

## Comparison of Suppressive Effects of Demethoxycurcumin and Bisdemethoxycurcumin on Expressions of Inflammatory Mediators *In Vitro* and *In Vivo*

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(Received December 7, 2007)

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Demethoxycurcumin and bisdemethoxycurcumin are the main active ingredients isolated from *Curcumae Longae Radix*. Recent studies demonstrated that both compounds exhibit antioxidative and anti-inflammatory effects as well as effects on cancer cell lines. In this study, we compared the activities of demethoxycurcumin and bisdemethoxycurcumin, and both compounds were evaluated on lipopolysaccharide (LPS)-induced nitric oxide (NO) production, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and nuclear factor-kappaB (NF- $\kappa$ B) activity in a RAW 264.7 macrophage cell line. The evaluation results suggested that the anti-inflammatory properties of demethoxycurcumin and bisdemethoxycurcumin were attributed to the inhibition of iNOS and COX-2 expression, as initiated by the inhibition of NF- $\kappa$ B activity. Additionally, both of them significantly inhibited carrageenan-induced paw edema in mice. Taken together, all of the results showed that the suppressive effect of demethoxycurcumin was stronger than that of bisdemethoxycurcumin, indicating that the methoxy group had enhanced demethoxycurcumin's anti-inflammation effects.

**Key words:** Demethoxycurcumin, Bisdemethoxycurcumin, LPS, NF- $\kappa$ B, Macrophage, Paw edema

**Abbreviations:** NO, nitric oxide; PGs, prostaglandins; LPS, lipopolysaccharide; NF- $\kappa$ B, nuclear factor-kappaB; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SEAP, secretory alkaline phosphates; 4-MUP, 4-methylumbelliferyl phosphate; TPCK, N-p-tosyl-L-phenylalanyl-chloromethyl ketone; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; I $\kappa$ B, inhibitor kappaB; RT-PCR, reverse transcriptase-polymerase chain reaction; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-6 $\beta$ , interleukin-6 $\beta$ ; NC membrane, Nitrocellulose membrane; CCK-8, Cell Counting Kit-8

### INTRODUCTION

Demethoxycurcumin and bisdemethoxycurcumin are the major components of *Curcuma longa* L., which is used in the food industry, for cooking, and in folkloric remedies. In recent biological studies, demethoxycurcumin and bisdemethoxycurcumin have been found to be cytotoxic as well as antioxidant and anti-inflammatory effects on cancer cell

lines (Ramsewak *et al.*, 2000; Ruby *et al.*, 1995; Anto *et al.*, 1996).

Inflammation is associated with a large number of mediators, such as iNOS and COX-2 (Nathan, 1992), which are regulated by NF- $\kappa$ B transcription factor (O'Neill, 2006). NF- $\kappa$ B plays a crucial role in inflammatory and immune responses (Chen *et al.*, 1999), which can be activated by LPS, IL-1 $\beta$ , and a variety of stimuli. After being stimulated, NF- $\kappa$ B increases the expressions of genes, many cytokines, enzymes and adhesion molecules in chronic inflammatory diseases. For example, cytokines are elevated in sepsis, trauma, neoplasia and burns as part of a systemic inflammatory response (Kushner, 1982). All of these products

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play a vital role in the pathogenesis of inflammatory diseases (Zhao *et al.*, 2005).

Prevention of carrageenan-induced paw edema in small animals is a means of evaluating anti-inflammatory activity. Edema develops in two phases. The first phase of edema is mediated through the release of histamine, 5-hydroxytryptamine and bradykinin, whereas the second phase has been attributed to the expression of COX-2 and the release of prostaglandin (Seibert *et al.*, 1994; Salvemini *et al.*, 1996).

To compare the anti-inflammatory activities of demethoxycurcumin and bisdemethoxycurcumin, we evaluated them on LPS-induced NO production, iNOS, COX-2, and NF- $\kappa$ B activity in a RAW 264.7 macrophage cell line, and tested their inhibitory effects on paw edema in mice.

## MATERIALS AND METHODS

### Materials

Demethoxycurcumin and bisdemethoxycurcumin (purity >96.0%) were isolated according to the procedure outlined in a previous report (Zhao and Yang, 1986). The chemical structures are shown in Fig. 1. The RAW 264.7 cells, murine macrophages, were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (D-PBS), *N*-*p*-tosyl-L-phenylalanyl-chloromethyl ketone (TPCK), lipopolysaccharide (*E. coli*, serotype 0127:B8; LPS), L-carrageenan, acetic acid, 4-methylumbelliferyl phosphate (4-MUP), and dimethyl sulfoxide were acquired from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Geneticin (antibiotic G-418) was purchased from Gibco BRL (Grand Island, NY, U.S.A.). A Cell-Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Tokyo, Japan). Other chemicals and solvents were acquired from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All of the samples, solutions and buffers were prepared from deionized water. Primary antibodies for COX-2, iNOS, I $\kappa$ B- $\alpha$ , pI $\kappa$ B- $\alpha$ , and

secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Nitrocellulose membrane (NC membrane) was obtained from Whatman GmbH (Germany). An enhanced chemiluminescence (ECL) detection kit was purchased from LabFrontier (Seoul, Korea). ICR mice (male, 4 weeks of age) were supplied by Samtaco (Osan, Korea). The animals were fed a standard laboratory diet and water *ad libitum* for one week (12 h light/dark cycle; temperature, 22 $\pm$ 2°C). The study as conducted was approved by the Animal Ethics Committee of Seoul National University, the guidelines of which are in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

### Cell culture and cell viability assay

The macrophage RAW264.7 cell line was incubated in Dulbecco's modified Eagle's medium (DMEM) at 37°C under 5% CO<sub>2</sub> humidified air. The cells were seeded into 96-well plates at the density of 1 $\times$ 10<sup>4</sup> cells/well and allowed to adhere for 24 h, also at 37°C under 5% CO<sub>2</sub>. After 24 h treatment with demethoxycurcumin and bisdemethoxycurcumin, 10  $\mu$ L of the CCK-8 solution was added to each well, followed by incubation for 2 h at 37°C. The resulting color was assayed at 450 nm using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

### Nitrite assay

The cells (1 $\times$ 10<sup>5</sup> cells/well) were pretreated with demethoxycurcumin (6.25, 12.5, and 25  $\mu$ M) and bisdemethoxycurcumin (12.5, 25, and 50  $\mu$ M) for 2 h, and then incubated for 24 h with LPS (1  $\mu$ g/mL). After incubation, the nitrite concentrations of supernatants (100  $\mu$ L/well) were measured by adding 100  $\mu$ L of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The optical density at 540 nm was measured using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The nitrite concentration was calculated by comparison with the absorbance at 540 nm of standard solutions of sodium nitrite prepared in culture medium.

### Western blot analysis

The cells (1 $\times$ 10<sup>6</sup> cells/well) were pretreated with demethoxycurcumin (6.25, 12.5, and 25  $\mu$ M) and bisdemethoxycurcumin (12.5, 25, and 50  $\mu$ M) for 2 h and then incubated with LPS (1  $\mu$ g/mL) for 5 min (pI $\kappa$ B- $\alpha$ ), 20 min (I $\kappa$ B- $\alpha$ ) and 18 h (iNOS, COX-2). After incubation, the total cytoplasmic extracts were lysed as previously described (Zhou *et al.*, 2007). Then, an SDS-PAGE was performed, and proteins were transferred onto NC membranes. After being blocked by 5% skim milk, the membrane was incubated with primary and secondary antibodies in turn.

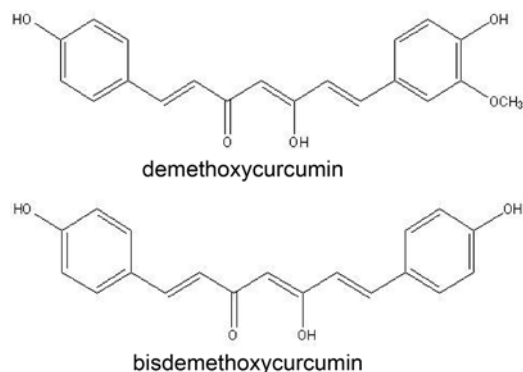


Fig. 1. Chemical structures of demethoxycurcumin and bisdemethoxycurcumin

Finally, the blot was developed for visualization using the ECL Plus detection kit.

### Reverse transcriptase polymerase chain reaction (RT-PCR)

RAW 264.7 cells were pretreated with demethoxycurcumin (6.25, 12.5, and 25  $\mu$ M) and bisdemethoxycurcumin (12.5, 25, and 50  $\mu$ M) for 2 h, and then incubated with LPS (1  $\mu$ g/mL) for 4 h. Briefly, the total RNA was extracted using the Easy-BLUE™ Total RNA Extraction Kit (Intron Biotechnology, Korea). The RT-PCR was performed using the ONE-STEP RT-PCR PreMix kit™ (Intron Biotechnology, Korea). The primers (iNOS, COX-2, and b-actin) were the same as previously described (Zhou *et al.*, 2007). The products of the RT-PCR were separated by electrophoresis using 1.5% agarose gel stained with ethidium bromide, and the gels were viewed under UV transillumination.

### Reporter gene assay

The reporter enzyme activity was measured using a cell-based assay system for monitoring NF- $\kappa$ B activity. Transfected RAW 264.7 cells ( $1.5 \times 10^5$  cells/well) were pretreated with the compounds for 2 h, and then treated with LPS (1  $\mu$ g/mL) alone or with LPS plus each indicated compound for 18 h. The fluorescence from the product of the SEAP/MUP (secretory alkaline phosphates/methylumbelliferyl phosphate) reaction, according to the excitation at 360 nm and the emission at 449 nm, was measured using a 96-well plate Gemini XS fluorometer (Molecular Devices, Sunnyvale, CA, U.S.A.) (Ahn *et al.*, 2003; Moon *et al.*, 2001a; Moon *et al.*, 2001b).

### Carrageenan-induced paw edema in mice

Sixty mice were randomly divided into 6 groups. Thirty minutes after demethoxycurcumin (40 and 80 mg/kg) and bisdemethoxycurcumin (40 and 80 mg/kg) were administered, the mice were treated with 0.05 mL of l-carrageenan (1%) by intraperitoneal injection into the right hind paw. The control group received the vehicle (olive oil), and the positive control group received dexamethasone (50 mg/kg). After the  $\lambda$ -carrageenan injection, the paw volumes were measured at 1, 2, 3, and 4 h by dial thickness gauge (Mitutoyo, Japan) (Henriques *et al.*, 1987).

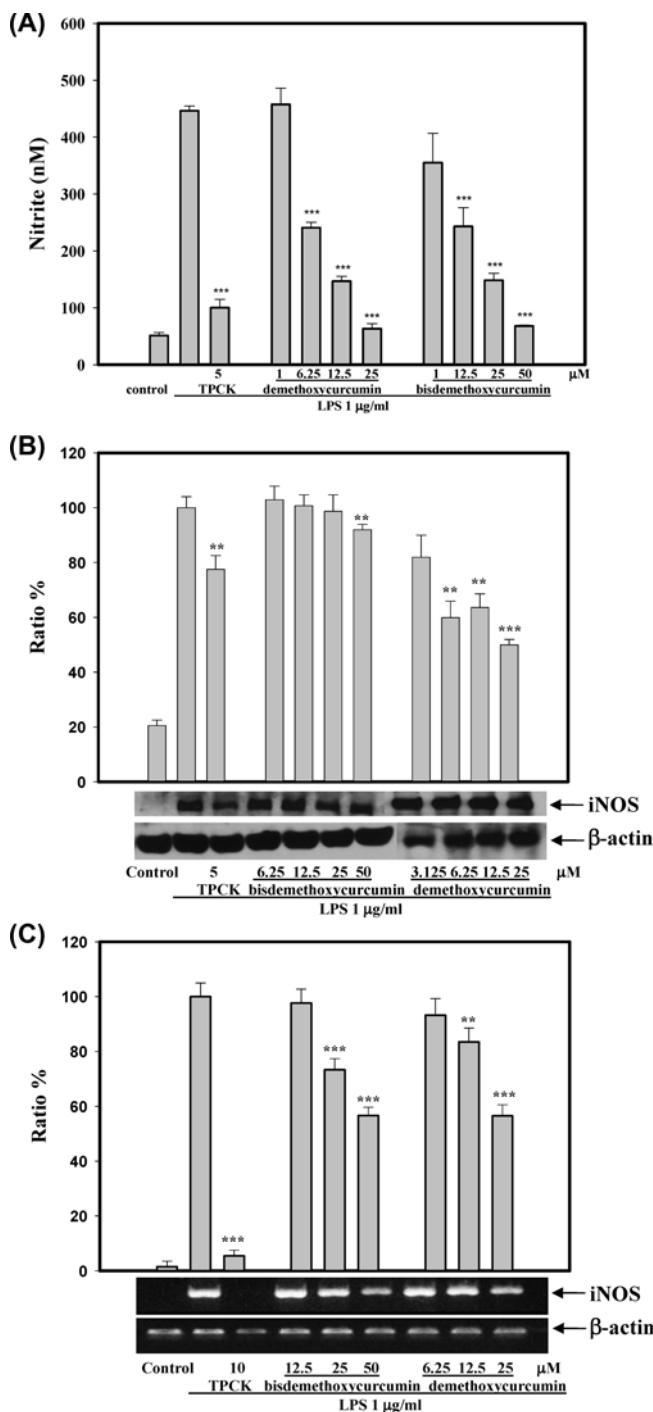
### Statistical analysis

The results were expressed as means  $\pm$  S.E.M. Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA) followed by a post-hoc Dunnett's test for multiple comparisons. Statistical significance was assessed as  $p < 0.05$  [ $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ ].

## RESULTS

### Inhibition of NO production, iNOS protein and mRNA expression in LPS-stimulated RAW 264.7 macrophages

Pretreatment of RAW 264.7 cells with demethoxycur-



**Fig. 2.** Effects of demethoxycurcumin and bisdemethoxycurcumin on LPS-induced NO production (A), iNOS protein (B) and mRNA expression (C) in macrophages

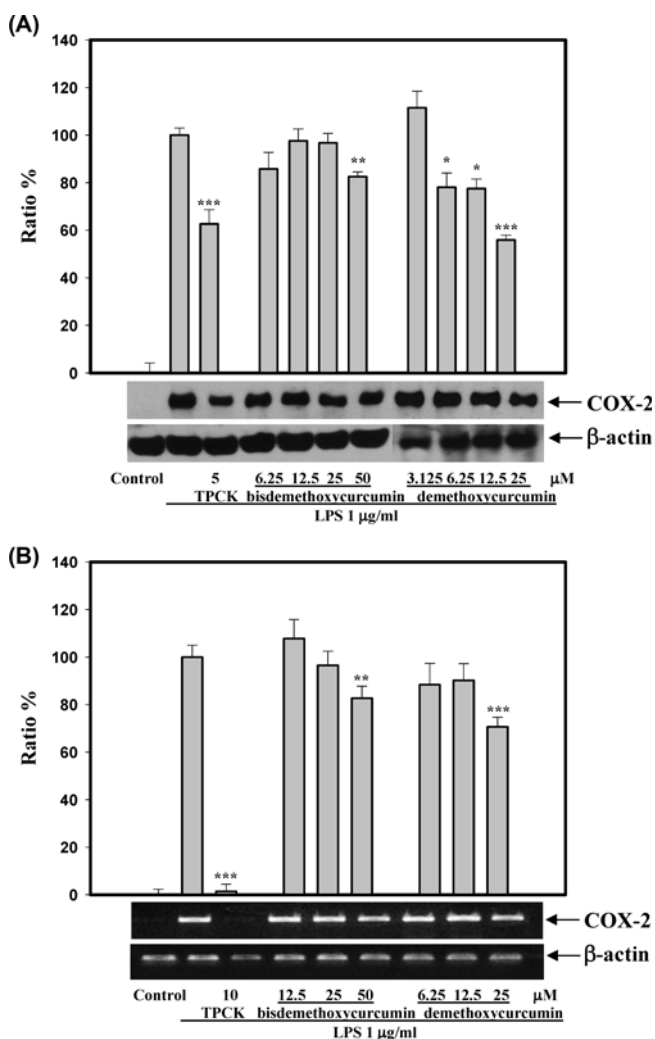
curcumin and bisdemethoxycurcumin inhibited LPS-stimulated NO production in a dose-dependent manner (Fig. 2A). This inhibitory effect was achieved with safe concentrations of the two compounds (Table I). The iNOS protein (Fig. 2B) and mRNA (Fig. 2C) expressions in the macrophages were markedly decreased by both compounds. As shown in Table I, the IC<sub>50</sub> values of demethoxycurcumin and bisdemethoxycurcumin inhibiting LPS-induced

NO production are 6.9 μM and 8.4 μM, respectively. Furthermore, the IC<sub>50</sub> values of demethoxycurcumin and bisdemethoxycurcumin inhibiting LPS-induced iNOS mRNA expression are 35.7 μM and 60 μM, respectively. Of the two compounds, then, the more active is demethoxycurcumin.

**Table I.** IC<sub>50</sub> values of curcuminoids

	NO	NF-κB	mRNA	
			iNOS	COX-2
demethoxycurcumin	6.9	10.2	35.7	52
bisdemethoxycurcumin	8.4	29.9	60	>100

Values are concentrations of curcuminoids required to lead to 50% inhibition of LPS-induced mediator production. Values are in μM.



**Fig. 3.** Effects of demethoxycurcumin and bisdemethoxycurcumin on LPS-induced COX-2 protein (A) and mRNA expression (B) in macrophages

**Inhibition of COX-2 protein and mRNA expressions in LPS-stimulated RAW 264.7 macrophages**

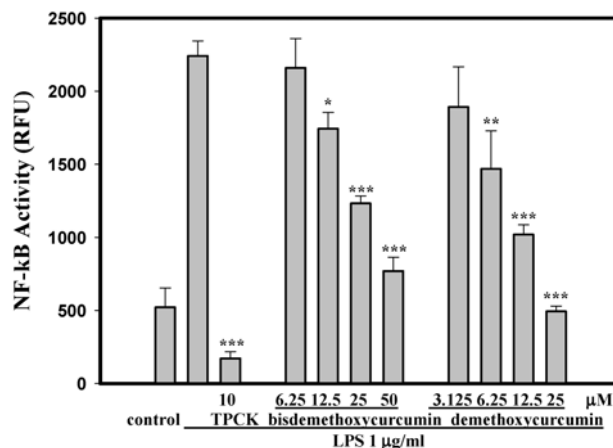
We studied the effects of demethoxycurcumin and bisdemethoxycurcumin on the expressions of COX-2 protein and mRNA. Our results suggest that the two compounds significantly inhibit COX-2 protein expression (Fig. 3A). Furthermore, they also inhibited LPS-induced COX-2 mRNA expression (Fig. 3B). The IC<sub>50</sub> value of demethoxycurcumin inhibiting LPS-induced COX-2 mRNA expression is 52 μM. However, the IC<sub>50</sub> value of bisdemethoxycurcumin is over 100 μM. It is evident that the effect of demethoxycurcumin is stronger than that of bisdemethoxycurcumin (Table I).

**Inhibition of activation of NF-κB in LPS-stimulated Transfected-RAW 264.7 macrophages**

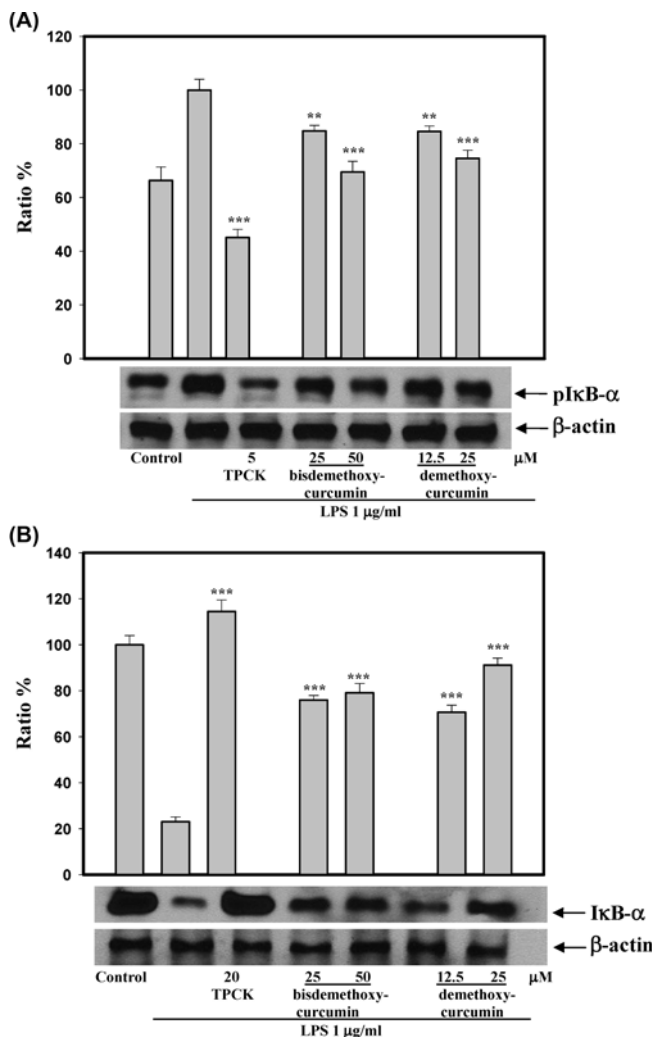
To further elucidate the effects of demethoxycurcumin and bisdemethoxycurcumin at the gene transcription level, their activation of NF-κB was evaluated. As shown in Table I, the IC<sub>50</sub> values of demethoxycurcumin and bisdemethoxycurcumin inhibiting LPS-induced NF-κB activity are 10.2 μM and 29.9 μM, respectively. This strongly suggests that the two compounds inhibit NF-κB activity in a dose-dependent manner and demethoxycurcumin is more active (Fig. 4).

**Effects of curcuminoids on phosphorylation and degradation of IκB-α**

Demethoxycurcumin and bisdemethoxycurcumin both



**Fig. 4.** Effects on NF-κB activity of macrophages treated with demethoxycurcumin and bisdemethoxycurcumin

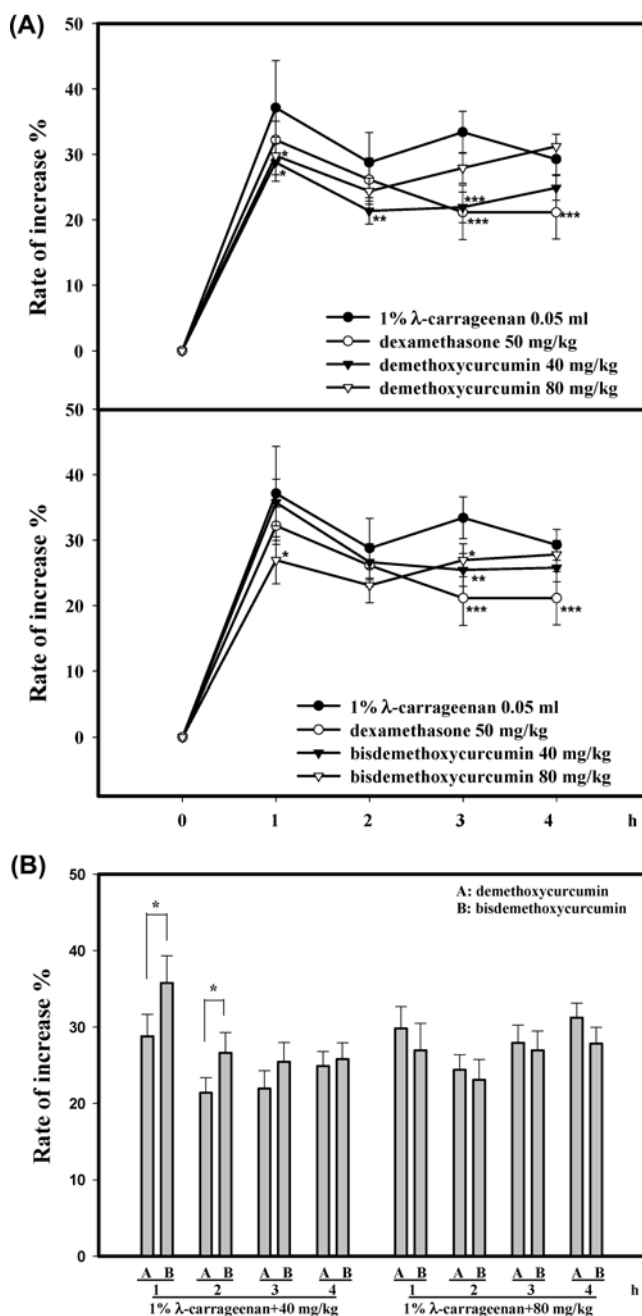


**Fig. 5.** Effects on p-IκB-α protein expression (A) and IκB-α protein degradation (B) of macrophages treated with demethoxycurcumin and bisdemethoxycurcumin. The data are shown as means ± S.E.M. from three independent experiments.

inhibited LPS-induced phosphorylation (Fig. 5A) and degradation of IκB-α (Fig. 5B), but the inhibitory effect of demethoxycurcumin is stronger than that of bisdemethoxycurcumin. Overall, this study has shown that the inhibition of NF-κB activity by curcuminoids is attributable to the prevention of IκB-α phosphorylation and degradation.

**Inhibitory effects of curcuminoids on carrageenan-induced paw edema**

In the paw edema assay, treatment of mice with curcuminoids (40 and 80 mg/kg) and dexamethasone (50 mg/kg) showed a significant reduction in carrageenan-induced paw edema. The inhibitory effects of curcuminoids and dexamethasone began at 1 h and sustained themselves for 4 h (Fig. 6A). At the low concentration, the inhibitory effect of demethoxycurcumin was significantly



**Fig. 6.** Effects of demethoxycurcumin and bisdemethoxycurcumin on carrageenan-induced mice-paw edema (A). Comparison of suppressive effects of demethoxycurcumin and bisdemethoxycurcumin on carrageenan-induced mice-paw edema (B).

better than that of bisdemethoxycurcumin. However, at the high concentration, the two compounds had a similar inhibitory effect on paw edema (Fig. 6B).

**DISCUSSION**

Curcuminoids have been shown to exhibit anti-inflammatory activities in many studies. And many reports in-

investigated the anti-inflammatory mechanism of curcumin (Sandur *et al.*, 2007; Ramsewak *et al.*, 2000). However, the anti-inflammatory mechanisms of demethoxycurcumin and bisdemethoxycurcumin have not been studied. In this study, demethoxycurcumin and bisdemethoxycurcumin inhibited NO production, COX-2 and iNOS expression and suppressed LPS-induced I $\kappa$ B- $\alpha$  phosphorylation and degradation in a dose-dependent manner. All of these anti-inflammatory effects could be attributed at least in part to the inhibition of LPS-induced NF- $\kappa$ B activation. These results may provide evidence for the mechanism of the anti-inflammatory effects of demethoxycurcumin and bisdemethoxycurcumin. We compared the effects and the concentrations of the two compounds on the cytotoxicity in the macrophages as well as their IC<sub>50</sub> values for inhibition of NO, NF- $\kappa$ B, iNOS and COX-2. All of the effects of demethoxycurcumin were found to be better than those of bisdemethoxycurcumin at the same concentration. It appears that the presence of the methoxy groups in the curcumin molecules is essential to anti-inflammatory activity (Sandur *et al.*, 2007). In a similar manner, the methoxy groups enhance cytotoxicity in macrophages (Ramsewak *et al.*, 2000). The safe concentration of demethoxycurcumin is lower than 43.7  $\mu$ M, and that of bisdemethoxycurcumin is lower than 61.3  $\mu$ M.

These inflammatory mediators are all associated with many acute and chronic inflammatory diseases. The paw edema assay indicated that the carrageenan-induced two-phase swelling was, from 1 h to 4 h, significantly inhibited by curcuminoids. Therefore, it can be posited that the inhibitory effects are due to the suppression of these inflammatory mediators. However, after 4 h, the inhibitory effects of the curcuminoids began to weaken, apparently as a result of rapid metabolism and rapid systemic elimination (Anand *et al.*, 2007). And, owing to the poor absorption that more curcumin does not result in higher absorption (Ravindranath and Chandrasekhara, 1981), the inhibitory effects of the curcuminoids did not obviously manifest themselves in a dose-dependent manner. We also compared the suppressive effects of the two compounds. Demethoxycurcumin showed better inhibition than bisdemethoxycurcumin at the low concentration, whereas, at the high concentration, the two compounds were similar, which latter result is also associated with the absorption of curcuminoids.

## ACKNOWLEDGEMENTS

This work was supported by a grant from the Korea Food and Drug Administration for Studies on the Identification of the Efficacy of Biologically Active Components from Oriental Herbal Medicines (2005).

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