Suppressive Effect of Tomentosin on the Production of Inflammatory Mediators in RAW264.7 Cells

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In this study, tomentosin, a sesquiterpene lactone was isolated from Inulae flos and its biological activities were investigated. The effects of tomentosin on the production of inflammatory mediators as well as on nuclear factor (NF)-κB and mitogen-activated protein (MAP) kinase activation were evaluated in RAW264.7 cells. Tomentosin decreased the production of nitric oxide (NO) and prostaglandin E₂ (PGE₂) by suppressing the protein expression of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2, respectively. Additionally, tomentosin reduced the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Tomentosin not only attenuated lipopolysaccharide (LPS)-induced NF-κB activation via the abrogation of inhibitory (I)κBα degradation and caused a subsequent decrease in nuclear p65 level, but it also suppressed the phosphorylation of MAP kinases (p38 and c-Jun N terminal kinase (JNK)). These results indicate that tomentosin exerts anti-inflammatory activities through the inhibition of inflammatory mediators (NO, iNOS, PGE₂, COX-2, TNF-α, and IL-6) by regulating NF-κB activation and phosphorylation of p38/JNK kinases in macrophages, thus suggesting that tomentosin could be a potential agent for the treatment of inflammatory diseases.

Key words tomentosin; inflammatory mediator; nuclear factor (NF)-κB; mitogen-activated protein (MAP) kinase

Inflammation is the host response to infection and injury, but if it is left uncontrolled, the inflammatory mediators become involved in the pathogenesis of many inflammatory disorders.1) Macrophages play a crucial role in both the specific and non-specific immune responses to microbial and viral infections. When activated by stimuli such as lipopolysaccharide (LPS), macrophages produce a variety of inflammatory mediators such as prostaglandin E₂ (PGE₂) and nitric oxide (NO) and pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6.

Expression of these inflammatory mediators is regulated by the activation of nuclear factor-kappaB (NF-κB).2) Also, inflammatory mediators including cytokines or mitogen-activated protein (MAP) kinases such as p38 and c-Jun N terminal kinase (JNK) stimulate the pathways by activating the inhibitor κB kinase (IKK).3,4) In unstimulated cells, NF-κB is located in the cytoplasm as an inactive complex bound to its inhibitory protein (IκB). During the process of inflammation, the IKK phosphorylates IκB, inducing its ubiquitination and degradation of IκBα. The free NF-κB then translocates to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes and regulates the expression of various genes, including inducible NO synthase (iNOS) and cyclooxygenase 2 (COX-2), and inflammatory cytokines.5–7)

Our previous studies have shown that the ethanol extract of Inulae flos is a potent inhibitor of inflammatory mediators.8) Recently, it was shown that britannin isolated from Inulae flos inhibited the production of inflammatory mediators and pro-inflammatory cytokines in RAW264.7 cells.9) As a continuing study, we evaluated the compounds isolated from Inulae flos extracts and tomentosin isolated from the ethyl acetate fraction showed anti-inflammatory activities.

To the best of our knowledge, no previous study has reported the anti-inflammatory activity of tomentosin. Therefore, we investigated the anti-inflammatory properties of tomentosin isolated from Inulae flos and its underlying mechanisms using RAW264.7 cells. In this study, we show for the first time that tomentosin significantly decreases the production of NO and PGE₂ by suppressing the protein expression of iNOS and COX-2, respectively and reduces the production of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells by inhibiting NF-κB and MAP kinase (p38 and JNK) activities.

MATERIALS AND METHODS

Preparation of Tomentosin Tomentosin (Fig. 1A) was isolated from the 70% ethanol extract of Inulae flos followed by partition with different solvents, thereby providing n-hexane, ethyl acetate, n-butanol, and water extracts.9) The ethyl acetate extract was evaporated in vacum and the residue was chromatographed on a silica gel column by using a step gradient (0%, 5%, and 50%) of ethyl acetate in hexane to obtain 18 fractions. Fraction 6 was separated on a Sephadex column with methanol, to afford 5 fractions. Finally, Fraction 3 was separated on a silica gel column with n-hexane-ethyl acetate (60:40 by volume) to yield tomentosin, which was identified on the basis of the 1H, 13C, and distortionless enhancement by polarization transfer nuclear magnetic resonance spectra in CDCl₃.

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Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) were purchased from Hyclone (Logan, UT, U.S.A.). Lipopolysaccharide (LPS), SP600125 (a specific inhibitor of JNK), SB203580 (a specific inhibitor of p38), PD98059 (a specific inhibitor of extracellular signal-
regulated kinase (ERK)), and Griess reagent were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Antibodies specific for iNOS and COX-2 were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Total or anti-phospho antibodies to JNK, ERK, p38, IκB-α/β, and actin were purchased from Cell Signaling (Beverly, MA, U.S.A.). Secondary antibodies (goat anti-rabbit and anti-mouse) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

**Measurement of NO and PGE$_2$** RAW264.7 cells (2×10$^5$ cells/well) were plated overnight in 24-well plates at 37°C in the medium. The cells were pre-incubated with various concentrations of tomentosin for 1h and then incubated for 18h with or without LPS. NO production was then monitored by measuring nitrite levels in the culture media using Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid). The absorbance was measured at 570nm and nitrite levels in the samples were calculated from a standard curve of sodium nitrite. The PGE$_2$ concentration in the culture supernatant was measured to determine the inhibitory activities of tomentosin against COX-2 using an EIA kit according to the manufacturer’s instructions.

**RNA Extraction and Real Time (RT)-Polymerase Chain Reaction (PCR)** Total RNA was isolated from LPS-stimulated RAW264.7 cells using TRI Solution according to the manufacturer’s instructions. cDNA was synthesized from 1µg of total RNA using OligodT$_{15}$ and Goscript Reverse transcription system kit (Promega). The PCR reaction was carried out on the StepOne Plus (Applied Biosystems, Foster City, CA, U.S.A.) using HotStart SYBR Green qPCR Master Mix (USB, Cleveland, OH, U.S.A.). Primer sequences were as follows: TNF-α sense 5′-GCA GAG AGG TTG ACT TTC-3′ and antisense 5′-CTA CTC CCA GGT TCT CTT CAA-3′; IL-6 sense 5′-TCA GAA TTG CCA TTG CAC A-3′ and antisense 5′-GTC GGA GGC TTA ATT ACA CAT G-3′; β-actin sense 5′-TGG ACA GTG AGG CCA GGA TAG-3′ and antisense 5′-TAC TGC CCT GGC TCC TAG CA-3’. Each PCR cycle

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![Fig. 3. Effect of Tomentosin on the Release and mRNA Expression of Pro-inflammatory Cytokines in LPS-Stimulated RAW264.7 Cells](image)

Cells were pre-treated with the indicated concentrations of tomentosin for 1h before LPS (200ng/mL) stimulation and incubated for 18h (ELISA) or 5h (RT-PCR). The cytokine levels in the culture media (A) and transcription levels (B) in the cells were measured using an ELISA kit and a real time RT-PCR. The data shown are means±S.E.M. of three different samples. *p<0.05, **p<0.01, and ***p<0.001 were compared to LPS-stimulated cells.
consisted of the following 3 steps: 95°C for 2 min, 95°C for 5 s, and 60°C for 30 s. The results of real time PCR are presented as the fold-change in cytokine gene (TNF-α and IL-6) induction, and these were calculated using β-actin, which was amplified under the same conditions, as an internal control.

**Measurement of Cytokine Level** The supernatants of cell cultures with or without treatment with tomentosin were used to measure the TNF-α and IL-6 levels using ELISA kits according to the manufacturer’s instructions. The cytokine concentrations in the samples were calculated from a standard curve created using a known concentration of recombinant TNF-α and IL-6.

**Western Blot Analysis** RAW264.7 cells were treated with various concentrations of tomentosin and stimulated with or without LPS for 18 h. For the total protein extract, cells were washed once with 10 mM PBS (pH 7.4) containing 150 mM NaCl and then lysed with PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Daejeon, Korea) according to the manufacturer’s protocol.

For the cytosolic and nuclear extractions, cells were collected, washed with PBS and extracts were prepared by using the NE-PER nuclear protein extract kit according to the manufacturer’s instructions. Thirty micrograms of protein (10 μg of nuclear extract) were applied to 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes with glycine transfer buffer (20% methanol/25 mM Tris/192 mM glycine). After blocking with 5% non-fat dry milk in TTBS (25 mM Tris–HCl, 150 mM NaCl, and 0.2% Tween-20), the membranes were probed with various first antibodies overnight followed by three washes. Then, the membranes were incubated for 1 h with a secondary horseradish peroxidase (HRP)-conjugated antibody. The protein bands were then visualized using an ECL system (Thermo Fisher Scientific Inc., IL, U.S.A.). The densities of the bands were measured with the ImageQuant LAS 4000 luminescent image analyzer and ImageQuant TL software system (GE Healthcare, Little Chalfont, U.K.).

**NF-κB Transcription Factor Assay** The activity of NF-κB p65 was examined by using Trans-AM NF-κB ELISA kit (Active Motif, Carlsbad, CA, U.S.A.). In brief, nuclear extracts (10 μg) from untreated or treated cells were added to each well coated with an oligonucleotide containing the NF-κB consensus region (5’-GGGACTTTC-3’) and incubated for 1 h. After washing, the plate was incubated with primary and secondary horseradish peroxidase (HRP)-conjugated antibodies. The colorimetric substrate was added to produce a signal quantifiable by spectrophotometry.

**Statistical Analysis** All values are expressed as the mean±S.D. Statistical significance was compared using ANOVA and Duncan’s multiple range tests. The p-values of less than 0.05 were considered statistically significant.

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**Fig. 4. Effect of Tomenosin on NF-κB Activation in LPS-Stimulated RAW264.7 Cells**

Cells were pre-treated with indicated concentrations of tomentosin for 1 h and then stimulated with LPS (200 ng/mL) for 30 min. Cytoplasmic and nuclear proteins were determined by Western blot analysis using NF-κB p65 and IκBα antibodies (A). To confirm the effect of tomentosin on NF-κB activation, the binding activity of NF-κB to DNA was determined by quantification of translocated NF-κB p65 in nuclear extract using an ELISA-based Trans-AM p65 assay (B). PDTC (pyrrolidine dithiocarbamate), a specific inhibitor of NF-κB; Jurkat, nuclear extracts from stimulated Jurkat cells. The data shown are means±S.E.M. of three different samples. *p<0.05 and **p<0.01 were compared to LPS-stimulated cells.
RESULTS AND DISCUSSION

Tomentosin, a sesquiterpene lactone, has been isolated from several plants such as Dittrichia viscosa, Carpesium faberi, Inula viscosa, and Carpesium macrocephalum.\textsuperscript{10–14} It has been shown to exhibit anti-fungal and anti-proliferative activities and to induce apoptosis in cancer cell lines. In this study, the ethanol extract of Inulae flos was partitioned with different solvents and the ethyl acetate fraction showed anti-inflammatory activities (data not shown). The ethyl acetate fraction was further subjected to silica gel column chromatography and HPLC to identify the active compounds, yielding several known compounds such as britanin, quercetin and tomentosin (Fig. 1A). RAW264.7 cells were incubated with different concentrations of tomentosin and cell viability was not affected up to a concentration of 10 \( \mu \text{M} \) (Fig. 1B).

Macrophages play an important role in both innate and acquired immune responses. RAW264.7 cells provide us with an excellent model for drug screening because macrophages activated by LPS induce the synthesis and production of NO/iNOS, PGE\textsubscript{2}/COX-2, and pro-inflammatory cytokines. In the present study, we first examined the effects of tomentosin on NO production and iNOS expression in LPS-stimulated RAW264.7 cells. As shown in Fig. 2A, LPS stimulation resulted in a marked induction of NO production when compared to that in the untreated cells. However, pre-treatment with tomentosin markedly decreased NO production. To explore the mechanisms underlying the inhibition, cells were stimulated with LPS in the presence or absence of tomentosin. The result showed that tomentosin significantly inhibited the LPS-induced iNOS protein expression, indicating that tomentosin inhibited NO production by decreasing iNOS protein expression (Fig. 2C). The effects of tomentosin on PGE\textsubscript{2} production and COX-2 expression following LPS stimulation in RAW264.7 cells were also examined. We demonstrated that tomentosin suppressed LPS-induced PGE\textsubscript{2} production and COX-2 expression (Fig. 2B, D). Collectively, these results demonstrated that tomentosin inhibited LPS-stimulated NO and PGE\textsubscript{2} production by decreasing the expression of iNOS and COX-2 at the protein levels in macrophages, thereby suggesting that the inhibition of NO and PGE\textsubscript{2} release might be attributable to the suppression of iNOS and COX-2 expression at the transcriptional level.

Pro-inflammatory cytokines that are usually released by macrophages play a critical role in initiating and sustaining the inflammatory response. Therefore, we also investigated the effect of tomentosin on the release of pro-inflammatory cytokines such as TNF-\( \alpha \), IL-6 in LPS-stimulated RAW264.7 cells. Figure 3 showed that the unstimulated RAW264.7 cells produced basal levels of cytokines, and the levels of TNF-\( \alpha \) and IL-6 were increased in the culture supernatant of the LPS-stimulated cells. However, pre-treatment with tomentosin resulted in a decrease in cytokine production (Fig. 3A). We also examined the mRNA levels of cytokines and demon-

![Fig. 5. Effect of Tomentosin on the Activation of MAP Kinases in LPS-Stimulated RAW264.7 Cells](image-url)

Cells were pre-treated with indicated concentrations of tomentosin for 1h and then stimulated with LPS (200 ng/mL) for 30 min. Whole cell lysates were prepared and analyzed for the phosphorylation of ERK, JNK and p38 kinases by Western blot analysis. Total ERK, JNK and p38 kinases were used as a control of the protein amount in the same samples. The data shown are means±S.E.M. of three different samples. *\( p<0.05 \) and **\( p<0.01 \) were compared to LPS-stimulated cells.
strated that treatment with tomentosin inhibited the increased mRNA levels of cytokines (Fig. 3B). The results of this study showed that tomentosin significantly decreased the release of these cytokines by suppressing their mRNA expression.

Upon LPS recognition of complex proteins including LPS binding protein (LBP), CD14, MD-2 and Toll-like receptor (TLR) 4, a serious of TLR-mediated signal pathways occurs through Toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP), IL-1 receptor-associated kinase (IRAK) family, TNF receptor-associated factor (TRAF) 6, and transforming growth factor-β activated kinase (TAK) 1 to activate downstream of IKK and MAP kinase pathways.\(^{15}\)

NF-κB is an important factor regulating the expression of inflammatory mediators.\(^2\) Many anti-inflammatory agents exhibit potent activity by suppressing NF-κB signaling.\(^{16-18}\)

Therefore, NF-κB-targeted therapeutic agents might be effective in treating inflammatory diseases since a variety of pharmacologic agents have been reported to inhibit one or more activation steps in the signaling pathway.\(^{19}\) In this study, we examined the effects of tomentosin on the NF-κB pathway. As shown in Fig. 4, tomentosin suppressed the LPS-induced IκB degradation and inhibited p65 translocation from the cytosol to the nucleus. To further investigate the activation of NF-κB, we examined the DNA-binding activity of NF-κB. Nuclear extracts were obtained from LPS-stimulated RAW264.7 cells that were pre-treated with tomentosin. The result showed that tomentosin reduced the binding activity of NF-κB consensus site (Fig. 4). Pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor inhibited both the translocation of the NF-κB p65 and the degradation of IκBα as well as the NF-κB p65 binding activity. The nuclear extracts from stimulated Jurkat cells were used as a positive reference in the assay for the NF-κB DNA binding activity. These results are consistent with those in other studies, which demonstrated that NF-κB responsive elements are present in the promoters of inflammatory mediators.\(^{20,21}\)

LPS is also a potent activator of MAP kinase pathways because NF-κB and MAP kinase pathways are the major intracellular signaling pathways that are activated by LPS through binding to its receptor TLR4 on the cell membrane.\(^{22,23}\)

Therefore, MAP kinases not only play an important role in the iNOS and COX-2 expression, but also they are also involved in the regulation of pro-inflammatory cytokine release.\(^4,24\)

Since tomentosin attenuated NF-κB activation, we examined the effects of tomentosin on the LPS-induced phosphorylation of ERK, p38 and JNK. As shown in Fig. 5, the phosphorylation levels of all three MAP kinases were increased after LPS stimulation and the activation of p38 and JNK was clearly attenuated by tomentosin, however; it seems that ERK activation was not affected by tomentosin. Although LPS activates all three MAP kinases that involved in the signaling pathways leading to expression of inflammatory mediators, it is speculated that each MAP kinase plays a role in the up-regulation of inflammatory mediators in LPS-stimulated macrophages. As previous reports that JNK and p38 MAP kinases are mainly involved in LPS-stimulated expression of iNOS and COX-2 in macrophages,\(^{25-27}\) our study shows similar results that tomentosin attenuated the increased levels of JNK and p38 MAP phosphorylation.

It seems that tomentosin is weaker than britain in the suppression of the inflammatory mediators and MAP kinase activities. Although tomentosin and britain belong to sesquiterpene lactons, they have distinct structural differences. For examples, tomentosin has a five member ring (C1–C5), but tomentosin does not have it. Also, the carbon structure of C8 and C10 for tomentosin is alpha configuration whereas that of britanin is beta. In addition, britanin has two acetyl moieties in C2 and C6. Therefore, it is difficult to compare both compounds in the aspect of biological activities. However, it may be speculated that the presence of the acetyl moieties increased the anti-inflammatory activities of sesquiterpene lactones.\(^{28}\)

In conclusion, our study provides the first evidence that tomentosin exerts anti-inflammatory effects by inhibiting the production and expression of LPS-induced NO/iNOS, PGE\(_2\)/COX-2, and pro-inflammatory cytokines via the suppression of transcription factor NF-κB and MAP kinase (p38/JNK) activation. In view of the fact that inflammatory mediators play important roles in mediating inflammatory response, it suggests that tomentosin might be a potential anti-inflammatory agent.

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REFERENCES


