

Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor- κ B in mouse macrophages

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Carnosol is a naturally occurring phytopolyphenol found in rosemary. Carnosol functions as antioxidant and anticarcinogen. In the present study, we compared the antioxidant activity of carnosol and other compounds extracted from rosemary. Carnosol showed potent antioxidative activity in α,α -diphenyl- β -picrylhydrazyl (DPPH) free radicals scavenge and DNA protection from Fenton reaction. High concentrations of nitric oxide (NO) are produced by inducible NO synthase (iNOS) in inflammation and multiple stages of carcinogenesis. Treatment of mouse macrophage RAW 264.7 cell line with carnosol markedly reduced lipopolysaccharide (LPS)-stimulated NO production in a concentration-related manner with an IC₅₀ of 9.4 μ M; but other tested compounds had slight effects. Western blot, reverse transcription-polymerase chain reaction, and northern blot analyses demonstrated that carnosol decreased LPS-induced iNOS mRNA and protein expression. Carnosol treatment showed reduction of nuclear factor- κ B (NF- κ B) subunits translocation and NF- κ B DNA binding activity in activated macrophages. Carnosol also showed inhibition of iNOS and NF- κ B promoter activity in transient transfection assay. These activities were referred to down-regulation of inhibitor κ B (I κ B) kinase (IKK) activity by carnosol (5 μ M), thus inhibited LPS-induced phosphorylation as well as degradation of I κ B α . Carnosol also inhibited LPS-induced p38 and p44/42 mitogen-activated protein kinase (MAPK) activation at a higher concentration (20 μ M). These results suggest that carnosol suppresses the NO production and iNOS gene expression by inhibiting NF- κ B activation, and provide possible mechanisms for its anti-inflammatory and chemopreventive action.

Introduction

Rosemary (*Rosmarinus officinalis* Labiatae) originally grows in southern Europe. Its herb and oil are commonly used as spice and flavoring agents in food processing for its desirable

Abbreviations: DPPH, α,α -diphenyl- β -picrylhydrazyl; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; I κ B, inhibitor κ B; IKK, I κ B kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; NO, nitric oxide; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; RNS, reactive nitrogen species; RT, reverse transcription.

flavor and high antioxidant activity (1). Rosemary was stated to act as a mild analgesic and antimicrobial agent in traditional herbal use (2). Rosemary contains flavonoids, phenols, volatile oil and terpenoids (2,3). The antioxidant activity of an extract from rosemary leaves is comparable with known antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, without the cytotoxic and carcinogenic risk of synthetic antioxidants (4,5). Among the antioxidant compounds in rosemary leaves, ~90% of the antioxidant activity can be attributed to carnosol and carnosic acid. Topical application of rosemary extract, carnosol or ursolic acid to mouse skin inhibited the covalent binding of benzo[a]pyrene to epidermal DNA, tumor initiation by 7,12-dimethylbenz[a]anthracene (DMBA), TPA-induced tumor promotion, ornithine decarboxylase activity and inflammation (4,6). Carnosol was stated to inhibit nitric oxide (NO) production in activated macrophages (7). Rosmarinic acid was widely studied for its antimicrobial and complement inhibition properties (8,9). Additional studies have revealed that rosemary extracts, carnosic acid and carnosol strongly inhibited phase I enzyme, CYP 450 activities and induced the expression of the phase II enzyme, glutathione S-transferase (GST) (10). These results give insight into the different mechanisms involved in the chemopreventive actions of rosemary.

NO is a short-lived small molecule free radical produced from L-arginine (11) in a reaction catalyzed by NO synthase (NOS). NO has many biological functions involved in vasodilatation, neurotransmission, tissue homeostasis, wound repair, inflammation and cytotoxicity (12–14). NO mediates diverse functions by acting on most cells of the body through the interaction with different molecular targets, which can either be activated or inhibited (15). Molecular cloning and sequencing analysis has revealed that there are at least three main types of NOS isoforms (16). Endothelial NOS and neuronal NOS are constitutively expressed and are Ca²⁺/calmodulin dependent. While stimulated by agonists that increase intracellular calcium, these isoforms produce small amounts of NO for only short periods. In sharp contrast, the high-output isoform, inducible NOS (iNOS), is expressed in cytokines [such as interferon (INF)- α , - β and - γ and interleukin (IL)-1 α and -1 β] and endotoxin lipopolysaccharide (LPS)-activated macrophages and endothelial cells following its transcriptional induction and new protein synthesis (17). Low concentrations of NO produced by iNOS possess beneficial roles in much of the antimicrobial activity of macrophages against pathogens (12,13,18,19). However, over production of NO and its derivatives, such as peroxynitrite and nitrogen dioxide, are found to be mutagenic *in vivo*, provoke the pathogenesis of septic shock and diverse autoimmune disorders (20–24). Furthermore, NO and its oxidized forms have also been shown to be carcinogenic (25,26). Excessive amounts of reactive nitrogen species (RNS) increase the oxidative stress in the body (27). RNS actively participate in the metabolic activation of procarcinogens, oxidative deterioration of lipids, proteins and DNA, altering

membrane fluidity, cellular homeostasis and gene expression. RNS are considered as major biodeterminants in the process of diseases and carcinogenesis (27,28). Therefore, inhibiting high-output NO production by blocking iNOS expression or its activity may be a useful strategy for treatment of NO-related disorders. The promoter region of the murine gene encoding iNOS contains two nuclear factor- κ B (NF- κ B) binding sites, located at 55 and 971 bp upstream of the TATA box (13,29). The binding of the potentially relevant transcription factor, NF- κ B, to the κ B sites has been shown to be functionally important for iNOS induction by LPS. The NF- κ B family of transcription factors regulates the expression of many genes, including the iNOS gene involved in immune and inflammatory responses. NF- κ B exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, I κ B (30,31). External stimuli for macrophage activation raise the phosphorylation of I κ B α , which causes its degradation and then simultaneously the activation and translocation of NF- κ B to the nucleus for binding to its cognate DNA binding site in the regulation region of a variety of genes. p38 and p44/42 mitogen-activated protein kinase (MAPK) are also reported to be involved in iNOS expression (32–35). The recent emphasis on the role of NO in pathological conditions has led to the discovery of new therapeutic agents. Antioxidants such as (–)-epigallocatechin-3-gallate (EGCG) (36), resveratrol (37) and naturally occurring flavonoids including apigenin and kaempferol (38) have been reported to suppress NO production and their inhibition mechanisms are based on their abilities to inhibit the activation of NF- κ B. Although the broad spectrum of research on rosemary and its extracts has concentrated on its action as a chemopreventive agent, the action mechanisms are not well established yet. Thus, here we compared the antioxidative properties of rosemary phytochemicals and examined their effects on NO generation, iNOS expression, NF- κ B, p38 and p44/42 MAPK activation in LPS-stimulated RAW 264.7 cells. The present data suggest that carnosol could protect against endotoxin-induced inflammation by blocking NF- κ B, p38 and p44/42 MAPK activation, thereby inhibiting the iNOS expression.

Materials and methods

Materials

Carnosol and ursolic acid were isolated from rosemary as described (4); briefly, carnosol was obtained by extracting rosemary leaves with hexane, evaporating solvent with rotary evaporator, dissolving the dried material with methanol, filtrating and evaporating the solvent again. Then the dry residue was dissolved by hexane:ether (3:1) and subjected to preparative column. Ursolic acid was obtained by extracting rosemary leaves with boiling ethanol. The final crystal formed was ursolic acid. The identities of these compounds were confirmed by particle bean liquid chromatography/mass spectrometry. Carnosic acid and rosmarinic acid were isolated from the ground dried leaves of rosemary sequentially by hexane and *n*-butanol extractions. The final products were purified by column chromatography on silica gel (1). LPS (*Escherichia coli* 026:B6), sulfanilamide and naphthylethylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St Louis, MO). Isotopes were obtained from Amersham (Arlington Heights, IL). Reverse transcription-polymerase chain reaction (RT-PCR) reagents were purchased from Promega (Madison, WI). Polynucleotide kinase was purchased from Pharmacia (Piscataway, NJ).

The mouse iNOS promoter plasmid was generously provided by Dr Charles J. Lowenstein, Johns Hopkins University (13). The plasmid construct contains a 1.75 kb *Hinc*III restriction fragment upstream of the macrophage NOS gene which was cloned into the GeneLight luciferase vector system (Promega). All constructs were then sequenced to characterize them definitively. Plasma DNA was purified by equilibrium centrifugation in CsCl/ethidium bromide gradients (13). The pNF κ B-Luc plasmid was purchased from Stratagene (La Jolla, CA).

DPPH free radicals scavenge activity

α , α -Diphenyl- β -picrylhydrazyl (DPPH) was purchased from Sigma. DPPH (100 μ M in 60% absolute alcohol) was mixed with vehicle (DMSO) only/or with different concentrations of tested compounds. Absorption at 517 nm was measured using an UV-visible spectrophotometer (Hitachi, u3201, Hitachi). The decrease in absorbance was shown when tested compounds possessed free radicals scavenge activity (39). DMSO did not interfere the reaction.

Protection of supercoiled DNA from strand breakage by Fenton reaction

pUC-19 plasmid DNA (200 ng) was incubated with 0.35% H₂O₂ and 10 μ M ferrous sulfate in the presence or absence of tested compounds at 37°C for 30 min. DNA relaxation to an open circular form was induced in the presence of hydroxyl radicals generated by H₂O₂ and Fe²⁺ (40). DNA was separated on 1% agarose gel and stained with ethidium bromide. The percentage of supercoiled forms of DNA among total DNA was calculated using a densitometer (IS-100 Digital Imaging System), and expressed as the ratio of supercoiled forms plasmid DNA to total plasmid DNA.

Cell culture

The mouse monocyte-macrophage cell line RAW 264.7 (ATCC TIB-71) was cultured in DMEM containing 10% heat-inactivated fetal bovine serum (Gibco-BRL, Life Technologies, Grand Island, NY). Except for the transient transfection assay, cells were plated in dishes at a density of 4 \times 10⁶/ml for 18–24 h before activation by LPS (1 μ g/ml). Tested compounds were co-treated with LPS and the final DMSO (as vehicle) concentration was <0.2% (v/v).

Nitrite determination

The nitrite accumulated in the culture medium was measured as an indicator of NO production according to the Griess reaction (41). Cells were plated in 24 well culture plates and stimulated with LPS (1 μ g/ml) in the presence or absence of various concentrations of tested compounds for 24 h. A 100 μ l sample of each media supernatant was mixed with 50 μ l of 1% sulphanilamide (in 5% phosphoric acid) and 50 μ l of 0.1% naphthylethylenediamine dihydrochloride (in dH₂O) at room temperature. The absorbance at 550 nm was measured with a NaNO₂ serial dilution standard curve and nitrite production was determined.

Western blots

Total cellular protein (for iNOS, α -tubulin, I κ B α , p38 and p44/42) was prepared using lysis buffer containing 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris-HCl pH 7.9, 100 μ M β -glycerophosphate, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin and 10 μ g/ml leupeptin. Cytosolic fraction (for PARP, α -tubulin, c-Rel, p65 and p50) and nuclear fraction (for PARP, α -tubulin, c-Rel, p65 and p50) were prepared according to a modified procedure of Xie *et al.* (29). Cells were suspended in hypotonic buffer containing 10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 1.5 mM MgCl₂, 1 mM PMSF, 1 μ g/ml aprotinin and 1 μ g/ml leupeptin. The nuclei were pelleted by centrifugation at 3000 g for 5 min. The supernatants containing cytosolic proteins were collected. Nuclei lysis was performed with hypertonic buffer containing 30 mM HEPES, 1.5 mM MgCl₂, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μ g/ml aprotinin and 1 μ g/ml leupeptin. The supernatants containing nuclear proteins were obtained by centrifugation at 12 000 g for 20 min. The nuclear proteins were also retained at –70°C for use in the DNA binding assay. Proteins (30–50 μ g) were separated on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (8% for iNOS and α -tubulin, 10% for PARP, α -tubulin, c-Rel, p65, p50, p38, p44/42 and I κ B α) and electro-transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon^P, Millipore, Bedford, MA). The membrane was pre-incubated in phosphate buffered-saline (PBS) containing 0.01% Tween-20, 1% bovine serum albumin (BSA) and 0.2% Na₂S₂O₃ overnight at 4°C, and then incubated with anti-iNOS monoclonal antibody (Transduction Laboratories, Lexington, KY), anti- α -tubulin monoclonal antibody (Oncogene Science, Cambridge, UK), anti-phospho (Ser32)-specific I κ B α , anti-phospho (Thr202/Tyr204)-specific p44/42 MAP kinase, anti-phospho (Thr180/Tyr182)-specific p38 MAP kinase antibody (New England Biolabs, Beverly, MA), anti-PARP (UBI, Lake Placid, NY) or anti-I κ B α , c-Rel, p65, p50 polyclonal antibodies (Santa Cruz Biochemicals, Santa Cruz, CA). After incubation with horseradish peroxidase- or alkaline phosphatase-conjugated anti-rabbit, -goat, -mouse IgG antibody, the immunoreactive bands were visualized with the enhanced chemiluminescence reagents (ECL, Amersham), or with the colorigenic substrates: nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as suggested by the manufacture (Sigma Chemical Co.). Data were quantified using a densitometer (IS-100 Digital Imaging System).

RT-PCR and northern blot

Cells were washed with ice-cold PBS and total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (42). Total RNA (5 μ g) was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase and oligo (dT)₁₈ primer by incubating the reaction mixture (15 μ l) at 37°C for 90 min. The PCR was performed as described previously (36) in a final volume of 25 μ l containing dNTPs (each at 200 μ M), 1 \times reaction buffer, 0.4 μ M each primer (iNOS; forward 5'-CCCTCCG-AAGTTTCTGGCAGCAGC-3' and reverse 5'-GGCTGTCAGAGAGCCTC-GTGGCTTTGG-3'; glyceraldehyde-3-phosphate dehydrogenase (G3PDH) 5'-TGAAGTCCGGTGTGAACGGATTGGC-3' and 5'-CATGTAGGCCA-TGAGGTCCACCAC-3'), 3 μ l of RT product, and 50 U/ml Super Taq DNA polymerase. After an initial denaturation for 5 min at 95°C, 30 cycles of amplification (95°C for 30 s, 65°C for 45 s and 72°C for 2 min) were performed followed by a 10 min extension at 72°C. A 5 μ l sample of each PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining (497 bp iNOS fragment and 983 bp G3PDH fragment). For northern blot analysis, total RNA (20 μ g) was denatured with formaldehyde/formamide and incubated at 65°C for 15 min, size fractionated on 1.2% formaldehyde-containing agarose gel, and transferred to Hybond-N nylon membrane (Amersham) in 20 \times standard sodium citrate (SSC) buffer. The blotted membrane was hybridized with [α -³²P]dCTP-labeled (Random Primer Labeling kit, Amersham) iNOS fragment at 42°C overnight. After a stringent wash, the membrane was dried and autoradiographed with Kodak X-ray film (Rochester, NY). Amplification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and ethidium bromide staining of ribosomal RNA (18S and 28S) served as a control for sample loading and integrity. Data were quantified using a densitometer (IS-100 Digital Imaging System), and represented one of three similar experimental results.

Immunoprecipitation and IKK kinase (IKK) assay in cell culture

Immunoprecipitation (IP) was performed on the whole-cell lysates (300 μ g) with anti-IKK α / β antibody and protein A/G plus agarose beads (Santa Cruz) for 18 h at 4°C in IP buffer containing 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 10 μ g/ml aprotinin and 1 μ g/ml leupeptin with gentle rotation. Immunoprecipitated IKK was washed three times with IP buffer and twice with kinase buffer containing 50 mM HEPES pH 7.4, 10 mM MgCl₂, 2.5 mM EDTA, 10 mM β -glycerophosphate, 1 mM NaF, 1 mM DTT, 10 mM *p*-nitrophenyl phosphate, 300 μ M sodium orthovanadate, 1 mM benzamide, 10 μ g/ml aprotinin and 1 μ g/ml leupeptin. IKK kinase activity was assayed by phosphorylation of GST-IKK α fusion protein (1 μ g in each sample, Santa Cruz) in a final volume of 40 μ l kinase buffer with 5 μ Ci [γ -³²P]dATP (5000 Ci/mmol, Amersham) for 30 min at 30°C. The reaction was terminated by the addition of 10 μ l 5 \times Laemmli's loading buffer and heated at 100°C for 10 min. The sample was resolved by 10% SDS-PAGE, dried and visualized by autoradiography. Data were quantified using a densitometer (IS-100 Digital Imaging System), and represented one of three similar experimental results.

Electrophoretic mobility shift assay

A 5 μ g sample of nuclear extract was mixed with the double-stranded NF- κ B oligonucleotide, 5'-ATGTGAGGGGACTTCCAGGC-3' end-labeled by [γ -³²P]dATP (underlining indicates a κ B consensus sequence or a binding site for NF- κ B/c-Rel homodimeric and heterodimeric complexes), and salmon sperm DNA in binding buffer containing 100 mM KCl, 30 mM HEPES, 1.5 mM MgCl₂, 0.3 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin and 10% glycerol at room temperature for 20 min. A DNA-protein complex was separated from the free probe on a 4.5% non-denaturing TBE polyacrylamide gel in 0.5 \times Tris-borate-EDTA buffer (TBE: 44.5 mM Tris, 44.5 mM boric acid and 1 mM EDTA). The specificity of binding was examined by competition with the unlabeled oligonucleotide. The gel was dried and subjected to autoradiography. Data were quantified using a densitometer (IS-100 Digital Imaging System), and represented one of three similar experimental results.

Transient transfection and luciferase assay

RAW 264.7 cells were seeded in a 60 mm dish. When the cells were confluent, the medium was replaced with serum-free Opti-MEM (Gibco-BRL). Then the cells were transfected with the pNF- κ B-Luc, or pGL2-iNOS plasmid reporter gene using LipofectAMINE™ (Gibco-BRL). After 24 h incubation, the medium was replaced with complete medium. After 24 h, the cells were trypsinized and equal numbers of cells were plated in 12 well tissue culture plates for 12 h. Cells were then treated with LPS (1 μ g/ml) alone or with carnosol (20 μ M) for 3 h. Each well was washed twice with cold PBS and harvested in 150 μ l of lysis buffer (0.5 M HEPES pH 7.8, 1% Triton X-100, 1 mM CaCl₂ and 1 mM MgCl₂). Aliquots (100 μ l) of cell lysate were used to assay luciferase activity with the Luc-Lite™-M luciferase reporter gene

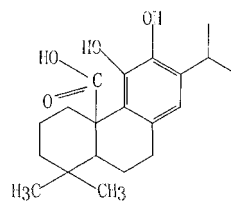
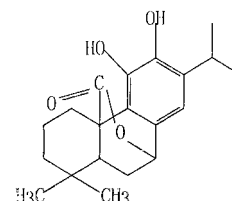
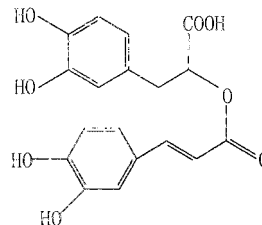
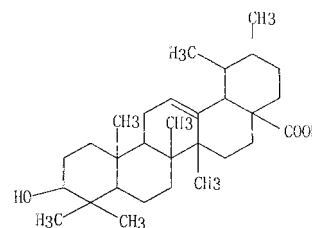
(A) Carnosic acid**(B) Carnosol****(C) Rosmarinic acid****(D) Ursolic acid**

Fig. 1. Structures of carnosic acid (A), carnosol (B), rosmarinic acid (C) and ursolic acid (D).

assay kit (Packard Instrument Co., Meriden, CT). Luminescence was measured in a TopCount® Microplate Scintillation and Luminescence Counter (Packard 9912V1; Meriden, CT) in single photon counting mode for 0.05 min/well, following 5 min adaptation in the dark. Luciferase activities were normalized to protein concentrations. Data represented one of three similar experimental results.

Statistical analysis

Data were presented as means \pm SE for the indicated number of independently performed experiments. Statistical significance ($P < 0.05$) was assessed by one-way analysis of variance (ANOVA) coupled with Dunnett's *t* tests.

Results*Antioxidative properties of rosemary phytochemicals*

The antioxidative activity of the four compounds (shown in Figure 1) isolated from rosemary was assayed in two different systems. In DPPH free radical scavenge activity, carnosic acid, carnosol and rosmarinic acid showed concentration-dependent scavenge ability (Figure 2A). They were more potent than vitamin C and vitamin E. The 50% DPPH scavenge concentrations are: carnosic acid 0.6 μ M, carnosol 0.59 μ M, rosmarinic acid 0.49 μ M and ursolic acid >10 μ M as approximation values coming from one of the three separated experiments. Rosmarinic acid was the most potent antioxidant over the same concentration range and ursolic acid showed no effects on DPPH scavenge. We further used the Fenton reaction by H₂O₂/FeSO₄ system to determine whether rosemary phytopolyphenols affected the hydroxyl radical-induced DNA damage. Treatment of pUC-19 plasmid with H₂O₂ and FeSO₄ concentration dependently relaxed the supercoiled form DNA (data not shown). However, co-treatment with rosemary phytochemicals exerted a protective effect from the H₂O₂/FeSO₄-induced DNA damage in a concentration-dependent manner (Figure 2B). The arbitrary values coming from densitometric analysis represent the supercoiled forms plasmid DNA to total plasmid DNA. Carnosic acid, carnosol, rosmarinic acid and ursolic acid were comparable with vitamin C and vitamin E. The IC₅₀ of DNA relaxation of tested compounds were: carnosic acid 0.13 μ M, carnosol 0.055 μ M, rosmarinic acid 0.51 μ M, ursolic acid 0.51 μ M as approximation values coming from three separated

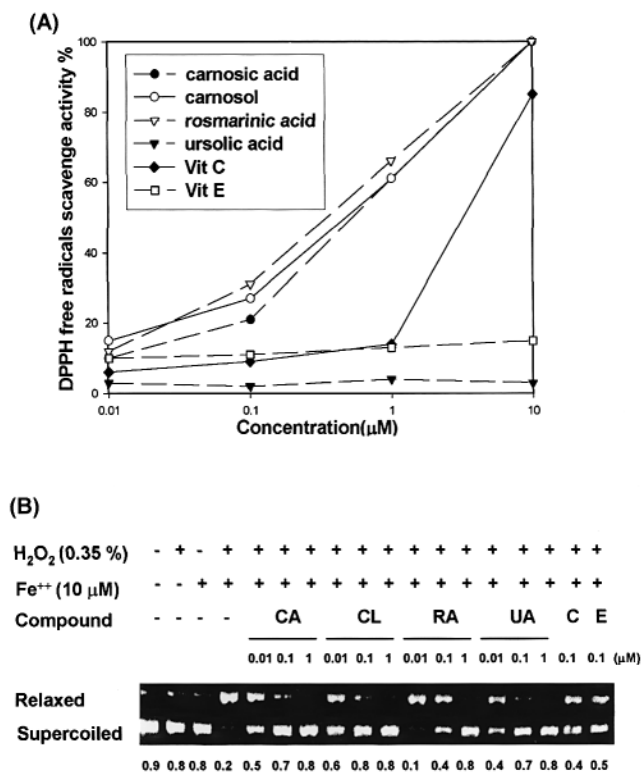


Fig. 2. Antioxidative properties of tested compounds. (A) DPPH free radicals scavenge activity of rosemary phytochemicals and vitamins C and E. Various concentrations of phytopolyphenols and vitamins were mixed with 100 µM (final) DPPH dissolved in 60% alcohol. Absorption at 517 nm was measured immediately; free radicals scavenge activity was expressed correlating to the decreased OD 517 nm value. Data represent one of three similar results. (B) Protection of plasmid DNA by rosemary phytochemicals. Abbreviations: CA (carnosic acid), CL (carnosol), RA (rosmarinic acid), UA (ursolic acid), C (vitamin C) and E (vitamin E). The products were analyzed by electrophoresis in 1% agarose gel. (Upper band) Relaxed forms plasmid DNA; (lower band) supercoiled forms plasmid DNA. Data represent one of three similar results. The ratio represents supercoiled forms plasmid DNA to total plasmid DNA coming from densitometric analysis.

experiments. These results suggested that rosemary phytochemicals reacted with and/or scavenged the hydroxyl radicals and reduced the DNA damage in the tested system.

Effects of carnosic acid, carnosol, rosmarinic acid and ursolic acid on LPS-induced NO production in activated macrophages

The half-life of NO is very short, so we used nitrite production as an indicator of NO released in LPS-activated macrophages to investigate the anti-inflammatory effects of the four rosemary phytochemicals. The concentration-response relationships (Figure 3) were determined 24 h after treatment of LPS (1 µg/ml) only or along with the tested compounds. Carnosol inhibited nitrite production by >50% at 10 µM (*P* < 0.01). Carnosol was found to reduce NO generation in a concentration-dependent manner. Carnosol reduced NO generation with an IC₅₀ of 9.4 µM as an approximation value coming from one of the three separated experiments. Carnosic acid and rosmarinic acid showed less inhibitory effect over the same concentration range. A cell viability assay verified that the inhibition of carnosol was not due to general cellular toxicity (data not shown). The inhibitory ability of ursolic acid might be due to its cytotoxic effect (data not shown). The phytochemicals dissolved in DMSO did not interfere with the Griess reaction.

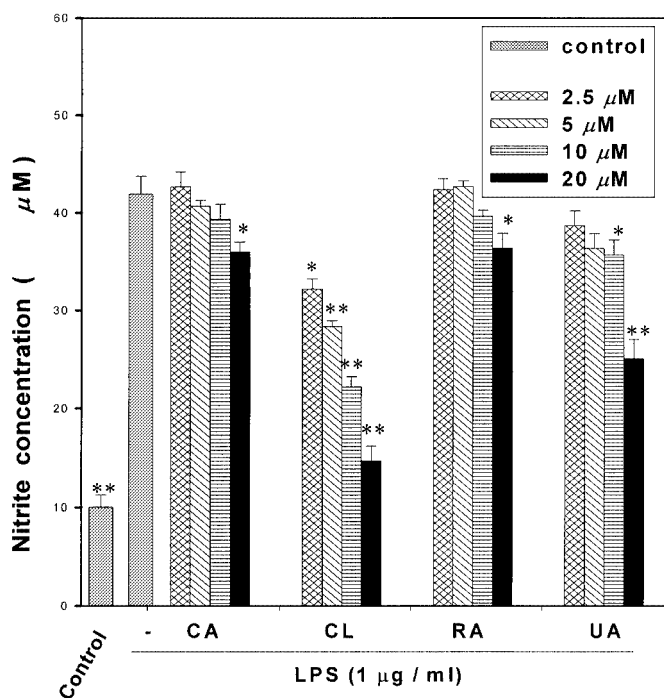


Fig. 3. Effects of tested compounds on LPS-induced nitrite formation in RAW 264.7 cells. Cells were treated with or without indicated concentrations of tested compounds and LPS (1 µg/ml) for 24 h. Abbreviations: CA (carnosic acid), CL (carnosol), RA (rosmarinic acid) and UA (ursolic acid). Amounts of nitrite released to culture medium were determined by Griess reagent. Data represent mean ± SE of triplicate tests. **P* < 0.05; ***P* < 0.01 versus LPS alone treatment.

Inhibition of iNOS protein and mRNA expression by carnosol

As shown in Figure 3, carnosol was the most potent in inhibiting the production of nitrite among the tested compounds. To elucidate the mechanisms involved in the inhibition of NO generation by carnosol in LPS-activated macrophages, we further studied the effect of carnosol on iNOS protein and gene expression. RAW 264.7 cells, maintained in normal conditions, express slightly detectable levels of iNOS mRNA and protein. After stimulating with LPS, the level of iNOS mRNA continually increases between 1 and 12 h following the increased iNOS protein level. Inhibition of iNOS protein by carnosol was detected in a concentration-dependent manner after 18 h treatment (Figure 4A). The relative levels of iNOS protein with the treatment of 2.5, 5, 10 and 20 µM carnosol were 0.8, 0.7, 0.4 and 0.2 versus LPS alone. The inhibitory concentration for iNOS protein expression was similar to that for reduction of nitrite formation. In order to investigate whether the suppression of iNOS protein by carnosol was due to reduced iNOS mRNA expression, RT-PCR analysis for total mRNA samples extracted from RAW 264.7 cells after 12 h treatment was carried out. The amplification of cDNA with primers specific for mouse iNOS and G3PDH (as control gene) was shown in Figure 4B. The results indicated that lower levels of iNOS mRNA were expressed in the presence of carnosol in LPS-activated macrophages. The relative level of iNOS mRNA with the treatment of 2.5, 5, 10 and 20 µM carnosol were 0.7, 0.5, 0.4 and 0.2 versus LPS alone. Similar results were obtained from the northern blot analysis of iNOS mRNA in cell extracts (Figure 4C). The data suggested that carnosol modulated iNOS expression at the transcriptional level. Consistent with previous findings, carnosol almost

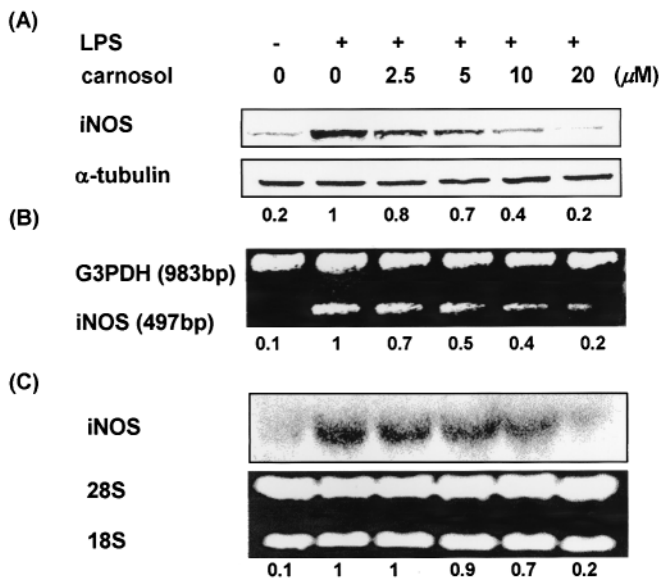


Fig. 4. Effects of carnosol on LPS-induced iNOS expression in RAW 264.7 cells. (A) Cells were treated with various concentrations of carnosol and LPS (1 μ g/ml) for 18 h. Total-cell lysate (30 μ g) was resolved in 8% SDS-PAGE then transferred to PVDF membrane and detected with specific antibodies as described in the Materials and methods. (B) Cells were treated with different concentrations of carnosol and LPS (1 μ g/ml) for 12 h. Total RNA (5 μ g) was subjected to RT-PCR and the PCR product was resolved in 1% agarose gel. (C) Total RNA was extracted from treated cells and separated on 1.2% formaldehyde-containing agarose gel then transferred onto nylon membrane. (Upper panel) The blots were hybridized by 32 P-labeled iNOS probe as described in the Materials and methods. (Lower panel) Ethidium bromide staining of ribosomal RNA (18S and 28S) was shown as controls. Data represented one of three similar results. The arbitrary values were obtained from densitometric analysis. The relative level observed with LPS alone is set at 1.

completely suppressed iNOS gene expression at 20 μ M (Figure 4) and thus inhibited the production of NO in LPS-stimulated RAW 264.7 cells (Figure 3). To further investigate the importance of LPS and carnosol in modulating the expression of iNOS, transient transfection was performed using mouse iNOS luciferase promoter constructs. LPS-induced iNOS promoter activity was inhibited by carnosol (Figure 5C). Carnosol might block LPS-mediated signal transduction.

Reduction of nuclear contents of NF- κ B/Rel family members by carnosol treatment in LPS-stimulated RAW 264.7 cells

Previous reports have demonstrated that the transcription factor NF- κ B is involved in the activation of iNOS by LPS induction. We tested whether carnosol perturbed the translocation of NF- κ B/Rel family subunits (c-Rel, p65 and p50) into the nucleus in LPS-stimulated RAW 264.7 cells. Nucleus and cytosolic extracts were prepared and subjected to immunoblot analysis. The amounts of nuclear c-Rel, p65 and p50 were increased 30 min after LPS treatment. However, co-incubation with LPS plus carnosol resulted in the reduction of nuclear contents of c-Rel and p65 proteins (Figure 5A). p50 nuclear localization was also reversed by carnosol to a lesser extent. PARP, a nuclear protein, and α -tubulin, a cytosolic protein, were used as controls to confirm that there was no contamination during extraction of each fraction (data not shown).

Inhibition of carnosol on LPS-induced NF- κ B activation

To investigate if carnosol specifically inhibited the activation of NF- κ B, a gel mobility shift assay was performed to analyze NF- κ B DNA binding activity. As shown in Figure 5B, the

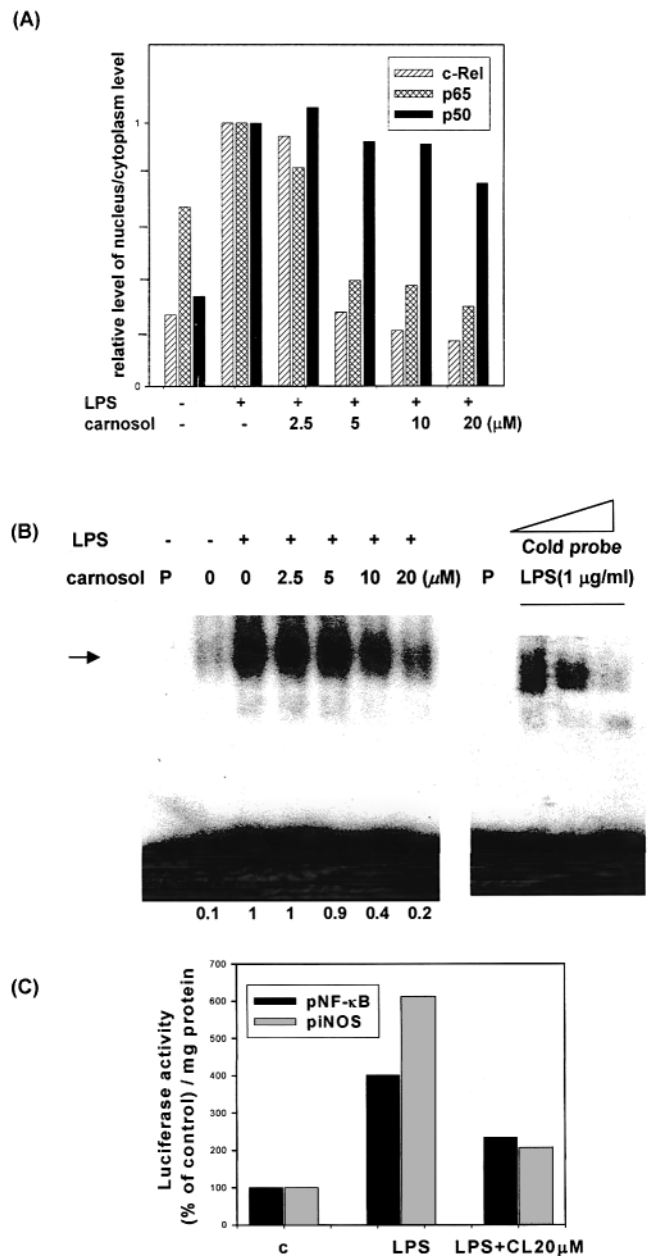


Fig. 5. Effects of carnosol on NF- κ B activation. (A) Carnosol reduced NF- κ B nuclear translocation levels. Cells were treated with LPS (1 μ g/ml) without or with carnosol and then incubated for 30 min. Cytosolic and nuclear fractions were prepared and analyzed for the content of NF- κ B subunits by western blot. Data represent one of three similar results. The relative level was calculated as the ratio of nuclear to cytosolic NF- κ B subunits coming from densitometric analysis. The ratio of NF- κ B nuclear translocation observed in LPS treated only group is set at 1. (B) EMSA for nuclear NF- κ B DNA binding activity. Cells were treated with LPS (1 μ g/ml) with or without carnosol for 30 min. Nuclear extract was subjected to DNA binding assay. The retarded bands were indicated. P, probe only. Data represent one of three similar results. The arbitrary values were obtained from densitometric analysis. The relative level observed with LPS alone is set at 1. Specificity of NF- κ B complex formation was verified in LPS-only sample by displacement with 25-, 2.5- or 0-fold excess unlabeled consensus oligonucleotide. (C) Effects of carnosol on LPS-induced NF- κ B and iNOS promoter activities in RAW 264.7 cells. The cells were transfected with 2.5 μ g of mouse iNOS promoter (-1592 to +160) or pNF κ B-Luc reporter plasmid. After transfection, cells were subcultured in 12 well plates, and then treated with LPS (1 μ g/ml) without or with carnosol for 3 h. Cells were harvested and the levels of luciferase activities were determined as described in the Materials and methods. C, control; CL, carnosol. Data represent one of three similar results.

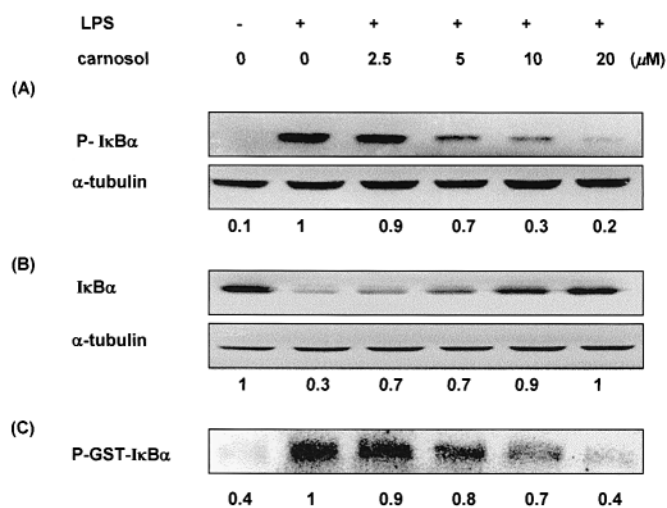


Fig. 6. Effects of carnosol on IkB α content and IkB kinase activity. Cells were treated with LPS (1 μ g/ml) without or with carnosol and incubated for 12 or 30 min. Total cell lysate were prepared for western blot analysis for the content of IkB α protein. After 12 min activation, the phosphorylated IkB α was detected by anti-phospho (Ser32)-specific IkB α antibody (A). Data represent one of three similar results. The arbitrary values were obtained from densitometric analysis. The relative level observed with LPS alone is set at 1. The content of IkB α protein was detected after 30 min activation by anti-IkB α antibody (B). The relative level observed with control is set at 1. (C) Cells were treated and incubated for 12 min. IKK-immuno complex was prepared from total cell lysate. The kinase activity was assayed with GST-IkB α as substrate and performed as described in the Materials and Methods. 32 P-Labeled GST-IkB was separated on 10% SDS-PAGE and subjected to autoradiography. The relative level observed with LPS alone is set at 1.

induction of specific NF- κ B DNA binding activity by LPS was inhibited by carnosol. The relative levels of NF- κ B DNA binding activity with the treatment of 2.5, 5, 10 and 20 μ M carnosol were 1, 0.9, 0.4 and 0.2 versus LPS alone. The specificity of binding was examined by competition with the addition of excess consensus unlabeled oligonucleotide. In an additional study, transient transfection with a NF- κ B-dependent luciferase reporter plasmid was done to confirm whether carnosol inhibited the NF- κ B binding activity in LPS-induced macrophages. As shown in Figure 5C, carnosol inhibited LPS-induced NF- κ B transcriptional activity.

Inhibitory effects of carnosol on LPS-induced phosphorylation and degradation of IkB α

As the LPS-mediated activation of NF- κ B is correlated with the hyperphosphorylation of IkB α and its subsequent degradation, we examined the phosphorylated and protein levels of IkB α by immunoblot analysis. After 12 min activation by LPS, the serine-phosphorylated IkB α protein was detected by anti-Ser32-phospho-specific IkB α antibody as illustrated in Figure 6A. Carnosol inhibited LPS-induced IkB α phosphorylation. Time course experiments showed that treatment with LPS caused the degradation of IkB α after 20–40 min (data not shown) and IkB α recovered to basal levels after 50 min. As shown in Figure 6B, the amount of IkB α was decreased by treatment with LPS for 30 min, and the treatment with carnosol effectively sustained the IkB α protein content. The pattern of inhibition on IkB α phosphorylation by carnosol was paralleled to the pattern of inhibition on its degradation. These results suggest that inhibition of NO production by carnosol (Figure 3) occurs via blocking the phosphorylation as well as degradation of IkB α protein (Figure 6), thus preventing the translocation and activation of NF- κ B in the nucleus (Figure 5).

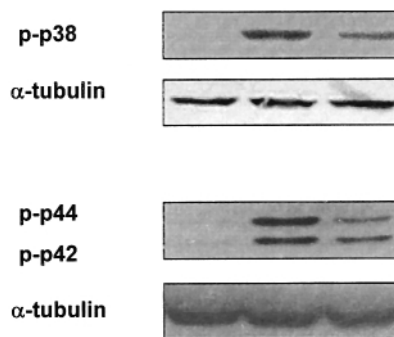


Fig. 7. Inhibition of p38 and p44/42 MAPK by carnosol in activated RAW 264.7 cells. Cells were treated with LPS (1 μ g/ml) with or without carnosol (20 μ M) for 30 min. Cell extracts were then prepared and subjected to western blotting with antibodies specific for phosphorylated forms of p38 or p44/42. C, control. Data represent one of three similar results.

Effects of carnosol on activation of IKK

To further confirm the inhibition of IkB α phosphorylation and degradation by carnosol and the correlation between phosphorylation of IkB α and IKK activity, we assayed IKK activity in cell culture. When we assayed the immunoprecipitated IKK activity by phosphorylation of GST-IkB α , IKK activity was inhibited by carnosol in LPS-induced RAW 264.7 cells (Figure 6C). There was no effect of carnosol on the level of IKK protein (data not shown). This result suggested that carnosol inhibited iNOS expression via down-regulated IKK activity and thus prevented NF- κ B activation.

Effects of carnosol on activation of p38 and p44/42 MAP kinase

Because p38 and p44/42 MAPK have been shown to be involved in iNOS induction mediated by LPS in mouse macrophages (34,35). We investigated the effects of carnosol on the activation of p38 and p44/42 MAPK in LPS-stimulated RAW 264.7 macrophages. Activation of MAPK requires phosphorylation at threonine and tyrosine residues (43). Immunoblot analysis with anti-phospho-specific antibody was performed. Time course experiments showed the activation of p38 and p44/42 by LPS peaked after 20–40 min of treatment and declined to basal level after 60 min of treatment (data not shown). When the cells were co-treated with carnosol (20 μ M) and LPS (1 μ g/ml) for 30 min, the LPS-induced activation of p38 and p44/42 MAPK was attenuated by carnosol (Figure 7).

Discussion

The important issue of relevance of the *in vitro* findings regarding carnosol to the *in vivo* dietary, or even pharmacological chemoprevention deserves further elaboration. Rosemary is widely used as a kitchen spice. There is no detailed pharmacokinetic data available in humans. The relative amount of carnosol in dry ground rosemary leaves is 3.8–4.6% (4). Huang *et al.* (4) have demonstrated the inhibitory effect of carnosol on skin tumorigenesis in mice. Singletary and Nelshopp (6) reported that administration of 1% crude extract of rosemary in the diet to female Sprague–Dawley rats for 3 weeks before a single oral dose of DMBA reduced mammary gland tumor incidence by 47% at 16 weeks after DMBA treatment. In the present study, carnosol, carnosic acid and rosmarinic acid showed effective DPPH free radicals scavenge activities. Carnosic acid, carnosol, rosmarinic acid and ursolic acid effectively inhibited pUC19 DNA strand break induced by Fenton reaction. The reaction of iron (II) with

hydrogen peroxide is generally considered to yield the hydroxyl radicals (44). These compounds may react with and/or scavenge the hydroxyl radicals produced by Fenton reaction. It has been reported that the antioxidative activity of phenolic compounds is related to their hydroxyl group and the presence of a second hydroxyl group in the *ortho* or *para* position is known to increase the antioxidative activity due to additional resonance stability and *o*-quinone or *p*-quinone formation (45,46). Carnosic acid, carnosol and rosmarinic acid have *o*-hydroxyl group and possessed high antioxidative activity.

Our group had reported that many antioxidants possessed anti-inflammatory effects (36–38). These findings provided a significant molecular basis on the mode of actions of dietary biochemically active compounds in preventing cancer and inflammation. In this study, we demonstrated that carnosol inhibited the LPS-induced NO production in the mouse macrophage RAW 264.7 cells with an IC₅₀ of 9.4 μM. Carnosic acid, rosmarinic acid and ursolic acid had less effect although they possessed different extents of antioxidant or DNA protection activity. The antioxidant activity of a given phytopolyphenol is related, but not really consistent with its effect on the LPS-induced NO production. To identify the direct scavenge effect of carnosol, we determined the amount of NO remaining in the supernatant shortly after the addition of carnosol to the supernatant of LPS-stimulated RAW 264.7 cells; however, the amount of NO pre-existed in the supernatant was not changed by carnosol (data not shown). From these results, we assumed that the inhibition of NO production in LPS-stimulated RAW 264.7 cells by carnosol occurred via modulation of iNOS.

Many compounds modulate LPS-stimulated iNOS at the transcription level. To determine the inhibitory effect of carnosol on the LPS-induced iNOS protein and mRNA expression, western blot, RT-PCR and northern blot analysis were performed. Carnosol concentration-dependently inhibited LPS-induced iNOS mRNA and protein expression. LPS positively regulates NF-κB/Rel for iNOS and other immune or inflammatory gene expression (47). As reported, the synergistic effect of IFN-γ will be needed to fully express iNOS gene in LPS-stimulated macrophages (48). The recently cloned promoter of the murine gene coding for iNOS also contains at least 22 elements homologous to consensus sequences for the binding of transcription factors involved in the inducibility of other genes by cytokines and LPS. Among those transcription factors, NF-κB is necessary to confer inducibility by LPS in mouse macrophages (29). Kleinert *et al.* (49) also reported that in 3T3 cells, at least three different signal transduction pathways could stimulate iNOS mRNA expression. All these pathways seem to converge with the activation of the essential transcription factor NF-κB, although a marked inter-cell variability exists (50–52). NF-κB is composed mainly of two proteins: p65 and p50. In unstimulated cells, NF-κB exists in the cytosol in a quiescent form bound to its inhibitory protein, IκB (30). After stimulating the cells with various agents, IκB becomes phosphorylated and goes to subsequent proteolytic degradation. Releasing IκB from NF-κB allows the activation and nuclear accumulation of NF-κB subunits (31). To identify the mechanisms involved in the effects of carnosol on iNOS expression, we investigated the transcriptional regulation of NF-κB. Electrophoretic mobility shift assay (EMSA) and promoter activity studies showed that carnosol treatment inhibited activation of these κB binding complexes. Immunoblot analysis also showed that the inhibition of NF-κB activity by carnosol might be the

result of the inhibition of IκBα phosphorylation and degradation then reduction of the translocation of NF-κB subunits. p50 forms heterodimers with p65 and c-Rel, the Rel family protein. Here we found that carnosol reversed the nuclear translocation of each subunit with different ratios and this may be due to the different degradation rates of subunits and this interesting phenomenon deserves further investigation. We also identified that inhibition of IKK activity was important in the anti-inflammatory action of carnosol.

Phosphorylation plays an important role in activating protein tyrosine kinase, MAPK, and protein kinase C in mediating LPS signaling. Reactive oxygen species have been proposed to be involved in the activation of NF-κB via regulating various redox-sensitive protein or tyrosine kinases (53–55). Antioxidants, thiols and iron chelators can specifically prevent activation of NF-κB (56). Furthermore, many antioxidants or phytopolyphenols such as pyrrolidine dithiocarbamate (57), *N*-acetyl-L-cysteine (58) and EGCG (36) inhibit NO production via regulating NF-κB activity. Recently, aspirin was also reported to possess antioxidative and DNA protection properties as well as inhibit NF-κB (40). Carnosol possessed high antioxidant activity in a previous study (59) and in our study. Our findings showed that carnosol can block activation of NF-κB by interfering with the signal-induced phosphorylation of IκB.

The p38 kinase is an important mediator of stress-induced gene expression (43). In particular, the p38 kinase is known to play a key role in the LPS-induced signal transduction pathway (32,60). Badger *et al.* (61) reported that infusion of the p38 inhibitor, SB203580, reduced mortality in LPS-treated mice. However, the involvement of p38 kinase and iNOS expression is controversial. Paul *et al.* (62) described no effect of the specific p38 kinase inhibitor, SB203580, on iNOS expression in LPS-induced RAW 264.7 macrophages. Also, Chan *et al.* (63) found no effect of SB203580 on INF-γ/tumor necrosis factor-α (TNF-α)-induced iNOS expression in mouse macrophages. In contrast, it was demonstrated that p38 MAPK activation is involved in iNOS expression in TNF-α and IL-1-stimulated mouse astrocytes, as well as in LPS-stimulated mouse macrophages (33,34). Jeon *et al.* (47) also showed that the p38 MAPK pathway is specifically involved in LPS-induced iNOS expression, while a specific inhibitor of p44/42 MAPK, PD98059, had no effect on iNOS expression. But Lahti *et al.* (35) reported that PD98059 suppressed endotoxin-induced iNOS expression. In this study we found that incubation of RAW 264.7 cells with LPS (1 μg/ml) caused activation of p38 and p44/42 MAP kinase and co-treatment of carnosol blocked these pathway. The relation between inhibition of p38 and p44/42 MAP kinase by carnosol and suppression of iNOS expression remains for further study.

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