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Peroxynitrite scavenging activity of lithospermate B from *Salvia miltiorrhiza*

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Abstract

Peroxynitrite ($ONOO^-$) is produced by the reaction of superoxide (O_2^-) with nitric oxide. $ONOO^-$ damages proteins through nitration or oxidation. For protection from $ONOO^-$ induced protein modifications, $ONOO^-$ scavengers should be supplemented. Evidence was obtained that lithospermate B extracted from *Salvia miltiorrhiza* showed the strongest scavenging activity among its constituents. Its $ONOO^-$ scavenging activity is via an electron donation mechanism. A dihydroxyl group and a double bond seem to be essential structure requirements. The data from the experiments further confirmed a protective effect of lithospermate B on bovine serum albumin and low-density lipoprotein against $ONOO^-$. This study demonstrated that lithospermate B with hydroxyl groups and double bonds exerts an anti-nitration effect by scavenging $ONOO^-$.

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Introduction

Oxidative stress is known to occur widespread throughout the body (Gutteridge 1999). Peroxynitrite ($ONOO^-$), a product of nitrogen-derived oxidative stress, is formed by the reaction of two ubiquitous free radical species, superoxide (O_2^-) and nitric oxide (NO) (Pryor and Squadrito 1995). $ONOO^-$ is a powerful oxidant that can cause damage to proteins through a subtle mechanism called nitration (Arteel et al 1999). Tyrosine has been proven to be extremely sensitive to $ONOO^-$. In fact, nitrotyrosine has served as a fingerprint for $ONOO^-$ mediated damage of cellular proteins in a variety of pathological conditions, including ischaemia/reperfusion injury, Alzheimer's disease and atherosclerosis (Smith et al 1997; He et al 1999; Luoma & Yla-Herttuala 1999).

Low molecular mass compounds such as glutathione, methionine, cysteine, melatonin and serotonin have been found to neutralize cytotoxic $ONOO^-$ (Gilad et al 1997; Sandoval et al 1997; Soung et al 1998). In addition, NO and O_2^- levels are shown to be controlled by oxyhaemoglobin and superoxide dismutase, respectively (Gilad et al 1997). However, in order to overcome excessive $ONOO^-$ produced during pathological conditions, scavengers are needed. Scavengers for $ONOO^-$ include the antioxidant ingredients found in natural products such as flavonoids and hydroxycinnamates (Haenen et al 1997; Pannala et al 1998). We have previously reported on the $ONOO^-$ scavenging effects of catechin polyphenols extracted from green tea and the phenolic compounds from *Eriobotrya japonica* (Chung et al 1998; Soung et al 1999).

Extracts from *Salvia miltiorrhiza* consisting of phenolic compounds have been widely used in China as herbal medicine for the clinical treatment of vascular system abnormalities (Tang & Eisenbrand 1992). The major active constituents of *S. miltiorrhiza* are identified as caffeic acid, trans-rosmarinic acid, lithospermate A, lithospermate B, magnesium lithospermate B, and ammonium-potassium lithospermate B (Tanaka et al 1989). However, to date, the $ONOO^-$ scavenging activity of *S. miltiorrhiza* and its major component, lithospermate B, has not been reported.

In the present study, we investigated the $ONOO^-$ scavenging effect of *S. miltiorrhiza* and its fractions using six components extracted from the subfraction A-2 in *S. miltiorrhiza*. We found lithospermate B to be the most potent $ONOO^-$ scavenger. In addition, we were able to estimate the scavenging activity of lithospermate B against $ONOO^-$ and the ability of lithospermate B to protect tyrosine, a cellular component,

against $ONOO^-$ by an electron donation mechanism. We also examined the possible protective effect of lithospermate B against $ONOO^-$ induced nitration of bovine serum albumin (BSA) and low-density lipoprotein (LDL).

Materials and Methods

Materials

S. miltiorrhiza and its components (caffeic acid, trans-rosmarinic acid, lithospermate A, lithospermate B, magnesium lithospermate B and ammonium-potassium lithospermate B) were isolated and provided by Dr Itsuo Nishioka, Kyushu University, Japan. $ONOO^-$ and dihydrorhodamine 123 (DHR 123) were obtained from Cayman Chemical Company (Ann Arbor, MI, USA) and Molecular Probes (Eugene, OR, USA), respectively. BSA and LDL were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Anti-nitrotyrosine antibody and horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep were obtained from Upstate Biotechnology (Lake Placid, NY, USA) and Amersham (Buckinghamshire, UK), respectively.

Isolation of caffeic acid analogues from *S. miltiorrhiza* Radix

Commercially available *S. miltiorrhiza* Radix (1.0 kg) produced in China was extracted twice with water (1.5 L) at 80 °C (fraction A) and then extracted twice with acetone (1.52 L) at room temperature (fraction B). After removal of the insoluble fraction by filtration, the filtrate was concentrated under reduced pressure (ca 40 °C) and subjected to MCI-gel CHP 20 P (7.5 cm i.d. × 35 cm) column chromatography. After washing the column with water, the samples were chromatographed over Sephadex LH-20 (5.0 cm i.d. × 42 cm) with water/EtOH to afford subfractions A-1, A-2 and A-3. Magnesium lithospermate B (7.56 g) was isolated from subfraction A-2. Further elution of the column with water containing increasing proportions of ethanol yielded rosmarinic acid (0.15 g), caffeic acid (0.03 g) and lithospermic acid B (1.86 g). Fraction 1 was dissolved in 0.5 M hydrochloric acid (5 mL), applied directly to a Sephadex LH-20 column (3.5 cm i.d. × 30 cm) and eluted with water containing increasing amounts of methanol to yield lithospermic acid B (0.5 g) and lithospermic acid (0.3 g). Lithospermic acid B and its salts were identified on the basis of chemical and spectroscopic data reported previously (Tanaka et al 1989). Lithospermic acid, rosmarinic acid and caffeic acid were identified by comparison of their physical and spectroscopic data (Tanaka et al 1989). The chemical structures of these constituents are illustrated in Figure 1.

Measurement of $ONOO^-$ scavenging activity

The method of Kooy et al (1994) was used for the determination of $ONOO^-$ scavenging activity. Briefly, a stock solution of 5 mM DHR 123 in *N*, *N*-dimethylformamide purged with nitrogen was prepared and stored at −20 °C. Each sample was added to a 96-well plate (Nunc, Denmark). A buffer solution of 50 mM sodium phosphate (pH 7.4) containing 90 mM sodium chloride, 5 mM potassium chloride and 100 μM diethylenetriaminepentaacetic acid, and 10 μM DHR 123 were then added to the plate with or without 10 μM $ONOO^-$. Changes in fluorescent intensity were measured after 5 min. The fluorescence intensity of oxidized DHR 123 was measured with a microplate fluorescence reader (FL 500; Bio-Tex Instruments, USA) at excitation and emission wavelengths of 500 nm and 536 nm, respectively, at room temperature. The scavenging efficacy was expressed as IC50, denoting the concentration of each sample required to induce 50% inhibition of DHR 123 oxidation.

Interaction of lithospermate B with $ONOO^-$

To indicate the neutralization mechanism, the interaction of lithospermate B with $ONOO^-$ was measured by spectrophotometric analysis as described by Pannala et al (1998). A total of 500 μM $ONOO^-$ in 0.3 M NaOH was added to a solution containing 100 μM lithospermate B in 50 mM phosphate buffer (pH 7.4), to give a final volume of 1 mL. Each mixed solution was incubated at 37 °C with shaking for 1 h and scanned between 190 nm and 600 nm on an Ultraspec 2000 UV/vis spectrophotometer (Pharmacia-Biotech, UK). The spectral changes in the visible region of lithospermate B in the presence of $ONOO^-$ were monitored at 430 nm to determine the existence of nitration. A tube containing no $ONOO^-$ was also included for comparative purposes.

Determination of 3-nitrotyrosine

The ability of lithospermate B to suppress $ONOO^-$ mediated tyrosine nitration was determined as described above. Briefly, a total of 500 μM $ONOO^-$ in 0.3 M NaOH was added to a solution containing 100 μM tyrosine in the presence of 100 μM lithospermate B in 50 mM phosphate buffer (pH 7.4), to give a final volume of 1 mL. The formation of 3-nitrotyrosine by the reaction of tyrosine with $ONOO^-$ was also included for comparative purposes. The spectrum of the peak displayed at 430 nm indicates the formation of 3-nitrotyrosine.

Sample preparation for the detection of nitrated proteins

A 2.5-μL sample of lithospermate B dissolved in 10% EtOH was added to 95 μL BSA (0.5 mg protein mL^{-1}) or LDL (1 mg protein mL^{-1}). The mixed samples were incubated with shaking at 25 °C for 1 h. Then, 2.5 μL of $ONOO^-$ (final concn: 100 μM for BSA, 200 μM for LDL) in 0.3 M NaOH was added. The samples were further incubated with shaking for 30 min at 25 °C.

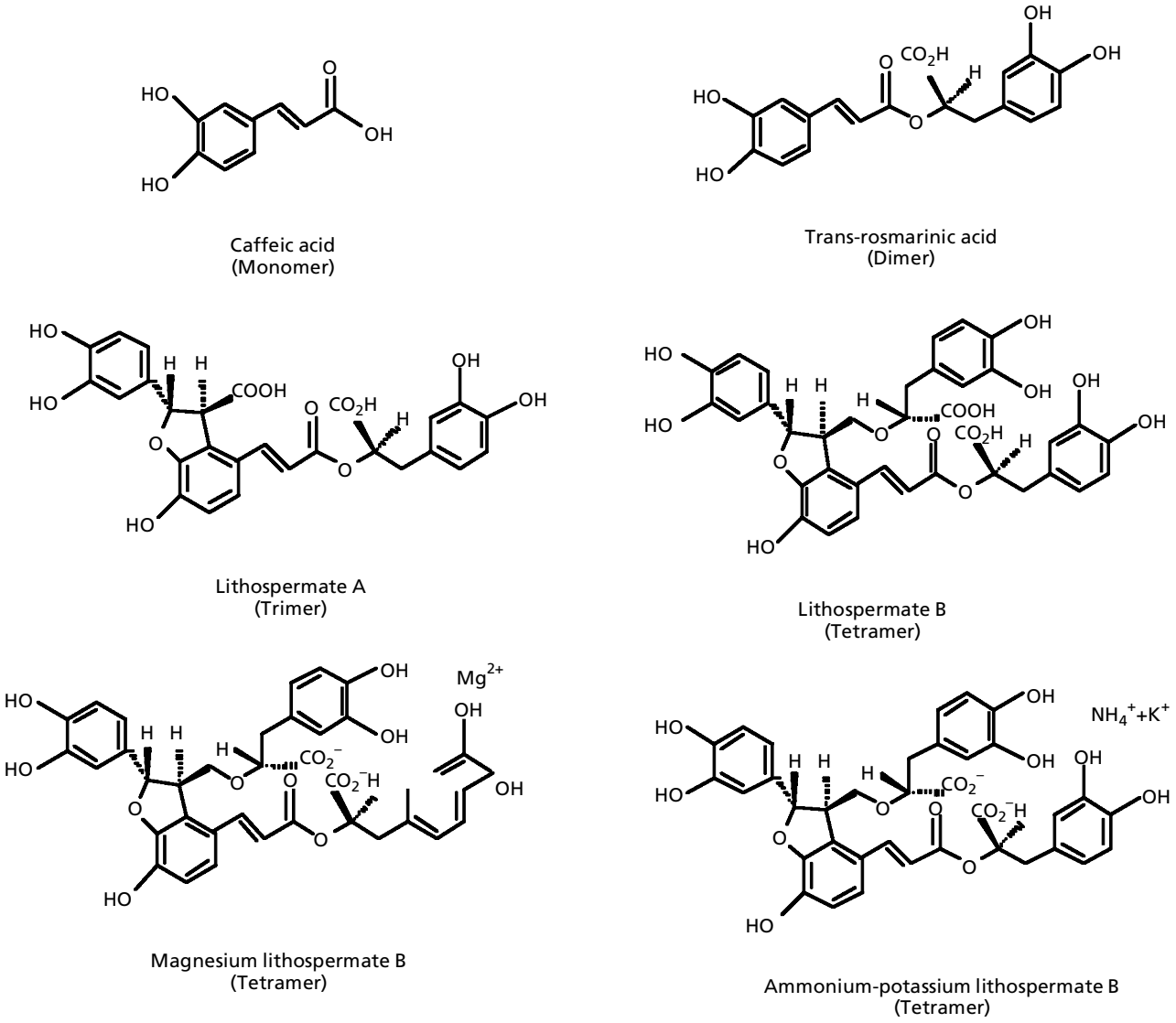

Figure 1 Structures of active components from *Salvia miltiorrhiza*.

Determination of nitrated proteins

The prepared samples in the gel loading buffer, pH 6.8, (0.125 M Tris(hydroxymethyl)aminomethane, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol and 0.2% bromophenol blue) in a ratio of 1:1 were boiled for 5 min. A total of 20 μL of each sample was separated on a sodium dodecyl sulfate polyacrylamide mini-gel (10% for BSA, 6% for LDL) at 100 V and transferred to a polyvinylidene fluoride membrane at 100 V for 1.5 h in a wet transfer system (Bio-Rad, Hercules, CA, USA). The membrane was immediately placed in a blocking solution (10% non-fat dry milk in TBS-T buffer containing 10 mM Tris, 100 mM NaCl and 0.1% Tween 20, pH 7.5) at 4 °C overnight. The membrane was washed in TBS-T buffer for 30 min and then incubated with a monoclonal anti-nitrotyrosine antibody (diluted 1:2000 in TBS-T buffer) at room temperature for 2 h. After two 15-min washings in TBS-T buffer, the membrane was reacted with a horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep (0.1% non-fat milk, diluted 1:2000 in TBS-T buffer) at room temperature for 2 h. After three 15-min washings in TBS-T buffer, antibody labelling was detected using enhanced chemiluminescence and exposed to radiographic film. Prestained blue protein markers were used for molecular weight determination.

Statistical analysis

Data are expressed as the mean $\pm$ s.d. Results were analysed by a one-factor analysis of variance. Differences between the means of individual groups were assessed by Fischer's protected LSD post-hoc test.

Table 1 $ONOO^-$ scavenging activity of crude extract and its fractions from *Salvia miltiorrhiza*.

Extract	Activity (%)
Crude extract	65.7 ± 1.9
Fraction A	45.5 ± 0.4
Subfraction A-1	8.7 ± 0.9
Subfraction A-2	74.4 ± 0.1
Subfraction A-3	73.5 ± 0.3
Fraction B	29.2 ± 0.8

Values are mean ± s.d.

Table 2 $ONOO^-$ scavenging activity of six components isolated from subfraction A-2.

Active component	IC_{50} (μM)
Caffeic acid	1.34 ± 0.11
Trans-rosmarinic acid	0.92 ± 0.12
Lithospermate A	1.11 ± 0.18
Lithospermate B	0.55 ± 0.03
Magnesium lithospermate B	0.60 ± 0.03
Ammonium-potassium lithospermate B	0.60 ± 0.02

Values are mean ± s.d.

Results

$ONOO^-$ scavenging effects of *S. miltiorrhiza* crude extract and its fractions

The $ONOO^-$ scavenging effects of *S. miltiorrhiza* and its fractions were measured using the DHR 123 method. The crude *S. miltiorrhiza* extract showed 65.7% scavenging activity, and its water-soluble fraction A and lipid-soluble fraction B showed scavenging activities of 45.5% and 29.2%, respectively. Fraction A was further subfractionated into A-1, A-2 and A-3. As seen in Table 1, the subfraction A-2, eluted with 50% aqueous MeOH, showed the highest $ONOO^-$ scavenging activity (74.4%).

$ONOO^-$ scavenging activity of six components isolated from subfraction A-2

Six active components purified using a water/EtOH mixture from subfraction A-2 were identified (see Figure 1). Table 2 presents the IC50 values of active $ONOO^-$ scavenging components measured at various concentrations (0.2, 0.4, 1.6, 3.2 and 6.4 μM). IC50 values were 1.34 μM for caffeic acid, 0.92 μM for trans-rosmarinic acid, 1.11 μM for lithospermate A, 0.60 μM for magnesium lithospermate B, 0.60 μM for ammonium-potassium lithospermate B, and 0.55 μM for lithospermate B. Among these components, lithospermate B, a tetramer of caffeic acid, showed the most potent scavenging efficacy.

Interaction of lithospermate B with $ONOO^-$

Scanning of the reaction mixture produced spectra of lithospermate B with and without $ONOO^-$ (data not shown). No peak was observed at 430 nm, indicating no nitration (data not shown). The absence of nitration suggested that the interaction of $ONOO^-$ with lithospermate B was mediated by an electron donation reaction.

Effect of lithospermate B on $ONOO^-$ mediated 3-nitrotyrosine

The spectrum of the reaction of tyrosine with $ONOO^-$ was observed. Absorbance at 430 nm indicated the formation of 3-nitrotyrosine, which disappeared when lithospermate B was incubated with tyrosine before the addition of $ONOO^-$, implying that lithospermate B blocked the formation of 3-nitrotyrosine (data not shown).

Effect of lithospermate B on nitration of BSA and LDL by $ONOO^-$

To investigate the action of lithospermate B on BSA and LDL nitration by $ONOO^-$, nitrotyrosine levels were measured using Western blotting (Figure 2). In the absence of $ONOO^-$, BSA and LDL showed no evidence of nitrotyrosine

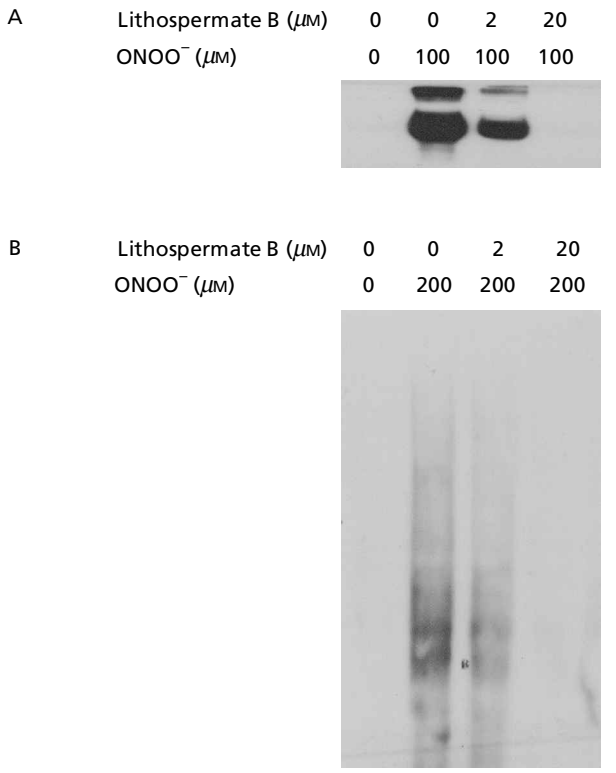

Figure 2 Effect of lithospermate B on nitration of bovine serum albumin (A) and low-density lipoprotein (B) by $ONOO^-$.

formation. Although the addition of $ONOO^-$ to BSA or LDL induced nitrotyrosine formation, the pre-incubation of lithospermate B with BSA or LDL led to reduced nitrotyrosine levels in a dose-dependent manner. The protection of lithospermate B against $ONOO^-$ was found to be effective.

Discussion

The inhibition of O_2^- and/or NO generation or the direct scavenging of O_2^- and/or NO are viable approaches to reducing $ONOO^-$ induced damage (Arteel et al 1999). So far, the prevention strategies against $ONOO^-$ damage are organized into two general approaches, one of which is to prevent the formation of $ONOO^-$. Previous studies on *S. miltiorrhiza* showed that when used as a herbal treatment, patients with non-insulin dependent diabetes (Jiang et al 1997) and coronary heart disease (Xing et al 1996) experienced increased superoxide dismutase levels and decreased lipid peroxidation. Glutathione peroxidase and catalase activities were also enhanced in chronic cor pulmonale patients pretreated with *S. miltiorrhiza* (Zhang & Chen 1994). In addition, *S. miltiorrhiza* was shown to suppress NO production and to scavenge O_2^- during xanthine and xanthine oxidase reactions in animals with ischaemia/reperfusion injury (Kuang et al 1996; Zhao et al 1996). Additional information on *S. miltiorrhiza* comes from studies of isolated components. For example, in mice, lithospermate B protected liver macrophages against D-galactosamine and lipopolysaccharide-induced tumour necrosis factor and O_2^- production (Hase et al 1997).

Magnesium lithospermate B, a salt form of lithospermate B, is reported to have strong antioxidant effects (Yokozawa et al 1997). Rats given magnesium lithospermate B after a subtotal nephrectomy showed restoration of superoxide dismutase and catalase activities to almost normal levels and a concomitant decrease in the hydroxyl radical level (Yokozawa et al 1997). Thus, *S. miltiorrhiza* and its component lithospermate B seem to be effective inhibitors and scavengers of both nitrogen- and oxygen-derived reactive species.

The second preventative strategy against $ONOO^-$ is to inactivate $ONOO^-$ itself. The present study showed that $ONOO^-$ was effectively scavenged by *S. miltiorrhiza* and its fractions. The six active components extracted from *S. miltiorrhiza* (caffeic acid, trans-rosmarinic acid, lithospermate A, lithospermate B, magnesium lithospermate B and ammonium-potassium lithospermate B) were shown to scavenge $ONOO^-$. In the present study, lithospermate B was found to be the most effective $ONOO^-$ scavenger among the six components. Other studies have shown that caffeic acid, the basic moiety of lithospermate B, has significant $ONOO^-$ scavenging ability (Pannala et al 1998).

Hydroxyl groups are known to be major contributors in scavenging $ONOO^-$. Most of the antioxidant activities of flavonoids, hydroxycinnamic acids and catechin polyphenols seem to depend on hydroxyl groups for $ONOO^-$ scavenging activity (Haenen et al 1997; Chung et al 1998; Pannala et al 1998). Therefore, hydroxyl groups and double bonds might play an important role in scavenging $ONOO^-$.

An important finding of the present study was the data showing the possible reaction mechanism of lithospermate B when exposed to $ONOO^-$. $ONOO^-$ scavengers are shown to react with $ONOO^-$ via two possible pathways: nitration or electron donation (Pannala et al 1998). Evidence obtained from the UV scan showed the reaction of lithospermate B with $ONOO^-$ produced no absorbance at 430 nm, implying that lithospermate B acts as an electron donor to neutralize $ONOO^-$. On the other hand, nitration, as observed in the conversion of tyrosine into 3-nitrotyrosine by $ONOO^-$, showed peak absorbance at 430 nm. Lithospermate B likely decreased the availability of $ONOO^-$, which resulted in blocking the formation of 3-nitrotyrosine by $ONOO^-$. This possibility was confirmed by evidence of no increased absorbance at 430 nm, when lithospermate B was exposed to $ONOO^-$ in the presence of tyrosine in the same concentration as lithospermate B.

The sources of $ONOO^-$ are wide-ranging. For example, stimulated macrophages, neutrophils, lymphocytes and endothelial cells are known to generate $ONOO^-$ (Deliconstantinos et al 1995; Hukkanen et al 1997; Gagnon et al 1998). Therefore, both the membrane and cytosol are potential targets for $ONOO^-$ attack. This is true also for proteins; their constituent amino acids, in particular, are among the preferred target molecules. Nitration reactions and modifications by $ONOO^-$ predominantly result from the nitration of tyrosine residues in proteins (Smith et al 1997; He et al 1999; Luoma & Yla-Herttuala 1999). In our study, BSA, a standard protein, was converted to nitrotyrosine-BSA by $ONOO^-$, and lithospermate B inhibited the albumin nitration. The formation of nitrotyrosine-LDL by $ONOO^-$ was also effectively blocked by lithospermate B.

Nitrated LDL is implicated in atherosclerosis (Panasenko et al 1997). In-vitro, nitrated LDL is known to be taken up and degraded by macrophages, which can lead to massive cholesterol deposition and foam cell formation, essential steps in lesion development (Panasenko et al 1997). It is suggested that macrophage cells have a macrophage scavenger receptor that is able to recognize chemically nitrated as well as oxidized LDL (Graham et al 1993). In this regard, the finding from the present study that pretreatment with lithospermate B reduces the formation of nitrated BSA and LDL is important. Also noteworthy is the finding that lithospermate B blocks the formation of nitration at a concentration as low as 20 μM.

In conclusion, it is suggested that lithospermate B exerts an anti-nitration effect by scavenging $ONOO^-$. Our results suggest that lithospermate B is a good candidate for a $ONOO^-$ scavenging agent to protect the cellular component, tyrosine, against $ONOO^-$ via $ONOO^-$ scavenging.

References

Arteel, G. E., Briviba, K., Sies, H. (1999) Protection against peroxynitrite. *FEBS Lett*. **445**: 226–230

Chung, H. Y., Yokozawa, T., Soung, D. Y., Kye, I. S., No, J. K., Baek, B. S. (1998) Peroxynitrite-scavenging activity of green tea tannin. *J. Agric. Food Chem*. **46**: 4484–4486

Deliconstantinos, G., Villiotou, V., Stavrides, J. C. (1995) Modulation of particulate nitric oxide synthase activity and peroxynitrite synthesis in cholesterol enriched endothelial cell membranes. *Biochem. Pharmacol*. **49**: 1589–1600

Gagnon, C., Leblond, F. A., Filep, J. G. (1998) Peroxynitrite production by human neutrophils, monocytes and lymphocytes challenged with lipopolysaccharide. *FEBS Lett*. **431**: 107–110

Gilad, E., Cuzzocrea, S., Zingarelli, B., Salzman, A. L., Szabo, C. (1997) Melatonin is a scavenger of peroxynitrite. *Life Sci*. **60**: PL169–PL174

Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V., Moncada, S. (1993) Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett*. **330**: 181–185

Gutteridge, J. M. (1999) Does redox regulation of cell function explain why antioxidants perform so poorly as therapeutic agents? *Redox Rep*. **4**: 129–131

Haenen, G. R., Paquay, J. B., Korthouwer, R. E., Bast, A. (1997) Peroxynitrite scavenging by flavonoids. *Biochem. Biophys. Res. Commun*. **236**: 591–593

Hase, K., Kasimu, R., Basnet, P., Kadota, S., Namba, T. (1997) Preventive effect of lithospermate B from *Salvia miltiorhiza* on experimental hepatitis induced by carbon tetrachloride or D-galactosamine/lipopolysaccharide. *Planta Med*. **63**: 22–26

He, K., Nukada, H., McMorran, P. D., Murphy, M. P. (1999) Protein carbonyl formation and tyrosine nitration as markers of oxidative damage during ischemia-reperfusion injury to rat sciatic nerve. *Neuroscience* **94**: 909–916

Hukkanen, M., Corbett, S. A., Batten, J., Konttinen, Y. T., McCarthy, I. D., Maclouf, J., Santavirta, S., Hughes, S. P., Polak, J. M. (1997) Aseptic loosening of total hip replacement. Macrophage expression of inducible nitric oxide synthase and cyclo-oxygenase-2, together with peroxynitrite formation, as a possible mechanism for early prosthesis failure. *J. Bone Joint Surg. Br*. **79**: 467–474

Jiang, Z. S, Zhang, S. L., Cai, X. J. (1997) Effect of *salvia miltiorrhiza* composita on superoxide dismutase and malonyldialdehyde in treating patients with non-insulin dependent diabetes mellitus. *Chung Kuo Chung Hsi I Chieh Ho Tsa Chih* **17**: 32–34

Kooy, N. W., Royall, J. A., Ischiropoulos, H., Beckman, J. S. (1994) Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radic. Biol. Med*. **16**: 149–156

Kuang, P., Tao, Y., Tian, Y. (1996) Effect of radix *Salviae miltiorrhizae* on nitric oxide in cerebral ischemic-reperfusion injury. *J. Tradit. Chin. Med*. **16**: 224–227

Luoma, J. S., Yla-Herttuala, S. (1999) Expression of inducible nitric oxide synthase in macrophages and smooth muscle cells in various types of human atherosclerotic lesions. *Virchows Arch*. **434**: 561–568

Panasenko, O. M., Briviba, K., Klotz, L. O., Sies, H. (1997) Oxidative modification and nitration of human low-density lipoproteins by the reaction of hypochlorous acid with nitrite. *Arch. Biochem. Biophys*. **343**: 254–259

Pannala, A. S., Razaq, R., Halliwell, B., Singh, S., Rice-Evans, C. A. (1998) Inhibition of peroxynitrite dependent tyrosine nitration by hydroxycinnamates: nitration or electron donation? *Free Radic. Biol. Med*. **24**: 594–606

Pryor, W. A., Squadrito, G. L. (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am. J. Physiol*. **268**: L699–L722

Sandoval, M., Henry, P. R., Ammerman, C. B., Miles, R. D., Littell, R. C. (1997) Relative bioavailability of supplemental inorganic zinc sources for chicks. *J. Anim. Sci*. **75**: 3195–3205

Smith, M. A., Richey Harris, P. L., Sayre, L. M., Beckman, J. S., Perry, G. (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci*. **17**: 2653–2657

Soung, D. Y., Chung, H. Y., Yokozawa, T. (1998) A novel action of 5-hydroxy-tryptamine as a peroxyonitrite scavenger. *Pharm. Pharmacol. Commun*. **4**: 583–586

Soung, D. Y., Kim, J. S., Chung, H. Y., Jung, H. A., Park, J. C., Choi, J. S. (1999) Flavonoids and chlorogenic acid from *Eriobotrya japonica* scavenge peroxynitrite. *Nat. Prod. Sci*. **5**: 80–84

Tanaka, T., Morimoto, S., Nonaka, G., Nishioka, I., Yokozawa, T., Chung, H. Y., Oura, H. (1989) Magnesium and ammonium-potassium lithospermates B, the active principles having a uremia preventive effect from *Salvia miltiorrhiza*. *Chem. Pharm. Bull*. **37**: 340–344

Tang, W., Eisenbrand, G. (1992) *Chinese drugs of plant origin*. Springer-Verlag, New York

Xing, Z. Q., Zeng, X. C., Yi, C. T. (1996) Effect of *Salvia miltiorrhiza* on serum lipid peroxide, superoxide dismutase of the patients with coronary heart disease. *Chung Kuo Chung Hsi I Chieh Ho Tsa Chih* **16**: 287–288

Yokozawa, T., Dong, E., Oura, H., Kashiwagi, H., Nonaka, G., Nishioka, I. (1997) Magnesium lithospermate B suppresses the increase of active oxygen in rats after subtotal nephrectomy. *Nephron* **75**: 88–93

Zhang, P. T., Chen, Z. R. (1994) Effect of *Salvia miltiorrhiza* on lipid peroxidation anti-oxidant enzymes activity in patients with chronic corpulmonale. *Chung Kuo Chung Hsi I Chieh Ho Tsa Chih* **14**: 474–477

Zhao, B. L., Jiang, W., Zhao, Y., Hou, J. W., Xin, W. J. (1996) Scavenging effects of *Salvia miltiorrhiza* on free radicals and its protection for myocardial mitochondrial membranes from ischemia-reperfusion injury. *Biochem. Mol. Biol. Int*. **38**: 1171–1182