Introduction

Ischemic heart diseases are the leading cause of death in modern society. It has been reported that nearly 23.6 million people will die from cardiovascular diseases by 2030 (1). Previous studies have showed that the inflammatory response, platelet aggregation and micro-embolization, and cell death contributed significantly during the process of myocardial ischemia/reperfusion (MI/R) injury (2).

Considerable attention is focused on searching for effective substances for the prevention and therapy of MI/R injury. Protocatechuic acid (3, 4-dihydroxybenzoic acid, PCA; Fig. 1), a simple phenolic compound, is plentiful in edible fruits and vegetables and also naturally present in many Chinese herbs such as Salvia miltiorrhiza (3), Sarcandra glabra (4), Lonicera japonica (5), and Hibiscus sabdariffa (6), etc. However, over the past years, little attention has been paid to the beneficial effects of PCA on health. Recent research found that PCA can be well absorbed by animals and humans and is one of the main metabolites of complex polyphenols such as anthocyanins and procyanidins that are reported

![Fig. 1. The chemical structure of PCA.](image)
to be closely associated with reductions of mortalities in cancer, neurodegeneration, and coronary heart disease (7, 8). This hints that PCA may be an active compound in treating diseases. Previous studies have revealed that PCA had beneficial effects on therapies of cancer (6, 9), neurodegenerative disease (10), and inflammation disease (11, 12). However, to date, little is known about the effects of PCA on MI/R injury.

In the present study, we investigated the therapeutic effect of PCA on MI/R injury and explored the possible mechanisms involved. To our best knowledge, this is the first report showing the importance and potential of PCA in the therapy of ischemic heart disease, which may be helpful for exploring the mechanism of the beneficial effects of polyphenols and aid in the search for effective drugs to treat ischemic heart disease. This study provides useful insight in understanding the pharmacological efficacy of traditional Chinese medicine.

Materials and Methods

Drugs and reagents

Protocatechuic acid (purity, > 98%; molecular weight, 154.12; characteristic, white powder) was purchased from National Institutes for Food and Drug Control (Beijing, China). Diltiazem was obtained from Tanabe Seiyaku Co., Ltd. (Tianjin, China). Clopidogrel was purchased from Sanofi Winthrop Industrie (Hauts de Seine, France). Tumor necrosis factor-α (TNF-α) quantikine ELISA kit was obtained from R&D (Minneapolis, MN, USA). FITC-annexin V/propidium iodide apoptosis detection kit was from BD Biosciences (San Jose, CA, USA). Nitroblue tetrazolium (N-BT) and rabbit polyclonal antibody specific for cleaved caspase-3 were products of Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal antibody to p-Akt and polyclonal antibody to Akt were from Cell Signaling (Danvers, MA, USA). All chemicals used were of analytical grade.

Animals and MI/R protocols

Male adult Wistar rats weighing 210 – 240 g were obtained from the Experimental Animal Research Institute, Chinese Academy of Medical Sciences, China. Animals were housed under standard conditions and supplied with drinking water and food ad libitum. Neonatal Sprague-Dawley rats (SPF degree, 1 – 2-day-old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. All animal experiments in this study were performed in accordance with the China Academy of Chinese Medical Sciences Guide for Laboratory Animals that conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publications No. 85-23, revised 1996).

Rats were randomized into 6 groups as follows (n = 10 for each group): 1) the sham group: rats were pretreated with 0.9% saline and only underwent left thoracotomy, 2) the MI/R model control group: rats were pretreated with 0.9% saline before MI/R injury, 3) the diltiazem (a positive control) + MI/R group: rats were pretreated with diltiazem (16 mg/kg) before MI/R injury, 4) the clopidogrel (another positive control) + MI/R group: rats were pretreated with clopidogrel (13.5 mg/kg) before MI/R injury, 5) the PCA + MI/R group: rats were pretreated with PCA (250 mg/kg) before MI/R injury, 6) the PCA + MI/R group: rats were pretreated with PCA (500 mg/kg) before MI/R injury. The doses of PCA were chosen on the basis of the previous study (13). Vehicle or drugs were administered orally once a day (10 mL/kg) for 3 consecutive days prior to the experiment.

At 1 h after the last drug treatment, the surgical protocol was performed according to the methods described previously (14). Briefly, rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). The animal’s trachea was exposed and cannulated to facilitate spontaneous respiration. Myocardial ischemia was performed by ligating the left anterior descending coronary artery (LAD) using a 5-0 silk suture with a section of plastic tubing placed over the LAD. After 40 min of ischemia, the plastic tubing was removed and myocardium was reperfused for 2 h.

Measurement of myocardial infarct size

The measurement of infarct size using N-BT staining was identical to the method described previously (15). The rats were then sacrificed at the end of 2-h reperfusion. The hearts were rapidly excised and cross-sectioned into 5 slices, which were then weighed and incubated in N-BT solution dissolved in saline at 25°C for 15 min. The viable tissue turns dark, whereas the ischemic area is red. The infarcted size, non-infarcted size, and total heart size were measured by a multimedia color pathological image analytical system (MPIAS-500; Beijing, China). The infarction percentage of the ventricle, infarction percentage of the heart, infarction area, and infarction weight were calculated.

Determination of serum TNF-α and platelet maximum aggregation rate

Serum TNF-α was measured with an ELISA kit according to the manufacturer’s protocol. Briefly, serum samples were collected at the end of reperfusion and reacted with the assay reagents and analyzed by a BioTek SYNERGY™ 4 microplate reader (BioTek, Winooski, VT, USA) at 450-nm absorbance.

Platelet aggregation was measured using an aggrego-
meter (BS634; Beijing, China) as previously described (16). In brief, after reperfusion, 4.5 mL blood was collected from the abdominal aorta using a disposable sterilized syringe, which contained 0.5 mL of the anticoagulant citrate (3.8%) in advance, and then transferred into a test tube. Blood was centrifuged at 1000 rpm at 25°C for 10 min to obtain platelet-rich plasma and the remaining blood was further centrifuged at 3000 rpm at 25°C for 10 min to prepare platelet-poor plasma. The platelets were adjusted to 4 × 10⁶ platelets/mL and then stimulated with the agonist adenosine diphosphate disodium (ADP) (1 mM, 10 μL) (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, China).

**Cell experiments**

Neonatal rat cardiomyocytes were prepared and cultured as previously described (17). In brief, the hearts were removed and then atria and aorta were discarded. The ventricles were minced into 1 mm³ fragments, which were then digested by gentle shaking at 37°C in PBS containing 0.625 g/L trypsin (Gibco, Grand Island, NY, USA) and 0.5 g/L collagenase II (Gibco). After digestion, the dispersed cells were incubated on a 100-mm culture dish for 1 h at 37°C in a humidified incubator with 5% CO₂. The non-adherent cells were harvested and then seeded into gelatin-coated 6-well plates and incubated in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) with 10% newborn calf serum (Tianjin Hao Yang biological manufacturer Co., Ltd., Tianjin, China), penicillin (100 U/mL), streptomycin (100 U/mL), and 5-bromo-2'-deoxyuridine (0.1 mM; Sigma), which was used to inhibit cardiac fibroblast growth.

Cells were randomly divided into four groups (n = 3 per group): 1) the normal control group (cultured in normal condition), 2) the model control group (subjected to 3 h of hypoxia (gas mixture of 95% N₂ and 5% CO₂) followed by 6 h of reoxygenation) (17, 18), 3) PCA (200 μg/mL) + hypoxia/reoxygenation (H/R) group, and 4) PCA (400 μg/mL) + H/R group. In all in vitro assays, PCA was dissolved in dimethyl sulfoxide (DMSO) and treated at the start of hypoxia and reoxygenation. For the normal control group and model control group, equivalent volumes of DMSO (final concentration: 0.5%) were added.

**Flow cytometric analysis for apoptosis**

Apoptosis was detected by the FITC–annexin V/propidium iodide apoptosis detection kit according to the manufacturer’s instructions. Briefly, after H/R injury, cells were harvested with trypsin (0.25%), washed with PBS, and then incubated with 5 μL FITC–annexin V and 5 μL propidium iodide in the dark at room temperature for 15 min. Samples were analyzed by Epics Elite flow cytometry (Beckman Coulter, Brea, CA, USA) immediately and data were analyzed using Expo32 software.

**Western blot analysis**

After H/R injury, cells were lysed in RIPA lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and phosphatase inhibitors mixture (Applygen Technologies, Inc., Beijing, China) on ice for 30 min. Cell lysates were centrifuged at 12,000 × g at 4°C for 5 min and proteins in the supernatants were determined by a BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Samples with equivalent amounts of total protein (20 μg) were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membranes.

The membranes were blotted with primary antibodies including rabbit anti-cleaved caspase-3 (1:500 dilution), anti-Akt (1:1000 dilution), anti-p-Akt (1:2000 dilution) and mouse anti-α-actin (1:2000 dilution) (Beijing Biosynthesis Biotechnology, Beijing, China) overnight at 4°C and then incubated with horseradish peroxidase–conjugated goat anti-rabbit or mouse secondary antibodies (1:40000 dilution) for 1 h at room temperature. The bands were detected with an enhanced chemiluminescence (ECL) system (Thermo, Waltham, MA, USA) and visualized with the Chem Doc XRS + gel documentation system (Bio-Rad, Hercules, CA, USA), and analyzed by using Image lab 3.0 software (Bio-Rad). The expression level of α-actin served as an internal control for protein loading.

**Statistical analyses**

Data were presented as the mean ± S.D. and analyzed by using one-way ANOVA analysis followed by the Student-Newman-Keuls test for multiple comparisons. Values of P < 0.05 were considered statistically significant. All experiments were performed at least three times.

**Results**

**PCA decreased myocardial infarct size**

First, we observed the effect of PCA on MI/R injury in rats. As shown in Table 1 and Fig. 2, 40 min of ischemia and 2 h of reperfusion induced a significant myocardial infarct in the model group (for the infarction percentage of the ventricle, the infarction percentage of the heart, infarction area, and infarction weight, vs. the sham group, P < 0.01 each). Compared with the model control group, both diltiazem and clopidogrel significantly reduced these indexes described above (P < 0.05 each,
respectively); PCA (250 mg/kg) had a tendency to reduce these indexes (for those indexes described above, $P = 0.067$, $P = 0.076$, $P = 0.082$, and $P = 0.088$, respectively), and PCA (500 mg/kg) significantly decreased these indexes ($P < 0.01$ each).

**PCA reduced production of serum TNF-α following myocardial ischemia/reperfusion**

For exploring the underlying mechanism, we next observed the effect of PCA on the production of TNF-α, a key inflammatory cytokine in MI/R injury (2, 19). As shown in Fig. 3, MI/R injury induced a significant rise of serum TNF-α level (17.50 ± 7.32 vs. 9.55 ± 2.25 pg/mL in the sham group, $P < 0.01$), which was significantly decreased by PCA (250 and 500 mg/kg) (9.67 ± 1.19 and 8.49 ± 1.4 pg/mL, respectively, $P < 0.01$ each).

**PCA inhibited platelet aggregation induced by ADP following myocardial ischemia/reperfusion**

We also observed the effect of PCA on platelet aggregation in MI/R injury. The results in Fig. 4 showed that MI/R injury significantly caused a higher rate of platelet aggregation, in comparison to the sham group (64.87% ± 10.95% vs. 51.01% ± 5.87%, $P < 0.05$). Compared with the model control group, PCA (250 mg/kg) had a tendency to reduce platelet aggregation rate to 52.39% ± 7.07% ($P = 0.051$), and PCA (500 mg/kg) significantly decreased platelet aggregation rate to 35.68% ± 18.95% ($P < 0.01$).

**PCA inhibited hypoxia/reoxygenation-induced cardiomyocytes apoptosis**

To examine whether PCA has a direct protective effect on cardiomyocytes in MI/R injury, we observed the anti-apoptotic effect of PCA on cultured ventricle cardiomyocytes subjected to H/R, which simulates MI/R in vivo. The toxicity of PCA in vitro had been evaluated by using the MTT assay. Compared with the normal control group, PCA at concentrations from 25 to 400 μg/mL did not induce any significant decrease in cell viability and apparent change in cell morphology (Supplementary Fig. 1: available in the online version only). As illustrated in Fig. 5, 3-h hypoxia and 6-h reoxygenation significantly induced an increase in cardiomyocyte apoptotic rate (25.83% ± 2.00% vs. 10.23% ± 1.42% in the normal control group, $P < 0.01$), which was significantly reduced by treatments with PCA (200 and 400 μg/mL) (20.77% ± 3.22% and 20.33% ± 2.02%, respectively, $P < 0.05$ each).

**The effects of PCA on the expressions of cleaved caspase-3 and phosphorylation of Akt in cardiomyocytes induced by hypoxia/reoxygenation**

We next investigated the expressions of cleaved caspase-3 and phosphorylation of Akt (Ser473). Treatments with PCA (200 and 400 μg/mL) in normal cardiomyocytes have no significant effects on the expressions of cleaved caspase-3 and phosphorylation of Akt (Supplementary Fig. 2 and 3: available in the online version only). As shown in Fig. 6, western blot analysis revealed that the expression of cleaved caspase-3 in cardiomyocytes subjected to 3-h hypoxia and 6-h reoxygenation injury was significantly upregulated ($P < 0.05$ vs. the normal control group), which was significantly inhibited by treatments with PCA (200 and 400 μg/mL) ($P < 0.05$ each). As illustrated in Fig. 7, western blot results demonstrated no significant difference in total Akt expression levels among all groups, and no significant difference of Akt phosphorylation levels between the normal control group and the model control group, while treatments with PCA (200 and 400 μg/mL) significantly increased Akt phosphorylation levels ($P < 0.05$ vs. the model control group, respectively).

**Discussion**

In the present study, our results first demonstrated that PCA could reduce myocardial infarcts and interfere with the following MI/R pathogenic procedures including the inflammatory response, platelet aggregation, and cardiomyocyte apoptosis.
Fig. 2. A representative N-BT staining of infarct size. The normal myocardium was stained dark, and the ischemic area was stained red.

Fig. 3. PCA decreased serum TNF-α level in rats subjected to myocardial ischemia/reperfusion. Data are shown as the mean ± S.D.  

\(^{##}P < 0.01\) vs. Sham, \(^{**}P < 0.01\) vs. Model control (n = 10).

Fig. 4. PCA inhibited platelet aggregation induced by ADP following myocardial ischemia/reperfusion. Data are shown as the mean ± S.D.  

\(^{#}P < 0.05\) vs. Sham, \(^{**}P < 0.01\) vs. Model control (n = 10).

Fig. 5. PCA inhibited hypoxia/reoxygenation-induced cardiomyocyte apoptosis. Cardiomyocytes were subjected to 3-h hypoxia and 6-h reoxygenation in the presence or absence of PCA. A. Flow cytometric analysis of cardiomyocyte apoptosis in each group. B. Percentage of apoptotic cells per total number of cardiomyocytes in each group. Data are shown as the mean ± S.D.  

\(^{##}P < 0.01\) vs. Normal control, \(^*P < 0.05\) vs. Model control (n = 3).
Cardioprotection of PCA on MI/R Injury

Diltiazem, a selective L-type Ca\(^{2+}\)-channel blocker, has been reported to be effective in treating myocardial infarction diseases (15). Clopidogrel, a thienopyridine derivative, and a selective and irreversible antagonist of ADP (P2Y\(_{12}\)) receptor, is a commonly used drug in the treatment of ischemic heart disease (20 – 22). Therefore, we chose these two drugs as positive controls in the in vivo experiments. As expected, our results showed that both diltiazem (16 mg/kg) and clopidogrel (13.5 mg/kg) could significantly reduce myocardial infarcts. A previous study indicated that PCA at 50 mg/kg (i.v.) reduced myocardial infarct size in anesthetized dogs that underwent left anterior descending coronary artery ligation for 24 h (13). Thus, we chose the doses of PCA as 250 and 500 mg/kg through dose conversion. Our in vivo experiment results showed that the effect of PCA (500 mg/kg) was a little stronger than the two positive controls, although the dose of PCA at 500 mg/kg used in this study was 31 and 37 times of the doses of diltiazem and clopidogrel, respectively. Furthermore, PCA has the characteristic of low toxicity in vivo. No apparent toxicity was observed in rats administrated PCA at doses of 250, 500, and 1000 mg/kg (23) or in mice given PCA at maximum dosage of 5 g/kg (24). In addition, PCA can be metabolized into prototype and vanillic acids, which were demonstrated to have protective effects in isoproterenol-induced cardiotoxic rats (25). Therefore, our results still furnish strong evidence that PCA has a novel therapeutic potential for MI/R injury.

Both the inflammatory response and enhanced platelet aggregation have been implicated in MI/R injury and are crucial factors in the pathogenesis of ischemic heart disease (26). The production and release of proinflammatory cytokines are significantly increased during MI/R. It is worth mentioning that TNF-\(\alpha\) is one of the most important proinflammatory cytokines. Platelet activation and aggregation usually leads to an enhanced rate of thromboembolic events in ischemic heart disease (27). Moreover, activated platelets express inflammatory mediators like sCD40L and CD62 P-selectin, resulting in the inflammatory response; thus the combined effects of these two factors further aggravated MI/R injury (20, 28). Considerable evidence demonstrates that PCA has the pharmacological effects of anti-inflammatory response and anti-platelet aggregation. Stumpf et al. (29) reported that PCA significantly inhibited TNF-\(\alpha\)-induced expression and release of adhesion molecules, cytokines, and chemokines as well as ADP-induced expression of platelet P-selectin in human vascular endothelial cells. A recent study also demonstrated that the anti-platelet and anti-thrombotic effect of PCA is mediated by selectively inhibiting shear-induced platelet aggregation through the blockade of the interaction between von Willebrand...
factor (vWF) and platelet receptor GPIb (5). Consistent with these studies, our data showed that PCA could also inhibit the inflammatory response and the enhanced platelet aggregation in MI/R. These findings further indicate that the cardioprotective effect of PCA may be partly related to its inhibition of the inflammatory response and platelet aggregation.

Cardiomyocyte apoptosis plays an important role in the process of cell death subsequent to MI/R injury. Lowering the occurrence of cardiomyocyte apoptosis could significantly prevent MI/R injury (30, 31). Previous studies indicate that PCA has double-edged effects on apoptosis depending on the type of cells: it induced apoptosis in many types of cancer cells, such as human breast cancer, lung cancer, and prostate cancer cell, etc. (9), while it inhibited apoptosis in neural stem cells (32). A recent study suggested that PCA prevented hypertension-enhanced cardiac Fas-dependent, and mitochondria-dependent apoptotic pathways and enhanced the cardiac pro-survival pathway in rat models (33). Our results showed that PCA significantly decreased hypoxia/reoxygenation-induced cardiomyocyte apoptotic rate and the expression of cleaved caspase-3. Akt protein kinase is an important mediator of cardiomyocyte growth and survival (34, 35). Previous studies indicated that activation of Akt could suppress apoptosis and promote survival of cardiomyocytes in ischemic heart disease (36). To investigate whether Akt is involved in PCA-induced cardioprotection, we evaluated the expression of Akt and its activated form, Akt phosphorylated at Ser473, in cardiomyocytes subjected to hypoxia/reoxygenation. Our results clearly showed that PCA significantly upregulated the expression of phosphorylated Akt. All these data in vitro support the conclusion that the cardioprotection of PCA is also related to preventing cardiomyocytes from apoptosis induced by MI/R through activation of Akt.

In summary, the present study demonstrates that PCA has a protective effect on MI/R injury, and the underlying mechanism may be associated with its anti-inflammatory response, anti-platelet aggregation, and preventing cardiomyocytes from apoptosis through activation of Akt.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

Cardioprotection of PCA on MI/R Injury