J. Toxicol. Pub. Health
Vol. 23, No. 2, pp. 123-126 (2007)

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Inhibition of the Induction of Nitric Oxide Synthase by Kobusin

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Received May 15, 2007; Accepted June 19, 2007

We isolated a lignan, kobusin from *Geranium thunbergii* and studied its effect on the expression of inducible nitric oxide synthase (*iNOS*) gene in a monocyte/macrophage cell line, RAW264.7 cells. Kobusin inhibited lipopolysaccharide (LPS)-stimulated NO production and the expression of iNOS in a concentration-dependent manner. To identify the mechanistic basis for its inhibition of iNOS induction, we examined the effect of kobusin on both the luciferase reporter activity using NF-κB minimal promoter and the nuclear translocation of p65. Kobusin suppressed the reporter gene activity and the LPS-induced movement of p65 in to nucleus. NF-κB activation is controlled by the phosphorylation and subsequent degradation of I-κBα, and in the present study, we found that I-κBα phosphorylation was also inhibited by kobusin. Our findings indicate that kobusin may provide a developmental basis for an agent against inflammatory diseases.

Key words: *Geranium thunbergii*, iNOS, Kobusin, NF-κB, Nitric oxide.

INTRODUCTION

Geranium thunbergii is widely used as an anti-diarrhetic agent in East Asia (Okuda *et al.*, 1975). Although it has been reported that kobusin (methylpiperitol) interacts with calmodulin and inhibits the activation of the calmodulin-dependent cAMP phosphodiesterase (Rojas *et al.*, 2003), other pharmacological effects of kobusin are still unknown. In our recent study, three lignans (kobusin, 7,7'-dihydroxybursehernin and 4-hydroxykobusin) were isolated from *Geranium thunbergii* and it has been found that kobusin and 4-hydroxykobusin are effective to inhibit interleukin-6 production in human osteosarcoma cell line (Liu *et al.*, 2006).

Excess amounts of nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS) in activated macrophages, are believed to be related with inflammatory responses. In some pathological conditions, NO induces various harmful responses including tissue injury, septic shock, and apoptosis (Kristof *et al.*, 1998; Strunk *et al.*, 2001). Thus, iNOS is a plausible target for the prevention or treatment of chronic inflammatory disorders.

Here, we aimed to estimate NO-blocking effect of kobusin and to elucidate its underlying mechanism in a murine macrophage cell line, RAW264.7 cells. We found that kobusin inhibits the activation of nuclear factor-κB (NF-κB) through the blocking of inhibitor-κBα (I-κBα) phosphorylation, and that these might be involved in its NO-blocking effect.

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; I-κBα, inhibitor-κBα; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; SDS, sodium dodecyl sulfate

MATERIALS AND METHODS

Materials. Kobusin was isolated from air-driec whole plant of *Geranium thunbergii* (Liu *et al.*, 2006). 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium solutions were purchased from Promega (Madison, WI); Anti-murine iNOS polyclonal antibody from Transduction Laboratories (Lexington, KY); Anti-phospho-I-κBα antibody from Cell Signaling Technology (Beverly, MA), and Anti-c-Rel (p65) from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and alkaline phosphatase-conjugated donkey anti-mouse IgGs were from Jackson Immunoresearch Laboratories (West Grove,

PA). All reagents used during this study were from Sigma (St. Louis, MO).

Cell culture. RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured at 37°C in 5% CO_2/95% air in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin. For all experiments, cells were grown to 80~90% confluency and subjected to no more than 20 cell passages. Kobusin was dissolved in dimethylsulfoxide and the final concentration of dimethylsulfoxide in culture medium was below 0.1%. The same volume of dimethylsulfoxide was added in the control samples as vehicle.

Nitrite determination. RAW264.7 cells (5×10^5 cells) were preincubated at 37°C for 12 h in serum-free medium and NO production was monitored by measuring nitrite levels in culture media using Griess reagent (Lee *et al.*, 2005). Absorbance was measured at 540 nm after incubating culture media with Griess reagent for 10 min.

Preparation of nuclear extract. Cells were preincubated for 10 min in culture medium in the presence or absence of kobusin, and then exposed to LPS (1 μg/ml). Cells were then removed using a cell scraper, centrifuged at 2,500 *g* at 4°C for 5 min, and swollen by adding 100 μl of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P40, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride]. Cells were vortexed to disrupt cell membranes, and samples were incubated for 10 min on ice and then centrifuged for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 60 μl of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride, and incubated for 30 min on ice. The samples were then centrifuged at 15,800 ×*g* for 10 min to obtain supernatant containing nuclear extracts, which were stored at -80°C until required.

Western blot analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analyses were performed as described previously (Woo *et al.*, 2005). Cells were lysed in buffer containing 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin. Lysates were centrifuged at 12,000 ×*g* for 10 min to remove debris, fractionated by 10% gel electrophoresis, electrophoretically transferred to nitrocellulose paper, and incubated with primary antibodies and then with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies. Finally papers were developed using either 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium or an ECL chemiluminescence detection kit.

Reporter gene assays. Cells were plated at a density of 3×10^5 cells/well in 12-well plate and transfected on the following day. A dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine promoter activity. Briefly, cells were transiently transfected with 1 μg of pNF-κB-Luciferase plasmid and 20 ng of the phRL-SV plasmid (Promega, Madison, WI) using the Genejuice® Reagent (Novagen, Madison, WI) and then exposed to LPS for 18 h. Firefly and *hRenilla* luciferase activities in cell lysates were measured using a luminometer (LB941, Berthold Tech., Bad Wildbad, Germany). Relative luciferase activities were calculated by normalizing NF-κB promoter-driven firefly luciferase activities versus that of *hRenilla* luciferase.

Scanning densitometry and statistics. Scanning densitometry was performed using an Image Scan & Analysis System (FLA-7000, Fujifilm, Tokyo, Japan), and the paired Student's *t*-test was used to assess significant inter-group differences. Statistical significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of kobusin on the induction of iNOS by LPS. The chemical structure of kobusin is presented in Fig. 1. To assess their NO-blocking effects, we determined nitrite levels in culture media after stimulating cells with LPS (1 μg/ml) in the presence of kobusin for 48 h. LPS stimulation caused a significant increase of nitrite in culture media at 12 h (2.8 fold), 24 h (6.0 fold) and 48 h (6.4 fold) (Fig. 2A). This enhancement in NO production was significantly suppressed by 30 or 100 μM kobusin.

We then investigated whether the inhibition of NO formation by the lignans was associated with the inhibition of iNOS gene expression. Western blot analysis using iNOS-specific antibody showed that exposure of RAW264.7 cells to LPS (1 μg/ml) for 12 h sharply increased iNOS protein levels (Fig. 2B). One hundred mM Kobusin almost completely blocked iNOS induction by LPS (Fig. 2B). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels were comparable among

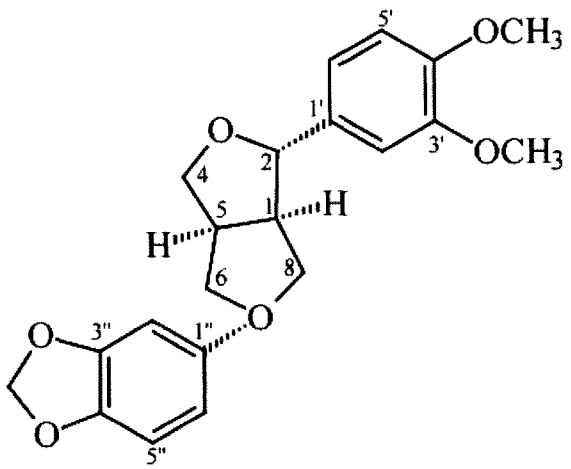

Fig. 1. Structure of kobusin isolated from *Geranium thunbergii* (Geraniaceae).

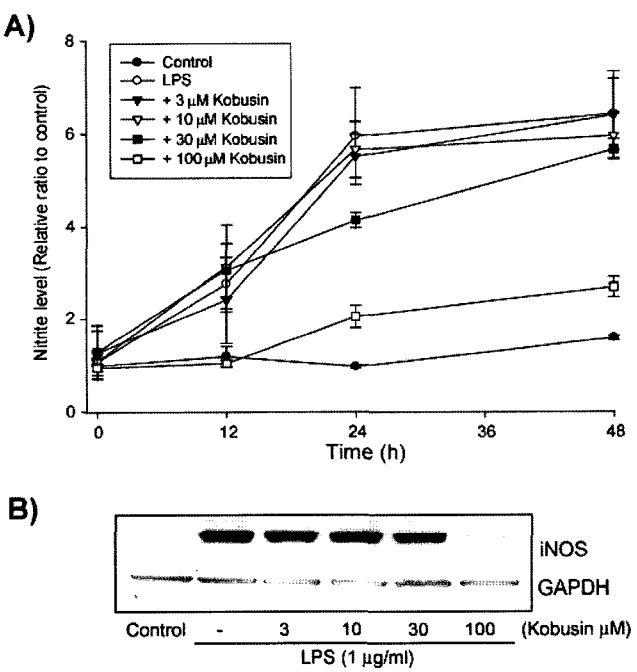

Fig. 2. Inhibition of LPS-inducible iNOS protein expression by kobusin. (A) Effect of kobusin on LPS-induced NO production. The RAW264.7 cells were incubated in a medium containing kobusin (3, 10, 30 and 100 μM) for 10 min and then treated with LPS at 1 μg/ml. The amount of nitrite in the medium was monitored for 48 h. The data represents means ± SD of 4 different samples. (B) Effect of kobusin on LPS-induced iNOS protein expression. The level of iNOS protein was monitored 12 h after treating cells with LPS (1 μg/ml) with or without kobusin treatment.

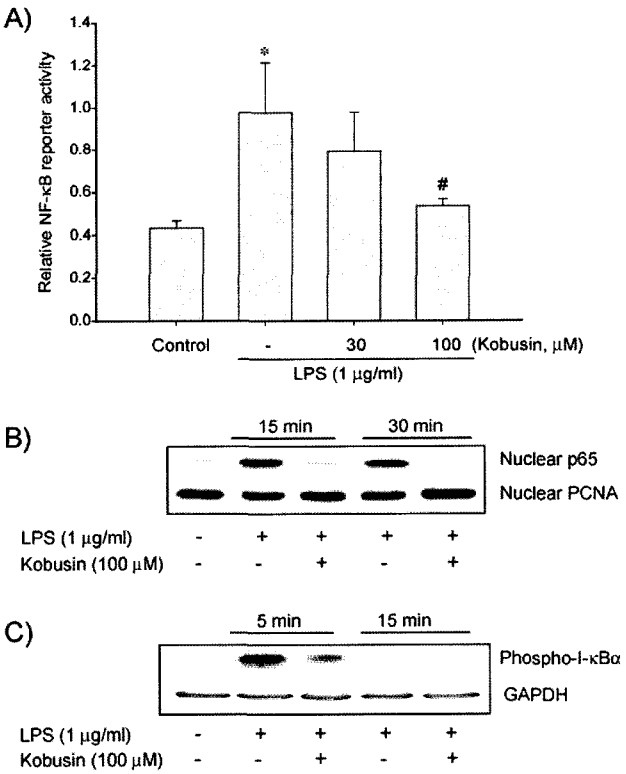

Fig. 3. Effect of kobusin on the LPS-inducible NF-κB activation. (A) NF-κB reporter gene analysis. A dual luciferase reporter gene assay was performed on the lysed cells co-transfected with pNF-κB-Luc plasmid (firefly luciferase) and phRL-SV (*Renilla* luciferase) (in a ratio of 100 : 1) after exposure to LPS (1 μg/ml) and kobusin (30 and 100 μM) for 18 h. Kobusin was pretreated 10 min before LPS treatment. The activation of the reporter gene was calculated as a relative change in the *Renilla* luciferase activity. Data represents the means ± SD of 3 separate samples (significant versus the control, $^{*}p < 0.05$; significant versus the LPS-treated group, $^{\#}p < 0.05$). (B) Effect of kobusin on the LPS-induced nuclear translocation of p65. RAW264.7 cells were treated with 1 μg/ml of LPS for 15 min or 30 min in the presence or absence of 100 μM kobusin, and nuclear p65 and proliferating cell nuclear antigen (PCNA) were immunochemically detected using specific antibodies. (C) Effect of kobusin on LPS-inducible I-κBα phosphorylation. The phosphorylated I-κBα was immunochemically assessed 5 or 15 min after 1 μg/ml LPS exposure to RAW264.7 cells. The cells were preincubated with 100 μM kobusin for 10 min.

the samples (Fig. 2B).

The productions of proinflammatory cytokines and NO by activated macrophages play critical roles in severe inflammatory diseases such as sepsis and arthritis (Szabo, 1998). Hence, the inhibition of *iNOS* gene expression by kobusin in inflammatory cells may offer a new therapeutic strategy against inflammation.

Effects of kobusin on the LPS-inducible NF-κB activation. NF-κB is an essential transcription factor for the induction of several inflammatory mediators

including, tumor necrosis factor-α, cyclooxygenase-2, and iNOS (Muller *et al.*, 1993; Guha and Mackman, 2001). Thus, the inhibition of iNOS expression by kobusin may result from the suppression of NF-κB activation. First, we performed reporter gene assay using a luciferase plasmid containing NF-κB minimal promoter. LPS treatment (1 μg/ml, 18 h) caused a 2.2-fold increase in NF-κB reporter activity (Fig. 3A), and 10 min pretreatment of cells with 100 μM of kobusin significantly inhibited the increase in NF-κB reporter activity by LPS (Fig. 3A).

NF-κB (a p65/p50 heterodimer) is sequestered in the cytoplasm as an inactive complex by the inhibitory protein I-κBα. Upon inflammatory stimulation, its inhibitory subunit, I-κBα is phosphorylated and degraded, and the liberated active p65 is then translocated into the nucleus (Gilmore, 1998). Thus, we measured nuclear p65 levels by subcellular fractionation and immunoblotting. Nuclear p65 protein levels increased from 15 min to 30 min after treating RAW264.7 cells with LPS (1 μg/ml). One hundred mM kobusin completely suppressed the LPS-induced nuclear translocation of p65 (Fig. 3B). p65 translocation is preceded by the phosphorylation and subsequent degradation of the I-κBα subunit (Wang *et al.*, 2002), and thus, we further examined phosphorylated I-κBα levels in macrophages. Immunoblot analysis using phospho-I-κBα antibody revealed that the LPS (1 μg/ml)-inducible phosphorylation of I-κBα was also inhibited by 100 μM kobusin at 5 min (Fig. 3C). These results combined with the data from NF-κB reporter gene assays suggested that the phosphorylation of I-κBα is a pharmacological target of kobusin. Phosphorylation of I-κBα bound to NF-κB is considered to be mediated with the I-κB kinase at two conserved serines within its N-terminal domain (Karin and Ben-Neriah, 2000). The I-κB kinase complex can be activated by a variety of upstream kinases such as NF-κB-inducing kinase and tyrosine kinase family (Huang *et al.*, 2003; Trushin *et al.*, 2003). Thus, kobusin may also act on these upstream kinases.

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