Cardiotonic 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-hydroxyeicosatetraenoic acid, both cytochrome P450 (P450) enzyme metabolites of arachidonic acid (AA), play important roles in cardiovascular diseases. For many years, the cardiotonic pill (CP), an herbal preparation derived from Salviae Miltiorrhiza Radix and 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 H9c2 cells (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 induced (P < 0.05); CYP2J and CYP2C11 proteins were upregulated (P < 0.05); and AA–epoxygenase activity was 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—leading to elevated concentrations of cardioprotective EETs after myocardial I/R—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-hydroxyeicosatetraenoic 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 epoxygenation 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 α-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+ channels, and Na+ channels in response to stimuli such as ischemia (Sato et al., 2011; Chen and Wang, 2013).
(Konkel and Schunck, 2011) and inhibit apoptosis by a caspase-dependent pathway (Wang et al., 2012). Notably, EETs inhibit the inflammatory response by repressing NF-κB activation and inhibiting TNF-α–induced VCAM-1 expression (Node et al., 1999). Moreover, EETs are potent endogenous angiogenic factors (Xu et al., 2011). However, 20-HETE has been demonstrated to have vasoconstrictive (Miyata and Roman, 2005), proinflammatory (Ishizuka et al., 2009) and proapoptosis (Nakahata et al., 2008) functions. Furthermore, although CYP2J2 and CYP2C also generate cardioprotective EETs from AA, studies have demonstrated that CYP2C also produces detrimental reactive oxygen species (ROS) (Fleming et al., 2001). In animal models, effective protection against MIRI has been shown by increasing EET concentrations through direct administration of EETs, upregulating the expression of AA epoxygenases using transgenic technology, or inhibiting the activity of iNOS (Seubert et al., 2004; Motoki et al., 2008; Batchu et al., 2011; Oni-Orisan et al., 2014), decreasing 20-HETE generation through CYP4A4F inhibition (Nithipatikom et al., 2004) and depressing ROS formation via CYP2C inhibition (Granville et al., 2004). AA-metabolizing P450 enzymes are thus considered to be therapeutic targets for MIRI and other CVDs.

For many years, the compound Danshen prescription (CDP), an herbal preparation derived from Salviae Milthorhize Radix et Rhizoma, Notoginseng Radix et Rhizoma, and Borneolum Syntheticum has been widely used in China to improve cardiac function and coronary circulation in the therapy of stable angina pectoris (Luo et al., 2013). Numerous CDP preparations, such as tablets, capsules, injectables, granules, oral liquid, and dripping pills, are commercially available in China. Among them, the cardiotonic pill (CP), also known as the dantonic pill or the compound Danshen dripping pill, is the best known. The CP is now available in countries such as Singapore, Korea, India, the United Arab Emirates, Russia, Cuba, and South Africa as a prescription or over-the-counter drug (Lu et al., 2008). It is in phase III clinical development in the United States (Lei et al., 2014). Although the CDP is recognized as clinically effective for coronary artery disease and is widely used, its pharmacological mechanism has not yet been fully documented. Moreover, to our knowledge, neither chronic CP’s protective effects against MIRI, nor the association between its pharmacological effects and AA P450 enzyme metabolism has been reported.

This study used the CP as the typical CDP preparation, investigating its effects on cardiac function, serum creatine kinase MB (CK-MB) and lactate dehydrogenase (LDH) concentrations, myocardial infarction and superoxide generation in ischemia-reperfusion (I/R) rats, and hypoxia-reoxygenation (H/R) injury in H9c2 cells. This study also examined the CP’s effects on the concentrations of AA P450 enzyme metabolites, and evaluated its influence on the cardiac expressions and activities of AA-metabolizing P450 enzymes in rats.

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-epoxyeicosapentaenoic 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 TRizol 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 CYP2C (sc-67276), CYP2E1 (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 Tansy 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 salvinorin acid A; 4.5 mg/g of salvinorin acid B; 8.2 mg/g of salvinorin acid D; 1.6 mg/g of lipoic 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/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, Sikerui 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 studies, 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 each day 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-dose group: 250 mg per person daily; high-dose 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 conditions. 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.

TABLE 1

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*Pharmacological study.

AA P450 enzyme metabolite determination.

AA-metabolizing P450 enzyme expression and activity.
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 (dp/dt-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 to occur during the first 15 minutes of reperfusion, HE fluorescence was determined at this period (after 15 minutes of reperfusion) (Khan et al., 2007). 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 15 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 (Aboubat 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. Thereafter, the final microsomal pellet was reconstituted in cold PBS containing 20% glycerol and stored at −80°C. The bicornichinic acid method using bovine serum albumin as a standard (BeyoTest, Nanjing, China) was used to determine the concentration of heart microsomal and cytosolic proteins.

Western Blotting Analysis

Next, 50 µg of cardiac microsomal (for P450 enzymes) or cytosolic (for sEH) protein from each treatment group was separated by 10% SDS-PAGE, and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4°C in blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris base (Tris-buffered saline), 5% skim milk, 2% bovine serum albumin, and 0.5% Tween 20. After blocking, the blots were incubated with the primary antibodies of CYP2C11 (1:1000), CYP2J, sEH, and glyceroldehyde-3-phosphate dehydrogenase (1:200) for 2 hours. Incubation with a peroxidase-conjugated secondary antibody was performed for 2 hours at room temperature. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer’s instructions. The intensity of the protein bands was quantified, relative to the signals obtained for glyceroldehyde-3-phosphate dehydrogenase, using the ImageJ software (version 1.48, National Institutes of Health).

Activity Determination of AA-Metabolizing P450 Enzymes

Rat heart microsomes (1 mg/ml) were incubated at 37°C in a shaking water bath (50 rpm). The incubation buffer consisted of 0.1 M potassium phosphate buffer containing 0.15 M KCl and 1 mM EDTA (pH 7.4). After pre-equilibration for 5 minutes, the reaction was initiated by the addition of 1 mM NADPH. AA was added to the incubation mixture with a final concentration of 50 µM and incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 200 µl of ice-cold acetonitrile followed by the internal standards, 14,15-EET-d1 (40 ng/ml) and 14,15-DHET-d11 (40 ng/ml). After a vortex for 1 minute, the incubation mixtures were centrifuged at 12,000 rpm for 5 minutes at 4°C, and an aliquot of 10 µl of the supernatant was injected for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. sEH activity was determined using its natural substrate 14,15-EET. The cytosolic protein was diluted to 0.4 mg/ml with potassium phosphate buffer (0.1 M, pH 7.4), supplemented with bovine serum albumin (2.5 mg/ml) (Alsaad et al., 2012). The reaction was initiated by adding 14,15-EET (final concentration 2 µg/ml) to the preheated cytosolic solution (37°C for 5 minutes), after which the reaction was conducted at 37°C for another 5 minutes. After incubation, the reaction was terminated by adding 1 ml of ice-cold ethyl acetate and 10 µl of 14,15-DHET-d11 (10 ng/ml). After shaking for 3 minutes, the tubes were centrifuged and the organic phase was transferred to a new tube. The organic phase was evaporated to dryness using a CentriVap Centrifugal Concentrator (Labconco, Kansas City, MO). The residue was dissolved in 120 µl of dehydrated alcohol and vortex mixed for 1 minute. The tubes were then centrifuged at 12,000g for 10 minutes, and 5 µl of the resulting supernatant was injected into the LC-MSMS system.

Cell Culture and H/R Treatments

H9c2 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium, without phenol red, supplemented with 0.45% glucose, 0.11% sodium pyruvate, 0.15% sodium bicarbonate, 20 µM L-glutamine, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in 75-cm² culture plates at 37°C in a 5% CO₂ humidified incubator (Thermo Fisher Scientific). To mimic hypoxic injury in vitro, the cells were incubated for 9 hours in a hypoxic solution containing 0.9 mM NaHPO₄, 6.0 mM NaHCO₃, 1.0 mM CaCl₂, 1.2 mM MgSO₄, 40 mM natrium lactium, 20 mM HEPES, 98.5 mM NaCl, and 10.0 mM KCl (pH adjusted to 6.8). The hypoxic condition was produced by transferring the plates of cultured cardiomyocytes in a hypoxic incubator (SANYO, Osaka, Japan); oxygen was adjusted to 1.0% and CO₂ was adjusted to 5.0%. After hypoxia, the cells were reincubated in a normal Dulbecco’s modified Eagle’s medium at 37°C in a 5% CO₂ humidified incubator for 3 hours.

Treatment of H9c2 Cells with the CP and 14,15-EEZE

to investigate the cardioprotective effect of the CP, cardiac-derived H9c2 cells were pretreated with various concentrations (0.025–0.02 mg/ml) of the CP.
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 of H2O/C176.

**Blood 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, C. Nitrogen was used as the nebulizing gas (50 psi), a flow rate of 0.6 ml/min: 0 minutes, 50% B; 3 minutes, 50% B; 3–5 minutes, 62% B; 5–10 minutes, 62% B; 10 minutes, 62%. The lower limits of quantification of AA P450 enzyme metabolites are shown in Supplemental Table 2.

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

The P450 enzyme metabolites of AA, EETs, DHETs, and 20-HETE were measured using a Picarro Mass Spectrometer (Picarro, CA) 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; 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]; 12,12-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 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/dt max (P < 0.001), and a significant increase in left-ventricular end-diastolic pressure (P < 0.001) and −dp/dt max (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/dt max, and −dp/dt max 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 (Fig. 1) 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...
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, after both 30 minutes and 3 hours of reperfusion in I/R rats, compared with either the sham or model group (Fig. 5B). Furthermore, the plasma concentrations of 14,15-DHET 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. 5B).

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, $\omega$-hydroxylases activity, and sEH activity, respectively. As shown in Fig. 5A, 5C, and 5D, 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*, *Cyp2e1*, *Cyp2el*, *Cyp2j2*, and *Cyp4f6* (Fig. 6A) were significantly induced compared with vehicle controls ($P < 0.05$). The western blotting results showed that expressions of CYPIJ 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 $\mu$M) 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 than those in the model group ($P < 0.05$). 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 the Epoxigenases Activity** **In Vivo**. The effects of the CP on the activities of AA epoxygenases were measured by 14,15-DHET generation. As shown in Fig. 7, D and E, the catalyzing 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 generation, was not different from the controls (Fig. 6D).
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% ± 4.2% to 84.3% ± 1.2%, 70.1% ± 2.6%, and 55.6% ± 4.3%, respectively. Furthermore, the effects of 14,15-EEZE (1 μM) on the cell survival rate in normal control, H/R control, and H/R CP-treated cells were also determined. The results (Fig. 8B) demonstrated that 14,15-EEZE (1 μM) had no effect on the cell survival rate in normal control cells, slightly reduced the cell survival rate in H/R control cells (63.6% ± 2.4% versus 59.9% ± 3.9%, P > 0.05), and significantly reduced the cell survival rate (85.8% ± 4.2% versus 72.2% ± 3.5%, P < 0.05) in CP-treated H/R cells (0.2 mg/ml).

Discussion

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).

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.

Fig. 3. Effects of the CP (10, 20, and 40 mg/kg/d; 7 days) on myocardial infarction in rats subjected to 45 minutes of ischemia and 24 hours of reperfusion. Myocardial infarction was determined by TTC staining. Data represent the mean ± S.E. obtained from six independent measurements.
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

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 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, CYP4F5, 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 \( \text{Cyp1a1}, \text{Cyp1b1}, \text{Cyp2b1}, \text{Cyp2c11}, \text{Cyp2e1}, \text{Cyp2j3}, \text{Cyp4a1}, \text{Cyp4a3}, \text{Cyp4f1}, \text{Cyp4f4}, \text{Cyp4f5}, \text{Cyp4f6}, \text{Ephx1}, \text{and Ephx2} \) in the rat heart were examined in this study. The results (Fig. 6A) showed that \( \text{Cyp1b1}, \text{Cyp2b1}, \text{Cyp2e1}, \text{and Cyp2j3} \) 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 CYP2E1 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 cardiac activity study (Fig. 6, C and D) demonstrated that the AA epoxygenase activity in CP-treated rats was significantly

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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 as EET-promoting substances under development, the CP has already been further studies using humansubjects would be necessary. 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, lipooxygenase, nitric oxide synthases, the mitochondrial respiratory chain, and P450 enzymes, have been reported in cardiac I/R studies (Ishihara et al., 2010).

The present study demonstrated that CYP2C11, which has been considered a potential ROS source during cardiac I/R (Sato et al., 2011), was induced after multiple doses of the CP in rats. However, the total superoxide anion generation in CP-treated rats was found to be significantly lower than those in model groups (Fig. 4). This suggests that despite CYP2C11 induction total ROS generation still decreased after CP administration.

Some inconsistencies in the results merit further consideration. First, 20-HETE formation was unchanged after CP administration in rats, but was significantly decreased in CP-treated H9c2 cells. The CP is an herbal formulation and contains many ingredients that may not be entirely absorbed into the plasma. This inconsistency may thus arise from how the concentrations and proportions of various components differ in vivo versus in vitro. Second, the ratio of DHETs/EETs was unchanged or slightly increased in I/R rats but was significantly decreased in H/R cells. The in vitro results suggest that sEH activity decreased after I/R in H9c2 cells, which is in line with previous studies. For example, hypoxia was found to significantly suppress sEH expression in mouse liver, pulmonary artery smooth muscle cells, and Hep3B and HEK 293 cells (Oguro et al., 2009; Keserü et al., 2010). However, the ratio of DHETs/EETs in rat plasma may reflect total sEH activity in vivo, not just in I/R hearts. Furthermore, after CP administration, the ratios of DHETs/EETs in vivo versus in vitro were both decreased, although in vivo sEH expression was unchanged. The final activity of an enzyme in vivo is the combination of its expression and its activity. Thus, the unchanged expression and decreased activity of sEH in vivo suggests that some absorbed components in the CP (or its metabolites) may inhibit sEH activity, a hypothesis that would require further investigation to confirm.

Increasing cardioprotective EET concentrations has been proposed as a therapeutic strategy not just for MIRI, but also for other CVDs such as heart failure, cardiac hypertrophy, stroke, and hypertension (Elbekai and El-Kadi, 2006). Over the last 35 years, although various synthetic sEH inhibitors have been explored, only one agent (AR9281) is now under clinical investigation (Xu et al., 2015). The instability of EETs limits their use as a therapeutic drug. However, EET analogs are still being tested using in vivo and in vitro models (Hye Khan et al., 2014). Thus, as a result of the present study, the CP is the first herbal preparation for CVD treatment that has been shown to increase EET concentrations in I/R rats. In contrast to the aforementioned EET-promoting substances under development, the CP has already been commercially available and widely used for many years. However, for these effects to be generalized to human cardiac AA P450 enzyme metabolism, further studies using human subjects would be necessary.

In conclusion, this study has demonstrated that after multiple doses of the CP in rats, certain cardiac AA epoxygenases were induced, and
cardioprotective EET concentrations were increased following IR. The results suggest that the mechanism underlying the CP’s protection in MIRI may be, at least in part, up-regulation of EET-generating P450 enzymes.

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Authorship Contributions

Participated in research design: Li, Hao, Ju, Wang.
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