Free Radical Research, February 2005; 39(2): 203–211

Taylor & Francis
Taylor & Francis Group

Glycyrrhizae Radix attenuates peroxynitrite-induced renal oxidative damage through inhibition of protein nitration

TAKAKO YOKOZAWA[1], EUN JU CHO[1], DONG YOUNG RHYU[1], NAOTOSHI SHIBAHARA[1], & KAZUMASA AOYAGI[2]

[1]*Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-0194, Japan, and* [2]*Tsukuba College of Technology, 4-12-7 Kasuga, Tsukuba 305-0821, Japan*

Accepted by Dr J. Yodoi

(Received 22 May 2004; in revised form 10 September 2004)

Abstract

We investigated the protective effects of Glycyrrhizae Radix extract against peroxynitrite ($ONOO^-$)-induced oxidative stress under *in vivo* as well as *in vitro* conditions. The extract showed strong $ONOO^-$ and nitric oxide (NO) scavenging effects under *in vitro* system, in particular higher activity against $ONOO^-$. Furthermore, elevations of plasma 3-nitrotyrosine levels, indicative of *in vivo* $ONOO^-$ generation and NO production, were shown using a rat *in vivo* $ONOO^-$-generation model of lipopolysaccharide injection plus ischemia-reperfusion. The administration of Glycyrrhizae Radix extract at doses of 30 and 60 mg/kg body weight/day for 30 days significantly reduced the concentrations of 3-nitrotyrosine and NO and decreased inducible NO synthase activity. In addition, the nitrated tyrosine protein level and myeloperoxidase activity in the kidney were significantly lower in rats given Glycyrrhizae Radix extract than in control rats. However, the administration of Glycyrrhizae Radix extract did not result in either significant elevation of glutathione levels or reduction of lipid peroxidation in renal mitochondria. Moreover, the *in vivo* $ONOO^-$ generation system resulted in renal functional impairment, reflected by increased plasma levels of urea nitrogen and creatinine, whereas the administration of Glycyrrhizae Radix extract reduced these levels significantly, implying that the renal dysfunction induced by $ONOO^-$ was ameliorated. The present study suggests that Glycyrrhizae Radix extract could protect the kidneys against $ONOO^-$ through scavenging $ONOO^-$ and/or its precursor NO, inhibiting protein nitration and improving renal dysfunction caused by $ONOO^-$.

Keywords: *Glycyrrhizae radix, peroxynitrite, nitric oxide, protein nitration, oxidative stress, renal function*

Introduction

Peroxynitrite ($ONOO^-$) has been suggested to be an important pathogenic agent produced from nitric oxide (NO) and the superoxide anion (O_2^-), which react with a variety of biomolecules, including proteins, lipids and DNA [1,2]. In particular, since the generation of $ONOO^-$ contributes to antioxidant depletion, alterations of protein structure and function by tyrosine nitration and oxidative damage are considered to be major factors in the pathogenesis and development of various diseases [3–7]. Thus, strategies to attenuate $ONOO^-$-induced damage have focused on scavenging $ONOO^-$ and/or its precursors NO and O_2^-, inhibiting protein modification and lipid peroxidation and defending against $ONOO^-$ by enhancing the antioxidative status.

Recently, as a result of considerable efforts to develop effective and safe antioxidants to attenuate oxidative damage, various Chinese traditional medicines have been suggested to be promising therapeutic agents for oxidative stress-related diseases, including

Correspondence: T. Yokozawa, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-0194, Japan. Tel: 81 76 434 7631. Fax: 81 76 434 4656. E-mail: yokozawa@ms.toyama-mpu.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Ltd
DOI: 10.1080/10715760400027888

renal failure. Glycyrrhizae Radix, which contains mainly glycyrrhizin and its aglycone glycyrrhetinic acid, is a Chinese traditional medicine commonly used for the treatment of gastric ulcer, fever, cough and hypercholesterolemia and it is also used as an expectorant, detoxifying agent, tonic and sudorific [8,9]. Moreover, it has been included in many Chinese medicinal prescriptions for various disorders. However, studies to determine whether Glycyrrhizae Radix is a potential therapeutic agent for oxidative stress-related disorders have not been carried out. We evaluate the protective roles from $ONOO^-$ under *in vitro* and *in vivo* $ONOO^-$ generation system in that rats subjected to lipopolysaccharide (LPS) injection plus ischemia-reperfusion, to investigate whether Glycyrrhizae Radix extract protects against $ONOO^-$-induced oxidative damage and could provide a novel pharmacological treatment for pathological conditions responsible for oxidative stress.

Materials and methods

Materials

LPS (from *Escherichia coli* serotype 055: B5), 3-nitro-L-tyrosine, hexadecyltrimethylammonium bromide (HETAB), pepstatin A, chymostatin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), flavin adenine dinucleotide (FAD), tetrahydrobiopterin, dithiothreitol (DTT), β-nicotinamide-adenine dinucleotide phosphate (reduced, NADPH), lactate dehydrogenase (LDH) and sodium pyruvate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dihydrorhodamine 123 (DHR 123) and $ONOO^-$ were from Molecular Probes (Eugene, OR, USA). Phenazium methylsulfate (PMS) and nitro blue tetrazolium (NBT) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Anti-nitrotyrosine antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA; catalog # 12-354). All other chemicals and reagents used were of analytical and obtained from commercial sources.

Preparation of Glycyrrhizae Radix extract

One hundred grams of Glycyrrhizae Radix (*Glycyrrhiza glabra* L. var. *glandulifera* Regel et Herder) was boiled gently in 1,000 ml water for 60 min and then concentrated and lyophilized under reduced pressure to yield a residue with a volume of about 25% of that of the original material. The residue was composed of glycyrrhizin (11.87%), 3-glycyrrhetinic monodesmoside (0.03%) and glycyrrhetinic acid (0.05%).

In vitro $ONOO^-$ and NO scavenging activities

$ONOO^-$ scavenging was measured by monitoring the oxidation of DHR 123 according to a modification of the method of Kooy et al. [10]. A stock solution of 5 mM DHR 123 in dimethylformamide was purged with nitrogen and stored at −80°C. A working solution with 5 μM DHR 123 diluted from the stock solution was placed on ice in the dark immediately before the experiment. The buffer (90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride) was purged with nitrogen and placed on ice before use. Just before use, 5 mM diethylenetriaminepenta acetic acid was added. The $ONOO^-$ scavenging ability, by the oxidation of DHR 123, was measured at room temperature on a microplate fluorescence Genious (Tecan, Austria) with excitation and emission wavelengths of 485 and 535 nm, respectively. The background and final fluorescent intensities were determined 5 min after treatment with or without authentic 10 μM $ONOO^-$ in 0.3 N sodium hydroxide (NaOH). Authentic $ONOO^-$ easily oxidized DHR 123 with its final fluorescent intensity being stable over time.

NO scavenging activity was determined by monitoring 4,5-diaminofluorescein (DAF-2) according to a modification of the method of Chung et al. [11] DAF-2 selectively traps NO and resultantly emits green fluorescence when excited at 490–495 nm [12]. In brief, 1 mg DAF-2 in 0.55 ml of dimethyl sulfoxide was diluted with 50 mM phosphate buffer (pH 7.4) to 1/400-fold. NO donor, 2 mM sodium nitroprusside, and 3.14 μM DAF-2 were added to a 96-well black microplate. The fluorescence intensity was dependent on the amount of NO trapped by DAF-2. The fluorescence signal caused by the reaction of DAF-2 with NO was measured using a fluorescence spectrometer (FL 500, Bio-Tek Instruments) at excitation and emission wavelengths of 495 and 515 nm, respectively, after 10 min.

Animal experiment

The "Guidelines for Animal Experimentation" approved by Toyama Medical and Pharmaceutical University were followed in these experiments. Five-week-old male Wistar rats (120–130 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). They were kept in wire-bottomed cages at a constant temperature (about 25°C) and humidity (about 60%) with a 12 h light-dark cycle and allowed free access to a commercial chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate) and water. Following 5 ~ 6 days of adaptation, the rats were divided into 4 groups, avoiding any inter group differences in body weight. Seven rats were used for each experimental group. Over the 30-day experimental period, two groups (control and sham) received plain drinking water and the other two were given an oral solution of Glycyrrhizae Radix extract at a dose of 30 or 60 mg/kg body weight/day via a stomach tube. To generate $ONOO^-$ enough in biological system, the established

animal model, LPS plus ischemia-reperfusion model, was employed [13,14]. Operative procedures were performed under general anaesthesia induced by 50 mg/kg sodium pentobarbital administered intraperitoneally. Using aseptic technique, bilateral flank incisions were made to expose the kidneys, both renal pedicles were isolated and occluded for 60 min with microvascular clamps. Then, the clamps were released and the incisions were closed with skin staples. Fifty minutes after the ischemia started, the rats received an intravenous LPS injection (5 mg/kg). The sham group was neither clamped renal pedicles nor injected LPS with exposure of kidneys by incision. Six hours after the LPS challenge, blood was collected by cardiac puncture and centrifuged immediately to prepare plasma. Subsequently, the renal arteries of each rat were 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.

Plasma 3-nitrotyrosine and nitrite/nitrate (NO_2^-/NO_3^-) levels

The plasma concentration of 3-nitrotyrosine was determined by HPLC, following the methods of both van der Vliet et al. [15] and Kaur et al. [16] with slight modifications. Briefly, the blood samples were centrifuged for 15 min at 17,300*g*, the resulting plasma samples were incubated with proteinase K (1 U/10 mg protein) for 18 h at 55°C, centrifuged for 15 min at 17,300*g* 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. Detection of the amino acid derivatives was accomplished by monitoring ultraviolet 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.

NO_2^-/NO_3^- levels, indices of NO production, were measured according to the method of Misko et al. [17]. Briefly, the NO_3^- in 20 μl plasma was reduced to NO_2^- by incubation with nitrate reductase (700 mU/ml), 200 μM NADPH solution was added to each sample and then the NO_2^-/NO_3^- levels were measured by a microplate assay method based on the Griess reaction [18]. The levels were calculated with reference to a standard solution of sodium nitrite.

Western blotting

One gram of kidney tissue was homogenized in 5 ml homogenization buffer (50 mM potassium phosphate buffer containing 1 mM EDTA, 500 μM PMSF, 1 μM pepstatin A, 80 mg/l trypsin inhibitor, pH 7.4) and then centrifuged at 900*g* at 4°C for 15 min. The freshly prepared homogenates were boiled for 5 min with equal volumes of gel loading buffer consisting of 125 mM Tris, 4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, pH 6.8, and 0.2% bromophenol blue. An aliquot (25 μg total protein equivalent) of each sample was separated on a SDS-polyacrylamide minigel (using 10% acrylamide gel) at 250 V for 1 h and then transferred to a poly vinylidene di-fluoride (PVDF) membrane at 50 V for 1 h in a semidry transfer system. The membrane was blocked with TBS buffer (pH 7.5, 10 mM Tris, 100 mM NaCl) containing 4% skim milk powder (TBS-milk) at 4°C overnight and then incubated overnight at 4°C with 1 μg/ml mouse anti-nitrotyrosine in freshly prepared TBS-milk. The membrane was washed three times for 10 min each with TBS-Tween buffer and incubated with sheep anti-mouse immunoglobulin G (IgG) linked to horseradish peroxidase (1:2,000 dilution in TBS-milk) at room temperature for 2 h. After four 10 min washes with TBS-Tween buffer, antibody labelling was detected using an enhanced chemiluminescence reagent (Amersham, New Jersey, USA) according to the manufacturer's instructions.

Enzyme activities in renal tissue

Renal myeloperoxidase (MPO) activity was determined using the tetramethylbenzidine method described by Suzuki et al. [19] and Laight et al. [20]. Kidney tissue was homogenized in five volumes of a solution comprising 0.5% HETAB (w/v) dissolved in 50 mM potassium phosphate buffer (pH 6.0) and then centrifuged for 30 min at 20,000*g* at 4°C. The supernatant was incubated for 2 h at 60°C and then centrifuged again at 4,000*g* for 12 min at 4°C. An aliquot of supernatant (40 μl) was removed and added to a reaction mixture (160 μl) comprising 1.6 mM 3,3′,5,5′-tetramethylbenzidine and 3 mM hydrogen peroxide (H_2O_2) in 80 mM phosphate buffer (pH 5.4) in a 96-well microplate. The rate of change in absorbance was measured spectrophotometrically at 650 nm and the MPO activity was expressed as absorbance (abs)/min/100 mg protein.

Inducible NO synthase (iNOS) activity, an initiator of NO production, was measured using the method described by Suh et al. [21]. Kidney tissue was homogenized in 4 volumes of cold 40 mM Tris-HCl (pH 8.0) containing 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin and 100 μM PMSF. The homogenate was centrifuged at 12,000*g* for 5 min at 4°C to discard nuclei and cell debris, and aliquots of the supernatant fraction were used in the experiments described below. Triplicate aliquots (10–20 μg protein) of each supernatant were incubated in 90 μl 20 mM Tris-HCl (pH 7.9) containing 4 μM FAD,

Table I. *In vitro* $ONOO^-$ scavenging activity of Glycyrrhizae Radix extract.

Treatment	Concentration (μg/ml)	Scavenging activity (%)
Glycyrrhizae Radix extract	2	41.3 ± 2.5
	10	83.8 ± 0.8
	50	97.7 ± 0.1

4 μM tetrahydrobiopterin, 3 mM DTT, 2 mM L-arginine and 2 mM NADPH in a 96-well plate for 180 min at 37°C. Residual NADPH was oxidized enzymatically with 10 units/ml LDH and 5 mM sodium pyruvate in a final volume of 130 μl, and quantified by the Griess assay [18].

Protein levels were determined by the microbiuret method with bovine serum albumin as the standard [22].

Oxidative damage in renal mitochondria

Renal mitochondria were prepared essentially by following the procedure of Jung and Pergande [23]. Briefly, renal tissue was homogenized in three volumes of ice-cold preparation medium comprising 210 mM mannitol, 70 mM sucrose, 500 μM EDTA and 10 mM Tris-HCl at pH 7.4. The homogenate was centrifuged for 10 min at 800*g* and 4°C in a refrigerated centrifuge and the resulting supernatant was centrifuged for a further 5 min at 12,000*g* and 4°C. The pellet was then resuspended in preparation medium to produce a concentration of about 10 μg mitochondrial protein/ml and stored on ice.

The lipid peroxidant level was estimated by determining the thiobarbituric acid (TBA)-reactive substance concentration, according to the method of Uchiyama and Mihara [24].

Glutathione (GSH) levels were measured by the method of Floreani et al., [25] using *o*-phthalaldehyde as the fluorescent reagent.

Plasma urea nitrogen and creatinine (Cr) levels

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

Data analysis

The results for each group are expressed as mean ± S.E. values. The effect of Glycyrrhizae Radix extract 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.

Table II. *In vitro* NO scavenging activity of Glycyrrhizae Radix extract.

Treatment	Concentration (μg/ml)	Scavenging activity (%)
Glycyrrhizae Radix extract	12.5	30.3 ± 1.1
	25	36.7 ± 2.4
	50	48.3 ± 0.3
	125	61.3 ± 1.4
	250	69.6 ± 1.9

Results

In vitro $ONOO^-$ and NO scavenging activities

As shown in Table I, Glycyrrhizae Radix extract at 10 and 50 μg/ml led to 83.8 and 97.7% of $ONOO^-$ scavenging activity, respectively. In addition, the extract also showed NO scavenging effect dose-dependently, exerting 30.3, 48.3 and 69.6% scavenging effects at 12.5, 50 and 250 μg/ml, respectively (Table II). The scavenging effect of $ONOO^-$ was stronger than that of its precursor, NO.

Plasma concentrations of 3-nitrotyrosine, NO and O_2^-

Table III shows the effect of Glycyrrhizae Radix extract on the plasma concentration of 3-nitrotyrosine, an indicator of *in vivo* $ONOO^-$ generation induced by the LPS plus ischemia-reperfusion process. While the plasma 3-nitrotyrosine level of the sham treatment group was undetectable, that of the control group subjected to LPS plus ischemia-reperfusion was elevated markedly to 626.2 pmol/ml. In contrast, oral administration of Glycyrrhizae Radix extract at doses of 30 and 60 mg/kg body weight/day for 30 days significantly reduced the levels to 118.2 and 100.5 pmol/ml, respectively. The effect of Glycyrrhizae Radix extract on plasma NO level is shown in Table IV. The control group subjected to LPS plus ischemia-reperfusion had a much higher NO level than the sham treatment group. However, the administration of Glycyrrhizae Radix extract at 30 and 60 mg/kg body

Table III. Effect of Glycyrrhizae Radix extract on plasma 3-nitrotyrosine level.

Group	3-Nitrotyrosine (pmol/ml)
Sham treatment	N.D.
LPS plus ischemic-reperfused	
Control	626.2 ± 135.5
Glycyrrhizae Radix extract (30 mg/kg B.W./day)	118.2 ± 26.9*
Glycyrrhizae Radix extract (60 mg/kg B.W./day)	100.5 ± 23.9*

N.D.: Not detectable.
* $p < 0.001$ compared with LPS plus ischemic-reperfused control rats.

Table IV. Effect of Glycyrrhizae Radix extract on plasma NO radical.

Group	NO (μM)
Sham treatment	1.1 ± 0.1
LPS plus ischemic-reperfused	
Control	27.1 ± 1.0⋆
Glycyrrhizae Radix extract (30 mg/kg B.W./day)	24.2 ± 1.1⋆,†
Glycyrrhizae Radix extract (60 mg/kg B.W./day)	23.5 ± 1.2⋆,†

⋆ $p < 0.001$ compared with sham treatment rats.
† $p < 0.001$ compared with LPS plus ischemic-reperfused control rats.

weight/day led to significant reductions of the NO level from 27.1 μM to 24.2 and 23.5 μM, respectively.

Renal nitrated tyrosine protein level and MPO and iNOS activities

As shown in Figure 1, 66 kDa of renal tyrosine protein was highly and specifically nitrated by the LPS plus ischemia-reperfusion procedure compared with that of sham treatment group. On the other hand, the smaller size of tyrosine protein was not nitrated under the $ONOO^-$ *in vivo* model. However, the administration of Glycyrrhizae Radix extract at 30 and 60 mg/kg body weight/day resulted in dose-dependent reductions in the renal nitrated tyrosine protein level.

Table V shows the effect of Glycyrrhizae Radix extract on renal MPO activity related to tyrosine nitration. While the control rats subjected to LPS plus ischemia-reperfusion showed a significant increase in MPO activity from 27.3 to 44.3 abs/min/100 mg protein, the activity decreased to 40.5 and 39.1 abs/min/100 mg protein after the administration of Glycyrrhizae Radix extract at 30 and 60 mg/kg body weight/day, respectively.

The activity of iNOS in renal tissue is shown in Table VI. The iNOS activity of the sham treatment group was 0.67 pmol/mg protein/min, whereas that of the control rats subjected to LPS plus ischemia-reperfusion increased significantly to 2.32 pmol/mg protein/min. However, the oral administration of 60 mg/kg body weight/day Glycyrrhizae Radix extract reduced this activity significantly to 1.70 pmol/mg protein/min.

Renal mitochondrial oxidative stress

Table VII shows the effects of Glycyrrhizae Radix extract on renal mitochondrial oxidative stress, estimated by measuring GSH and TBA-reactive substance levels. The renal mitochondrial GSH level of control rats subjected to LPS plus ischemia-reperfusion decreased significantly from 5.50 to 4.59 nmol/mg protein/min, but the oral administration of Glycyrrhizae Radix extract at both doses of 30 and 60 mg/kg body weight/day did not lead to significant changes in the GSH level. However, the renal mitochondrial TBA-reactive substance level of control rats subjected to LPS plus ischemia-reperfusion increased significantly to 2.78 nmol/mg protein/min compared with the value of the sham group, 1.18 nmol/mg protein/min. The administration of Glycyrrhizae Radix extract at doses of 30 and 60 mg/kg body weight/day reduced this level slightly, but the difference failed to reach statistical significance.

Plasma urea nitrogen and Cr levels

Table VIII shows the effects of Glycyrrhizae Radix extract on parameters of renal function. The plasma

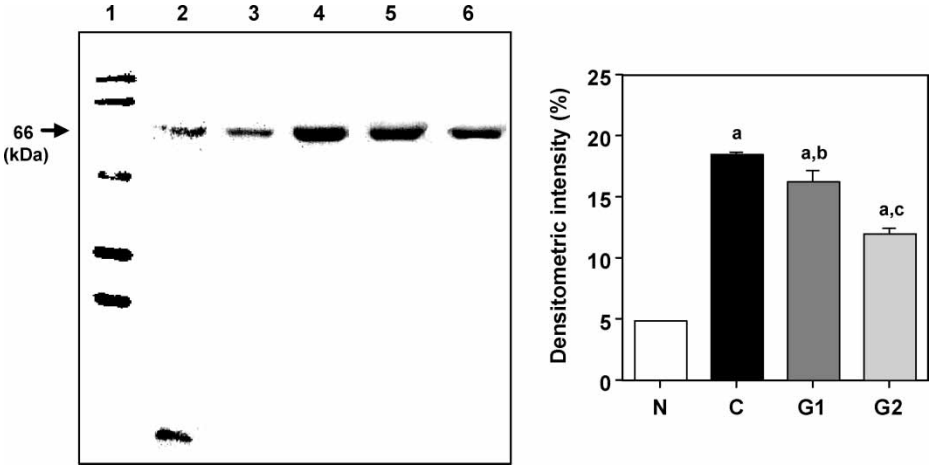

Figure 1. Effect of Glycyrrhizae Radix extract on renal nitrated tyrosine protein. 1: Marker, 2: Nitrotyrosine immunoblotting control, 3: Sham operation, 4: LPS plus ischemic-reperfused control, 5: LPS plus ischemic-reperfused Glycyrrhizae Radix extract (30 mg/kg B.W./day), 6: LPS plus ischemic-reperfused Glycyrrhizae Radix extract (60 mg/kg B.W./day). N: Sham operation, C: LPS plus ischemic-reperfused control, G1: LPS plus ischemic-reperfused Glycyrrhizae Radix extract (30 mg/kg B.W./day), G2: LPS plus ischemic-reperfused Glycyrrhizae Radix extract (60 mg/kg B.W./day). [a] $p < 0.001$ compared with sham treatment rats; [b] $p < 0.01$, [c] $p < 0.001$ compared with LPS plus ischemic-reperfused control rats.

Table V. Effect of Glycyrrhizae Radix extract on renal MPO activity.

Group	MPO (abs/min/100 mg protein)
Sham treatment	27.3 ± 2.1
LPS plus ischemic-reperfused	
Control	44.3 ± 0.6★
Glycyrrhizae Radix extract (30 mg/kg B.W./day)	40.5 ± 0.8★,†
Glycyrrhizae Radix extract (60 mg/kg B.W./day)	39.1 ± 1.8★,‡

★$p < 0.001$ compared with sham treatment rats.
†$p < 0.01$, ‡$p < 0.001$ compared with LPS plus ischemic-reperfused control rats.

urea nitrogen and Cr levels of the control rats subjected to LPS plus ischemia-reperfusion increased significantly by about 4.5- and 5.7-fold, respectively, in comparison with those of the sham group. In contrast, the plasma urea nitrogen and Cr levels of rats given Glycyrrhizae Radix extract 60 mg/kg body weight/day for 30 days prior to LPS and ischemia-reperfusion declined from 61.4 to 56.0 mg/dl and 1.60 to 1.35 mg/dl, respectively.

Discussion

Reactive nitrogen species are considered to be crucial targets for therapeutic interventions for the prevention and treatment of oxidative stress-related diseases, including renal failure, since the pathological effects of $ONOO^-$ and the hydroxyl radical, the decomposition product of $ONOO^-$, lead to oxidative stress by suppressing the antioxidative defence status and altering protein function by tyrosine nitration. Thus, the elucidation of a protective effect of Glycyrrhizae Radix extract against $ONOO^-$-induced tissue damage suggests a novel means of treating various disorders related to oxidative stress.

To evaluate the scavenging effect of $ONOO^-$, the oxidation of DHR 123 to fluorescent rhodamine 123 was determined in the treatment of Glycyrrhizae Radix extract under *in vitro*. Furthermore, DAF-2 was employed as a specific NO indicator, which selectively traps NO between two amino groups in its molecule, and yields triazolofluorescein [12]. As shown in Tables I and II, Glycyrrhizae Radix extract exerted strong $ONOO^-$ and NO scavenging effects under *in vitro* system, in particular higher activity against $ONOO^-$. This result indicates that the extract would probably exert more protective role from $ONOO^-$ rather than its precursor, NO.

Table VI. Effect of Glycyrrhizae Radix extract on renal iNOS activity.

Group	iNOS (pmol/mg protein/min)
Sham treatment	0.67 ± 0.27
LPS plus ischemic-reperfused	
Control	2.32 ± 0.17★
Glycyrrhizae Radix extract (30 mg/kg B.W./day)	1.93 ± 0.24★
Glycyrrhizae Radix extract (60 mg/kg B.W./day)	1.70 ± 0.18★,†

★$p < 0.001$ compared with sham treatment rats.
†$p < 0.01$ compared with LPS plus ischemic-reperfused control rats.

On the basis of *in vitro* result, the protective effects against $ONOO^-$-induced oxidative damage were investigated under the *in vivo* $ONOO^-$ generation model. The LPS plus ischemia-reperfusion model used in the present study is clearly useful and valid, as confirmed by our previous studies,[13,14,26] since it generates excessive $ONOO^-$ by the reactions of NO and O_2^- and results in $ONOO^-$-induced oxidative stress through antioxidant depletion, as well as protein modification and lipid peroxidation.

$ONOO^-$ in biological fluids was detected by identifying nitrated tyrosine as a marker of $ONOO^-$ formation *in vivo* or a stable end-product of $ONOO^-$ oxidation. The existence of nitrotyrosine and its relationship to tissue damage in a wide variety of disease states have been demonstrated in various lesions, such as atherosclerotic plaques and those seen in ischemia-reperfusion and acute renal failure models [27,28]. Consistent with this evidence, the present study also showed high levels of 3-nitrotyrosine produced by the *in vivo* LPS plus ischemia-reperfusion model, which is an $ONOO^-$ generation system. Although the present study could not give the direct evidence of $ONOO^-$ scavenging effect of Glycyrrhizae Radix because of short half life and reactivity, the ability to inhibit $ONOO^-$-dependent nitration provides a useful assay for screening various compounds for their ability to scavenge $ONOO^-$ and the nitrating species derived from it [29]. The administration of Glycyrrhizae Radix extract reduced the plasma 3-nitrotyrosine concentration significantly. Together with the result of $ONOO^-$ scavenging activity *in vitro* determined by the oxidation of DHR 123, Glycyrrhizae Radix extract would play the protective role from $ONOO^-$ as the scavenger of $ONOO^-$.

Elevated 3-nitrotyrosine levels under pathological conditions are also responsible for the interactions of secondary oxidants spontaneously derived from $ONOO^-$, such as MPO-catalyzed oxidation of NO_2^- [30,31]. Thus, the activation of MPO, as well as an increase in the nitrotyrosine level, is also a crucial indicator of nitrosative stress [32]. To evaluate the effect of Glycyrrhizae Radix extract on the renal oxidative stress induced by $ONOO^-$, we measured the MPO activity and nitrated protein level of kidney tissue. Glycyrrhizae Radix administration resulted in a reduction in the renal nitrated tyrosine protein level and inhibition of MPO activity, suggesting that it protected against renally generated $ONOO^-$. Noiri et al. [28]

Table VII. Effect of Glycyrrhizae Radix extract against oxidative damages of renal mitochondria.

Group	GSH (nmol/mg protein)	TBA-reactive substance (nmol/mg protein)
Sham treatment	5.50 ± 0.25	1.18 ± 0.06
LPS plus ischemic-reperfused		
Control	4.59 ± 0.03★	2.78 ± 0.56★
Glycyrrhizae Radix extract (30 mg/kg B.W./day)	4.64 ± 0.06★	2.71 ± 0.26★
Glycyrrhizae Radix extract (60 mg/kg B.W./day)	4.68 ± 0.04★	2.62 ± 0.36★

★$p < 0.001$ compared with sham treatment rats.

observed that suppression or scavenging of $ONOO^-$ in ischemic acute renal failure improved renal function, consequently preventing lipid peroxidation and oxidative DNA damage. The reduction of $ONOO^-$ levels by Glycyrrhizae Radix extract suggests that Glycyrrhizae Radix could attenuate $ONOO^-$-induced oxidative damage by several mechanisms that we infer on the basis of reports of its influence on protein nitration by $ONOO^-$ [33–36]. Nitration of tyrosyl residues in proteins interferes with signalling pathways that rely on tyrosine phosphorylation/dephosphorylation and regulate cellular proliferation and apoptosis. In addition, oxidation and nitration of proteins mediated by $ONOO^-$ disturb the tertiary structure of proteins and, in the presence of severe damage, render them inaccessible to degradation by the proteasome. Such proteins accumulate and form insoluble aggregates, resulting in the development of several diseases, including renal failure.

The attenuation of $ONOO^-$-induced oxidative damage by Glycyrrhizae Radix extract involves scavenging NO and/or O_2^-, the precursors of $ONOO^-$, as well as scavenging $ONOO^-$ directly. The LPS plus ischemia-reperfusion process increased the NO level and iNOS activity that is attributable to NO formation. However, our present investigation clearly indicated that the administration of Glycyrrhizae Radix extract led to a decline in the NO level with inhibition of iNOS activity, although the inhibitory effect on NO was weaker than that on $ONOO^-$. From these results, we conclude that the protective activity of Glycyrrhizae Radix against $ONOO^-$ is mainly attributable to direct scavenging of $ONOO^-$ and, at least in part, its precursor NO.

On the other hand, the O_2^- level decreased in the rats subjected to LPS plus ischemia-reperfusion (data not shown). Several studies have demonstrated that the activity of xanthine oxidase (XOD), which plays the main role in the generation of O_2^-, decreased following the burst of $ONOO^-$ generation, although XOD activity initially increased during ischemia [37–40]. Therefore, we propose that the reduction in the O_2^- level we observed with the $ONOO^-$ generation system was mainly due to the inhibition of XOD activity as a result of feedback in response to the burst of $ONOO^-$. Although the O_2^- level in the rats given Glycyrrhizae Radix extract increased dose-dependently, it was lower than that in the sham treatment group (data not shown). Since the half life of O_2^- is too short to measure it, the O_2^- level determined after separation of serum might reflect the O_2^- generation activity in 20% oxygen in the air. Thus, the effect of Glycyrrhizae Radix extract on O_2^- is obscure, therefore, the direct scavenging activity and its possible relationship to XOD activity need further study.

$ONOO^-$ promotes mitochondrial oxidative stress that results from the shift between the oxidant and antioxidant balance, [1] indicating that depletion of the antioxidant defense system plays important roles in $ONOO^-$-mediated oxidative injury. $ONOO^-$ reacts directly with various mitochondrial targets [41] and mitochondria can partially detoxify $ONOO^-$ as a result of the contribution of one or more scavenging systems, including cytochrome *c* oxidase, GSH and ubiquinol [42–44]. $ONOO^-$-mediated depletion of cellular antioxidants can lead to positive feedback cycles of intracellular oxidant generation and exacerbation of oxidative cellular injury [45]. Our previous study also demonstrated a decrease in the GSH level and increased lipid peroxidation in the renal mitochondria in the LPS plus ischemia-reperfusion model [13,14]. While mitochondrial oxidative stress due to $ONOO^-$ was also

Table VIII. Effect of Glycyrrhizae Radix extract on plasma urea nitrogen and Cr levels.

Group	Urea nitrogen (mg/dl)	Cr (mg/dl)
Sham treatment	13.5 ± 0.3	0.28 ± 0.03
LPS plus ischemic-reperfused		
Control	61.4 ± 2.6★	1.60 ± 0.08★
Glycyrrhizae Radix extract (30 mg/kg B.W./day)	56.1 ± 1.8★,†	1.36 ± 0.05★,†
Glycyrrhizae Radix extract (60 mg/kg B.W./day)	56.0 ± 1.2★,†	1.35 ± 0.04★,†

★$p < 0.001$ compared with sham treatment rats.
†$p < 0.001$ compared with LPS plus ischemic-reperfused control rats.

observed in the present study, attenuation of oxidative stress resulting from the administration of Glycyrrhizae Radix extract through the inhibition of lipid peroxidation and elevation of GSH levels was slight and failed to reach significance. Thus, we propose that the protective activity of Glycyrrhizae Radix extract against $ONOO^-$ is mainly attributable to inhibition of protein nitration rather than lipid peroxidation. In addition, GSH does not appear to be involved to any great extent in the protective effect of Glycyrrhizae Radix against $ONOO^-$. Further study on the changes of the antioxidative defense status involving GSH, antioxidative enzyme activities and other antioxidants is needed to elucidate the effects of Glycyrrhizae Radix on the antioxidative defense status depleted by $ONOO^-$.

The excessive generation of $ONOO^-$ in the kidneys clearly causes impairment of renal function by reducing the excretion of final metabolites from the kidneys and causing accumulation of uremic toxins, such as urea nitrogen and Cr [13,14,26]. Our present investigation showed that urea nitrogen and Cr accumulated in the rats subjected to the procedure by which excessive $ONOO^-$ was generated, indicating that renal dysfunction had occurred. The reduction of plasma urea nitrogen and Cr levels by Glycyrrhizae Radix extract implies that this extract ameliorated the renal dysfunction and damage caused by uremic toxins resulting from $ONOO^-$ generation.

Since Glycyrrhizae Radix has been reported to have various biological activities, including anti-inflammatory, anti-allergy, anti-gastric ulcer and anti-viral activities, it occupies an important place in traditional Oriental medicine [46–49]. In addition, the present study demonstrated that Glycyrrhizae Radix scavenges $ONOO^-$ and NO and inhibits protein modification caused by $ONOO^-$. This protective activity against $ONOO^-$ indicates that this extract could be a promising treatment for various oxidative stress-related disorders.

References

[1] Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys 1991;288:481–487.

[2] Douki T, Cadet J, Ames BN. An adduct between peroxynitrite and 2′-deoxyguanosine: 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine. Chem Res Toxicol 1996;9:3–7.

[3] Fukuyama N, Takebayashi Y, Hida M, Ishida H, Ichimori K, Nakazawa H. Clinical evidence of peroxynitrite formation in chronic renal failure patients with septic shock. Free Radic Biol Med 1997;22:771–774.

[4] Ischiropoulos H. Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. Arch Biochem Biophys 1998;356:1–11.

[5] Nakazawa H, Fukuyama N, Takizawa S, Tsuji C, Yoshitake M, Ishida H. Nitrotyrosine formation and its role in various pathological conditions. Free Radic Res 2000;33:771–784.

[6] Ceriello A, Mercuri F, Quagliaro L, Assaloni R, Motz E, Tonutti L, Taboga C. Detection of nitrotyrosine in the diabetic plasma: Evidence of oxidative stress. Diabetologia 2001;44:834–838.

[7] Cuzzocrea S, Reiter RJ. Pharmacological action of melatonin in shock, inflammation and ischemia-reperfusion injury. Eur J Pharmacol 2001;426:1–10.

[8] Guoji Y, Orita M, Tashiro K, Abe H. Effects of glycyrrhetinic acid on aminonucleoside nephrosis in rats. Arch Pharmacol 1994;349:318–323.

[9] Shibata S. A drug over the millennia: Pharmacognosy, chemistry, and pharmacology of licorice. Yakugaku Zasshi 2000;120:849–862.

[10] Kooy NW, Royall JA, Ishiropoulos H, Beckman JS. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radic Biol Med 1994;16:149–156.

[11] Chung HY, Choi HR, Park HJ, Choi JS, Choi WC. Peroxynitrite scavenging and cytoprotective activity of 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether from the marine alga *Symphyocladia latiuscula*. J Agric Food Chem 2001;49:3614–3621.

[12] Nagata N, Momose K, Ishida Y. Inhibitory effects of catecholamines and anti-oxidants on the fluorescence reaction of 4,5-diaminofluorescein, DAF-2, a novel indicator of nitric oxide. J Biochem 1999;125:658–661.

[13] Yokozawa T, Chen CP, Rhyu DY, Tanaka T, Park JC, Kitani K. Potential of sanguiin H-6 against oxidative damage in renal mitochondria and apoptosis mediated by peroxynitrite *in vivo*. Nephron 2002;92:133–141.

[14] Yokozawa T, Rhyu DY, Cho EJ, Aoyagi K. Protective activity of (-)-epicatechin-3-*O*-gallate against peroxynitrite-mediated renal damage. Free Radic Res 2003;37:561–571.

[15] van der Vliet A, O'Neill CA, Halliwell B, Cross CE, Kaur H. Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite: Evidence for hydroxyl radical production from peroxynitrite. FEBS Lett 1994;339:89–92.

[16] Kaur H, Lyras L, Jenner P, Halliwell B. Artefacts in HPLC detection of 3-nitrotyrosine in human brain tissue. J Neurochem 1998;70:2220–2223.

[17] Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 1993;214:11–16.

[18] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal Biochem 1982; 126:131–138.

[19] Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal Biochem 1983;132:345–352.

[20] Laight DW, Lad N, Woodward B, Waterfall JF. Assessment of myeloperoxidase activity in renal tissue after ischemia-reperfusion. Eur J Pharmacol 1994;292:81–88.

[21] Suh N, Honda T, Finlay HJ, Barchowsky A, Williams C, Benoit NE, Xie QW, Nathan C, Gribble GW, Sporn MB. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. Cancer Res 1998;58:717–723.

[22] Itzhaki RF, Gill DM. A micro-biuret method for estimating proteins. Anal Biochem 1964;9:401–410.

[23] Jung K, Pergande M. Influence of cyclosporin A on the respiration of isolated rat kidney mitochondria. FEBS Lett 1985;183:167–169.

[24] Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978;86:271–278.

[25] Floreani M, Petrone M, Debetto P, Palatini P. A comparison between different methods for the determination of reduced and oxidized glutathione in mammalian tissues. Free Radic Res 1997;26:449–455.

[26] Rhyu DY, Yokozawa T, Cho EJ, Park JC. Prevention of peroxynitrite-induced renal injury through modulation of peroxynitrite production by the Chinese prescription Wen-Pi-Tang. Free Radic Res 2002;36:1261–1269.

[27] Depre C, Havaux X, Renkin J, Vanoverschelde JL, Wijns W. Expression of inducible nitric oxide synthase in human coronary atherosclerotic plaque. Cardiovasc Res 1999; 41:465–472.

[28] Noiri E, Nakao A, Uchida K, Tsukahara H, Ohno M, Fujita T, Brodsky S, Goligorsky MS. Oxidative and nitrosative stress in acute renal ischemia. Am J Physiol 2001;281:F948–F957.

[29] Whiteman M, Halliwell B. Protection against peroxynitrite-dependent tyrosine nitration and α_1-antiproteinase inactivation by ascorbic acid. A comparison with other biological antioxidants. Free Radic Res 1996;25:275–283.

[30] Brennan ML, Wu W, Fu X, Shen Z, Song W, Frost H, Vadseth C, Narine L, Lenkiewicz E, Borchers MT, Lusis AJ, Lee JJ, Lee NA, Abu-Soud HM, Ischiropoulos H, Hazen SL. A tale of two controversies: Defining both the role of peroxidases in nitrotyrosine formation *in vivo* using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. J Biol Chem 2002;277:17415–17427.

[31] Hurst JK. Whence nitrotyrosine? J Clin Invest 2002; 109:1287–1289.

[32] Chatterjee PK, Cuzzocrea S, Brown PA, Zacharowski K, Stewart KN, Mota-Filipe H, Thiemermann C. Tempol, a membrane-permeable radical scavenger, reduces oxidant stress-mediated renal dysfunction and injury in the rat. Kidney Int 2000;58:658–673.

[33] Kong SK, Yim MB, Stadtman ER, Chock PB. Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: Lymphocyte-specific tyrosine kinase fails to phosphorylate nitrated cdc2(6–20)NH_2 peptide. Proc Natl Acad Sci USA 1996;93:3377–3382.

[34] Grune T, Blasig IE, Sitte N, Roloff B, Haseloff R, Davies KJ. Peroxynitrite increases the degradation of aconitase and other cellular proteins by proteasome. J Biol Chem. 1998; 273:10857–10862.

[35] Grune T, Klotz LO, Gieche J, Rudeck M, Sies H. Protein oxidation and proteolysis by the nonradical oxidants singlet oxygen or peroxynitrite. Free Radic Biol Med 2001; 30:1243–1253.

[36] Naylor DJ, Hartl FU. Contribution of molecular chaperones to protein folding in the cytoplasm of prokaryotic and eukaryotic cells. Biochem Soc Symp 2001;68:45–68.

[37] Xia Y, Zweier JL. Substrate control of free radical generation from xanthine oxidase in the postischemic heart. J Biol Chem 1995;270:18797–18803.

[38] Wang P, Zweier JL. Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. J Biol Chem 1996;271:29223–29230.

[39] Houston M, Chumley C, Radi R, Rubbo H, Freeman BA. Xanthine oxidase reaction with nitric oxide and peroxynitrite. Arch Biochem Biophys 1998;355:1–8.

[40] Lee CI, Liu X, Zweier JL. Regulation of xanthine oxidase by nitric oxide and peroxynitrite. J Biol Chem 2000; 275:9369–9376.

[41] Radi R, Cassina A, Hodara R. Nitric oxide and peroxynitrite interactions with mitochondria. Biol Chem 2002; 383: 401–409.

[42] Castro LA, Robalinho RL, Cayota A, Meneghini R, Radi R. Nitric oxide and peroxynitrite-dependent aconitase inactivation and iron-regulatory protein-1 activation in mammalian fibroblasts. Arch Biochem Biophys 1998;359:215–224.

[43] Pearce LL, Pitt BR, Peterson J. The peroxynitrite reductase activity of cytochrome *c* oxidase involves a two-electron redox reaction at the heme α_3-Cu_B site. J Biol Chem 1999;274:35763–35767.

[44] Poderoso JJ, Carreras MC, Schopfer F, Lisdero CL, Riobo NA, Giulivi C, Boveris AD, Boveris A, Cadenas E. The reaction of nitric oxide with ubiquinol: Kinetic properties and biological significance. Free Radic Biol Med 1999; 26:925–935.

[45] Cuzzocrea S, Zingarelli B, O'Connor M, Salzman AL, Szabo C. Effect of L-buthionine-(S,R)-sulphoximine, an inhibitor of γ-glutamylcysteine synthetase on peroxynitrite- and endotoxic shock-induced vascular failure. Br J Pharmacol 1998;123:525–537.

[46] Pompei R, Flore O, Marccialis MA, Pani A, Loddo B. Glycyrrhizic acid inhibits virus growth and inactivates virus particles. Nature 1979;281:689–690.

[47] Ohuchi K, Kamada Y, Levine L, Tsurufuji S. Glycyrrhizin inhibits prostaglandin E_2 production by activated peritoneal macrophages from rats. Prostaglandins Med 1981;7:457–463.

[48] Baba M, Shigeta S. Antiviral activity of glycyrrhizin against varicella-zoster virus *in vitro*. Antiviral Res 1987;7:99–107.

[49] Inoue H, Mori T, Shibata S, Saito H. Pharmacological activities of glycyrrhetinic acid derivatives: Analgesic and anti-type IV allergic effects. Chem Pharm Bull 1987;35:3888–3893.