Cardiotoxic Pill Reduces Myocardial Ischemia-Reperfusion Injury via Increasing EET Concentrations in Rats

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ABSTRACT

Accumulating data suggest that epoxyeicosatrienoic acids (EETs) and 20-hydroxyicosatetraenoic acid, both cytochrome P450 (P450) enzyme metabolites of arachidonic acid (AA), play important roles in cardiovascular diseases. For many years, the cardiotoxic pill (CP), an herbal preparation derived from Salviae Miltiorrhiza Radix et Rhizoma, Notoginseng Radix et Rhizoma, and Borneolum Syntheticum, has been widely used in China for the treatment of coronary artery disease. However, its pharmacological mechanism has not been well elucidated. The purpose of this study was to investigate the chronic effects of the CP on myocardial ischemia-reperfusion injury (MIRI) and AA P450 enzyme metabolism in rats (in vivo) and the protective effects of the CP on myocardial ischemia-reperfusion injury (MIRI) and AA P450 enzyme metabolism in rats (in vitro). The results showed that CP dose dependently (10, 20, and 40 mg/kg/d; 7 days) mitigated MIRI in rats. The plasma concentrations of EETs in CP-treated ischemia-reperfusion (I/R) rats (40 mg/kg/d; 7 days) were significantly higher (P < 0.05) than those in controls. Cardiac Cyp1b1, Cyp2b1, Cyp2e1, Cyp2j3, and Cyp4f6 were significantly increased (P < 0.05); CYP2J and CYP2C11 proteins were upregulated (P < 0.05); and AA-epoxygenase activities were significantly increased (P < 0.05) after CP (40 mg/kg/d; 7 days) administration in rats. In H9c2 cells, the CP also increased (P < 0.05) the EET concentrations and showed protection in hypoxia-reoxygenation (H/R) cells. However, an antagonist of EETs, 14,15-epoxyeicosa-5(Z)-enoic acid, displayed a dose-dependent depression of the CP’s protective effects in H/R cells. In conclusion, upregulation of cardiac epoxygenases after multiple doses of the CP may be the underlying mechanism, at least in part, for the CP’s cardioprotective effect in rats.

Introduction

Cardiovascular disease (CVD), including coronary artery disease and acute myocardial infarction, is a human health crisis that remains one of the leading causes of death worldwide (Elbekai and El-Kadi, 2006). Increasing evidence has shed light on the roles of the cytochrome P450 (P450) enzymes in the onset, progression, and prognosis of CVD. Over the past three decades, P450 enzymes have been found expressed in the cardiovascular system (Enayetallah et al., 2004; Delozier et al., 2007). Endogenous P450 enzyme metabolites, such as epoxyeicosatrienoic acids (EETs), aldosterone, and sex hormones, are shown to be implicated in the maintenance of cardiovascular health (Elbekai and El-Kadi, 2006).

Arachidonic acid (AA) is a polyunsaturated fatty acid located in body cell membranes in an inactive state, and can be released by phospholipase A2 in response to stimuli such as ischemia (Sato et al., 2011). Recent investigations indicate that the P450 enzyme metabolites of AA, EETs, and 20-hydroxyicosatetraenoic acid (20-HETE), are involved in many cardiovascular pathophysiological mechanisms, and play important roles in myocardial ischemia-reperfusion injury (MIRI) (Elbekai and El-Kadi, 2006; Sato et al., 2011; Chen and Wang, 2013). EETs, mainly generated by members of the CYP2 family, including CYP2C8, CYP2C9, and CYP2J2, are the epoxidation metabolites of AA. Their actions are terminated by conversion to the less biologically active dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). 20-HETE is the ω-hydroxylation metabolite of AA, and is primarily produced by members of the CYP4 family, including CYP4A and CYP4F isoforms (Sato et al., 2011). EETs act as vasodilatory substances in several vascular beds via activation of Ca2+-activated K channels (Campbell et al., 1996), endothelial nitric oxide synthase (Wang et al., 2003), and the TRPV4 channel (Vriens et al., 2005). In the heart, EETs regulate L-type Ca2+, ATP-sensitive K+, and Na+ channels...
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Materials and Methods

Chemicals and Reagents

14,15-EET; 11,12-EET; 8,9-EET; 5,6-EET; 14,15-DHET; 11,12-DHET; 8,9-DHET; 5,6-DHET; 14,15-epoxyeicosanoic acid (14,15-EEZE); 14,15-DHET-d11; 14,15-EET-d11; and 20-HETE were obtained from Cayman Chemicals (Ann Arbor, MI). AA, NADPH, MgCl2, 2,3,5-triphenyltetrazolium chloride (TTC), and dihydroethidium were obtained from Sigma-Aldrich Co. (St. Louis, MO). The CK-MB isozyme and LDH assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The TRZol reagent and Prime Script RT Master Mix Perfect Real Time Kit were bought from Takara (Tokyo). The primary antibody of CYP2C11 (ab3571) was purchased from Abcam (Cambridge, MA), and antibodies of CYP2D (sc-67276), SEH (sc-24797), and glyceraldehyde-3-phosphate dehydrogenase (sc-365062) were obtained from Santa Cruz Biotechnology, Inc. (Paso Robles, CA). The Pierce ECL Western Blotting Kit was bought from Thermo Fisher Scientific (Grand Island, NY). The CP used in this research (27 mg each pill) was obtained from Tasyi Pharmaceutical Group Co., Ltd. (Tianjin, China). The content levels of various Danshen components were determined in a previous study (Liu et al., 2014) using three different CP batches. The average levels were as follows: 30.4 mg/g of Danshensu; 10.2 mg/g of protocatechuic aldehyde; 8.1 mg/g of salvianolic acid A; 4.5 mg/g of salvianolic acid B; 8.2 mg/g of salvianolic acid D; 1.6 mg/g of luteolin acid; 4.1 mg/g of rosmarinic acid; 0.74 mg/g of tanshinone I; 0.63 mg/g tanshinone II A; 0.39 mg/g of cryptotanshinone; 0.63 mg/g of dihydrotanshinone I; 22.0 mg/g of ginsenosides Rg1; 19.1 mg/g of ginsenosides Rb1; and 10.6 mg/g of ginsenosides Rb1 (see Supplemental Table 1). High-performance liquid chromatography-grade formic acid, ammonium acetate, and acetonitrile were obtained from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q water system (Millipore, Bedford, MA). All other chemicals used were the highest grade commercially available.

Animals

Adult male Sprague-Dawley rats (240 ± 20 g body weight) were supplied by the Nanjing Qinglongshan Experimental Animal Co. Ltd. (Nanjing, China). All experimental procedures and protocols were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/dot/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf) and approved by the Animal Ethics Committee of China Pharmaceutical University. The animals were maintained in a room with a temperature of 25°C ± 2°C, a 12-hour day-night cycle, and 50% ± 10% relative humidity. Water and food (laboratory rodent chow pellets, Skenrei Biological Technology Co. Ltd., Nanjing, China) were provided ad libitum.

Groups and Administration of the CP in Rats

As shown in Table 1, the present in vivo study was comprised of three substudies, using separate rat samples. Rats were divided into groups at random (randomized block experiment design) and gavage administered normal saline or CP solution. The CP solution was freshly prepared before use each day by crushing the pills with a mortar and then dissolving them in normal saline with the help of ultrasound. No organic solvent was added. The animal doses were derived according to the daily dose of the CP applied in the phase II clinical trials (low-dosage group: 250 mg per person daily; high-dosage group: 375 mg per person daily), which was calculated by the body surface area difference between humans and rats, with minor adjustments to conform to pharmacological research conventions. All assessors in the study were blinded to the group assignment of the specimens they were assessing.

Establishment of the MIRI Model

After 7 days of administration of normal saline or CP, the rats were anesthetized with intraperitoneal injections of chloral hydrate (300 mg/kg). Following endotracheal intubation, the heart was exposed via a left thoracotomy in the fourth intercostal space. For induction of the myocardial ischemia model, the left anterior descending coronary artery, together with a silica rubber cannula, was ligated with a 6-0 silk suture. After 45 minutes of occlusion, the coronary artery was reperfused by cutting the suture. The length of reperfusion was 15 minutes for superoxide generation measurements, 30 minutes and 3 hours for AA P450 enzyme metabolites determinations, and 24 hours for cardiac injury.
measurements. Alterations of color in the myocardium and electrocardiographic changes were used to confirm successful surgery. Rats in the sham group experienced the same surgical progress without the ligation.

**Assessment of Myocardial Injury**

After 45 minutes of myocardial ischemia and 24 hours of reperfusion, all rats were narcotized by urethane (1 g/kg). Hemodynamics and cardiac function were measured using a biologic mechanism experiment system (BL420, Taimeng Co. Ltd., Chengdu, China) as described previously (Wei et al., 2014). A pressure-volume catheter, filled with heparin saline (500 U/ml), was inserted into the right carotid artery and then advanced into the left ventricle for monitoring heart rate, left-ventricular end-diastolic pressure, left-ventricular systolic pressure, and maximum/minimum rates of developed left ventricular pressure (\(2 \text{ dP/dt}_{\text{max}}\)).

Subsequently, all rats were sacrificed to harvest the hearts immediately for infarct size determination.

The size of the infarcted myocardium was determined through TTC staining. The hearts were kept at 20°C for 10 minutes, and then cut into five 1- to 2-mm-thick slices and incubated for 10 minutes in 1% (w/v) TTC in buffer (pH 7.4) at 37°C. Next, the slices were fixed in 10% formalin and pictures were taken. The sizes of infarcted area (white coloration) and at-risk areas (the entire scanned section) were determined by the Image-Pro Plus image analysis software (version 4.1, Media Cybernetics, LP, Silver Spring, MD). The infarct size was calculated as a percentage of the risk area for assessing the degree of myocardial infarction.

Myocardial injury was assessed by measuring the amount of CK-MB and LDH in serum collected after 24 hours of reperfusion. CK-MB and LDH are expressed in the heart muscle and released during tissue damage, thus they are used as indicators of cardiac injury (Liu et al., 2014). The activities of CK-MB and LDH in serum were assessed by commercially available kits, and measured spectrophotometrically at 340 and 450 nm, respectively. All procedures adhered to the manufacturer’s instructions.

**Superoxide Generation Measurement in Heart Tissues**

Superoxide generation in the I/R heart tissue was determined as previously reported (Khan et al., 2007) with dihydroethidium, which can be oxidized to fluorescent ethidium (HE) by superoxide and then intercalated into DNA. Since superoxide generation in hearts subjected to I/R was reported (Khan et al., 2007) with dihydroethidium, which can be oxidized to superoxide, fluorescence intensity was quantified using ImageJ software (version 1.48, National Institutes of Health).

**Real-time Polymerase Chain Reaction (PCR) Analysis of P450 Enzymes in the Rat Heart**

Total RNA from the heart tissues was isolated using a TRizol reagent according to the manufacturer’s instructions. Next, first-strand cDNA synthesis was performed by using the Prime Script RT Master Mix Perfect Real Time Kit (Takara) according to the manufacturer’s instructions. Quantitative analysis of specific mRNA expression was performed with real-time PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems, Foster City, CA). The primers employed in the current study (Table 2) were chosen from previously published studies (Zordoky et al., 2011; Alsaad et al., 2012). No-template controls were incorporated into the same plate to test for contamination of any assay reagents. An optical adhesive cover was used to seal the plate; thereafter, thermocycling conditions were initiated at 95°C for 10 minutes, followed by 40 PCR cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 30 seconds. Dissociation curves were performed at the end of each cycle to confirm the specificity of the primers and purity of the final PCR product.

**Microsomal Protein Preparation**

Cardiac microsomal protein was prepared from heart tissues as described previously (Aboutabl et al., 2009). In brief, heart tissues were washed with ice-cold phosphate-buffered saline (PBS), cut into pieces, and homogenized using an IKA T10 basic ULTRA-TURRAX homogenizer (IKA, Königswinter, Germany) in additional cold PBS solution. The homogenates from three separate hearts in the same group were mixed, and the microsomal and cytosolic proteins were separated by differential ultracentrifugation from the homogenized tissues. Subsequently, all rats were sacrificed to harvest the hearts immediately for infarct size determination.

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(dissolved in the cell culture with the help of ultrasonic and vortex mixing) for 12 hours. Following H/R, the cell viability was determined. To investigate the actions of 14,15-EEZE on the cardioprotective effect of the CP in H9c2 cell H/R injury, various concentrations of 14,15-EEZE (0.01, 0.1, and 1 μM) were added to the hypoxia solution and incubated with H9c2 cells in a hypoxic condition. 14,15-EEZE was dissolved in ethanol, in which the final concentration was less than 1% (v/v) in the mixture, and the same amount of ethanol was added to the control group. After H/R, the cell viability was determined by a Cell Counting Kit-8 assay (CCK-8, Dojindo, Shanghai, China).

Sample Pretreatment for AA P450 Enzyme Metabolite Determination

Plasma Sample. Next, 300 μl of rat plasma was added with 315 μl methanol, 30 μl internal standard (14,15-DHET-d11, 5 ng/ml), and 30 μl butylated hydroxytoluene (10 mg/ml). After vortex mixing for 1 minute, the mixture was then centrifuged at 12,000 rpm at 4°C for 5 minutes. The supernatant was drawn off and treated with a solid-phase extraction (SPE) method described previously (Zhang et al., 2007; Martin-Venegas et al., 2011) with minor modification. Briefly, the supernatant was applied to a 3-ml Oasis HLB SPE column (3 cc/60 mg, Waters, Milford, MA) that had been preconditioned with 3 ml methanol and 3 ml water. The columns were washed with 3 ml of water and allowed to run dry. Then, AA P450 enzyme metabolites were eluted from the column with 5 ml of ethyl acetate. Because there was still a small amount of water left on the SPE column, the elution liquid was divided into two layers. The ethyl acetate layer was carefully removed from the water layer and dried using a CentriVap Centrifugal Concentrator (Labconco). The sample was then redissolved in 125 μl ethanol. After being vortexed for 1 minute, the content was centrifuged at 12,000 rpm at 4°C for 10 minutes, and an aliquot of 5 μl of the supernatant was injected for LC-MS/MS analysis.

Cell Sample. After control or CP treatment, the cells were washed with ice-cold PBS, collected in 1 ml PBS, and disrupted with an ultrasonic crusher (Haishu Kesheng Ultrasonic Equipment, Ningbo, China). After being centrifuged at 12,000 rpm for 5 minutes at 4°C, the supernatant was loaded onto the SPE column, with the same extraction procedure as was used for the plasma sample.

Measurement of AA P450 Enzyme Metabolites Using LC-MS/MS

The P450 enzyme metabolites of AA, EETs, DHETs, and 20-HETE were simultaneously quantitated using the LC-MS/MS system (API 4000 LC-MS/MS; Applied Biosystems Sciex, Ontario, Canada) equipped with an electrospray ionization interface. Chromatography was performed using a C18 column (2.6 μm, 100 × 2.1 mm; Kinetex, Phenomenex, Torrance, CA) and an Agilent (Agilent Technologies, Foster City, CA) 1200 high-performance liquid chromatography system. The mobile phase, which consisted of a 0.1% formic acid aqueous solution (A) and acetonitrile (B), was delivered with a gradient elution at a flow rate of 0.6 ml/min: 0 minutes, 50% B; 3–5 minutes, 62% B; 6–10 minutes, 85% B; and 11–18 minutes, 50% B. The column temperature was maintained at 40°C. Ion spray voltage was set at 4.5 kV for negative ionization, and the heater gas temperature was 650°C. Nitrogen was used as the nebulizing gas (50 psi), auxiliary gas (70 psi), and curtain gas (15 psi). The Multiple Reaction Monitoring experiments were conducted by monitoring the precursor ion to product ion transitions for 20-HETE [mass-to-charge ratio (m/z) 319.2–289.3]; 14,15-DHET (m/z 337.2–207.0); 11,12-DHET (m/z 337.2–208.1); 8,9-DHET (m/z 337.2–127.1); 5,6-DHET (m/z 337.2–145.1); 14,15-EET (m/z 319.2–219.2); 11,12-EET (m/z 319.2–208.1); 8,9-EET (m/z 319.2–155.1); 14,15-DHET-d11 (m/z 348.3–207.2); and 14,15-EET-d11 (m/z 330.3–219.2). The lower limits of quantification of AA P450 enzyme metabolites are shown in Supplemental Table 2.

Statistical Analysis

The results are presented as the mean ± S.E. Data were subjected to statistical analysis using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). The Student’s t test was used for data comparison between two groups. One-way analysis of variance with the Dunnett’s post hoc test was carried out for comparison of more than two groups. In all cases, a value of P < 0.05 was considered significant.

Results

Effect of the CP on MIRI in Rats. The effects of the CP on the heart rate, hemodynamics, and cardiac function were determined after hearts were subjected to 45 minutes of ischemia followed by 24 hours of reperfusion. There was no significant difference (P > 0.05) in the heart rate among all groups (Fig. 1A). Hearts in the model group showed a significant decrease in left-ventricular systolic pressure (P < 0.001) and dp/dtmax (P < 0.001), and a significant increase in left-ventricular end-diastolic pressure (P < 0.001) when compared with those in the sham group (Fig. 1). However, hearts in CP-treated rats (10, 20, and 40 mg/kg/d; 7 days) showed a dose-dependent recovery (P < 0.05) of left-ventricular systolic pressure, left-ventricular end-diastolic pressure, dp/dtmax, and −dp/dtmax when compared with those in the model group (Fig. 1).

The serum levels of LDH and CK-MB were regarded as significant biomarkers of MIRI. As presented in Fig. 2, levels in the model group were significantly higher (P < 0.001) than those in the sham group. However, treatment with the CP (10, 20, and 40 mg/kg/d; 7 days) significantly decreased the levels of LDH and CK-MB compared with the model group.

As shown in Fig. 3, TTC staining of hearts in the sham group showed no infarct and ischemic area. Hearts in the model group showed an infarction of 59.7% ± 4.1% in the risk area. However, infarct size in CP-treated rats (10, 20, and 40 mg/kg/d; 7 days) was significantly reduced to 48.0% ± 5.4% (P < 0.01), 33.1% ± 4.2% (P < 0.001), and 21.2% ± 8.0% (P < 0.001), respectively.

Effects of the CP on Myocardial Superoxide Generation. As shown in Fig. 4, the HE fluorescence intensity was significantly higher

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>AGGACGAGTGGTCTAAGTTG</td>
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in the model group than in the sham group (P < 0.001). However, hearts in CP-treated rats (10, 20, and 40 mg/kg/d; 7 days) showed a dose-dependent decrease (P < 0.05) in HE fluorescence intensity compared with the model group.

**Effects of the CP on AA P450 Enzyme Metabolite Concentrations In Vivo.** As shown in Fig. 5A, after reperfusion of 30 minutes there was no significant difference in EET, DHET, and 20-HETE concentrations between the model and sham groups. However, the concentrations of 20-HETE, 14,15-DHET, 11,12-DHET, and 8,9-EET in the model group were significantly higher (P < 0.05) than in the sham group after reperfusion (Fig. 5B). Treatment with the CP (40 mg/kg/d; 7 days) resulted in significantly higher (P < 0.01) plasma concentrations of 14,15-EET, 11,12-EET, and 8,9-EET, after both 30 minutes and 3 hours of reperfusion in I/R rats, compared with either the sham or model group (Fig. 5, A and B). Furthermore, the plasma concentrations of 14,15-DHET and 8,9-DHET in CP-treated rats (40 mg/kg/d; 7 days) were significantly lower than those in the model group after reperfusion of 3 hours (Fig. 5B). The individual concentrations of AA P450 enzyme metabolites in the sham, model, and CP-treated groups are given in Supplemental Table 3.

To evaluate the effects of the CP on the activities of AA P450 metabolizing-related enzymes, the sum of EETs and DHETs (EETs + DHETs), the concentration of 20-HETE, and the ratio of DHETs to EETs (DHETs/EETs) were used to determine the total AA epoxygenase activity, ω-hydroxylases activity, and sEH activity, respectively. As shown in Fig. 5, C and D, these values are significantly higher in the model group than in the sham group at 3 hours after reperfusion (P < 0.05). However, after CP administration at both 30 minutes and 3 hours of reperfusion, the concentration of EETs + DHETs was significantly higher (P < 0.05), while the ratio of DHETs/EETs was significantly lower (P < 0.05), than those of either the sham or model groups.

**Effects of the CP on the Expression and Activity of AA-Metabolizing P450 Enzymes In Vivo.** The effects of the CP on the expressions and functions of AA-metabolizing P450 enzymes in normal rat hearts were investigated using real-time PCR, western blotting, and LC-MS/MS assay. The results indicate that after administration of the CP (40 mg/kg/d) for 7 days, expressions of cardiac Cyp1b1, Cyp2b1, Cyp2c11, Cyp2j3, and Cyp4f6 (Fig. 6A) were significantly induced compared with vehicle controls (P < 0.05). The western blotting results showed that expressions of CYP2J1 and CYP2C11 proteins were significantly elevated (Fig. 6B) compared with controls (P < 0.05). The catalyzing activity of AA-metabolizing P450 enzymes was determined using AA (50 μM) as the substrate in heart microsomes prepared from vehicle or CP-treated rats (detailed results are given in Supplemental Table 4). As shown in Fig. 6C, no significant difference was found in the concentrations of 20-HETE, DHETs, and the ratio of DHETs/EETs between the two groups. However, the concentrations of DHETs + EETs and EETs of CP-treated rats were significantly higher (P < 0.05) than those of the controls, indicating that the total activity of AA epoxygenases was increased after CP administration. However, the sEH activity in cardiac cytosols of CP-treated rats, evaluated by 14,15-DHET reoxygenation, was not different from the controls (Fig. 6D).

**Effects of the CP on the H/R Injury and Concentrations of AA P450 Enzyme Metabolites In Vitro.** The protective effect of the CP on H/R injury in cells was investigated in vitro. The results (Fig. 7A) demonstrated that the CP (0.025, 0.05, 0.1, and 0.2 mg/ml) caused a dose-dependent improvement (66.2% ± 2.4% versus 76.1% ± 1.8%, 77.9% ± 1.4%, 84.5% ± 2.1%, and 90.9% ± 1.9%) in the cell survival rate in H9c2 cells subjected to 9 hours of hypoxia and 3 hours of reoxygenation. The concentrations of AA P450 enzyme metabolites in normal or H/R H9c2 cells were also determined (see Supplemental Table 5). As shown in Fig. 7, B and C, the concentrations of 20-HETE, DHETs + EETs, and EETs in cells after H/R were significantly higher (P < 0.05), than those of either the normal or H/R H9c2 cells.
Effects of 14,15-EEZE on the CP’s Protection against H/R Injury in H9c2 Cells. 14,15-EEZE, a structural analog of 14,15-EET, can compete with EETs for the same action site and play a role in antagonism of EETs. As shown in Fig. 8A, 14,15-EEZE (0.1, 1, and 10 μM) dose dependently reduced the cell survival rate in CP-treated H/R cells (0.2 mg/ml) from 85.8% to 72.2% ± 3.5%, P < 0.05) in CP-treated H/R cells (0.2 mg/ml).

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Fig. 2. Effects of the CP (10, 20, and 40 mg/kg/d; 7 days) on the I/R-induced variations in the myocardial zymogram. Data show the following: (A) LDH and (B) CK-MB of hearts subjected to 45 minutes of ischemia followed by 24 hours of reperfusion. Data represent the mean ± S.E. obtained from six independent measurements. **P < 0.01 and ***P < 0.001 compared with the sham group [one-way analysis of variance (ANOVA), Dunnett’s test]; ****P < 0.001 compared with the model group (one-way ANOVA, Dunnett’s test).

In this study, multiple doses of the CP were first found to have the following dose-dependent effects in rats subjected to 45 minutes of myocardial ischemia and 24 hours of reperfusion (I/R rats): improved hemodynamics and cardiac function, moderated variation in the myocardial zymogram, and decreased infarct size (Figs. 1–3). These results suggest that chronic administration of the CP may have protective effects on MIRI.

To explore the mechanism underlying this protection, the CP’s effect on AA P450 enzyme metabolite concentrations was then investigated. A previous study in dogs (Nithipatikom et al., 2001) found that plasma concentrations of EETs, DHETs, and 20-HETE changed during reperfusion periods of 5–120 minutes. Accordingly, levels of these AA metabolites were measured at 30 minutes and 3 hours of reperfusion in I/R rats. The results of the present study (Fig. 5) found that at 30 minutes of reperfusion, plasma concentrations of AA P450 enzyme metabolites in the model group were slightly (but not significantly) higher than those in the sham group. However, after 3 hours of reperfusion the concentrations of 20-HETE, DHETs, and EETs + DHETs in the model group were significantly higher (P < 0.05) than the sham group, suggesting that these metabolites increase in I/R rats in a similar pattern to that found in dogs (Nithipatikom et al., 2001). After chronic CP administration, the concentration of EETs in I/R rats increased earlier and more dramatically than in the model group (Fig. 5).

Because of the vasodilatory, anti-inflammatory, proangiogenic, and antiapoptosis actions of EETs, augmented EET concentrations during I/R has proven beneficial in numerous studies. For example, CYP2J2 transgenic mice exhibited not only increased EET biosynthesis in coronary arteries, but also improved postischemic recovery of left ventricular function after myocardial I/R (Seubert et al., 2004). sEH inhibition through gene deletion or pharmacological suppression was protective against MIRI in mice (Motoki et al., 2008). Administration of exogenous 14,15-EET was found to reduce MIRI in rats and dogs (Nithipatikom et al., 2006; Gross et al., 2007).
et al., 2007). Likewise, exogenous administration of EET analogs, UA-
8, provided significant protection against MIRI (Batchu et al., 2011).
The results of the present study (Fig. 5) have demonstrated that after CP
administration, total activity of AA epoxygenases (EETs + DHETs)
increased, while sEH activity (DHETs/EETs) decreased, ultimately
leading to elevated EET plasma concentrations. The results also indicated
that multiple doses of the CP had little effect on AA ω-hydroxylation
(20-HETE) in I/R rats.

The protection of the CP against H/R injury and its effect on AA P450
enzyme metabolism were further investigated in H9c2 cells. The H9c2
cell H/R model is a common cell model for simulating myocardial I/R in
rats in vitro (Batchu et al., 2011). In line with the present in vivo study,
the CP exhibited dose-dependent protection on H/R injury and a similar
impact on AA P450 enzyme metabolism (Fig. 7). In drug-untreated
cells, concentrations of 20-HETE and EETs were significantly higher
after H/R, compared with non-H/R controls. As expected, this suggested
an increased generation of AA P450 enzyme metabolites after H/R.
However, in CP-pretreated cells EET concentrations were significantly
higher than in control cells under both normal and H/R conditions
(Fig. 7B). Furthermore, sEH activity was found to decrease after CP
pretreatment. 14,15-EEZE, a structural analog of 14,15-EET, behaves as
a direct EET antagonist at a receptor binding site, and selectively
antagonizes many EET actions (Gross et al., 2008). The results of this
study found that 14,15-EEZE dose-dependently reversed the CP's

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**Fig. 4.** Effects of the CP (10, 20, and 40 mg/kg/d; 7 days) on the superoxide generation in the rat’s heart subjected to 45 minutes of ischemia and 15 minutes of reperfusion. Cryosections of hearts after reperfusion were incubated with dihydroethidium (10 μM) at 37°C in the dark for 30 minutes, and the formation of HE was determined by fluorescence microscopy. (A) Three representative images (magnification: 20×) from each group are shown. (B) Mean fluorescence intensity of each group. Data represent the mean ± S.E. obtained from three independent measurements. ***P < 0.001 compared with the sham group [one-way analysis of variance (ANOVA), Dunnett’s test]; *P < 0.05 and **P < 0.01 compared with the model group (one-way ANOVA, Dunnett’s test).

**Fig. 5.** Effects of the CP (40 mg/kg/d; 7 days) on the plasma concentrations of AA P450 enzyme metabolites in rats subjected to 45 minutes of ischemia, and 30 minutes (A) or 3 hours (B) of reperfusion. (C) Summarized concentrations of the AA P450 enzyme metabolites and (D) DHET/EET ratios of each group. The concentrations of AA P450 enzyme metabolites were determined using the LC-MS/MS method. Data represent the mean ± S.E. obtained from seven independent measurements (hearts). *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the sham group [one-way analysis of variance (ANOVA), Dunnett’s test] at the same time; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the model group (one-way ANOVA, Dunnett’s test) at the same time; #P < 0.05 compared with the value of 30 minutes (one-way ANOVA, Dunnett’s test) in the same group.
protective effects against H/R injury in H9c2 cells, suggesting that this protection is mediated via an EET pathway.

To investigate why AA P450 enzyme metabolism was influenced by multiple doses of the CP, the expressions and catalyzing activities of AA-related P450 enzymes in the rat heart were studied. CYP2C11 and CYP2J3 are two main AA epoxygenases in the rat heart (El-Sherbeni et al., 2013). Cardiac CYP1A1, CYP2B1, and CYP2E1 have also been reported to display AA epoxygenase activity (El-Sherbeni and El-Kadi, 2014). CYP1B1 has been found able to metabolize AA into both EETs and 20-HETE simultaneously (Choudhary et al., 2004). As for the AA \( \omega \)-hydroxylases, CYP4A1, CYP4A3, CYP4F1, CYP4F4, and CYP4F6 have all been found to be expressed in the rat heart (Xu et al., 2004). Epoxide hydrolase can be classified into microsomal epoxide hydrolase or sEH, according to its distribution. Microsomal epoxide hydrolase, encoded by \( \text{Ephx1} \), is distributed in the endoplasmic reticulum and mainly metabolizes the xenobiotic epoxide. sEH, encoded by \( \text{Ephx2} \), is distributed in the cytoplasm and primarily metabolizes endogenous epoxides. Although investigations into cardiovascular protection mainly focus on sEH, both microsomal epoxide hydrolase and sEH have been found able to produce DHETs from EETs (Morisseau and Hammock, 2013). Drawing from the aforementioned research, expressions of \( \text{Cyp}1a1 \), \( \text{Cyp}1b1 \), \( \text{Cyp}2b1 \), \( \text{Cyp}2c11 \), \( \text{Cyp}2e1 \), \( \text{Cyp}2j3 \), \( \text{Cyp}4a1 \), \( \text{Cyp}4a3 \), \( \text{Cyp}4f1 \), \( \text{Cyp}4f4 \), \( \text{Cyp}4f5 \), \( \text{Cyp}4f6 \), \( \text{Ephx}1 \), and \( \text{Ephx}2 \) in the rat heart were examined in this study. The results (Fig. 6A) showed that \( \text{Cyp}1b1 \), \( \text{Cyp}2b1 \), \( \text{Cyp}2e1 \), and \( \text{Cyp}2j3 \), all of which encode EET-generating enzymes, were significantly induced after CP administration (\( P < 0.05 \)). In line with the mRNA results, the protein expression of CYP2J was also found to be significantly higher than in controls (\( P < 0.05 \)). In contrast, while CYP2C11 was significantly induced (\( P < 0.05 \)), its mRNA was not significantly upregulated (\( P > 0.05 \)). This implies that the CP may have little effect on CYP2C11 gene expression but may significantly impact its post-transcriptional translation process.

The effects of the CP (40 mg/kg/d; 7 days) on mRNA (A), protein (B), activity of AA-metabolizing P450 enzymes (cardiac microsomes) (C), and activity of sEH (cardiac cytosols) (D) in the rat heart were studied. Total RNA was isolated from the hearts of vehicle controls and rats treated with the CP for 7 days, and all of the gene expressions were determined by real-time PCR. Cardiac microsomal and cytosolic proteins were isolated from the hearts of controls and rats treated with the CP for 7 days, and proteins were separated on 10% SDS-PAGE. The catalyzing activity of AA-metabolizing P450 enzymes was determined using AA (50 \( \mu \)M) as the substrate in heart microsomes, while the activity of sEH was measured using 14,15-EET as the substrate in heart cytosols. After incubation of 30 minutes in microsomes and 5 minutes in cytosols, the concentrations of AA P450 enzyme metabolites, 20-HETE, EETs, and DHETs were determined using the LC-MS/MS method. Data represent the mean ± S.E. obtained from nine hearts. * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \) compared with the vehicle controls (Student’s t test).

**Fig. 6.** Effects of the CP (40 mg/kg/d; 7 days) on mRNA (A), protein (B), activity of AA-metabolizing P450 enzymes (cardiac microsomes) (C), and activity of sEH (cardiac cytosols) (D) in the rat heart. Total RNA was isolated from the hearts of vehicle controls and rats treated with the CP for 7 days, and all of the gene expressions were determined by real-time PCR. Cardiac microsomal and cytosolic proteins were isolated from the hearts of controls and rats treated with the CP for 7 days, and proteins were separated on 10% SDS-PAGE. The catalyzing activity of AA-metabolizing P450 enzymes was determined using AA (50 \( \mu \)M) as the substrate in heart microsomes, while the activity of sEH was measured using 14,15-EET as the substrate in heart cytosols. After incubation of 30 minutes in microsomes and 5 minutes in cytosols, the concentrations of AA P450 enzyme metabolites, 20-HETE, EETs, and DHETs were determined using the LC-MS/MS method. Data represent the mean ± S.E. obtained from nine hearts. * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \) compared with the vehicle controls (Student’s t test).
enzymes, have been reported in cardiac I/R studies (Ishihara et al., 2010). Nitric oxide synthases, the mitochondrial respiratory chain, and P450 as NADPH oxidase, xanthine oxidase, cyclooxygenase, lipoxygenase, and induce myocardial damage. Numerous potential ROS sources, such leading to increased EET concentration in I/R rats.

It is well known that ROS are widely generated during reperfusion and induce myocardial damage. Numerous potential ROS sources, such as NADPH oxidase, xanthine oxidase, cyclooxygenase, lipoxygenase, nitric oxide synthases, the mitochondrial respiratory chain, and P450 enzymes, have been reported in cardiac I/R studies (Ishihara et al., 2010).

higher \((P < 0.05)\) than the controls. Based on these observations, one can infer that multiple doses of the CP may have induced the expression of cardiac AA epoxygenases, such as CYP2J3 and CYP2C11, ultimately leading to increased EET concentration in I/R rats.

In conclusion, this study has demonstrated that after multiple doses of the CP may have induced the expression of cardiac AA epoxygenases, such as CYP2J3 and CYP2C11, ultimately leading to increased EET concentration in I/R rats.
cardioprotective EET concentrations were increased following I/R. The results suggest that the mechanism underlying the CP’s protection in MIRI may be, at least in part, upregulation of EET-generating P450 enzymes.

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References


CP Reduces MIRI via Increasing EET levels in Rats


