Anti-inflammatory Activity of Gumiganghwaltang through the Inhibition of Nuclear Factor-κB Activation in Peritoneal Macrophages

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Gumiganghwaltang (GMGHT) is an Oriental herbal prescription, which has been commonly used to treat a cold and inflammatory diseases in Korea. However, the mechanism of GMGHT is not clear. In this study, we investigated the anti-inflammatory mechanism of GMGHT in mouse peritoneal macrophages. GMGHT exerted an anti-inflammatory action through inhibiting lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF-α) and interleukin (IL)-6 production in mouse peritoneal macrophages. The maximum inhibition rate of TNF-α and IL-6 production by GMGHT (1 mg/ml) was 52.31 ± 2.8% and 56.3 ± 3.1%, respectively. In the inflammatory process, cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) increased in peritoneal macrophages. GMGHT decreased the protein level of COX-2 and iNOS in LPS-stimulated mouse peritoneal macrophages. In addition, GMGHT inhibited nuclear factor-κB activation and IκB-α degradation. Our study suggests that an important molecular mechanism by GMKHT reduce inflammation, which might explain its beneficial effect in the regulation of inflammatory reactions.

Key words Gumiganghwaltang; tumor necrosis factor-α; interleukin-6; cyclooxygenase-2; inducible nitric oxide synthase; nuclear factor-kappa B

Gumiganghwaltang (GMGHT) consists of 9 different herbs and is a Korean herbal prescription, which has been commonly used to treat a cold and inflammatory diseases in Korea. However, it is still unclear how it regulates the immune or inflammatory responses.

Macrophage activation is known to play an important role in the inflammatory process and produce potent proinflammatory cytokines such as tumor necrosis factor (TNF)-α, and interleukin (IL)-6 which induce inflammation and recruit other immune cells, e.g., neutrophils and T lymphocytes. Although these proinflammatory cytokines are beneficial to the host defense, they can also trigger pathological conditions when expressed in excess. For example, massive stimulation of macrophages after a severe Gram-negative bacterial infection leads to excessive production of proinflammatory cytokines and the development of fatal septic shock syndrome, and multiple organ failure. In addition, higher levels of proinflammatory cytokines are also implicated in a variety of chronic inflammatory diseases including rheumatoid arthritis, psoriasis, and Crohn’s disease.

Nitric oxide (NO) produced by the inducible NO synthase (iNOS) isoform is an essential component of the host innate immune and inflammatory response to a variety of pathogens, such as intracellular bacteria, viruses, fungi, and parasites. Nevertheless, as for other components of the host inflammatory and immune response, excessive activation of iNOS results in cardiovascular and organ dysfunction in clinical or experimental situations of inflammatory disease of both septic and nonseptic etiology.

Cyclooxygenases (COX) produce various types of prostaglandins (PGs), which are implicated in various physiological events including progression of inflammation, immunomodulation, and transmission of pain. Two COX isoenzymes were identified: COX-1, the constitutive enzyme makes PGs that protect the stomach and kidney from damage and COX-2, the inducible enzyme induced by inflammatory stimuli such as cytokines produces PGs that contribute to the pain and swelling of inflammation. We examined the effects of GMGHT on lipopolysaccharide (LPS)-induced cytokines (TNF-α and IL-6) production, COX-2, and iNOS protein expression from mouse peritoneal macrophages.

Nuclear factor-kappa B (NF-κB) plays a critical role in the expression of many genes involved in immune and inflammatory responses. In unstimulated cells, Rel protein dimers, mainly p50 and p65 subunits, are sequestered in the cytoplasm in complex with one of the several inhibitors of NF-κB. The activation of NF-κB is the consequence of phosphorylation of two specific serines near the N terminus of IκB-α and its degradation. The phosphorylation of IκB-α lead to the ubiquitination, resulting in the degradation, which targets the protein for degradation by the 26S proteasome and the translocation of NF-κB to the nucleus.

The present study was designed to investigate whether GMGHT could modulate expression of cytokines (TNF-α and IL-6) and enzymes (COX-2 and iNOS) regulated by a transcription factor, NF-κB.

MATERIALS AND METHODS

Reagents Dulbeccos Modified Eagles Medium (DMEM) and LPS were purchased from Sigma (St. Louis, MO, U.S.A.). Rabbit polyclonal antibody (Ab) to iNOS was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-mouse TNF-α, biotinylated anti-mouse TNF-α and recombinant mouse TNF-α were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Anti-mouse IL-6, bi-
otinylated anti-mouse IL-6 and recombinant mouse IL-6 were purchased from Pharmingen (San Diego, CA, U.S.A.). Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI, U.S.A.). DMEM containing L-arginine (84 mg/l), Hanks balanced salt solution (HBSS), fetal bovine serum (FBS) and other tissue culture reagents were purchased from Life Technologies (Grand Island, NY, U.S.A.). Male C57BL/6 mice were purchased from Daehan Experimental Animal Center (Eumseong, Republic of Korea).

Peritoneal Macrophage Cultures TG-elicited macrophages were harvested 3—4 d after i.p. injection of 2.5 ml TG to the mice and isolated, as reported previously.21) Using 8 ml of HBSS containing 10 U/ml heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 4-well tissue culture plates (2.5×10^6 cells/well) incubated for 3 h at 37 °C in an atmosphere of 5% CO_2, washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Preparation of GMGHT GMGHT which is a mixture of nine traditional drugs as shown in Table 1 was obtained from the Oriental drug store, Daehak Oriental Drugstore (Iksan, South Korea) and classified and identified by local experts. Extract of GMGHT was prepared by decocting the dried prescription of herbs with boiling distilled water. The extraction decocted for approximately 3 h has been filtered, lyophilized, and kept at 4 °C. Dilutions were made in saline then filtered through 0.45-μm syringe filter.

MTT Assay Cell viability was determined using MTT assay. Briefly, 500 μl of peritoneal macrophage cells suspension (3×10^6 cells) was cultured in 4-well plates for 24 h after treatment with various concentrations of GMKHT. 50 μl of MTT solution (5 mg/ml) was added and then cells were incubated for 4 h at 37 °C. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, optical density of 96-well culture plates was measured using enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

Cytokines and Prostaglandins E_2 (PGE_2) Assay Cytokines assay was performed by a modified ELISA, as described previously.21) The ELISA was devised by coating 96-well plates with mouse monoclonal Ab specific to TNF-α, and IL-6. Before subsequent steps in the assay, coated plates were washed with PBS containing 0.05% Tween 20. All reagents used in this assay were incubated for 2 h at 37 °C. Recombinant TNF-α, and IL-6 were diluted and used as a standard. Serial dilutions starting from 10 ng/ml were used to establish the standard curve. Assay plates were exposed sequentially to biotinylated mouse TNF-α, and IL-6 avidin peroxidase, and ABTS substrate solution containing 30% H_2O_2. The plates were read at 405 nm. The PGE_2 level was quantified by immunoassay kits according to the manufacturer’s protocols (Stressgen Biotechnologies, U.S.A.).

RT-PCR Analysis Total RNA was isolated from mouse peritoneal macrophage cells according to the manufacturers specification using easy-BLUE RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.5 μg) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using cDNA synthesis kit (AmershamPharmacia, U.S.A.). PCR was performed with the following primers for mouse (m) TNF-α (5′ ATG AGA ACA GAA AGC ATG ATC-3′; 5′ TAC AGG CTT GTC ACT CGA ATT 3′), IL-6 (5′ CGG GAT CCA TGT TTC CTA CTT CAC AA 3′; 5′ CCC AAG CTT GGT TTG CCG AGT AGA-3′), COX-2 (5′ GGA GAG ACT AAG ATA GTG ATC-3′; 5′ GTC ATG AGT AGA CTT TTA CGA CTA 3′), and GAPDH (5′ GCC ATG GAC TGT CTT GAG AAG AGC-3′; 5′ TTC ACC ACC ATG GAG AAG GC 3′) to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 55 °C for COX-2, 60 °C for TNF-α, 50 °C for IL-6 and 62 °C for GAPDH, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Measurement of Nitrite (NO) Concentration Peritoneal macrophages (3×10^6 cells/well) were pretreated with GMGHT for 30 min, and then treated with LPS (10 μg/ml) for 48 h. To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a plate reader. NO_2 was determined using sodium nitrite as a standard. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Preparation of Cytoplasmic and Nuclear Extract Nuclear and cytoplasmic extracts were prepared as described previously (Schoonbroodt et al., 2001). Briefly, after cell activation for the times indicated cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in 60 μl of buffer A (10 mM Hepes/KOH, 2 mM MgCl_2, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 μl of 10% Nonidet P (NP)-40, and centrifuged at 2000 g for 10 min at 4 °C. The supernatant was collected and used as the cytoplasmic extracts. The nuclei pellet was resuspended in 40 μl of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), left on ice for 20 min, inverted and the nuclear debris was spun down at 15000 g for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at −70 °C until ready for analysis.

Western Blot Analysis Peritoneal macrophages (5×10^6 cells/well) were stimulated with LPS (10 μg/ml).

Table 1. The Ratio of the Component in GMGHT

<table>
<thead>
<tr>
<th>Component</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>1. Angelicae koreanae Radix (Angelica koreana Maxim.)</td>
<td>15</td>
</tr>
<tr>
<td>2. Saposhnikoviae Radix (Saposhnikovia divaricata Schlecht.)</td>
<td>15</td>
</tr>
<tr>
<td>3. Cnidii Rhizoma (Cnidium officinale Makino)</td>
<td>12</td>
</tr>
<tr>
<td>4. Angelicae Dahuricae Radix</td>
<td>12</td>
</tr>
<tr>
<td>5. Atractylodes lancea (Angelica dahurica Bessm. et Hooker)</td>
<td>12</td>
</tr>
<tr>
<td>6. Scutellariae Radix (Scutellaria baicalensis Georgi)</td>
<td>12</td>
</tr>
<tr>
<td>7. Rehmanniae Radix (Rehmannia glutinosa Liboschitz var. purpurea Makino)</td>
<td>12</td>
</tr>
<tr>
<td>8. Asiasari Radix (Asiasarum sieboldi F. Maekawa)</td>
<td>5</td>
</tr>
<tr>
<td>9. Glycyrrhizae Radix (Glycyrrhiza uralensis Fischer)</td>
<td>5</td>
</tr>
</tbody>
</table>

- 2. SAP: 15
- 3. C: 12
- 4. A: 12
- 5. S: 12
- 6. R: 12
- 7. A: 12
- 8. A: 5
- 9. G: 5

Vol. 28, No. 2
Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk in PBS-tween-20 for 1 h at room temperature and then incubated with anti-COX-2, iNOS, NF-κB, and IkB-α. After washing in PBS-tween-20 three times, the blot was incubated with secondary Ab for 1 h and the Ab-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp. Newark).

**Immunofluorescence** The cells were washed with PBS, fixed with 3.7% paraformaldehyde for 30 min, and cells were blocked with wash buffer containing 5% BSA for 30 min and provided with Ab, anti-NF-κB at a 1 : 500 dilution (Santa Cruz Biotechnology, Inc.). After washing, cells were incubated with secondary Ab (Antirabbit, fluorescein isothiocyanate conjugated). After washing, cover-slips were placed. Slides were scanned under fluorescence with Olympus confocal microscope (New Hyde Park, NY).

**Statistical Analysis** The experiments shown are a summary of the data from at least-three experiments and are presented as the mean±S.E.M. Statistical evaluation of the results was performed by independent t-test.

**RESULTS**

**Effects of GMGHT on Cytokine Production and mRNA Expression** The effect of GMGHT was tested on TNF-α and IL-6 production from LPS-treated mouse peritoneal macrophages. As shown in Fig. 1A, TNF-α and IL-6 production in response to LPS was inhibited by pre-treatment with 0.01—1 mg/ml GMGHT in a dose-dependent manner, the maximal inhibition rate of TNF-α and IL-6 production by GMGHT (1 mg/ml) was 52.31±2.8% and 56.31±3.1%, respectively. Cell cytotoxicity by GMGHT was not observed (data not shown). To determine GMGHT can modulate LPS-induced TNF-α and IL-6 mRNA expression in peritoneal macrophages, RT-PCR was performed. Data in Fig. 1B, enhanced level of TNF-α, and IL-6 mRNA by stimulation of LPS was decreased by GMKHT in a dose-dependent manner.

**Effects of GMGHT on PGE2 production and COX-2 Expression** To investigate the effect of GMGHT on LPS-induced PGE2 production, cells were pretreated with GMGHT (0.01—1 mg/ml) for 30 min and then treated with LPS for 24 h. We showed that GMGHT decreased PGE2 production in dose-dependent manner (Fig. 2A). The maximal inhibition rate of PGE2 production by GMGHT (1 mg/ml) was 40.31±3.8%.

To determine the effect of GMGHT on LPS-induced COX-2 expression in peritoneal macrophages, RT-PCR and western blotting were performed. As shown in Fig. 2B, treatment with LPS caused a significant increase of COX-2 mRNA expression. Pretreatment of GMGHT (0.01—1 mg/ml) resulted in the inhibition of the COX-2 mRNA in a dose-dependent manner and expression of COX-2 protein was also inhibited compared to that of LPS treated cells (Fig. 2C).

**Effects of GMGHT on NO Production and iNOS Expression**
pression. To investigate the effect of GMGHT on LPS-induced NO production, cells were pretreated with GMGHT (0.01—1 mg/ml) for 30 min and then treated with LPS for 48 h. We showed that GMGHT decreased NO production in a dose-dependent manner (Fig. 3A). The maximal inhibition rate of NO production by GMGHT (1 mg/ml) was 59.11 ± 3.1%.

To determine the effect of GMGHT on LPS-induced iNOS expression in peritoneal macrophages, western blotting was performed. Data in Fig. 3B, treatment with LPS caused a significant increase of iNOS expression. Pretreatment of GMGHT decreased the expression level of Rel/p65 in LPS-stimulated cells, the expression level of Rel/p65 was increased. However, pretreatment with 1 mg/ml GMGHT inhibited LPS-stimulated translocation of Rel/p65 from cytoplasm to nucleus levels of Rel/p65 was examined after LPS-stimulation by western blot analysis. As shown in Fig. 3C, pretreatment with 1 mg/ml GMGHT significantly inhibited LPS-stimulated translocation of Rel/p65 from cytoplasm to nucleus in mouse peritoneal macrophages (Fig. 3B).

Effect of GMGHT on NF-κB Expression Since NF-κB activation requires nuclear translocation of Rel/p65 subunit of NF-κB, we examined the effect of GMGHT on the expression of Rel/p65 in both unstimulated cells and LPS-stimulated cells, the expression level of Rel/p65 was increased. However, pretreatment of GMGHT decreased the expression level of Rel/p65 in LPS-stimulated cells (Fig. 3A). To confirm this result, nuclear translocation of p65 subunits of NF-κB in mouse peritoneal macrophages was visualized using immunofluorescent confocal microscopy. Before LPS treatment, p65 was localized mainly in the cytoplasm. After LPS treatment, there was extensive nuclear staining for the p65 protein. However, pretreatment with 1 mg/ml GMGHT inhibited LPS-stimulated translocation of Rel/p65 from cytoplasm to nucleus in mouse peritoneal macrophages (Fig. 3B).

Effect of GMGHT on IκB-α Degradation Most of the inhibitor of NF-κB activation mediated their effect through suppressing IκB-α degradation.23) To determine whether the inhibitory action of GMGHT was due to its effects on IκB-α degradation, the cytosol levels of IκB-α was examined after LPS-stimulation by western blot analysis. As shown in Fig. 4, we showed that LPS treatment effectively caused an induction of IκB-α degradation and that pretreatment of GMKHT (1 mg/ml) significantly inhibited LPS-induced IκB-α degradation in mouse peritoneal macrophages.

DISCUSSION

Proinflammatory cytokines such as TNF-α and IL-6 mediate the development of various inflammatory reactions.24,25) In this study, we showed that GMGHT effectively inhibited the production and mRNA expression of TNF-α, and IL-6 cytokines on LPS-stimulated peritoneal macrophages. These results suggest that GMGHT might have an anti-inflammatory activity.

COX-2 plays a role in the pathophysiological processes including inflammation.26,27) The present study showed that GMGHT effectively inhibited the mRNA and protein expression of COX-2 in a dose dependent manner. It suggests that GMGHT may provide its beneficial effect on anti-inflammation.

Although NO played an important role in the host defense against various pathogens, the overproduction of NO can be harmful and result in septic shock, rheumatoid arthritis, and autoimmune diseases.28) Therefore, therapeutic agents that inhibit the iNOS may be useful for the relieving these inflammatory conditions. In this study, GMGHT inhibited iNOS expression in a dose dependent manner. We suggest that the anti-inflammatory action of GMGHT may be associated with the reduction of iNOS protein expression.

NF-κB is a transcription factor that is important for the activation of many inflammatory mediators, cytokines (e.g., TNF-α and IL-6), COX-2, and iNOS enzyme.29) In inactivated state, NF-κB is sequestered in the cytoplasm bound to its inhibitory protein, IκB-α, which, with stimulation, is de-
graded thus allowing NF-κB to translocate into the nucleus and activate proinflammatory genes. Degradation of IκB-α is a key step for NF-κB-induced transcription of certain proinflammatory genes including inducible COX-2 and iNOS. Other study reported that anti-inflammatory agents suppressed NF-κB activation through stabilization of IκB-α. In this study, GMGHT inhibited transcription factor, activation of NF-κB by blocking the Rel/p65 translocation to the nucleus. Recent study demonstrated that aspirin activated NF-κB by increasing the phosphorylation and degradation of IκB-α in colon cancer cell lines, but had no effect on IκB-α in embryonic kidney. It is suggesting that the activation of NF-κB by aspirin is cell-type specific. GMGHT inhibited NF-κB activation through suppression of IκB-α degradation and Rel/p65 translocation in mouse peritoneal macrophages. Our results provide a novel mechanism by GMGHT regulate NF-κB activation in mouse peritoneal macrophages.

The promoter region of COX-2 contains two putative NF-κB binding sites. Thus, NF-κB has been shown to be a positive regulator of COX-2 expression in murine macrophages exposed to LPS. Other research reported that serine protease inhibitor (N-α-p-tosyl-L-lysine chloromethylketone) which is inhibitors of NF-κB activation inhibited the PGE2 production, and COX-2 expression. In this study, we suggested that COX-2 inhibitory mechanism of GMKHT is through suppression of NF-κB activation.

In conclusion, the anti-inflammatory activity of GMGHT in peritoneal macrophages could be was attributed at least in part to inhibition of the proinflammatory cytokines production, COX-2, and iNOS protein expression. These effects of GMGHT cause through the inhibition of LPS-induced NF-κB activation and IκB-α degradation. These results may provide evidence for novel mechanism of the anti-inflammatory effect of GMGHT.

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