Palmatine from *Coptidis rhizoma* reduces ischemia–reperfusion-mediated acute myocardial injury in the rat

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**ABSTRACT**

The aim of the present study was to evaluate the protective effect of palmatine, one of active ingredients of *Coptidis rhizoma*, against myocardial ischemia–reperfusion (I/R) injury is due to its antioxidant and anti-inflammatory action. Adult male rats were subjected to 30 min of ischemia and 6 or 24 h of reperfusion. Rats were randomized to receive vehicle or palmatine 1 h before reperfusion. Infarct size, myocardial function, and the antioxidant enzyme activity, such as malonaldehyde (MDA), lactate dehydrogenase (LDH), creatine phosphokinase (CK), superoxide dismutase (SOD) and catalase (CAT) were measured. Palmatine significantly improved I/R-induced myocardial dysfunction by increasing the values of the first derivative (±dp/dt) of left ventricular pressure and decreased infarct size by 50% (*P* < 0.01 versus vehicle). As expected, palmatine markedly increased the activity of LDH, CK, and MDA contents in I/R rat serum, and it also significantly inhibited the decline of the activity of SOD and CAT in I/R cardiac tissues. In addition, COX-2 and iNOS expression in I/R myocardium was significantly reduced. Interestingly, plamatine increased heme oxygenase (HO)-1 induction in human aortic endothelial cells. We concluded that palmatine protects hearts from I/R injury in rats possibly by reducing oxidative stress and modulating inflammatory mediators.

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1. Introduction

Myocardial infarction is one of common ischemic heart diseases, which is the leading cause of death in developed countries. In the ischemic heart, initial cardiac damage is prompted by diminished blood supply. However, swift restoration of normal blood supply is imperative to minimize cardiac injury. Unfortunately, reperfusion itself can lead to additional injury in the form of cardiac dysfunction, reperfusion arrhythmias, and exacerbated myocardial infarction. Oxidant stress seems to play a major role for cardiac dysfunction, reperfusion arrhythmias, and exacerbated myocardial infarction. Oxidant stress seems to play a major role for organ injury during ischemia and reperfusion (I/R) (Cross et al., 1987; Halliwell et al., 1992; McCord, 1985; Jeroudi et al., 1994). Indeed, increased production of reactive oxygen species (ROS) and accumulation of calcium in the cytosol and mitochondria are two major causal factors of ischemia and reperfusion (I/R) injury (Murphy and Steenbergen, 2008; Zucchi et al., 2007). The well-known protection of flavonoids against I/R injury is conferred by their direct antioxidant activities (Kim et al., 2009; Han et al., 2008). Palmatine, the protoperoberine class of isoquinoline alkaloids, has been found in plants of various families (Küpeli et al., 2002) and mainly presents in the rhizomes of *Coptidis* (Jung et al., 2008). Recently we extracted and isolated seven bioactive alkaloids from *Coptidis rhizoma* and found that these alkaloids have various pharmacological activities (Jung et al., 2008). Although *C. rhiza* is reported to possess anti-inflammatory (Schinella et al., 2002), anti-proliferative (Tse et al., 2006), antioxidant (Schinella et al., 2002; Yokozawa et al., 2005), anti-hypertensive activities (Ko et al., 2000), and hypoglycemic and hypcholesterolemic effects (Yuan et al., 2006), many studies have been focused on berberine (Hely et al., 2009; Liang et al., 2008; Xu et al., 2008; Lee et al., 2007). It is unknown whether palmatine has cardioprotective effect against myocardial I/R injury. Thus, we tested our hypothesis that palmatine has cardioprotective effect against myocardial I/R injury due to its antioxidant and anti-inflammatory actions.
2. Materials and methods

2.1. Materials

Palmatine was isolated from C. rhizoma and identified as described in [Jung et al., 2008]. Human aortic endothelial cells (HAEC), endothelial cell basal medium 200, and low serum growth supplements were purchased from Invitrogen (Portland, OR), Dulbecco’s modified Eagles medium (DMEM), fetal bovine serum (FBS) and antibiotics were supplied by Gibco-BRL (Rockville, MD). Lactate dehydrogenase (LDH) and creatine phosphokinase (CK) activity assay kit were purchased from Bio-Assay Systems (Hayward, CA). Anti-INOS antibody was purchased from Transduction Laboratories (Lexington, KY), and Anti-COX-2, Anti-HO-1, Anti-HMGBl antibodies and horseradish peroxidase labeled anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All chemicals otherwise not mentioned were obtained from Sigma Co. (St. Louis, MO).

2.2. Experimental animals

Male Sprague–Dawley rats weighing 200–250 g (Samtako Inc. Osan, Korea) were used for the study. All animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals Published by the USA National Institutes of Health (NIH publication No. 85-23, revised in 1996). The protocol of the animal study was approved by Animal Research Committee of the Gyeongsang National University, Korea.

2.3. Experiment protocol

The animals were randomly divided into two sets of experiment each consisting of four groups. In the first set of experiment, animals were divided as follows: (1) sham-operated rat group (Sham), in which no tightening of the left anterior descending (LAD) coronary artery sutures was performed (n = 6), (2) I/R control group (I/R), in which rats were pretreated with vehicle (DMSO 0.25 mL, L-I, 1 h before ischemia, n = 10), (3) I/R + palmatine 25 mg/kg group (I/R + pal 25), in which rats were given palmatine at a dose of 25 mg/kg (L-I, 1 h before ischemia, n = 10), (4) I/R + palmatine 50 mg/kg group (I/R + pal 50), in which rats were given palmatine at a dose of 50 mg/kg (L-I, 1 h before ischemia, n = 10). The animals were exposed to a 30 min ischemia followed by 24 h of reperfusion after which infarct size and heart functions were estimated.

For the second set of experiment, animals were exposed to a 30 min ischemia followed by 6 h of reperfusion for biochemical analysis: (1) Sham group (Sham, n = 24), (2) I/R control group (I/R, n = 24), (3) I/R + pal 25 mg/kg group (I/R + pal 25, n = 24), (4) I/R + pal 50 mg/kg (I/R + pal 50, n = 24). Blood serum samples or myocardial tissues from ischemic zone were collected after 6 h reperfusion to measure the antioxidant marker enzyme activities.

2.4. Surgical procedures

The animals were anesthetized with ketamine (100 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.), and intubated through a midline tracheotomy and mechanically ventilated using a positive pressure respirator for small animals (Type 7025, Ugo Basile, Varese, Italy; 60–80 strokes/min, 8 ml/kg body weight) at a rate adjusted to keep blood pH and blood gases within normal range. The chest was opened via a left thoracotomy, followed by a pericardiotomy. A 4–0 black silk suture was placed around the LAD coronary artery, and the ends were pulled through a small vinyl tube to form a snare and then tightened. After 30 min of ischemia, the myocardium was reperfused by loosening the snare for 6 or 24 h. The body temperature was measured by an electric thermometer placed in the rectum and maintained at 37 °C by a heating pad placed under the rats. Sham-operated rats were subjected to the same procedures without LAD occlusion. After surgery all animals were housed in cages and maintained on a 12 h night/day cycle and allowed free access to food and water at all times until the experiments begin.

2.5. Hemodynamic measurements

The right common carotid artery was cannulated with a 2 F Millar catheter (Millar Instruments, Houston, TX) into the ascending aorta to measure systolic blood pressure (SBP), diastolic pressure (DBP), mean arterial pressure (MAP) and heart rate (HR). The pressure transducer was then advanced into the left ventricle (LV) to measure left ventricular end diastolic pressure (LVEDP), and positive and negative maximal values of the first derivative of left ventricular pressure (±dp/dt). LVEDP was digitally processed using a hemodynamic analyzing system (Powerlab Hardware; AD Instruments, Charlotte).

2.6. Determination of area at risk and infarct size

After measurements of heart function, before harvesting the hearts, the LAD was reoxygenated. Two mL of 1% Evans blue dye was injected into the femoral vein route to stain the normally perfused region blue and outline the area at risk. Right ventricle and atrium tissues were removed, and the LV was cut into transverse slices. The area at risk was separated from the non-ischemic zone and incubated for 20 min at 37 °C in a 1% solution of triphenyltetrazolium chloride (TTC) to visualize the infarct area. The area at risk as a percent of the LV mass (AAR at risk, %) and the infarct area as a percent of the area at risk mass (Infarct size, %) were calculated.

2.7. Biochemical analyses

LDH and CK activities were determined in a Perkin–Elmer lambda EZ210 Spectrophotometer by using commercial kit supplied from BioAssay Systems (CA). Thiobarbituric acid reactant malondialdehyde (MDA) level was measured by colorimetric analysis using a spectrophotometer with the associated detection kits (Cayman Chemical, MI). Catalase and SOD activity were measured using the colorimetric assay (Biosytech, Catalase 520, Oxir Research).

2.8. Cell culture

Human aortic endothelial cells (HAEC) were grown in medium 199 supplemented with 20% fetal bovine serum (FBS), 100 IU/ml penicillin, 10 μg/ml streptomycin, and 50 μg/ml ECGS. RAW 264.7 cells were grown in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in 100 mm dishes and grown in a humidified 5% CO2 incubator.

2.9. Western blot analysis

To detect iNOS, or COX-2, myocardial tissues were homogenized in ice cold lysis buffer containing 0.5% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–Cl (pH 7.5), and protease inhibitors. Homogenates were centrifuged at 12,000 rpm for 20 min, and supernatants were collected. For detecting HO-1, HEACs were treated dose dependently for 8 h, and then cells were harvested and lysed in lysis buffer. Protein concentration was determined by the Bradford method. Aliquots of the total protein (40 μg) were electrophoresed on 12% polyacrylamide gel. For detecting high mobility group box 1 (HMGB1), culture medium from RAW 264.7 cells were collected after 16 h incubation with lipopolysaccharide (LPS, 1 μg/ml) and concentrated (10×) by means of centrifric columns (Millipore, Billerica, MA) and supernatants were loaded on a 12% polyacrylamide gel. The electrophoresed proteins were transferred to polyvinylidene difluoride membranes by semidy electrophoretic transfer at 15 V for 60–75 min. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 2 h at room temperature. Next, the membranes were incubated with anti-iNOS, anti-COX-2, anti-HO-1 antibodies or anti-HMGB1-antibody at a 1:500 concentration (4°C) by means

2.10. Statistical analysis

All data were expressed as mean ± SD. Differences between groups were assessed by one-way ANOVA followed by Student–Newman-keuls test. Values of p < 0.05 were considered to be statistically significant.

3. Results

3.1. Palmatine increases functional recovery from ischemia/ reperfusion heart

Baseline values of hemodynamic parameters such as LVEDP, ±dp/dt and HR were similar between I/R and I/R + palmatine (Pal). Treatment with palmatine (50 mg/kg) significantly inhibited decrease of ±dp/dt (P < 0.05) compared to I/R (Table 1). Moreover, the elevated LVEDP in I/R control significantly (P < 0.05) attenuated by palmatine (50 mg/kg). However, lower dose of palmatine (25 mg/kg) did not show protective effective against myocardial I/R injury.

3.2. Palmatine reduces myocardial injury

As shown in Fig. 1, the ratio of AAR/LV did not differ between I/R and palmatine-treated groups. However, palatine (50 mg/kg) significantly reduced the area of myocardial infarction as a ratio of area at risk for infarction (IA/AAR) compared to I/R groups (P < 0.05).
3.3. Palmatine decreases serum levels of LDH, CK and MDA

The level of LDH in serum was used to monitor the damage of myocardial tissues. As shown in Fig. 2A, I/R caused a significant increase in the serum level of LDH compared with sham group. The release of LDH was significantly reduced by treatment with palmatine (25 or 50 mg/kg). In I/R control group, the activity of CK was also markedly elevated than sham group. As expected, palmatine (25 or 50 mg/kg). In I/R control group, the activity of CK was also significantly reduced compared to I/R controls. Administration of 50 mg/kg palmatine significantly reduced the infarct size. Results are mean ± SD. \( P<0.05 \) in each groups. \( n=10 \).

3.4. Palmatine decreases SOD and CAT activity

As shown in Fig. 3A and B, the levels of enzymatic activity (SOD and CAT) in myocardial tissues were significantly reduced due to I/R, respectively. However, compared with I/R control group, administration of palmatine significantly decreased not only SOD but CAT activity. In addition, administration of palmatine reduced I/R-induced expression of iNOS and COX-2 proteins in myocardial tissues (Fig. 3C).

3.5. Palmatine induces HO-1 in HAEC and reduces HMGB1 release in LPS-stimulated RAW 264.7 cells

Finally, we addressed whether palmatine induces HO-1 in HAEC. Fig. 4A shows that palmatine induced HO-1 expression concentration-dependently in HAEC. Furthermore, palmatine concentration-dependently reduced the release of HMGB1 in LPS-stimulated RAW 264.7 cells (Fig. 4B).

4. Discussion

We clearly demonstrated that palmatine, an active ingredient of \( C. rhizoma \), reduced myocardial injury and improved function of left ventricle in I/R rat. The protective effect of palmatine on I/R injury comes from reducing the burden of oxidant stress through antioxidant and possibly anti-inflammatory action. In fact, a growing body of evidence indicates that oxidative stress, such as ROS, and free radicals, and reactive nitrogen species, have long been recognized to act as the major mediators of I/R injury. Many substances such as antioxidants and/or free radical scavengers have been shown to minimize reperfusion injury and indirectly support the key role played by oxygen radicals in I/R injury (Akhlagli and Bandy, 2009; Kim et al., 2006). To support this, palmatine ameliorated myocardial injury against I/R in the present study which has been known to possess antioxidant activity (Yokozawa et al., 2004, 2005). Consistent with others, we found that palmatine significantly increased SOD and CAT concentration in I/R rat myocardial tissues. SOD is an antioxidant enzyme that catalyzes the conversion of \( O_2^- \) into \( H_2O_2 \) and \( O_2 \). Thus, it can be speculated that palmatine could decrease the release of free radicals during I/R injury. Moreover, MDA, a lipid peroxidation end product, has been used to assess oxygen free radical-mediated injury of I/R myocardium (Ozer et al., 2005). The reduced the increase of serum MDA content in I/R rats by palmatine reflect indirectly that accumulation of polymorphonuclear cells can be prevented in infarct area. In addition, palmatine reduced CK and
LDH, which are also important metabolic enzymes in cardiomyocytes. Thus, CK and LDH have been regarded as reliable indices with which to evaluate myocardial I/R injury. Because anti-inflammatory action of palmatine was reported (Yasukawa et al., 1991), to understand anti-inflammatory action of palmatine more, we used LPS-stimulated RAW 264.7 cells to investigate whether palmatine reduces HMGB1 protein release, which known as a novel inflammatory cytokine that orchestrates the induction of other pro-inflammatory cytokines such as tumor necrosis factor-α, IL-1β, and IL-8 (Yamada and Maruyama, 2007). HMGB1 plays an important role in the activation of neutrophils during inflammation (Park et al., 2003) such as I/R condition. As expected, palmatine significantly reduced HMGB1, suggesting it may reduce inflammation during I/R, thus limiting infiltration of neutrophils into the infarct tissues. In addition, we measured two key important inflammatory genes namely, COX-2, and iNOS. Because it has been well recognized that compounds which block the expression of both COX-2 and iNOS can effectively treat inflammatory diseases. We found that palmatine reduced the expression of iNOS and COX-2 in the ischemic zone of I/R tissues. A significant improvement of myocardial function and reduction of myocardial injury were shown only dosage of 50 mg/kg palmatine in vivo, although 25 mg/kg reduced iNOS protein expression as much as 50 mg/kg in I/R myocardium, which may suggest that inhibition of iNOS expression would not be major factor to improve myocardial function by palmatine, but it indicates that it has anti-inflammatory action. Indeed, there are reports suggesting that increased iNOS expression can be beneficial rather than detrimental in ischemic preconditioning heart (Guo et al., 1999).

**Fig. 2.** Effect of palmatine on serum LDH, CK, and MDA levels after I/R injury in each group rats. The levels of LDH (A), CK (B), and MDA (C) in serum were measured by using commercial kit supplied from BioAssay Systems. The blood serum samples were collected after 6 h reperfusion to measure the activity of LDH, CK, and MDA. Results are mean ± SD (n = 6 in each groups). *P < 0.05 vs. sham, #P < 0.05 vs. I/R control, **P < 0.01 vs. I/R control.

**Fig. 3.** Effect of palmatine on antioxidant enzyme activity and induction of inflammatory markers in myocardial I/R tissues. After 6 h reperfusion in the sham, I/R, and palmatine (25 mg/kg, 50 mg/kg) groups, the myocardial tissue from ischemic zone after I/R with or without palmatine (25 mg/kg, 50 mg/kg) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with anti-iNOS, anti-COX-2, or β-actin antibody (C). Results are mean ± SD. (n = 6 in each groups). *P < 0.05 vs. sham, #P < 0.05 vs. I/R control.
is extremely cytotoxic (Kim et al., 1999; Beckman and Koppenol, 1996; Ferdinandy et al., 2000). In deed, NO itself is not toxic and does not produce significant tissue injury even at a very high concentration (Kim et al., 1999). However, the NO/O_2 reaction and the subsequent production of peroxynitrite reverses its biological protective properties and results in oxidative/nitrative tissue injury (Beckman and Koppenol, 1996). It should be noted that C. rhizoma extract and active ingredients including palmatine scavenged peroxynitrite and protected cells from peroxynitrite-induced damage (Yokozawa et al., 2004, 2005). Another interesting finding in the present study was that palmatine induced heme oxygenase (HO-1) expression in human aortic endothelia cells (HAEC). HO-1 is known to possess antioxidant and anti-inflammatory action. Thus, palmatine may induce HO-1 in cardiac muscles or coronary artery endothelial cells during I/R, which needs further investigation. Our finding suggests that the enhancement of antioxidant activity and the inhibition of peroxidation of free radicals in the myocardium, may be, at least partially, involved in the cardioprotective mechanism of palmatine against myocardial I/R injury.

In conclusion, our study is the first attempt to investigate the cardioprotective effect of palmatine and the possible underlying mechanisms. Palmatine significantly improved myocardial function and reduced infarct size against myocardial I/R injury possibly due to its strong antioxidant action and anti-inflammatory action. Thus, it may be beneficial in cardiovascular disorders, such as myocardial infarction.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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**References**


