Apigenin attenuates myocardial ischemia/reperfusion injury via the inactivation of p38 mitogen-activated protein kinase

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Abstract. Apigenin (Api) is a plant monomer associated with reducing the risk of heart disease. However, the mechanism of action remains to be fully elucidated. In the present study, it was hypothesized that API has cardioprotective effects by attenuating myocardial ischemia/reperfusion (I/R) injury. Rats were randomly subjected to sham operation, myocardial I/R alone or I/R + Api. Cardiac function was measured, and infarct size was evaluated by triphenyltetrazolium chloride staining following reperfusion. The myocardial enzyme leakage was analyzed for lactate dehydrogenase (LDH) and creatine kinase (CK). The myocardium was also assessed for total superoxide dismutase (SOD) activity and malondialdehyde (MDA) content. The phosphorylation of p38 mitogen-activated protein kinase (MAPK) was analyzed by western blotting. The present study reported for the first time, to the best of our knowledge, that I/R significantly increased infarct size, induced CK and LDH release, inhibited the activity of SOD and increased the levels of MDA, all of which were prevented by treatment with Api. In addition, I/R increased the phosphorylation of p38 MAPK, which was significantly decreased in the Api-treated heart tissue samples following I/R, compared with the untreated heart tissue samples. In conclusion, the results of the present study demonstrated that Api inhibited the p38 MAPK signaling pathway to protect cardiomyocytes from I/R-induced injury.

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Introduction

Apigenin (4,5,7-trihydroxyflavone or Api) is a non-mutagenic flavone subclass of flavonoids exhibiting low levels of toxicity, which is isolated from the leaves of *Apium graveolens* L. var. *dulce* DC (a traditional Chinese medicinal herb). Api is also present in a variety of shrubs, vegetables, plants, fruits and herbs, a number of which are widely marketed as dietary and herbal supplements (1-3). Previous studies have demonstrated that Api possesses a wide range of biological activities, including anti-carcinogenic, antiviral, antibacterial, antioxidant and anti-inflammatory effects (4-8). Following the ingestion of Api with food, Api becomes widely distributed in various tissues and provides several protective effects. A previous study demonstrated that Api protects the endothelium-dependent relaxation of the rat aorta against oxidative stress (9). In addition, the intake of Api-rich foods can significantly increase the levels of antioxidant enzymes *in vivo* (10), and Api is correlated with a reduced incidence of cardiovascular disease (11). However, to the best of our knowledge, no studies have been performed to determine whether Api can inhibit I/R induced myocardial injury.

p38 mitogen-activated protein kinase (MAPK) is an important signaling pathways, which regulates various pathological conditions, including myocardial I/R (12). The activation of MAPK results in increased I/R-induced myocardial injury (13) and impaired cardiac function (14). In addition, Api reduces the activation of p38 MAPK, having beneficial effects in various tissues (15-17).

The present study aimed to investigate the protective effects of Api following I/R injury and to explore the mechanisms underlying these effects.

Materials and methods

Materials. Api (purity >98%; Fig. 1) was purchased from Xi'an XiaoCao Botanical Development Co., Ltd. (Xi'an, China). 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The superoxide dismutase (SOD), malondialdehyde (MDA), creatine kinase (CK) and lactate dehydrogenase (LDH) activity assay kits were

purchased from Nanjing Jiancheng Biochemical Reagent Co. (Nanjing, China). Anti-p38 MAPK and anti-phosphorylated (p)-p38 MAPK antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All other chemicals and reagents were commercially available and of standard biochemical quality.

Animal model of I/R. Sprague-Dawley (SD) male rats weighing ~250-300 g were obtained from The Animal Center of The General Hospital of Lanzhou Command (Lanzhou, China) and were maintained on a 12 h light/dark cycle and administered standard rodent chow at a constant room temperature (22˚C±1˚C). The rats (n=22) were randomly divided into three groups: i) Sham group (n=6), ii) I/R group (n=8), iii) Api+I/R group (n=8). Pentobarbital sodium (30 g/l; 40 mg/kg) was used to anesthetize the rats. The carotid artery was cannulated to monitor mean blood pressure (MBP) using a pressure transducer, and a Lead-II electrocardiogram (ECG-300G; Shanghai Tai Yi Medical Equipment Co., Ltd., Shanghai, China) was used to monitor the heart rate (HR) using subcutaneous stainless steel electrodes (Shanghai Tai Yi Medical Equipment Co., Ltd.). The HR and MBP were recorded at different time-points. The rat limbs were supinely fixed, and subcutaneous electrodes were connected to monitor ECG changes. The chest was opened through the left border of the sternum, and the heart was exposed by cutting the pericardium. I/R was produced by passing a 5-0 silk suture underneath the left anterior descending coronary artery (LAD) and forming a ligature. Significant ECG changes, including widening of the QRS complex and elevation of ST segment were considered to determine successful coronary occlusion and reperfusion. The present study was performed in accordance with the National Institutes of Health Guidelines for the Use of Experimental Animals, and approved by the Committee for the Ethical Use of Experimental Animals at the General Hospital of Lanzhou Command. All experiments were performed in adherence with the National Institute of Health Guidelines for the Use of Experimental Animals (National Institutes of Health, Bethesda, MA, USA) and all animal protocols were approved by the Committee for the Ethical Use of Experimental Animals at the General Hospital of Lanzhou Command (Lanzhou, China).

Experimental protocol. The SD rats were randomly divided into three groups: (1) A Sham group, in which silk sutures were placed underneath the LAD but the LAD was not ligated; (2) I/R group, in which the LAD was ligated for 45 min and then allowed to reperfuse for 180 min with intravenously administered 0.9% NaCl vehicle; (3) Api+I/R group, in which 5 mg/kg Api was intravenously administered prior to ischemia. The experiments were performed in a double-blinded manner.

Evaluation of CK and LDH. Following myocardial I/R, the myocardial enzyme leakage levels of CK and LDH, which were collected from blood samples (0.5 ml) from the ventricular chambers, were measured to assess the level of myocardial injury. The levels of CK and LDH in blood samples correlate positively with the extent of myocardial injury (18), therefore, serum levels of CK and LDH from each group were measured using corresponding kits and analyzed using a DU640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

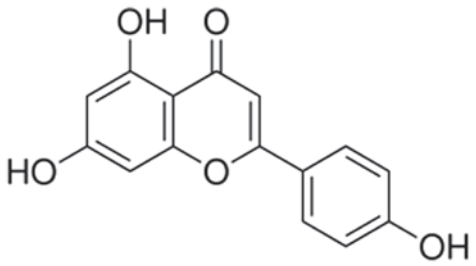

Figure 1. Chemical structure of apigenin.

Determination of myocardial infarction. Following a reperfusion period, the rat was euthanized by overdose with pentobarbital sodium anesthesia. The heart was then removed and washed using a Langendorff apparatus (Radnoti Glass Technology Inc., Monrovia, CA, USA). Subsequently, 1% Evans blue dye (Sigma-Aldrich), which stains normal non-I/R myocardium, the area not at risk (ANAR) and does not stain I/R myocardium, the area at risk (AAR), was injected into the hearts, and the hearts were frozen at -20°C for 3 h. Subsequently, 2-3 mm-thick slices of the frozen ventricle area were made and incubated in 1% TTC solution (Sigma-Aldrich) in 0.1 M Tris buffer (pH 7.8) for 15 min at 37°C. TTC can stain the viable areas of the I/R myocardium a brick red color, whereas the infarct area is not stained with TTC. Myocardial infarct size (INF) was calculated as INF / AAR x 100%.

Estimation of SOD and MDA. Oxidative stress was estimated by measuring the levels of SOD and MDA in the damaged myocardium. At the end of reperfusion, an SOD activity assay kit and MDA assay kit (Nanjing Jiancheng Bioengineering Institute) were used to measure the activity of SOD and the level of MDA in the cardiac tissues. For biochemical analysis, cardiac tissues were washed twice with cold saline solution and stored at -80˚C until analysis. MDA was reacted with thiobarbituric acid by incubating for 1 h at 95-100˚C and the fluorescence intensity was measured in the n-butanol phase via fluorescence spectrophotometry (DU 640; Beckman Coulter), by comparing with a standard solution of 1,1,3,3-tetramethoxypropane. SOD activity was measured according to reduction of nitro-blue tetrazolium via the xanthine-xanthine oxidase system.

Western blot analysis. As described previously (19), tissue samples, which were placed in lysis buffer (20 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l Na2 EDTA, 1 mmol/l EDTA, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate, pH 7.4), were mechanically minced. The homogenates were centrifuged at 15,000 x g for 20 min at 4˚C. The Bradford method was used for protein quantification. Proteins were extracted from the hearts using standard tissue lysates, including a protease inhibitor cocktail (P8340; Sigma-Aldrich) and phosphatase inhibitor cocktail (P5726; Sigma-Aldrich). The protein content of the homogenates was determined according to the Bradford method. Protein concentrations were estimated by reference to absorbances at 595 nm obtained for a series of standard

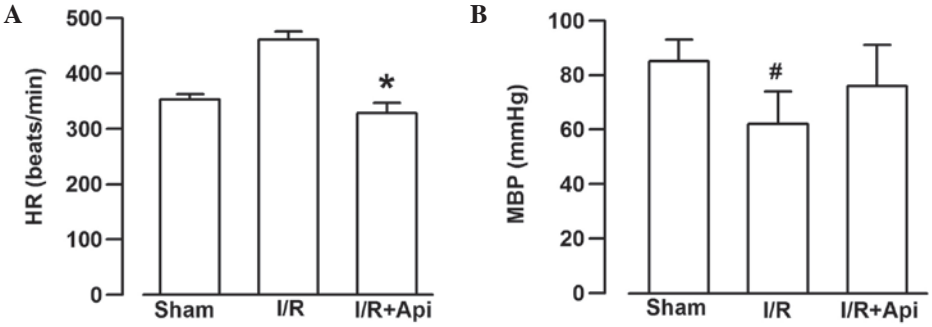

Figure 2. Hemodynamics in the rat treatment groups (n=8-10). The (A) HR and (B) MBP of the three groups are shown. The data are expressed as the mean ± standard error of the mean of three independent experiments. *P<0.05, vs. I/R group; #P<0.05 vs. Sham group. HR, heart rate; MBP, mean blood pressure. I/R, ischemia reperfusion; Api, apigenin.

protein dilutions, which are assayed alongside the unknown samples. Homogenate samples representing 20 μg of total protein were run on 12% SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc.). Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc.). Nonspecific binding of antibodies was blocked by washing with 5% fat-free milk for 1 h. The blot was then subjected to two brief washes with Tris-buffered saline plus 0.1% Tween-20, incubated with the primary antibody against p38 MAPKS (cat. no. sc-535; 1:500; rabbit polyclonal IgG; Santa Cruz Biotechnology, Inc.) and p-p38 MAPKS (cat. no. sc-17852-R; 1:500; rabbit polyclonal IgG; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. Samples were then incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG; cat. no. A0208; 1:1,000; Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 1 h. Chemiluminescence was used to detect the bands (ECL; Bio-Rad ChemiDoc XRS, Bio-Rad Laboratories, Inc.). The band densities were analyzed using the Quantity One v4.62 software package (Bio-Rad Laboratories, Inc.).

Statistical analysis. The data are expressed as the mean ± standard error of the mean. Statistical comparisons were performed using Student's *t*-test, and differences between multiple groups were assessed using a two-way analysis of variance. Data analysis was performed with a personal computer statistical software package (Prism v5.0, GraphPad Software, La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Measurement of hemodynamics. As shown in Fig. 2, the HR and MBP of the rats in all groups were determined. The HR in the I/R group increased significantly following I/R, compared with the Api+I/R group (P<0.05; Fig. 2). The MBP in the I/R group, monitored at the same time-point, was significantly lower than that in the Sham group (P<0.05; Fig.. 2). The MBP in the I/R group at the same time-point was also lower, compared with that in the Api+I/R group, however, no statistical significance was observed.

Effects of Api on myocardial infarct size. Evans blue staining does not stain the AAR in tissues, and the size of the AAR

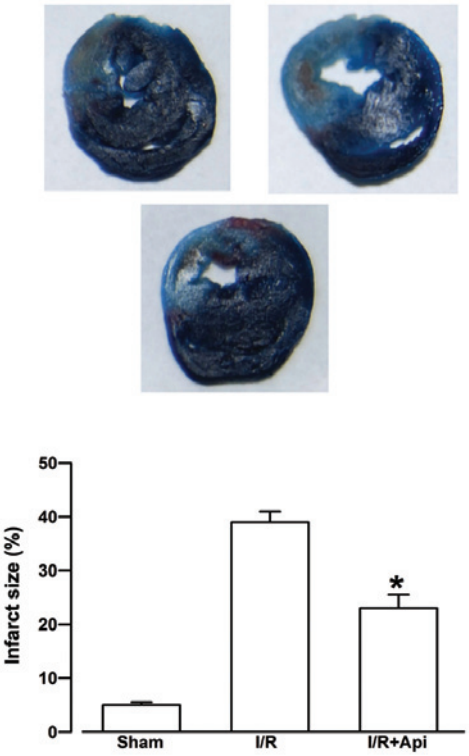

Figure 3. Api decreases myocardial infarction size following I/R. Representative heart tissue samples from the Sham and experimental groups were subjected to 45 min of ischemia and 180 min of reperfusion (n=10). The infarct size is expressed as the percentage of the AAR. An ideograph was created using Photoshop CS3 software. The blue area represents myocardial tissue with normal blood supply (ANAR), the white and the red areas represents the I/R myocardial tissue (AAR) and the infarcted myocardium is represented by the white area. Data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05, vs. I/R group. Api, apigenin; I/R, ischemia reperfusion; AAR, area at risk; ANAR, area not at risk.

depends predominantly on the area of blood supply of the blocked artery. As shown in Fig. 3, the infarct size in the I/R group was 39.16±1.98%. Treatment with Api was observed to significantly reduce myocardial infarction size (23.52±2.53%; P<0.05).

Effects of Api on LDH and CK release. To examine the effects of Api on I/R-induced injury, the present study measured the extent of LDH and CK leakage. I/R markedly increased the

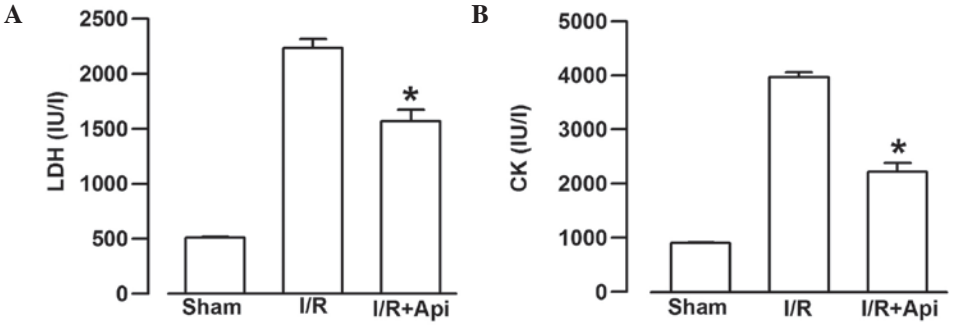

Figure 4. Effects of Api on myocardial enzyme leakage (n=8-10). The concentrations of (A) LDH and (B) CK in the three groups are shown. The data are expressed as the mean ± standard error of the mean of three independent experiments. *P<0.05, vs. I/R group. Api, apigenin; I/R, ischemia reperfusion; LDH; lactate dehydrogenase; CK, creatine kinase.

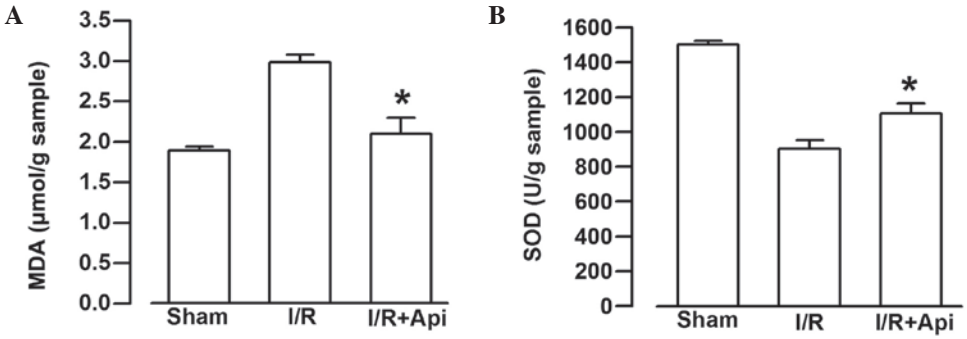

Figure 5. Effects of Api on the activity of SOD and MDA in cardiomyocytes following I/R in isolated rats hearts (n=8-10). The activity levels of (A) MDA and (B) SOD in cardiomyocytes in the three groups are shown. The data are expressed as the mean ± standard error of the mean of three independent experiments. *P<0.01, vs. I/R group. I/R, ischemia reperfusion; Api, apigenin; SOD, superoxide dismutase; MDA, malondialdehyde.

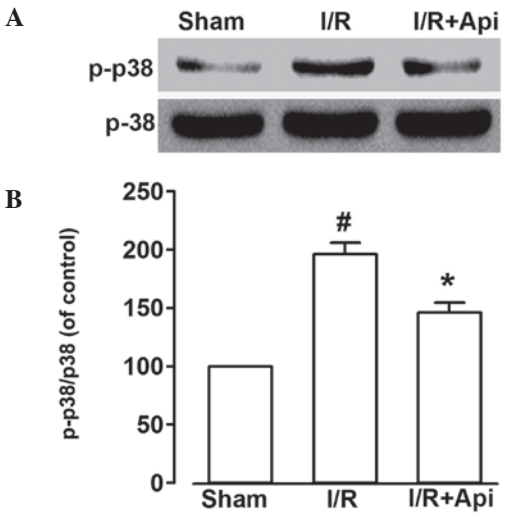

Figure 6. Api modulates I/R-induced phosphorylation of p38 MAPK. (A) During 45 min of myocardial ischemia and 180 min of reperfusion, Api was administered prior to sustained ischemia. Western blot analysis was performed to examine the phosphorylation of p38 MAPK. (B) Effects of Api on the phosphorylation of p38 MAPKS. Data are expressed as the mean ± standard error of the mean of three independent experiments. *P<0.05, vs. I/R group; #P<0.05, vs. Sham group. Api, apigenin; I/R, ischemia reperfusion; MAPK, mitogen-activated phosphorylated kinase; p-, phosphorylated.

extent of CK and LDH leakage from the myocardium (Fig. 4). As shown in Fig. 4A, treatment of the rats with Api significantly decreased the extent of LDH leakage, compared with the I/R group (P<0.05). In addition, Api also significantly decreased the extent of CK leakage, compared with the I/R group (P<0.05, Fig. 4B).

Effects of Api on SOD activity and MDA content. To examine the effects of Api on I/R--induced injury, the present study also measured the SOD activity and the content of MDA. I/R markedly decreased the SOD activity and significantly increased the content of MDA (Fig. 5). As shown in Fig. 5A, treatment of the rats with Api significantly decreased the content of MDA, compared with the I/R group (P<0.05). By contrast, Api markedly increased the activity of SOD, compared with the I/R group (P<0.05; Fig. 5B).

Effects of the inhibition of p38 MAPKS on Api and protection against isolated myocardial I/R injury. As shown in Fig. 6, I/R markedly increased the phosphorylation of p38 MAPKS. Treatment with Api decreased the level of I/R-induced p38 MAPKS phosphorylation (P<0.05). These findings indicated that Api may protect the myocardium by suppressing the phosphorylation of p38 MAPKS.

Discussion

The results of the present study demonstrated that Api protected the heart against I/R injury. The following novel findings were revealed: Administration of Api attenuated myocardial I/R injury, which was evidenced by a decrease in myocardial

enzyme leakage, a reduction in myocardial infarct size and an improvement in cardiac function; Api pre-treatment induced endogenous anti-oxidative enzyme activity and inhibited oxidative stress; and Api prevented the cardiac dysfunction caused by I/R injury by downregulating the expression of myocardial p-p38 MAPKS.

Api, a plant flavone predominantly isolated from *Apium graveolens* L. var. dulce DC leaves, a traditional Chinese medicinal herb, is of significant value due to its health-promoting benefits, and, it is widely distributed in the plant kingdom (1). Api has several biological activities, including antiaggregatory, antibacterial, antioxidant and anti-inflammatory effects. Consistent with lower prevalence of cardiovascular diseases (11), the present study demonstrated that Api exerted significant cardioprotective effects against I/R injury in a rat heart model.

A previous study demonstrated that an increase in LDH is observed following an increase in infarct size in the heart (20). The increase in infarct size is also accompanied by increased levels of CK (21). In the present study, treatment with Api treatment resulted in a decrease in myocardial infarct size. In all the Api-treated groups, the serum levels of LDH and CK were reduced, which suggested that Api had cardioprotective effects.

The present study further confirmed the anti-oxidative effect of Api treatment. Increased levels of oxidants have been reported in I/R-induced mitochondrial injury, in addition to reduced levels of oxidant-eliminating agents. Several studies have reported that I/R-induced oxidative stress increases the production of MDA and inhibits the activity of SOD in the heart (4,6). In addition, studies have reported that Api decreases the content of MDA and improves the activity of SOD in brain and intestine following I/R (22,23). The present study demonstrated that I/R upregulated the content of MDA and downregulated the activity of SOD. However, pre-treatment of the experimental animal with Api inhibited the production of MDA and improved the activity of SOD in following I/R-induced myocardial injury. These data suggested that Api may protect the myocardium against I/R injury by inhibiting oxidative stress.

Oxidative stress and inflammatory cytokines, which occur following myocardial I/R, can activate the p38 MAPKS pathway (24). Increasing evidence has demonstrated that p38 MAPKS is important in I/R-induced myocardial injury, and its targeted inhibition can reduce myocardial I/R injury (25,26).

The phosphorylation of p38 MAPKS, which is observed with an increase in adhesion molecules and cytokines, contributes to cell death in I/R injury (27). To the best of our knowledge, the present study is the first to report that Api exerted a cardioprotective effect against I/R injury via inhibiting the p38 MAPKS pathway. Therefore, Api-induced inactivation of p38 MAPKS may contribute to the cardioprotective effect following I/R-induced injury. These findings indicated the potential use of Api as a potential therapeutic agent to attenuate myocardial I/R injury.

In conclusion, the present study suggested that Api protects heart from I/R injury via inhibiting the p38 MAPKS pathway. However, the investigations in the present study only partially revealed the cardioprotective mechanisms of Api. Therefore, further investigations are required to investigate the cardioprotective mechanisms of Api.

Acknowledgements

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