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Brief Article

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A Novel Naturally Occurring Salicylic Acid Analogue Acts as an Anti-Inflammatory Agent by Inhibiting Nuclear Factor-kappaB Activity in RAW264.7 Macrophages

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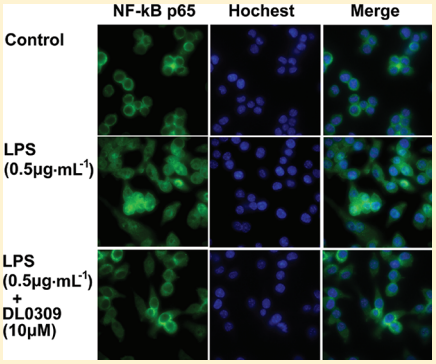
ABSTRACT: Methyl salicylate 2-*O*-*β*-D-lactoside (DL0309), is a molecule chemically related to salicylic acid that is isolated from *Gaultheria yunnanensis* (FRANCH.) REHDER (*G. yunnanensis*). *G. yunnanensis*, a traditional Chinese herbal medicine, is widely used for treating rheumatoid arthritis, swelling, pain, trauma, and chronic tracheitis. In the present study, we explored the mechanism whereby DL0309 exerts anti-inflammatory effects, using the model of lipopolysaccharide (LPS)-treated RAW264.7 cells. We examined the effects of DL0309 on LPS-induced nuclear factor-kappaB (NF-κB) activity by Western blot analysis, cell imaging analysis and an electrophoretic mobility shift assay (EMSA). Production of pro-inflammatory cytokines was also measured. Our observations indicate that DL0309 suppressed production of nitric oxide (NO), reactive oxygen species (ROS) and the pro-inflammatory cytokines, such as tumor necrosis factor-*α* (TNF-*α*), interleukin-6 (IL-6), and interleukin-1*β* (IL-1*β*), in a concentration-dependent manner. The phosphorylation of IKK-*β* and degradation of IκB-*α* by LPS were both inhibited by DL0309 in the cytoplasm. The increased protein level of NF-κB by LPS in the nucleus was also reduced by DL0309. Consistent with these results, we found that DL0309 prevents the nuclear translocation and DNA binding activity of NF-κB. Finally, our results demonstrate that DL0309 exerts anti-inflammatory effects, by inhibiting the production of pro-inflammatory cytokines and suppressing of the activation of the NF-κB signaling pathway in LPS-treated macrophage cells. Therefore, DL0309 may have therapeutic potential for treating inflammatory diseases by regulating the NF-κB pathway and pro-inflammatory cytokine production.



KEYWORDS: *Gaultheria yunnanensis, anti-inflammation, NF-κB signaling pathway, methyl salicylate 2-O-β-D-lactoside, TNF-α*

INTRODUCTION

In the eighteenth century, salicylic acid was first identified as an active anti-inflammatory compound.[1] Acetylsalicylic acid (aspirin), which was synthesized to eliminate the irritant and bitter-tasting properties of salicylic acid, retains the anti-inflammatory action of salicylic acid, and has been widely used for its analgesic, anti-inflammatory and antipyretic actions for over 100 years.[2] However, its unwanted effects, particularly gastric bleeding, are a serious disadvantage.[3]

NF-κB, a critical regulator of the innate early pathogen response, regulates transcription of multiple inflammatory factors and cytokines. NF-κB, mainly the p50/p65 heterodimer, normally exists as an inactive state tightly bound to the inhibitory protein of IκB in the cytoplasm.[4] The NF-κB/IκB complex is activated by numerous diverse factors, including UV light, cytokines, viral infection, LPS, and reactive oxygen intermediates. The dissociation of the NF-κB/IκB complex is mediated when IκB is phosphorylated by IκB kinase (IKK), an enzyme that is activated by various stimuli associated with tissue damage. The phosphorylation of IκB leads to the ubiquitination and degradation of IκB. As a result, NF-κB is released from its inhibition by IκB, and activated NF-κB is then translocated into the nucleus.[5] Kopp and Ghosh[6] first reported that acetylsalicylic acid and sodium salicylate inhibited the activation of NF-κB transcription factor. Kopp and Ghosh[6] also suggested that other nonsteroidal anti-inflammatory drugs may also exert anti-inflammation by acting at the transcriptional level.

Gaultheria yunnanensis (FRANCH.) REHDER (*G. yunnanensis*) is a traditional Chinese herbal medicine that is a member of the Ericaceae family and is grown in the southern regions of China. It has been widely used in these districts as a folk medicine for the treatment of various painful inflammatory conditions.[7] Methyl salicylate 2-*O*-*β*-D-lactoside (DL0309, Figure 1) is a natural product isolated from *G. yunnanensis*.

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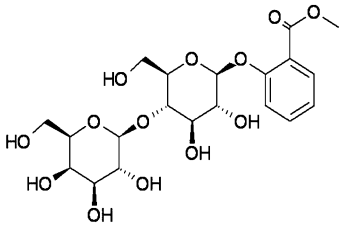

Figure 1. The chemical structure of DL0309 (methyl salicylate 2-*O*-*β*-D-lactoside).

The DL0309 molecule chemically related to salicylic acid is a 2-*O*-lactoside of methyl salicylate. In previous studies, we found that DL0309 showed anti-inflammatory and analgesic properties in a mouse model of croton oil-induced ear swelling.[8] However, the molecular mechanisms underlying its anti-inflammatory effect remain unclear. In the present study, we investigated the anti-inflammatory effects and action mechanism of DL0309 on LPS-induced RAW264.7 macrophage cells. Our findings suggest that DL0309 blocks LPS-induced NF-*κ*B activity by interfering with IKK-*β* phosphorylation.

EXPERIMENTAL SECTION

Chemical and Biological Materials. DL0309 (methyl salicylate 2-*O*-*β*-D-lactoside) was synthesized by the Institute of Materia Medica at the Chinese Academy of Medical Sciences.[8] The DL0309 used in this paper is of 99% purity as confirmed by HPLC. Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and the antibiotic−antimycotic solution were purchased from Gibco (Aukland, NZ). The ELISA kits for TNF-*α*, IL-1*β* and IL-6 were obtained from Jiamei Biotech Co. (Beijing, China). The LightShift chemiluminescence EMSA kit (Pierce) was purchased from Thermo Scientific Co.

Antibodies against NF-*κ*B, I*κ*B-*α*, phosphorylated or total IKK-*β* were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies and primary antibodies against GAPDH or histone 3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture. The RAW264.7 murine macrophage cell line (ATCC TIB-71; American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU mL^{-1} penicillin and 100 $\mu g\ mL^{-1}$ streptomycin in a 37 °C incubator with 5% CO_2. For all experiments, the cells were grown to 80−90% confluence, with no more than 20 passages. Cells were stimulated by LPS (0.5 $\mu g\ mL^{-1}$) in the presence or absence of DL0309 for the measurement of the production of pro-inflammatory cytokines, accumulation of nitric oxide (NO), reactive oxygen species (ROS) release, and NF-*κ*B expression.

Cell Viability Assay. Cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described by Lombardi et al.[9] RAW264.7 cells were seeded at a density of 10^4 cells per well in a 96-well plate to determine any potential cytotoxicity. Cells were serum-starved for 12 h and then treated with DL0309 (0.01−100 μM) for a further 24 h at 37 °C in a 5% CO_2 humidified incubator, and then incubated in 0.5 mg mL^{-1} MTT solution. After 3 h incubation, the supernatant was taken and measured at 540 nm using a microplate reader (SpectraMax M5; Molecular Devices).

Enzyme-Linked Immunosorbent Assay. RAW264.7 cells were cultured in 96-well plates (1×10^4 cell mL^{-1}) and preincubated with DL0309 for 1 h, followed by a further 12 h treatment with LPS for measurement of TNF-*α* and IL-6, or for 18 h for measurement of IL-1*β*. TNF-*α*, IL-1*β* and IL-6 contents in the culture medium were measured by ELISA using anti-mouse TNF-*α*, IL-1*β* or IL-6 antibodies and a biotinylated secondary antibody, according to the manufacturer's instructions. The optical density of each well was measured at 450 nm with an ELISA reader (Molecular Devices 5, Menlo Park, CA, USA).

NF-*κ*B Nuclear Translocation Assay. The nuclear translocation of NF-*κ*B p65 was examined by an indirect immunofluorescence assay using a cellular NF-*κ*B activation kit (Thermo Fisher, Rockford, IL, USA). Briefly, RAW264.7 cells were cultured in 96-well plates (1×10^4 cells per well) and pretreated with DL0309 (0.1, 1.0, and 10 μM) for 1 h prior to incubation with LPS for 30 min. The cells were then washed and fixed with a 4% paraformaldehyde for 30 min at 37 °C, permeabilized with 0.3% Triton X-100 for 10 min, and blocked with 3% BSA for 30 min at room temperature. Next, cells were incubated with primary anti-NF-*κ*B p65 antibody for 1 h at room temperature, followed by DyLight 488-conjugated secondary antibody. After being washed with PBS, cells were incubated in 10 μM Hoechst solution for 10 min in the dark. The fluorescence of the p65 protein and nuclei is green and blue, respectively, and live cell imaging was simultaneously viewed using a microscopy system (DeltaVision core, API Co., Issaquah, WA, USA) at an excitation wavelength of 350 nm for Hoechst and 494 nm for DyLight 488.

Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA). RAW264.7 cells were plated in 100 mm dishes (1×10^6 cells per dish) and grown in complete DMEM until confluent. The cells were preincubated with different concentrations of DL0309 (0.1, 1.0, and 10 μM) for 1 h and then stimulated with 0.5 $\mu g\ mL^{-1}$ LPS for 30 min. The cells were washed once with cold phosphate buffered saline (PBS) and then scraped off and centrifuged to collect the pellets. Nuclear extracts were prepared according to the methods described by Wang et al.[10] The EMSA assay was performed as previously described with minor modifications.[11] Briefly, oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′, IDTDNA Technologies; Coralville, IA) was synthesized as a probe for the gel retardation assay, and the probe was labeled with biotin (Pierce). The binding reactions contained 10 μg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40 and 2.5% glycerol), 50 ng of poly(dI-dC) and 20 fM of biotinylated DNA. The samples were incubated for 20 min at room temperature in a final volume of 20 μL. In all experiments, DNA-binding specificity was verified using 50-fold excess of unlabeled probe to the reaction mixture. The reaction mixture was analyzed by electrophoresis in a 5% polyacrylamide gel in 0.5 × Tris-borate buffer (Tris-HCl 89 mM, boric acid 89 mM, EDTA 2 mM, pH 8.0). The reactions were transferred to nylon membranes, and the biotinylated DNA was detected using a LightShift chemiluminescent EMSA kit (Pierce).

Western Blot Assay. The RAW264.7 cells were seeded in 6-well plates. The cells were pretreated with DL0309 at different concentrations of 0.1, 1.0, and 10 μM for 1 h and then

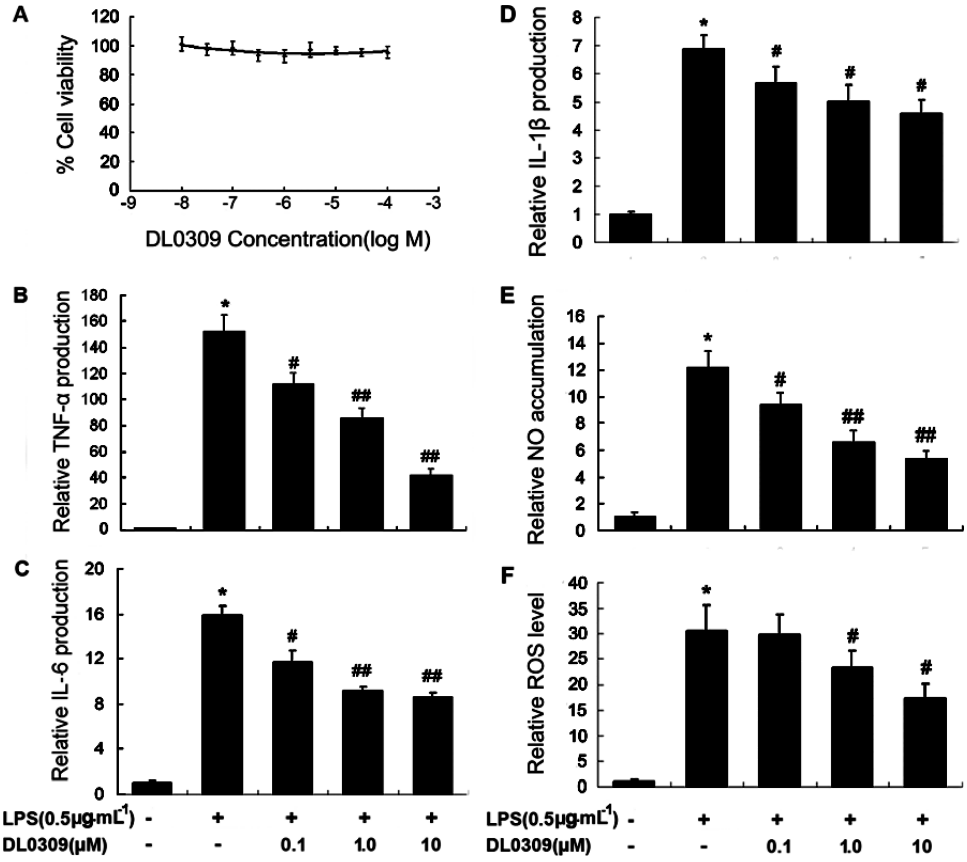

Figure 2. The effects of DL0309 on the production of pro-inflammatory factors by LPS-induced RAW264.7 cells. (A) Cell viability was assessed by MTT assays, RAW264.7 cells were treated with the indicated concentrations of DL0309 (0.01−100 μM) for 24 h, and the results are expressed by percentage of surviving cells over control cells (no addition of DL0309). (B−D) Measurement of secreted TNF-α, IL-6, IL-1β by ELISA; cells were treated with 0.1, 1.0, 10 μM DL0309 for 1 h and continuously incubated with LPS (0.5 μg mL^{-1}) for 12 h (TNF-α and IL-6) or 18 h (IL-1β). (E, F) Measurement of NO and ROS contents in culture medium monitored as described in the Experimental Section. Data represent the means ± SEM from three separate experiments. *$p < 0.01$, significant compared with untreated control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant compared with LPS alone.

exposed to LPS (0.5 μg mL^{-1}) for 30 min. Cytosolic and nucleic protein extracts were prepared as described by Schreiber et al.[12] 10 μg of a protein extract was resolved on a 10% SDS−PAGE gel, transferred onto a membrane, and blocked with 5% nonfat milk in Tris-buffered saline (TBS), with 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were washed three times in TBS-T for 5 min and then incubated at 4 °C overnight with anti-NF-κB p65, polyclonal anti-IKK-β, antiphospho-IKK-β, rabbit polyclonal anti-IκB-α, and antiphospho-IκB-α. Membranes were washed three times in TBS-T for 5 min and incubated with the appropriate peroxidase-conjugated secondary antibodies in TBS. After another three washes with TBS-T for 5 min, the membranes were reacted with the enhanced chemiluminescence system (Amersham Pharmacia), according to the manufacturer's protocol, and they were then exposed to films. Protein levels were quantified by scanning densitometry using ImageJ analysis systems (Scion, Frederick, MD, USA).

Measurement of NO Release. RAW264.7 cells were pretreated by DL0309 for 1 h and stimulated by LPS (0.5 μg mL^{-1}) after 12 h of incubation. NO production was estimated from the amount of stable nitrite produced in the cell culture supernatants measured photometrically by the Griess assay[13] against a standard curve obtained with different concentrations of sodium nitrite. Each experiment was performed at least four times in duplicate.

Production of Reactive Oxygen Species (ROS). Intracellular ROS production was measured by incubating the cells with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), as previously described.[14] This method is based on the oxidation of DCFH-DA by ROS resulting in the formation of the fluorescent compound 2′,7′-dichlorofluorescein (DCF). RAW264.7 cells were seeded (1×10^4 cells per well) in 96-well plates and treated with increasing concentrations (0.1−10 μM) of DL0309 for 1 h before stimulation with LPS (0.5 μg mL^{-1}) for 12 h. Then, DCFH-DA (10 μM) was added to incubate for an additional 45 min. Accumulation of DCF was measured as an increase in the fluorescence at 525 nm, when the samples were excited at 488 nm using a microplate spectrofluorometer (Molecular Devices 5, Menlo Park, CA, USA).

Statistical Analysis. Results are expressed as the mean ± SEM of at least three experiments. Statistical significance was evaluated by one-way ANOVA followed by Student's t test for paired populations. P values <0.05 are considered statistically significant.

RESULTS

DL0309 Is Not Cytotoxic on RAW264.7 Cells. Exposure of RAW264.7 cells to DL0309 (0.01−100 μM) for 24 h caused no reduction of cell viability as measured by the MTT assay

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(Figure 2 A). In the subsequent experiments, concentrations of DL0309 did not exceed 100 μM.

Production of Pro-Inflammatory Cytokines. RAW264.7 cells were treated with LPS (0.5 μg mL^{-1}) with or without pretreatment with DL0309 in different concentrations. Release of cytokines in the cell culture medium was measured by ELISA assays. As shown in Figure 2B–D, LPS treatment for 12 h increased the production of TNF-α and IL-6, and treatment for 18 h increased IL-1β production. Preincubated cells with DL0309 at concentrations of 0.1, 1.0, and 10 μM dose-dependently inhibited the production of TNF-α by 26, 44, and 72%, respectively, and inhibited IL-6 production by 26, 40, and 45%, respectively, and reduced IL-1β level by 17, 27, and 33%, respectively.

DL0309 Inhibits NF-κB Translocation to Nucleus. We also investigated the effect of DL0309 on LPS-induced NF-κB nuclear translocation, as translocation of NF-κB to the nucleus has been shown to be required for NF-κB-dependent transcription following LPS stimulation. NF-κB p65 translocation was analyzed by an indirect immunofluorescence assay. As shown in Figure 3, compared to untreated cells (shown as Control), intracellular p65 clearly translocated into the nucleus from the cytoplasm of cells induced with LPS alone. The DL0309 pretreated cells showed an inhibition of this translocation.

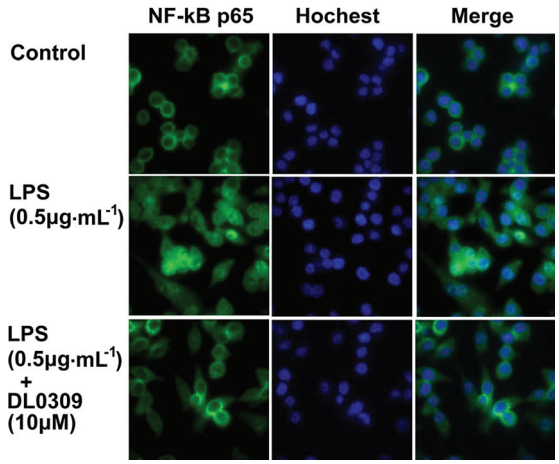

Figure 3. Effects of DL0309 on the nuclear translocation of the p65 protein. For the immunofluorescence assay for NF-κB p65 nuclear translocation, cells were pretreated with DL0309 for 1 h, and then 0.5 μg mL^{-1} of LPS was treated for 30 min. Images, from left to right, represent the p65 protein, nucleus and merged images with p65 protein and nucleus. p65 was detected by DyLight 488-labeled immunostaining (green); nuclei were stained by Hoechst (blue).

DL0309 Abolished the LPS-Mediated DNA Binding Activity of NF-κB. The NF-κB pathway plays a crucial role in LPS-induced macrophage activation, mediating the transcription of a number of genes involved in inflammation, and the NF-κB-DNA binding is directly initial trigger. The inhibition of LPS-induced NF-κB DNA binding activity by DL0309 was detected by EMSA in RAW264.7 cells. As shown in Figure 4A, LPS (0.5 μg mL^{-1}, 30 min) treatment caused a significant increase in the DNA binding activity of NF-κB, while a concentration-dependent reduction of NF-κB DNA binding complexes was observed in cells pretreated (1 h before LPS) with DL0309. 10 μM DL0309 almost abolished the LPS-induced NF-κB DNA binding.

Dose-Dependent Reduction of Expression of Proteins in the NF-κB Pathway. Phosphorylation of IκB-α kinase (IKK-β) and degradation of IκB-α are known to be involved in the process of the activation of NF-κB.[15] To determine the effect of DL0309 on the NF-κB pathway, the relative cytoplasmic levels of NF-κB, IKK-β, and IκB-α, and the nuclear level of NF-κB were examined by Western blot analysis. As shown in Figure 4B–E, after 30 min of LPS stimulation, the phosphorylated IKK-β was detected to be significantly increased and the degradation of IκB-α was observed in cytoplasma. LPS also decreased the NF-κB level in the cytoplasm, but increased the NF-κB level in the nucleus. Preincubation of the cells with DL0309 for 1 h before the addition of LPS for 30 min markedly inhibited the phosphorylation of IKK-β, the degradation of IκB-α in the cytoplasm, and the nuclear level of NF-κB in a dose-dependent manner.

Effect of DL0309 on NO Production. NO production was determined by measuring nitrite released into the culture medium using the Griess reagent. The result showed that incubation with LPS alone markedly increased (about 12-fold) NO production from the cells, compared with that generated under control conditions. However, preincubated cells with DL0309 at concentrations of 0.1, 1.0, and 10 μM dose-dependently inhibited the production of NO by 25, 49 and 58%, respectively. (Figure 2E).

Antioxidant Effect of DL0309. To determine the effect of DL0309 on intracellular ROS, the LPS-induced ROS level was detected in RAW264.7 cells in the presence or absence of DL0309. The production of ROS induced by LPS was increased (over 30-fold) significantly compared to LPS-untreated cells. However, as shown in Figure 2F, LPS-induced ROS production in RAW264.7 cell was inhibited by DL0309 in concentration of 1.0 and 10 μM of 20% and 49%, respectively.

DISCUSSION

Current clinical studies have raised concerns for long-term use of NSAIDs. In general, this is not only due to the gastrointestinal toxicity of NSAIDs but also because of an increased risk of cardiovascular adverse events in patients who used NSAIDs.[16,17] There is, therefore, a need for new drug candidates. Our previously unpublished study indicated that DL0309 not only has a significant anti-inflammatory effect but also possesses a reduced gastrointestinal tract toxicity compared with aspirin, according to investigation of a gastric mucosal lesion by a single administration of DL0309 or aspirin in rat. Here, we aimed to explore the mechanism of DL0309, a natural salicylate derivative extracted from *G. yunnanensis*, underlying the anti-inflammatory effects in LPS-treated RAW264.7 cells. We demonstrated that DL0309 suppressed the LPS-induced IKK/NF-κB activation *in vitro*.

Inflammation is a host response to harmful stimuli and is initiated by complex processes triggered by microbial pathogens such as LPS, which is a prototypical endotoxin.[18] LPS can directly activate macrophages, in which the production of inflammatory cytokines is increased.[19] Pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are small secreted proteins that mediate and regulate immunity and inflammation. LPS acts on macrophages to release TNF-α, and subsequently, the secreted TNF-α or LPS further induces the cells to release IL-1β and IL-6.[20] On the one hand, NF-κB activation mediates the transactivation of pro-inflammatory genes, including TNF-α, IL-1β and IL-6.[21,22] On the other hand, TNF-α and IL-1β

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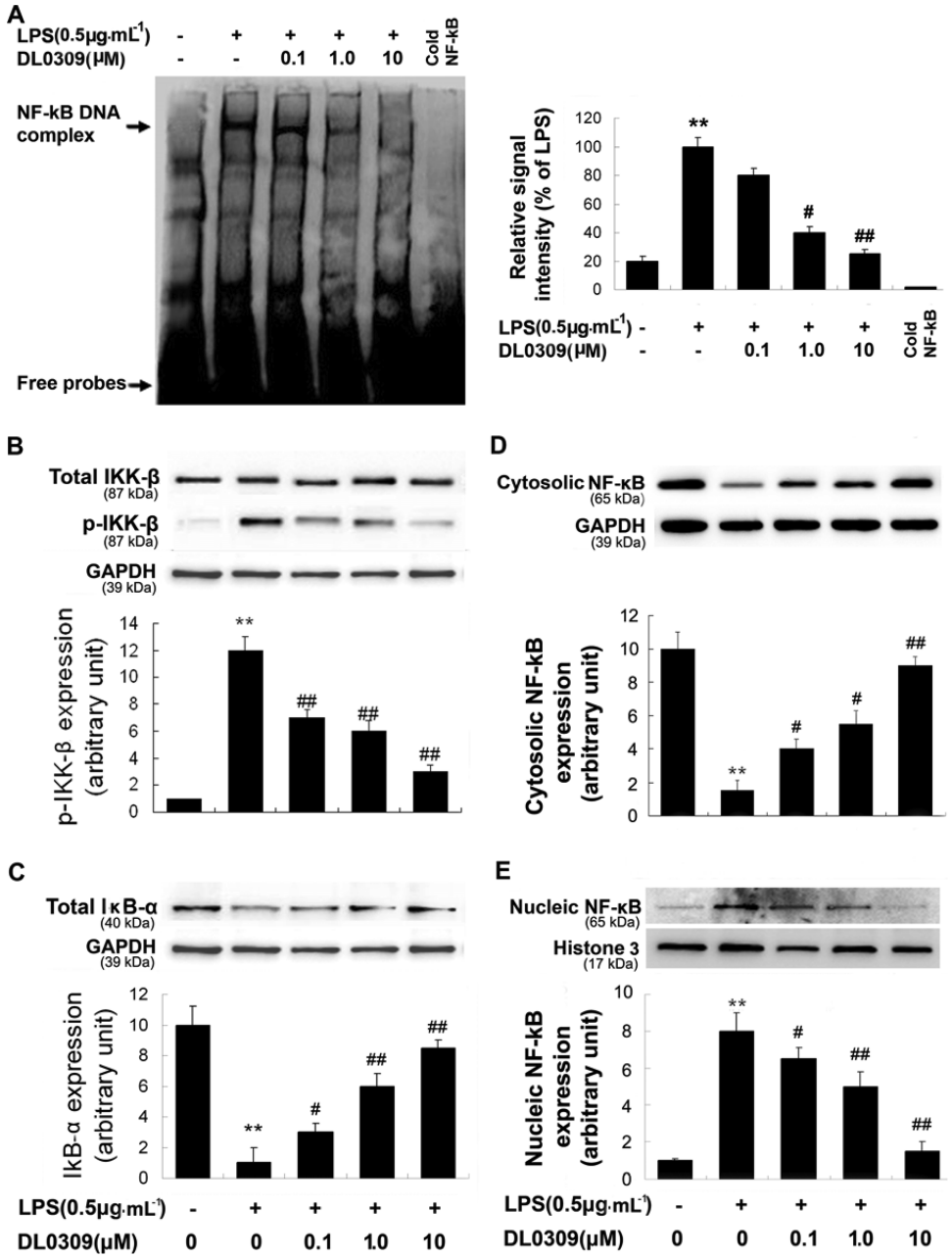

Figure 4. Effects of DL0309 on LPS-induced NF-κB DNA binding and the activation of the IKK/NF-κB signal pathway. RAW264.7 cells were pretreated with DL0309 at different concentrations of 0.1, 1.0, and 10 μM for 1 h and then exposed to LPS (0.5 μg mL^{-1}) for another 30 min. For NF-κB DNA binding (A), nuclear extracts were prepared and assayed for NF-κB by EMSA on a 5% polyacrylamide gel with a biotin-labeled double-stranded oligonucleotide containing the NF-κB consensus sequence. Binding competition assays were performed with a 50-fold excess of unlabeled NF-κB oligonucleotide as the competitor (cold NF-κB). Cytosolic and nucleic protein activation of the IKK/NF-κB signal pathway was analyzed by Western blot. Effects of DL0309 on total and phosphor-IKK-β (B), total IκB-α (C), cytosolic (D) and nucleic (E) NF-κB expression. The bands for phospho-IKK-β, IκB-α, and cytosolic NF-κB were normalized to GAPDH. Nucleic NF-κB was normalized to histone 3. Data represent the means ± SEM from three separate experiments. *$p < 0.01$, significant compared with untreated control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant compared with LPS alone.

directly activate NF-κB and increase the initial inflammatory responses.[23] Here, we measured the inhibitory effect of DL0309 on LPS-treated macrophages. The results showed that DL0309 significantly inhibits the production of TNF-α, IL-1β and IL-6.

Since the first report on the inhibition of NF-κB activation by sodium salicylate and acetylsalicylic acid (aspirin) by Kopp and Ghosh,[6] much interest has focused on this transcription factor as an important regulator of various genes involved in immune and inflammatory responses.[24] In resting cells, the NF-κB protein is retained in the cytoplasm as a complex with the inhibitory proteins of IκB, which masks the nuclear localization signal. Upon stimulation of the cells with stimuli such as LPS or TNF-α, the activated IKK induces the phosphorylation of IκB and the subsequent degradation to generate transcriptionally active NF-κB. Free NF-κB is translocated into the nucleus, where it binds to its cognate sites present in the pro-inflammatory genes and activates the transcription of these genes. In the present study, the translocation and binding of

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NF-κB were investigated. We observed that the p65 protein is significantly translocated into the nucleus after 30 min of LPS treatment, and this translocation is suppressed by DL0309, especially when a high dosage is used. The EMSA assay further confirmed that DL0309 inhibited p65 DNA binding activity.

The IκB kinase (IKK) complex contains two catalytic subunits, IKK-α and IKK-β, and controls the activation of NF-κB transcription factors, which play a pivotal role in inflammation. Ample evidence indicates that IKK-β mediates NF-κB activation in response to pro-inflammatory cytokines.[25] In this study, the cytoplasmic levels of NF-κB, IκB-α, and total and phosphorylated IKK-β were examined by Western blot. Our findings showed that LPS significantly increases the phosphorylation of IKK-β in RAW264.7 cells after 30 min of stimulation and decreases the abundance of IκB-α, and DL0309 treatment significantly depletes the LPS-induced phosphorylation of IKK-β and suppresses the degradation of IκB-α.

Many natural and synthetic agents, although diverse in effects and differences in structures, were reported to inhibit NF-κB activation.[26] These agents target different step(s) in the NF-κB signal pathway including phosphorylation, ubiquitination, degradation, nuclear translocation, and DNA binding. For example, sanguinarine (a benzophenanthridine alkaloid), a known anti-inflammatory agent, is a potent inhibitor of NF-κB activation by blocking the phosphorylation and degradation of IκBα.[27] Cilostazol is a specific inhibitor of 3′-5′-cyclic adenosine monophosphate (cAMP) phosphodiesterase, which is widely used for treatment of ischemic symptoms of peripheral vascular disease. It has been shown that cilostazol exhibits anti-inflammatory effects by inhibiting the DNA binding and transcriptional activity of NF-κB.[28] However, our results indicated that the inhibitory effect of DL0309 on the NF-κB signal pathway is mediated by preventing the phosphorylation of IKK-β. Other NSAIDs, such as aspirin and salicylate, were also reported to exert a similar action on the IKK/NF-κB signal pathway by inhibiting phosphorylation of IKK-β.[29] Of note, sodium salicylate or aspirin inhibits NF-κB activation at concentrations more than 5 mM.[30] However, 10 μM DL0309 exerts even greater potency of inhibition.

Induction of nitric oxide (NO) synthesis has been identified as one of the major responses to inflammatory stimuli in macrophages.[31] Several studies have indicated a superpharmacological concentration of aspirin or sodium salicylate can inhibit the NO accumulation in LPS-treated macrophage cells.[32,33] To evaluate the effect of DL0309 on LPS-treated RAW264.7 cells, we examined the NO synthesis. The results indicated that DL0309 significantly inhibited the generation of NO with concentrations of 0.1, 1.0, and 10 μM in RAW264.7 cells. It is well-known that the transcription of NF-κB is critical for the transcriptional regulation of iNOS.[34] Although nitrite accumulation, which represents the cumulative effect of iNOS expression from induction of the enzyme, does not directly assess the effects of pharmacologic agents, the reason for the NO reduction is partially due to the results of the DL0309 inhibition on NF-κB activation.

ROS have been associated with the initiation or aggravation of diverse pathological states. The production of ROS by monocytes/macrophages at inflammatory sites affects the process of inflammation. ROS is known to affect the expression of a number of genes through their effects on NF- κB.[35] As a second messenger, the first signaling proteins to be recognized as oxidative stress-sensitive molecules were actually transcription factors, such as NF-κB.[36] Pro-inflammatory expression and NF-κB activation are strongly influenced by ROS.[37] In this study, we measured the effect of DL0309 on ROS generation in macrophage cells by using the 2′,7′-dichlorodihydrofluorescein diacetate method, and our findings indicated that DL0309 critically reduced the ROS accumulation in LPS-treated RAW264.7 cells. This may at least partially explain why salicylic acid-like molecules can protect the activation of macrophage cells.

In conclusion, our present study demonstrates that DL0309, a natural salicylate derivative from the traditional Chinese herb *G. yunnanensis*, protects against LPS-induced inflammation by inhibition of the IKK/NF-κB signal pathway. DL0309 inhibits the nuclear translocation and DNA binding of the NF-κB by suppressing the activation of IKK-β and degradation of IκB-α, leading to a reduction in the production of proinflammatory cytokines. Furthermore, the results demonstrate that DL0309 may prevent macrophages from generating ROS.

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