Nakao *et al* (45) found that 0.08% AST had a significantly positive effect on cardiac function of mice. AST treatment reversed enhanced lipid peroxidation and reduced antioxidant enzyme activities

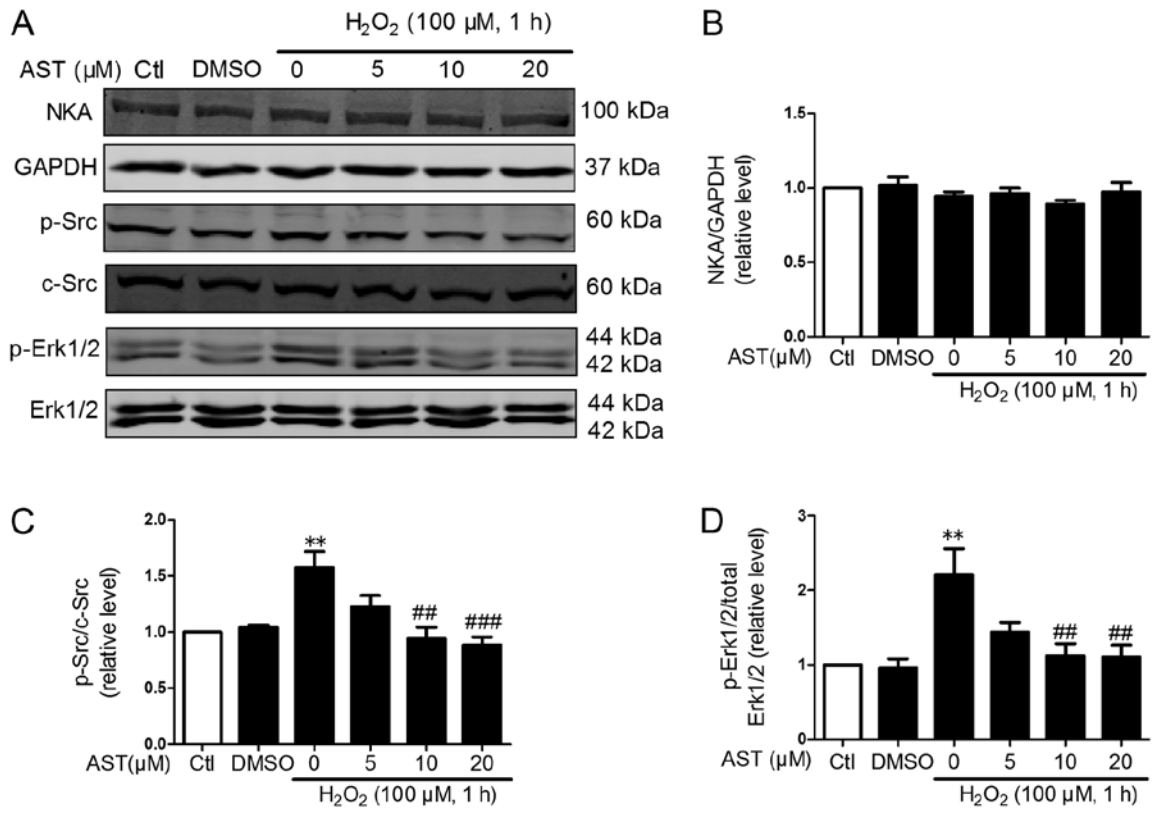

Figure 4. Effects of 3S, 3'S-AST on the NKA/Src/Erk1/2 signaling pathway. (A) Levels of NKA, p-Src and p-Erk1/2 were determined by western blot analysis in H9c2 cells treated with H_2O_2 (100 μM, 1 h) and pretreated with 3S, 3'S-AST. Densitometric analysis of (B) NKA, (C) p-Src and (D) p-Erk1/2. Data are expressed as the mean ± standard error of the mean. n=4. **P<0.01 vs. Ctl; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H_2O_2. AST, 3S, 3'S-ASTaxanthin; Ctl, control; p, phosphorylated; NKA, Na^+/K^+-ATPase.

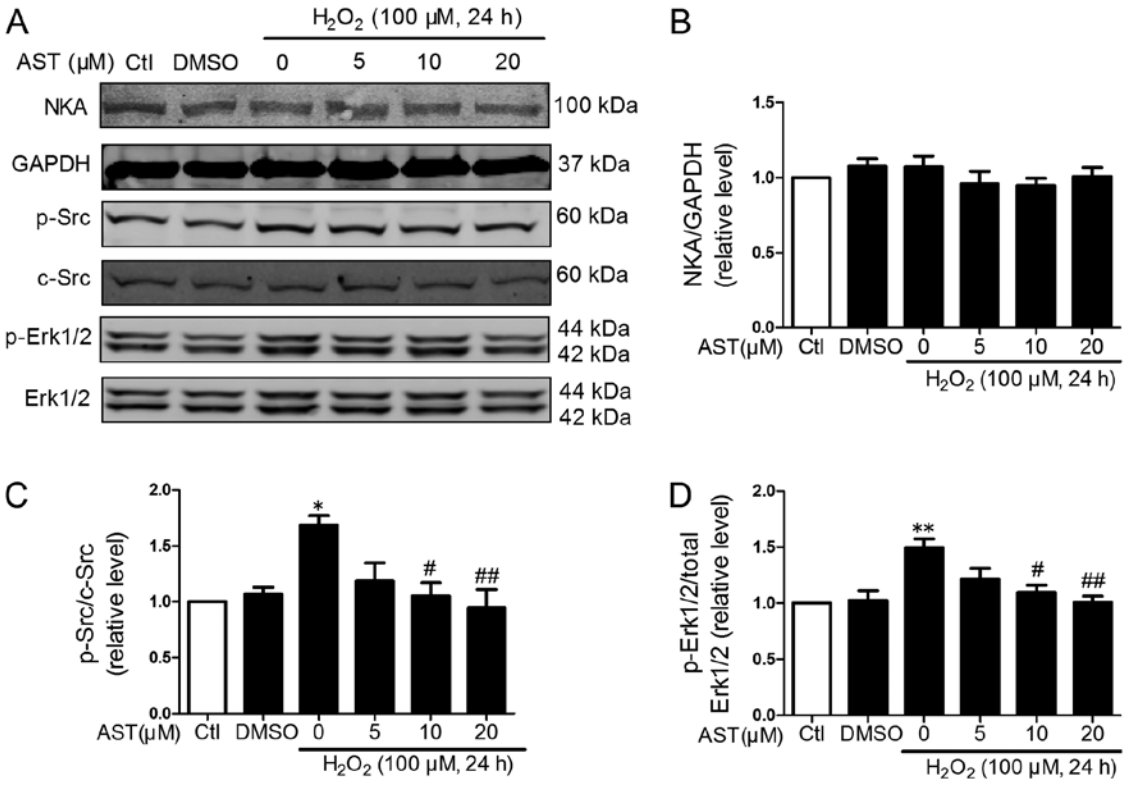

Figure 5. Effects of 3S, 3'S-AST on the NKA/Src/Erk1/2 signaling pathway. (A) Levels of NKA, p-Src and p-Erk1/2 were determined by western blot analysis in H9c2 cells treated with H_2O_2 (100 μM, 24 h) and pretreated with 3S, 3'S-AST. Densitometric analysis of (B) NKA, (C) p-Src and (D) p-Erk1/2. Data are expressed as the mean ± standard error of the mean. n=3. *P<0.05 and **P<0.01 vs. Ctl; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H_2O_2. AST, 3S, 3'S-ASTaxanthin; Ctl, control; p, phosphorylated; NKA, Na^+/K^+-ATPase.

in heart tissues caused by isoproterenol (ISO) administration in rats, which suggested that AST may protect cardiac tissue in ISO-administered rats by suppressing oxidative stress and enhancing antioxidant enzyme functions (9). However, the effects and mechanism of 3S, 3'S-AST on oxidative stress injury in myocardial cells caused by I/R was unknown. In the current study, oxidative stress injury in the H9c2 cells induced by H_2O_2 was used to mimic I/R injury. It was found that 3S, 3'S-AST significantly increased cell viability in H_2O_2-treated H9c2 cells. LDH and CK-MB are known to be markers of cell injury, and release of LDH and content of CK-MB were significantly reduced by 3S, 3'S-AST.

AST shows effective anti-apoptosis, anti-inflammatory, anti-cancer, and cardioprotective capacities, which were associated with its antioxidant activity (14,29). ROS is a universal inducer of myocardial apoptosis in reperfusion injury (46). Excessive ROS production has been considered to be important to induce I/R injury (46). MDA is the end product of the lipid peroxidation reaction; therefore, the content of MDA could reflect the degree of lipid peroxidation (47). To protect molecules from ROS, cells stimulate antioxidant defense systems, including GSH-px, GSH and GR, which play important roles in cellular antioxidant activity (48,49). The present study found that 3S, 3'S-AST could decrease the content of MDA and increase GSH-px, GSH and GR activity levels in the H9c2 cells. The results indicated that 10 and 20 μM 3S, 3'S-AST could improve the antioxidant ability of cardiomyocytes, inhibit excessive ROS accumulation and the occurrence of lipid peroxidation induced by oxidative damage. Apoptosis-related proteins, such as Bax, Bcl-2, caspase-3 and p53 play vital roles in cardiomyocyte damage (50,51). The present study showed that myocardial apoptosis was significantly inhibited by 3S, 3'S-AST. In addition, the pro-apoptotic proteins Bax and caspase-3 were significantly upregulated, while the anti-apoptotic protein Bcl-2 was significantly decreased in H_2O_2-treated H9c2 cells. Treatment with 3S, 3'S-AST inhibited the increase of Bax, caspase-3 and cleaved-caspase-3 and the decrease of Bcl-2 protein expression.

The NKA was discovered as an ion pump for moving Na^+ and K^+ across the cell membrane ~60 years ago (52). It is a key enzyme in maintaining the cellular Na^+ and K^+ ion gradient in human cardiac myocytes (53). In addition to its ion pumping function, NKA also serves as a scaffold protein, interacting with neighboring proteins and facilitating multiple cell signaling pathways. Wang *et al* (22) reported that endogenous cardiotonic steroids could activate the NKA/Src/Erk1/2 receptor complex to increase ROS levels, and ROS act as endogenous cardiotonic steroids to stimulate NKA/Src/Erk1/2 activation. Inhibition of the NKA/Src/Erk1/2/ROS amplification loop exhibited a beneficial effect on I/R injury (23). The present study found that 3S, 3'S-AST could reduce ROS generation induced by H_2O_2, and exerted antioxidant effects by restoring the activation of antioxidant enzymes.

The NKA receptor not only acts as an ion pump, maintains cell membrane potential and stabilizes cell volume, but also participates in a variety of protein-protein interactions, which activates a number of protein cascades and participate in a variety of signal transductions, including Raf/MEK/ERK, phospholipase C/protein kinase C, PI3K/Akt, Ca^{2+} signal transduction and ROS production (54). However, a number of studies have shown that these signal transduction pathways can often cross-react and generate specific cell regulation according to different signal stimulation. Wu *et al* (55) PI3K signaling could be stimulated by NKA with or without the presence of Src. Src inhibitors can downregulate PI3K signaling in cells treated with ouabain. Haas *et al* (56) showed that Src regulate interactions between NKA and EGFR, resulting in activated Ras/MAPK kinases cascade reaction. Further study will be performed to investigate the effects of AST on other signaling pathways, such as Raf/MEK/ERK or PI3K/Akt.

Decrease of Src and Erk1/2 phosphorylation levels could inhibit ROS generation, and p-Src and p-Erk1/2 were reduced by pNaKtide, which showed a cardioprotective effect in I/R injury (23). In the present study, treatment with H_2O_2, for 1 and 24 h significantly upregulated the levels of p-Src and p-Erk1/2. Pretreatment with 3S, 3'S-AST attenuated the activation of Src and Erk1/2 induced by H_2O_2; however, NKA protein expression levels were not changed. The present study demonstrated that 3S, 3'S-AST could inhibit oxidative stress injury of myocardial cells by reducing the NKA/Src/Erk1/2/ROS amplification signaling pathway.

In conclusion, 3S, 3'S-AST could inhibit ROS production and apoptosis by inhibiting the activation of Src and Erk1/2, and associated oxidant amplification signaling in myocardial cells following ROS treatment. The current study showed the therapeutic effects of 3S, 3'S-AST and its potential to be developed as a clinical approach for oxidative stress-related myocardial cell injury.

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Authors' contributions

XQ and YW designed the project and supervised all research. XQ wrote the manuscript. ZZ, WH, ML, BZ, LZ, SM, ZH, DL, ZL and JC performed all experiments and analyzed data. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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