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 Miltiorrhizae 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 chronic effects of the CP on myocardial ischemia-reperfusion (I/R) 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—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 (Cyp) enzymes in the onset, progression, and prognosis of CVD (Sato et al., 2011). EETs, 20-hydroxyeicosatetraenoic acid (20-HETE), 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 epoxydiol 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 $\omega$-hydroxylation metabolite of AA, and is primarily produced by members of the CYP4 family, including CYP4A and CYP4F isozymes (Sato et al., 2011). EETs act as vasodilatory substances in several vascular beds via activation of Ca$^{2+}$-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 Ca$^{2+}$, ATP-sensitive K$^+$, and Na$^+$ channels 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 epoxide 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 $\omega$-hydroxylation metabolite of AA, and is primarily produced by members of the CYP4 family, including CYP4A and CYP4F isozymes (Sato et al., 2011). EETs act as vasodilatory substances in several vascular beds via activation of Ca$^{2+}$-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 Ca$^{2+}$, ATP-sensitive K$^+$, and Na$^+$ channels.
used in this research (27 mg each pill) was obtained from Tasyli 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 Danshen; 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 liriospermic acid; 4.1 mg/g of rosinanici; 0.74 mg/g of tanshinone I; 0.63 mg/g tanshinone IIIA; 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.

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). DHET-d11; 14,15-EET-d11; and 20-HETE generation through CYP2C inhibition (Granville et al., 2011; Oni-Orisan et al., 2014); decreasing 20-HETE concentrations through direct administration of EETs, upregulating EETs are potent endogenous angiogenic factors (Xu et al., 2011).

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-dose 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 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

<table>
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<th>Group</th>
<th>Number of Rats</th>
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<tr>
<td>CP (high dose)</td>
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1Pharmacological study
2AA P450 enzyme metabolite determination
3AA-metabolizing P450 enzyme expression and activity.
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 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. Thereafter, the final microsomal pellet was reconstituted in cold PBS containing 20% glycerol and stored at -80°C. The bichromonic acid method using bovine serum albumin as a standard (Beyostain, 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 phosphate, 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), CYP2J2, sEH, and glyceraldehyde-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 glyceraldehyde-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-d11 (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 ethanol at 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-MS/MS 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 10 µg/ml streptomycin. Cells were grown in 75-cm² culture plates at 37°C in 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 placing 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
ionization interface. Chromatography was performed using a C18 column (Applied Biosystems Sciex, Ontario, Canada) equipped with an electrospray ionization interface. The ion spray voltage was 5500 V, the temperature of the ion source was 650°C, auxiliary gas (70 psi), and curtain gas (15 psi). The Multiple Reaction Monitoring (MRM) method was used to detect and quantify the AA P450 enzyme metabolites. The precursor ion was monitored for each ion of interest, and the production ions were detected. The detection mass range was between 300 and 800 m/z.

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

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 centrifuged at 12,000 rpm at 4°C for 5 minutes. The supernatant was loaded onto an 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 dried using a CentriVap Centrifugal Concentrator (Labconco). The sample was subjected to LC-MS/MS analysis.

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 treated with the CP (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/dtmin 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...
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 Metabolic 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-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. 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, Cyp2c1, Cyp2j3, and Cyp4f6 (Fig. 6A) were significantly induced compared with vehicle controls (P < 0.05). The western blotting results showed that expressions of CYP2J 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 generation, 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...
CP Reduces MIRI via Increasing EET levels in Rats

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 underlining 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).

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

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).

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. ****P < 0.01 and ####P < 0.001 compared with the sham group [one-way analysis of variance (ANOVA), Dunnett’s test].

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).

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. ****P < 0.001 compared with the sham group [one-way analysis of variance (ANOVA), Dunnett’s test]; **P < 0.01 and ***P < 0.001 compared with the model group (one-way ANOVA, Dunnett’s test).
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
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 ω-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 Ephx1, is distributed in the endoplasmic reticulum and mainly metabolizes the xenobiotic 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 Cyp1a1, Cyp1b1, Cyp2b1, Cyp2c11, Cyp2e1, Cyp2j3, Cyp4a1, Cyp4a3, Cyp4f1, Cyp4f4, Cyp4f5, Cyp4f6, Ephx1, and Ephx2 in the rat heart were examined in this study. The results (Fig. 6A) showed that Cyp1b1, Cyp2b1, Cyp2e1, 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 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 cardiac activity study (Fig. 6, C and D) demonstrated that the AA epoxide activity in CP-treated rats was significantly

**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 μM) 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 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). 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). 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).

In conclusion, this study has demonstrated that after multiple doses of the CP in rats, certain cardiac AA epoxygenases were induced, and 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. 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 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, upregulation of EET-generating P450 enzymes.

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