Cardiotonic Pill reduces myocardial ischemia-reperfusion injury via increasing EETs concentrations in rats

Meijuan Xu, Haiping Hao, Lifeng Jiang, Yidan Wei, Fang Zhou, Jianguo Sun, Jingwei Zhang, Hui Ji, Guangji Wang, Wenzheng Ju and Ping Li

State Key Laboratory of Natural Medicines (M.X., H.H., L.J., Y.W., P.L.) and Key Laboratory of Drug Metabolism and Pharmacokinetics (H.H., F.Z., J.S., J.Z., H.J., G.W.), China Pharmaceutical University, Nanjing, China; and Department of Clinical Pharmacology (M.X., W.J.), Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, China
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Address correspondence to: Prof. Ping Li, China Pharmaceutical University, No.24 Tongjia Lane, Nanjing 210009, China.

Tel: 86 25 8327 1379.

Fax: 86 25 8327 1379

E-mail: liping2004@126.com

Address correspondence to: Prof. Wenzheng Ju, Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, No.155 Hanzhong Road, Nanjing 210029, China.

Tel: 86 25 86617141 50518

Fax: 86 25 86555033

E-mail: ju_njxmj@126.com

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Abbreviations

AA, arachidonic acid; CAD, coronary artery disease; CP, Cardiotonic Pill; CDP, Compound Danshen Prescription; CK-MB, creatine kinase MB; CYPs, Cytochrome P450 enzymes; CVD, cardiovascular disease; DHE, dihydroethidium; EETs, epoxyeicosatrienoic acids; HE, ethidium; HETE, hydroxyeicosatetraenoic acid; LC-MS/MS, liquid chromatography/tandem mass spectrometry; H/R, hypoxia and reoxygenation; HR, heart rate; I/R, ischemia-reperfusion; LVEDP, left ventricular end-diastolic pressure; LDH, lactate dehydrogenase; LVSP, left ventricular systolic pressure; MIRI, myocardial ischemia reperfusion injury; NS, normal saline; ROS, reactive oxygen species; sEH, soluble epoxide hydrolases; TTC, 2,3,5-triphenyltetrazolium chloride
Abstract

Accumulating data suggest that EETs and 20-HETE, cytochrome P450 enzymes (CYPs) metabolites of arachidonic acid (AA), play important roles in cardiovascular diseases. For many years, 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 (CAD). However, its pharmacological mechanism has not been well elucidated. The purpose of this study was to investigate the chronic effects of CP on myocardial ischemia-reperfusion injury (MIRI) and AA CYPs 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 (40 mg/kg/d, 7 days) ischemia-reperfusion (I/R) rats 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 up-regulated (P<0.05), and AA-epoxygenases activity was significantly increased (P<0.05) after CP (40 mg/kg/d, 7 days) administration in rats. In H9c2 cells, CP also increased (P<0.05) the EETs concentrations and showed protection in hypoxia-reoxygenation (H/R) cells. However, an antagonist of EETs, 14,15-EEZE, displayed a dose-dependent depression of CP’s protective effects in H/R cells. In conclusion, up-regulation of cardiac epoxygenases after multiple-dose of CP, leading to elevated concentrations of cardioprotective EETs after myocardial I/R, may be the underlying mechanism, at least in part, for CP’s cardioprotective effect in rats.
Introduction

Cardiovascular disease (CVD), including coronary artery disease (CAD) 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 enzymes (CYPs) in the onset, progression, and prognosis of CVD. Over the past three decades, CYPs have been found expressed in the cardiovascular system (Enayetallah et al., 2004; Delozier et al., 2007). Endogenous CYPs 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 CYPs 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 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 hydrolases (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), the eNOS (Wang et al., 2003) and the TRPV4 channel (Vriens et al., 2005). In the heart, EETs regulate L-type Ca\textsuperscript{2+}-, ATP-sensitive K\textsuperscript{+}, and Na\textsuperscript{+}-channels (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-\kappa B activation and inhibiting TNF-\alpha induced VCAM-1 expression (Node et al., 1999). Moreover, EETs are potent endogenous angiogenic factors (Xu et al., 2011). 20-HETE, however, has been demonstrated to have vasoconstrictive (Miyata and Roman, 2005), pro-inflammatory (Ishizuka et al., 2008) and pro-apoptosis (Nilakantan et al., 2008) functions. Furthermore, though CYP2J2 and CYP2C both generate cardioprotective EETs from AA, studies have demonstrated that CYP2C also produces the detrimental reactive oxygen species (ROS) (Fleming et al., 2001). In animal models, effective protection against MIRI has been shown by: increasing EETs concentrations through direct administration of EETs, up-regulating the expression of AA epoxygenases using transgenic technology or inhibiting the activity of sEH (Seubert et al., 2004; Motoki et al., 2008; Batchu et al., 2011; Oni-Orisan et al., 2014); decreasing 20-HETE generation through CYP4A/4F inhibition(Nithipatikom et al., 2004); and depressing ROS formation via CYP2C inhibition (Granville et al., 2004). AA-metabolizing CYPs are thus considered to be therapeutic targets for MIRI and other CVDs.

For many years, Compound Danshen Prescription (CDP), an herbal preparation derived from Salviae Miltiorrhizae 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, Cardiotonic Pill (CP, also known as “Dantonic Pill” or “Compound Danshen Dripping Pill”), is the best-known. 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 CDP is recognized as clinically effective for CAD and 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 CYPs metabolism has been reported.

This study used 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 and reoxygenation (H/R) injury in H9c2 cells. It also examined CP’s effects on the concentrations of AA CYPs metabolites, and evaluated its influence on the cardiac expressions and activities of AA-metabolizing CYPs in rats.
Materials and Method

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-EEZE, 14, 15-DHET-d11, 14, 15-EET-d11 and 20-HETE were obtained from Cayman Chemicals (Ann Arbor, MI, USA). AA, NADPH, MgCl$_2$, 2,3,5-triphenyltetrazolium chloride (TTC), and dihydroethidium (DHE) were obtained from Sigma-Aldrich Co. (St.Louis, MO, USA). CK-MB isoenzyme assay kit and LDH assay kit 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, Japan). The primary antibody of CYP2C11 (ab3571) was purchased from Abcam (MA, USA), and antibodies of CYP2J (sc-67276), sEH (sc-24797), and GAPDH (sc-365062) were obtained from Santa Cruz Biotechnology, Inc (CA, USA). Pierce ECL Western Blotting Kit was bought from Thermo Fisher Scientific (CA, USA). CP used in this research (27 mg each pill, Lot: 110419) was obtained from Tasly Pharmaceutical Group Co., Ltd (Tianjin, China). The content levels of various danshen components were determined in our previous study (Liu et al., 2014) using three different CP batches (Lot: 100824, 110419, and 110510); average levels were: 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 lithospermic acid, 4.1 mg/g of rosmarinci acid, 0.74 mg/g of tanshinone I, 0.63 mg/g tanshinone IIA, 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 Rh1 (see Supplementary Table 1). HPLC-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, USA). All other chemicals used were the highest grade commercially available.

**Animals**

Adult male Sprague-Dawley rats (240±20 g body weight) were supplied by Nanjing Qinglongshan Experimental Animal Co. Ltd (Nanjing, China). All experiment procedures and protocols were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and approved by the Animal Ethics Committee of China Pharmaceutical University. The animals were maintained in a room with a temperature of 25±2 °C, a 12 h day-night cycle, and 50±10 % relative humidity. Water and food (laboratory rodent chow pellets, Nanjing, China) were provided ad libitum.

**Groups and Administration of CP in Rats**

As shown in Table 1, the present *in vivo* study was comprised of three sub-studies, using separate rat samples. Rats were divided into the groups at random (randomized block experiment design) and gavage-administered the normal saline (NS) or CP solution. CP solution was freshly prepared before use each day, by crushing the pills with mortar, then dissolving them in NS with the help of ultrasound. No organic solvent was added. The animal doses were derived according to the daily dose of 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 Myocardial Ischemia-Reperfusion Injury Model

After seven days administration of NS 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 (LAD) coronary artery, together with a silica rubber cannula, was ligated with a 6-0 silk suture. After 45 min of occlusion, the coronary artery was reperfused by cutting the suture off. The length of reperfusion was 15 min for superoxide generation measurements, 30 min and 3 h for AA CYPs metabolites determinations, and 24 h 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 min of myocardial ischemia and 24 h of reperfusion, all rats were narcotized by urethane (1 g/kg). Hemodynamics and cardiac function were measured using a biological 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 (HR), left ventricular end-diastolic pressure (LVEDP), left ventricular...
systolic pressure (LVSP), and maximum/minimum rates of developed left ventricular pressure
(±dP/dtmax). Subsequently, all rats were sacrificed to harvest the hearts immediately for
infarct size determination.

The size of infarcted myocardium was determined through 2,3,5-triphenyltetrazolium
chloride (TTC) staining. The hearts were kept at -20 °C for 10 min, and then cut into five 1-2
mm thick slices and incubated for 10 min in 1 % (w/v) TTC in pH 7.4 buffer at 37 °C. After
that, the slices were fixed in 10 % formalin, and pictures were taken. The sizes of infarcted
area (white coloration) and at risk area (the entire scanned section) were determined by the
Image-Pro Plus image analysis software (Version 4.1, Media Cybernetics, LP, USA). 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 h reperfusion. CK-MB and LDH are expressed in the heart muscle and
released during tissue damage, so 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 nm and 450 nm, respectively. All
procedures adhered to 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 DHE, 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 min 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, MD, USA).

**Real-time PCR Analysis of CYPs in the Rat Heart**

Total RNA from the heart tissues was isolated using a TRIzol reagent according to the manufacturer’s instructions. After that, first-strand cDNA synthesis was performed by using the Prime Script RT Master Mix Perfect Real Time Kit according to 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, CA, USA). 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 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 30 s. 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 PBS, cut into pieces, and homogenized using an IKA T10 basic ULTRA-TURRAX® homogenizer (IKA, 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. BCA method using bovine serum albumin as a standard (Beyotime, Nanjing, China) was used to determine the concentration of heart microsomal and cytosolic proteins.

**Western Blotting Analysis**

50 μg of cardiac microsomal (for CYPs) 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 GAPDH (1:200) for 2 h. Incubation with a peroxidase-conjugated secondary antibody was performed for 2 h at room temperature. The bands were visualized using the enhanced chemiluminescence method according to manufacturer’s instructions. The intensity of the protein bands was quantified, relative to the signals obtained for GAPDH, using ImageJ software (Version 1.48, National Institutes of Health, MD, USA).

**Activity Determination of AA-metabolizing CYPs**

Rat heart microsomes (1 mg/mL) were incubated at 37°C in a shaking water bath (50 r.p.m.). 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 min, 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 min. 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 min, the incubation mixtures were centrifuged at 12,000 rpm for 5 min 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 BSA (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 min), after which the reaction was conducted at 37 °C for another 5 min. 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 min, 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, USA). The residue was dissolved in 120 μL of dehydrated alcohol and vortex-mixed for 1 min. The tubes were then centrifuged at 12,000×g for 10 min. 5 μL of the resulting supernatant was injected into the LC-MS/MS system.

**Cell Culture and Hypoxia/Reoxygenation Treatments**

H9c2 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM, 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 a 5% CO₂ humidified incubator (Thermo Fisher Scientific, CA, USA). To mimic hypoxic injury in vitro, the cells were incubated for 9 h in a hypoxic solution containing 0.9 mM NaH₂PO₄, 6.0 mM NaHCO₃, 1.0 mM CaCl₂, 1.2 mM MgSO₄, 40 mM Natrium lacticum, 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) and oxygen was adjusted to 1.0 % and CO₂ to 5.0 %. After hypoxia, the cells were re-incubated in a normal DMEM at 37 °C in a 5% CO₂ humidified incubator for 3h.

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

To investigate the cardioprotective effect of CP, cardiac-derived H9c2 cells were pretreated with various concentrations (0.025–0.02 mg/mL) of CP (dissolved in the cell culture with the help of ultrasonic and vortex mixing) for 12 h. Following hypoxia and reoxygenation (H/R), the cell viability was determined. To investigate the actions of 14,15-EEZE on the cardioprotective effect of 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, whose 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 CYPs Metabolites Determination**

**Plasma sample:** 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 BHT (10 mg/mL). After a vortex mixer for
1 min, the mixture was then centrifuged at 12000 rpm at 4 °C for 5 min. 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 (3cc/60mg, Waters, USA) 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 CYPs 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
removed from the water layer carefully and dried using a CentriVap® Centrifugal
Concentrator (Labconco, USA). The sample was then redissolved in 125 μL ethanol. After
vortexed for 1 min, the content was centrifuged at 12000 rpm at 4 °C for 10 min, 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 centrifuged at 12,000 rpm for 5 min at 4°C, the
supernatant was loaded onto the SPE column, with the same extraction procedure as the
plasma sample.

**Measurement of AA CYPs Metabolites Using LC-MS/MS**

The CYPs metabolite of AA, EETs, DHETs, and 20-HETE, were simultaneously
quantitated using an 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, USA) and an
Alliance 2695 HPLC 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: 0min, 50%B; 3-5min, 62%B; 6-10min, 85%B; 11-18min, 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 nebulizing gas (50 p.s.i), auxiliary gas (70 p.s.i) and curtain gas (15 p.s.i). The MRM experiments were conducted by monitoring the precursor ion to product ion transitions for 20-HETE m/z 319.2-289.3, for 14,15-DHET m/z 337.2-207.0, for 11,12-DHET m/z 337.2-208.1, for 8,9-DHET m/z 337.2-127.1, for 5,6-DHET m/z 337.2-145.1, for 14,15-EET m/z 319.2-219.2, for 11,12-EET m/z 319.2-208.1, for 8,9-EET m/z 319.2-155.1, for 14,15-DHET-d11 m/z 348.3-207.2, and for 14,15-EET-d11 m/z 330.3-219.2. The lower limits of quantification of AA CYPs metabolites are shown in Supplementary Table 2.

**Statistical analysis**

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

Effect of CP on MIRI in Rats

The effects of CP on HR, hemodynamics, and cardiac function were determined after hearts were subjected to 45 min of ischemia followed by 24 h of reperfusion. There was no significant difference (P>0.05) in HR among all groups (Fig.1A). Hearts in the model group showed a significant decrease in LVSP (P<0.001) and dp/dt max (P<0.001), and a significant increase in LVEDP (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 (10, 20, and 40 mg/kg/d, 7 days) rats showed a dose-dependent recovery (P<0.05) of LVSP, LVEDP, 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 were significantly higher (P<0.001) than those in the sham group. However, treatment with 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% of the risk area. However, infarct size in CP-treated (10, 20, and 40 mg/kg/d, 7 days) rats 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 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). Hearts in CP-treated (10, 20 and 40 mg/kg/d, 7 days)
rats, however, showed a dose-dependent decrease (P<0.05) in HE fluorescence intensity compared with the model group.

**Effects of CP on AA CYPs Metabolites Concentrations in vivo**

As shown in Fig.5A, after reperfusion of 30 min, there was no significant difference in EETs, DHETs, and 20-HETE concentrations between the model group and the sham group. However, the concentrations of 20-HETE, 14,15-DHET, 11,12-DHET and 8,9-DHET in the model group were significantly higher (P<0.05) than those in the sham group after reperfusion of 3 h (Fig.5B). Treatment with 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 min and 3 h reperfusion in I/R rats, compared with either the sham group or the model group (Fig.5A and Fig.5B). Furthermore, the plasma concentrations of 14,15-DHET and 8,9-DHET in CP-treated (40 mg/kg/d, 7 days) rats were significantly lower than those in the model group (P<0.05) after reperfusion of 3 h (Fig. 5B). The individual concentrations of AA CYPs metabolites in the sham, model, and CP-treated groups are shown in Supplementary Table 3.

To evaluate the effects of CP on the activities of AA CYPs 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.5C and Fig.5D, these values are significantly higher in the model group than in the sham group at 3 h after reperfusion (P<0.05). After CP administration, however, at both 30 min and 3 h after 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 CP on the Expression and Activity of AA-metabolizing CYPs in vivo**

The effects of CP on the expressions and functions of AA-metabolizing CYPs in normal rat hearts were investigated using real-time PCR, Western blotting, and LC-MS/MS assay. The results indicate that after administration of CP (40 mg/kg/d) for 7 days, expressions of cardiac *Cyp1b1*, *Cyp2b1*, *Cyp2e1*, *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 CYPs was determined using AA (50 μM) as the substrate in heart microsomes prepared from vehicle or CP-treated rats (detailed results in Supplementary 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 CP on the H/R Injury and Concentrations of AA CYPs Metabolites in vitro**

The protective effect of CP on H/R injury in cells was investigated in vitro. The results (Fig.7A) demonstrated that CP (0.025, 0.05, 0.1 and 0.2 mg/mL) caused a dose-dependent
improvement (66.2±2.4% vs. 76.1±1.8%, 77.9±1.4%, 84.5±2.1% and 90.9±1.9%) in cell survival rate in H9c2 cells subjected to hypoxia of 9h and reoxygenation of 3 h. The concentrations of AA CYPs metabolites in normal or H/R H9c2 cells were also determined (see Supplementary Table 5). As shown in Fig.7B and Fig.7C, the concentrations of 20-HETE, (DHETs+EETs), and EETs in cells after H/R were significantly higher (P<0.05), while the values of DHETs and DHETs/EETs were significantly lower (P<0.01), than those in normal controls. After incubation with CP (0.2 mg/mL) for 12 h, the values of 20-HETE and DHETs/EETs in normal H9c2 cells were significantly lower (P<0.001), while the concentrations of EETs and (DHETs+EETs) were significantly higher (P<0.001) than those in normal controls (Fig.7B and Fig.7C). As for H/R cells subjected to hypoxia of 9h and reoxygenation of 3h after pretreatment with CP (0.2 mg/mL) for 12h, the concentrations of EETs and (DHETs+EETs) were significantly higher (P<0.01), while the values of 20-HETE and DHETs/EETs were significantly lower (P<0.001) than those in the hypoxic controls.

**Effects of 14,15-EEZE on 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 (0.2 mg/mL) H/R cells from 85.8±4.8% to 84.3±1.2%, 70.1±2.6% and 55.6±4.3%, respectively. Furthermore, effects of 14,15-EEZE (1 μM) on cell survival rate in normal control, H/R control, and H/R CP-treated cells were also determined. 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% vs. 59.9±3.9%, $P>0.05$), and significantly reduced the cell survival rate (85.8±4.2% vs. 72.2±3.5%, $P<0.05$) in CP-treated (0.2 mg/mL) H/R cells.
Discussion

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

To explore the mechanism underlining this protection, CP’s effect on AA CYPs 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 to 120 min. Accordingly, levels of these AA metabolites were measured at 30 min and 3 h of reperfusion in I/R rats. Results of the present study (Fig.5) found that at 30 min of reperfusion, plasma concentrations of AA CYPs metabolites in the model group were slightly (but not significantly) higher than those in the sham group. By 3 h of reperfusion, however, the concentrations of 20-HETE, DHETs, and (EETs+DHETs) in the model group were significantly higher \( (P<0.05) \) than the sham group, suggesting 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 EETs concentration in I/R rats increased earlier and more dramatically than in the model group (Fig.5).

Because of the vasodilatory, anti-inflammatory, pro-angiogenic and anti-apoptosis actions of EETs, augmented EETs concentrations during I/R has been considered a protective mechanism (Seubert et al., 2004). Increased EETs levels during I/R has proven beneficial in
numerous studies. For example, CYP2J2 transgenic mice exhibited not only increased EETs biosynthesis in coronary arteries, but also improved post-ischemic 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). Likewise, exogenous administration of EETs analogues, UA-8, provided significant protection against MIRI (Batchu et al., 2011). Results of the present study (Fig.5) demonstrated that after CP administration, total activity of AA epoxygenases (EETs+DHETs) increased, while sEH activity (DHETs/EETs) decreased, ultimately leading to elevated EETs plasma concentrations. Results also indicated that multiple-dose of CP had little effect on AA ω-hydroxylation (20-HETE) in I/R rats.

The protection of CP against H/R injury and its effect on AA CYPs 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, CP exhibited dose-dependent protection on H/R injury and a similar impact on AA CYPs metabolism (Fig.7). In drug-untreated cells, concentrations of 20-HETE and EETs were significantly higher after H/R, compared to non-H/R controls. As expected, this suggested an increased generation of AA CYPs metabolites after H/R. In CP-pretreated cells, however, EETs 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 EETs
antagonist at a receptor binding site, and selectively antagonizes many EETs actions (Gross et al., 2008). Results of this study found that 14,15-EEZE dose-dependently reversed CP’s protective effects against the H/R injury in H9c2 cells, suggesting that this protection is mediated via an EETs pathway.

To investigate why AA CYPs metabolism was influenced by multiple-dose of CP, expressions and catalyzing activities of AA-related CYPs 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, CYP4F5, and CYP4F6 have all been found expressed in the rat heart (Xu et al., 2004). Epoxide Hydrolase (EH) can be classified into mEH or sEH, according to its distribution. mEH, encoded by Ephx1, is distributed in the endoplasmic reticulum and mainly metabolizes the xenobiotic epoxide. sEH, encoded by Ephx2, is distributed in the cytoplasm and primarily metabolizes endogenous epoxides. Though investigations into cardiovascular protection mainly focus on sEH, both mEH and sEH have been found able to produce DHETs from EETs (Morisseau and Hammock, 2013). Drawing from the above 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. Results (Fig.6A) showed that Cyp1b1, Cyp2b1, Cyp2e1, and Cyp2j3, all of which encode EETs-generating enzymes, were
significantly induced after CP administration ($P<0.05$). In line with mRNA results, the protein expression of CYP2J was also found significantly higher than in controls ($P<0.05$). In contrast, while CYP2C11 was significantly induced ($P<0.05$), its mRNA was not significantly up-regulated ($P>0.05$). This implies that CP may have little effect on CYP2C11 gene expression, but significant impact on its post-transcriptional translation process. The cardiac activity study (Fig.6C and Fig.6D) demonstrated that the AA epoxygenase activity in CP-treated rats was significantly ($P<0.05$) higher than the controls. Based on these observations, one can infer that multiple-dose of CP may have induced the expression of cardiac AA epoxygenases, such as CYP2J3 and CYP2C11, ultimately leading to increased EETs 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 CYPs, 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 sources during cardiac I/R (Sato et al., 2011), was induced after multiple-dose of 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. Firstly, 20-HETE formation was unchanged after CP administration in rats, but significantly decreased in
CP-treated H9c2 cells. 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. Secondly, the ratio of DHETs/EETs was unchanged or slightly increased in I/R rats; but 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 the previous studies. For example, hypoxia was found to significantly suppress sEH expression in mouse liver, pulmonary artery smooth muscle cells, Hep3B and HEK 293 cells (Oguro et al., 2009; Keseru et al., 2010). The DHETs/EETs ratio in rat plasma, however, may reflect total sEH activity in vivo, not just in the I/R hearts. Furthermore, after CP administration, the ratios of DHETs/EETs in vivo and 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 CP (or its metabolites) may inhibit sEH activity, a hypothesis that would require further investigation to confirm.

Increasing cardioprotective EETs 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, though 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. EETs analogs, however, are still being tested using in vivo and in vitro models (Hye Khan et
al., 2014). Now, as a result of the present study, CP is the first herbal preparation for CVD treatment that has been shown to increase EETs concentrations in I/R rats. In contrast to the aforementioned EETs-promoting substances under development, CP has already been commercially available and widely used for many years. However, for these effects to be generalized to human cardiac AA CYPs metabolism, further studies using human subjects would be necessary.

In conclusion, this study demonstrated that after multiple-dose of CP in rats, certain cardiac AA epoxygenases were induced, and cardioprotective EETs concentrations were increased following I/R. Results suggest that the mechanism underlying CP’s protection in MIRI may be, at least in part, upregulation of EETs-generating CYPs.
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Author contributions

Participated in research design: Ping Li, Haiping Hao, Wenzheng Ju and Guangji Wang.

Conducted experiments: Meijuan Xu, Lifeng Jiang, Yidan Wei, and Jingwei Zhang

Contributed new reagents or analytic tools: Jianguo Sun and Hui Ji.

Performed data analysis: Meijuan Xu and Fang Zhou.

Wrote or contributed to the writing of the manuscript: Meijuan Xu and Haiping Hao.


Gross GJ, Gauthier KM, Moore J, Falck JR, Hammock BD, Campbell WB, and Nithipatikom K (2008) Effects of the selective EET antagonist, 14,15-EEZE, on cardioprotection...


Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids.


phosphatase domains in soluble epoxide hydrolase to vascular endothelial growth


Study of the protective mechanisms of Compound Danshen Tablet (Fufang Danshen
Pian) against myocardial ischemia/reperfusion injury via the Akt-eNOS signaling


postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial

cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium.

*Circ Res* **97**:908-915.


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M.X. and H.H. contributed equally to this work.
Figure legend

Fig.1 Effects of CP (10, 20 and 40mg/kg/d, 7 days) on the I/R induced cardiac dysfunction.

Data show the (A) HR, (B) LVSP, (C) LVEDP, (D) dp/dt max, and (E) -dp/dt max of hearts subjected to 45 min of ischemia followed by 24 h of reperfusion. Data represent Mean±S.E. obtained from six independent measurements. *P<0.05 **P<0.01 ***P<0.001 compared with the sham group (One-way ANOVA, Dunnett test); *P<0.05 **P<0.01 ***P<0.001 compared with the model group (One-way ANOVA, Dunnett test).

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

Fig.3 Effects of CP (10, 20 and 40mg/kg/d, 7 days) on the myocardial infarction in rats subjected to 45 min of ischemia and 24 h of reperfusion. The myocardial infarction was determined by TTC staining. Data represent Mean±S.E. obtained from six independent measurements. ***P<0.001 compared with the sham group (One-way ANOVA, Dunnett test); **P<0.01 ***P<0.001 compared with the model group (One-way ANOVA, Dunnett test).
Fig.4 Effects of CP (10, 20 and 40mg/kg/d, 7d) on the superoxide generation in rat’s heart subjected to 45 min of ischemia and 15 min of reperfusion. Cryosections of hearts after reperfusion were incubated with DHE (10 μM) at 37 °C in dark for 30 min, 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 Mean±S.E. obtained from three independent measurements. ***P<0.001 compared with the sham group (One-way ANOVA, Dunnett test); *P<0.05 **P<0.01 compared with the model group (One-way ANOVA, Dunnett test).

Fig.5 Effects of CP (40mg/kg/d, 7days) on the plasma concentrations of AA CYPs metabolites in rats subjected to 45 min of ischemia, and (A) 30 min or (B) 3h of reperfusion. (C) The summarized concentrations of the AA CYPs metabolites and (D) the DHETs/EETs ratios of each group. The concentrations of AA CYPs metabolites were determined using an LC-MS/MS method. Data represent Mean±S.E. obtained from seven independent measurements (hearts). * P<0.05 **P<0.01 ***P<0.001 compared with the sham group (One-way ANOVA, Dunnett test) at the same time; * P<0.05 **P<0.01 ***P<0.001 compared with the model group (One-way ANOVA, Dunnett test) at the same time; *P<0.05 compared with the value of 30 min (One-way ANOVA, Dunnett test) in the same group.

Fig.6 Effects of CP (40mg/kg/d, 7days) on (A) mRNA, (B) protein, (C) activity of AA-metabolizing CYPs (cardiac microsomes), and (D) activity of sEH (cardiac cytosols) in rat heart. Total RNA was isolated from the hearts of vehicle controls and rats treated with CP
for 7 days, and all 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 CP for 7 days, and proteins were separated on 10% SDS-PAGE. The catalyzing activity of AA-metabolizing CYPs 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 min in microsomes and 5 min in cytosols, the concentrations of AA CYPs metabolites, 20-HETE, EETs, and DHETs were determined using an LC-MS/MS method. Data represent Mean±S.E. obtained from nine hearts. * P<0.05 **P<0.01 ***P<0.001 compared with the vehicle controls (Student’s t-test).

Fig.7 Effects of CP on the (A) H/R injury, and (B and C) AA CYPs metabolism in H9c2 cells. The cells were incubated in a hypoxic condition for 9 h and then re-incubated in a normal condition for 3 h to mimic the H/R injury, and CP was administered 12h before H/R. H/R injury was determined using a CCK-8 assay. AA CYPs metabolism was estimated by the concentrations of AA CYPs metabolites determined in cells. Data represent Mean±S.E. obtained from three to four independent measurements. *P<0.05 **P<0.01 ***P<0.001 compared with the normal controls (One-way ANOVA, Dunnett test); ##P<0.01 ###P<0.001 compared with the ischemia controls (One-way ANOVA, Dunnett test).

Fig.8 Effects of 14,15-EEZE on the protection of CP (0.2 mg·mL⁻¹) on H/R injury in H9c2 cells. (A) dose-dependent attenuation of 14,15-EEZE (0.1, 1 and 10 μM) on the protection of CP on H/R cells; (B) effects of 14,15-EEZE on the cell survival rate in normal control, H/R
control, and H/R CP-treated cells. The cells were incubated in a hypoxic condition for 9 h and then re-incubated in a normal condition for 3 h to mimic the H/R injury, and CP (0.2 mg/mL) was administered 12 h before H/R. Data represent Mean±S.E. obtained from three to four independent measurements. * P<0.05 ** P<0.01 *** P<0.001 compared with the H/R controls (One-way ANOVA, Dunnett test); * P<0.05 # P<0.01 compared with the CDP-treated H/R cells (One-way ANOVA, Dunnett test).
Table 1 Groups, numbers and administration of CP in rats

<table>
<thead>
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<td>CP-Middle</td>
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<td>CP-High</td>
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<sup>a</sup> Pharmacological study; <sup>b</sup> AA CYPs metabolites determination; <sup>c</sup> AA metabolizing CYPs expression and activity
Table 2 Primer sequences used for real-time PCR reactions.

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</table>
Figure 1
Figure 2
Figure 3

Infarct size (%)

- Sham
- Model
- CP 10mg/kg
- CP 20mg/kg
- CP 40mg/kg

Significance levels:
- **: p < 0.01
- ***: p < 0.001

### Additional Comments

The bar chart illustrates the infarct size (%) across different groups: Sham, Model, CP 10mg/kg, CP 20mg/kg, and CP 40mg/kg. The Model group shows a significantly higher infarct size compared to the Sham group. The CP 10mg/kg group shows a decreased infarct size compared to the Model group, although the significance is not marked. The CP 20mg/kg and CP 40mg/kg groups show further decreases in infarct size, with CP 40mg/kg being the most significant. The images below the bars provide a visual representation of the infarct areas.
Figure 4

(A)  Images showing different conditions: Sham, Model, CP 10mg/kg, CP 20mg/kg, and CP 40mg/kg.

(B)  Graph showing HE Fluorescence (a.u.)

- Sham:  
- Model:  
- CP 10mg/kg:  
- CP 20mg/kg:  
- CP 40mg/kg:  

Significance levels:  
- #***: Significant at p < 0.001
- *: Significant at p < 0.05
- **: Significant at p < 0.01
Figure 5
Figure 8