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Coptidis Rhizoma: protective effects against peroxynitrite-induced oxidative damage and elucidation of its active components

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Abstract

We have investigated the protective effects of Coptidis Rhizoma against peroxynitrite ($ONOO^-$)-induced oxidative damage and have elucidated the active components of this preparation. In an in-vitro system, Coptidis Rhizoma extract scavenged $ONOO^-$ and its precursors, nitric oxide (NO) and superoxide anion (O_2^-). This scavenging activity was more marked for $ONOO^-$ than its precursors. In addition, against 3-morpholinosydnonimine-induced cellular damage, this extract significantly reduced cellular $ONOO^-$ formation and increased cell viability. In an in-vivo lipopolysaccharide plus ischaemia-reperfusion system that generated $ONOO^-$, the administration of Coptidis Rhizoma extract at 50 and 100 mg kg^{-1}/day for 30 days exerted greater inhibition of $ONOO^-$ than NO and O_2^-. This suggested that it acted as a direct scavenger of $ONOO^-$ rather than as a scavenger of its precursors. Moreover, the suppression of the activities of the antioxidative enzymes superoxide dismutase, catalase and glutathione peroxidase was significantly attenuated by the administration of Coptidis Rhizoma extract. Furthermore, the extract ameliorated renal dysfunction judged by decreasing serum urea nitrogen and creatinine levels. To elucidate the active components of Coptidis Rhizoma extract, we evaluated and compared the effects of the phenol plus alkaloid and alkaloid fractions on $ONOO^-$-induced damage. We found that the alkaloid fraction consisting of berberine, palmatine and coptisine was the most effective at protecting against $ONOO^-$. We confirmed that berberine (10 and 20 mg kg^{-1}/day for 10 days), the main and most active alkaloid in Coptidis Rhizoma extract, was also protective, exerting NO-, O_2^-- and $ONOO^-$-scavenging activities. This study suggested that Coptidis Rhizoma could protect against $ONOO^-$-induced oxidative damage and that this effect was mainly attributable to the constituent alkaloids, especially berberine. This study is the first to demonstrate an antioxidative effect of alkaloids, including berberine, against $ONOO^-$-induced damage.

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Introduction

Great effort has been made to search for safe and effective therapeutic agents for oxidative stress-related diseases, including renal failure. In particular, the development of agents with protective activity against peroxynitrite ($ONOO^-$) suggests that there is great potential for treating oxidative stress-related pathological conditions that play crucial roles in the development of tissue injury. $ONOO^-$ formed in-vivo from the superoxide anion (O_2^-) and nitric oxide (NO) has been suggested to be an important pathogenic causative agent of cellular damage and renal dysfunction (Radi et al 1991; Douki et al 1996). This is because the pathological effects of $ONOO^-$ and the hydroxyl radical (·OH), the decomposition product of $ONOO^-$, contribute to antioxidant depletion, alterations of protein structure and function by tyrosine nitration and oxidative damage (Fukuyama et al 1997; Ischiropoulos 1998; Nakazawa et al 2000; Ceriello et al 2001). Therefore, reactive nitrogen species should be considered as potential targets for therapeutic intervention to prevent and treat oxidative stress-related diseases, including renal failure.

Our screening tests of crude drugs for NO-scavenging activity showed that Coptidis Rhizoma possessed strong NO-scavenging activity (Yokozawa et al 1999). In addition, we demonstrated that Coptidis Rhizoma extract attenuated oxidative damage in a

renal ischaemia-reperfusion animal model (Cho et al 2003). Moreover, Coptidis Rhizoma has long been considered to have antiphlogistic, sedative, antidotal, haemostatic, stomachic and antitumour properties and it has been used as a therapeutic agent for gastrointestinal disorders and infectious and/or inflammatory diseases in East Asia.

Although the active components of Coptidis Rhizoma that exert these bioactivities have not been fully elucidated, it has generally been considered that its alkaloids, such as berberine, palmatine and coptisine, contribute to these activities. In particular, berberine, a major alkaloid component of Coptidis Rhizoma, has been reported to exhibit several types of biological activities and interest has been focused on its antioxidative potential (Chun et al 1979; Rabbani et al 1987; Marin-Neto et al 1988; Jin et al 2000; Choi et al 2001). However, protective activity of alkaloids, including berberine, against $ONOO^-$ has not been demonstrated yet. On the basis of these reports, elucidation of the effects of Coptidis Rhizoma and its main active components against $ONOO^-$-induced oxidative damage is expected to provide valuable evidence that will lead to the development of safe and effective therapeutic agents for oxidative stress-related disorders. Therefore, the aim of this study was to investigate the effects of Coptidis Rhizoma extract and its active alkaloids in an in-vivo lipopolysaccharide (LPS) plus ischaemia-reperfusion model that generated $ONOO^-$.

Materials and Methods

Reagents and medium

LPS (from *Escherichia coli* serotype 055:B5), 3-morpholinosydnonimine (SIN-1) and dihydrorhodamine 123 were purchased from Sigma Chemical Co. (St Louis, MO). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12) and fetal calf serum (FCS) were purchased from Life Technologies Inc. (Grand Island, NY) and Cell Culture Laboratories (Cleveland, OH), respectively. The other chemicals and reagents used were of high quality and obtained from commercial sources.

Preparation and analysis of Coptidis Rhizoma extract

Extract preparation

Coptidis Rhizoma (*Coptis chinensis* Franchet), grown in China and supplied by Uchida Wakan-yaku Co. Ltd (Tokyo, Japan) was finely powdered and extracted with H_2O at 100 °C for 1 h (rhizome:water = 1:10, w/v). After removal of the insoluble matter by filtration, the filtrate was concentrated under reduced pressure and then lyophilized to yield a residue. The yield of the extract was 20.3% by weight of the original material.

Fractionation of Coptidis Rhizoma extract

The extract (25 g) was dissolved with H_2O, and then subjected to fractionation using a MCl-gel CHP 20 P column ($7\phi \times 22$ cm). Elution with H_2O containing increasing MeOH concentrations and monitoring by thin-layer chromatography yielded fraction I (eluted by 0–40% MeOH) containing polysaccharides and phenolics and fraction II (eluted by 40–100% MeOH) containing phenolics and alkaloids. Subsequent elution with 2% HCl–MeOH furnished fraction III consisting of alkaloids. The yields of fractions I, II and III were 38.8%, 44.8% and 16.0% w/w, respectively.

Analysis of the extract and its fractions by HPLC

The contents of berberine, coptisine and palmatine were analysed by HPLC using a system consisting of a Hitachi L-6300 + L-6000 gradient HPLC pump, an Inertsol ODS-2 column ($4.6\phi \times 250$ mm, GL Science), a Hitachi L-4200 UV detector (345 nm), a column oven (Model 556, GL Science) and a Hitachi D-2500 Chromato-Integrator. The mobile phase was a solution of CH_3CN and H_2O (1:1, v/v) containing 25 mM KH_2PO_4 and 5.9 mM $CH_3(CH_2)_{11}OSO_3Na$. The analysis was carried out with an eluent flow rate of 1.0 mL min^{-1} at 40 °C and the contents of berberine, coptisine and palmatine were determined quantitatively by the peak area method. The HPLC results and the chemical structures of these constituents are illustrated in Figures 1 and 2. The amount of each alkaloid was as follows; extract:

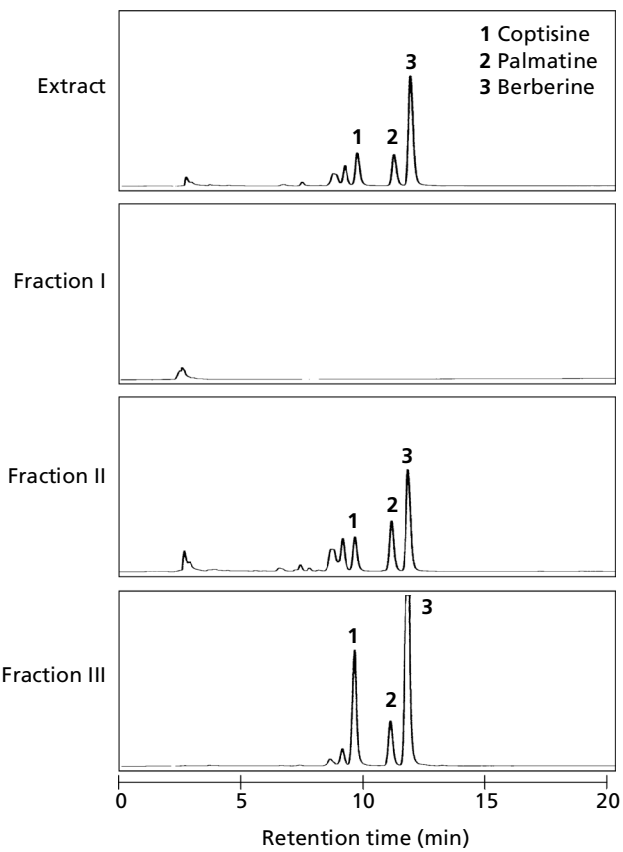

Figure 1 HPLC of Coptidis Rhizoma extract and its fractions.

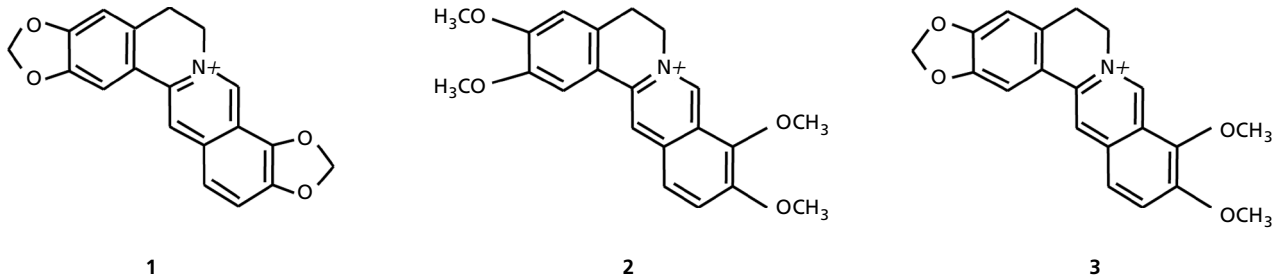

Figure 2 Chemical structures of coptisine (**1**), palmatine (**2**) and berberine (**3**).

23.2% berberine, 7.4% coptisine, 5.3% palmatine; fraction II: 20.9% berberine, 7.7% coptisine, 8.3% palmatine; fraction III: 64.2% berberine, 27.4% coptisine, 8.1% palmatine.

In vitro experiment

NO-scavenging effect

According to the method of Sreejayan & Rao (1997), sodium nitroprusside (SNP, 5 mM) in phosphate-buffered saline was mixed with different concentrations of Coptidis Rhizoma extract dissolved in 50 mM phosphate buffer, before being incubated at 25 °C for 150 min. The amount of NO produced by SNP was assayed by measuring the accumulation of nitrite, using a microplate assay method based on the Griess reaction (Green et al 1982).

O_2^--scavenging activity

O_2^- levels were measured by following the method described by Ewing & Janero (1995). For the assay, the Coptidis Rhizoma extract was pipetted into microplate wells containing 200 μL freshly-prepared 125 μM EDTA, 62 μM nitro blue tetrazolium (NBT) and 98 μM NADH in 50 mM phosphate buffer, pH 7.4. The reaction was initiated by adding 25 μL freshly-prepared 33 μM 5-methylphenazium methyl sulfate in 50 mM phosphate buffer, pH 7.4. The absorbance at 540 nm, as an index of NBT reduction, was monitored continuously over 5 min using a Microplate Reader (Model 3550-UV, Bio-Rad, Tokyo, Japan).

$ONOO^-$-scavenging activity

$ONOO^-$-scavenging effects in the in-vitro system were evaluated by the method of Kooy et al (1994). The Coptidis Rhizoma extract and $ONOO^-$ solution were added to the dihydrorhodamine solution, left to stand for 5 min at 25 °C and then the fluorescence at 480 nm excitation and 525 nm emission was measured.

Cell culture experiment

The porcine kidney cell line LLC-PK_1 was maintained in culture plates containing 5% FCS-supplemented DMEM/F-12 medium at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were subcultured weekly with 0.05% trypsin–EDTA. To measure $ONOO^-$, NO and O_2^- formation, LLC-PK_1 cells were seeded at a density of 10^5 cells/well in 24-well culture plates and incubated at 37 °C for 48 h. In addition, to determine cell viability, the same numbers of cells were seeded in 96-well plates and incubated at 37 °C for 2 h to enable them to adhere. Subsequently, 5 or 25 $\mu g\,mL^{-1}$ (final concentration) Coptidis Rhizoma extract or an equivalent volume (mL^{-1}) of control vehicle was added to each well and incubation was continued for 24 h. After this time, all the cells were treated with 800 μM SIN-1 for 4 h before the $ONOO^-$, NO and O_2^- levels and cell viability were measured.

Cell viability

Cell viability was assessed using the MTT colorimetric assay (Mosmann 1983). A 50-μL sample of MTT solution (1 $mg\,mL^{-1}$) was added to each well of a 96-well culture plate, incubated for 4 h at 37 °C, and then the medium containing MTT was removed. The formazan crystals incorporated in the viable cells were solubilized with 100 μL dimethyl sulfoxide and the absorbance at 540 nm of each well was read using a Microplate Reader (Model 3550-UV, Bio-Rad, Tokyo, Japan).

Cellular $ONOO^-$, NO and O_2^- formation

The $ONOO^-$-dependent oxidation of dihydrorhodamine 123 to rhodamine 123 was measured based on the principles of the method described by Haddad et al (1994). A 1.25-μM sample of dihydrorhodamine 123 was added to each well of a 24-well plate, which was incubated for 4 h at 37 °C, and the absorbance at 500 nm of rhodamine 123 in the medium was measured. The cellular level of NO was determined on the basis of the Griess reaction by measuring the absorbance at 540 nm (Green et al 1982). The cellular level of O_2^- was measured using the method for determining the in-vitro O_2^- level described above.

Animal experiment

The Guidelines for Animal Experimentation, approved by Toyama Medical and Pharmaceutical University, were followed in these experiments. Male Wistar rats aged five weeks (120–130 g) were obtained from Japan SLC Inc. (Hamamatsu, Japan). They were kept in wire-bottomed cages and exposed to a 12-h light/dark cycle. The room temperature and humidity were maintained automatically at approximately 25 °C and 60%, respectively. They were allowed access to commercial laboratory chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0% protein,

3.5% lipid and 60.5% carbohydrate) and water was freely available. Following several days of adaptation, the rats were divided into four to six groups for each experiment, avoiding any intergroup differences in body weight. In each experiment, two groups (sham and control) were given water, while the other groups were given Coptidis Rhizoma extract, fractions II or III or berberine dissolved in their drinking water, orally. Seven rats were used for each experimental group. The extract was administered orally, at a dose of 50 or 100 mg kg^{-1}/day, for 30 consecutive days. The fractions and berberine were administered, at a dose of 10 or 20 mg kg^{-1}/day, for 10 days through a stomach tube. To generate adequate $ONOO^-$ in a biological system, the established animal model of LPS plus ischaemia-reperfusion in rats, was used (Yokozawa et al 2002, 2003). Operative procedures were performed under general anaesthesia induced by 50 mg kg^{-1} sodium pentobarbital administered intraperitoneally. Using aseptic techniques, bilateral flank incisions were made, the renal artery and vein of each kidney were occluded with microvascular clamps for 60 min, the clamps were released and then the kidneys were subjected to reperfusion for 350 min. Fifty minutes after the ischaemia began, the rats received an intravenous injection of LPS (5 mg kg^{-1}). The sham group underwent sham surgery (incisions were made to expose the kidneys, but the renal pedicles were not clamped). Six hours after the LPS challenge, blood was collected by cardiac puncture and centrifuged immediately. Subsequently, the renal artery of each rat was perfused with ice-cold perfusion buffer comprising 50 mM sodium phosphate, 10 mM EDTA-2Na and 120 mM NaCl and the kidneys were removed, quickly frozen and kept at −80 °C until analysis.

Serum $ONOO^-$ level

The concentration of 3-nitrotyrosine, as an indicator of in-vivo $ONOO^-$ formation, was determined by HPLC, following the methods of van der Vliet et al (1994) and Kaur et al (1998) with slight modifications. Briefly, the blood samples were centrifuged for 15 min at 17 300 *g* at 4 °C, incubated with proteinase K (1 U/10 mg protein) for 18 h at 55 °C, centrifuged for 15 min at 17 300 *g* at 4 °C, and passed through a 10 000-Da molecular mass cut-off filter. The samples were loaded onto a reversed-phase column (Nucleosil 5 μC-18, 250 × 46 mm) at 25 °C and eluted with 50 mM KH_2PO_4–H_3PO_4 (pH 3.01) in 10% MeOH (v/v) at a flow rate of 0.8 mL min^{-1}. Detection of the amino acid derivatives was accomplished by monitoring UV absorbance at 365 nm. The peaks were identified by comparing their retention times with those of authentic standards added to additional samples and quantified according to their peak areas relative to known amounts of the external standards.

Serum NO level

NO_2^-/NO_3^- levels, as indices of NO levels, were measured according to the method of Misko et al (1993). Briefly, the nitrate in 20 μL serum was reduced to nitrite by incubation with nitrate reductase (700 mU mL^{-1}), 200 μM NADPH solution was added to each sample and then NO_2^-/NO_3^- levels were measured by a microplate assay method based on the Griess reaction (Green et al 1982). The levels were calculated with reference to standard solutions of sodium nitrite.

Serum O_2^- level

Serum O_2^- levels were measured by following the method for measuring O_2^- levels in-vitro described above (Ewing & Janero 1995).

Renal antioxidative enzyme activities

The kidney tissue was homogenized with a 4-fold volume of ice-cold physiological saline and the activities of enzymes in the homogenate were determined. Superoxide dismutase (SOD) activity was determined according to the nitrous acid method described by Elstner & Heupel (1976) and Oyanagui (1984), which was based on the inhibition of nitrite formation by hydroxylamine in the presence of O_2^- generators. Catalase activity was evaluated by following the decomposition of hydrogen peroxide (H_2O_2) directly by monitoring the decrease in extinction at 240 nm (Aebi 1974). Glutathione peroxidase (GSH-Px) activity was measured by a colorimetric assay that determined the concentration of 2-nitro-5-thiobenzoic acid, a compound produced by the reaction between glutathione and 5,5′-dithiobis(2-nitrobenzoic) acid (Hafeman et al 1974). Protein levels were determined by the micro-biuret method with bovine serum albumin as the standard (Itzhaki & Gill 1964).

Renal functional parameters

Serum urea nitrogen and creatinine (Cr) levels were determined using the commercial reagents BUN Kainos and CRE-EN Kainos, respectively (Kainos Laboratories, Inc., Tokyo, Japan).

Data analysis

The results for each group were expressed as mean ± s.e. values. The effect on each parameter was examined using the one way analysis of variance. Individual differences between groups were evaluated using Dunnett's test and those at $P < 0.05$ were considered to be statistically significant.

Results

In-vitro free radical-scavenging activity

Figure 3 shows the NO-, O_2^-- and $ONOO^-$-scavenging effects of Coptidis Rhizoma extract in the in-vitro system. The extract exerted inhibitory effects on these radicals in a dose-dependent manner. The $ONOO^-$-scavenging activity was very high, even at low concentration, showing more than 80% inhibition at 5 μg mL^{-1}, whereas the scavenging effects on NO and O_2^-, the precursors of $ONOO^-$, were relatively low compared with that on $ONOO^-$.

Cellular protective activity against SIN-1

The protective activity of Coptidis Rhizoma extract in the cellular $ONOO^-$ generation system is shown in Figure 4.

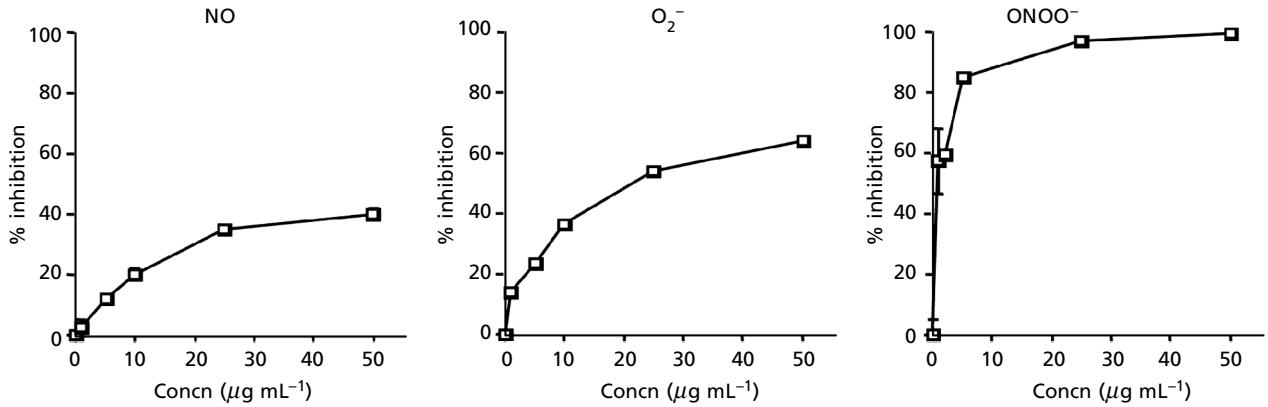

Figure 3 Free radical-scavenging effects of Coptidis Rhizoma extract in the in-vitro system.

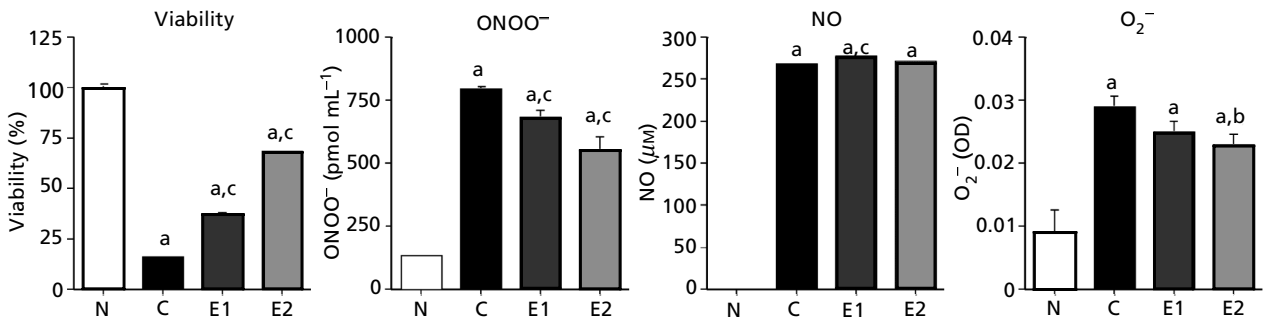

Figure 4 Effect of Coptidis Rhizoma extract on SIN-1-induced cell viability and free radical formations in renal epithelial cells, LLC-PK$_1$. N, no treatment; C, SIN-1 (800 μM) treatment; E1, SIN-1 (800 μM) and Coptidis Rhizoma extract (5 μg mL^{-1}) treatment; E2, SIN-1 (800 μM) and Coptidis Rhizoma extract (25 μg mL^{-1}) treatment. [a] $P < 0.001$ compared with no-treatment values; [b] $P < 0.05$, [c] $P < 0.001$ compared with SIN-1-treatment values.

Treatment of the control LLC-PK$_1$ cells with 800 μM SIN-1 led to a decrease in the cell viability, but the viability of the cells treated with Coptidis Rhizoma extract at the concentrations of 5 and 25 μg mL^{-1} increased significantly and dose-dependently. In addition, the magnitude of the enhancement of $ONOO^-$ generation by SIN-1 was reduced by the extract in a concentration-dependent manner. However, treatment with Coptidis Rhizoma extract did not inhibit NO formation by LLC-PK$_1$ cells treated with SIN-1. O_2^- generation was markedly increased in SIN-1-treated control cells compared with normal cells, whereas the addition of Coptidis Rhizoma extract at 25 μg mL^{-1} led to a significant decline in the cellular O_2^- level.

The protective activities of Coptidis Rhizoma extract, fractions and berberine in the LPS plus ischaemia-reperfusion rat model

Coptidis Rhizoma extract

Table 1 (upper panel) shows the effects of Coptidis Rhizoma extract on serum parameters reflecting free radical levels in the LPS plus ischaemia-reperfusion rat model. The magnitudes of the elevation of the serum levels of 3-nitrotyrosine, an indicator of $ONOO^-$ formation in-vivo, of rats subjected to LPS plus ischaemia-reperfusion were decreased by the administration of Coptidis Rhizoma extract at daily doses of 50 and 100 mg kg^{-1} from 687.4 pmol mL^{-1} to 468.0 and 343.8 pmol mL^{-1}, respectively. In addition, the increased serum NO level of the control rats subjected to LPS plus ischaemia-reperfusion was decreased significantly by the administration of Coptidis Rhizoma extract 100 mg/day. Conversely, the elevation of the O_2^- level induced by LPS plus ischaemia-reperfusion was increased by Coptidis Rhizoma extract. Furthermore, the activities of the free radical-scavenging enzymes, SOD, catalase and GSH-Px in the kidneys of rats subjected to LPS plus ischaemia-reperfusion were inhibited compared with the rats subjected to the sham operation, as shown in Table 2 (upper panel). However, the administration of Coptidis Rhizoma extract enhanced these activities significantly. In particular, the increase in SOD activity induced by the 100 mg kg^{-1}/day dose of the extract was particularly marked; the activity almost reached that of the rats that underwent the sham operation. The serum urea nitrogen and Cr levels of

Table 1 Effect of Coptidis Rhizoma extract, fractions and berberine on free radicals in serum.

Group	$ONOO^-$ (pmol mL^{-1})	NO (μM)	O_2^- (OD)
Sham treatment	N.D.	14.2 ± 0.5	0.065 ± 0.021
LPS plus ischaemia-reperfusion			
Control	687.4 ± 64.8	259.4 ± 7.4[a]	0.105 ± 0.012[a]
Coptidis Rhizoma extract (50 mg kg^{-1}/day)	468.0 ± 52.0[d]	250.8 ± 9.2[a]	0.130 ± 0.005[a,b]
Coptidis Rhizoma extract (100 mg kg^{-1}/day)	343.8 ± 20.6[d]	238.0 ± 8.8[a,c]	0.141 ± 0.005[a,c]
Sham treatment	N.D.	16.6 ± 0.7	0.049 ± 0.002
LPS plus ischaemia-reperfusion			
Control	724.5 ± 60.5	253.8 ± 7.2[a]	0.100 ± 0.003[a]
Fraction II (10 mg kg^{-1}/day)	683.4 ± 35.7	242.5 ± 6.6[a]	0.093 ± 0.004[a]
Fraction II (20 mg kg^{-1}/day)	682.5 ± 59.3	235.2 ± 6.3[a,d]	0.087 ± 0.006[a,d]
Fraction III (10 mg kg^{-1}/day)	494.0 ± 40.6[d]	228.0 ± 7.3[a,d]	0.092 ± 0.005[a]
Fraction III (20 mg kg^{-1}/day)	380.8 ± 25.4[d]	216.9 ± 6.5[a,d]	0.070 ± 0.005[a,d]
Sham treatment	N.D.	13.3 ± 0.8	0.053 ± 0.004
LPS plus ischaemia-reperfusion			
Control	632.5 ± 56.0	246.6 ± 7.5[a]	0.118 ± 0.005[a]
Berberine (10 mg kg^{-1}/day)	494.9 ± 31.8[d]	225.5 ± 9.7[a,c]	0.100 ± 0.006[a,d]
Berberine (20 mg kg^{-1}/day)	281.8 ± 26.7[d]	201.9 ± 9.3[a,d]	0.093 ± 0.002[a,d]

[a] $P < 0.001$ compared with sham treatment values; [b] $P < 0.05$, [c] $P < 0.01$, [d] $P < 0.001$ compared with LPS plus ischaemia-reperfusion control values.

Table 2 Effect of Coptidis Rhizoma extract, fractions and berberine on radical scavenging enzyme activities in renal tissue.

Group	SOD (U (mg protein)$^{-1}$)	Catalase (U (mg protein)$^{-1}$)	GSH-Px (U (mg protein)$^{-1}$)
Sham treatment	34.09 ± 0.51	228.2 ± 7.5	251.7 ± 6.9
LPS plus ischaemia-reperfusion			
Control	22.98 ± 1.74[a]	123.9 ± 8.5[a]	150.7 ± 3.9[a]
Coptidis Rhizoma extract (50 mg kg^{-1}/day)	25.69 ± 1.29[a,b]	145.5 ± 2.4[a,d]	169.9 ± 6.3[a,d]
Coptidis Rhizoma extract (100 mg kg^{-1}/day)	33.18 ± 1.01[d]	151.5 ± 3.9[a,d]	168.6 ± 4.1[a,d]
Sham treatment	37.55 ± 1.82	240.0 ± 10.3	264.3 ± 6.8
LPS plus ischaemia-reperfusion			
Control	22.23 ± 1.78[a]	140.0 ± 8.4[a]	161.7 ± 3.1[a]
Fraction II (10 mg kg^{-1}/day)	24.08 ± 2.02[a]	142.2 ± 9.5[a]	163.6 ± 3.1[a]
Fraction II (20 mg kg^{-1}/day)	25.22 ± 1.08[a]	147.0 ± 12.7[a]	160.9 ± 4.0[a]
Fraction III (10 mg kg^{-1}/day)	26.08 ± 1.37[a,c]	155.7 ± 8.5[a]	167.1 ± 2.6[a]
Fraction III (20 mg kg^{-1}/day)	29.59 ± 1.63[a,d]	174.4 ± 10.3[a,d]	177.3 ± 2.3[a,d]
Sham treatment	38.30 ± 1.89	235.5 ± 7.4	271.1 ± 5.9
LPS plus ischaemia-reperfusion			
Control	24.66 ± 1.75[a]	147.0 ± 7.2[a]	156.0 ± 4.8[a]
Berberine (10 mg kg^{-1}/day)	27.55 ± 1.07[a]	162.1 ± 8.5[a,b]	175.9 ± 5.7[a,d]
Berberine (20 mg kg^{-1}/day)	31.01 ± 1.93[a,d]	181.1 ± 9.3[a,d]	187.5 ± 4.2[a,d]

[a] $P < 0.001$ compared with sham treatment values; [b] $P < 0.05$, [c] $P < 0.01$, [d] $P < 0.001$ compared with LPS plus ischaemia-reperfusion control values.

control rats subjected to LPS plus ischaemia-reperfusion were augmented significantly by approximately 4.7- and 4.0-fold, respectively, compared with sham-operated rats. In contrast, the serum urea nitrogen and Cr levels of rats given Coptidis Rhizoma extract 100 mg kg^{-1}/day declined from 61.9 to 53.8 mg dL^{-1}, and 1.41 to 1.00 mg dL^{-1}, respectively (Table 3, upper panel).

Fractions II and III

Tables 1 to 3 (middle panel) reveal the differences between the protective effects of the fractions from Coptidis Rhizoma extract. The generation of $ONOO^-$ was significantly inhibited by the administration of fraction III (alkaloid fraction), while fraction II (phenol plus alkaloid fraction) did not show such activity. Fractions II and III

Table 3 Effect of Coptidis Rhizoma extract, fractions and berberine on renal function.

Group	Urea nitrogen (mg dL^{-1})	Cr (mg dL^{-1})
Sham treatment	13.1 ± 0.5	0.35 ± 0.02
LPS plus ischaemia-reperfusion		
Control	61.9 ± 1.3[a]	1.41 ± 0.06[a]
Coptidis Rhizoma extract (50 mg kg^{-1}/day)	56.4 ± 0.8[a,d]	1.12 ± 0.04[a,d]
Coptidis Rhizoma extract (100 mg kg^{-1}/day)	53.8 ± 2.4[a,d]	1.00 ± 0.12[a,d]
Sham treatment	12.6 ± 0.5	0.32 ± 0.01
LPS plus ischaemia-reperfusion		
Control	66.0 ± 2.7[a]	1.45 ± 0.06[a]
Fraction II (10 mg kg^{-1}/day)	66.4 ± 1.8[a]	1.52 ± 0.07[a]
Fraction II (20 mg kg^{-1}/day)	65.9 ± 1.8[a]	1.53 ± 0.07[a]
Fraction III (10 mg kg^{-1}/day)	66.4 ± 1.2[a]	1.40 ± 0.05[a]
Fraction III (20 mg kg^{-1}/day)	63.1 ± 1.1[a]	1.29 ± 0.06[a,c]
Sham treatment	13.9 ± 0.9	0.34 ± 0.01
LPS plus ischaemia-reperfusion		
Control	61.8 ± 1.5[a]	1.39 ± 0.04[a]
Berberine (10 mg kg^{-1}/day)	61.0 ± 2.3[a]	1.25 ± 0.07[a,c]
Berberine (20 mg kg^{-1}/day)	60.9 ± 1.8[a]	1.29 ± 0.07[a,b]

[a] $P < 0.001$ compared with sham treatment values; [b] $P < 0.05$, [c] $P < 0.01$, [d] $P < 0.001$ compared with LPS plus ischaemia-reperfusion control values.

both exerted NO- and O_2^--scavenging activity, but fraction III showed the stronger activities. Fraction III elevated the SOD, catalase and GSH-Px activity significantly, but fraction II did not lead to increases in these free radical-scavenging enzyme activities. In addition, the increase in the serum Cr level caused by LPS plus ischaemia-reperfusion was reduced significantly by fraction III, but not by fraction II. The elevation in the serum urea nitrogen level did not change significantly after the administration of fraction II or III.

Berberine

The effects of berberine, the main and active component of Coptidis Rhizoma extract, in the in-vivo $ONOO^-$-generating LPS plus ischaemia-reperfusion model are shown in Tables 1 to 3 (lower panel). The administration of berberine led to decreases in the serum levels of 3-nitrotyrosine, NO and O_2^- and in the three free radical-scavenging enzyme activities. The increased 3-nitrotyrosine level (to 632.5 pmol mL^{-1}) was reduced to 494.9 and 281.8 pmol mL^{-1} by the administration of berberine 10 and 20 mg kg^{-1}/day, respectively. The effect was greater on $ONOO^-$ than on its precursors NO and O_2^-. After the oral administration of berberine 10 and 20 mg kg^{-1}/day, the reduced antioxidative enzyme activities were elevated. In addition, the administration of berberine reduced the serum level of Cr, whereas it did not show this effect on the serum urea nitrogen level.

Discussion

Since traditional crude drugs have excellent pharmacological actions without side effects and toxicity, they could lead to the development of new classes of safe and effective therapeutic agents for oxidative stress-mediated pathological conditions. In our previous fundamental studies aimed at developing new medicaments for renal disease, focusing particularly on Chinese medicines and medical prescriptions, we found Coptidis Rhizoma extract had high free radical-scavenging activity (Yokozawa et al 1999). In addition, our previous study indicated that Coptidis Rhizoma had a protective effect against renal ischaemia-reperfusion injury, in that tissue damage due to oxidative stress was reduced, thus ameliorating renal functional impairment (Cho et al 2003). This study indicated that Coptidis Rhizoma extract exerted greater scavenging effect on $ONOO^-$ itself than on its precursors NO and O_2^- in in-vitro and cellular systems (Figures 3 and 4). In addition, the decrease in cell viability caused by $ONOO^-$ was reversed by treatment with Coptidis Rhizoma extract (Figure 4). On the basis of these pieces of evidence, this study focused on the protective activity of this extract against $ONOO^-$ generated by an in-vivo LPS plus ischaemia-reperfusion animal model.

We analysed the biological parameters and morphological changes in the LPS plus ischaemia-reperfusion rat model. Compared with rats that underwent a sham operation, the rats subjected to LPS plus ischaemia-reperfusion showed elevated serum concentrations of $ONOO^-$, NO and O_2^- and eventually developed renal dysfunction shown by the increases in the serum urea nitrogen and Cr levels (Yokozawa et al 2002, 2003). It is well established that excessive production of reactive oxygen species, such as O_2^-, ·OH and NO, plays a critical role in the development of various diseases, including renal failure (Tominaga et al 1993; Dirnagl et al 1995). These reactive oxygen species may induce tissue damage either directly through interacting

with and destroying cellular proteins, lipids and DNA, or indirectly by affecting normal cellular signalling pathways and gene regulation (Traystman et al 1991). Our preliminary study showed that the activities of the antioxidative enzymes SOD, catalase and GSH-Px decreased and lipid peroxidation increased significantly, indicating destruction of the antioxidative defense mechanism by the process. Furthermore, photomicrographs of renal tissue from rats subjected to LPS plus ischaemia-reperfusion showed that this process caused acute renal injury, resulting in sclerosis of renal distal tubules through expansion of the mesangial regions. These histological renal lesions were not evident in the rats subjected to the sham operation. These observations suggested that the LPS plus ischaemia-reperfusion animal model would be useful for evaluating the effects of promising therapeutic agents against $ONOO^-$-induced renal oxidative damage.

To confirm that Coptidis Rhizoma extract could protect against $ONOO^-$-induced renal failure, the effects of this extract on the formation of $ONOO^-$ and its precursors, renal antioxidative capacities and renal impairment resulting from the LPS plus ischaemia-reperfusion process were evaluated. Similarly to the in-vitro results, Coptidis Rhizoma extract exerted higher inhibitory activity against $ONOO^-$ than its precursors in the in-vivo model. In addition, the scavenging effect was stronger as the dose administered increased. This result suggested that the protective effect of Coptidis Rhizoma extract was due to direct scavenging of $ONOO^-$ rather than of its precursors NO and O_2^-.

High concentrations of $ONOO^-$ generated by the reaction between NO and O_2^- are toxic and the decomposition product of $ONOO^-$, ·OH, is also highly reactive and toxic. Therefore, detoxification of oxygen radicals by SOD, catalase and GSH-Px is regarded as playing a major role in improving the pathological conditions induced by oxidative stress. Our results suggested that the depletion of the antioxidative status induced by $ONOO^-$ was attenuated by the administration of Coptidis Rhizoma extract. The protective effect of SOD is directly related to scavenging of O_2^- by enzymatic dismutation. The finding that the administration of Coptidis Rhizoma extract increased the SOD activity markedly, almost to the level of the rats that underwent the sham operation, suggested that the increased enzyme activity would correspond with the increase in the O_2^- level. Coptidis Rhizoma extract increased the serum level of O_2^-, but this might not lead to O_2^--induced damage as a balance between O_2^- generation and antioxidative enzyme activity might be achieved. In addition, Coptidis Rhizoma extract increased the activities of catalase and GSH-Px enzymes that are specifically involved in the elimination of H_2O_2 and inhibition of ·OH production. The results of this study implied that Coptidis Rhizoma would result in protection against the oxidative stress caused by LPS plus ischaemia-reperfusion through the enhancement of antioxidative defense enzyme activities. Eventually, the Coptidis Rhizoma extract ameliorated the renal dysfunction induced by free radical generation and depletion of the renal antioxidative capacity.

To elucidate the active components of Coptidis Rhizoma that exhibited protective activity against $ONOO^-$, we fractionated the extract into a phenol fraction (fraction I), phenol plus alkaloid fraction (fraction II) and alkaloid fraction (fraction III). In the in-vitro study, the NO-, O_2^-- and $ONOO^-$-scavenging activities of fractions II and III were high, whereas fraction I had no such activity (data not shown). Although it remains unclear which of the Coptidis Rhizoma components exhibited the pharmacological actions, alkaloids present in Coptidis Rhizoma, berberine, coptisine, jateorrhizine and palmatine, were considered to be active constituents (Otsuka et al 1974; Peng et al 1997; Schmeller et al 1997). Therefore, we evaluated and compared the protective properties of fractions II and III in an in-vivo system. The results demonstrated that the fraction composed only of alkaloids, such as berberine, palmatine and coptisine (fraction III), exerted higher $ONOO^-$-scavenging activity and enhanced antioxidative enzyme activities more than the phenol plus alkaloid fraction (fraction II). These results indicated that the alkaloids, such as berberine, palmatine and coptisine, were mainly responsible for the protective activity of Coptidis Rhizoma against $ONOO^-$.

Fraction III contained mainly berberine (approximately 64%) and, therefore, we investigated the effects of berberine on $ONOO^-$-induced renal damage. As expected, berberine showed $ONOO^-$-, NO- and O_2^--scavenging effects significantly. In particular, the activity was stronger against $ONOO^-$ than its precursors, whereas the activities of the antioxidant enzymes, SOD, catalase and GSH-Px, were significantly increased by the administration of berberine. However, in comparison with Coptidis Rhizoma extract, berberine had a relatively weak effect on renal dysfunction. In the light of these results, we formulated two hypotheses. Firstly, the administration period affected the amelioration of renal dysfunction. Coptidis Rhizoma extract was given for 30 days, while berberine was given for 10 days. The duration of administration of berberine might have been too short to improve the renal dysfunction induced by $ONOO^-$. Our previous study demonstrated that the administration period played an important role in the protection against the damage caused by renal ischaemia-reperfusion, in that the protective activity was greater with a longer administration period (Cho et al 2003). Secondly, components other than berberine played a role in the improvement of renal dysfunction. Although the protective effect of berberine against renal dysfunction caused by oxidative stress was weak, this alkaloid definitely demonstrated $ONOO^-$-, NO- and O_2^--scavenging activities. Other studies support the effect of berberine on oxidative stress. Choi et al (2001) demonstrated that berberine effectively inhibited single-strand cleavage of DNA and this effect was concentration-dependent. Moreover, the inhibitory activity of berberine against DNA cleavage was stronger than that of caffeic acid or ascorbic acid. In addition, benzylisoquinoline alkaloids, including berberine, have been shown to interact with oxygen radicals and inhibit lipid peroxidation (Amin et al 1969; Martinez et al 1992; Jin et al 2000). Furthermore, berberine possesses a wide range of pharmacological and biological activities, including anti-bacterial, anti-tumour, anti-diarrhoeal, anti-inflammatory and anti-HIV effects, and, therefore, berberine chloride or sulfate isolated from plants or synthesized chemically has been used clinically

(Sharda 1970; Otsuka et al 1981; Kumazawa et al 1984; Vlietinck et al 1998). However, we believe that we are first to demonstrate the protective activity of alkaloids, including berberine, against $ONOO^-$-induced oxidative damage.

The results suggested that Coptidis Rhizoma could protect against $ONOO^-$-induced oxidative damage and its effects were mainly attributable to its constituent alkaloids, especially berberine.

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