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ABSTRACT

The Experimental Study on anti-inflammatory Effect of GamiJihwangTang

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목적: 이 연구는 천식, 기관지염, 폐렴, 결핵, 산후감모 등의 호흡기 질환에 사용되는 加味地黃湯의 抗炎作用의 효과에 대해 알아보기 위한 실험이었다.

방법: 加味地黃湯의 抗炎作用의 효과를 평가하기 위해 세포독성에 미치는 영향, NO, TNF-α, IL-1β, IL-6 생성량에 미치는 영향, TNF-α, IL-1β, IL-6 유전자 발현에 미치는 영향, iNOS, COX-2 유전자 및 단백질 발현에 미치는 영향, PGE2 합성에 미치는 영향 및 COX-2, NF-κB 활성을 미치는 영향에 대한 실험을 진행하였다.

결과: 加味地黃湯은 MTT 분석을 통한 RAW 264.7 세포주의 생존력 평가에서 세포독성이 없었고, LPS로 유도된 RAW 264.7 세포주에서 NO, TNF-α, IL-1β 및 IL-6 생성량을 높도 의존적으로 억제하였다. 加味地黃湯은 400 g/ml 농도에서 LPS로 유도된 RAW 264.7 세포주에 대해 TNF-α, IL-1β 및 IL-6 유전자 발현을 높도 의존적으로 억제하였고, LPS로 유도된 RAW 264.7 세포주에서 iNOS와 COX-2 유전자 및 단백질 발현은 높도 의존적으로 억제하였다. 또한 그 농도에 따라 PGE2 생성량이 현저하게 억제하였고, LPS로 유도된 COX-2 및 NF-κB 전사활성을 높도 의존적으로 억제함으로써 iNOS와 COX-2 유전자 발현을 억제하였다.

결론: 이 연구의 실험을 통해 加味地黃湯은 iNOS나 COX-2와 같은 cytokine이 있는 혈소에 의해 억제되고 천식에서 증가하는, 혈관과 기관지 건강을 위해 필요로 하는 NO와 PGE2 생성량을 억제하고, 염증과 관련된 TNF-α, IL-1β, IL-6의 생성량을 억제하였다. 또한 NF-κB 활성을 억제함으로써 iNOS 및 COX-2 유전자 발현을 억제하였으므로 부인과 염증에 있어서도 산호감소, 만성해수 및 천식 등의 기관지의 염증철환에 응용할 수 있을 것으로 사료된다.

Key words: GamiJihwangTang (GJT), anti-inflammatory Effect, inflammatory diseases

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I. Introduction

Asthma is a major public health problem worldwide and is recognized today as an inflammatory disease of the lung characterized by acute non-specific airway hypersensitivity in association with chronic pulmonary inflammation\(^1\).

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\(^2\). 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) have been well documented\(^3\). 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 growing children. Corticosteroids suppress both Th1 and Th2 responses, which can result in increased susceptibility to infections. In view of this, the search for alternative safe and effective asthma treatments is intensifying. The chronic airway inflammation of asthma is unique in that the airway wall is infiltrated by lymphocytes of the T-helper (Th) type 2 phenotype, eosinophils, macrophages/ monocytes, and mast cells. Many of the effector cells, including mast cells in asthma, produce a variety of cytokines\(^4\). The concentrations of tumor necrosis factor (TNF)-α and interleukin (IL)-6 has been reported to be significantly high in bronchial asthma patients\(^5\). Another common theme in asthma and its associated inflammation of the airway is the increased presence of the pro-inflammatory cytokine IL-1β.

A prescription for Gamijihwangtang (GJT) was adapted from Daejeon University Oriental Medicinal Hospital, and is used for the treatment of post-delivery coughing in the gynecology department. GJT is the Yukmijihwangtang (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) and prostanoids are mediators of vascular and bronchial tone that are postulated to be involved in asthma. Increased levels of both are found in asthmatic subjects and are synthesised by enzymes that have
cytokine inducible forms: inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. Mucosal inflammation in asthma is characterized by infiltration and activation of immune cells—principally mast cells, eosinophils, T lymphocytes and monocytes/macrophages, and activation of structural cells such as airway epithelial cells and subepithelial myofibroblasts. These various cell populations are believed to release biochemical mediators whose biological properties determine the clinical and pathophysiological expression of the disease. Prominent among these putative mediators are nitric oxide (NO) and prostanoids.

Nitric oxide (NO) is synthesized from L-arginine in the human respiratory tract by enzymes of the NO synthase (NOS) family. Levels of NO in exhaled air are increased in asthma, and measurement of exhaled NO has been advocated as a noninvasive tool to monitor the underlying inflammatory process. NOS plays a major role in regulating vascular tone, neurotransmission, the killing of microorganisms and tumor cells and other homostatic mechanisms. 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. 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 of NO synthesis is the key to eliciting its biological activity. NO production by iNOS is mainly regulated at the transcriptional level. In macrophages, LPS activates the transcription factor nuclear factor-κB (NF-κB), which leads to the induction of expression of many immediate early genes. The presence of the cis-acting NF-κB element has been demonstrated in the 5′-flanking regions of the iNOS genes. NF-κB is an obvious target for new types of anti-inflammatory treatment.

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. There are two isoforms of COX-1 that catalyze the formation of prostaglandins (PGs) from arachidonic acid. COX-1 is a housekeeping gene that is expressed constitutively. COX-2 is an immediate, early response gene that is highly inducible by mitogenic and inflammatory stimuli. The differences in the regulation of COX-1 and COX-2 gene expression reflect differences in the regulatory elements in the 5′-flanking regions of the two genes. Considerable evidence has accumulated to suggest that COX-2 is important for tumorigenesis. For example, COX-2 is
up-regulated in transformed cells\textsuperscript{12} and various forms of cancer\textsuperscript{13}. whereas levels of COX-1 remain essentially unchanged. COX-2 deficiency also protected against the formation of extraintestinal tumors. Thus, COX-2 knockout mice developed approximately 75% fewer chemically induced skin papillomas than control mice\textsuperscript{14}. A selective inhibitor of COX-2 caused nearly complete suppression of azoxymethane-induced colon cancer. Many cell types associated with inflammation, such as macrophages, endothelial cells and fibroblasts, express the COX-2 gene upon induction\textsuperscript{15}. 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\textsuperscript{15}. 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\textsuperscript{16}. Therefore, the pathways leading to NF-κB activation are frequent targets for a variety of anti-inflammatory drugs\textsuperscript{17}. It is increasingly being acknowledged that foods and beverages containing non-nutritional constituents may have beneficial health effects, such as anti-inflammatory and anti-carcinogenic properties\textsuperscript{18}.

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.

II. 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.; pGL3-4κB-Luc, pCMV-β-gal, and the luciferase assay system from Promega; Methyl thiazol tetrazolium assay (MTT assay) for cell viability from Sigma-Aldrich (St. Louis, MO, USA); The enzyme-linked immunosorbent assay (ELISA) kit for PGE\textsubscript{2} 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
The mouse macrophage cell line, RAW 264.7 cells, were 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 μg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. GJT was dissolved 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.

3. Cell viability
The RAW 264.7 cells (2 × 10⁴ cells/ml) were seeded on 96-well plates in RPMI-1640 medium, and methyl thiazol tetrazolium (MTT) assay was performed for cell viability. Cells were incubated with 0.25 μg/ml MTT for 4h at 37°C and the reaction was terminated by the addition of 100% dimethylsulfoxide. The amount of MTT formazon product was determined by using a microplate reader, and the absorbance was measured at 560 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

4. Nitrite assay
RAW 264.7 cells (5 × 10⁵ 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% naphthylethlenediamine 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.

5. PGE2 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 PGE₂ concentration in the culture medium was determined using an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer’s instructions.

6. Immunoblot analysis
The cells were cultured with the GJT for 24 h and 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. 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.

7. 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. 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. 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.

8. Transfection and luciferase and β-galactosidase assays

The RAW 264.7 cells (5 × 10⁵ cells/ml) were plated in each well of a 12-well plate, and transiently co-transfected with the plasmids, pGL3-4xB-Luc, pGL3-COX-2-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 g of pGL3-4xB-Luc, 0.5 g of pGL3-COX-2-Luc and 0.2 g 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 elsewhere. The luciferase activity was normalized with respect to the β-galactosidase activity and is expressed relative to the activity of the control.

9. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as previously described. Two double-stranded deoxyoligonucleotides containing the NF-κB binding site 5′-CAGAGGGAC TTTCCGAGAG-3′, bold and underlined indicates NF-κB core consensus sequences) were end-labeled with [-32P]dATP. Nuclear extracts (5 g) were incubated with 2 g of poly (dIdC) and the 32P-labeled DNA probe in binding buffer (100 mM NaCl, 30 mM
HEPES, 1.5 mM MgCl2, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml concentration each of aprotinin and leupeptin) for 10 min on ice. DNA was separated from the free probe using a 4.8% polyacrylamide gel in 0.5 TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). Following electrophoresis, the gel was dried and subjected to autoradiography.

10. 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. p < 0.01 was considered significant.

III. Results

1. No effects of GJT on cytotoxicity in RAW 264.7 cells
To test the cytotoxicity of GJT, we performed MTT assay in RAW 264.7 cells. Fig. 1. shows the cell viability at 24 h incubation after treatment with GJT (50 ~ 400 μg/ml). A MTT assay was used to examine the cytotoxicity of GJT in the macrophages. The results showed that the GJT itself had no effect on viability of RAW 264.7 cells (Fig. 1).

Fig. 1. Effects of GJT on cell viability. MTT viability staining assay for cells either untreated or exposed to 100, 200, and 400 M GJT for 24 h. After 24 h, cell viability was evaluated by the MTT assay. Values for MTT assays are expressed as % control value (> 95% cell viability).

2. 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. 2 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. GJT alone did not affect NO production. However, GJT inhibited LPS-induced NO production in a dose-dependent manner in RAW 264.7 cells (Fig. 2).
by ELISA method. GJT dose-dependently inhibited the production of TNF-α in LPS-stimulated RAW 264.7 cell (Fig. 3-1).

4. Inhibitory effect of IL-1β production by GJT

We examined the inhibitory effect of GJT on the LPS-induced production of IL-1β from RAW 264.7 cells. Culture cell lysates were assayed for IL-1β levels by ELISA method. GJT dose-dependently inhibited the production of IL-1β in LPS-stimulated RAW 264.7 cells (Fig. 3-2).

5. Inhibitory effect of IL-6 production by GJT

We examined the inhibitory effect of GJT on the LPS-induced production of IL-6 from RAW 264.7 cells. Culture cell lysates were assayed for IL-6 levels by ELISA method. GJT dose-dependently inhibited the production of IL-6 in LPS-stimulated RAW 264.7 cells (Fig. 3-3).
6. Inhibition of TNF-α gene expression by GJT

In order to determine whether GJT regulates TNF-α production at the mRNA level, a RT-PCR assay was conducted with LPS as a positive control. Consistent with the results obtained from the proinflammatory cytokines production assays, LPS-inducible TNF-α mRNA levels were found to be markedly suppressed by GJT treatment (Fig. 4-1). The control β-actin was constitutively expressed, and was unaffected by the GJT treatment. Therefore, a decrease in the TNF-α levels by GJT is believed to be regulated by the transcriptional activation.

7. Inhibition of IL-1β gene expression by GJT

In order to determine whether GJT regulates IL-1β production at the mRNA level, a RT-PCR assay was conducted with LPS as a positive control. Consistent with the results obtained from the proinflammatory cytokines production assays, LPS-inducible IL-1β mRNA levels were found to be markedly suppressed by GJT treatment (Fig. 4-2). The control β-actin was constitutively expressed, and was unaffected by the GJT treatment. Therefore, a decrease in the IL-1β levels by GJT is believed to be regulated by the transcriptional activation.

8. Inhibition of IL-6 gene expression
9. Inhibition of iNOS gene expression by GJT

In order to determine whether GJT regulates regulated NO production at the mRNA level, a RT-PCR assay was conducted with LPS as a positive control. Consistent with the results obtained from the proinflammatory cytokines production assays, LPS-inducible iNOS mRNA levels were found to be markedly suppressed by GJT treatment (Fig. 5-1). The control β-actin was constitutively expressed and was unaffected by the GJT treatment. Therefore, a decrease in the iNOS levels by GJT is believed to be regulated by the transcriptional activation.

10. Inhibition of COX-2 gene expression by GJT

In order to determine whether GJT regulates PGE2 production at the mRNA level, a RT-PCR assay was conducted with LPS as a positive control. Consistent with the results obtained from the PGE2 production assays, LPS-inducible COX-2 mRNA levels were found to be markedly suppressed by GJT treatment (Fig. 5-2). The control β-actin was
constitutively expressed and was unaffected by the GJT treatment. Therefore, a decrease in the COX-2 levels by GJT is believed to be regulated by the transcriptional activation.

Fig. 5-1. Effects of GJT on iNOS mRNA expression

Fig. 5-2. Effects of GJT on COX-2 mRNA expression

Fig. 5. Effects of GJT on iNOS mRNA. COX-2 mRNA expression. RAW 264.7 cells (1×10⁶ cells/ml) were treated with LPS (0.5 µg/ml) and/or GJT (100, 200, and 400 µg/ml) for 6 h. The cells were lysed, and the total RNA was analyzed by RT-PCR. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel, and stained with ethidium bromide. (A) Quantified iNOS, COX-2 levels are shown as iNOS, COX-2 / β-actin, and (B) mRNA expression reported as mean SD. The ratio of the RT-PCR products of iNOS, COX-2 to β-actin was calculated. Induction-fold is represented as mean SD of three separate experiments. *P<0.01, significantly different from the LPS.

11. Inhibition of iNOS protein expression by GJT

The effects of the GJT on iNOS protein expression in RAW 264.7 macrophages were examined by Western blotting. As shown in Fig. 6-1, the cells expressed extremely low levels of iNOS protein in an un-stimulated condition. However, iNOS 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 protein expression.

12. Inhibition of COX-2 protein expression by GJT

The effects of the GJT on COX-2 protein expression in RAW 264.7
macrophages were examined by Western blotting. As shown in Fig. 6-2, the cells expressed extremely low levels of COX-2 protein in an un-stimulated condition, however, 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 COX-2 protein expression.

Fig. 6-1. Effect of GJT on LPS-induced expression of LPS-induced iNOS protein levels

Fig. 6-2. Effect of GJT on LPS-induced expression of LPS-induced COX-2 protein levels

Fig. 6. Effect of GJT on LPS-induced expression of LPS-induced iNOS, 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 g protein) were subjected to 8% SDS-PAGE, and expression of iNOS was determined by Western blotting using specific anti-iNOS. α-tubulin was used as an internal control. (A) Quantified iNOS, COX-2 levels are shown as iNOS, COX-2 / α-tubulin, and (B) protein expression reported as mean SD. The ratio of the RT-PCR products of iNOS, COX-2 to α-tubulin was calculated. Induction-fold is represented as mean SD of three separate experiments. *P<0.01, significantly different from the LPS.

13. Inhibition of LPS-induced PGE2 synthesis in RAW 264.7 macrophages by GJT

We investigated the possibility that GJT could inhibit LPS-induced PGE2 synthesis in RAW 264.7 macrophages (Fig. 7). When they were incubated with vehicle alone, the cells yielded 2.58 ± 0.12 ng/ml of PGE2. Treatment of the cells with 0.5 μg/ml LPS produced 25.4 ± 2.6 ng/ml of PGE2, a 10 fold increase of PGE2 production compared
to the control. When they were treated with LPS following pre-treatment with GJT (100–400 µg/ml), however, the cells showed markedly decreased production of PGE2. Suppression of PGE2 production by concentration of GJT was significant as compared to cells receiving LPS treatment alone.

![Graph](image)

**Fig. 7.** Effects of GJT on PGE2 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 PGE2 production. PGE2 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.

14. Inhibition of LPS-induced COX-2 activation by GJT

Transient transfections were performed to determine whether differences in amounts of COX-2 mRNA reflect altered rates of transcription. As shown in Fig. 8, a marked increase in rates of synthesis of nascent COX-2 mRNA was detected after treatment with LPS, and it was consistent with the differences observed by RT-PCR. Transient transfection with a COX-2-dependent luciferase reporter plasmid was done to confirm whether GJT inhibited the COX-2 binding activity in LPS-activated macrophages. As shown in Fig. 8, GJT inhibited the LPS-activated COX-2 transcriptional activity in a dose-dependent manner. These results suggest that the suppression of COX-2 gene expression by GJT occurred via the prevention of COX-2 transcriptional activation.

![Graph](image)

**Fig. 8.** Effects of GJT on COX-2-dependent luciferase gene expression in macrophages. The RAW 264.7 cells (5 x 10^5 cells/ml) were transiently co-transfected with pGL3-COX-2-Luc and pCMV-β-gal. After 18 h, the cells were treated with the indicated concentrations of GJT (100, 200, and 400 µg/ml) and/or LPS (0.5 µg/ml). The cells were then harvested, and their luciferase and β-galactosidase activities were determined. The luciferase activity was normalized with respect to the β-galactosidase activity, and expressed relative to the activity of the control. Each bar shows the mean SD of three independent experiments, and performed in triplicate. *P<0.01, significantly different from the LPS.

15. 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. 9, 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.

16. Inhibition of LPS-induced NF-κB activation using electrophoretic mobility shift assay by GJT

This study investigated whether or not GJT could suppress NF-κB activation in the LPS-activated macrophages using an electrophoretic mobility shift assay. The induction of the NF-κB binding activity by LPS was markedly inhibited by GJT in a dose-dependent manner (Fig. 10). The addition of an excessive quantity of an unlabeled wild type probe completely prevented the NF-κB binding, demonstrating the binding specificity of the NF-κB complex.

Fig. 9. Effects of GJT on NF-κB-dependent luciferase gene expression in macrophages. The RAW 264.7 cells (5 × 10⁵ cells/ml) were transiently co-transfected with pGL3-4κB–Luc and pCMV–β–gal. After 18 h, the cells were treated with the indicated concentrations of GJT (100, 200, and 400 μg/ml) and/or LPS (0.5 μg/ml). The cells were then harvested, and their luciferase and β-galactosidase activities were determined. The luciferase activity was normalized with respect to the β-galactosidase activity, and expressed relative to the activity of the control. Each bar shows the mean SD of three independent experiments, and performed in triplicate. *P<0.01. significantly different from the LPS.

Fig. 10. Effects of GJT on NF-κB-binding in macrophages. The RAW 264.7 cells were treated with GJT (100, 200, and 400 μg/ml) or LPS (0.1 μg/ml) for 1 h. The nuclear extracts were isolated, and used in an electrophoretic mobility shift assay with 32P-labeled NF-κB oligonucleotide as a probe, as described in Materials and Methods. The arrow indicates the NF-κB binding complex. Excess NF-κB: 200-fold molar excess of non-labeled NF-κB probe.
IV. Discussion

In this study, we demonstrated that GJT inhibited the TNF-α, IL-1β, and IL-6 production from LPS-stimulated RAW 264.7 cells. GJT also suppressed the LPS-activated expression of iNOS and COX-2, which have NF-κB binding sites in its promoter, and regulate its transcription in macrophages.

A prescription for Gamijihwangtang (GJT) is used for the treatment of post-delivery coughing in the gynecology. GJT is the Yukmjihwangtang (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.

This study showed that GJT inhibit NO and PGE₂ production in LPS-stimulated macrophages in a dose-dependent manner. This suggests the possible suppression of COX-2 induction by this compound. From this point of view, we suggest that the presence of GJT is responsible for their strong anti-inflammatory properties. Therefore, the effect of GJT on COX-2 expression was investigated in order to obtain a better understanding of the inhibitory mechanism of PGE₂ production. GJT appears to 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. One of 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, and 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. 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. 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 is especially potent inhibitor of LPS-induced iNOS and COX-2 gene expression in RAW 264.7 macrophages, and this inhibition is apparently mediated by the blocking of NF-κB activation. Because the suppression of NF-κB has been implicated in chemoprevention, it is also possible that the anti-carcinogenic effects of GJT are mediated via the suppression of NF-κB-dependent gene expression. Some anti-inflammatory drugs have actually been tested in human clinical trials to prevent cancer, and numerous pharmaceutical companies are developing new drugs targeting NF-κB, iNOS, COX-2, etc. Many signaling pathways and molecules (e.g. NF-κB, iNOS, etc.) however, play dual roles in inflammation and carcinogenesis. For example, inhibition of NF-κB activation or iNOS promotes inflammation and carcinogenesis under certain circumstances. The expression of iNOS and COX-2 in murine macrophages has been shown to be dependent on NF-κB activation. The possibility that GJT might inhibit the activity of NF-κB was examined. The results indicate that the inhibition by GJT on the expression of the iNOS and COX-2 proteins, and iNOS, COX-2 and inflammatory cytokines mRNA was most likely due to the suppression of NF-κB. This is consistent with previous reports that NF-κB response elements are present on the promoters for the iNOS, COX-2 and inflammatory cytokine genes. NF-κB is primarily composed of two proteins, p50 and p65, which are also referred to as aRel and cRel, respectively. These observations suggest that GJT exert an anti-inflammatory action through regulation of the NF-κB inactivation. In conclusion, these results indicate that GJT is a potent inhibitor of the LPS-induced NO, PGE₂ and inflammatory cytokines production via gene expression, and this inhibition was found to be caused by the blocking of NF-κB activation in RAW 264.7 macrophages. The beneficial effect of GJT in the treatment of asthma seems to be due to its actions as an anti-inflammatory agent. Further studies are clearly needed to identify the molecular mechanisms by which chronic inflammation increases cancer risk, and to develop new and more effective strategies for cancer prevention on the basis of these findings.

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