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Mollugin-mediated Inhibition of Proinflammatory Biomarkers in Lipopolysaccharide-stimulated RAW264.7 Cells

Jin-Kyung Kim[1,†], Geun-Mook Park[1] and Jong-Gab Jun[2]

[1]Department of Biomedical Science, Catholic University of Daegu, Gyeongsan-Si 700-712, Korea;
[2]Department of Chemistry and Institute of Natural Medicine, Hallym University, Chuncheon 200-702, Korea

Mollugin is the active compound of *Rubia cordifolia*, a well known herb widely used in alternative medicines for the treatment of various inflammatory diseases including arthritis and uteritis. In the present study, we investigated the anti-inflammatory effects of mollugin in lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophage cells. Treatment with mollugin significantly inhibited LPS-induced release of nitric oxide, prostaglandin E_2, and inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6. In addition, mollugin suppressed LPS-induced nuclear factor-kappa B (NF-κB) transcriptional activity. These results suggest that mollugin inhibits LPS-induced expression of inflammatory molecules via NF-κB, at least in part, and indicate the potential value of mollugin as a valuable new drug candidate for the treatment of various inflammatory diseases.

Key Words: Inflammation, Mollugin, IL-6, Nitric oxide

INTRODUCTION

Phytochemicals are natural compounds derived from fruits, vegetables, and medicinal plants, which exhibit inhibitory activity in various diseases and which have been extensively studied in recent years. Mollugin ($C_{17}H_{16}O_4$, methyl 2,2-dimethyl-6-hydroxy-2H-naphtho[1,2-b]pyran-5-carboxylate) is a phytochemical isolated from the roots of *Rubia cordifolia* L., which has been used widely as a traditional herbal medicine to treat various diseases (Jun et al., 2011). Several studies demonstrated that mollugin possesses anti-tumor activity, anti-mutagenic effect (Kawasaki et al., 1992; Marec et al., 2001) and activity against the hepatitis B virus (Ho et al., 1996). In addition, mollugin significantly inhibits arachidonic acid-induced and collagen-induced platelet aggregation (Chung et al., 1994).

Inflammation is a normal physiological response of a tissue to injury against various stimuli. It is a defense mechanism aimed to remove the injurious stimulus and initiate the tissue healing process. However, the process of inflammation can produce inflammatory mediators that have insidious effects and can potentially be harmful (Kundu and Surh, 2008). These various inflammatory mediators, such as nitric oxide (NO), prostanoids, interleukins, and tumor necrosis factor-alpha (TNF-α), are mainly produced by activated macrophages. Among the stimuli to induce the activation of macrophages, lipopolysaccharide (LPS), an endotoxin from the outer membrane of gram-negative bacteria, can activate macrophages and secrete various inflammatory mediators (Rossol et al., 2011).

NO is synthesized from the amino acid L-arginine by three isoforms of nitric oxide synthase (NOS) enzymes: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Ho et al., 1996). iNOS is not present in resting cells and its expression is inducible (Förstermann and Sessa, 2012). Cyclooxygenase (COX) is the key enzyme required for the conversion of arachidonic acid to pro-

stanoids, and exists as two isoforms: COX-1 and COX-2. Under many circumstances, the COX-1 enzyme is produced constitutively, while COX-2 is inducible (Smith et al., 2011). When macrophages are stimulated by LPS, iNOS and COX-2 are activated, leading to overproduction of NO and various prostanoids, respectively (Smith et al., 2011; Förstermann and Sessa, 2012).

Nuclear factor-kappa B (NF-κB) transcription factor family has been considered essential for the inflammatory process and a key participant in innate and adaptive immune responses. During the processes of inflammation, NF-κB regulates the expression of a variety of genes including iNOS, COX-2, and TNF-α, and so has drawn much attention as an attractive therapeutic target for anti-inflammatory drugs (Stoffel, 2005).

In this study, we examined the potency of mollugin as an anti-inflammatory agent by using RAW264.7 mouse macrophages. We demonstrate that mollugin decreases LPS-induced pro-inflammatory mediators. Further, we provide evidence to support the view that NF-κB pathways play an important role in mediating the anti-inflammatory effects of mollugin.

MATERIALS AND METHODS

Chemicals and reagents

Mollugin was synthesized as previously described (Jung et al., 2007) and dissolved in 0.01% dimethylsulfoxide (DMSO) in order to use in the present study. LPS derived from *Escherichia coli* (O111:B4), DMSO was obtained from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Hyclone (Logan, UT, USA).

Cell culture and cell viability assay

RAW264.7 murine macrophages obtained from the Korean Cell Bank (Seoul, Korea) were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37℃ in 5% CO_2. The effects of mollugin on cell viability were tested using the CellTiter 96$^®$ AQ$_{ueous}$ One Solution cell proliferation assay (Promega, Madison,

WI, USA). RAW264.7 cells were plated at a density of 2 × 10^4 cells in a 96-well flat-bottom plate, and mollugin was added to each plate at the indicated concentrations. After a 24 h incubation period, the number of viable cells was counted according to the manufacturer's instructions. This assay is based on the reduction of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS), to formazan, which has an optimum absorption at 490 nm. Thus, the quantity of the product in the cell culture is indicated by the optical density of formazan at 490 nm, which is directly proportional to the number of living cells.

Measurement of nitrite, prostaglandin E$_2$ (PGE$_2$) and cytokines

The amount of nitrite, PGE$_2$, interleukin (IL)-6, and TNF-α produced by the mouse macrophages was measured in RAW264.7 cell culture supernatant. RAW264.7 cells were plated at a density of 2.5 × 10^5 cells in a 48-well cell culture plate with 500 μl of culture medium and incubated for 12 h. They were then treated with indicated concentrations of mollugin plus LPS (100 ng/ml) and incubated for another 24 h. The amount of nitrite and PGE$_2$ produced was measured using the Griess reagent system (Promega) and an enzyme-linked immunosorbent assay (ELISA) kit (ENZO Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instruction, respectively. IL-6 and TNF-α were measured using an ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Western blotting analysis

RAW264.7 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in ice-cold PRO-PREP^{TM} Protein Extraction Solution (iNtRON Biotechnology, Seongnam-Si, Korea). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were electrophoretically transferred onto nitrocellulose membranes. The membrane was blocked with 5% skim milk in Tris-buffered saline/Tween 20 solution. The blots were incubated with the anti-iNOS rabbit polyclonal and anti-

COX-2 monoclonal antibodies (Cell Signaling (Technology, Danvers, MA, USA). β-actin (Sigma-Aldrich) was performed as an internal control. Immunoreactive bands were detected by incubating the samples with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using a WEST-ZOL plus Western Blot Detection System (iNtRON Biotechnology).

Transfection and luciferase assay

RAW264.7 cells were transiently transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with pNF-κB-Luc (firefly luciferase, Promega). After 18 h following transfection, fresh medium was replaced, then incubated for 24 h. Cells were either left untreated or were treated with mollugin at different doses for 30 min. They were then stimulated with LPS for an additional 12 h. Luciferase activity was determined using Luciferase Assay System (Promega) and luminometer TD-20/20 (Promega). The concentration of protein was measured from different aliquots of the same lysates by microBCA assay (Thermo Fisher Scientific Inc., Rockford, IL USA). The intensity of the luciferase reactions measured in the lysates of the transient transfectants was normalized to their protein concentration.

Statistical analysis

The data are depicted as the mean ± SEM. Student t-test was performed using the GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA) and $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of mollugin on the release of NO and PGE_2

To evaluate mollugin-induced anti-inflammatory effects, we used an *in vitro* model with the murine RAW264.7 macrophage cell line. Cytotoxic potential of mollugin on RAW264.7 cells was tested using the MTS assay. In the presence of up to 40 μM of mollugin with or without LPS, the viability of RAW264.7 cells was not significantly lower than in non-treated cells (Fig. 1B). Since mollugin showed no cytotoxicity with concentrations up to 40 μM in RAW264.7 cells, we used up to 40 μM mollugin for the
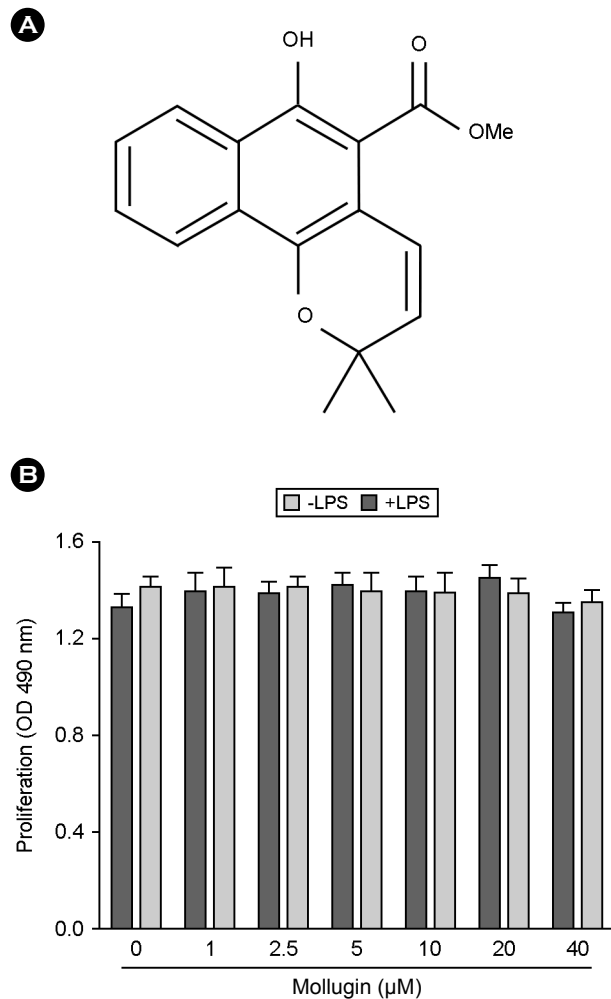
Fig. 1. Effects of mollugin on murine macrophage viability. (A) Chemical structure of mollugin. (B) RAW264.7 cells were treated with indicated concentrations of mollugin for 24 h, and proliferation was determined. The results are reported as mean ± SEM of three independent experiments in triplicate.

rest of the experiments.

We first sought to determine the effect of mollugin on the LPS-induced release of the inflammatory mediators, NO and PGE_2, by RAW264.7 cells. As shown in Figs. 2A and 2B, mollugin inhibited LPS-induced NO and PGE_2 production in a dose-dependent manner. The nitrite concentrations in LPS-stimulated cells and in those exposed to 20 μM mollugin were 29.7 ± 0.3 μM and 12.0 ± 0.3 μM, respectively. The inhibitory effects of mollugin on PGE_2 production in LPS-exposed cells were similar to their effects on NO production (Fig. 2B). The PGE_2 concentrations in LPS-stimulated cells and in those exposed to 20 μM
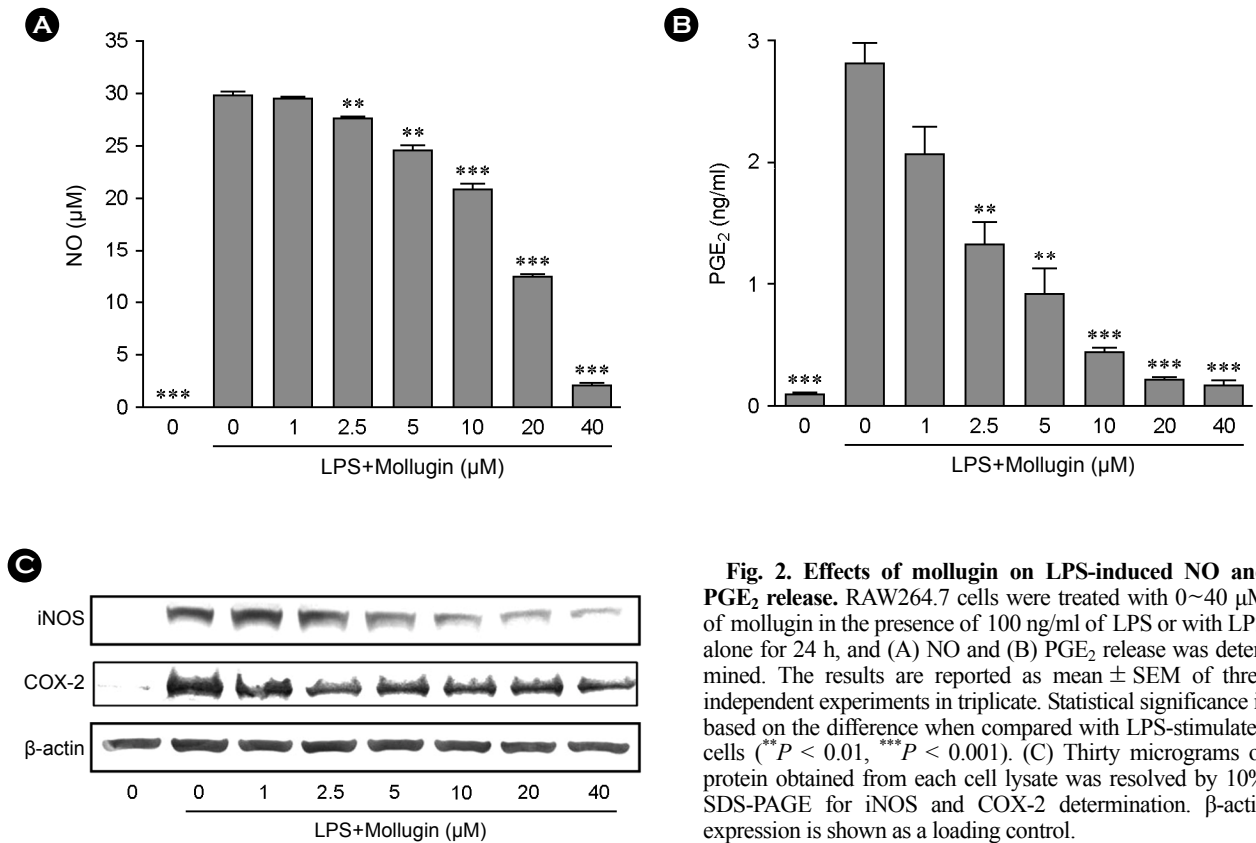
A

B

C

mollugin were 2808 ± 182.4 pg/ml and 167 ± 30.6 pg/ml, respectively.

iNOS activation induces massive NO production at the site of inflammation. On the other hand, COX-2 is the key enzyme regulating the production of PG, the central mediators of inflammation. We thus investigated the effect of mollugin on LPS-induced iNOS and COX-2 expression by Western blot analysis. Consistent with the findings related to NO and PGE₂ production, the protein expression of iNOS and COX-2 induced by LPS in RAW264.7 cells was also reduced by mollugin treatment (Fig. 2C).

Mollugin inhibits the release of pro-inflammatory cytokines in murine macrophages

We next examined if mollugin reduced the release of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells. Although the concentrations of TNF-α and IL-6 were not detected in vehicle (0.01% DMSO)-treated RAW264.7 cells, LPS treatment elevated the levels of TNF-α (842.8 ± 57.2 pg/ml) and IL-6 (1132.0 ± 144.1 pg/ml) in LPS-

treated RAW264.7 cells (Fig. 3). Mollugin induced marked suppression of increases induced by LPS in these cytokines. LPS-treated RAW264.7 cells exposed to mollugin at concentrations of 1, 10, and 40 μM displayed a dose-dependent inhibited production TNF-α (0%, 36%, and 55%, respectively) and IL-6 production (2%, 82.6%, and 100%, respectively). These results indicate that mollugin suppressed various inflammatory mediators including NO and PGE₂, as well as pro-inflammatory cytokines.

Mollugin reduced the NF-κB activation in murine macrophages

The anti-inflammatory potential of various phyto-chemicals has been linked to their capacity to suppress NF-κB signaling (Aggarwal and Shishodia, 2004) which coordinates the expression of pro-inflammatory enzymes and cytokines including iNOS, COX-2, and IL-6 (Aggarwal and Shishodia, 2004; Puangpraphant and de Mejia, 2009; Salminen et al., 2012). Mollugin was thus evaluated *in vitro* for its ability to inhibit LPS-mediated NF-κB transcriptional
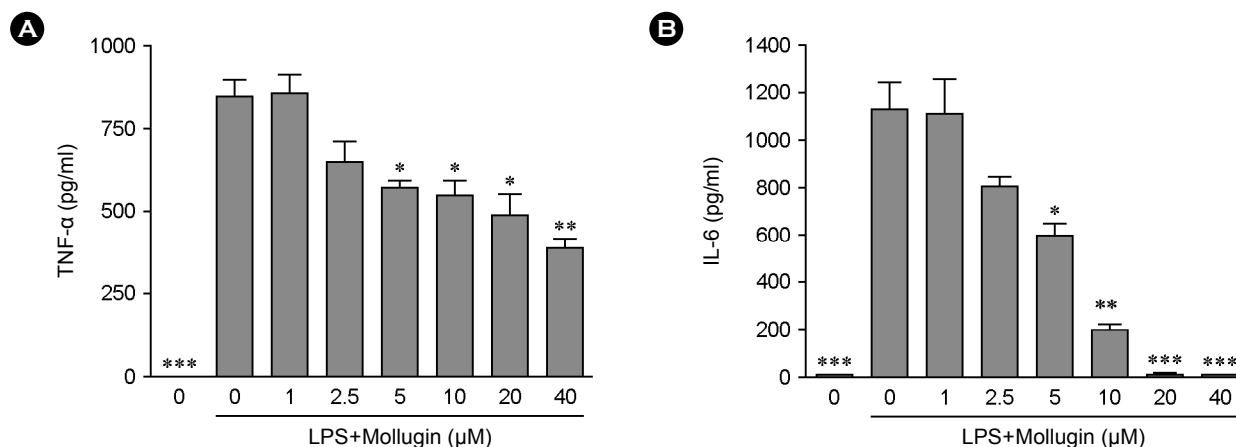
Fig. 3. Effects of mollugin on LPS-induced inflammatory cytokine production in murine macrophages. RAW264.7 cells were treated with 0~40 μM of mollugin in the presence of 100 ng/ml LPS or with LPS alone for 24 h. The cell culture media were then collected, and the amount of (A) TNF-α and (B) IL-6 released was measured. The results are reported as mean ± SEM of three independent experiments in triplicate. Statistical significance is based on the difference when compared with LPS-stimulated cells ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).
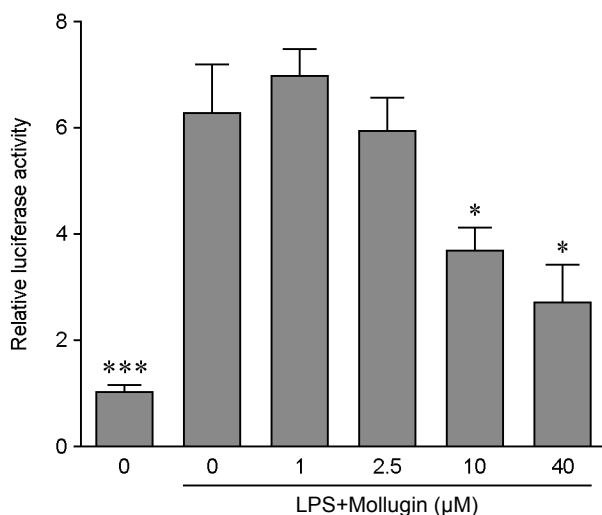

Fig. 4. Effects of mollugin on LPS-induced NF-κB activity in RAW264.7 cells. To evaluate the effects of mollugin on NF-κB transcriptional activity, cells were transfected with the pNF-κB-Luc plasmid, and pretreated with mollugin for 0.5 h before being stimulated for an additional 12 h with LPS (500 ng/ml). Luciferase activity was determined by luminometry. The results are reported as mean ± SEM of three independent experiments in triplicate. Statistical significance is based on the difference when compared with LPS-stimulated cells ($^*P < 0.05$, $^{***}P < 0.001$).

activity to investigate the precise molecular mechanism of the anti-inflammatory effect. As shown in Fig. 4, NF-κB transcriptional activity was significantly increased by 6.2-fold in cells stimulated with LPS alone. The pre-treatment with mollugin prior to LPS stimulation significantly decreased LPS-induced NF-κB transactivation (Fig. 4). This result shows that the anti-inflammatory effect of mollugin correlates with the suppression of NF-κB activation.

DISCUSSION

The present investigation tested the effects and mechanisms of action of mollugin on LPS-induced inflammatory reaction. Our results suggest that the inhibition of NF-κB activation by mollugin contributes to its anti-inflammatory action in reducing the induction of iNOS, COX-2, IL-6, and TNF-α protein.

NF-κB is sequestered in the cytoplasm through an avid association with its inhibitory protein, inhibitor of κB (IκB). The activation of NF-κB occurs through site-specific phosphorylation of IκB by IκB kinase (IKK). IκB is subsequently degraded by the 26S proteasome. NF-κB is freed from IκB and translocates into the nucleus for binding to NF-κB-specific DNA-binding sites, regulating target gene transcription (Li and Vederas, 2002). Many types of stimulators such as carcinogens, tumor promoters, stress, endotoxin, apoptosis inducers, infection, and cytokines, lead to the activation of NF-κB (Kleinert et al., 2003; Wu, 2003; Chen, 2006; Falvo et al., 2010; Checker et al., 2012). Activated NF-κB controls the expression of genes involved in the control of cell proliferation, differentiation, apoptosis,

inflammation, stress response, angiogenesis, tumor promotion, and metastasis, and other cellular and physiological processes (Kleinert et al., 2003; Wu, 2003; Chen, 2006; Falvo et al., 2010; Checker et al., 2012). Because of its critical effects in inflammatory diseases and cancer, NF-κB has been described as a therapeutic target (Wang and Cho, 2010; Vitiello et al., 2012). In addition, phytochemicals that could regulate NF-κB activation could be potent agents to treat various inflammatory diseases and cancer (Chen, 2006; Luqman and Pezzuto, 2010).

Recently, profound interest has been generated for a wide range of medical plants, following the published demonstration of their role in the modulation of inflammatory responses, including flavonoids, phenolics, alkaloids, and terpenoids (Kim et al., 2004, Kuete, 2010; Qi et al., 2010). These natural products have long contributed to the development of modern therapeutic drugs. To date, non-steroidal anti-inflammatory drugs, steroids, suppressants, or inhibitors of the release of mediators and the like have been used as anti-inflammatory agents. However, some of them lack immediate effectiveness or have central side effects (Whitehouse, 2011; Gerstein et al., 2012). Development of new drugs from medical plants requires various approaches including botanical, ethno-botanical, and biological techniques. In spite of these difficulties, several phytochemicals are in clinical use and some are undergoing Phase II and Phase III clinical trials (Li and Vederas, 2009; Yuan et al., 2011; Zauderer and Krug, 2012). A major effort was directed toward discovery of novel anti-inflammatory agents, which resulted in the invention of several patented formulations.

In conclusion, mollugin exerts anti-inflammatory effects by suppressing NF-κB activation, at least in part, which leads to the downregulation of the expression of inflammation-related proteins. In view of these results, we suggest that the utility range of mollugin can be expanded as an anti-inflammatory therapeutic agent.

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