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Inhibitory mechanism of chroman compound on LPS-induced nitric oxide production and nuclear factor-κB activation☆

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Abstract

6-Hydroxy-7-methoxychroman-2-carboxylic acid phenylamide (KL-1156) is a novel chemically synthetic compound. In the present study, the chroman KL-1156 compound was found to inhibit lipopolysaccharide (LPS)-induced nitric oxide production in macrophages RAW 264.7. KL-1156 compound attenuated LPS-induced synthesis of both mRNA and protein of inducible nitric oxide synthase (iNOS), in parallel, and inhibited LPS-induced iNOS promoter activity, indicating that the chroman compound down-regulated iNOS expression at transcription level. As a mechanism of the anti-inflammatory action shown by KL-1156 compound, suppression of nuclear factor (NF)-κB has been documented. KL-1156 compound exhibited a dose-dependent inhibitory effect on LPS-induced NF-κB transcriptional activity in macrophages RAW 264.7. Furthermore, the compound inhibited LPS-induced nuclear translocation of NF-κB p65 and DNA binding activity of NF-κB complex, in parallel, but did not affect IκBα degradation. Taken together, this study demonstrated that chroman KL-1156 compound interfered with nuclear translocation step of NF-κB p65, which was attributable to its anti-inflammatory action.

Keywords: Novel chroman compound; Nitric oxide; Inducible nitric oxide synthase; Nuclear factor-κB; Anti-inflammation

Nitric oxide (NO) plays an important role in the regulation of many physiological functions such as vasodilation, neurotransmission, and inflammation [1,2]. NO is produced from L-arginine by a chemical reaction catalyzed by NO synthase (NOS) in living systems [3]. Endothelial NOS and neuronal NOS produce moderate amounts of NO, which primarily mediate the physiological responses [1,2]. Meanwhile, NO is also synthesized in the immune system by inducible NOS (iNOS), where it facilitates the killing of invading microorganisms [4]. However, high-output NO by iNOS can provoke septic shock, autoimmune disorders, and inflammatory diseases [5–7]. Indeed, NO production in macrophages is related to the level of iNOS expression, which is dependent on several transcription factors including nuclear factor (NF)-κB [8].

NF-κB transcription factor is functional as heterodimer or homodimer of Rel family proteins such as RelA (p65), RelB, cRel, p50, and p52, and is sequestered in the cytoplasm, bound to inhibitory κB (IκB) protein [9,10]. Lipopolysaccharide (LPS), a major component of the outer membranes of Gram-negative bacteria, can trigger a variety of inflammatory reactions by binding to Toll-like receptor 4 (TLR4) [11]. Downstream signaling components of the receptor include MyD88, interleukin-1 receptor-associated kinase, and tumor

☆ *Abbreviations:* IκB, inhibitory κB protein; iNOS, inducible nitric oxide synthase; KL-1156 compound, 6-hydroxy-7-methoxychroman-2-carboxylic acid phenylamide; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO, nitric oxide; PDTC, pyrrolidine dithiocarbamate; SEAP, secretory alkaline phosphatase.

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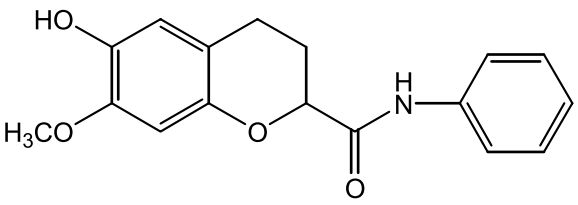

Fig. 1. Chemical structure of chroman KL-1156 compound, 6-hydroxy-7-methoxychroman-2-carboxylic acid phenylamide.

necrosis factor receptor-associated factor 6, which activate IκB kinase (IKK) complex [12–14]. Activation of IKK complex results in phosphorylation of IκB, which marks for ubiquitination followed by proteasome-mediated degradation [15,16]. IκB degradation unmasks the nuclear localization signal (NLS) of NF-κB, allowing the transcription factor to move to the nucleus, and then NF-κB binds to the promoter region of immune and inflammatory genes for transcriptional regulation [16,17].

In our ongoing study to discover anti-inflammatory agent, chemically synthetic 6-hydroxy-7-methoxychroman-2-carboxylic acid phenylamide (Fig. 1), named as chroman KL-1156 compound, was found to inhibit NO production in LPS-stimulated macrophages RAW 264.7. The chroman KL-1156 compound showed down-regulatory effect on LPS-induced iNOS expression at transcription level. As a mechanism of the anti-inflammatory action, KL-1156 compound inhibited LPS-induced NF-κB activation, specifically nuclear translocation of NF-κB p65 without affecting IκBα degradation.

Materials and methods

Materials and cell culture. LPS (*Escherichia coli* 055:B5) was purchased from Sigma–Aldrich (St. Louis, MO), and fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA). Antibodies against iNOS, NF-κB p65, and IκBα were obtained from Santa Cruz Biotech (Santa Cruz, CA), and antibody against phospho-IκBα (Ser-32/36) was from Cell Signaling Tech (Beverly, MA). Chemical preparation of chroman KL-1156 compound (purity, ⩾98%) was described elsewhere [18]. Macrophages RAW 264.7 were obtained from ATCC (Manassas, VA), 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, and 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 NF-κB-secretory alkaline phosphatase (SEAP)-NPT plasmid [19] were also grown under the same conditions except supplement of 500 μg/ml geneticin to the media.

Nitrite quantification. Macrophages RAW 264.7 were treated with 1 μg/ml LPS plus sample for 24 h. Nitrite content in the cell-free culture media was measured using Griess reagent [20]. Briefly, cell-free culture media (100 μl) were reacted with 1:1 mixture (100 μl) of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine in distilled water, and then absorbance at wavelength 540 nm was measured.

Western immunoblot analysis. Macrophages RAW 264.7 were treated with 1 μg/ml LPS plus sample for 5 min (phospho-IκBα), 10–50 min (IκBα), 1 h (NF-κB p65) or 18 h (iNOS). Western immunoblot analysis for phospho-IκBα, IκBα or iNOS 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 [21]. The blots were finally reacted with ECL detection reagent (Amersham–Pharmacia, San Francisco, CA) and exposed to X-ray film.

Reverse transcription-polymerase chain reaction. Macrophages RAW 264.7 were treated with 1 μg/ml LPS plus sample for 6 h. Total RNA was purified from the cells using a FastRNA kit (Bioneer, Daejeon, Korea) and subjected to semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) using an RNA PCR kit (Bioneer, Daejeon, Korea). Oligonucleotides used for quantification of iNOS and β-actin transcripts, and semi-quantitative RT-PCR conditions were described in our previous work [22]. RT-PCR products were resolved on 1.5% agarose gel by electrophoresis and stained with ethidium bromide.

Measurement of iNOS promoter activity. Macrophages RAW 264.7 were transiently transfected with iNOS-luciferase reporter plasmid [8] and pSV-β-galactosidase control vector (Promega, Madison, WI) using LipofectAMINE (Invitrogen, Carlsbad, CA). The transfected RAW 264.7 cells were treated with 1 μg/ml LPS plus sample for 16 h. Equal amounts (40 μg) of total protein from the cells were subjected to luciferase assay using Luciferase Reporter Assay System (Promega, Madison, WI) and β-galactosidase assay using β-Galactosidase Enzyme Assay System (Promega, Madison, WI).

Measurement of NF-κB transcriptional activity. Macrophages RAW 264.7 transfected stably with NF-κB-SEAP-NPT plasmid [19] were treated with 1 μg/ml LPS plus sample for 16 h. Aliquots of the

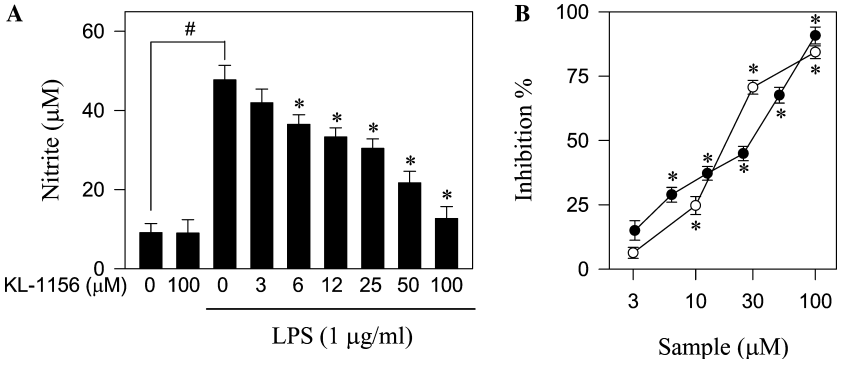

Fig. 2. LPS-induced NO production. Macrophages RAW 264.7 were treated with LPS (1 μg/ml) plus KL-1156 compound for 24 h. Amount of nitrite, a stable metabolite of NO, was measured with the cell-free media (A). Effects of KL-1156 compound (●) and PDTC (○) on NO production are represented as inhibition percentage (B). Values are means ± SEM ($n = 5$). $^{\#}p < 0.01$ vs. media alone-treated group. $^{*}p < 0.01$ vs. LPS alone-treated group.

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

Electrophoretic mobility shift assay (EMSA). Macrophages RAW 264.7 were treated with 1 μg/ml LPS plus sample for 1 h. Equal amounts (10 μg) of nuclear protein from the cells were reacted with ^{32}P-labeled oligonucleotide (Promega, Madison, WI) specific to NF-κB in a binding buffer (10 mM Tris–HCl, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 μg/ml poly(dI–dC), and 4% glycerol, pH 7.5) for 10 min on ice. The complexes between oligonucleotide and nuclear protein were resolved on non-denaturing 6% polyacrylamide gel by electrophoresis. The gels were dried and exposed to X-ray film.

Statistical analysis. Results are expressed as means ± SEM. Data were analyzed by ANOVA followed by Student's t test. A value of $p < 0.01$ was considered significant.

Results

Inhibitory effect of chroman KL-1156 compound on LPS-induced NO production in macrophages RAW 264.7

Resting macrophages RAW 264.7 released 9.1 ± 2.3 μM nitrite, a stable metabolite of NO, during incubation for 24 h, whereas the RAW 264.7 cells markedly increased NO production to 47.7 ± 3.7 μM nitrite by treatment of LPS alone (Fig. 2A). No significant difference in the NO production was found between resting RAW 264.7 cells and the cells treated with KL-1156 compound (100 μM) alone (Fig. 2A). KL-1156 compound inhibited LPS-induced NO production in a dose-dependent manner, corresponding to 28.9 ± 2.9% inhibition at 6 μM, 37.2 ± 2.7% at 12 μM, 44.9 ± 2.8% at 25 μM, 67.6 ± 3.1% at 50 μM, and 90.8 ± 3.3% at 100 μM, showing an IC_{50} value of 30.6 μM (Fig. 2B). As a positive control, pyrrolidine dithiocarbamate (PDTC) [23] also inhibited LPS-induced NO production in a dose-dependent manner with an IC_{50} value of 21.4 μM (Fig. 2B).

Down-regulation of iNOS expression by chroman KL-1156 compound in LPS-stimulated macrophages RAW 264.7

To examine whether inhibitory effect of KL-1156 compound on LPS-induced NO production was attributed to its influence on iNOS expression, Western immunoblot analysis was carried out. iNOS protein was hardly detectable in resting macrophages RAW 264.7, but pronounced amount of iNOS protein was induced upon exposure to LPS for 18 h (Fig. 3A). However, synthesis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping protein was not affected by treatment of LPS and KL-1156 compound (Fig. 3A). Treatment of KL-1156 compound to RAW 264.7 cells decreased LPS-induced synthesis of iNOS protein in a dose-dependent manner, corresponding to 51.4 ± 2.9% inhibition at 25 μM, 74.6 ± 3.5% at 50 μM, and 87.3 ± 4.6% at 100 μM (Fig. 3A).

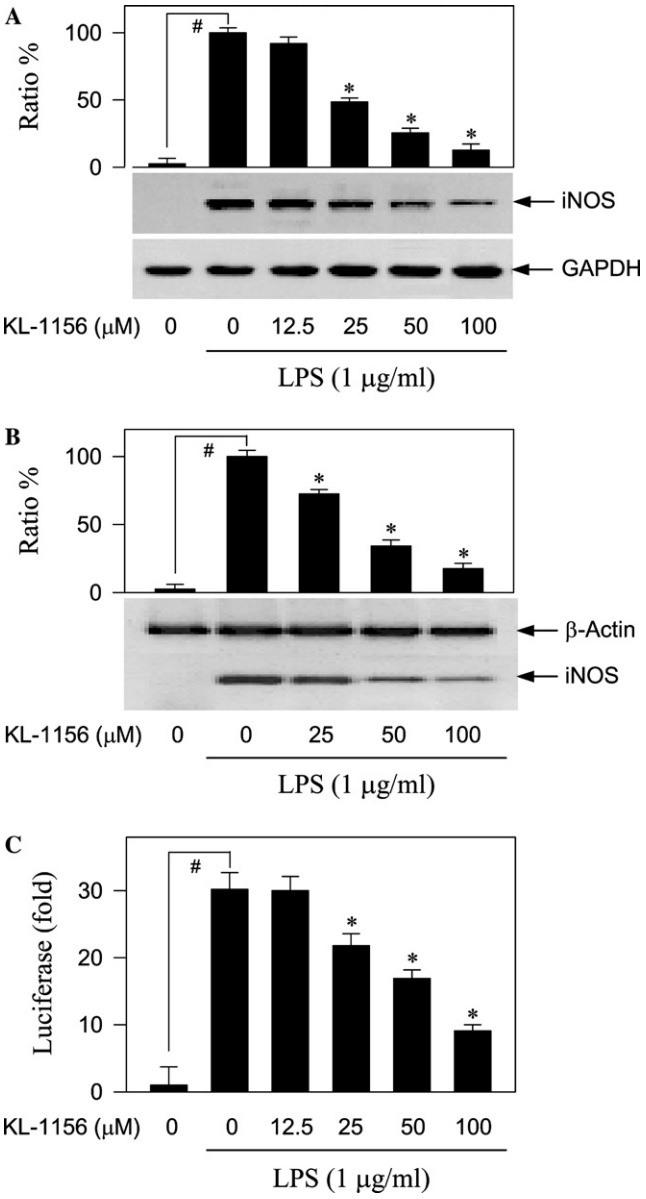

Fig. 3. LPS-induced iNOS expression. Macrophages RAW 264.7 were treated with LPS (1 μg/ml) plus KL-1156 compound for 18 h. Lysates of the cells were subjected to Western immunoblot analysis with anti-iNOS antibody. One of similar results is represented and relative ratio percentage is also shown, where iNOS signal was normalized to GAPDH signal (A). The cells were treated with LPS (1 μg/ml) plus KL-1156 compound for 6 h. Total RNA of the cells was subjected to semi-quantitative RT-PCR. One of similar results is represented and relative ratio percentage is also shown, where iNOS signal was normalized to β-actin signal (B). Macrophages RAW 264.7 transfected transiently with iNOS-luciferase reporter plasmid and pSV-β-galactosidase control vector were treated with LPS (1 μg/ml) plus KL-1156 compound for 16 h. Luciferase and β-galactosidase activities were measured with lysates of the cells. Luciferase expression as iNOS promoter activity is represented as relative fold, where luciferase activity was normalized to β-galactosidase activity (C). Values are means ± SEM ($n = 3$). $^{\#}p < 0.01$ vs. media alone-treated group. $^{*}p < 0.01$ vs. LPS alone-treated group.

To further understand whether inhibitory effect of KL-1156 compound on iNOS expression was influenced at transcription level, semi-quantitative RT-PCR for iNOS transcript was carried out. KL-1156 compound inhibited LPS-induced synthesis of iNOS transcript in a dose-dependent manner, corresponding to $27.4 \pm 3.1\%$ inhibition at 25 μM, $65.8 \pm 4.5\%$ at 50 μM, and $82.4 \pm 3.8\%$ at 100 μM (Fig. 3B). However, synthesis of housekeeping β-actin transcript was not affected by treatment of LPS and KL-1156 compound (Fig. 3B). Transcriptional regulation of iNOS expression by KL-1156 compound was further documented by iNOS promoter activity. The promoter activity was analyzed in macrophages RAW 264.7 transfected transiently with iNOS-luciferase plasmid containing murine iNOS promoter (−1592/+183) fused to luciferase as the reporter [8]. Treatment of LPS to the transfected cells increased luciferase expression to 30-fold over the basal level, and KL-1156 compound inhibited LPS-induced luciferase expression in a dose-dependent manner, corresponding to $28.9 \pm 2.9\%$ inhibition at 25 μM, $45.8 \pm 2.2\%$ at 25 μM, and $72.8 \pm 1.8\%$ at 100 μM (Fig. 3C).

Inhibitory effect of chroman KL-1156 compound on LPS-induced NF-κB transcriptional activity in macrophages RAW 264.7

NF-κB transcription factor has been evidenced to play an important role in LPS-induced expression of pro-inflammatory proteins including iNOS [8,11,24]. NF-κB transcriptional activity was monitored using macrophages RAW 264.7 transfected stably with NF-κB-SEAP-NPT plasmid containing four copies of κB sequence fused to SEAP as the reporter [19]. Treatment of LPS to the transfected cells increased SEAP expression to about 3-fold over the basal level, indicating that cellular NF-κB is transcriptionally functional (Fig. 4A). No significant difference in the SEAP expression was found between resting RAW 264.7 cells and the cells treated with KL-1156 compound (100 μM) alone (Fig. 4A). KL-1156 compound inhibited LPS-induced SEAP expression in a dose-dependent manner, corresponding to $38.8 \pm 3.6\%$ inhibition at 25 μM, $57.0 \pm 3.1\%$ at 50 μM, and $88.7 \pm 3.3\%$ at 100 μM, showing an IC_{50} value of 40.4 μM (Fig. 4B). PDTC [23] also inhibited LPS-induced SEAP expression in a dose-dependent manner with an IC_{50} value of 37.2 μM (Fig. 4B).

Inhibitory mechanism of chroman KL-1156 compound on NF-κB activation in LPS-stimulated macrophages RAW 264.7

To elucidate inhibitory mechanism on NF-κB activation, we next determined whether KL-1156 compound could interfere with DNA binding activity of NF-κB in LPS-stimulated macrophages RAW 264.7, that was analyzed by EMSA with a ^{32}P-labeled oligonucleotide corresponding to the κB sequence. Treatment of LPS to RAW 264.7 cells markedly increased DNA binding activity of NF-κB complex, NF-κB p65/50 and p50/p50, within 1 h (Fig. 5A). KL-1156 compound decreased LPS-induced DNA binding activity of NF-κB complex in a dose-dependent manner (Fig. 5A). To further investigate whether KL-1156 compound could affect 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 nuclear NF-κB p65 was markedly increased upon exposure to LPS for 1 h (Fig. 5B). KL-1156 compound inhibited LPS-induced nuclear translocation of NF-κB p65 in a dose-dependent manner, corresponding to $55.1 \pm 4.8\%$ inhibition at 50 μM and $73.5 \pm 3.7\%$ at 100 μM (Fig. 5B).

Another Western immunoblot was carried out with cytoplasmic extracts of LPS-stimulated macrophages RAW 264.7. IκBα degradation dramatically happened within 20 min upon exposure to LPS, and amount of IκBα at cytoplasm was recovered to basal level at

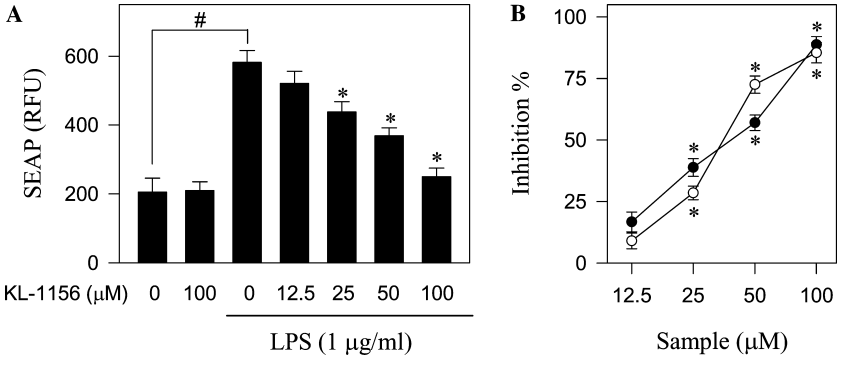

Fig. 4. LPS-induced NF-κB transcriptional activity. Macrophages RAW 264.7 harboring NF-κB-SEAP-NPT reporter plasmid were treated with LPS (1 μg/ml) plus KL-1156 compound for 16 h. SEAP activity as NF-κB transcriptional activity was measured with the cell-free media, and is represented as relative fluorescence unit (RFU) (A). Effects of KL-1156 compound (●) and PDTC (○) on NF-κB transcriptional activity are represented as inhibition percentage (B). Values are means ± SEM ($n = 5$). $^{\#}p < 0.01$ vs. media alone-treated group. $^{*}p < 0.01$ vs. LPS alone-treated group.

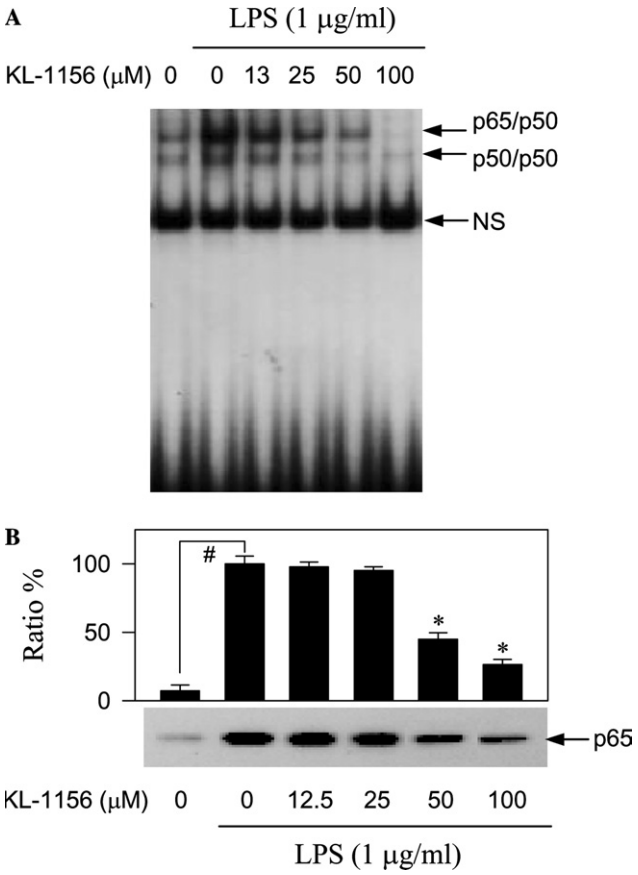

Fig. 5. DNA binding activity of NF-κB complex and nuclear translocation of NF-κB p65. Macrophages RAW 264.7 were treated with LPS (1 μg/ml) plus KL-1156 compound for 1 h. Nuclear extracts of the cells were reacted with ^{32}P-labeled oligonucleotide specific to NF-κB. EMSA is represented, where NF-κB complex of p65/p50 or p50/p50, and non-specific signal (NS) are indicated by an arrow (A). The nuclear extracts were subjected to Western immunoblot analysis with anti-NF-κB p65 antibody. One of similar results is represented and relative ratio percentage is also shown, where NF-κB p65 was normalized to nuclear protein content (B). Values are means ± SEM ($n = 3$). $^{\#}p < 0.01$ vs. media alone-treated group. $^{*}p < 0.01$ vs. LPS alone-treated group.

50 min after LPS stimulation (Fig. 6A). Under the same conditions, KL-1156 compound (100 μM) did not inhibit or delay LPS-induced IκBα degradation and recovery (Fig. 6A). Phosphorylation of IκBα at Ser-32 and 36 residues was also analyzed by Western immunoblot analysis. Phosphorylated forms of IκBα were hardly detectable in resting macrophages RAW 264.7, but treatment of LPS to the cells induced IκBα phosphorylation within 5 min (Fig. 6B). KL-1156 compound (12.5–100 μM) did not inhibit LPS-induced IκBα phosphorylation, at all (Fig. 6B).

Discussion

In the present study, novel chroman KL-1156 compound (Fig. 1) was discovered to show an IC_{50} value of 30.6 μM on LPS-induced NO production in macrophages RAW 264.7 (Fig. 2). KL-1156 compound inhibited LPS-induced mRNA and protein syntheses of iNOS, in parallel (Figs. 3A and B). Furthermore, KL-1156 compound attenuated LPS-induced iNOS promoter activity (Fig. 3C), indicating that KL-1156 compound down-regulated iNOS expression at transcription level.

NF-κB activation has been evidenced as major mechanism for LPS-induced iNOS expression [8,24]. KL-1156 compound inhibited LPS-induced NF-κB transcriptional activity in a dose-dependent manner with an IC_{50} value of 40.4 μM (Fig. 4). KL-1156 compound showed inhibitory effects on LPS-induced DNA binding activity of NF-κB complex and nuclear translocation of NF-κB p65, in parallel (Fig. 5). However, KL-1156 compound did not influence LPS-induced IκBα degradation (Fig. 6). These results indicate that KL-1156 compound could inhibit LPS-induced nuclear translocation of NF-κB p65 without affecting IκBα

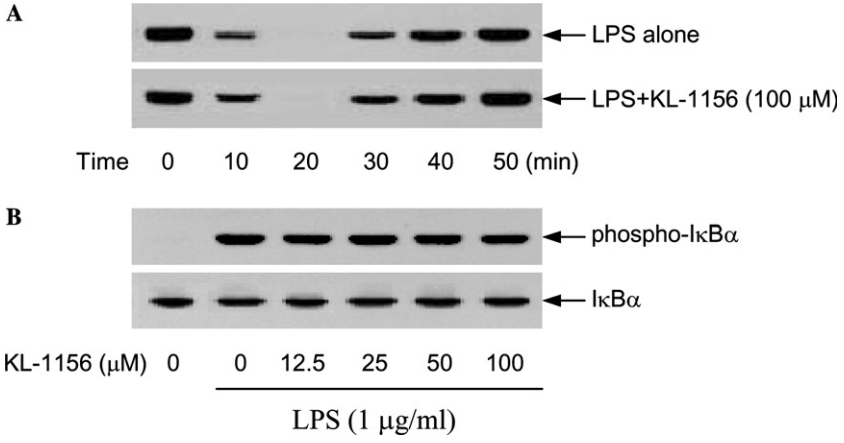

Fig. 6. Degradation and phosphorylation of IκBα. Macrophages RAW 264.7 were treated with LPS (1 μg/ml) alone or LPS (1 μg/ml) plus KL-1156 compound (100 μM) for indicated times, and cytoplasmic extracts of the cells were subjected to Western immunoblot analysis with anti-IκBα antibody (A). The cells were treated with LPS (1 μg/ml) plus KL-1156 compound for 5 min. Cytoplasmic extracts of the cells were subjected to Western immunoblot analysis with anti-phospho-IκBα (Ser-32/36) antibody or anti-IκBα antibody (B).

degradation, which is a rare mechanism as the control point of NF-κB activation.

Synthetic peptide SN50, containing a hydrophobic membrane-translocating region and the NLS of NF-κB p50, was reported to inhibit nuclear translocation of NF-κB p50 in response to LPS and TNF-α [25]. NF-κB free of IκB is recognized by karyopherin α, and they interact with karyopherin β and GTP-binding Ran protein [26]. The complex bound to nuclear pore and then NF-κB is transported from cytoplasm to nucleus. Target of synthetic peptide SN50 is the NLS on NF-κB p50 that is recognized by karyopherin α [25]. Even though molecular target of chroman KL-1156 compound would be elucidated, this study demonstrated that non-peptide compound could interfere with nuclear localization step of NF-κB p65 without affecting IκBα degradation.

In conclusion, KL-1156 compound inhibited LPS-induced NO production by down-regulatory action on iNOS expression at transcription level. As a mechanism of the anti-inflammatory action shown by KL-1156 compound, interference of LPS-induced NF-κB activation, specifically nuclear translocation step of NF-κB p65, has been demonstrated in this study.

Acknowledgments

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