4-Hydroxykobusin Inhibits the Induction of Nitric Oxide Synthase by Inhibiting NF-κB and AP-1 Activation

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We recently isolated a novel lignan, 4-hydroxykobusin from Geranium thunbergii (Liu et al., Arch. Pharm. Res., 29, 1109–1113, 2006). Here, we studied its effect on the expression of inducible nitric oxide synthase (iNOS) gene in RAW264.7 cells. 4-Hydroxykobusin inhibited nitric oxide (NO) production in a concentration-dependent manner and blocked the lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS). To identify the mechanistic basis for its inhibition of iNOS induction, we examined the effect of 4-hydroxykobusin on the transactivation of iNOS gene by luciferase reporter activity using −1.59 kb flanking region. The lignan suppressed the reporter gene activity and the LPS-induced reporter activations of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) were also significantly blocked by 4-hydroxykobusin. These findings suggest that the inhibition of LPS-induced NO formation by 4-hydroxykobusin is due to its inhibition of NF-κB and AP-1 activation.

Key words 4-hydroxykobusin; activator protein-1 (AP-1); Geranium thunbergii; inducible nitric oxide synthase (iNOS); nuclear factor-κB (NF-κB); nitric oxide

Geranium thunbergii, which is widely used as an anti-diarrheatic agent in East Asia, has been reported to have anti-mutagenicity, anti-inflammation and anti-oxidative effects. One of representative tannin in Geraniaceae, geraniin shows diverse effects including anti-bacterial, anti-fungal and anti-hypertension. We recently isolated three lignans (kobusin, 7,7′-dihydroxybursehernin and 4-hydroxykobusin) from Geranium thunbergii. Among them, 4-hydroxykobusin has been identified as a new furofuran lignan suppressed the reporter gene activity and is effective to inhibit interleukin-6 production in MG-63, a human osteosarcoma cell line.

Nitric oxide (NO) is a bioactive radical produced from L-arginine via three types of nitric oxide synthases (NOS) and also plays as an important cellular second messenger. Small amounts of NO produced by the constitutive NOS (cNOS) is essential for maintaining the cellular function. Since inducible NOS (iNOS) can sustainably produce a high output of NO, iNOS induction in activated macrophages, is believed as one of the most important inflammatory reactions. Physiologically, NO induces various harmful responses including tissue injury, septic shock, and apoptosis during acute or chronic inflammation. Thus, iNOS is a plausible target for the prevention or treatment of chronic inflammatory disorders. The ability of plant-derived compounds to inhibit inflammatory processes has been well documented, and a variety of flavonoids have suppressive functions in the expression of iNOS gene. However, it has not been intensively studied to identify lignans to act on iNOS induction.

The aims of the present study were 1) to determine the NO-blocking effects of 4-hydroxykobusin and 2) to elucidate its underlying mechanism in a murine macrophage cell line, RAW264.7 cells. We found that 4-hydroxykobusin inhibits nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) activation, and that these were essentially required for its NO-blocking effect.

MATERIALS AND METHODS

Extraction and Isolation of Lignans from Geranium thunbergii The air-dried whole plant of Geranium thunbergii (460 g) was cut and extracted with MeOH (3×3) at 60°C for 4 h (×3). The MeOH extract (82.92 g) was suspended in water (1.0 l) and then partitioned sequentially with equal volumes of dichloromethane, ethyl acetate, and n-butanol. Each fractions were evaporated in vacuo to yield the residues of CH2Cl2 (12.18 g), EtOAc (20.97 g), n-BuOH (12.43 g), and water (22.49 g) extract. A portion of the CH2Cl2 soluble fraction (9.0 g) were subjected to chromatography over a silica gel column over a silica gel eluting with a CHCl3–MeOH gradient system to afford four subfractions (12.43 g), and water (22.49 g) extract. A portion of the CH2Cl2 soluble fraction (9.0 g) were subjected to chromatography over a silica gel column. The products were identified by 1H and 13C NMR, IR, and MS. The major compound 4-hydroxykobusin (3.5 mg) was purified by repeated Sephadex LH-20 column chromatography with a silica gel (400 g) eluting with a n-hexane–EtOAc (100:0 →1:4), CHCl3-MeOH=1:0 →1:1 in gradient system. The fractions were combined based on their TLC pattern to yield subfraction designated as C1—C6. Subfraction C3 (1.67 g) was further purified by column chromatography over a silica gel (300 g) eluting with a CHCl3–MeOH gradient system to afford four subfractions (C31—C34). The subfraction C33 (101.6 mg) was purified by preparative TLC (Si Gel F254 plates, 0.5 mm, n-hexane:acetone=1:1, Rf=0.6) to give of 4-hydroxykobusin (3.5 mg).

4-Hydroxykobusin: Colorless oil, [α]D31 42.0° (c=0.63, MeOH); UV (MeOH) λmax nm: 232, 284; IR νmax (KBr) cm−1: 3400, 1610, 1595, 1505, 1250; EI-MS m/z (rel. int.): 386 ([M]+, 1), 339 (20.0), 267(12), 177(60); HR-EI-MS m/z: 386.1363 (Calcd for C16H12O5: 386.1366); 1H-NMR (500 MHz, CD3OD) δ: 7.21 (1H, d, J=2.0 Hz, H-2′), 6.98 (1H, dd, J=2.0, 8.0 Hz, H-6′), 6.91 (1H, d, J=8.0 Hz, H-5′), 6.88 (1H, d, J=2.0 Hz, H-2′), 6.85 (1H, dd, J=2.0, 8.0 Hz, H-6′), 6.79 (1H, d, J=8.0 Hz, H-5′), 5.94 (2H, s, –OCH2O–), 5.50 (1H, d, J=1.0 Hz, H-4), 4.90 (1H, d, J=7.0 Hz, H-2), 4.84 (1H, d, J=7.0 Hz, H-6), 4.21 (1H, dd, J=6.0, 9.0 Hz, H-8eq), 4.02 (1H, dd, J=2.5, 9.0 Hz, H-8ax), 3.85 (3H, s, –OCH3), 3.82 (3H, s, –OCH3), 3.14 (1H, m, H-1), 2.84 (1H, brt, J=7.5 Hz, H-5); 13C-NMR (125 MHz, CD3OD) δ:

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150.80 (s, C-4’), 150.19 (s, C-3’), 149.65 (s, C-4’), 148.83 (s, C-3’), 137.25 (s, C-1’), 137.04 (s, C-1’), 120.55 (d, C-6’), 120.12 (d, C-6’), 112.81 (d, C-5’), 111.55 (d, C-2’), 109.97 (d, C-5’), 107.37 (d, C-3’), 102.97 (d, C-4’), 102.58 (t, –OCH3), 88.69 (d, C-2’), 85.07 (d, C-6’), 73.24 (t, C-8), 63.79 (d, C-5’), 56.69 (q, –OCH3), 56.57 (q, –OCH3), 55.09 (d, C-1’).

Materials 5-Bromo-4-chloro-3-indolylphosphosphate and nitroblue tetrazolium solutions were purchased from Promega (Madison, WI, U.S.A.); Anti-murine iNOS polyclonal antibody from Transduction Laboratories (Lexington, KY, U.S.A.); Anti--phospho-1-κBζ antibody from Cell Signaling Technology (Beverly, MA, U.S.A.), and Anti-c-Rel (p65) antibody from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-phospho-IκB antibody from Cell Signaling Technology (Beverly, MA, U.S.A.), and Anti-murine iNOS polyclonal antibody from Transduction Laboratories (Lexington, KY, U.S.A.).

Cell Culture RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and cultured at 37 °C in 5% CO2/95% 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% confluence and subjected to no more than 20 cell passages.

MTT Cell Viability Assay Viable adherent cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (2 μg/ml) for 4 h. Media were then removed and the formazan crystals produced were dissolved by adding 200 μl of dimethylsulfoxide. Absorbance was assayed at 540 nm and cell viabilities were expressed as ratios versus untreated control cells.

Measurement of NO RAW264.7 cells (5×105 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. Absorbance was measured at 540 nm after incubation 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 4-hydroxykobusin and then exposed to LPS (1 μg/ml). Cells were then removed using a cell scraper, centrifuged at 2500 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% Nonident-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 15800 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. 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 12000 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-indolylphosphate and nitroblue tetrazolium or an ECL chemiluminescence detection kit.

Reporter Gene Assays pGL-miNOS-1588 luciferase reporter was generated by ligating pGL3-basic vector (Promega, Madison, WI, U.S.A.) with the −1588 bp promoter region of the murine iNOS gene. Cells were plated at a density of 3×105 cells/well in 12-well plate and transfected on the following day. A dual-luciferase reporter assay system (Promega, Madison, WI, U.S.A.) was used to determine promoter activity. Briefly, cells were transiently transfected with 1 μg of pGL-miNOS1588, pNF-κB-Luciferase, or pAP-1-Luciferase plasmid and 20 ng of the pRL-SV plasmid (Promega, Madison, WI, U.S.A.) using the Genejuice® Reagent (Novagen, Madison, WI, U.S.A.) and then exposed to LPS for 18 h. Firefly and Renilla luciferase activities in cell lysates were measured using a luminometer (Turner Designs; TD-20, CA, U.S.A.). Relative luciferase activities were calculated by normalizing iNOS, NF-κB, or AP-1 promoter-driven firefly luciferase activities versus that of Renilla luciferase.

Scanning Densitometry and Statistics Scanning densitometry was performed using an Image Scan & Analysis System (FLA-7000, Fujifilm, Japan), and one-way analysis of variance (ANOVA) was used to assess the significant differences between the treatment groups. For each significant effect of treatment, the Newman–Keuls test was used to compare the multiple group means. The criterion for statistical significance was set at either p<0.05 or p<0.01.

RESULTS

Effects of 4-Hydroxykobusin on the Induction of iNOS by LPS The chemical structures of 4-hydroxykobusin is presented in Fig. 1A. Viability of RAW264.7 cells was not significantly affected by 4-hydroxykobusin at up to 100 μM (Fig. 1B). Thus, we treated cells with the lignan in the concentration range 3–100 μM during subsequent experiments. To assess its NO-blocking effects, we determined nitrite levels in culture media after stimulating cells with LPS (1 μg/ml) in the presence or absence of both the lignans for 48 h. LPS stimulation caused a significant increase of nitrite in culture media at 12 h (2.5 fold), 24 h (6.2 fold) and 48 h (7.3 fold) (Fig. 2A). This enhancement in NO production was significantly suppressed by 4-hydroxykobusin in a concentration dependent manner. Especially, LPS-inducible NO production was ca. 90% blocked by 100 μM 4-hydroxykobusin (Fig. 2A).

We then investigated whether the inhibition of NO formation by 4-hydroxykobusin was the result of the inhibition of iNOS gene expression. The inhibitory effects of different concentrations of 4-hydroxykobusin on iNOS protein expression induced by LPS (1 μg/ml) were estimated. Western blot
analysis using iNOS-specific antibody showed that exposure of RAW264.7 cells to LPS (1 μg/ml) for 18 h increased iNOS protein levels versus un-stimulated controls (Fig. 2B). Pre-treatment of RAW264.7 cells with 4-hydroxykobusin (10 min) significantly inhibited iNOS protein expression at 30—100 μM (Fig. 3A). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels were comparable among the samples (Fig. 2B). These results suggest that 4-hydroxykobusin is effective to block iNOS induction and NO production in macrophages.

Effects of 4-Hydroxykobusin on Transcriptional Activation of iNOS Gene and LPS-Inducible AP-1 Activation

iNOS expression is mainly regulated at the transcriptional level. To determine whether the process of iNOS gene transcription is affected by 4-hydroxykobusin, reporter gene analysis was performed using RAW264.7 cells transfected with the mammalian cell expression vector pGL-miNOS1588 (1588 bp iNOS promoter sequences), which contained a dual luciferase reporter gene construct, and pRL-SV (Renilla luciferase). The increase in luciferase activity by LPS was 51% inhibited by 100 μM 4-hydroxykobusin (Fig. 3A), but 30 μM 4-hydroxykobusin did not

One of key transcription factors involved in the transcription of iNOS gene is AP-1. AP-1 activation was assessed by reporter gene assay using luciferase plasmid containing AP-1 minimal promoter. The increase in AP-1 reporter activity by LPS was 51% inhibited by 100 μM 4-hydroxykobusin (Fig. 3B), but 30 μM 4-hydroxykobusin did not
suppress AP-1 reporter activity enhancement by LPS (1 μg/ml) (Fig. 3B). These findings suggest that blocking of AP-1 activation may be partly associated with the mechanism of iNOS expression inhibition by 4-hydroxykobusin.

**Effect of 4-Hydroxykobusin on 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.20,21) Thus, the inhibition of iNOS expression by 4-hydroxykobusin 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 3-fold increase in NF-κB reporter activity (Fig. 4A), and pretreatment of cells with 100 μM of 4-hydroxykobusin completely inhibited the increase in NF-κB reporter activity by LPS (Fig. 4A).

NF-κB (a p65/p50 heterodimer) is sequestered in the cytoplasm as an inactive complex by the inhibitory protein IκBα. Upon inflammatory stimulation, its inhibitory subunit, IκBα is phosphorylated and degraded, and the liberated active p65 is then translocated into the nucleus.22) Thus, we measured nuclear p65 levels by subcellular fractionation and immunoblotting. Nuclear p65 protein levels increased from 15 to 30 min after treating RAW264.7 cells with LPS (1 μg/ml) and peaked at 30 min after LPS treatment. Pretreatment of cells with 100 μM 4-hydroxykobusin for 10 min suppressed the LPS-induced nuclear translocation of p65 (Fig. 4B). p65 translocation is preceded by the phosphorylation and subsequent degradation of the IκBα subunit.23) and thus, we further examined phosphorylated IκBα levels in macrophages. Immunoblot analysis using specific antibodies revealed that the IκBα phosphorylation by LPS (1 μg/ml) were also reversed by 100 μM 4-hydroxykobusin (Fig. 4C). These results indicate that the inhibition of NF-κB activation by 4-hydroxykobusin is due to the prevention of IκBα phosphorylation and the subsequent nuclear translocation of p65.

We additionally assessed the effect of 4-hydroxykobusin on the LPS-inducible COX-2 expression. COX-2 expression was not altered by up to 100 μM 4-hydroxykobusin (Fig. 4D). The expression of both iNOS and COX-2 genes is dependent on NF-κB activation.24,25) However, other cis-acting elements such as C/EBPβ binding site and cAMP response element (CRE) are also involved in the transcriptional regulation of the COX-2 gene.26,27) Although NF-κB binding in the promoter region of COX-2 gene can be blocked by the lignan, other cis-acting elements may be still active. Hence, the minimal effect of 4-hydroxykobusin on COX-2 expression may result from the discrepancy of active transcription factors between iNOS and COX-2 genes.

**DISCUSSION**

In the present study, we isolated a lignan, 4-hydroxykobusin from *Geranium thunbergii* and found that 4-hydroxykobusin inhibits iNOS expression in macrophages, suggesting that 4-hydroxykobusin is a naturally-occurring iNOS inhibitor. The productions of proinflammatory cytokines and NO by activated macrophages play critical roles in severe inflammatory diseases such as sepsis and arthritis.28) Hence, the inhibition of iNOS gene expression by 4-hydroxykobusin in inflammatory cells may offer a new therapeutic strategy against inflammation. We showed that IC_{50} value of 4-hydroxykobusin on the nitrite production is ca. 30 μM. Park et al. recently reported that a lignan, lappaol F isolated from *Arctium lappa* more potently inhibited NO production (IC_{50}=9.5 μM) in comparison to 4-hydroxykobusin.29) The potency discrepancy between lappaol F and 4-hydroxykobusin would be due to the structure difference. Because lappaol F is classified as a di-lignan, lappaol F may metabolized to two different compounds containing active lignan moiety.

We further revealed that 4-hydroxykobusin mainly acted on the transcriptional process of iNOS gene. A reporter gene analysis using iNOS gene expression by 4-hydroxykobusin promoter, showed that the LPS-inducible transactivation of the iNOS gene was significantly suppressed by 4-hydroxykobusin, thus indicating the lignan targets the transcription of iNOS gene.

The iNOS gene promoter contains several homologous consensus sequences as binding sites for transcription factors including NF-κB and AP-1.30,31) Since NF-κB and AP-1 are believed to be essentially required for iNOS gene transcription,17,32,33) we performed reporter gene analyses using NF-κB and AP-1 minimal promoters. Reporter gene assays showed that 4-hydroxykobusin inhibited activation process of...
both NF-κB and AP-1. We further found that 4-hydroxykobusin completely blocked the nuclear translocation of p65 and the lignan was effective at blocking the phosphorylation of I-κBα protein. These results combined with the data from NF-κB reporter gene assays suggested that the phosphorylation of I-κBα is a pharmacological target of 4-hydroxykobusin. Since I-κBα is serially phosphorylated by diverse upstream kinases such as I-κB kinase, NF-κB-inducing kinase, protein kinase C and the tyrosine kinase family,22–36 the possible molecular target(s) of the lignan for the blocking of NF-κB seem to be one of the upstream kinases. It has been reported that a dibenzylbutyrolactone lignan, arctigenin concomitantly inhibits the activation of NF-κB and AP-1 in LPS-treated macrophages.37,38 In this study, we also showed that 4-hydroxykobusin acted on the activation of both NF-κB and AP-1. Cho et al. revealed that MAP kinases and their upstream kinases MKK1 were inhibited by arctigenin and suggested that AP-1 inhibition by arctigenin resulted from its kinase blocking activity.39 Therefore, the inhibitory effect of 4-hydroxykobusin on AP-1 activity also may be related with its actions on the upstream kinases regulating MAP kinases. The exact molecular target(s) affected by 4-hydroxykobusin remains to be identified. We recently showed that 7,7′-dihydroxybursehernin from Geranium thunbergii inhibited LPS-inducible iNOS expression.39 The inhibitory potency of 4-hydroxykobusin against iNOS induction was very similar to that of 7,7′-dihydroxybursehernin (Complete inhibition was seen in 100 μM of both the lignans). However, the mechanism of iNOS inhibitory action by 7,7′-dihydroxybursehernin is distinct from that by 4-hydroxykobusin. The pharmacological target of 7,7′-dihydroxybursehernin is physical binding of NF-κB to DNA. Thus, both the lignans in Geranium thunbergii have identical functions to control transcription of iNOS gene, but their mechanistic bases would be different. It was also found that iNOS inhibitory activity of kobusin was almost comparable to that of 4-hydroxykobusin (data not shown). Hence, the existence of hydroxyl group in 4-hydroxykobusin may not be critical to its NO blocking activity. In summary, the present study shows that 4-hydroxykobusin isolated from G. thunbergii inhibits the expressions and activities of inducible NOS in macrophages. The iNOS inhibitory effects of 4-hydroxykobusin are associated with both NF-κB inactivation via the blockade of I-κBα phosphorylation and AP-1 inactivation. Since NF-κB and AP-1 are critical transcription factors that regulate the transcriptions of many genes associated with inflammation, inhibition of these transcription factors with 4-hydroxykobusin offers a possible approach to the treatment of severe inflammatory diseases.

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