

Anti-inflammatory Effect of Protocatechuic Aldehyde on Myocardial Ischemia/Reperfusion Injury *In Vivo* and *In Vitro*

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Abstract—Myocardial ischemia/reperfusion (MI/R) injury, in which inflammatory response plays a vital role, is frequently encountered in clinical practice. The present study was aimed to investigate the anti-inflammatory effect and the possible mechanism of protocatechuic aldehyde (PAI) on MI/R injury both *in vivo* and *in vitro*. The rat model of MI/R injury was induced by ligation of the left anterior descending coronary artery for 30 min, followed by 3-h reperfusion, and pretreatment with PAI could protect the heart from MI/R injury by reducing myocardial infarct size and the activities of creatine kinase-MB and cardiac troponin I (cTn-I) in serum. Also, PAI administration markedly reduced cellular injury induced by simulated ischemia/reperfusion (SI/R) in cultured neonatal rat cardiomyocytes, which was evidenced by increased cell viability, reduced lactate dehydrogenase and cTn-I activities in the culture medium, and greatly decreased percentage of cell apoptosis. Moreover, the levels of tumor necrosis factor- α , interleukin-6, intracellular adhesion molecule-1, phosphorylated I κ B- α , and the nuclear translocation of nuclear factor-kappa B (NF- κ B) were all evidently decreased by PAI both *in vivo* and *in vitro*. Taken together, these observations suggested that PAI could exert great protective effects against MI/R injury in rats and SI/R injury in cultured neonatal rat cardiomyocytes, and the cardioprotective mechanism might be involved in the suppression of inflammatory response via inhibiting the NF- κ B signaling pathway.

KEY WORDS: protocatechuic aldehyde; anti-inflammatory; cardiomyocytes; myocardial ischemia/reperfusion; nuclear factor-kappa B.

INTRODUCTION

Myocardial ischemia/reperfusion (MI/R) injury is a common clinical entity, which occurs in many situations including angioplasty, coronary bypass surgery, cardiac transplantation, and thrombolytic therapy. Despite the multiple mechanisms proposed to underlie the pathology, including energy metabolic disturbance, oxidative damage, and calcium overload [1], inflammatory responses have

gotten increased attention. It has been established that MI/R injury is closely associated with a strong inflammatory response [2, 3]. A vast body of evidence suggested a critical role for inflammatory cascades mediated by cytokines and adhesion molecules including tumor necrosis factor (TNF)- α , interleukin (IL)-6, and intracellular adhesion molecule (ICAM)-1 in MI/R process [4–6]. In addition, numerous experimental studies have shown excellent cardioprotection with the use of specific anti-inflammatory measures [7–9]. Therefore, therapeutic strategies aimed at preventing the inflammatory process may be a reasonable choice for the treatment of related heart diseases.

The root of *Salvia miltiorrhiza* (Lamiaceae), commonly known as Danshen, is one of the most popular traditional herbal medicines in China. Ample clinical and experimental evidences have implicated a critical role for Danshen in the pathogenesis of MI/R injury [10–12]. Protocatechuic aldehyde (PAI) {benzaldehyde,

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3, 4-dihydroxy} (Fig. 1) is one of the active water-soluble compounds derived from Danshen. Studies have demonstrated that PAI can exert a strong anti-inflammatory effect via suppressing the expression of ICAM-1, vascular cell adhesion molecule (VCAM)-1, and CD40 protein in human umbilical vein endothelial cells [13, 14]. However, whether PAI plays a direct role against the inflammation mediated by cardiomyocytes in ischemia/reperfusion (I/R) process has not been clarified so far, as well as whether activation of nuclear factor-kappa B (NF- κ B) plays a pivotal role in inflammatory response during MI/R injury [15]. Thus, in the present study, we sought to investigate (1) whether PAI may inhibit inflammation mediated by cardiomyocytes in MI/R injured rat model and simulated ischemia/reperfusion (SI/R) injured cell model, (2) whether the anti-inflammatory effect of PAI is involved in the NF- κ B signaling pathway, and (3) whether suppression of the inflammatory response afforded by PAI may have a beneficial effect on MI/R injury.

MATERIALS AND METHODS

Materials and Animals

PAI (molecular weight 138.12, purity >99 %) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's Modified Eagle's Medium (DMEM) and other cell culture supplies were purchased from Gibco BRL (Grand Island, NY, USA). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], TTC (2, 3, 5-triphenyltetrazolium chloride), and Evans blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit monoclonal antibodies to NF- κ B (p65), ICAM-1, and rabbit polyclonal antibodies specific

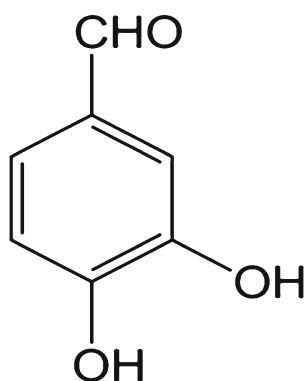


Fig. 1. Chemical structure of PAI.

for β -actin, PCNA, were the products of Cell Signaling (Beverly, MA, USA). All materials for sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA).

Sprague-Dawley rats including neonatal rats were obtained from the animal research center at the Fourth Military Medical University, Xi'an, China. The experiments were performed in adherence with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care.

Rat Model of Myocardial Ischemia/Reperfusion Injury

Rat model of MI/R injury was produced by ligation of the left anterior descending coronary artery (LAD), as described previously [16]. Briefly, rats were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally). They were artificially ventilated using a volume-regulated respirator. The heart was exposed via a left thoracotomy and the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium using a 6-0 silk suture. After 30 min of ischemia, reperfusion was established by releasing the slipknot for 3 h.

Experimental Protocol *In Vivo*

Sixty rats weighing 200–250 g were randomly divided into five experimental groups. There were 12 rats in each group: (1) Sham group, silk was drilled underneath the LAD but the LAD was not ligated; (2) Model group, the LAD was ligated for 30 min and then allowed 3-h reperfusion with receiving vehicle (saline, intravenously); and (3) three PAI-treated groups, different doses (5, 10, and 20 mg/kg body weight, respectively) of PAI were administered via tail vein injection twice daily for three consecutive days and the last dose was given 30 min before the coronary occlusion.

After 3-h reperfusion, blood samples were collected from abdominal aorta (six rats in each group) for enzyme-linked immunosorbent assay (ELISA) to detect the release of CK-MB, cTn-I, TNF- α , IL-6, and ICAM-1 (Jiancheng Bioengineering Institute, Nanjing, China) in serum according to the manufacturers' instructions. The animals were then sacrificed and the hearts were harvested for Western blot analysis. Myocardial infarct size was measured in the remaining six rats in each group.

Measurement of Myocardial Infarct Size

Myocardial infarct size was measured by means of a double-staining technique adapted from a previous method [17] using 3 % Evans blue and 2 % TTC. Specifically, at the end of reperfusion, the ligature around the LAD was retied and 2 ml of 3 % Evans blue dye was injected into the jugular vein. The dye was circulated and uniformly distributed, except in the portion of the heart previously perfused by the occluded coronary artery. The heart was quickly excised, frozen at -20°C , and the entire ventricular tissue was cross-sectioned into five slices. The slices were then counterstained with 2 % TTC prepared with phosphate buffer (pH 7.8) for 15 min at 37°C and photographed after overnight storage in 4 % paraformaldehyde. The areas stained with Evans blue (area not at risk), TTC (red staining, ischemic but viable myocardium, area at risk, AAR), and TTC-negative area (white area, infarct size, IS) were measured digitally using Image Pro Plus software (Media Cybernetics, USA). The myocardial infarct size was measured and expressed as a percentage of infarct size over total AAR.

Cell Culture and Simulated Ischemia/Reperfusion Injury Model

Neonatal rat cardiomyocytes from 1- to 2-day-old Sprague–Dawley rats were obtained and purified as the previous method [18]. Cells were routinely cultured in high-glucose DMEM with 10 % fetal bovine serum (v/v), 100 U/ml penicillin, and 100 mg/ml streptomycin, and maintained in an atmosphere of 95 % air and 5 % CO_2 at 37°C . The cells were seeded at a density of 5×10^4 cells/ml in 24-well plates for Hoechst 33258 staining and 5×10^5 cells/ml in six-well plates for biochemical indicators determination and Western blot analysis. The medium was replaced every 2 days, and cells were subjected to experimental procedures at 80–90 % confluence.

Simulated ischemia/reperfusion was performed as described previously [19]. Briefly, cells were first transferred into “ischemic buffer”, which was used to simulate the extracellular milieu of myocardial ischemia *in vivo*. Then plates of cultured cardiomyocytes were placed in a hypoxic incubator (37°C , 95% N_2 , 5% CO_2). After 2 h of incubation, the cells were subjected to reperfusion by replacing the “ischemic buffer” with routine culture medium, followed by incubation under normal conditions (37°C , 95 % air, 5 % CO_2) for 4 h.

Experimental Protocol *In Vitro*

The neonatal rat cardiomyocytes were randomly divided into different groups. (1) In the control (Con) group, cardiomyocytes were cultured under normal conditions for 6 h. (2) The SI/R group was operated on as described previously. (3) In the PAI-treated groups (SI/R + PAI), PAI (0.25, 0.5, and 1.0 mM) was added to the cell medium 24 h prior to the start of ischemia.

Cell Viability Assay

Cell viability was determined by a Microculture Tetrazolium (MTT) assay. Cells were seeded at a density of 1×10^4 cells/well in 96-well cell culture plates. Following exposure, 150 μl of the MTT solution (0.5 mg/ml) was added into each well, and then the plates were incubated for an additional 4 h at 37°C . The medium was then removed and the metabolized MTT was solubilized with 150 μl DMSO. The absorbance of the solubilized blue formazan crystals was read at 490 nm. The percent viability was defined as the relative absorbance of treated versus that of untreated control cells.

Determination of LDH, cTn-I, TNF- α , and IL-6 Releases in Culture Medium

LDH [20] and cTn-I [21] are two reliable biomarkers of myocyte injury. In order to confirm the degree of cardiomyocytes injury in SI/R, we determined the activities of LDH and cTn-I in the culture medium using corresponding bi-antibodies sandwich ELISA kits according to the manufacturers’ instructions (Jiancheng Bioengineering Institute). The activities of LDH and cTn-I in different groups were expressed as percentage of control.

TNF- α and IL-6 are two key proinflammatory cytokines released by cardiomyocytes in response to SI/R [22]. The levels of TNF- α and IL-6 in the culture medium were also determined by using an ELISA method (Jiancheng Bioengineering Institute) and expressed as picograms per milliliter.

Apoptosis Assay

Apoptosis is an important form of cell death in MI/R injury [23]. In the present study, apoptotic cells were confirmed by Hoechst 33258 staining. The staining process is as follows: after different treatments, cells were fixed with 4 % paraformaldehyde and then stained with Hoechst 33258 (1 $\mu\text{g/ml}$ in PBS) for 10 min at

room temperature. After three rinses with PBS, the nuclear morphology of the cells was detected under a fluorescence microscope with an appropriate filter. The cells showed distinctive condensed and/or fragmented nuclear structure were categorized as apoptotic cells.

To further quantify the level of apoptosis in different groups, double staining with Annexin V-FITC and propidium iodide (PI) was performed according to the manufacturer's instructions (Keygen Biotech, Nanjing, China). Cells were harvested and washed twice with PBS after the experimental procedures. Then cells (1×10^6) were resuspended in binding buffer, into which 5 μ l Annexin V-FITC and PI were subsequently added. The mixture was incubated for 15 min in the dark at room temperature. Cellular fluorescence was then measured by bivariate flow cytometry using a FACS-SCAN (FACSCalibur; BD Biosciences, San Jose, CA, USA) and analyzed by CellQuest software. Quadrant dot plot was introduced to identify living and apoptotic cells and cells in early or late phase of apoptosis. Living cells were identified as double negative for Annexin V-FITC and PI. Early apoptotic cells were branded as Annexin V-FITC positive only and cells in late apoptosis were recognized as double positive for Annexin V-FITC and PI. The rate of apoptosis was determined as the percentage of Annexin V-FITC-positive cells.

Preparation of Protein Extractions and Western Blot Analysis

Cytosolic and nuclear proteins were extracted from heart tissue and cultured myocytes, using NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer's instructions (Thermo Scientific, USA). The protein concentration of each sample was measured using a Bio-Rad Protein assay kit (Bio-Rad, CA, USA). After quantitation of protein concentration, denatured protein was separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. After being blocked with 5 % (w/v) non-fat milk at 37 °C for 30 min, membranes were incubated overnight at 4 °C with primary antibodies to NF- κ B (p65), I κ B- α , ICAM-1, β -actin, and PCNA, respectively. After three washings in TBS-T, the membranes were incubated for 30 min with a horseradish peroxidase conjugated secondary antibody diluted in TBS-T. The blots were visualized using the enhanced chemiluminescence method, and the bands were scanned and quantified by densitometric analysis.

Statistical Analysis

Data were expressed as mean \pm SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by an LSD test for multiple comparisons, using SPSS version 18.0 statistical software. A value of $P < 0.05$ was considered statistically significant.

RESULTS

PAI Protected the Rats from MI/R Injury

As reliable indicators of MI/R injury, infarct size, and the activities of CK-MB, cTn-I in serum were measured. And as illustrated in Fig. 2a and b, 30-min ischemia followed by 3-h reperfusion resulted in development of substantial myocardial infarcts, which were significantly attenuated by PAI (10 and 20 mg/kg) treatment ($P < 0.01$, compared with Model group). Besides, Fig. 2c and d showed that the elevated activities of CK-MB and cTn-I in serum resulted from MI/R were also decreased markedly by the administration of PAI (10 and 20 mg/kg) ($P < 0.05$, $P < 0.01$).

PAI Reduced the Serum Levels of Inflammatory Mediators Following MI/R

Considering the key roles of TNF- α , IL-6, and ICAM-1 in the pathophysiology of inflammatory injury caused by MI/R, we measured the levels of these three inflammatory mediators in serum. As shown in Table 1, the levels of TNF- α , IL-6, and ICAM-1 were all notably increased in the Model group compared with the Sham group ($P < 0.01$), but decreased significantly, though to different degrees, by PAI (10 and 20 mg/kg) ($P < 0.05$, $P < 0.01$).

Appropriate Doses of PAI for *In Vitro* Experiments were Confirmed

To explore suitable doses of PAI employed in subsequent *in vitro* study, we first examined the depression effect of different concentrations of PAI (0–3.0 mM) on the viability of neonatal rat cardiomyocytes cultured in normal conditions, using an MTT assay. As seen in Fig. 3a, PAI did not significantly affect cell viability at concentrations below 1.0 mM, and this dose was selected as the maximal concentration for the subsequent studies.

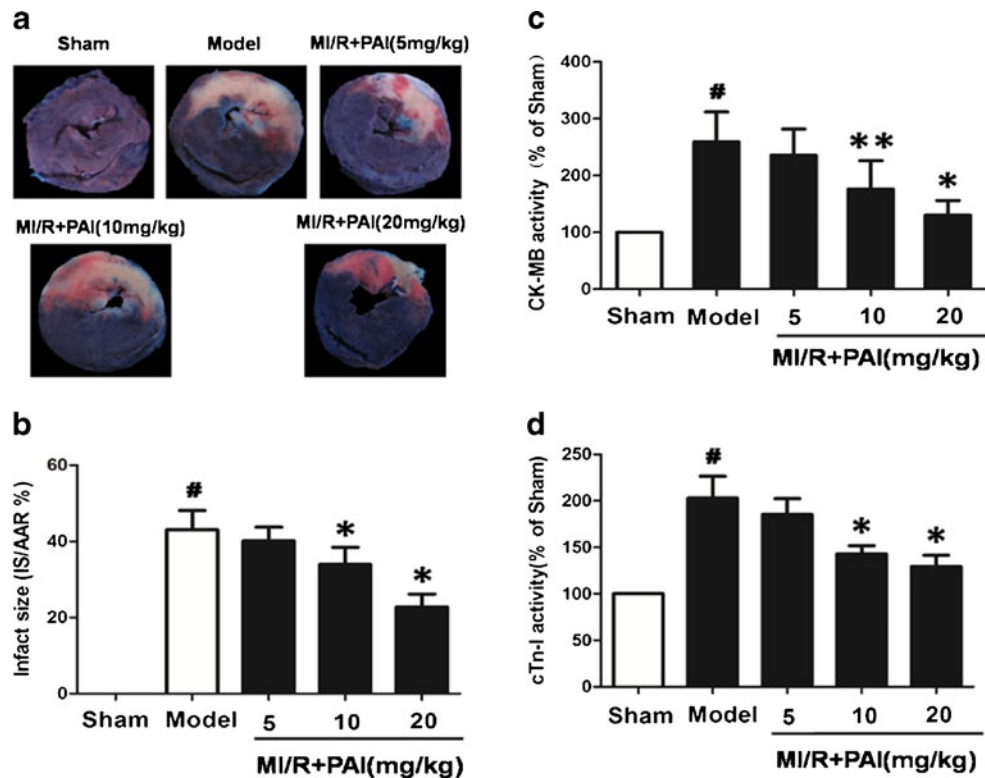


Fig. 2. The effects of PAI on myocardial damage in rats subjected to MI/R. **a** Representative photomicrographs of heart sections stained by Evans blue and TTC in different treatment groups. **b** The effect of PAI on myocardial infarct sizes (IS) expressed as percent of total ischemic–reperused area (area-at-risk, AAR). **c** The effect of PAI on the activity of CK-MB in serum of rats subjected to MI/R. **d** The effect of PAI on the activity of cTn-I in serum of rats subjected to MI/R. The activities of LDH and cTn-I were detected by ELISA and expressed as percentage of Sham. Data are presented as mean \pm SD ($n=6$). [#] $P<0.01$ vs. Sham group; ^{**} $P<0.05$, ^{*} $P<0.01$ vs. Model group.

PAI Alleviated SI/R-Induced Cell Viability Loss

Cell viability was determined by MTT assay. The result is shown in Fig. 3b. Exposed to SI/R, there were only 59.42 ± 3.04 % viable cells ($P<0.01$, compared with the Con group). However, PAI (0.5 and 1.0 mM), to a great degree, protected the neonatal rat cardiomyocytes against SI/R-induced cell viability loss, and restored cell survival to 75.45 ± 3.56 % and 80.35 ± 2.59 % ($P<0.01$, compared with SI/R group).

PAI Attenuated the Activities of LDH and cTn-I in the Culture Medium

As two markers of cardiomyocyte injury, the LDH and cTn-I activities in the culture medium were markedly elevated in the SI/R group compared with the Con group ($P<0.01$), but were significantly decreased by PAI (0.25, 0.5, and 1.0 mM) ($P<0.05$, $P<0.01$) (Fig. 4).

Table 1. Effect of PAI on serum levels of TNF- α , IL-6, and ICAM-1

Treatment (mg/kg)	TNF- α (pg/ml)	IL-6 (pg/ml)	ICAM-1 (ng/ml)
Sham	21.16 \pm 9.31	100.45 \pm 14.36	0.53 \pm 0.12
Model	68.28 \pm 11.41 ^{***}	235.00 \pm 28.54 ^{***}	1.68 \pm 0.26 ^{***}
MI/R+PAI (5)	63.95 \pm 13.52	218.45 \pm 26.51	1.37 \pm 0.16
MI/R+PAI (10)	58.60 \pm 8.88	189.63 \pm 33.87 ^{**}	1.26 \pm 0.24 [*]
MI/R+PAI (20)	44.40 \pm 7.42 [*]	142.12 \pm 18.57 [*]	0.68 \pm 0.12 [*]

Values are expressed as the mean \pm SD ($n=6$)

^{*} $P<0.01$, ^{**} $P<0.05$ vs. Model group; ^{***} $P<0.01$ vs. Sham group.

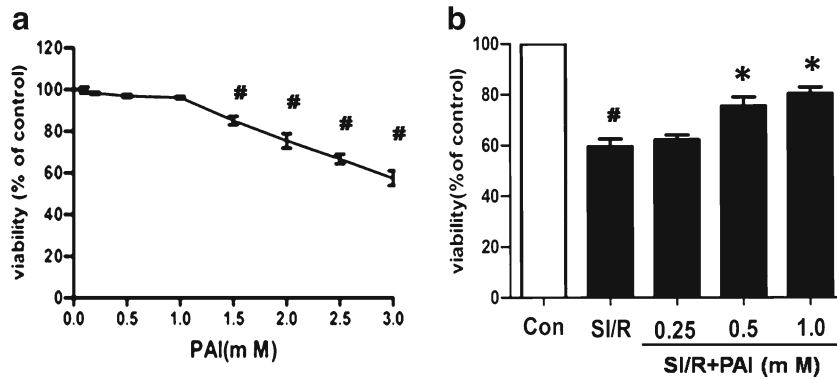


Fig. 3. The results of MTT assay. **a** Viability of neonatal rat cardiomyocytes incubated for 24 h with PAI (0–3 mM) in normal conditions. **b** Protective effect of PAI (0.25, 0.5, and 1.0 mM) against SI/R-induced cell viability loss. Cells in the Control group (0 mM group or Con group) were considered 100 % viable. Data were presented as mean \pm SD ($n=6$). $^{\#}P<0.01$ vs. Control group, $*P<0.01$ vs. SI/R group.

PAI Protected Cardiomyocytes Against Apoptosis Induced by SI/R

The anti-apoptotic effect of PAI was assessed by Hoechst 33258 staining and flow cytometry in the present study. Compared with the Con group, SI/R significantly increased the number of apoptotic cells with distinctive morphological changes including chromatin and/or cell nuclear fragmentation. However, the morphological changes were markedly attenuated by the pretreatment with different concentrations of PAI (Fig. 5). A similar result was also observed in the result of a determination by flow cytometry. In the Con group, only a small number of cells were inviable. SI/R simulation, however, increased the percentage of apoptotic cells markedly ($P<0.01$, compared with the Con group) while pretreating the cardiomyocytes with PAI (0.25, 0.5, and 1.0 mM) reduced the apoptosis ratio significantly ($P<0.01$, compared with SI/R group) (Fig. 6).

PAI Suppressed the Production of Inflammatory Mediators in the Culture Medium

As before, the levels of TNF- α and IL-6 in the culture medium were analyzed by ELISA. Compared with the Con group, SI/R resulted in notably increased levels of TNF- α and IL-6 in the supernatant ($P<0.01$). However, the release of these two cytokines was greatly inhibited at the presence of PAI (0.25, 0.5, and 1.0 mM) compared with the SI/R group ($P<0.05$, $P<0.01$) (Fig. 7).

Likewise, as a crucial adhesion molecule involved in inflammatory cascades induced by I/R, the cell surface expression of ICAM-1 was also detected. The Western blot result showed that ICAM-1 expression was markedly upregulated in the SI/R group ($P<0.01$, compared with the Con group), but decreased significantly by the administration of PAI ($P<0.05$, $P<0.01$, compared with SI/R group). As shown in Fig. 8, PAI induced significant dose-dependent inhibition of ICAM-1 expression.

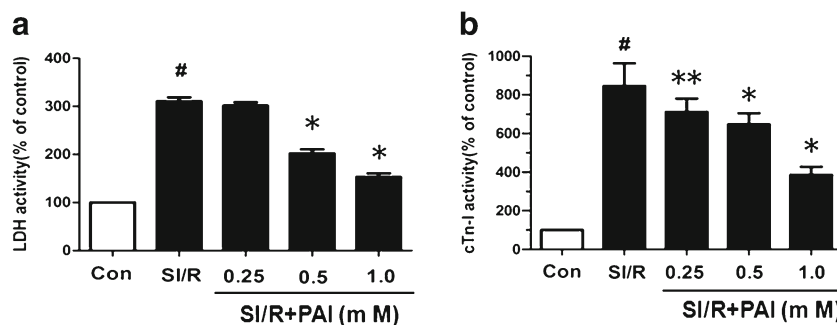


Fig. 4. The effects of PAI on LDH and cTn-I activities in the culture medium of cardiomyocytes subjected to SI/R. **a** LDH activity; **b** cTn-I activity. The activities of LDH and cTn-I were determined by ELISA and expressed as percentage of control. Data are presented as mean \pm SD ($n=6$). $^{\#}P<0.01$ vs. Con group; $**P<0.05$, $*P<0.01$ vs. SI/R group.

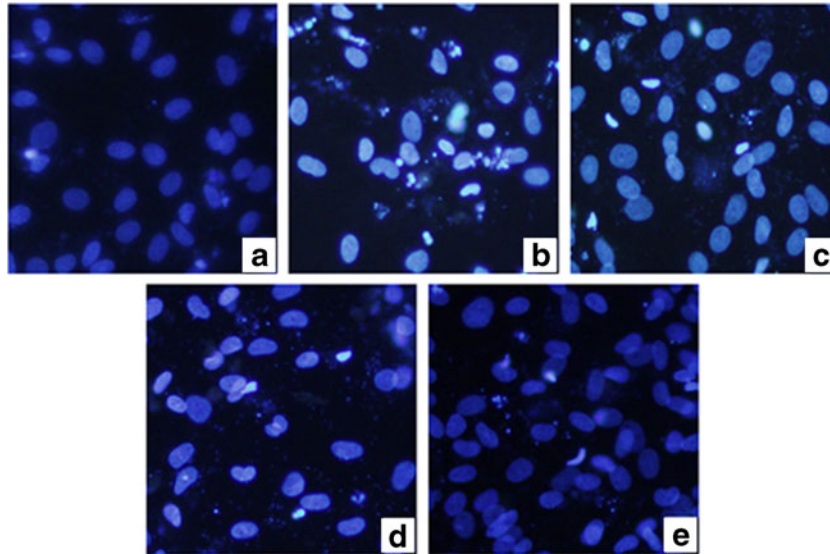


Fig. 5. The effect of PAI on nuclear morphology changes of cardiomyocytes subjected to SI/R. Apoptotic cells were confirmed by Hoechst 33258 staining ($\times 400$). **a** Con group; **b** SI/R group; **c** SI/R + PAI (0.25 mM) group; **d** SI/R + PAI (0.50 mM) group; **e** SI/R + PAI (1.0 mM) group.

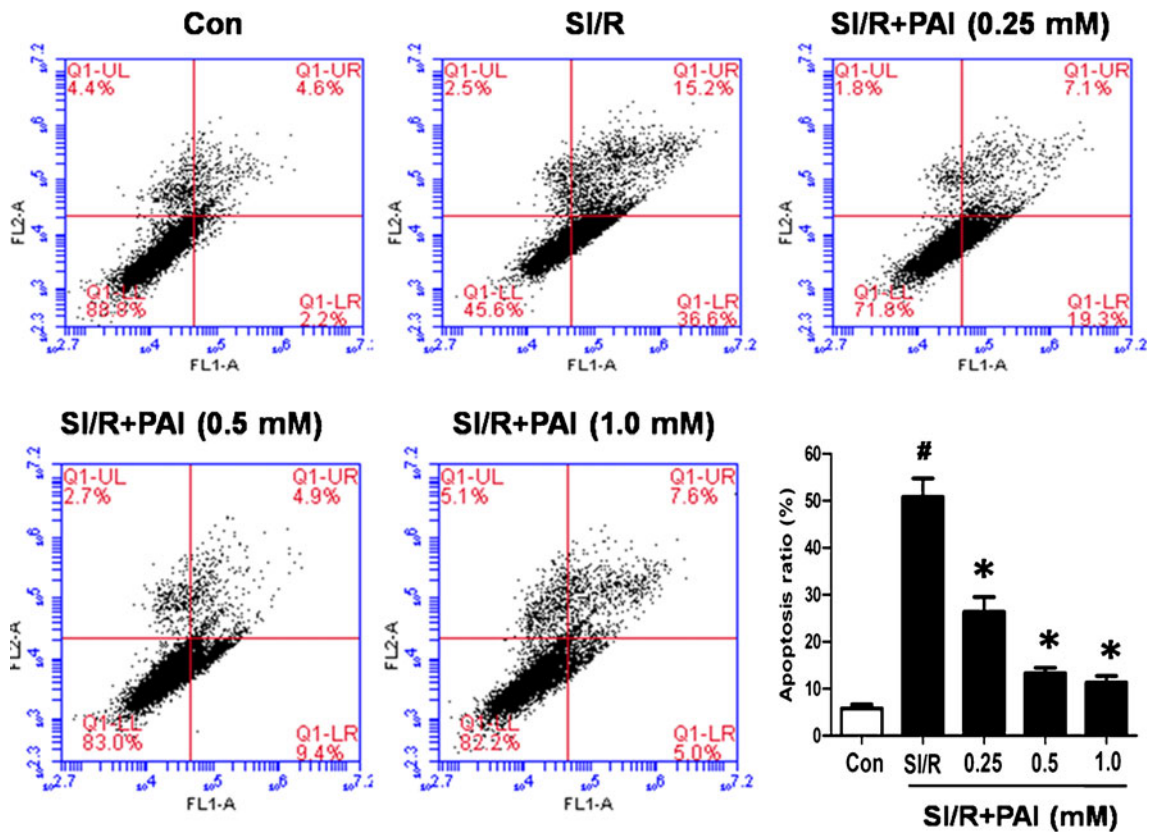


Fig. 6. The effect of PAI on SI/R-induced cardiomyocytes apoptosis. Flow cytometric detection was performed to detect apoptotic ratio of neonatal rat cardiomyocytes in different groups. Data are presented as mean \pm SD of three independent experiments. [#] $P < 0.01$ vs. Con group, ^{*} $P < 0.01$ vs. SI/R group.

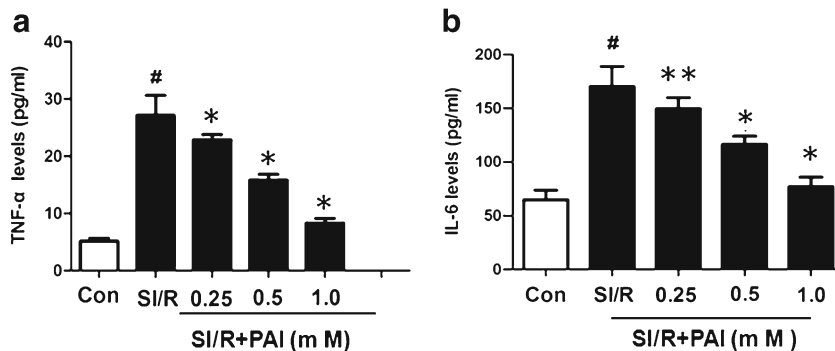


Fig. 7. The effects of PAI on TNF- α and IL-6 levels in the culture medium of cardiomyocytes subjected to SI/R. **a** TNF- α level; **b** IL-6 level. The levels of TNF- α and IL-6 were measured by ELISA and expressed as picograms per milliliter. Data were presented as mean \pm SD ($n=6$). # $P<0.01$ vs. Con group; ** $P<0.05$, * $P<0.01$ vs. SI/R group.

PAI Modulated NF- κ B Signaling Pathway Both *In Vivo* and *In Vitro*

The central role of NF- κ B makes it an appealing focus for intervention by many drugs commonly used in cardiovascular disease. In the present study, the effect of PAI on the activation of NF- κ B pathway was examined both in rats subjected to MI/R and in neonatal rat cardiomyocytes subjected to SI/R. *In vitro*, the changes in expression of NF- κ B in cytosolic and nuclear extractions were first examined (Fig. 9a). In the SI/R

group, the cytosolic expression of NF- κ B was significantly attenuated but enhanced notably in the nuclear fraction. However, this change was inhibited by pretreatment with PAI (1.0 mM). In addition, the phosphorylation of I κ B- α was also examined. The data (Fig. 9b) showed that the phosphorylation of I κ B- α increased significantly in the SI/R group compared with the Con group ($P<0.01$), but was blocked to a large extent by the administration of PAI (1.0 mM) ($P<0.01$, compared with the SI/R group). Interestingly, *in vivo* results (Fig. 10) showed an obvious similarity. MI/R not only increased the nuclear expression of NF- κ B dramatically but also markedly degraded I κ B- α in cytoplasm ($P<0.01$, compared with the Sham group). However, different doses of PAI (5, 10, and 20 mg/kg) could inhibit the MI/R-induced changes obviously ($P<0.05$, $P<0.01$, compared with the Model group).

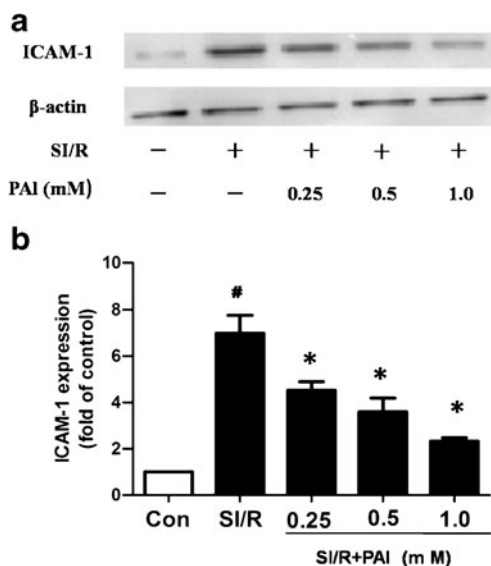


Fig. 8. The effect of PAI on ICAM-1 expression on neonatal rat cardiomyocytes subjected to SI/R. ICAM-1 expression was measured by Western blot analysis. ICAM-1 levels were corrected by β -actin and demonstrated as a fold of control. Data were presented as mean \pm SD of three independent experiments. # $P<0.01$ vs. Con group, * $P<0.01$ vs. SI/R group.

DISCUSSION

Nowadays, studies of inflammatory lesions due to MI/R have been notably focused on endothelial cells and inflammatory cells [2, 3], but little attention has been paid to myocardial cells which can be also induced to undergo inflammation in the MI/R process. In the current study, we combined *in vivo* experiments with rats and *in vitro* tests with cultured neonatal rat cardiomyocytes to explore the anti-inflammatory effect of PAI on myocardial injury induced by I/R. To the best of our knowledge, this is the first study to estimate the role of PAI against cellular injury induced by cardiomyocyte-mediated inflammation both *in vivo* and *in vitro*. The results showed that PAI could reduce MI/R

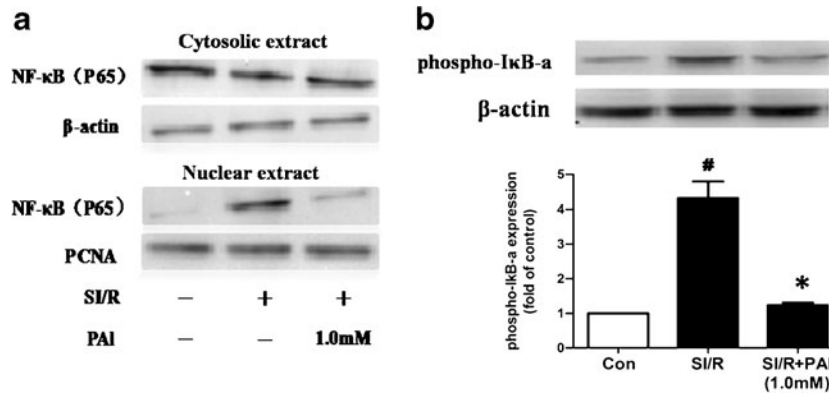


Fig. 9. The effect of PAI on the activation of NF- κ B pathway in neonatal rat cardiomyocytes subjected to SI/R. **a** The effect of PAI on SI/R-induced nuclear translocation of NF- κ B. The changes in expression of NF- κ B in cytosolic and nuclear extractions were examined by Western blot analysis. **b** The effect of PAI on SI/R-induced I κ B- α phosphorylation. Phospho-I κ B- α levels were also measured by western blot analysis and corrected by β -actin and demonstrated as a fold of control. Data are presented as mean \pm SD of three independent experiments. [#] P <0.01 vs. Con group, ^{*} P <0.01 vs. SI/R group.

injury in rat and SI/R injury in cultured cardiomyocytes on one hand and decrease the levels of inflammatory mediators in serum and culture medium on the other hand. In addition, the transcription factor NF- κ B was closely involved in these therapeutic effects. This was manifested by increased NF- κ B nuclear translocation and promoted phosphorylation or degradation of I κ B- α .

As mentioned before, many inflammatory mediators are involved in myocardial damage due to MI/R. TNF- α , IL-6, and ICAM-1 are three essential ones, which have tight interactions with each other. The synthesis and release of TNF- α is the initial event of inflammatory responses induced by I/R [4]. It is capable of inducing different kinds of proinflammatory mediators, such as IL-6 [24] and

ICAM-1 [25] to express in/on cardiac myocytes exposed to I/R. Also, IL-6 synthesis is an integral part of the response to injury resulting from I/R and relates to the induction of ICAM-1 on cardiac myocytes. Studies demonstrated that the expression of ICAM-1 on cardiac myocytes was upregulated by IL-6 [24, 26]. The formation of mass ICAM-1 conducted to enhanced neutrophil adhesion and leukocyte infiltration to myocardium subjected to I/R [27]. Then the activated neutrophils release large amounts of oxygen radicals, reactive oxygen species, and cytotoxic molecules, resulting in cardiac dysfunction and myocardial cell death [28].

Obviously, inflammatory cascades mediated by cytokines and adhesion molecules including TNF- α ,

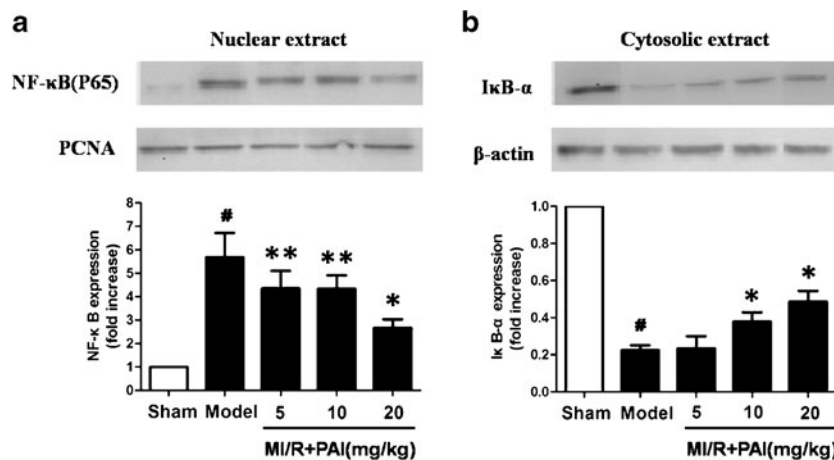


Fig. 10. The effect of PAI on the activation of NF- κ B pathway in rats subjected to MI/R. **a** The effect of PAI on nuclear expression of NF- κ B in myocardial tissue from rats subjected to MI/R. **b** The effect of PAI on the degradation of I κ B- α in cytosolic fractions from rats subjected to MI/R. The levels of NF- κ B in nuclear fractions and I κ B- α in cytosolic fractions were examined by Western blot analysis and corrected by PCNA and β -actin respectively. Data are presented as mean \pm SD of three independent experiments. [#] P <0.01 vs. Sham group; ^{**} P <0.05, ^{*} P <0.01 vs. Model group.

IL-6, and ICAM-1 are closely related to MI/R injury. Thus, inhibiting this process will contribute to cardioprotection. In our study, PAI significantly decreased the levels of TNF- α , IL-6, and ICAM-1 both *in vivo* and *in vitro*. This fact indicated that PAI may have a pivotal role in alleviating the acute inflammatory injury caused by I/R. Interestingly, our research confirmed that PAI preserved cardiomyocytes against the injury induced by MI/R *in vivo* and SI/R *in vitro*. In the study, PAI pretreatment significantly reduced myocardial infarct size and serum levels of CK-MB and cTn-I after I/R injury. Not only that, the elevated LDH and cTn-I activities in the culture medium and increased percentage of apoptotic cells induced by SI/R were also markedly decreased by PAI administration. Based on all of the observations mentioned above, we can conclude that PAI can exert a strong anti-inflammatory effect both in rats subjected to MI/R and cultured neonatal rat cardiomyocytes suffering SI/R, and suppression of the inflammatory response afforded by PAI may be responsible for its beneficial effect on myocardial injury.

Since the anti-inflammatory effect and the protection on cardiomyocyte of PAI were defined, further study was performed to investigate the possible mechanisms. Given the fact that NF- κ B is a ubiquitously expressed transcription factor [29] and has a significant position both in inflammatory response and cell survival in the MI/R process [15, 30], our study was centered on NF- κ B signaling pathway. In fact, NF- κ B has been the focus of intense investigation for nearly two decades. Considerable progress has been made in determining the regulation of NF- κ B. As we know, in its inhibited form, NF- κ B dimer is segregated in the cytoplasm bound to an inhibitory protein known as I κ B. Inhibition is relieved by various stimuli including I/R, which firstly results in the phosphorylation of I κ B and its subsequent degradation via the proteosomes. After that, the released NF- κ B is translocated to the nucleus and binds to the promoter or enhancer of specific genes, leading to the activation of those encoding cytokines, and intercellular adhesion molecules [8, 31, 32]. Thus, it can be perceived that a curb on the NF- κ B signaling pathway is bound to be implicated in the regulation of I/R-induced inflammatory reactions. In the present study, both MI/R *in vivo* and SI/R *in vitro* promoted the nuclear translocation of NF- κ B as well as phosphorylation or degradation of I κ B- α markedly; however, PAI administration greatly inhibited these changes. The results indicated that the anti-inflammatory effect and myocardial protection of PAI relied heavily on the inhibition of NF- κ B signaling pathway.

It is sure that our research may have some limitations. First, although PAI clearly reduced the inflammatory markers, as well as alleviated myocardial injury significantly, it could not directly prove that PAI exerted its myocardial protection solely through anti-inflammatory effect; more compelling evidence should be provided to support the hypothesis. Second, we have not proved in which way PAI may regulate the NF- κ B signaling pathway; further studies are needed to clarify its effect on upstream regulation of NF- κ B activation.

In summary, the present study has demonstrated that PAI could provide cardioprotection against MI/R injury in rat and SI/R injury in myocardial cells. The results indicated that PAI reduced myocardial infarct size, the releases of CK-MB, cTn-I in serum, and the activities of LDH, cTn-I in the culture medium, as well as decreased the ratio of cell apoptosis. These protective effects of PAI were associated with decreased levels of TNF- α , IL-6, ICAM-1, phosphorylated I κ B- α , and activated NF- κ B proteins. These observations suggested that the protective effects of PAI could be due, in large part, to the suppression of the inflammatory response via inhibition of NF- κ B activation. These findings pointed to the therapeutic potential of PAI as a useful anti-inflammatory lead compound in MI/R injury.

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Conflicts of Interest. The authors have no conflicting financial interests.

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