Inhibition of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 by Gamijihwang-tang Via Suppression of Nuclear Factor-B Activation in RAW 264.7 cells

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Asthma is recognized today as an inflammatory disease of the lung characterized by acute non-specific airway hypersensitiveness in association with chronic pulmonary inflammation. Gamijihwang-tang(GJT), a fortified prescription of YMJHT, is applied for the treatments of chronic coughing and asthma, and post-delivery coughing and asthma in the gynecology. Also in the clinical practice, GJT is known to be very effective for controlling coughing and asthma as a cold sequela. In this study, we investigated the effects of GJT on the lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production, and on the level of inducible nitric oxide synthase (iNOS) and Cyclooxygenase-2 expression in murine macrophage RAW 264.7 cells. We found that GJT inhibited LPS-induced NO and PGE_2 production in a dose dependent manner. Furthermore, GJT inhibited the expression of LPS-induced iNOS and COX-2 protein and mRNA expression in RAW 264.7 macrophages. Treatment with GJT of RAW 264.7 cells transfected with a reporter construct indicated a reduced level of LPS-induced nuclear factor-κB (NF-κB) activity and effectively lowered NF-κB binding as measured by transient transfection assay. These results suggest that the main inhibitory mechanism of the GJT may be the reduction of iNOS and COX-2 gene expression through blocking of NF-κB activation.

Key words : Gamijihwang-tang(GJT), inducible nitric oxide synthase, cyclooxygenase-2, NF-κB

Introduction

Asthma isa major public health problem worldwide and is recognized today as an inflammatory disease of the lung characterized by acute non-specific airway hypersensitiveness in association with chronic pulmonary inflammation[4].

The previous emphasis on bronchodilator therapy, which does not treat the underlying inflammation, may be misplaced. Effective suppression of airway inflammation reduces the need for bronchodilator therapy and may reduce the morbidity and, perhaps, mortality of asthma[4]. Although the precise mechanisms that lead to the development of allergic asthma are not fully understood, associations among antigen-induced bronchial inflammation and airway hyper reactivity (AHR) has been well documented[11]. Since inflammation is a principal factor in bronchial inflammation and AHR, attention has focused on suppressing inflammatory processes. In asthmatic children inhaled corticosteroids are widely used. However, there are some concerns about the systemic adverse effects of these drugs, especially in the growing child. Corticosteroids suppress both Th1 and Th2 responses, which can result in increased susceptibility to infections[16]. In view of this, the search for alternative safe and effective asthma treatments is intensifying.

A prescription for Gamijihwang-tang (GJT) was adapted from Daejeon University Oriental Medicinal Hospital, and is used for the treatment of post-delivery coughing in the gynecology. GJT is the Yulmijihwang-tang (YMJHT) prescription fortified with the additional ingredients known to be effective for halting descending 'qi', expiring the cold-wind, and stopping coughing by removing phlegm. GJT is thus widely used for diverse pulmonary diseases caused by 'eumheo' meaning a state of lack in body's essential fluids including blood and semen, or 'hyulheo' meaning a state of deficiency in blood.

Nitric oxide (NO) is produced from L-arginine by nitric

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oxide synthase (NOS), a family of ubiquitous enzymes. NOS play a major role in regulating vascular tone, neurotransmission, the killing of microorganisms and tumor cells and other homeostatic mechanisms[15]. iNOS activation catalyzes the formation of a large amount of NO, which plays a key role in a variety of pathophysiological processes including various forms of inflammation and carcinogenesis[14]. Therefore, the amount of NO produced by iNOS may be a reflection of the degree of inflammation, and therefore provide a means of assessing the effect of drugs on the inflammatory process. Because cells cannot sequester and regulate the local concentration of NO, the regulation ofNO synthesis is the key to eliciting its biological activity. NO production by iNOS is mainly regulated at the transcriptional level[13]. In macrophages, LPS activates the transcription factor nuclear factor-κB (NF-κB), which leads to the induction of expression of many immediate early genes[3]. The presence of the cis-acting NF-κB element has been demonstrated in the 5'-flanking regions of the iNOS genes[3]. NF-κB is an obvious targetfor new types of anti-inflammatory treatment[2].

Cyclooxygenase (COX) catalyzes the synthesis of prostaglandins (PGs) from arachidonic acid. Two isozymes, COX-1 and COX-2, have been identified but are encoded by separate genes. The COX-1 isozyme is believed to be a housekeeping protein in most tissues and appears to catalyze the synthesis of PGs for normal physiological functions. In contrast, COX-2 is not present under normal physiological conditions but is rapidly induced in various cell types by tumor promoters, growth factors, cytokines and mitogens[18]. Many cell types associated with inflammation, such as macrophages, endothelial cells and fibroblasts, express the COX-2 gene upon induction[17]. It is well established that COX-2 is important in carcinogenesis, and is over-expressed in transformed cells as well as in various forms of cancer[18]. Because the targeted inhibition of COX-2 is a promising approach to inhibiting inflammation and carcinogenesis as well as to prevent cancer, various chemopreventive strategies have focused on inhibitors of the COX-2 enzyme activity. In macrophages, LPS activates NF-κB, eventually induces the expression of many immediate early genes[8]. Therefore, the pathways leading to NF-κB activation are frequent targets for a variety of anti-inflammatory drugs[19]. It is increasingly being acknowledged that foods and beverages contain non-nutritional constituents that may have beneficial health effects, such as anti-inflammatory and anti-carcinogenic properties[5].

In the present study, we investigated the effect of GJT on iNOS and COX-2 in LPS-induced RAW 264.7 macrophages. We provide evidence to support to GJT induced down-regulation of LPS-induced iNOS and COX-2 gene expression and that suppression is mediated through the NF-κB inactivation of these genes.

Materials and methods

1. Materials

The chemicals and cell culture materials were obtained from the following sources: Escherichia coli 0111:B4 lipopolysaccharide (LPS) from Sigma Co.; MTT-based colorimetric assay kit from Roche Co.; LipofectAMINE Plus, RPMI 1640, fetal bovine serum, and penicillin-streptomycin solution from Gibco BRL-Life Technologies, Inc. pGL3-4B-Luc, pCMV--gal, and the luciferase assay system from Promega; The enzyme-linked immunosorbent assay (ELISA) kit for PGE2 from R&D systems. Antibodies to iNOS, COX-2, and α-tubulin from Santa Cruz Biotechnology, Inc. Western blotting detection reagents (ECL) from Amersham Pharmacia Biotech.; the other chemicals were of the highest commercial grade available.

2. Cell culture and cell viability assay.

The mouse macrophage cell line, RAW 264.7 cells, was obtained from the American Type Culture Collection (Bethesda, MD), and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ㎍/ml streptomycin at 37 ℃ in a 5% CO_2 humidified incubator. GJT wasdissolved in dimethylsulfoxide and added directly to the culture media. The control cells were treated with the solvents only, the final concentration of which never exceeded 0.1%, which is a concentration that did not have any noticeable effect on the assay systems. The cell viability was assessed using a MTT assay according to the manufacturer's instructions.

3. Nitrite assay.

RAW 264.7 cells (5 X 10^5 cells/ml) were cultured in 48-well plates. After incubating for the cells for 24 h, the level of NO production was determined by measuring the nitrite level in the culture supernatants, which is a stable reaction product of a reaction between NO and molecular oxygen, using a Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water), and the absorbance of the mixture at 550 nm was determined with a microplate fluorometer. LPS was used as a positive control.

4. PGE_2 production

RAW 264.7 cells were subcultured in 24-well plates and

were incubated with the chemicals and/or LPS (0.5 μg/ml) for 24 h. After incubating the cells, the PGE_2 concentration in the culture medium was determined using an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions.

5. Immunoblot analysis

The cells were cultured with the GJT for 24 hand the cell lysates were then prepared by treating the cells with a lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1 % Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin). The protein concentration of the supernatant was measured using the method reported by Bradford[6]. SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels. The resolved proteins were transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with COX-2 polyclonal antiserum or monoclonal anti-α-tubulin. The secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

6. RNA preparation and mRNA analysis by reverse transcription-polymerase chain reaction (RT-PCR).

The cells were cultured with GJT and/or LPS (0.5 μg/ml) for 2 or 6 h. Total cellular RNA was isolated by the acidic phenol extraction procedure of Chomczynski and Sacchi[7]. cDNA synthesis, semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) for iNOS, COX-2, and β -actin mRNA, and the analysis of the results were all performed as described previously[10]. PCR reactions were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. Prior to analysis the PCR product band intensities were checked to ensure that they had not reached the saturation intensity.

7. Transfection and luciferase and -galactosidase assays.

The RAW 264.7 cells (5 X 10^5 cells/ml) were plated in each well of a 12-well plate, and transiently co-transfected with the plasmids, pGL3-4κB-Luc and pCMV-β-gal 12 h later using the LipofectAMINE Plus according to the manufacturer's protocol. Briefly, a transfection mixture containing 0.5 μg of pGL3-4κB-Luc and 0.2 μg of pCMV-β-gal was mixed with the LipofectAMINE Plus reagent and added to the cells. After 18 h, the cells were treated with LPS and/or GJT for 18 h, and then lysed. The luciferase and β-galactosidase activities were determined using a method described elsewhere12). The luciferase activity was normalized with respect to the β -galactosidase activity and is expressed relative to the activity of the control.

8. Statistical Analysis.

All the experiments were repeated at least three times. The data is presented as mean ± SD of at least three different sets of plates and treatment groups. A Student's t-test was used to examine the statistical significance of the differences. A $p < 0.01$ was considered significant.

Results

1. Inhibition of LPS-induced NO production in RAW 264.7 macrophages by GJT

To evaluate the effect of GJT on NO production in LPS-induced RAW 264.7 macrophages, nitrite accumulation was examined by the Griess assay. Fig. 1. shows that LPS (0.5 μg/ml) treatment for 24 h triggered significant nitrite accumulation, which was effectively inhibited in a dose-dependent manner by treatment with GJT. None of the GJT had any effect on cell viability at the various concentrations used, as indicated by the modified MTT assay.

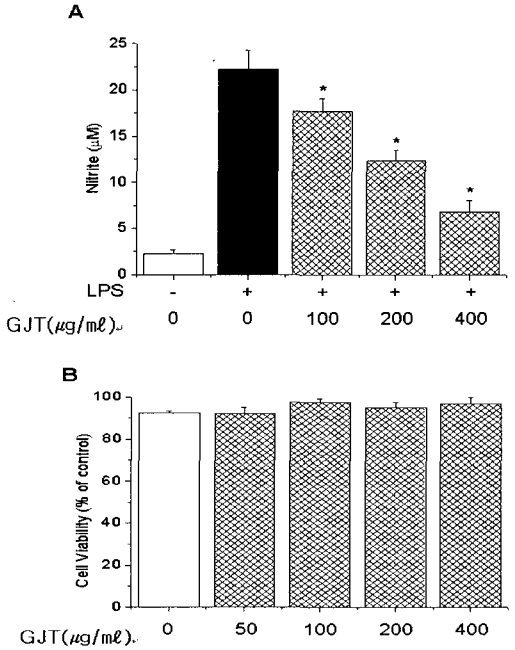

Fig. 1. Effects of GJT on NO production. (A). RAW 264.7 cells (5×10^5 cells/ml) were treated with GJT in the presence of LPS (0.5 μg/ml). Nitrite production was measured by the Griess reaction assay as described in the methods section. Data were obtained from three independent experiments and were expressed as means ± SD. *P < 0.01, significantly different from the LPS. (B). Cell viability was determined by MTT assay and expressed as a percentage of the control without the addition of indicated GJT.

2. Inhibition of LPS-induced PGE_2 synthesis in RAW 264.7 macrophages by GJT

We investigated the possibility that GJT could inhibit LPS-induced PGE_2 synthesis in RAW 264.7 macrophages (Fig. 2). When incubated with vehicle alone, the cells yielded 2.58 ± 0.12 ng/ml of PGE_2. Treatment of the cells with 0.5 μg/ml LPS produced 25.4 ± 2.6 ng/ml of PGE_2, an 10 fold increase of PGE_2 production compared to the control. When treated with LPS following pre-treatment with GJT (100~400 μg/ml), however, the cells showed markedly decreased production of PGE_2. Suppression of PGE_2 production by concentration of GJT was significant as compared to cells receiving LPS treatment alone.

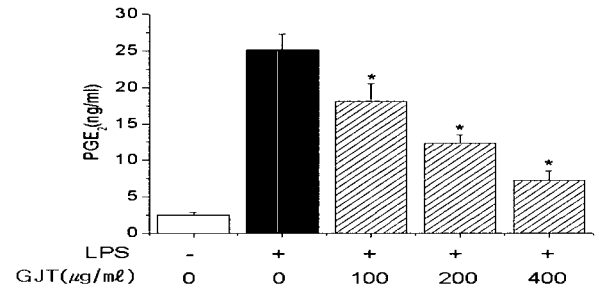

Fig. 2. Effects of GJT on PGE_2 production. RAW 264.7 cells were treated with GJT in the presence of LPS (0.5 μg/ml). The supernatants were harvested 24 h later and assayed for PGE_2 production. PGE_2 concentrations in the culture medium were measured by ELISA as described in Materials and methods. The values are expressed as means ± SD of triplicate experiments. *P<0.01, significantly different from the LPS.

3. Inhibition of LPS-induced iNOS and COX-2 gene expression by GJT

The effects of the GJT on iNOS and COX-2 protein expression in RAW 264.7 macrophages were examined by Western blotting. As shown in Fig. 3, the cells expressed extremely low levels of iNOS and COX-2 protein in an un-stimulated condition; however, iNOS and COX-2 protein expression was markedly increased in response to LPS (0.5 μg /ml) after 20 h. Treatment with GJT caused dose dependent decreases in LPS-induced iNOS and COX-2 protein expression.

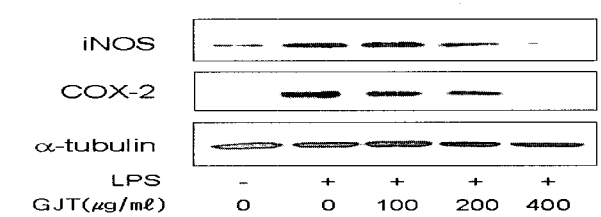

Fig. 3. Effect of GJT on LPS-induced expression of LPS-induced iNOS and COX-2 protein levels. RAW 264.7 cells were treated with GJT in the presence of LPS (0.5 μg/ml). After 24 h of incubation, the cell lysates (80 μg protein) were subjected to 8% (for iNOS) and 10% (for COX-2) SDS-PAGE, and expression of iNOS and COX-2 protein was determined by Western blotting using specific anti-iNOS and anti-COX-2 antibodies. α-Tubulin was used as an internal control.

4. Inhibition of LPS-induced iNOS and COX-2 mRNA expression by GJT

· We have attempted to determine whether the expression of iNOS and COX-2 proteins paralleled their mRNA levels. Following 30 min pre-treatment with GJT, RAW 264.7macrophages were activated with LPS, then harvested and assayed for COX-2 (2 h) and iNOS (6 h) mRNA expression by RT-PCR. Pre-treatment of cells with GJT prior to LPS treatment resulted in a complete suppression of LPS-induced iNOS and COX-2 mRNA induction(Fig. 4).

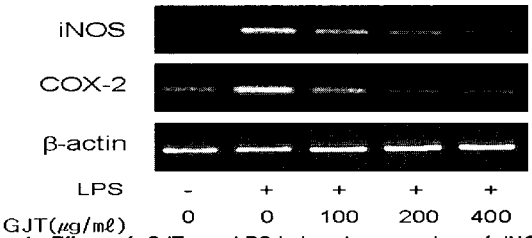

Fig. 4. Effect of GJT on LPS-induced expression of iNOS and COX-2 mRNA. RAW 264.7 cells were treated with GJT in the presence of LPS (0.5 μg/ml). Total RNA was prepared and RT-PCR was performed as described in Materials and methods. The PCR products were separated on a 2.5 % agarose gel and stained with ethidium bromide. β-actin was used as an internal control.

5. Inhibition of LPS-induced NF-κB activation by GJT

The transcription factor NF-κB is activated in response to stimulation by LPS, and this activation is an essential step in the induction of iNOS and COX-2 gene expression. Transient transfection with a NF-κB-dependent luciferase reporter plasmid was done to confirm whether GJT inhibited the NF-κB binding activity in LPS-activated macrophages. As shown in Fig. 5. GJT inhibited the LPS-activated NF-κB transcriptional activity in a dose-dependent manner. These results suggest that the suppression of iNOS and COX-2 gene expression by GJT occurred via the prevention of NF-κB activation.

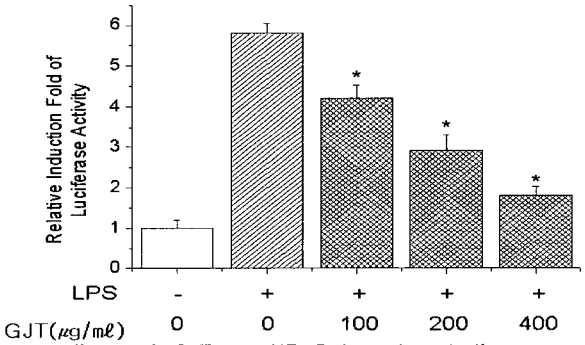

Fig. 5. Effects of GJT on NF-κB-dependent luciferase gene expression. RAW 264.7 cells were transiently co-transfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, the cells were treated with LPS (0.5 μg/ml) and/or GJT (100, 200, 400 μg/ml) for 18 h, harvested and their luciferase and β-galactosidase activities determined. Luciferase activities were expressed relative to the control. Each bar shows the mean±S.D. of three independent experiments performed in triplicate. *P<0.01, significantly different from the LPS.

Discussion

In this study, we demonstrated that GJT inhibited the iNOS and COX-2 gene expression from LPS stimulated RAW 264.7 cells. GJT was adapted from Daejeon University Oriental Medicinal Hospital. It has been used for prevention and treatment of cough and bronchial asthma in Korea. And also it is widely used for diverse pulmonary diseases caused by 'eumheo'(陰虛) meaning a state of lack in body's essential fluids including blood and semen, or 'hyulheo' meaning a state of deficiency in blood.

GJT was the most potent inhibitors of LPS induced NO and PGE_2 production. From this point of view, we suggest that the presence of GJT is responsible for their strong anti-inflammatory properties. GJT appearsto decrease the protein levels of iNOS and COX-2 by reducing the expression of iNOS and COX-2 mRNAs. At the mRNA level, the expression of iNOS and COX-2 in murine macrophages is largely regulated by transcriptional activation. The promoter of the iNOS gene contains two major and discrete regions that function synergistically in the binding of transcription factors[20]. Among these transcription factors NF-κB, which is a primary transcription factor activated by LPS, and regulates various genes, is important in immune response and inflammation. GJT also inhibited LPS-induced NF-κB activation therefore inhibited expression of iNOS and COX-2 expression. Because NF-κB plays a key role in regulating the genes involved in the initiation of the immune, acute phase, and inflammatory responses, there is growing interest in modulating its activity. Transcription factors, such as NF-κB and AP-1, play an important role in the orchestration of the airway inflammation in asthma[1].

The role of NF-κB should be seen as an amplifying and perpetuating mechanism that will exaggerate the disease-specific inflammatory process. There is evidence for activation of NF-κB in the bronchial epithelial cells of patient with asthma[9]. NF-κB is an activator of multiple inflammatory cytokines, chemokines and adhesion molecules, which are important in inflammatory diseases such as asthma, and is consequently considered as an attractive therapeutic target. In conclusion, our results demonstrate that the GJT isespecially potent inhibitors of LPS-induced iNOS and COX-2 gene expression in RAW 264.7 macrophages, and that this inhibition is apparently mediated by the blocking of NF-κB activation. These observations suggest that GJT exert an anti-inflammatory action through regulation of the NF-κB inactivation. The beneficial effect of GJT in the treatment of asthma seems to be due to its actions as an anti-inflammatory agent.

Acknowledgments

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