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ORIGINAL ARTICLE

In Vivo Cardioprotective Effects and Pharmacokinetic Profile of *N*-Propyl Caffeamide Against Ischemia Reperfusion Injury

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Abstract Caffeic acid derivatives constitute a class of potent anti-inflammatory and cardioprotective drug candidates. We recently synthesized a new caffeic acid derivative *N*-propyl caffeamide (PCA). Our pilot experiments demonstrated that PCA enhanced the survival of rat cardiomyocyte H9c2 cells against oxygen glucose deprivation and reoxygenation challenge in a concentration-dependent manner. Interestingly, PCA exhibited better cardioprotective potential than caffeic acid phenethyl ester and propyl caffeate. Thus, we hypothesized that PCA could protect heart against ischemia reperfusion (I/R) injury in mice. We first determined the stability and pharmacokinetic profile of PCA in male Sprague–Dawley rats by ultra-performance liquid chromatography coupled with UV and MS/MS detections. The stability of PCA in rat plasma was defined by the half-life of 31.39, 7.19 and 1.37 h in rat plasma at 25, 37 and 60 °C, respectively. To study the pharmacokinetic profiles, PCA was injected into male SD rats at the dose of 15 mg/kg via intravenous bolus administration. PCA showed the elimination half-life of approximate 235 min in rats. We subsequently evaluated the cardioprotective potential of PCA in mice model of myocardial infarction. Our results demonstrated that PCA effectively reduced infarct size and release of myocardial enzymes (e.g., CK, CK-MB and LDH). Biochemical analyses suggested that PCA increased the activities of antioxidant enzymes (e.g., CAT and SOD) while attenuated lipid peroxidation. Moreover, PCA profoundly reduced the number of apoptotic cells in infarcted myocardium. Consistently, PCA increased the expression level of anti-apoptotic protein Bcl2 whereas suppressed the expression of pro-apoptotic protein Bax in cardiac tissues. Collectively, PCA appears to be a novel bioavailable and stable pharmacological treatment for myocardial infarction.

Keywords *N*-Propyl caffeamide · Bioavailability · Pharmacokinetics · Cardioprotection · Ischemia reperfusion injury

Abbreviations

AAR	Area at risk
BOP	Benzo-triazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate
BSA	Bovine serum albumin
CAPE	Caffeic acid phenethyl ester
CAT	Catalase
CK	Creatine kinase
CK-MB	Myocardial muscle creatine kinase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
ESI-MS	Slectrospray ionization-mass spectrometry
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HRP	Horseradish peroxidase
IgG	Immunoglobulin G

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LC/MS/MS	Liquid chromatography/mass spectrometry/mass spectrometry
LAD	Left anterior descending coronary artery
LDH	Lactate dehydrogenase
MPO	Myeloperoxidase
OGD	Oxygen glucose deprivation
PCA	*N*-Propyl caffeate amide
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SOD	Superoxide dismutase
TTC	Triphenyltetrazolium chloride

Introduction

Myocardial infarction is a leading cause of morbidity and mortality worldwide (Bonow et al. 2002; Yellon and Hausenloy 2007). In the patients with myocardial infarction, ischemia and reperfusion trigger a sequence of inflammatory reactions involving the infiltration, activation, apoptosis and clearance of inflammatory leukocytes (e.g., neutrophils and macrophages) (Jordan et al. 1999). Neutrophils and macrophages not only cause tissue damage but also contribute to wound healing, cardiac remodeling and scar formation (Jordan et al. 1999; Nahrendorf et al. 2010). Upon reperfusion, neutrophils are rapidly recruited into infarcted hearts and activated to release reactive oxygen species (ROS), reactive nitrogen species (RNS), proteases, and specific chemoattractant mediators (Jordan et al. 1999). Different forms of ROS cause oxidative damage of proteins and lipids, leading to apoptotic cell death in myocardium and dysfunctions of heart. Previous studies suggested that neutrophil depletion dramatically reduced infarct size in animal models of myocardial infarction (Jolly et al. 1986; Litt et al. 1989).

Caffeic acid and related derivatives are well-known to exhibit anti-oxidant, anti-inflammatory, chemopreventive, anticancer and antibacterial properties in a structure-dependent and cell-type-specific manner (da Cunha et al. 2004; Fiuza et al. 2004; Rajan et al. 2001). As an example, caffeic acid phenethyl ester (CAPE) was initially isolated from propolis, a honeybee hive product as a specific NF-κB inhibitor (Natarajan et al. 1996). Several recent studies demonstrated that CAPE could effectively attenuate ischemia reperfusion (I/R) injury in different in vitro and in vivo models (Kart et al. 2009; Koltuksuz et al. 1999; Wei et al. 2004). However, caffeic acid esters are not stable enough and have short life-time in male Sprague–Dawley (SD) rats (Wang et al. 2007). For this reason, several different caffeic acid amide derivatives including *N*-methyl, *N*-propargyl, *N*-anilide, *N*-phenethyl and pyrrolidinyl caffeamide have been developed and partly evaluated for cardioprotective potential in the treatment of myocardial infarction (Ho et al. 2014; Lee et al. 2015; Rajan et al. 2001). Nevertheless, the pharmacological and toxicological profiles of these caffeic acid derivatives have not been fully characterized.

In this study, we developed a chemical procedure to synthesize *N*-propyl caffeate amide (PCA) as a potential structurally simple and pharmacologically stable caffeic acid derivative as described in Fig. 1a. We investigated the cardioprotective effect of PCA in cardiomyocyte H9c2 cells against oxygen glucose deprivation (OGD) and reoxygenation challenge, and also in a rat model of acute myocardial infarction by the ligation of left coronary artery. We also developed a rapid ultra-performance liquid chromatography (UPLC) method and liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) method to determine the stability and pharmacokinetic profile of PCA.

Materials and Methods

Biochemical Reagents

Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), caspase-3, Bax and Bcl2 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Protein Assay Dye Reagent Concentrate was purchased from Bio-Rad (Hercules, CA, USA). Select chemiluminescence (ECL) detection kit was purchased from GE Healthcare (Uppsala, Sweden). PCE was a synthetic product from Hangzhou Yuhao Chemical Technology Co., Ltd (Zhejiang, China). CAPE was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Caffeic acid and other biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Animal Husbandry

Male C57BL/6N mice (6–8 weeks, 20 ± 5 g) and male SD rats (7–8 weeks, 250 ± 20 g) were obtained from Laboratory Animal Unit, University of Hong Kong (China). All experimental procedures were in compliance with the guidelines of the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Animals were housed in a temperature- and humidity-controlled environment on a 12 h light–dark cycle, and allowed free access to standard laboratory mice chow and drinking water. Animals were acclimated in the laboratory

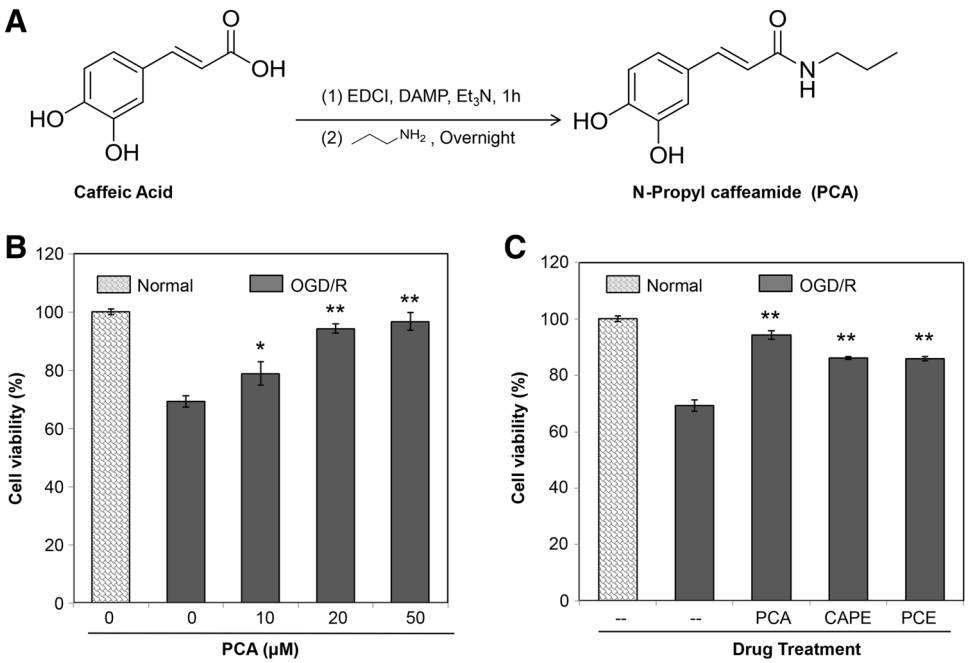


Fig. 1 Chemical synthesis and in vitro cytoprotective effect of PCA. **a** Synthesis of PCA via direct amidation of caffeic acid with propylamine. *BOP* benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate, CH_2Cl_2 dichlormethane, Et_3N trimethylamine. **b** Effect of PCA on the survival of H9c2 cells against OGD and reoxygenation challenge. H9c2 cells were exposed to OGD condition and subsequent reoxygenation under normal growth condition in the absence or presence of PCA with varying concentrations. The cell viability was determined by colorimetric MTT assay. The results were expressed as mean ± SD from three independent experiments. $*p < 0.05$ and $**p < 0.01$ (PCA + OGD/R vs OGD/R). **c** Different cytoprotective effect of PCA, CAPE and PCE. H9c2 cells were exposed to OGD condition and subsequent reoxygenation under normal growth condition in the absence or presence of caffeic acid derivatives at the concentration of 20 μM. The results were expressed as mean ± SD of three independent experiments. $*p < 0.05$ and $**p < 0.01$ (Drug + OGD/R vs OGD/R)

for 7 days prior to the experiment and fasted for 12 h but allowed water before experiments.

Heparinized male SD rat plasma was obtained from Laboratorial Animal Unit, The University of Hong Kong. Water used in the experiments was passed through a Millipore Milli-Q water purification system.

Synthesis and Chemical Characterization of PCA

Mixtures of 100 mg caffeic acid, 1.2 eq propylamine and 0.08 mL Et_3N were dissolved in 1 mL dichloromethane. While stirring on ice, a solution of benzotriazol-1-yloxytris (dimethylamino)phosphonium hexafluorophosphate (BOP) (1.2 equiv) in 5 mL dichloromethane was subsequently added into caffeic acid solution. The reaction mixture was stirred at 0 °C for 30 min and at room temperature overnight. At the end of reaction, the solvent was removed by rotary evaporator under vacuum. The residues were extracted five times with ethyl acetate (EtOAc). The organic phase was sequentially washed with 3 M HCl aqueous solution, and 10 % $NaHCO_3$ in water. The product PCA was purified by column chromatography on silica gel with chloroform and methanol (PE:EtOAc = 1:1.5) to yield a yellow powder (86 mg, 70 %). PCA was further characterized by mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy. MS analysis was performed on an ABI/Sciex triple quadrupole 3200 QTRAP mass spectrometer (Framingham, MA, USA) equipped with an TurboV Source operating in positive ionization mode under the control of Analyst v1.4.2 data system (Applied Biosystems/MDS Sciex, Concord, ON, Canada). ESI–MS (m/z): 222.1 $[M+H]^+$. HRMS for $C_{12}H_{15}NO_3$: calculated, 222.1125 $[M+H]^+$; experimental, 222.1127 $[M+H]^+$. NMR spectra were recorded in DMSO-d6 on a *Varian Unity plus NMR* 600 MHz spectrometer (Varian Inc., Palo Alto, CA, USA). ^{1}H NMR (DMSO-d6, 600 MHz): 9.22 (s, 2-OH), 7.95 (t, $J = 5.6$ Hz, 1-NH), 7.22 (d, $J = 15.7$ Hz, 1H), 6.94 (d, $J = 1.9$ Hz, 1H), 6.83 (dd, $J = 8.2$, 1.9 Hz, 1H), 6.74 (d, $J = 8.2$ Hz, 1H), 6.33 (d, $J = 15.7$ Hz, 1H), 3.11 (m, 2H), 1.46 (m, 2H), 0.87 (t, $J = 7.4$, 3H); and ^{13}C NMR (DMSO-d6, 150 MHz): 165.8, 147.7, 146.0, 139.3, 126.9, 120.7, 119.2, 116.2, 114.3, 40.9, 22.9, 11.9.

Measurement of Cell Viability

Rat cardiomyocyte H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with

2 mM glutamine, antibiotics (100 U/mL of penicillin A and 100 U/mL of streptomycin), and 10 % heat-inactivated fetal bovine serum (Gibco/BRL, Gaithersburg, MD, USA) and maintained in a 37 °C humidified incubator containing 5 % CO_2. For drug treatment, H9c2 cells were seeded at the density of 1.0×10^5 in a 96-well plate. After overnight incubation, the cells were incubated in degassed and glucose-free DMEM, and subsequently exposed to OGD condition in an anaerobic chamber saturated with 5 % CO_2 and 95 % N_2 (v/v) at 37 °C for 10 h. The cells were subsequently treated with PCA, CAPE and PCE in complete growth medium under normoxia condition for another 14 h. The cell viability was evaluated by a colorimetric assay of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction. Briefly, at the end of drug treatment, MTT (final concentration of 0.5 mg/mL) was added to cell culture in a 96-well plate. After 4 h incubation at 37 °C, the supernatant was removed. The formazan products in viable cells were solubilized in 150 μL of DMSO and quantified by measuring the absorbance at 570 nm on a Bio-Rad microplate reader (Hercules, CA, USA).

In Vitro Stability of PCA

The in vitro stability of PCA was studied by incubating the drug with rat plasma as previously described (Yang et al. 2012). Plasma was collected from male SD rats (body weight 250 ± 20 g). PCA (10 μL, 2 mg/mL) was incubated with rat plasma (180 μL) at 25, 37 and 60 °C for different times. After adding 10 μL of the internal control resveratrol solution (400 μg/mL), the samples were immediately extracted with 600 μL of ethyl acetate. The solvent was removed by passing a stream of nitrogen at 25 °C. The samples were reconstituted and mixed in 200 μL of MeOH. After brief centrifugation, the supernatants were transferred to glass tubes with caps (Agilent Inc, USA). The methanolic solution (5 μL) was analyzed through a C_{18} reverse phase UPLC column (5 μ, 4.6 × 150 mm) under the control of a Thermo (Thermo Scientific, USA) DIONEX series LC system. The UPLC system was equipped with a ultimate 3000 RS pump, 3000 RS auto-sampler, 3000 RS column compartment along with an 3000 RS diode array DAD detector. The flow rate was set at 0.4 mL/min. Detection wavelength was set to UV 320 nm. The concentration of intact PCA was determined at minimal five time points for each temperature condition. Three replicates were analyzed at each time point. The stability of PCA was correlated with the half-life of PCA at each respective temperature and the energy of activation was also calculated.

Pharmacokinetics of PCA

The pharmacokinetic profile of PCA was studied in male SD rats. A solution of PCA (0.8 mL, 15 mg/kg) in 80 % sterile saline and 20 % ethanol was administrated intravenously over a period of 20 s via femoral vein. Blood samples (0.3 mL) were collected into 1.5 mL tubes before injection, at 5, 15, 30 min, 1, 2, 4, 6 and 8 h after injection. After immediate centrifugation at 5700 rpm for 10 min, the plasma samples were recovered and stored at −80 °C until analysis.

For LC/MS/MS analysis, plasma sample (100 μL) was mixed with 200 ng of resveratrol as internal standard (IS) in 200 μL acetonitrile. The samples were vortexed for 5 min and then centrifuged at 13,000 rpm for 10 min. The supernatants were evaporated and reconstitution with 100 μL of MeOH. The methanolic solution (20 μL) was subsequently analyzed on a Synergi hydro-RP C18 column (150 × 4.6 mm, 4 μm) at the flow rate of 0.8 mL/min. The separation was operated under the control of an Agilent HPLC system equipped with a 1525 Separations Module and a 2998 DAD Unit. Mobile phase compositions were (A) water with 0.4 % (v/v) formic acid and (B) acetonitrile. Gradient was set as follows: 0–2 min, 80 % A; 2–5 min, 80–50 % A; 5–7 min, 50–80 % A; 7–8 min, 80 % A. The elution was analyzed on an ABI/Sciex triple quadrupole mass spectrometer 3200 QTRAP system (Framingham, MA, USA). The MS system was equipped with an ESI-Turbo V source operating in positive ionization mode under the control of Analyst v1.4.2 data system (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Mass spectrometry was operated under the conditions as follows: drying gas N_2, 10 L/min; capillary voltage, 20 V; pressure of nebulizer, 30 psi; ion spray voltage, 5.5 kV; and capillary temperature, 325 °C. Multiple reaction monitoring (MRM) parameters were optimized for analysis of PCA and IS, respectively. Analyst 1.5.1 software (AB Sciex, Foster, CA, USA) was used for the control of equipment, data acquisition and analysis. Plasma PCA concentration vs time data for each rat was analyzed by Kinetica 2000 software (Version 3.0).

Evaluation of Infarct Size and Cardiac Damage after Myocardial Infarction

Myocardial infarction was induced by the ligation of the left anterior descending coronary artery (LAD) in male C57BL/6N mice as previously described (Xu et al. 2014). Mice were randomly divided into the following four groups ($n = 13$): (1) Sham, receiving sham surgery and intraperitoneal (i.p.) injection of vehicle (a mixture of 50 % saline and 50 %1,2-propanodiol); (2) I/R, receiving 30 min ligation of coronary artery, 24 h reperfusion and i.p.

injection of vehicle (i.p.); (3) I/R + PCA5, receiving 30 min ligation of coronary artery, 24 h reperfusion and i.p. injection of 5 mg/kg PCA at a time point of 10 min after ischemia; (4) I/R + PCA20, receiving 30 min ligation of coronary artery, 24 h reperfusion and i.p. injection of 20 mg/kg PCA at a time point of 10 min after ischemia. To induce myocardial infarction, mice were anesthetized by i.p. injection of a mixture of midazolam (5 mg/kg), fentanyl (0.5 mg/kg) and medetomidine (0.05 mg/kg) and ventilated under a mouse volume-control ventilator. Following thoracotomy between the third and fourth intercostal space, a surgery was performed to expose the heart. The left main coronary artery was ligated with a 6–0 silk suture for 30 min. At 10 min after ligation, PCA was administered via i.p. injection, whereas equal volume of vehicle was injected into the animals in Sham and I/R groups. After 30 min ischemia, the suture was loosened to allow reperfusion in the mice for the recovery from anesthetization for 24 h. At the end of I/R operation, the LAD was relegated and Evans blue dye was injected to visualize the area at risk (AAR) of infarction showing no infiltration of Evans blue dye. Five slices produced by cutting the myocardium were staining in 0.5 % triphenyltetrazolium chloride (TTC) for 15 min, and infarct area (IA), which was not stained with TTC, was assessed. The areas of AAR and IA were quantified by computerized planimetry of digital images using a free downloadable NIH Image J software.

For hematoxylin and eosin (H&E) staining, the fresh heart tissues in each group ($n = 3$) were fixed in 4 % formalin and embedded in paraffin. The embedded tissues were sectioned at the thickness of 4 μm and stained with H&E stain for detailed observation of the potential pathological changes.

Determination of the Cardiac Enzymes and Oxidative Stress Biomarkers

To determine the release of myocardial muscle creatine kinase (CK-MB), creatine kinase (CK) and lactate dehydrogenase (LDH), at the end of reperfusion, the blood samples ($n = 3$) were collected in heparinized tubes and centrifuged at 5700 rpm for 10 min. The supernatants were recovered for the measurement of CK-MB with a commercial ELISA kit from USCN Life Science Inc (Wuhan, China), CK and LDH using corresponding detection kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. To determine the levels of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and myeloperoxidase (MPO), on the other hand, the heart tissues ($n = 3$) were harvested and homogenized in 0.9 % NaCl saline buffer. After centrifugation, the supernatants were collected for the measurement of SOD, CAT, MDA and MPO with specific commercial assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. The activities of SOD and CAT were expressed as U/mg proteins. The MDA level was represented in nmol/mg proteins. MPO activity was expressed as U/mg wet tissues.

TUNEL Staining of Myocardial Apoptosis

Formalin-fixed heart tissues were embedded in paraffin and sectioned into 4 μm thick slices ($n = 3$/group). The sections were deparaffinized, rehydrated and stained with TUNEL kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The sections were imaged on a Leica DME Basic Microscope (Hicksville, NY, USA) with Photometrics CoolSNAP Camera (Tucson, AZ, USA). The apoptotic cells and the overall cell nuclei in the heart tissue were counted. The positive-cells were calculated from five random field of view on the images with 400× magnification. All these images were collected and counted in a blinded fashion.

Western Blot Analysis of Apoptosis Biomarkers

Myocardial tissues were homogenized in ice-cold RIPA buffer (Sigma St. Louis, MO, USA) containing proteinase inhibitor for protein extraction. For the detection of protein expression, 70 μg of the cardiac proteins were resolved in 12 % SDS-PAGE, and subsequently transferred onto polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were incubated in tris-buffered saline buffer (pH 7.6) containing 0.1 % Tween-20 and 5 % BSA for 2 h at room temperature. After several washes, membranes were probed with specific primary antibodies against Bcl-2, Bax and GAPDH (1:500 dilution) overnight at 4 °C. Following the removal of excessive non-bound antibodies, membranes were detected with HRP-conjugated secondary antibody (1:1000 dilution) for another 3 h at 4 °C. The bound antibodies were visualized with enhanced chemiluminescence reagents (GE Healthcare, Uppsala, Sweden).

Statistical Analysis

The results were presented as mean ± SD from the indicated experiments. The data were compared by one-way analysis of variance (ANOVA), followed by least significant difference post hoc test using IBM software SPSS version 20.0 (Amonk, NY, USA). Differences with $p < 0.05$ were considered as statistically significant.

Results

PCA Enhanced the Survival of Cardiomyocytes Under OGD and Reoxygenation Condition

To develop new structurally simple and pharmacologically stable caffeic acid derivatives, we synthesized PCA in 70 % yield by direct amidation of caffeic acid with propylamine as outlined in Fig. 1a. The chemical structure of PCA was verified by NMR and MS techniques. We explored the effect of PCA on the survival of cardiomyocyte H9c2 cells under OGD and reoxygenation condition as an in vitro model of I/R injury. In practical, H9c2 cells were exposed to OGD condition for 10 h, and subsequent reoxygenation condition with/without drugs in normal cell culture medium for 14 h. The cell viability of H9c2 was determined by a colorimetric MTT assay as described (Cheng et al. 2016). As shown in Fig. 1b, PCA enhanced the survival of H9c2 cells against OGD and reoxygenation challenge in a concentration-dependent manner. Interestingly, by examining the cytoprotective effects of PCA with CAPE and PCE at the same concentration (20 μM), PCA showed better cytoprotective effect than CAPE and PCE (Fig. 1c).

Characterization of Pharmacological Stability by an Ultra-Fast UPLC-UV Method

To characterize the stability of PCA in plasma, we developed an ultra-fast UPLC-UV method to determine the intact PCA after the incubation with fresh rat plasma for different time points at 25, 37 and 60 °C. We excluded any endogenous interference that could be co-eluted with PCA and IS under the experimental chromatographic conditions. Prior to the actual chromatographic determination, we established a calibration curve (slope = 0.4608, $R = 0.9995$) by five replicated determinations of PCA at six concentrations of 2.5, 5, 10, 50, 100 and 250 μg/mL. We satisfied with the overall reproducibility of the method for the deviation within 13.01 %. By calculating the ratio of analyte response from plasma extracted samples to unextracted samples, the absolute recovery of PCA was ranged 91.8–98.9 %. The intra-day and inter-day precisions were below 9.98 % for PCA in terms of relative standard deviation (RSD; shown in Table 1). By plotting the natural logarithm (Ln) of the percentage remaining PCA (Ln % Remaining PCA) against time, we obtained the stability profiles of PCA as shown in Fig. 2a. Based on linear regression on these data points, we calculated the first-order rate constant of decomposition (k, h^{-1}) as 0.0302, 0.1181, 0.4879 for 25, 37 and 60 °C, respectively. Similarly, we obtained the half-life ($t_{1/2}$, h) of PCA in Table 2 as 31.39, 7.19, 1.37 for 25, 37 and 60 °C, respectively. By applying the Arrhenius equation, we calculated the energy of activation of 16.7 kcal/mol for PCA ($R^2 = 0.981$). In addition, the extraction of 100 μg/mL PCA in plasma allowed the recovery of drug at 97.9 % (1.11 % RSD) following three freeze–thaw cycles compared with the analysis of samples prior to freezing. In the study of long term stability, PCA samples were recovered at 96.4 % (3.72 % RSD).

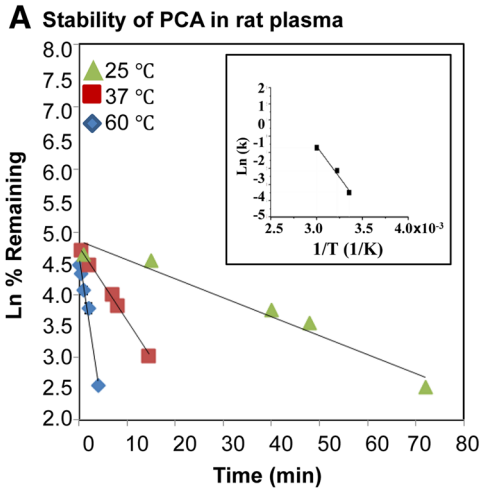
Determination of Pharmacokinetic Profile by a LC–MS/MS Method

To determine the pharmacokinetic profile of PCA, we developed a LC–MS/MS method for the detection of PCA (m/z, 222 to >163) and IS (m/z, 229 to >135) in MRM mode (shown in Table 3). We first confirmed that the detection was highly selective for PCA and IS at the retention time of 5.93 and 6.45 min, respectively, while no interference was detectable from the endogenous substances. We subsequently established the calibration curve with good linearity ($y = 31.926x$, $R^2 = 0.9948$) over the concentration range of 2–3000 ng/mL in rat plasma. Based on the assessment of the sensitivity, precision and accuracy in Table 4, we concluded that such a LC–MS/MS assay was accurate and reproducible for the determination of PCA in rat plasma. Finally, we successfully applied the LC–MS/MS method to study the pharmacokinetics of PCA in SD rats following i.v. administration of 15 mg/kg PCA. By plotting the PCA concentrations against the detection time as shown in Fig. 2b, we obtained the pharmacokinetic parameters for PCA in Table 5 as follows: elimination half-life ($t_{1/2}$), 235.02 ± 56.67 min; $AUC_{0-\infty}$, 1225.14 ± 162.39 ng h/mL; AUC_{0-t}, 1346.06 ± 161.99 ng h/mL;

Table 1 Method validation for HPLC–DAD detection of PCA

Nominal concentration (μg/mL)	Observed concentration (μg/mL; average ± SD)	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Accuracy (% deviation)	Absolute recovery (% RSD)
2.5	2.19 ± 0.127	2.00–9.60	9.98	13.01	91.8 (4.7)
50	45.65 ± 0.95	0.98–2.01	3.63	4.21	95.1 (5.7)
250	252.47 ± 1.78	1.5–2.09	4.36	1.44	98.9 (0.5)

HPLC–DAD high performance liquid chromatography–diode array detector



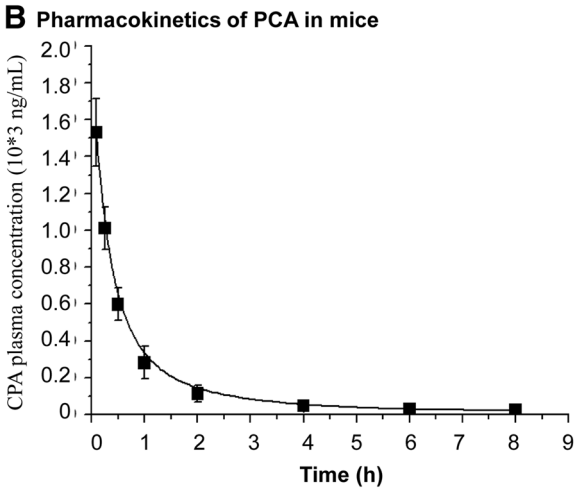


Fig. 2 Stability and pharmacokinetic profiles of PCA. **a** Stability of PCA in rat serum. PCA was incubated in rat serum at different temperature [i.e., 25 °C (*solid triangles*), 37 °C (*solid squares*), and 60 °C (*solid rhombus*)] for different time points. The remaining PCA was quantified by HPLC–UV method. **b** Pharmacokinetic profile of PCA in male Sprague–Dawley rats. PCA (15 mg/kg) was administrated to male Sprague–Dawley rats via intravenous injection. Plasma samples were collected at different time points and determined by HPLC–MS method. Data are presented as mean ± SD ($n = 3$)

Table 2 Rate constant, half-life, and activation energy of PCA

Temperature (°C)	k (h^{-1})	$t_{1/2}$ (h)	*Ea* (kcal/mol)
25	0.0302	31.39	16.7
37	0.1181	7.19	
60	0.4879	1.37	

Ea energy of activation

Table 3 Parameters for MRM-based analysis

Components	RT	MRM	CE (eV)	DP (eV)
CPA	5.93	222–163	19	51
RE	6.45	229–135	29	236

total plasma clearance (CL_t), 53.06 ± 7.79 mL/min; mean volume of distribution (V_d), 17.99 ± 6.12 L; MRT_{0-t}, 2.73 ± 0.64 h.

PCA Reduced Infarct Size and Cardiac Damage in Mouse Model of Myocardial Infarction

The in vivo cardioprotective potential of PCA was evaluated in a mouse model of myocardial infarction as illustrated in Fig. 3a. PCA was administrated by i.p. injection 10 min after ischemia was induced by the ligation of left coronary artery. Twenty minutes later, animals were subjected to reperfusion for 24 h. Based on TTC staining of viable myocardium in Fig. 3b, ischemia and reperfusion (I/R) induced ~42 % of myocardial infarction relative to the AAR. PCA treatment attenuated myocardial infarction in a dose-dependent manner (Fig. 3c). PCA at the dose of 20 mg/kg effectively reduced myocardial infarction to ~16 % relative to the AAR. Consistently, H&E staining in Fig. 3d showed that I/R challenge disrupted the organization of myocardial fibers compared with sham surgery. I/R challenge appeared to induce the infiltration of polymorphonuclear leukocytes into the myocardial interstitial space compared with sham surgery. Interestingly, PCA not only preserved the structure of myocardial fibers but also appeared to limit the infiltration of polymorphonuclear leukocytes compared to I/R challenge.

PCA Diminished the Release of Cardiac Enzymes and Oxidative Stress

To verify the effect of PCA on I/R-induced cardiac damage, we determined the release of cardiac enzymes including CK-MB, CK and LDH into the circulation. As shown in Fig. 4a–c, I/R challenge indeed markedly increased the serum levels of CK-MB, CK and LDH compared with sham surgery. However, administration of PCA significantly suppressed the level of LDH, CK and CK-MB (I/R-PCA vs I/R).

To examine the antioxidant effects of PCA against myocardial injury, we harvested the hearts from each experimental group, and analyzed for the levels of CAT, SOD and MDA. As shown in Fig. 4d–f, I/R challenge markedly decreased the enzymatic activities of CAT and SOD while increased the production of MDA in heart tissues. PCA pre-treatment could prevent the loss of CAT and SOD activities, and diminish the production of MDA.

Table 4 Method validation for LC/MS/MS detection of PCA

Nominal concentration (ng/mL)	Observed concentration (ng/mL; average ± SD)	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Accuracy (% deviation)
20	20.93 ± 0.92	4.44–7.12	4.35	11.6
500	520.1 ± 9.21	1.58–6.73	2.66	9.40
3000	2959 ± 8.21	0.75–1.53	1.20	1.42

LC/MS/MS liquid chromatography/mass spectrometry/mass spectrometry

Table 5 Pharmacokinetic parameters of PCA

$t_{1/2}$ (min)	235.02 ± 56.67
$AUC_{0-\infty}$ (ng h/mL)	1225.14 ± 162.39
AUC_{0-t} (ng h/mL)	1346.06 ± 161.99
CL_T (mL/min)	53.06 ± 7.79
V_d (L)	17.99 ± 6.12
MRT_{0-t} (h)	2.73 ± 0.64

To further clarify the effects of PCA on the infiltration of polymorphonuclear leukocytes, we harvested the hearts from each experimental group, and analyzed for the levels of MPO activity. As shown in Fig. 4g, I/R challenge markedly increased the enzymatic activity of MPO in heart tissues. PCA pre-treatment ameliorated the infiltration of polymorphonuclear leukocytes in response to I/R challenge.

PCA Reduced Myocardial Apoptosis via Regulating Bcl2 and Bax Expression

To understand the potential cardioprotective mechanism, we examined the effects of PCA on I/R-induced myocardial apoptosis by TUNEL assay. As shown in Fig. 5a, I/R challenge triggered apoptosis in cardiomyocytes. PCA pretreatment effectively protected cardiomyocytes against I/R-induced injury in a dose-dependent manner. Based on TUNEL staining, PCA profoundly reduces the ratio of

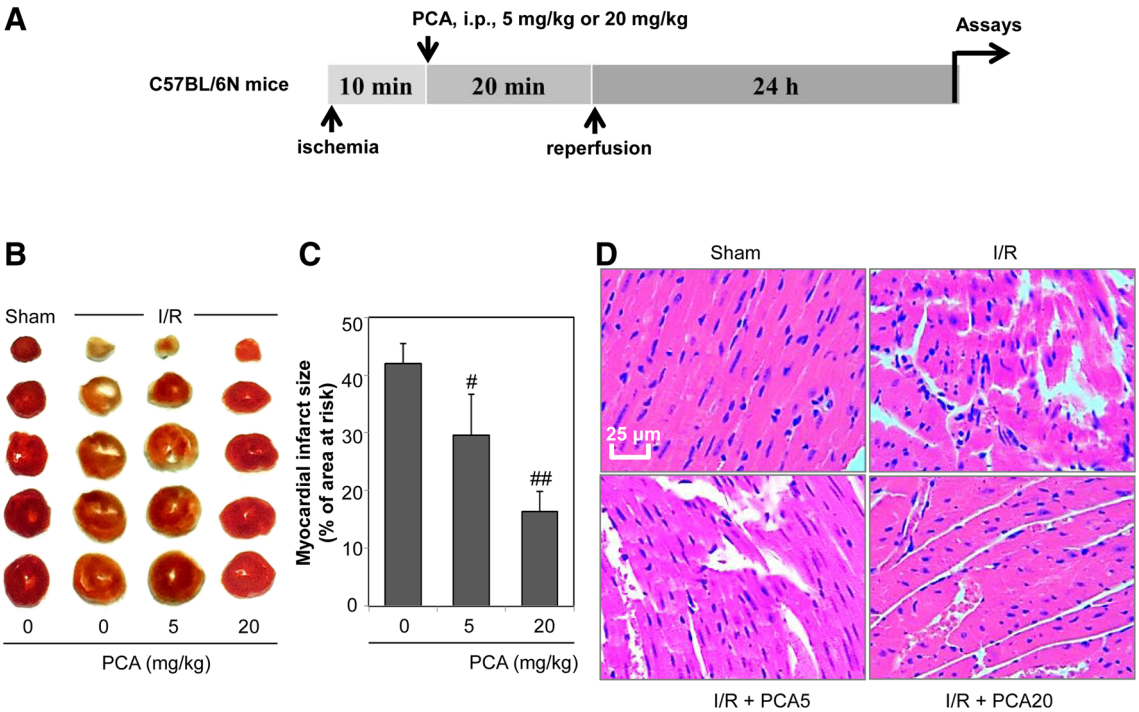


Fig. 3 Effect of PCA on infarct size and myocardial histology in mouse model of myocardial infarction. **a** Experimental design. The animals were randomly divided into four groups: Sham, I/R, I/R + PCA5 and I/R + PCA5. Acute myocardial infarction was induced by the ligation of the LAD artery as outlined. After the animals were euthanized, the hearts and blood samples were recovered for histological and biochemical assessments. **b** Detection of viable myocardium by TTC staining. **c** Quantification of I/R injury in hearts. Values represent mean ± SD (n 4). *$p < 0.05$; **$p < 0.01$ (PCA + I/R vs I/R). **d** Histological evaluation of I/R injury in hearts by H&E staining. *Scale bar* 25 μm

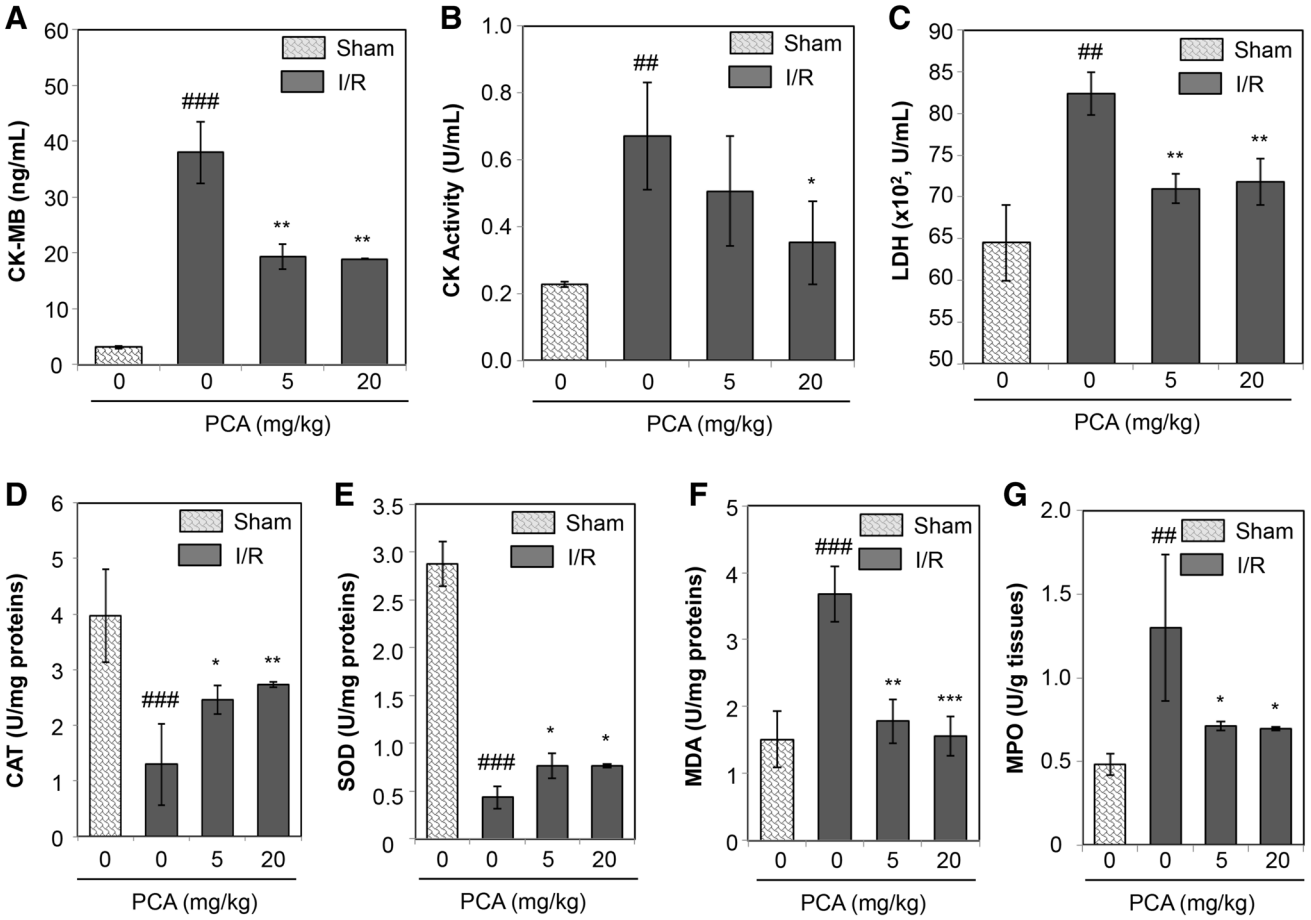


Fig. 4 Effects of PCA on the biomarkers of cardiac damage, oxidative stress and leukocyte infiltration. After 30 min ischemia and 24 h reperfusion, blood samples and heart tissues were collected. Serum levels of CK-MB (**a**), CK (**b**) and LDH (**c**) were determined by commercial assay kits as described in "Materials and methods". On the other hand, cardiac tissues were homogenized and determined for the levels of CAT (**d**), SOD (**e**), MDA (**f**) and MPO (**g**) by commercial assay kits as described in "Materials and methods". ($n = 3$) $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.01$ (I/R vs Sham); $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ (PCA + I/R vs I/R)

apoptotic nuclei in overall cardiomyocytes from 33.7 % for I/R group to 26.8 % for I/R + PCA5 group and 14.7 % for I/R + PCA20 group. To further explore the apoptotic mechanisms, we analyzed the protein levels of Bcl2 and Bax by Western blotting using specific antibodies. As shown in Fig. 4b, I/R challenge down-regulated the expression of anti-apoptotic protein Bcl2 while up-regulated the expression of pro-apoptotic protein Bax, causing a significant decrease in the ratio of Bcl2/Bax. PCA pre-treatment markedly up-regulated the expression of Bcl-2 while suppressed the expression of Bax, conferring a significant increase in the ratio of Bcl2/Bax for I/R + PCA20 group.

Discussion

The biological activities of caffeic acid derivatives are highly dependent on the chemical structures. For example, caffeic acid amide derivatives were found to be more stable in various biological systems. In this study, we synthesized a simple caffeic acid amide derivative PCA, and subsequently discovered that PCA exhibited better protection of cardiomyocyte H9c2 cells against OGD and reoxygenation challenge, compared with CAPE and PCE. These results stimulated us to evaluate the in vivo cardio-protective effects and pharmacokinetics of PCA. We further discovered that PCA effectively reduced cardiac damages in a mouse model of myocardial infarction possibly via suppressing oxidative stress and infiltration of polymorphonuclear leukocytes.

Ischemia induces oxidative stress in myocardial cells by stimulating ROS generation, but reperfusion recruits enormous polymorphonuclear leukocytes and further amplifies the production of toxic ROS (Chen and Zweier 2014). ROS is known to directly damage proteins and membrane lipids in cardiac tissues (Hori and Nishida 2009). Thus, the cardiac damage is often assessed by assaying different biomarkers. First, CK-MB, CK and LDH

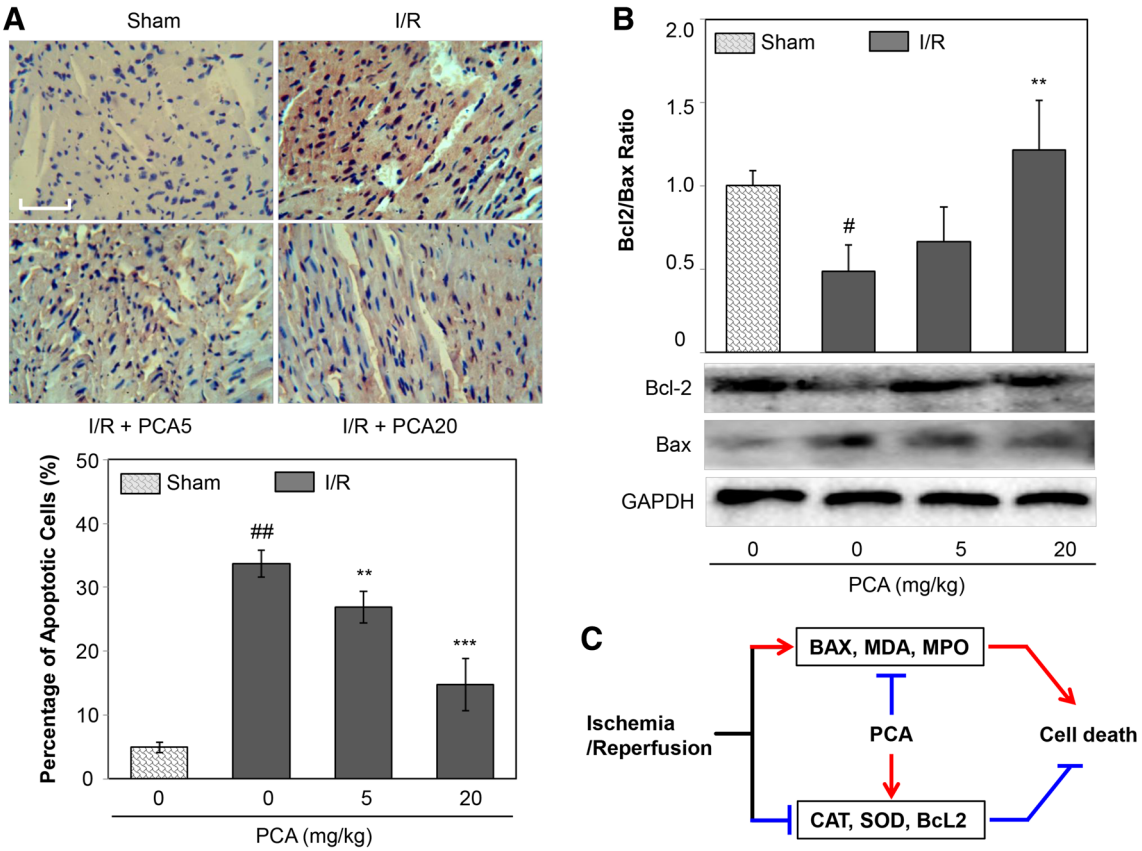


Fig. 5 Effects of PCA on the apoptosis of myocardial cells and the expression of Bcl-2 and Bax. **a** Representative images of TUNEL-stained myocardial tissues in different groups. *Scale bar* 50 μm. The images were quantified for TUNEL-positive cells ($n = 5$). $^{***}p < 0.001$ (treatment vs Sham group); $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.01$ (treatment vs I/R group). **b** Western blot analyses for Bcl-2 and Bax expression. The results were expressed as the ratio of Bcl-2/Bax ($n = 3$). $^{**}p < 0.01$ and $^{***}p < 0.001$ (treatment vs Sham group); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.01$ (treatment vs I/R group). **c** Proposed mechanisms underlying the cardioprotective effects of PCA. *Red arrow* activation, *blue line* inhibition

in serum have widely used as the biomarkers for the assessment of myocardial damage. In this study, I/R challenge elevated the serum levels of CK-MB, CK and LDH. PCA prevented the release of cardiac enzymes into the blood. Consistently, PCA effectively reduced infarct size and preserved the structure of myocardial fibers. Second, the oxidative damage of cell membrane is mainly mediated by the peroxidation of lipids into MDA. For this reason, MDA is another important biomarker to evaluate the oxidative damage of myocardial cells (Del Rio et al. 2005). Our results showed that I/R challenge significantly increased the formation of MDA. PCA almost abolished the production of MDA. Third, ROS overproduction overwhelms the antioxidant capacity in the heart. CAT and SOD are two key antioxidant enzymes for scavenging free radicals, serving as the first line defenders against oxidative tissue damage (Rashid et al. 2013). I/R challenge profoundly down-regulated the expression of CAT and SOD. PCA could rescue the loss of CAT and SOD activities to certain extent, helping the scavenger of free radicals.

Polymorphonuclear leukocytes are progressively recruited and persistently activated within infarcted myocardium. Neutrophils not only produce proteases and different forms of toxic ROS and RNS to destroy the surrounding tissues, but also secret various mediators to recruit more pro-inflammatory cells (Schofield et al. 2013). Neutrophils infiltration is often represented by assaying MPO activity in injured cardiac tissues (Goldmann et al. 2009). In this study, we observed that I/R challenge induced while PCA markedly attenuated the deposition of polymorphonuclear leukocytes in myocardium by H&E staining. We detected that PCA effectively suppressed I/R-induced increase in cardiac MPO activity. However, little is known about the mechanisms by which PCA regulates the infiltration of polymorphonuclear leukocytes against I/R challenge. Current anti-inflammatory drugs mainly target the production and the signaling pathways of various proinflammatory mediators (Hakonarson et al. 2005; Werz and Steinhilber 2006). Leukotriene B4 (LTB4) is one of the key pro-inflammatory mediators for regulating the

infiltration and survival of neutrophils in myocardial injury (Borgeat and Naccache 1990; Helgadottir et al. 2006; Yokomizo et al. 2001). We recently discovered that plant natural products (e.g., gallic acid and calycosin) could induce LTB4DH to convert LTB4 into less bioactive 12-oxo-LTB4. We demonstrated that LTB4DH induction might attenuate neutrophil-mediated myocardial injury via inactivating LTB4. Interestingly, natural product curcumin as a structural analogue of PCA could also induce LTB4DH expression (Yu et al. 2012). Thus, we speculated that PCA might limit neutrophils infiltration partly via the induction of LTB4DH expression.

I/R injury is well-known to damage myocardial tissues via activating apoptosis cascade in hearts (Buja et al. 2007). The anti-apoptotic proteins are commonly suppressed whereas pro-apoptotic protein Bax is activated in myocardial mitochondria during I/R treatment (Kumar and Jugdutt 2003). Dysregulated Bcl-2/Bax ratio induces the activation of caspase-3 and caspase-9 leading to apoptosis (Dong et al. 2003). On the other hand, Bcl-2 could inhibit myocardial apoptosis through suppressing the opening of mitochondrial permeability transition pore, blocking the release of cytochrome c and decreasing the activities of various caspases (Cook et al. 1999). In our study, we used TUNEL assay to detect myocardial apoptosis. As shown in Fig. 5a, PCA treatment could significantly reduce myocardial apoptosis. We further determined the protein levels of Bcl-2 and Bax in the infracted hearts compared with that in untreated hearts. As shown in Fig. 5b, PCA treatment markedly up-regulated the ratio of Bcl-2/Bax whereas I/R operation negatively regulated Bcl-2/Bax ratio in myocardial tissues. Collectively, the pharmacological actions of PCA could be summarized in Fig. 5c. In acute myocardial I/R model, PCA exhibited cardioprotective effects via promoting the expression of antioxidant enzymes (1.e., CAT, SOD) and anti-apoptotic Bcl-2, and suppressing the expression of pro-apoptotic Bax, lipid peroxidation, and neutrophil infiltration. Ultimately, PCA could effectively reduce cell apoptosis.

In conclusion, PCA not only protected H9c2 cells against OGD-induced damage in a concentration-dependent manner but also exhibited superior cardioprotective effects over CAPE and PCE. Based on the previous studies on CAPE (Yang et al. 2012), PCA appeared to be more stable in rat plasma and with longer half-life at 25 and 37 °C. Similarly, pharmacokinetic study revealed that PCA showed longer half-life in elimination relative to CAPE as described previously (Yang et al. 2014). By investigating the cardioprotective effects of PCA in acute myocardial I/R model, we discovered that PCA could profoundly reduce infarct size via increasing antioxidant capacity, suppressing oxidative stress and inflammatory response. Thus, our results suggest that PCA may be a potent drug candidate for the treatment of I/R injury.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest about the article.

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