**Natural Product Sciences**
10(6) : 315-320 (2004)

# Inhibitory Effect of Benzofuran Compound on Cyclooxygenase

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**Abstract** – Alpha-viniferin was previously isolated as a cyclooxygenase (COX)-2 inhibitor from *Carex humilis* (Cyperaceae) and is an oligomeric stilbene compound with benzofuran (BF) moieties in its chemical structure. In the present study, a chemically synthetic BF compound, named as 3,3-dimethyl-2,3,4,6,7,8,9,10,11,12,13,14,15,16,17,18-hexadecahydro-1*H*-benzo[*b*] cyclopentadeca[*d*]furan-1-one, was discovered to inhibit bacterial lipo polysaccharide (LPS)-induced prostaglandin $E_2$ ($PGE_2$) production in macrophages RAW 264.7. The BF compound exhibited a selectively preferred inhibitory effect on COX-2 activity over COX-1 activity. Furthermore, BF compound inhibited LPS-induced COX-2 expression at transcription level. As a down-regulatory mechanism of COX-2 expression shown by BF compound, suppression of nuclear factor (NF)-κB activation has been demonstrated. BF compound inhibited LPS-induced NF-κB transcriptional activity and nuclear translocation of NF-κB p65, in parallel, but did not affect LPS-induced degradation of inhibitory κBα protein (IκBα). Taken together, anti-inflammatory effect of BF compound on $PGE_2$ production was ascribed by its down-regulatory action on LPS-induced COX-2 synthesis in addition to inhibitory action on enzyme activity of COX-2.
**Keywords** – Benzofuran compound, Cyclooxygenase, Nuclear factor-κB, Macrophages

## Introduction

Cyclooxygenase (COX) is a key enzyme in the biosynthesis of prostaglandins (PGs). In the early 1990s, COX was identified to exist as two distinct isoforms. COX-1, a housekeeping enzyme, is constitutively expressed in nearly all tissues, and mediates physiological responses such as cytoprotection of the stomach and regulation of renal blood flow (Vane and Botting, 1996). In contrast, COX-2 expressed by inflammatory cells such as macrophages and synoviocytes has been recognized to produce prostanoids involved in pathological processes such as acute and chronic inflammatory states (Needelman and Isakson, 1997; Simon, 1999). Many of the side effects, such as gastrointestinal ulceration and bleeding, of nonsteroidal anti-inflammatory drugs (NSAIDs) have been ascribed to the inhibition of COX-1-derived prostanoids production, whereas inhibition of COX-2-dependent PG synthesis accounts for the anti-inflammatory, analgesic, and antipyretic effects of these drugs (Marret *et al.*, 2003; Sadikot *et al.*, 2004). Growth factors and cytokines in addition to bacterial lipopolysaccharide (LPS) are reported to provoke transcriptional activation of COX-2 (Nagano *et al.*, 2002; Stamp *et al.*, 2004). A promoter region of COX-2 gene contains a canonical TATA box and various transcriptional regulatory elements, such as nuclear factor (NF)-κB, NF-interleukin (IL) 6 and cAMP responsive element (CRE) (Inoue *et al.*, 1994; Rao *et al.*, 1997; Iniguez *et al.*, 2000). Depending on the stimulus and the cell type, these transcription factors can modulate COX-2 expression. As a pharmacological event, specific inhibition of enzyme activity and/or expression of COX-2 could provide therapeutic effects similar to those of NSAIDs without causing unwanted side effects.

In our ongoing study to discover anti-inflammatory agents, alpha-viniferin (Fig. 1) was isolated as an inhibitor of enzyme activity of COX-2 from *Carex humilis* (Cyperaceae), and later identified to inhibit LPS-induced COX-2 expression (Lee *et al.*, 1998; Chung *et al.*, 2003). Alpha-viniferin is a naturally occurring oligomeric stilbene compound with benzofuran (BF) moieties in its chemical structure. In the present study, a chemically

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synthetic BF compound named as 3,3-dimethyl-2,3,4,6,7,8,9,10,11,12,13,14,15,16,17,18-hexadecahydro-1*H*-benzo[*b*]cyclopentadeca[*d*]furan-1-one (Fig. 1) was found to inhibit LPS-induced $PGE_2$ production in macrophages RAW 264.7. As mechanism of the anti-inflammatory effect shown by BF compound, not only inhibitory action on enzyme activity of COX-2 but also down-regulatory action on COX-2 expression have been demonstrated in this study.

## Materials and Methods

**Materials and cell culture** – Alpha-viniferin (purity, >98%) was isolated from *Carex humilis* (Cyperaceae) as described in our previous work (Lee *et al.*, 1998). The BF compound (purity, >98%) was chemically synthesized as described elsewhere (Lee *et al.*, 2001). Antibodies against COX-1, COX-2, NF-κB p65 and IκBα were purchased from Santa Cruz Biotech (Santa Cruz, USA), and fetal bovine serum (FBS) from Invitrogen (Carlsbad, USA). The other reagents including LPS (*E. coli* 055:B5) were otherwise purchased from Sigma-Aldrich (St. Louis, USA). Macrophages RAW 264.7 were cultured in DMEM (13.4 mg/ml Dulbecco's modified Eagle's medium, 24 mM $NaHCO_3$, 10 mM HEPES, 143 U/ml benzylpenicillin potassium, 100 μg/ml streptomycin sulfate, pH 7.1) containing 10% FBS and maintained at 37°C with 5% $CO_2$. The RAW 264.7 cells harboring pNF-κB-secretory alkaline phosphatase (SEAP)-NPT reporter construct (Moon *et al.*, 2001) were also grown under the same conditions except supplement of geneticin (500 μg/ml) to the media.

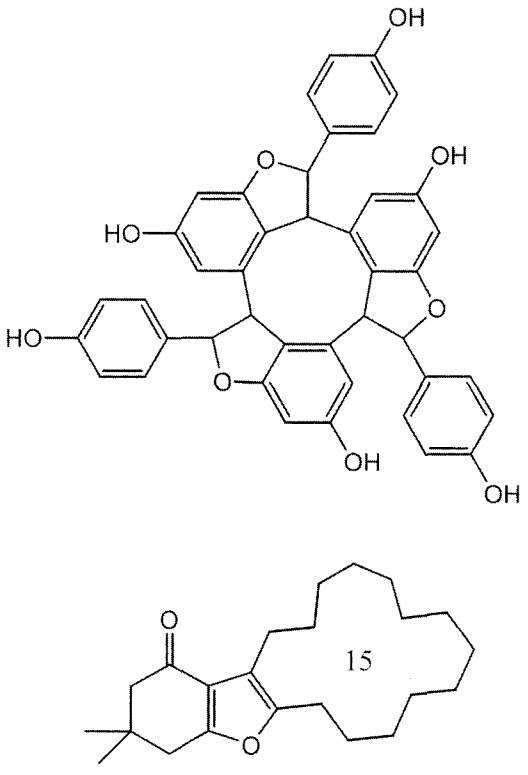


**Fig. 1.** Chemical structures of alpha-viniferin (top) and 3,3-dimethyl-2,3,4,6,7,8,9,10,11,12,13,14,15,16,17,18hexadecahydro-1*H*-benzo[*b*]cyclopentadeca[*d*]furan -1-one (BF compound, bottom).

**Enzyme-linked immunosorbent assay** (ELISA) – Macrophages RAW 264.7 were treated with BF compound for 2 h and then stimulated with LPS (1 μg/ml) for 24 h. Amount of $PGE_2$ in the cell-free culture media was quantified using an ELISA kit according to the supplier's protocol (Amersham-Pharmacia, San Francisco, USA).

**Measurement of enzyme activity of COX-1 or 2** – COX activity was analyzed using chemiluminescence in a total of 0.2-ml reaction mixture consisting of Tris-HCl (0.1 M, pH 8.0) containing arachidonic acid (100 μM) and luminol (25 μM) (Lee *et al.*, 1998). COX-1 source was prepared from resting macrophages RAW 264.7 and COX-2 source from LPS-stimulated RAW 264.7 cells. The harvested RAW 264.7 cells were subjected to sonication followed by centrifugation to obtain supernatants as sources of COX-1 or 2.

**Western immunoblot analysis** – Macrophages RAW 264.7 were treated with BF compound for 2 h and then stimulated with LPS (1 μg/ml) for 15-75 min (IκBα), 1 h (NF-κB p65) or 18 h (COX). Western immunoblot analysis for IκBα, COX-1 or COX-2 was carried out with cytoplasmic extracts of the RAW 264.7 cells, and that for NF-κB p65 with nuclear extracts. Western immunoblot conditions were described in our previous work (Shin *et al.*, 2004). The blots were finally reacted with ECL detection reagent (Amersham-Pharmacia, San Francisco, USA) and exposed to X-ray film.

**Measurement of COX-2 promoter activity** – Macrophages RAW 264.7 were transiently transfected with pCOX-2-luciferase reporter construct (Yeo *et al.*, 2003) and pSV-β-galactosidase control vector (Promega, Madison, USA) using LipofectAMINETM (Invitrogen, Carlsbad, USA). The transfected RAW 264.7 cells were treated with BF compound for 2 h and then stimulated with LPS (1 μg/ml) for 16 h. Lysates of the cells were subjected to luciferase assay using Luciferase Reporter Assay System (Promega, Madison, USA) and to β-galactosidase assay using β-Galactosidase Enzyme Assay System (Promega, Madison, USA).

**Measurement of NF-κB transcriptional activity** – Macrophages RAW 264.7 transfected stably with pNF-κB-SEAP-NPT reporter construct were treated with BF

compound for 2 h and then stimulated with LPS (1 μg/ml) for 16 h. Aliquots of the cell-free culture media were heated at 65°C for 5 min, and then reacted with SEAP assay buffer (500 μM 4-methylumbelliferyl phosphate, 2 M diethanolamine, 1 mM $MgCl_2$) in the dark at room temperature for 1 h. As a reporter, SEAP activity was measured as relative fluorescence units (RFU) with emission 449 nm and excitation 360 nm.

**Statistical analysis** – Results are expressed as mean ±S.E. Data were analyzed by ANOVA followed by the Student's *t* test. A value of $p < 0.01$ was considered significant.

## Results

**Effect of BF compound on LPS-induced $PGE_2$ production** – Macrophages RAW 264.7 in resting state released 550±92 pg/ml of $PGE_2$ to culture media during incubation for 24 h, whereas the macrophages markedly increased $PGE_2$ production to 2,081±188 pg/ml by treatment of LPS alone (Fig. 2A). No significant difference was identified in the $PGE_2$ production between resting RAW 264.7 cells and the cells treated with BF compound (10 μM) alone (Fig. 2A). BF compound inhibited LPS-induced $PGE_2$ production in a dose-dependent manner, corresponding to 36.8±3.7% inhibition at 1 μM, 63.2±4.9% at 3 μM and 93.4±3.5% at 10 μM (Fig. 2B). As a positive control, alpha-viniferin also exhibited a dose-dependent inhibitory effect with 44.9±6.4% inhibition at 3 μM, 63.1±4.2% at 10 μM and 92.6±3.6% at 30 μM (Fig. 2B). Neither BF compound (0.3-10 μM) nor alpha-viniferin (1-30 μM) showed any cytotoxic effects to the RAW 264.7 cells (data not shown).

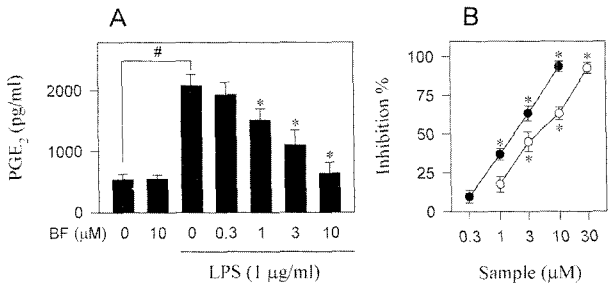


**Fig. 2.** Inhibition of LPS-induced $PGE_2$ production by BF compound. Macrophages RAW 264.7 were treated BF compound for 2 h and then stimulated with LPS for 24 h. Amount of $PGE_2$ was measured with the cell-free culture media (A). Effects of BF compound (•) and alpha-viniferin (○) on $PGE_2$ production are represented as inhibition % (B). Values are mean±S.E. ($n$=3). # $p < 0.01$ vs. media alone-treated group. * $p < 0.01$ vs. LPS alone-treated group.

**Effect of BF compound on enzyme activity of COX-1 or 2** – COX catalyzes a rate-limiting step in $PGE_2$ biosynthetic pathway staring from arachidonic acid (Vane and Botting, 1996). We next investigated whether BF compound could inhibit enzyme activity of COX isozyme. BF compound exhibited inhibitory effects with 35.3±3.8% inhibition at 40 μM and 58.5±3.0% at 100 μM on COX-1 activity (Fig. 3). BF compound inhibited COX-2 activity in a dose-dependent manner, corresponding to 33.8±3.4% inhibition at 5 μM, 58.0±5.7% at 20 μM and 87.7±4.2% at 80 μM (Fig. 3).

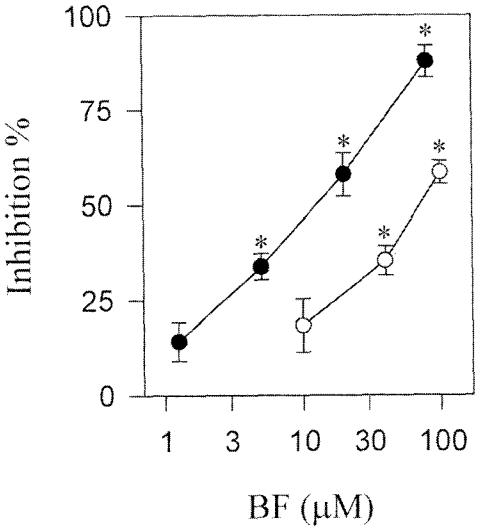


**Fig. 3.** Inhibition of enzyme activity of COX-1 or 2 by BF compound. Effects of BF compound on COX-2 activity (•) or COX-1 activity (○) are represented as inhibition %. Values are mean±S.E. ($n$=3). * $p < 0.01$ vs. the control, enzyme source only.

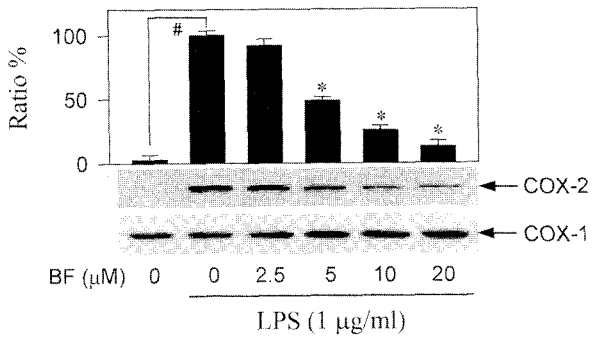


**Fig. 4.** Inhibition of LPS-induced COX-2 synthesis by BF compound. Macrophages RAW 264.7 were treated with BF compound for 2 h and then stimulated with LPS for 18 h. Lysates of the cells were subjected to Western immunoblot analysis with anti-COX-2 antibody or anti-COX-1 antibody. One of similar results is represented and relative ratio % is also shown, where COX-2 signal was normalized to COX-1 signal. Values are mean±S.E. ($n$=3). # $p < 0.01$ vs. media alone-treated group. * $p < 0.01$ vs. LPS alone-treated group.

**Effect of BF compound on LPS-induced COX-2 expression** – To examine whether BF compound could affect LPS-induced COX expression, Western immunoblot analysis was carried out. COX-2 protein was hardly detectable in resting macrophages RAW 264.7, but pronounced amount of COX-2 protein was induced upon exposure to LPS (Fig. 4). However, synthesis of housekeeping COX-1 was not affected by treatment of LPS and/or BF compound (Fig. 4). Treatment of BF compound to the RAW 264.7 cells decreased LPS-induced synthesis of COX-2 protein in a dose-dependent manner, corresponding to 52.3(2.9% inhibition at 5 μM, 71.1(3.5% at 10 μM and 89.7(4.6% at 20 μM (Fig. 4). To further understand whether inhibitory effect of BF compound on COX-2 expression was influenced at transcription level, COX-2 promoter activity was analyzed using macrophages RAW 264.7 transfected transiently with pCOX-2-luciferase construct containing murine COX-2 promoter (–963/+1) fused to luciferase as a reporter (Yeo *et al.*, 2003). Treatment of LPS to the transfected RAW 264.7 cells increased luciferase expression to 30-fold over the basal level, and BF compound inhibited LPS-induced luciferase expression in a dose-dependent manner, corresponding to 29.7±3.9% inhibition at 5 μM, 46.4±3.2% at 10 μM and 75.8±2.8% at 20 μM (Fig. 5).

**Effect of BF compound on LPS-induced NF-κB transcriptional activity** – NF-κB transcription factor has been evidenced to play an important role in LPS-induced expression of pro-inflammatory enzymes including COX-2 (Nagano *et al.*, 2002). NF-κB transcriptional activity was monitored using macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct containing four copies of κB sequence fused to SEAP gene as a reporter (Moon *et al.*, 2001). Treatment of LPS to the transfected RAW 264.7 cells increased SEAP expression to 3-fold over the basal level, indicating cellular NF-κB is transcriptionally functional (Fig. 6). No significant difference was identified in SEAP expression between resting RAW 264.7 cells and the cells treated with BF compound (20 μM) alone (Fig. 6). BF compound inhibited LPS-induced SEAP expression in a dose-dependent manner, corresponding to 39.1±4.2% inhibition at 5 μM, 56.8±3.9% at 10 μM and 89.2±4.4% at 20 μM (Fig. 6).

**Effect of BF compound on LPS-induced nuclear translocation of NF-κB p65 and degradation of IκBα** – To elucidate inhibitory mechanism on NF-κB activation, we next determined whether BF compound could influence nuclear translocation of NF-κB, Western immunoblot analysis for NF-κB p65 was carried out with nuclear extracts of LPS-stimulated macrophages RAW 264.7. Amount of NF-κB p65 in the nucleus was markedly increased upon exposure to LPS (Fig. 7). BF compound inhibited LPS-induced nuclear translocation of NF-κB p65 in a dose-dependent manner, corresponding to 54.8±4.2% inhibition at 10 μM and 76.4±4.4% at 20 μM (Fig. 7). Another Western immunoblot analysis was carried out with cytoplasmic extracts of LPS-stimulated

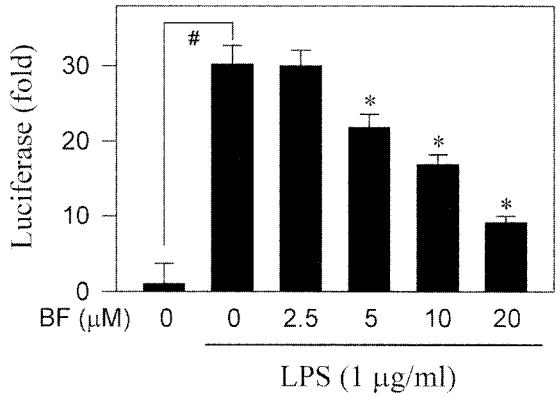


**Fig. 5.** Inhibition of LPS-induced COX-2 promoter activity by BF compound. Macrophages RAW 264.7 transfected transiently with pCOX-2-luciferase reporter construct and pSV-β-galactosidase control vector were treated with BF compound for 2 h and then stimulated with LPS for 16 h. Luciferase and β-galactosidase activities were measured with lysates of the cells. Luciferase expression as the iNOS promoter activity is represented as relative fold, where luciferase activity was normalized to β-galactosidase activity. Values are mean±S.E. ($n$=3). $^{\#}p < 0.01$ vs. media alone-treated group. * $p < 0.01$ vs. LPS alone-treated group.

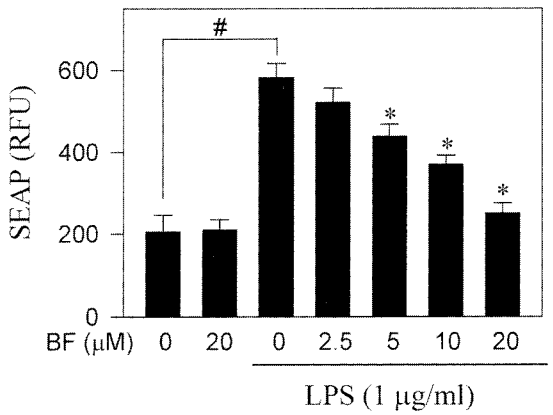


**Fig. 6.** Inhibition of LPS-induced NF-κB Transcriptional activity by BF compound. Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT reporter construct were treated with BF compound for 2 h and then stimulated with LPS for 16 h. SEAP activity as NF-κB transcriptional activity was measured with the cell-free media, and is represented as relative fluorescence units (RFU). Values are mean±S.E. ($n$=3). $^{\#}p < 0.01$ vs. media alone-treated group. * $p < 0.01$ vs. LPS alone-treated group.

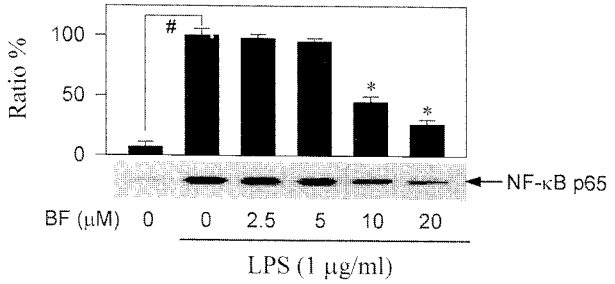


**Fig. 7.** Inhibition of LPS-induced nuclear translocation of NF-κB p65 by BF compounds. Macrophages RAW 264.7 were treated with BF compound for 2 h and then stimulated with LPS for 1 h. Nuclear extracts of the cells were subjected to Western immunoblot analysis with anti-NF-κB p65 antibody. One of similar results is represented and relative ratio % is also shown, where NF-κB p65 was normalized to nuclear protein content. Values are mean±S.E. ($n$=3). # $p < 0.01$ vs. media alone-treated group. * $p < 0.01$ vs. LPS alone-treated group.

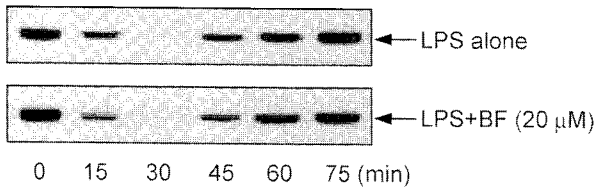


**Fig. 8.** No inhibition of LPS-induced IκBα degradation by BF compound. Macrophages RAW 264.7 were treated with either LPS (1 μg/ml) alone or LPS (1 μg/ml) plus BF compound (20 μM) for indicated times. Cytoplasmic extracts of the cells were subjected to Western immunoblot analysis with anti-IκBα antibody.

macrophages RAW 264.7, in order to understand whether BF compound could affect IκB degradation, a signal upstream nuclear translocation of NF-κB. Upon exposure of LPS to the RAW 264.7 cells, IκBα degradation was dramatically happened with 30 min, and amount of IκBα in the cytoplasm was recovered to the normal level at 75 min (Fig. 8). BF compound (20 μM) did not inhibit LPS-induced IκBα degradation, at all (Fig. 8).

## Discussion

In the present study, novel synthetic BF compound (Fig. 1) was discovered to show a dose-dependent inhibitory effect with an $IC_{50}$ value of 2.1 μM on LPS-induced $PGE_2$ production in macrophages RAW 264.7 (Fig. 2). The BF compound inhibited not only COX-1 activity with an $IC_{50}$ value 85.7 μM but also COX-2 activity with an $IC_{50}$ value 15.1 μM (Fig. 3), suggesting that a selectively preferred inhibition of COX-2 activity. Furthermore, BF compound inhibited LPS-induced COX-2 synthesis, which was documented by Western immunoblot analysis (Fig. 4), and attenuated LPS-induced COX-2 promoter activity (Fig. 5), indicating that a down-regulation of COX-2 expression at transcription level.

NF-κB activation has been evidenced as major mechanism of LPS-induced COX-2 expression (Nagano *et al.*, 2002). BF compound inhibited LPS-induced NF-κB transcriptional activity (Fig. 6) and showed inhibitory effect on LPS-induced nuclear translocation of NF-κB p65 (Fig. 7), in parallel. However, BF compound did not influence LPS-induced IκBα degradation, at all (Fig. 8). These results indicate that BF compound could inhibit LPS-induced nuclear translocation of NF-κB p65 without affecting IκBα degradation, which is a rare mechanism for the control of NF-κB activation.

In the nuclear import system, nuclear localization signal (NLS) motif of NF-κB binds to karyopherin α, which are docked to karyopherin β at cytoplasmic face of the nuclear pore (Moroianu, 1999). Once docking has occurred, the complex is subsequently translocated through the pore and into the nucleus by an energy-dependent process involving GTPase Ran/TC4 and the Ran interacting factor NTF2/p10 (Moroianu, 1999). Synthetic peptide SN50, containing a hydrophobic membrane-translocating region and the NLS motif of NF-κB p50, was reported to inhibit nuclear translocation of NF-κB p50 in response to LPS and TNF-α (Kolenko *et al.*, 1999). Target of synthetic peptide SN50 is the NLS motif on NF-κB p50 that is recognized by karyopherin α. Even though molecular target of BF compound on nuclear import machinery would be elucidated, this study demonstrated that a non-peptide compound could interfere nuclear localization step of NF-κB p65 without affecting IκBα degradation as the control mechanism of LPS-induced NF-κB activation.

Taken together, BF compound inhibited LPS-induced $PGE_2$ production, which was ascribed by its inhibitory effects on LPS-induced COX-2 expression in addition to enzyme activity of inducible COX-2. As a mechanism of the down-regulatory action on COX-2 expression by BF compound, suppression of LPS-induced NF-κB activation, specifically to nuclear translocation step of NF-κB p65, has been documented in this study.

## Acknowledgements

pCOX-2-luciferase reporter construct was kindly supplied by Dr. A.-K. Yi (University of Tennessee Health Science Center). This work was financially supported by a research fund (Haksoolyeonkoo 2002) from Chungbuk National University (K.R. Min).

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(Accepted November 30, 2004)