The Anti-inflammatory Effect of Gigukjiwhangwhangami through the Inhibition of Nuclear Factor-κB Activation in the Peripheral Blood Mononuclear Cells of Patients with Cerebral Infarction

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The Korean genuine medicine “Gigukjiwhangwhangami (GJWGM)” has long been used for various cerebrovascular diseases. However, the exact mechanism that accounts for the anti-inflammatory effect of GJWGM is not completely understood. The aim of the present study is to elucidate how GJWGM modulates the inflammatory reaction in lipopolysaccharide (LPS)-stimulated peripheral mononuclear cells from patients with cerebral infarction. Production of cytokine was measured by the ELISA and RT-PCR method. The level of nuclear factor-kappaB (NF-κB)/Rel A protein and NF-κB DNA binding activity were determined by the Western blot analysis and TF-EIA method. We showed that GJWGM inhibited the production of tumor necrosis factor (TNF)-κ, interleukin (IL)-1α, IL-6, and IL-8 induced by LPS in dose dependent manner (p<0.05). Maximal inhibition rate of TNF-κ, IL-1β, IL-6 and IL-8 production by GJWGM was about 54.34%, 41.37%, 44.04%, and 54.46%, respectively. GJWGM inhibited the TNF-κα and IL-8 mRNA expression. In addition, we showed that the inhibitory mechanism of GJWGM is through the suppression of NF-κB pathway. Our study suggests that an important molecular mechanism by GJWGM reduce inflammation, which may explain its beneficial effect in the regulation of inflammatory reactions.

Key words Gigukjiwhangwhangami; cerebral infarction; cytokine; nuclear factor-κB

Gigukjiwhangwhangami (GJWGM), a prescription of traditional Korean medicine, has long been used as a specific prescription for cerebral infarction (CI) to increase cerebral blood flow and to recover an injured brain cell. However, the pharmacological mechanisms of GJWGM have not been well defined.

Recently, it has become increasingly evident that the inflammatory response plays an important role in the pathogenesis of CI. Much of this inflammatory response appears to be mediated by proinflammatory cytokines.1–3) Proinflammatory cytokines are involved in hemostatic and immunological imbalance, which lead to enlargement of brain damage.4,5) Tumor necrosis factor (TNF-κ) is a major inflammatory cytokine due to their ability to stimulate the synthesis of nitric oxide and other inflammatory mediators that derive chronically delayed hypersensitivity reaction.6) Interleukin (IL)-1β is increased markedly during global and focal cerebral infarction.7–9) IL-6, which is one of the main inflammation-associated cytokines, is produced by a variety of cells in the central nervous system (CNS).10) The spontaneous production of inflammatory cytokines by mononuclear cells and the level of cytokines in serum are significantly increased in patients with CI.11) IL-8, as a pivotal mediator of cerebral reperfusion, is increased in brain tissues and a neutralizing anti-IL-8 antibody (Ab) significantly reduced brain edema and infarct size in comparison to rabbits receiving a control Ab.12) These results implicate that IL-8 is a novel target for the intervention of injury.

Other researches have shown that nuclear factor (NF)-κB plays an important role in inflammatory responses through the regulation of genes encoding proinflammatory cytokines and inducible enzyme.13–15) The prototype of NF-κB is a heterodimer consisting of p50 and p65 bound by members of the 1xB family, including 1xB-κ, in the cytoplasm.16,17) Phosphorylation of 1xB by bacterial products, viruses, drugs, and cytokines rapidly leads to 1xB degradation and translocation of NF-κB to the nucleus. Activation of NF-κB results in the binding of specific promoter elements and the expression of mRNAs for proinflammatory cytokine genes.18) Therefore, NF-κB is an appropriate target for inflammatory disease.

In this study, to elucidate the mechanism of GJWGM that accounts for its anti-inflammatory effect, we examined the effect of GJWGM on the production of TNF-κα, IL-1β, IL-6, IL-8 and the NF-κB pathway in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) from CI patients.

MATERIALS AND METHODS

Reagents Ficoll-Hypaque, avidin-peroxidase, 2-AZINO-bis (3-ethylbenzothiazoline-6-sulfonic acid) tablets substrate (ABTS), and LPS were purchased from Sigma (St. Louis, MO, U.S.A.). RPMI 1640, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Anti-human TNF-κ and IL-1β antibody (Ab), biotinylated anti-human TNF-κ, IL-1β, and recombinant human TNF-κ and IL-1β were purchased from R& D Systems (Minneapolis, MN, U.S.A.). Anti-human IL-6, IL-8
biotinylated anti-human IL-6, IL-8, and recombinant (r) human IL-6, IL-8 were purchased from Pharmingen (San Diego, CA, U.S.A.).

**Patients** Patients with CI were examined at the Department of Neurology, Wonkwang University (Iksan, Republic of Korea) from June 2004 to October 2004. The diagnosis of CI was confirmed with computerized tomography (CT) and magnetic resonance imaging (MRI) and clinical signs (hemiparesis, slurred speech, facial palsy etc.). For cytokine assay, blood samples were obtained from 12 patients (7 males and 5 females, age range 55—70) with CI. Ethics approval for human subject’s experiments was obtained from Wonkwang University. All patients have signed an informed consent.

**Preparation of GJWGM** GJWGM, which is a mixture of 13 traditional drugs, was obtained from the College of Oriental Medicine, Wonkwang University. Extract of GJWGM was prepared by decocting the dried prescription of herbs with boiling distilled water (84 g/l). The duration of decoction was about 3 h. The decoction was filtered, lyophilized and kept at 4 °C. The yield of extraction was about 11.8% (w/w). Amounts of the 13 traditional drugs studied in this work were shown in Table 1.

**BPMC Isolation and Culture** PBMC (patients with CI) from heparinized venous blood were isolated by Ficoll-gradient centrifugation, washed three times in phosphate-buffered saline (PBS) solution and resuspended in RPMI 1640 medium (Gibco) supplemented with 2 mM l-glutamin, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 10% FBS inactivated for 30 min at 56 °C. PBMC were adjusted to a concentration of 3 × 10^6 cells/ml in 30 ml Falcon tube, and 100 µl aliquots of cell suspension were placed in a four-well cell culture plate. PBMC were cultured for 24 h in 95% humified air containing 5% CO₂ (37 °C), in the presence or the absence of LPS, and the supernatants were collected by centrifugation and stored at −20 °C.

**MTT Assay** Cell viability was determined by MTT assay. Briefly, 500 µl of PBMC suspension (3 × 10^5 cells) was cultured in 4-well plates for 24 h after treatment by each concentration of GJWGM. 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 cell culture plate. PBMC were cultured for 24 h in 95% humified air containing 5% CO₂ (37 °C), in the presence or the absence of LPS, and the supernatants were collected by centrifugation and stored at −20 °C.

**Cytokines Assay** ELISA for TNF-α, IL-1β, IL-6, and IL-8 was carried out in duplicate in 96 well ELISA plates (Nunc, Denmark). The plates were coated with each of 100 µl aliquots of anti-human TNF-α, IL-1β, IL-6, and IL-8 monoclonal antibodies at 1.0 µg/ml in PBS at pH 7.4 and was incubated overnight at 4 °C. The plates were washed in PBS containing 0.05% Tween 20 (Sigma) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN₃ for 1 h. After additional washes, samples or TNF-α, IL-1β, IL-6, and IL-8 standards were added and incubated at 37 °C for 2 h. After 2 h incubation at 37 °C, the wells were washed and then each of 0.2 µg/ml of biotinylated anti-human TNF-α, IL-1β, IL-6, and IL-8 were added and again incubated at 37 °C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 20 min at 37 °C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. The inhibition percentage of cytokine release was calculated using the following equation:

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\%\text{ inhibition} = \left(\frac{A - B}{A}\right) \times 100
\]

where \(A\) is cytokine release without GJWGM and \(B\) is cytokine release with GJWGM.

**RNA Isolation and RT-PCR** Total RNA was isolated from PBMC according to the manufacturer’s specifications using an easy-BLUE RNA extraction kit (iNtRON Biotech, Republic of Korea). The concentration of total RNA in the final elutes was determined by spectrophotometer. Total RNA (2.0 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 a cDNA synthesis kit (Amersham Pharmacia, NJ, U.S.A.). RT-PCR was carried out with 1 µl of a cDNA mixture, in 20 µl final volume with 2.5 mM MgCl₂, 200 mM dNTPs, 25 µM of cytokine primers, and 2.5 U of Taq DNA polymerase in the reaction buffer (50 mM KCl, 10 mM Tris- HCl, pH 9, and 0.1% Triton X-100). PCR was performed with the following primers for human TNF-α (5’-CAC CAG CTG GTT A TC TCT CA-3’; 5’-CGG GAC GTG GAG GTG CTC GGC GAG GAG-3’), IL-8 (5’-CGA TGT CAG TGC ATA AAG ACA-3’; 5’-TGA ATT CTC AGC CCT CTT CAA AAA-3’), and GAPDH (5’-CAA AAG GGT CAT CAT CTC TG-3’; 5’-CCT GCT GCA CCA CCT TCT TG-3’), which were used to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 60 °C for TNF-α, and 82 °C for GAPDH. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The relative mRNA amounts were estimated by an image analyzer (VILBER LOURMAT FC-26WL, France).

**Preparation of Cyttoplasmic and Nuclear Extract** Nuclear and cytoplasmic extracts were prepared as described previously. Briefly, after cell activation for the times indicated, cells were washed with ice-cold PBS and resuspended in 60 µl of buffer A (10 mM HEPES/KOH, 2 mM MgCl₂, 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-40, and centrifuged at 2000 g for 10 min at 4 °C. The supernatant was

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**Table 1. Description of GJWGM**

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radix rehmanniae preparat</td>
<td>16</td>
</tr>
<tr>
<td>Rhizoma dioscorea</td>
<td>8</td>
</tr>
<tr>
<td>Fructus corn</td>
<td>8</td>
</tr>
<tr>
<td>Poria cocos</td>
<td>4</td>
</tr>
<tr>
<td>Cortex moutan radici</td>
<td>4</td>
</tr>
<tr>
<td>Rhizoma alismati</td>
<td>4</td>
</tr>
<tr>
<td>Fructus lycii</td>
<td>4</td>
</tr>
<tr>
<td>Flos chrysanthem</td>
<td>4</td>
</tr>
<tr>
<td>Fructus crataeg</td>
<td>8</td>
</tr>
<tr>
<td>Radix salviae miliorrhiza</td>
<td>4</td>
</tr>
<tr>
<td>Rhamulus uncariae cam unci</td>
<td>8</td>
</tr>
<tr>
<td>Rhizoma gastrodia</td>
<td>4</td>
</tr>
<tr>
<td>Fructus ligustri lucid</td>
<td>4</td>
</tr>
<tr>
<td>Total amounts</td>
<td>84</td>
</tr>
</tbody>
</table>
collected and used as the cytoplasmic extracts. The nuclei pellet was resuspended in 40μl 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, and inverted. The nuclear debris was then spun down at 15000g for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at −70°C until conducting the analysis.

**Western Blot Analysis** For analysis of the levels of NF-κB, phospho-IκB-α, and IκB-α, stimulated cells were rinsed twice with ice-cold PBS and were then lysed in ice-cold lysis buffer (1% Triton, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate in PBS). Cell lysates were centrifuged at 15000g for 5 min at 4°C; the supernatant was then mixed with an equal volume of 2×SDS sample buffer, boiled for 5 min, and then separated through 10% SDS–PAGE gels. After electrophoresis, the protein was transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% skim milk for 2 h, rinsed, and incubated overnight at 4°C with primary antibodies in PBS/0.5% Tween 20. Excess primary antibody was then removed by washing the membranes four times in PBS/0.5% Tween 20, and the membranes were incubated for 1 h with HRP-conjugated secondary antibodies (against mouse, goat, or rabbit). After three washes in PBS/0.5% Tween 20, the protein bands were visualized by an enhanced chemiluminescence assay (Amersham Pharmacia Biotech, NJ, U.S.A.) following the manufacturer’s instructions.

**Transcription Factor Enzyme-Linked Immunoassay (TF-EIA)** Avidin peroxidase was coated at 96 well ELISA plate. The plate was then washed with PBST and then blocked with 3% skim milk solution. Coated plate was incubated with 1μg/ml of 5’-biotinylated 21 single strand DNA oligonucleotide sequence for 1 h at the room temperature. This sequence contains the previously described NF-κB binding motif. The sequences used here were: 5’- AGT GGG GAC TTT CCC AGG- 3’. DNA binding reaction was carried out in tissue protein containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 5% glycerol, 1 mM EDTA, and 1 mM DTT, for 1 h at room temperature and then washed. NF-κB antibodies were then added at a 1:500 concentration in PBST containing 3% BSA for 1 h, followed by the addition of the corresponding alkaline phosphatase (AP)-coupled secondary antibody. Between each addition, wells were extensively washed in PBST. AP activity was then detected by the addition of p-nitrophenyl phosphate (PNPP) solution (Sigma). After a 10 min incubation period, the reaction was arrested by the addition of 0.5 M H2SO4. Color intensity was detected at 405 nm using ELISA reader. AP activity was normalized to control values (unstimulated cells). Protein was determined using a bicinchoninic acid (Sigma, St. Louis, MO, U.S.A.).

**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 ANOVA with a Tukey post hoc test. The results were considered significant at a value of p<0.05.

RESULTS

**Effects of GJWGM on LPS-Induced Cytokine Production** To assess the effect of GJWGM in LPS-induced TNF-α, IL-1β, IL-6, and IL-8 production from PBMC of CI patients, the cells were pretreated with various concentrations of GJWGM (0.01—1 mg/ml) for 1 h, and then treated with LPS for 24 h. As shown in Fig. 1, LPS enhanced the production of TNF-α, IL-1β, IL-6, and IL-8. These increases were inhibited by GJWGM in a dose-dependent manner. The maximal inhibition rate for TNF-α, IL-1β, IL-6, and IL-8 production by GJWGM (1 mg/ml) was about 54.34% (p<0.05), 41.37% (p<0.05), 44.04% (p<0.05), and 54.46% (p<0.05), respectively. Cell cytotoxicity by GJWGM was not observed (data not shown).

**Effects of GJWGM on LPS-Induced NF-κB and IL-8 mRNA Expression** The inhibition rate of TNF-α and IL-8 production by GJWGM was higher than the IL-1β and IL-6 production. Therefore, we examined whether the IL-8 gene can affect the mRNA expression of TNF-α and IL-8 in LPS-stimulated PBMC. The cells were pretreated with various concentrations of GJWGM for 1 h prior to LPS treatment of 8 h. As shown in Fig. 2, the level of TNF-α and IL-8 mRNA expression was increased by LPS however, pretreatment with GJWGM decreased the TNF-α and IL-8 mRNA level. The relative mRNA amounts were estimated using image analyzer.

**Effect of GJWGM on LPS-Induced NF-κB Activation** Since NF-κB activation requires nuclear translocation of RelA/p65 subunit of NF-κB, we examined the effect of GJWGM on the cytosolic and nuclear pool of RelA/p65 protein by Western blot analysis. As shown in Fig. 3A, LPS treatment considerably increased the nuclear RelA/p65 protein level and decreased the cytosolic RelA/p65, which is an indication of the nuclear translocation of RelA/p65. Pretreatment of GJWGM (1 mg/ml) inhibited the LPS-stimulated increase and decrease of the nuclear and cytosolic RelA/p65 levels, respectively. These results suggested that GJWGM blocks the nuclear translocation of the RelA/p65 from cytoplasm.

The NF-κB TF-EIA method was performed to investigate the effect of GJWGM on NF-κB binding activity. This assay has the advantage of being ten times more sensitive than...
Most agents that activate NF-κB mediate their effects through suppressing IκB-α phosphorylation and degradation. Therefore, we examined whether the GJWGM can modulate the phosphorylation and degradation of IκBα in cytoplasm. As shown in Fig. 3C, treatment of LPS effectively caused an induction of IκB-α phosphorylation, and degradation in PBMC, but the increased IκB-α phosphorylation and degradation were inhibited by treatment of GJWGM (1 mg/ml). The relative levels of pIκB-α, and IκB-α were estimated using image analyzer.

DISCUSSION

GJWGM is an oriental medicine, which consist of 13 different herbs. GJWGM can be hot water-extracted from the herb medicines. Other studies have reported that each herb medicine has a different effect. Some examples includes; Radix rehmanniae preparata has an anti-inflammatory activity in the CNS through the suppression of TNF-α secretion in LPS-stimulated astrocytes; Rhizoma dioscorea has an effect in rheumatoid arthritis through the inhibition of the production of TNF-α and IL-1β as well as down-regulating the expression of cyclooxygenase-2; Poria cocos has an anti-inflammation activity in arachidonic acid-induced ear inflammation in mice; and Fructus lycii has beneficial effects in the treatment of the immunodeficient diseases including rheumatoid arthritis. GJWGM is composed on the basis of the theory of Korean medicine to maximize its efficacy. In this study, we investigated the anti-inflammatory mechanism of GJWGM on LPS-stimulated PBMC isolated from CI patients.

Cytokines in stroke patients have been extensively studied during recent years. There are early inflammatory responses as indicated by the up-regulation of pro-inflammatory cytokines in brain autopsies after acute stroke. TNF-α is known to trigger a proinflammatory/prothrombotic reaction that is produced mainly by activated mononuclear leukocytes. IL-1β is produced rapidly in the brains of rodents exposed to CI, and enhances ischaemic and other forms of injury. Several investigators characterized the role of TNF-α and IL-1β in experimental cerebral nervous system (CNS) ischemia and found a therapeutic benefit of IL-1 receptor antagonist (IL-1RA) treatment. IL-6 is involved in modulating the acute expression of other proinflammatory cytokines in the brain after ischemia. These cytokines are involved in inflammation and exerting pathophysiological effects. Brain cells produce chemokines during the inflammatory process after stroke both in animal models and patients. IL-8, a major chemokine known to attract and activate leukocytes, has recently been under focused investigation due to its possible participation in the evolution of CI. In this study, we showed that GJWGM effectively inhibited the productions of TNF-α, IL-1β, IL-6, and IL-8 in LPS-stimulated PBMC. Inhibition rate of TNF-α and IL-8 production by GJWGM was observed to be higher than the IL-1β and IL-6 production. We also showed that GJWGM inhibited the TNF-α and IL-8 mRNA expression. These results indicate that GJWGM has an anti-inflammatory effect through the regulation of these cytokines, which may explain its benefic-
The suppression of NF-κB activation has been linked with anti-inflammation.30,31 We postulated that GJWGM mediates its effects at least partly through the suppression of NF-κB activation. The activation of NF-κB is dependent on the phosphorylation and degradation of IκB-α, an endogenous inhibitor that binds to NF-κB in the cytoplasm. Many other researchers reported that NF-κB might indeed be a good target for brain inflammation therapy. For example, Pyrrolidinedithiocarbamate (PDTC), a NF-κB inhibitor, suppressed the induction of IL-1β, TNF-α, and NF-κB activation during human stroke, therefore, PDTC and its structurally modified derivatives with possible greater efficacy may represent potential therapies against stroke.32 Previously, we reported that aucubin inhibited NF-κB activation via phosphorylation and degradation of IκB-α.33 In this study, we showed that GJWGM suppresses NF-κB transcriptional activation and translocation to the nucleus induced by LPS in PBMC isolated from CI patients. This suppression is also mediated through the inhibition of IκB-α phosphorylation and degradation. Therefore, our results suggest that the anti-inflammatory effect of GJWGM is similar to the mechanism of aucubin. Hence, it is hypothesized that GJWGM may act as a potent NF-κB inhibitor on the PBMC activation induced by LPS.

In conclusion, we have shown that GJWGM can regulate the inflammatory response induced by LPS in PBMC isolated from CI patients. GJWGM affected the production and expression of inflammatory cytokines through the regulation of the NF-κB/IκB-α pathway. These results provide new insight into the pharmacological actions of GJWGM as a potential molecule for therapy against inflammatory diseases such as CI.

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REFERENCES