*Free Radical Research*, September 2005; 39(9): 995–1003

Taylor & Francis
Taylor & Francis Group

# Rosmarinic acid inhibits the formation of reactive oxygen and nitrogen species in RAW264.7 macrophages

SHANLOU QIAO, WEIHUA LI, RYOKO TSUBOUCHI, MIYAKO HANEDA, KEIKO MURAKAMI, FUMIO TAKEUCHI, YUKIO NISIMOTO, & MASATAKA YOSHINO

*Department of Biochemistry, Aichi Medical University School of Medicine, Nagakute, Aichi 480-1195, Japan*

Accepted by Professor N. Taniguchi

*(Received 25 May 2005)*



**Abstract**

Antioxidant action of Rosmarinic acid (Ros A), a natural phenolic ingredient in many Lamiaceae herbs such as *Perilla frutescens*, sage, basil and mint, was analyzed in relation to the Iκ-B activation in RAW264.7 macrophages. Ros A inhibited nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) protein synthesis induced by lipopolysaccharide (LPS), and also effectively suppressed phorbol 12-myristate 13-acetate (PMA)-induced superoxide production in RAW264.7 macrophages in a dose-dependent manner. Peroxynitrite-induced formation of 3-nitrotyrosine in bovine serum albumin and RAW264.7 macrophages were also inhibited by Ros A. Moreover, Western blot analysis demonstrated that LPS-induced phosphorylation of Iκ-Bα was abolished by Ros A. Ros A can act as an effective protector against peroxynitrite-mediated damage, and as a potent inhibitor of superoxide and NO synthesis; the inhibition of the formation of reactive oxygen and nitrogen species are partly based on its ability to inhibit the serine phosphorylation of Iκ-Bα.

**Keywords:** *Rosmarinic acid, superoxide, nitric oxide, peroxynitrite, RAW264.7 macrophages, Iκ-Bα*

## Introduction

Free radicals such as superoxide anions, peroxides, hydroxyl radicals and nitrite radicals generated by activated macrophages for defense mechanisms of the host can also act as mediators of inflammation, if produced in an uncontrolled manner [1]. Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NOS) *in vivo*, and excessive NO production by up-regulation of iNOS during chronic infection or inflammation has been implicated in cancer development. Inducible NOS (iNOS or NOS2) is an inducible form of NOS (iNOS) first isolated from murine macrophages, and generates a large quantity of NO, which is partially responsible for the tumoricidal, bactericidal, and possibly immune regulatory activities. Intracellular NO can react with superoxide anions, resulting in the formation of the potent oxidizing and nitrating molecule, peroxynitrite ($ONOO^-$). Reactive nitrogen species (RNS), including peroxynitrite, can possess toxic effects on mitochondria and damage macromolecules, such as DNA, proteins and lipids.

RNS are believed to be involved in carcinogenesis because they influence signal transduction and can damage DNA [2–4]. Therefore, precise regulation of superoxide and NO production under pathophysiological conditions would be critical for the survival of host cells.

It is known that many spices and herbs exhibit various pharmacological virtues including antioxidant and anti-inflammatory effects, which are thought to contribute to their anti-mutagenic and

Correspondence: S. Qiao, Department of Biochemistry, Aichi Medical University School of Medicine, Nagakute, Aichi 480-1195, Japan. Fax: 81 561 614056. E-mail: qiaosl@aichi-med-u.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10715760500231836

anti-carcinogenic properties [5–7]. Rosmarinic acid (Ros A), a natural phenolic substance contained in many Lamiaceae herbs such as *Perilla frutescens*, sage, basil and mint, inhibits complement-dependent inflammatory processes [8,9] and may have therapeutic potential [10]. Ros A in *Perilla* extract inhibits allergic reactions in mice [11]. The medicinal value of Ros A has been well recognized, especially in regard to its antioxidant and anti-inflammatory activities [12,13], although other researchers reported that Ros A induces p56lck-dependent apoptosis in Jurkat and peripheral T cells [14]. A recent study has shown that Ros A inhibits inflammation in animal models of liver injury via downregulation of ROS [15]. However, very little information is known regarding the effects of Ros A on superoxide, NO and its derivatives that are thought to be related to inflammation processes. In this study, we examined the effects of Ros A on superoxide, NO production and iNOS enzyme induction in phorbol 12-myristate 13-acetate (PMA) or lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and their possible inhibition mechanisms were also considered. Ros A was also found to show an effective scavenging effect on the peroxynitrite-induced oxidation and nitration reactions.



## Materials and methods

### *Reagents*

Ros A was from Cayman Chemical (MI, USA). Lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolidum bromide (MTT) were obtained from Sigma (St Louis, MO). Anti-Cu/Zn SOD and anti-Nitric Oxide Synthase II (iNOS) were from Stressgen Biotechnologies Corp (Vic., BC, Canada). Phosphory-Iκ-Bα (Ser32/36) (5A5) monoclonal antibody was bought from cell signaling technology (Heidelberg, Germany). Anti β-actin antibody and peroxidase-labeled anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### *Cell culture*

RAW264.7 macrophages, a murine macrophage cell line were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$. RAW264.7 macrophages were plated at a density of $5 \times 10^5\,ml^{-1}$ and preincubated for 24 h at 37°C and incubated with and without the indicated concentration of Ros A (10, 50 μM) for 12 h, and then incubated with or without LPS or PMA.

### *Measurement of cell viability*

Cell viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)2,5-diphenyltetrazolidum bromide MTT assay. Cells plated in 96-well plates were treated with different concentrations of Ros A and LPS at different concentrations. After 24 h incubation, MTT (1 mg/ml) was added for 60 min, the culture medium was removed, and cells were then dissolved in DMSO and shaken for 10 min. OD values at 550 and 630 nm were measured using a microplate reader. The net absorbance ($OD_{550}-OD_{630}$) indicates the enzymatic activity of mitochondria and provides information on cell viability.

### *Determination of superoxide production by chemiluminescence assay*

Superoxide production by RAW264.7 macrophages was determined as described by de Mendez et al. [16]. The $1 \times 10^5$ cells were pre-incubated with Ros A at different concentrations for 12 h, washed to remove Ros A, and resuspended in 50 μl of HBSS without $Ca^{2+}$, $Mg^{2+}$. After the addition of 50 μl of the enhanced luminol-based substrate (DIOGENES, National Diagnostics), the cells were stimulated with different concentrations of PMA and incubated at 37°C in 96-well plates. The chemiluminesence was assayed using a luminoskan luminometer (Fluoroskan Ascent FL, Labsystems, Nethland). For each data point, a parallel reaction containing 0.1 μg of superoxide dismutase was used as a control.

### *Nitrite assay*

RAW264.7 macrophages were plated at a density of $5 \times 10^5$ cells/ml in 6-well plates for 12 h, followed by treatment with LPS and Ros A at different concentrations for an additional 12 h. The amount of NO production in the medium was detected with the Griess reaction. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The absorbance of the mixture at 570 nm was determined with a plate reader (Dynatech MR-1200; Dynatech Laboratories), and the nitrite concentration was determined using a dilution of sodium nitrite as a standard [17].

### *Tyrosine nitration*

Additions of peroxynitrite at a final concentration of 100 μM were made to BSA (1 mg/ml) with varying concentrations of Ros A in PBS containing 100 μM DTPA, then the reaction solution was incubated for 30 min at 37°C. Tyrosine nitration was measured by Western blotting and densitometric analysis.

The reaction solution (10 μl) was treated by 10% SDS-polyacrylamide minigel electrophoresis, followed by blotting onto a nitrocellulose membrane. The membrane was analyzed by the method described in the subsection Western blot.

For RAW264.7 macrophages ($1 \times 10^6$), additions of peroxynitrite at a final concentration of (50, 200 and 500 μM) were made to RAW264.7 macrophages with Ros A (1, 10 and 100 μM) in Hanks' balanced salt solution (1.26 mM $CaCl_2$, 5.37 mM KCl, 0.44 mM $KH_2PO_4$, 0.81 mM $MgSO_4$, 137 mM NaCl, 4.17 mM $NaHCO_3$, 0.33 mM $Na_2HPO_4$ and 5.55 mM D-(+)-glucose), and incubated for 30 min at 37°C. The nitration of protein tyrosyl residues in RAW264.7 macrophages was measured by Western blotting and densitometric analysis. RAW264.7 macrophages exposed to peroxynitrite were lysed with 2% SDS, and then the lysates containing 25 μg of total protein in SDS-PAGE sample buffer were applied onto a nitrocellulose membrane. The membrane was analyzed by the method described in the subsection Western blot.

### *Western blotting*

Western blotting was taken by a procedure as described previously [18]. In brief, cytosolic extracts were used for immunoblotting. Proteins were electrophoretically separated through 8% polyacrylamide gels containing 0.1% SDS in running buffer (25 mM Tris base, 190 mM L-glycine, 1% SDS). Proteins were electrotransferred to polyvinylidene difluoride membranes in transfer buffer (20 mM Tris base, 150 mM L-glycine, 10% methanol, 0.01% SDS) for 2 h at 180 mA. Membranes were blocked for 1 h at room temperature in PBST containing 1% bovine serum albumin and were incubated overnight at 4°C with anti-iNOS, β-actin, phospho-Iκ-B-α(ser32/36) and Cu/Zn SOD antibodies (1:1000 dilution) in the same buffer. Membranes were then washed with PBST three times for 5 min, incubated for 30–60 min at room temperature with horseradish peroxidase-conjugated anti-mouse polyclonal antibody (1:1000 dilution), and then washed again with PBST twice for 30 min. Signal detection was done with the chemiluminescence ECL detection kit (Amersham, USA).

### *Statistical analysis*

All experiments were performed three times. Data were expressed as mean ± standard error of the mean (SEM) and were determined by one-way analysis of variance (ANOVA) and Student–Newman–Keul's test for individual comparisons. *P* values less than 0.05 were considered as significant.

## Results

### *Ros A shows low toxicity in RAW264.7 macrophages*

Ros A is rapidly eliminated from the blood circulation after intravenous administration ($t_{1/2} = 9$ min) and shows a very low toxicity with an $LD_{50}$ in mice of 561 mg $kg^{-1}$ after intravenous application (Parnham and Kesselring 1985). In order to investigate the potential cytotoxicity of Ros A in RAW264.7 macrophages, cell viability was examined using MTT assay. We incubated RAW264.7 macrophages with different concentrations of Ros A (1–100 mM) for 24 h; the viability of cells at different concentrations was >95% (Figure 1). This result shows Ros A has a low toxicity in RAW264.7 macrophages.

### *Effects of Ros A on superoxide generation of RAW264.7 macrophages induced by PMA*

We used PMA as a stimulant to induce superoxide generation of RAW264 cells. PMA induced significant production of superoxide at concentrations higher than 0.02 μM, and this concentration-response curve reached a plateau at concentrations higher than 2 μM (Figure 2). Interestingly, the peak of the curve stimulated with 2 μM PMA appeared at an early stage, and then 0.02 μM PMA and 0.002 μM PMA induced weak and delayed superoxide generation. The chemiluminesence was also photographed at indicated time points (Figure 2B). In this study, 0.2 μM PMA was used in the stimulation of superoxide production. As shown in Figure 3, at all the concentrations tested (ranging 1, 10 and 50 μM), Ros A did not increase superoxide formation by unstimulated RAW264.7 macrophages. Inhibition of superoxide production in PMA-stimulated cells was observed readily at concentrations of Ros A higher than 10 μM, whereas at concentrations higher than 50 μM we observed an almost complete inhibition of superoxide production induced by 0.2 μM PMA. These results showed Ros A can inhibit the PMA-induced superoxide production in RAW264.7 macrophages in a dose-dependent manner (Figure 3A). The chemiluminesence was also photographed (Figure 3B).

### *Effect of Ros A on LPS-induced NO release and iNOS protein expression*

The effect of Ros A on NO production in LPS-activated RAW264.7 macrophages was investigated (Figure 4). The nitrite levels in the culture medium were measured as an index of NO production using Griess reaction [19]. NO production (24 h) stimulated with LPS (1 μg/ml) was inhibited by treating the macrophages with Ros A (10 and 50 μM) in a dose-dependent manner. The Griess reaction was not affected by Ros A in this concentration range.

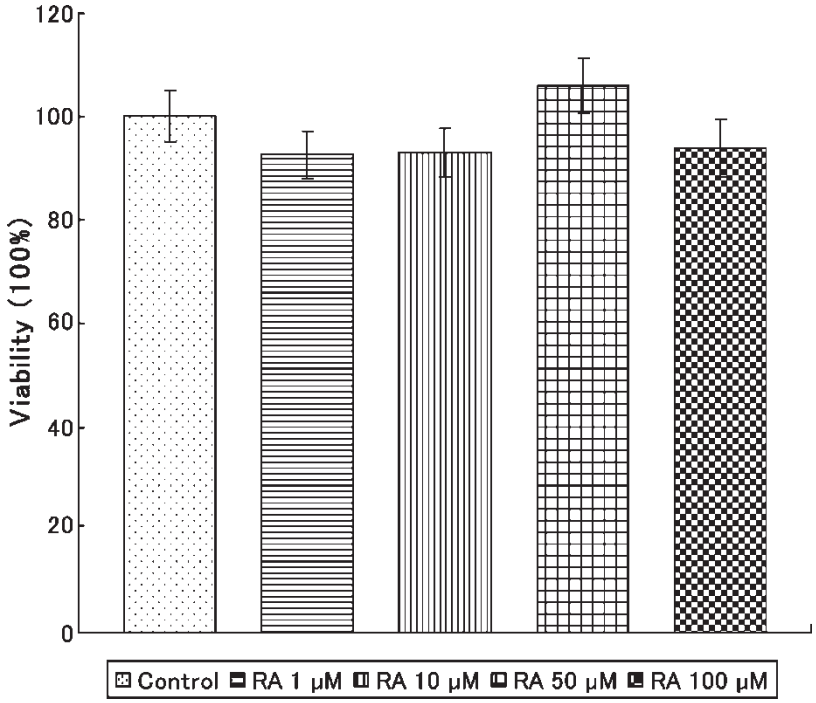


Figure 1. Effect of Ros A on cell viability of RAW264.7 macrophages. RAW264.7 macrophages were incubated with Ros A at indicated concentrations for 24 h. Cell viability was measured as described in "Materials and methods" section. Each value is shown as mean ± S. D. from three independent experiments.

The Western blot analysis of iNOS in RAW264.7 macrophages is shown. LPS-stimulated macrophages were cultured for 24 h in the presence and absence of Ros A (10 and 50 μM). Ros A itself did not influence the iNOS expression. iNOS protein levels in Ros A-treated cells were lower than the LPS-stimulated control, although β-actin levels remained unchanged during incubations of LPS-stimulated RAW264.7 macrophages with different amounts of Ros A and/or LPS (Figure 5). Densitometric analysis showed that iNOS protein levels decreased to 80 and 22% in the cells treated with of 10 and 50 μM Ros A, respectively. However, LPS tended to increase the Cu/Zn SOD proteins in LPS-treated cells, and Ros A had no effect on the LPS-induced Cu/Zn SOD increase (Figure 5).

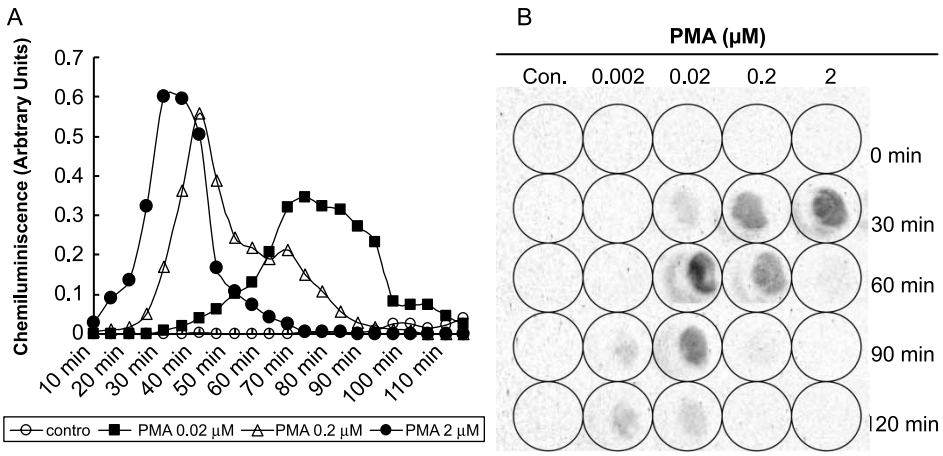


Figure 2. Dose and time-dependent generation of superoxide from RAW264 cells induced by phorbol myristate acetate. Cells ($1 \times 10^4$) were washed to remove PMA, and resuspended in 50 μl of HBSS without $Ca^{2+}$, $Mg^{2+}$. After addition of 50 μl of enhanced luminol-based substrate (DIOGENES; National Diagnostics), cells were stimulated with different concentration of PMA and incubated at 37°C in 96-well plates. Chemiluminesence was assayed using a luminoskan luminometer (Labsystem). Each column and bar represents the mean ± S.E.M. of three experiments. * Significantly different from control value by ANOVA ($p < 0.05$). For each data point, a parallel reaction containing 0.1 μg of superoxide dismutase was used as a control (A). Chemiluminesence was also photographed at indicated time points (B).

*Ros A inhibits peroxynitrite-induced protein tyrosine nitration*

Peroxynitrite is not only a powerful oxidant but also a strong nitrating agent [20]. Protein tyrosine residues are especially susceptible to peroxynitrite-dependent nitration reactions forming 3-nitrotyrosine [21]. In order to test for the protective effect of Ros A against tyrosine nitration mediated by peroxynitrite, we chose bovine serum albumin (BSA) as a model target (Figure 6).Peroxynitrite-induced nitration of tyrosine residues in BSA was examined by Western blotting using anti-3-nitrotyrosine antibody. Exposure of BSA to peroxynitrite resulted in dose-dependent nitrotyrosine immunoreactivity (Figure 6); the intensity of this band decreased with increasing amounts of Ros A (Figure 6), thereby indicating the protection of BSA by Ros A against peroxynitrite-induced tyrosine nitration. Next, as a cellular model for evaluating the protective action of Ros A against peroxynitrite, RAW264.7 macrophages were used. *In vivo*, macrophages are likely to come into contact with

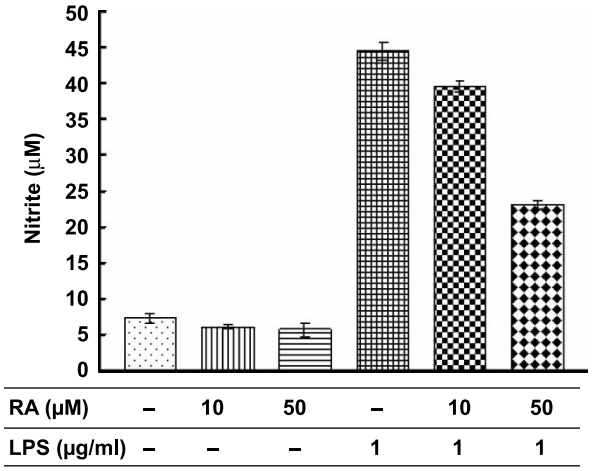


Figure 4. Effect of Ros A on NO production of LPS-activated RAW264.7 macrophages. RAW264.7 macrophages were pre-incubated with Ros A at indicated concentrations, and then stimulated with 1 μg/ml LPS for 24 h. Nitrite concentrations in the culture medium were determined by Griess reaction. Each value is shown as mean ± S.D. from three independent experiments. Asterisks indicate significant difference ($p < 0.05$) by ANOVA, compared with that of LPS-stimulated cells.

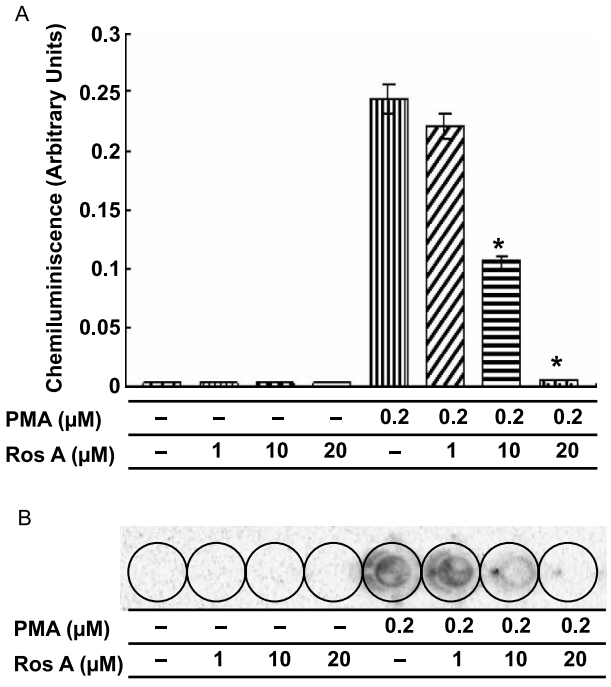


Figure 3. Concentration-dependent inhibitory effects of Ros A on generation of superoxide from RAW264 macrophages induced by PMA (0.02 μM). Cells ($1 \times 10^4$) were pre-incubated with or without Ros A at different concentration for 6 h, washed to remove Ros A, and resuspended in 50 μl of HBSS without $Ca^{2+}$, $Mg^{2+}$. After addition of 50 μl of enhanced luminol-based substrate (DIOGENES; National Diagnostics), cells were stimulated with 0.2 μM PMA and incubated at 37°C for 50 min in 96-well plates. Chemiluminesence was assayed using a luminoskan luminometer (Labsystem). For each data pint, a parallel reaction containing 0.1 μg of superoxide dismutase was used as a control. Results shown represent three independent experiments. * $p < 0.05$, indicates statistical significance to control (A). The chemiluminesence was also photographed after treatment with PMA (B).

peroxynitrite formed in a rapid reaction of nitric oxide and superoxide as generated by themselves [22]. Nitration of protein tyrosyl residues in RAW264.7 macrophages mediated by peroxynitrite was detected by Western blotting in a dose-dependent manner (Figure 7A). Addition of Ros A decreased the extent of peroxynitrite-induced protein damage in a dose-dependent manner (Figure 7A).

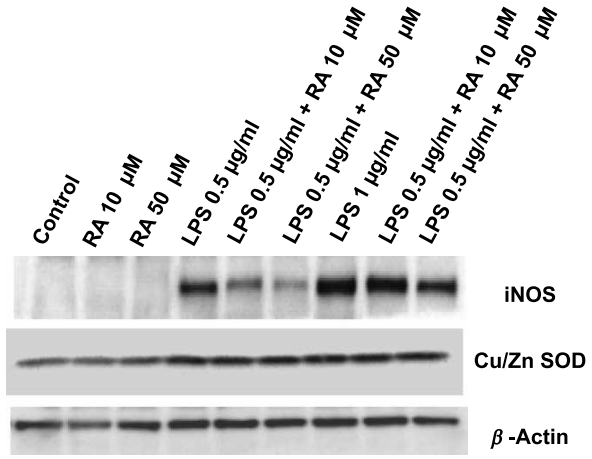


Figure 5. Western blot analysis of iNOS and Cu/Zn SOD proteins in LPS-stimulated RAW264.7 macrophages. Cells were pre-incubated with Ros A at indicated concentration, and then stimulated with 1 μg/ml LPS for 24 h. Whole-cell lysate was subjected to SDS-PAGE and immunoreactivities of iNOS were measured by immunoblotting with a rabbit polyclonal anti-iNOS antibody. To confirm that equivalent proteins were loaded, the same membrane was probed with an anti-β-actin antibody. Two additional experiments yielded similar results.

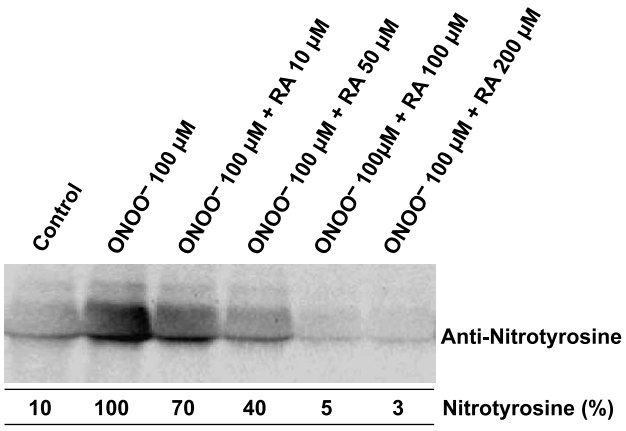


Figure 6. Protective effect of Ros A on tyrosine nitration of bovine serum albumin (BSA) protein. BSA (1 mg/ml) in PBS was incubated with 100 μM peroxynitrite for 10 min at 37°C with or without increasing concentration of Ros A, then 5 μl of each sample was separated on a 10 ~ 20% SDS/PAGE gel and transferred onto a PVDF membrane. Nitrotyrosine-containing proteins were detected by using a mouse monoclonal anti-nitrotyrosine antibody as detailed in "Materials and Methods" section. The level of nitrotyrosinated protein is expressed as a percentage of control value.

*Ros A inhibits LPS-induced Ik-B alpha phosphorylation*

The iNOS gene is known to be regulated by transcription factors such as nuclear transcription factor kB (NF-kB). Because phosphorylation of Ik-B at ser32 and ser36 is essential for release of active NF-kB, in order to investigate whether the inhibitory action of Ros A on iNOS expression can be induced by regulating the phosphorylation level of Iκ-B at ser32 and ser36, we used a specific phospho-Iκ-B-alpha monoclonal antibody, and the WB results demonstrated that Ros A inhibited LPS-mediated phosphorylation of Iκ-B effectively (Figure 8), indicating that Ros A inhibits the production of iNOS probably through the down-regulation of phosphorylation of Ik-B at ser32 and ser36.

## Discussion

NO is generated by a family of NOS isozymes that convert L-arginine to L-citruline and NO. Certain cytokines, microbes, or microbial products trigger the expression of an iNOS, which results in a high output of NO production from macrophages. By reacting with DNA, proteins and lipids, NO impairs normal cellular functions and thus exerts its cytotoxic effects [23,24]. Because the cytotoxic effect of NO is nonspecific, the consequences of overproduction of NO can be detrimental to the host instead of its pivotal role in the normal function of the host defense system. Therefore, a precise regulation of NO production under pathophysiological conditions would be critical for the survival of host cells. In this aspect, multiple ways to decrease NO production, including inhibition of iNOS enzyme activity [24], depletion of arginine substrate by arginase [25,26] and transcriptional down-regulation of iNOS gene expression by endogenous or exogenous manipulations, have been documented, with great interest, as strategies for

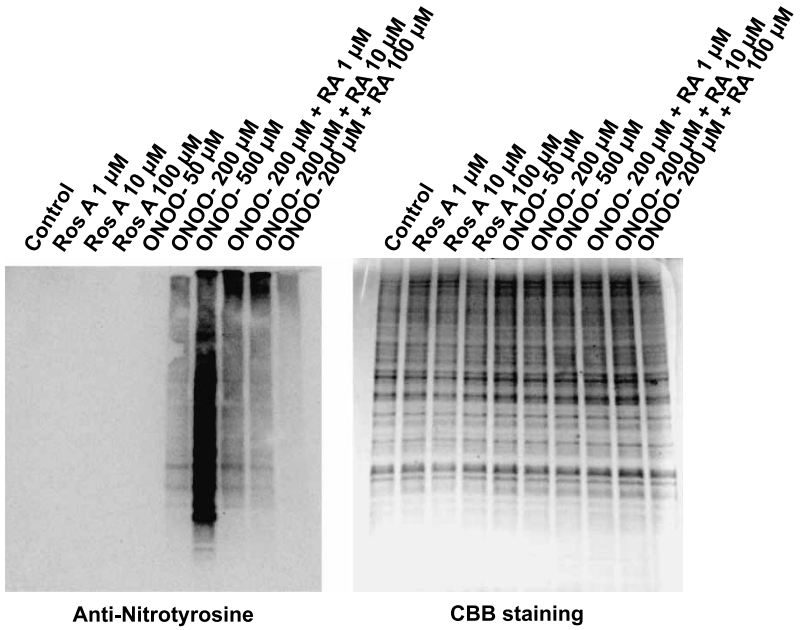


Figure 7. Protective effect of Ros A on nitration of protein tyrosyl residues in RAW264.7 macrophages. RAW264.7 macrophages were pre-incubated with Ros A at indicated concentration in PBS, and then incubated for 30 min at 37°C in the presence of peroxynitrite. Cell lysate (20 μg) was separated on a 10–20% SDS/PAGE gel and transferred onto a PVDF membrane. Nitrotyrosine-containing proteins were detected by using a mouse monoclonal anti-nitrotyrosine antibody as detailed in "Materials and Methods" section. The same gel was stained and showed equal amounts of proteins were loaded.

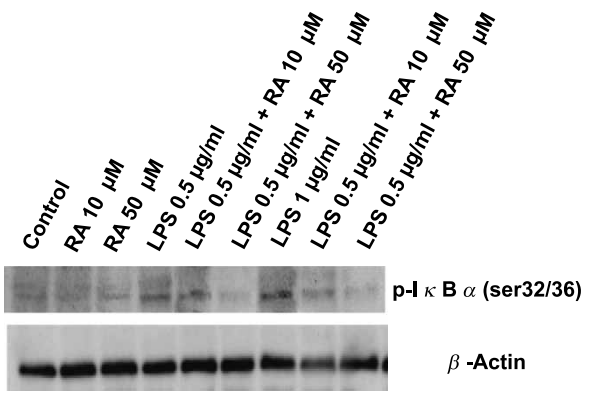


Figure 8. Western blot analysis of phosphorylated Iκ-Bα protein in LPS-stimulated RAW264.7 macrophages. Cells were pre-incubated with Ros A at indicated concentration, and then stimulated with 1 μg/ml LPS for 24 h. Whole cell lysate was subjected to SDS-PAGE and immunoreactivities of iNOS were measured by immunoblotting with a specific antibody, phosphorylated Iκ-Bα (ser32/36). To confirm that the equivalent proteins were loaded, the same membrane was probed with an anti-β-actin antibody. Two additional experiments yielded similar results.

developing anti-inflammatory agents. In this point, Ros A inhibits complement-dependent inflammatory processes [8,9] and may have therapeutic potential [10]. However, very little information is known regarding the effects of Ros A on superoxide, NO and their derivatives, reactive nitrogen species (RNS) that are thought to be related to antioxidant and anti-inflammatory effects. This study investigated for the first time the effects of Ros A on the responses of RAW264.7 macrophages, namely on the production of reactive oxygen species (ROS) and RNS. The results suggest that Ros A is a potent inhibitor of superoxide and NO syntheses, and also an effective protector against peroxynitrite-mediated damage.

Curcumin interacted directly with superoxide anions and hydroxyl radicals by electron paramagnetic resonance, quenching the interaction of the radicals with the spin trap, Tempone-H [27]. Because Ros A shares common structural features with curcumin, we can speculate that Ros A acts as a similar mechanism on the inhibition of PMA-induced superoxide production in this study. However, in activated macrophages and neutrophils, ROS are generated by NADPH oxidase in a process called the respiratory burst. This key enzyme catalyzes the generation of superoxide and hydrogen peroxide using electrons provided by the hexose monophosphate shunt [28,29]. The enzyme appears to play an important role in the regulation of internal redox equilibrium in response to external stimuli [1,30]. Therefore, we cannot exclude the possibility that the reduction of superoxide by Ros A may also be indirectly or directly attributed to inhibition of NADPH oxidase. However, this remains to be determined in the future studies.

The inhibitory mechanism of Ros A on iNOS induction in activated macrophages remains unclear. Ros A shares common structural features found in chemopreventive diarylheptanoid compounds including curcumin, a widely-used spice and coloring agent in food extracted from the rhizome of turmeric (*Curcuma longa* Linn, ginger family) [31]. Curcumin also inhibits NO production and induction of iNOS in RAW 264.7 macrophages activated with LPS and interferon-γ. The promoter of the gene encoding iNOS contains consensus sequences for binding of several transcription factors including NF-κB. NF-κB, an inducible transcription factor, exists in a latent form in the cytoplasm of unstimulated cells, comprising a transcriptionally active dimer bound to an inhibitor protein, Iκ-B. NF-κB is activated in response to various extracellular stimuli, including cytokines, LPS [32], and oxidative stress [33], translocated to the nucleus, and regulates the expression of many target genes involved in immune and inflammatory responses. It is well known that phosphorylation of Iκ-Bα (i.e. degradation of Iκ-Bα) is required for NF-κB activation. The effective suppression of LPS-induced Iκ-B activation by Ros A infers that the inhibitory effect of Ros A on the iNOS induction is mediated through the inhibition of iNOS transcription by suppressing Iκ-B activation. Bode et al. reported that Ros A inhibits epidermal growth factor (EGF)-induced AP-1 DNA binding activity in mouse epidermal JB6 cells in a dose-dependent manner [7]. From these facts, we speculate that the inhibition of Iκ-B activation may be involved in the suppression of iNOS induction in LPS-stimulated RAW264.7 macrophages by Ros A.

Both Ros A and curcumin have exhibited antimicrobial, antiviral, antioxidantive, anti-inflammatory and anti-carcinogenic activities. There are, however, some differences between these two agents. Curcumin generally induces apoptosis in a wide variety of tumor cells, including colon carcinoma, breast carcinoma and B and T cell leukemia [34–37], and mainly involves the mitochondria-mediated pathway. Interestingly, Ros A induces T and NK cell apoptosis through the mitochondrial pathway in an Lck-dependent manner, but not in Lck-deficient cells, including B cells, monocytes, Chang liver cells, COS cells, and Lewis lung carcinoma cells, even up to 500 μM Ros A [14]. Curcumin did not induce Lck-dependent apoptosis. These reports show these two agents induce apoptosis in a different manner which still requires further study.

Peroxynitrite, a highly reactive molecule generated from the reaction between NO and superoxide anion, produces altered DNA bases such as 8-hydroxyguanine and 8-nitroguanine which can cause mutations if unrepaired, and can cause DNA single strand breaks. Peroxynitrite also inhibits classic DNA repair enzymes [3]. In addition, 3-nitrotyrosine, formed from the nitration reaction of peroxynitrite to proteins containing tyrosine residues, has been

detected in various diseases (e.g. atherosclerotic lesions of human coronary arteries) using specific antibodies recognizing 3-nitrotyrosine in proteins. Therefore, peroxynitrite is thought to be involved in their pathogenesis [38]. We observed that Ros A effectively prevents peroxynitrite-induced oxidation reaction of nitration of protein tyrosyl residues in BSA and RAW264.7 macrophages. These observations show that Ros A protects against oxidative damage induced by peroxynitrite. Ros A treatment would have decreased peroxynitrite formation by inhibiting production of superoxide and iNOS and hence reduced superoxide and nitric oxide production in addition to the direct peroxynitrite scavenging. In this study, we also showed the LPS-induced increase in Cu/Zn SOD protein, and these findings are consistent with the report that LPS stimulation of macrophages increases expression of Cu/Zn SOD, Mn SOD and catalase up to 2-fold [39]. Most importantly, Ros A inhibits the production of superoxide and NO, but shows no action on the LPS-induced Cu/Zn SOD increases, which suggests Ros A has a favorable role in protecting RAW264.7 macrophages from the inflammatory response-induced ROS damage.

In summary, Ros A inhibited NO production and iNOS protein syntheses induced by LPS, and also suppressed PMA-induced superoxide production in RAW264.7 macrophages in a dose-dependent manner, suggesting that Ros A is a potent inhibitor of reactive oxygen and nitrogen species, and their inhibition mechanisms are partly based on the ability to inhibit the serine phosphorylation of Iκ-Bα, in addition to the direct peroxynitrite scavenging. Moreover, because of its low toxicity, Ros A could potentially lead to attractive anti-inflammatory drugs.



## Acknowledgements

We thank Mr Makoto Naruse and Mr Minoru Fukayama for their skillful technical assistance.

## References

[1] Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 2004;4:181–189.
[2] Moncade F, Fortier A, Guyon P, Darrieus H, Pitre J, Pailler JL. Peritoneal puncture dialysis in the monitoring and treatment of hemoperitoneum of traumatic origin? J Chir (Paris) 1991;128:285–289.
[3] Payne CM, Bernstein C, Bernstein H, Gerner EW, Garewal H. Reactive nitrogen species in colon carcinogenesis. Antioxid Redox Signal 1999;1:449–467.
[4] Yermilov V, Rubio J, Becchi M, Friesen MD, Pignatelli B, Ohshima H. Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite *in vitro*. Carcinogenesis 1995;16:2045–2050.
[5] Qiao S, Li W, Tsubouchi R, Haneda M, Murakami K, Yoshino M. Involvement of peroxynitrite in capsaicin-induced apoptosis of C6 glioma cells. Neurosci Res 2005;51:175–183.
[6] Yoshino M, Haneda M, Naruse M, Htay HH, Tsubouchi R, Qiao SL, Li WH, Murakami K, Yokochi T. Prooxidant activity of curcumin: Copper-dependent formation of 8-hydroxy-2′-deoxyguanosine in DNA and induction of apoptotic cell death. Toxicol in Vitro 2004;18:783–789.
[7] Bode AM, Ma WY, Surh YJ, Dong Z. Inhibition of epidermal growth factor-induced cell transformation and activator protein 1 activation by [6]-gingerol. Cancer Res 2001;61:850–853.
[8] Sahu A, Rawal N, Pangburn MK. Inhibition of complement by covalent attachment of rosmarinic acid to activated C3b. Biochem Pharmacol 1999;57:1439–1446.
[9] Zupko I, Hohmann J, Redei D, Falkay G, Janicsak G, Mathe I. Antioxidant activity of leaves of Salvia species in enzyme-dependent and enzyme-independent systems of lipid peroxidation and their phenolic constituents. Planta Med 2001;67:366–368.
[10] al-Sereiti MR, Abu-Amer KM, Sen P. Pharmacology of rosemary (*Rosmarinus officinalis* Linn.) and its therapeutic potentials. Indian J Exp Biol 1999;37:124–130.
[11] Makino T, Furuta A, Fujii H, Nakagawa T, Wakushima H, Saito K, Kano Y. Effect of oral treatment of *Perilla frutescens* and its constituents on type-I allergy in mice. Biol Pharm Bull 2001;24:1206–1209.
[12] Ito H, Miyazaki T, Ono M, Sakurai H. Antiallergic activities of rabdosiin and its related compounds: Chemical and biochemical evaluations. Bioorg Med Chem 1998;6:1051–1056.
[13] Nakamura Y, Murakami A, Ohto Y, Torikai K, Tanaka T, Ohigashi H. Suppression of tumor promoter-induced oxidative stress and inflammatory responses in mouse skin by a superoxide generation inhibitor 1′-acetoxychavicol acetate. Cancer Res 1998;58:4832–4839.
[14] Hur YG, Yun Y, Won J. Rosmarinic acid induces p56lck-dependent apoptosis in Jurkat and peripheral T cells via mitochondrial pathway independent from Fas/Fas ligand interaction. J Immunol 2004;172:79–87.
[15] Osakabe N, Yasuda A, Natsume M, Sanbongi C, Kato Y, Osawa T, Yoshikawa T. Rosmarinic acid, a major polyphenolic component of *Perilla frutescens*, reduces lipopolysaccharide (LPS)-induced liver injury in D-galactosamine (D-GalN)-sensitized mice. Free Radic Biol Med 2002;33:798–806.
[16] de Mendez I, Adams AG, Sokolic RA, Malech HL, Leto TL. Multiple SH3 domain interactions regulate NADPH oxidase assembly in whole cells. EMBO J 1996;15:1211–1220.
[17] Chen YC, Shen SC, Lee WR, Hou WC, Yang LL, Lee TJ. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. J Cell Biochem 2001;82:537–548.
[18] Qiao S, Iwashita T, Furukawa T, Yamamoto M, Sobue G, Takahashi M. Differential effects of leukocyte common antigen-related protein on biochemical and biological activities of RET-MEN2A and RET-MEN2B mutant proteins. J Biol Chem 2001;276:9460–9467.
[19] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131–138.
[20] Radi R, Peluffo G, Alvarez MN, Naviliat M, Cayota A. Unraveling peroxynitrite formation in biological systems. Free Radic Biol Med 2001;30:463–488.
[21] Pannala AS, Razaq R, Halliwell B, Singh S, Rice-Evans CA. Inhibition of peroxynitrite dependent tyrosine nitration by hydroxycinnamates: Nitration or electron donation? Free Radic Biol Med 1998;24:594–606.
[22] Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem 1991;266:4244–4250.
[23] Nathan C. Inducible nitric oxide synthase: What difference does it make? J Clin Investig 1997;100:2417–2423.

[24] Albina JE, Reichner JS. Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. Cancer Metastasis Rev 1998;17:39–53.
[25] Boucher JL, Moali C, Tenu JP. Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization. Cell Mol Life Sci 1999;55:1015–1028.
[26] Gotoh T, Mori M. Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. J Cell Biol 1999;144:427–434.
[27] Biswas SK, McClure D, Jimenez LA, Megson IL, Rahman I. Curcumin induces glutathione biosynthesis and inhibits NF-kappaB activation and interleukin-8 release in alveolar epithelial cells: Mechanism of free radical scavenging activity. Antioxid Redox Signal 2005;7:32–41.
[28] Bastian NR, Hibbs JB, Jr. Assembly and regulation of NADPH oxidase and nitric oxide synthase. Curr Opin Immunol 1994;6:131–139.
[29] Jones RD, Hancock JT, Morice AH. NADPH oxidase: A universal oxygen sensor? Free Radic Biol Med 2000;29:416–424.
[30] Morre DJ, Brightman AO. NADH oxidase of plasma membranes. J Bioenerg Biomembr 1991;23:469–489.
[31] Motterlini R, Foresti R, Bassi R, Green CJ. Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. Free Radic Biol Med 2000;28:1303–1312.
[32] Schreck R, Meier B, Mannel DN, Droge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. J Exp Med 1992;175:1181–1194.
[33] Bonizzi G, Piette J, Schoonbroodt S, Greimers R, Havard L, Merville MP, Bours V. Reactive oxygen intermediate-dependent NF-kappaB activation by interleukin-1beta requires 5-lipoxygenase or NADPH oxidase activity. Mol Cell Biol 1999;19:1950–1960.
[34] Chen H, Zhang ZS, Zhang YL, Zhou DY. Curcumin inhibits cell proliferation by interfering with the cell cycle and inducing apoptosis in colon carcinoma cells. Anticancer Res 1999;19:3675–3680.
[35] Mehta K, Pantazis P, McQueen T, Aggarwal BB. Antiproliferative effect of curcumin (diferuloylmethane) against human breast tumor cell lines. Anticancer Drugs 1997;8:470–481.
[36] Han SS, Chung ST, Robertson DA, Ranjan D, Bondada S. Curcumin causes the growth arrest and apoptosis of B cell lymphoma by downregulation of egr-1, c-myc, bcl-XL, NF-kappa B, and p53. Clin Immunol 1999;93:152–161.
[37] Piwocka K, Zablocki K, Wieckowski MR, Skierski J, Feiga I, Szopa J, Drela N, Wojtczak L, Sikora E. A novel apoptosis-like pathway, independent of mitochondria and caspases, induced by curcumin in human lymphoblastoid T (Jurkat) cells. Exp Cell Res 1999;249:299–307.
[38] Tsuda T, Kato Y, Osawa T. Mechanism for the peroxynitrite scavenging activity by anthocyanins. FEBS Lett 2000;484:207–210.
[39] Ferret PJ, Soum E, Negre O, Fradelizi D. Auto-protective redox buffering systems in stimulated macrophages. BMC Immunol 2002;3:3.