

清肺化痰湯의 抗炎作用에 대한 實驗的 研究

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ABSTRACT

The Experimental Study on anti-inflammatory Effect of ChengpyeHwadamtang

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목 적: 이 연구는 천식, 기관지염, 폐렴, 결핵, 산후감모 등의 호흡기 질환에 사용되는 清肺化痰湯의 抗炎作用의 효과에 대해 알아보기 위해 시행되었다.

방 법: 清肺化痰湯의 抗炎作用의 효과를 평가하기 위해 세포독성에 미치는 영향, NO, TNF- α , IL-1 β , IL-6 생성량에 미치는 영향, TNF- α , IL-1 β , IL-6 유전자 발현에 미치는 영향, iNOS, 염증cytokine 유전자 및 단백질 발현에 미치는 영향, PGE₂ 합성에 미치는 영향 및 NF- κ B 활성화에 미치는 영향에 대한 실험평가를 하였다.

결 과: 清肺化痰湯은 MTT 분석을 통한 RAW 264.7 세포주의 생존력 평가에서 세포독성이 없었고, LPS로 유도된 RAW 264.7 세포주에서 NO 생성량을 농도 의존적으로 억제하였다. 清肺化痰湯은 400 g/ml 농도에서 LPS로 유도된 RAW 264.7 세포주에 대해 TNF- α , IL-1 β , IL-6 생성량을 각각 41.86 \pm 2.26 %, 61.11 \pm 2.54 %, 55.33 \pm 3.65 % 억제하였으며 TNF- α , IL-1 β 및 IL-6 유전자 발현을 농도 의존적으로 억제하였고, LPS로 유도된 RAW 264.7 세포주에서 iNOS와 COX-2 유전자 및 단백질 발현은 농도 의존적으로 억제하였다. 또한 그 농도에 따라 PGE₂ 생성량이 현저하게 억제하였고, LPS로 유도된 NF- κ B 전사활성을 농도 의존적으로 억제함으로써 iNOS와 염증Cytokine 유전자 발현을 하향조절 하였다.

결 론: 이상의 실험을 통해 清肺化痰湯은 LPS로 유도된 macrophage에서 NO와 염증Cytokine 생성량을 억제하였고 murine macrophage에서 NF- κ B 활성을 억제함으로써 iNOS와 염증Cytokine 유전자 발현을 하향조절 하였다. 이러한 清肺化痰湯의 항염작용으로 천식, 기관지염, 폐렴, 결핵, 산후감모 등의 호흡기 질환에 응용할 수 있으리라 사료된다.

Key Words: Cheongpyehwadamtang (CHT), anti-inflammatory Effect, inflammatory diseases

I. Introduction

Cheongpyehwadamtang (CHT) is composed of *Puerariae Radix*, *Bupleuri Radix*, *Pinelliae Rhizoma*, *Citri Pericarpium*, *Poria*, *Perillae Folium*, *Armeniaca Amarum Semen*, *Mori Cortex*, *Platycody Radix*, *Aurantii Fructus*, *Glycyrrhizae Radix*, *Farfarae Flos* and *Gleditsiae Spina*. CHT is known to be effective for the cure of asthma by regaining normal lung function, and to lower the body temperature and eliminate the phlegm. Thus, it has long been used for the treatment of inflammatory diseases in the lung and bronchus such as bronchitis, bronchial asthma, pneumonia and tuberculosis. Asthma is an inflammatory disease of the airways caused by a range of factors including reactions to inhaled allergens, pollutants and infections with respiratory viruses. Asthma is a chronic inflammatory disorder of the airways in which many cells and elements play a role. The chronic inflammation is associated with increased airway hyper responsiveness leading to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning¹⁾. The chronic airway inflammation of asthma is unique in that the airway wall is infiltrated by lymphocytes of the T-helper (T_h) type 2 phenotype, eosinophils, macrophages/monocytes, and mast

cells²⁾. Many of the effector cells, including mast cells in asthma, produce a variety of cytokines³⁾. The concentrations of tumor necrosis factor (TNF)- α and interleukin (IL)-6 has been reported to be significantly high in bronchial asthma patients⁴⁾. Another common theme in asthma and its associated inflammation of the airway is the increased presence of the pro-inflammatory cytokine IL-1 β ⁵⁾.

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

II. Materials and methods

Materials.

The chemicals and cell culture materials were obtained from the following sources: *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) from Sigma Co. (St Louis, MO); MTTbased colorimetric assay kit from Roche Co. (Indianapolis, IN); LipofectAMINE Plus, Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin

solution from Gibco BRL-Life Technologies, Inc. (Grand Island, NY); pGL3-4κB-Luc, pCMV-β-gal and the luciferase assay system from Promega (Madison, WI); The enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, IL-6, and TNF-α from R&D Systems (Minneapolis, MN, USA). Methyl thiazol tetrazolium assay (MTT assay) for cell viability from Sigma-Aldrich (St. Louis, MO, USA): 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.

Cell cultures.

The RAW 264.7 cells, which are a mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD), and grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. The CHT was dissolved in dimethylsulfoxide to make a stock solution, and added directly to the culture media. The control cells were treated with the solvent only at a final concentration that never exceeded 0.1%, which is a

concentration that did not have any adverse effect on the assay systems. The cell viability was assessed using a MTT assay according to the manufacturer's instructions.

Cell viability

The RAW 264.7 cells (2×10^4 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 mg/ml MTT for 4h at 37°C and the reaction was terminated by the addition of 100% dimethylsulfoxide. The amount of MTT fromazon 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.

Nitrite assay.

The nitrite accumulated in the culture medium was measured as an indicator of NO production bases on Griess reaction⁶¹. RAW 264.7 cells (5×10^5 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. One hundred microliters of each medium supernatant was mixed with 50 μl of 1% sulphanilamide (in 5% phosphoric acid) and 50 μl of 0.1% of

naphtylenediamine dihydrochloride (in distilled water), and then incubated at room temperature for 10 min. the absorbance at 550nm was measured using the NaNO₂ serial dilution standard curve, and nitrite production was determined.

PGE₂ production

RAW 264.7 cells were subcultured in 24-well plates and were incubated with the chemicals and/or LPS (0.5 g/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.

Immunoassay of TNF- α , IL-1 β , and IL-6 production

For TNF- α , IL-1 β , and IL-6 cytokine production was measured by modification of ELISA as described previously⁷⁾. The cells were stimulated with LPS (0.5 g/ml) for 3 or 24 h in the absence or presence of CHT. The ELISA was performed by coating 96-well plates with 6.25 ng/well of human monoclonal antibody with specificity for TNF- α , IL-1 β , and IL-6, respectively. Before use and between subsequent steps in the assay, the coated plates were washed three times with PBS containing 0.05% Tween-20 (PBST). All reagents used in this assay and the coated wells were incubated for 1 h at room temperature. For the

standard curve, rhTNF- α , rh IL-1 β , and rhIL-6 were added to serum previously determined to be negative for endogenous TNF- α , IL-1 β , and IL-6. After exposure to the medium, the assay plates were exposed sequentially to biotinylated anti-human TNF- α , IL-1 β , and IL-6, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) tablet substrates. Optical density readings were made within 10 min of the addition of the substrate. Then, the optical density was measured using an ELISA reader at 405 nm.

Immunoblot analysis

The cells were cultured with the CHT 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 g/ml aprotinin, 10 g/ml trypsin inhibitor, and 10 g/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 iNOS, 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.

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

The RAW 264.7 cells (2×10^5 cells/ml) were cultured with either CHT or LPS (0.5 g/ml) for 2 or 6 h. Total cellular RNA was isolated by the acidic phenol extraction procedure of Chomczynski and Sacchi⁸⁾. The methods used for cDNA synthesis, semiquantitative RT-PCR for IL-1 β , IL-6, TNF- α , COX-2, iNOS and β -actin mRNA, and the analysis of the results are described elsewhere⁷⁾. The 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.

Transfection and luciferase and β -galactosidase assays.

The RAW 264.7 cells (5×10^5 cells/ml) were plated in each well of a 12-well plate, and transiently co-transfected with the plasmids, pGL3-4 κ B-Luc and pCMV- β -gal 18 h later using the LipofectAMINE Plus according to the manufacturer's protocol. Briefly, a transfection mixture containing 0.5 g of pGL3-4 κ B-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 CHT for 18 h, and then lysed. The luciferase and β -galactosidase activities were determined using a method described elsewhere²⁰⁾. The luciferase activity was normalized with respect to the β -galactosidase activity and is expressed relative to the activity of the control.

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

No effects of CHT on cytotoxicity in RAW 264.7 cells

To test the cytotoxicity of CHT, we performed MTT assay in RAW 264.7 cells. Fig. 1. shows the cell viability at 24 h incubation after treatment with CHT (100 ~ 400 g/ml). A MTT assay was used to examine the cytotoxicity of CHT in the macrophages. The results showed that the CHT concentration used in these experiments did not adversely affect the cell viability (Fig. 1).

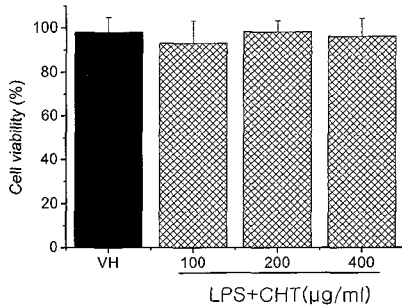


Fig. 1. Effects of CHT on cell viability. MTT viability staining assay for cells either untreated or exposed to 100, 200, and 400 M CHT for 24 h. Cell viability was evaluated by the MTT assay. Values for MTT assays are expressed as % control value (> 95% cell viability).

Effect of the CHT on NO production

We performed to investigate the effects of CHT on NO production, and its effects on the levels of iNOS gene expression in mouse macrophages. NO production was assessed using the Griess reaction. In the present study, the potent macrophage activator LPS increased NO production compared to the control. CHT alone did not affect NO production. However, CHT inhibited LPS-induced NO production in a dose-dependent manner in RAW 264.7 cells (Fig. 2).

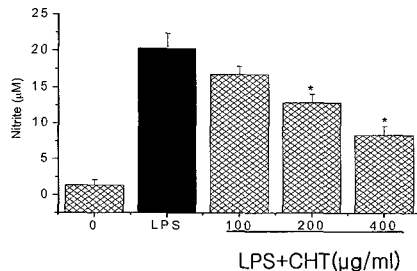


Fig. 2. Effects of CHT on NO

production in macrophages. RAW 264.7 cells (5×10^5 cells/ml) were treated with CHT in the presence of LPS (0.5 µg/ml). The medium was harvested 24 h later and assayed for nitrite production. The cell viability was evaluated with the MTT assay (solid line connecting solid squares). The results are presented as a percentage of the control value obtained from non-treated cells. Values are expressed as mean S.D. of three individual experiments, performed in triplicate. * $P < 0.01$, significantly different from the LPS.

Effects of the CHT on macrophage-related TNF-α production

We examined the inhibitory effect of CHT on the LPS-induced production of TNF-α, pro-inflammatory cytokine, from RAW 264.7 cells. Culture supernatants was assayed for each cytokine levels by ELISA method. CHT dose-dependently inhibited the production of TNF-α in LPS-stimulated RAW 264.7 cells. CHT treatment at 400 µg/ml blocked TNF-α production by $41.86 \pm 2.26\%$, as compared with no treatment of CHT ($P < 0.001$), respectively (Table. 1).

Table 1. Effects of CHT on LPS-stimulated TNF-α production in RAW 264.7 cells.

Treatment ^a	TNF-α (ng/ml)
Control	0.55 ± 0.17^b
LPS (0.1 µg/ml)	11.58 ± 1.17
LPS plus CHT (100 µg/ml)	$10.22 \pm 0.05^*$
LPS plus CHT (200 µg/ml)	$7.12 \pm 1.21^*$
LPS plus CHT (400 µg/ml)	$4.85 \pm 1.30^*$

^a : The RAW 264.7 cells (5×10^5 cells/ml)

were pretreated with CHT for 30 min, and the challenged with CHT for either 3 h. The amount of TNF- α released to the culture medium was measured by an immunoassay.
^b : The Results are expressed as a mean SD of four independent experiments, performed in triplicate. * $P < 0.01$, significantly different from the LPS.

Effects of the CHT on macrophage-related IL-1 β production

We examined the inhibitory effect of CHT on the LPS-induced production of IL-1 β , pro-inflammatoiry cytokine, from RAW 264.7 cells. Cell lysates were assayed for each cytokine levels by ELISA method. CHT dose-dependently inhibited the production of IL-1 β production in LPS-stimulated RAW 264.7 cells. CHT treatment at 400 g/ml blocked IL-1 β production by 61.11 \pm 2.54% , as compared with no treatment of CHT ($P < 0.001$), respectively (Table. 2).

Table 2. Effects of CHT on LPS-stimulated IL-1 β production in RAW 264.7 cells.

Treatment ^a	IL-1 β (ng/ml)
Control	0.28 \pm 0.01 ^b
LPS (0.1 μ g/ml)	4.88 \pm 0.65
LPS plus CHT (100 μ g/ml)	4.21 \pm 0.33*
LPS plus CHT (200 μ g/ml)	3.72 \pm 0.40*
LPS plus CHT (400 μ g/ml)	2.70 \pm 0.23*

^a : The RAW 264.7 cells (5×10^5 cells/ml) were pretreated with CHT for 30 min, and the challenged with CHT for either 24 h. The amount of IL-1 β released to the cell lysates was measured by an immunoassay.
^b : The Results are expressed as a mean SD of four independent experiments, performed in triplicate. * $P < 0.01$, significantly different

from the LPS.

Effects of the CHT on macrophage-related IL-6 production

We examined the inhibitory effect of CHT on the LPS-induced production of IL-6, pro-inflammatoiry cytokine, from RAW 264.7 cells. Culture supernatants were assayed for each cytokine levels by ELISA method. CHT dose-dependently inhibited the production of IL-6 production in LPS-stimulated RAW 264.7 cells. CHT treatment at 400 g/ml blocked IL-6 production by 55.33 \pm 3.65%, as compared with no treatment of CHT ($P < 0.001$), respectively (Table. 3).

Table 3. Effects of CHT on LPS-stimulated IL-6 production in RAW 264.7 cells.

Treatment ^a	IL-6 (ng/ml)
Control	0.31 \pm 0.03 ^b
LPS (0.1 μ g/ml)	3.85 \pm 0.13
LPS plus CHT (100 μ g/ml)	3.57 \pm 0.24
LPS plus CHT (200 μ g/ml)	2.95 \pm 0.37*
LPS plus CHT (400 μ g/ml)	2.37 \pm 0.27*

^a : The RAW 264.7 cells (5×10^5 cells/ml) were pretreated with CHT for 30 min, and the challenged with CHT for either 24 h. The amount of IL-6 released to the culture medium was measured by an immunoassay.
^b : The Results are expressed as a mean SD of four independent experiments, performed in triplicate. * $P < 0.01$, significantly different from the LPS.

Effects of the CHT on macrophage-related TNF- α gene expression

In order to determine whether CHT regulates TNF- α , proinflammatory

cytokine. 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 CHT treatment (Fig. 3). The control β -actin was constitutively expressed, and was unaffected by the CHT treatment. Therefore, a decrease in the TNF- α levels by CHT is believed to be regulated by the transcriptional activation.

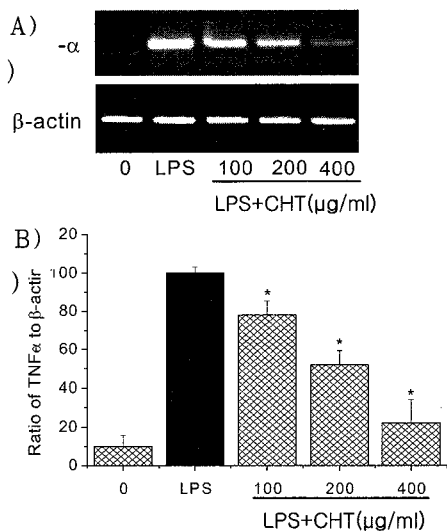


Fig. 3. Effects of CHT on TNF- α mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were treated with LPS (0.5 μ g/ml) and/or CHT (100, 200, and 400 μ g/ml) for 6 h. The cells were lysed, and the total RNA was analyzed by RT-PCR. PCR amplification of the housekeeping gene, β -actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel, and

stained with ethidium bromide. One of three representative experiments is shown. (A) Quantified TNF- α levels are shown as TNF- α / β -actin, and (B) mRNA expression reported as mean SD. The ratio of the RT-PCR products of TNF- α to β -actin was calculated. Induction-fold is represented as mean SD of three separate experiments. * $P < 0.01$, significantly different from the LPS.

Effects of the CHT on macrophage-related IL-1 β gene expression

In order to determine whether CHT regulates IL-1 β , proinflammatory cytokine, 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-1 β mRNA levels were found to be markedly suppressed by CHT treatment (Fig. 4). The control β -actin was constitutively expressed, and was unaffected by the CHT treatment. Therefore, a decrease in the IL-1 β levels by CHT is believed to be regulated by the transcriptional activation.

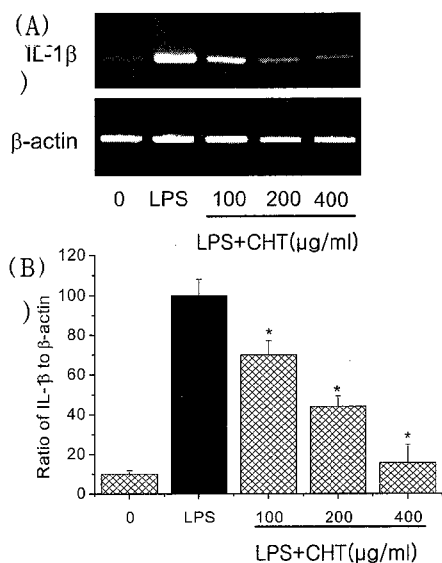


Fig. 4. Effects of CHT on IL-1 β mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were treated with LPS (0.5 μ g/ml) and/or CHT (100, 200, and 400 μ g/ml) for 6 h. The cells were lysed, and the total RNA was analyzed by RT-PCR. PCR amplification of the housekeeping gene, β -actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel, and stained with ethidium bromide. One of three representative experiments is shown. (A) Quantified IL-1 β levels are shown as IL-1 β / β -actin, and (B) mRNA expression reported as mean SD. The ratio of the RT-PCR products of IL-1 β to β -actin was calculated. Induction-fold is represented as mean SD of three separate experiments. * $P < 0.01$, significantly different from the LPS.

Effects of the CHT on macrophage-related IL-6 gene expression

In order to determine whether CHT regulates IL-6, proinflammatory cytokine, 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-6 mRNA levels were found to be markedly suppressed by CHT treatment (Fig. 5). The control β -actin was constitutively expressed, and was unaffected by the CHT treatment. Therefore, a decrease in the IL-6 levels by CHT is believed to be regulated by the transcriptional activation.

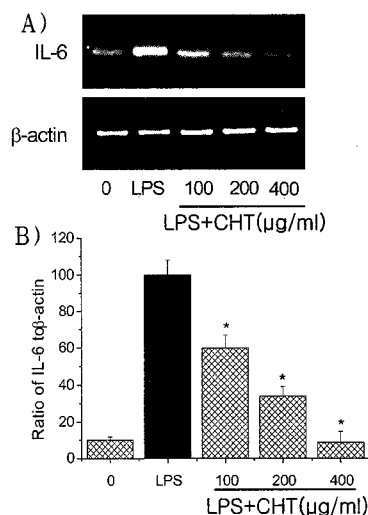


Fig. 5. Effects of CHT on IL-6 mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were treated with LPS (0.5 μ g/ml) and/or CHT (100, 200, and 400 μ g/ml) for 6 h. The cells were lysed, and the total RNA was analyzed by RT-PCR. PCR amplification of the housekeeping gene, β -actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel, and stained with ethidium bromide. One of three representative experiments is shown. (A) Quantified IL-6 levels are shown as IL-6 / β -actin, and (B)

mRNA expression reported as mean SD. The ratio of the RT-PCR products of IL-6 to β -actin was calculated. Induction-fold is represented as mean SD of three separate experiments. * $P < 0.01$, significantly different from the LPS.

Effects of the CHT on macrophage-related iNOS gene expression

In order to determine whether CHT 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 CHT treatment (Fig. 6). The control β -actin was constitutively expressed and was unaffected by the CHT treatment. Therefore, a decrease in the iNOS levels by CHT is believed to be regulated by the transcriptional activation.

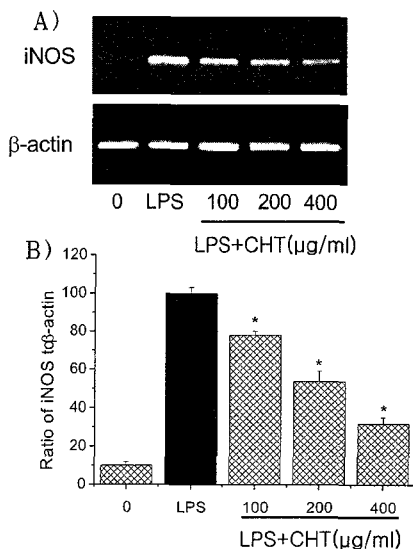


Fig. 6. Effects of CHT on iNOS mRNA

expression. RAW 264.7 cells (1×10^6 cells/ml) were treated with LPS (0.5 μ g/ml) and/or CHT (100, 200, 400 μ g/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. One of three representative experiments is shown. (A) Quantified iNOS levels are shown as iNOS / β -actin, and (B) mRNA expression reported as mean SD. The ratio of the RT-PCR products of iNOS to β -actin was calculated. Induction-fold is represented as mean SD of three separate experiments. * $P < 0.01$, significantly different from the LPS.

Effects of the CHT on iNOS protein expression

The effects of the CHT on iNOS protein expression in RAW 264.7 macrophages were examined by Western blotting. As shown in Fig. 7, 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 μ g/ml) after 20 h. Treatment with CHT caused dose dependent decreases in LPS-induced iNOS protein expression.

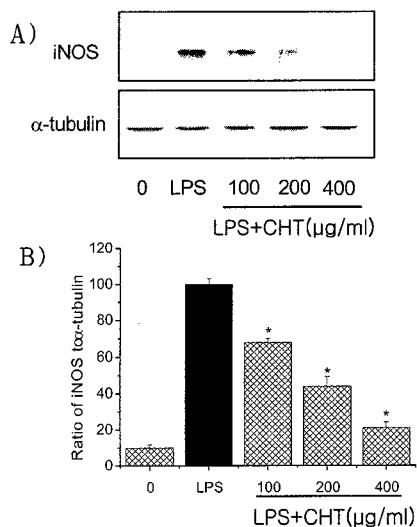


Fig. 7. Effect of CHT on LPS-induced expression of LPS-induced iNOS protein levels. RAW 264.7 cell lysates were extracted after the treatment with LPS and/or CHT(100, 200, 400 µg/ml) and western blot analysis was performed. (A) Cell lysates (80 µg protein) were subjected to 8% SDS-PAGE, and expression of iNOS was determined by Western blotting using specific anti-iNOS. α-tubulin was used as an internal control. (B) Densitometry analysis of iNOS-protein expression. Expression of iNOS protein was induced by LPS treatment (0.5 µg/ml) and co-incubation with 100, 200, and 400 µg/ml of CHT. Mean densitometry units SD of three separate experiments. * $P < 0.01$, significantly different from the LPS.

Effects of CHT on PGE₂ synthesis

We investigated the possibility that CHT could inhibit LPS-induced PGE₂ synthesis in RAW 264.7 macrophages (Fig. 8). 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 g/ml LPS produced

25.4 ± 2.6 ng/ml of PGE₂, an 10 fold increase of PGE₂ production compared to the control. When they were treated with LPS following pre-treatment with CHT (100~400 g/ml), however, the cells showed markedly decreased production of PGE₂. Suppression of PGE₂ production by concentration of CHT was significant as compared to cells receiving LPS treatment alone.

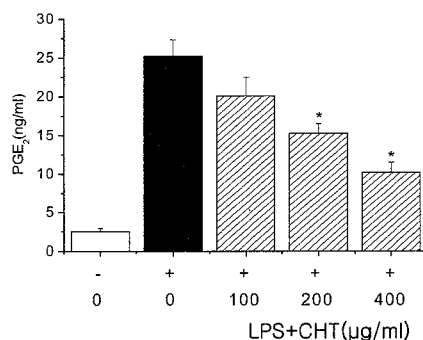


Fig. 8. Effects of CHT on PGE₂ production. RAW 264.7 cells were treated with CHT in the presence of LPS (0.5 µg/ml). The supernatants were harvested 24 h later and assayed for PGE₂ production. PGE₂ concentrations in the culture medium were measured by ELISA as described in Materials and methods. The values are expressed as means S.D. of triplicate experiments. * $P < 0.01$, significantly different from the LPS.

Effects of the CHT on macrophage-related COX-2 gene expression

In order to determine whether CHT 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 CHT treatment (Fig. 9.). The control β -actin was constitutively expressed and was unaffected by the CHT treatment. Therefore, a decrease in the COX-2 levels by CHT is believed to be regulated by the transcriptional activation.

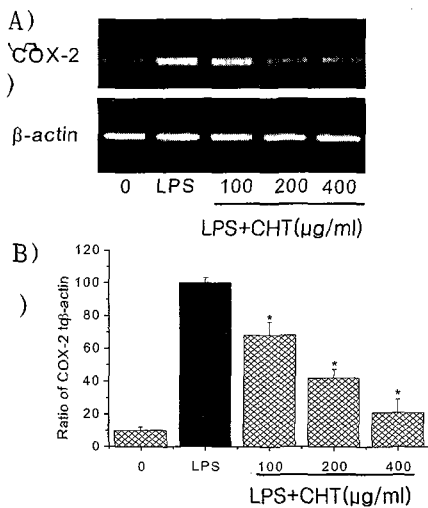


Fig. 9. Effects of CHT on COX-2 mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were treated with LPS (0.5 μ g/ml) and/or CHT (100, 200, and 400 μ g/ml) for 2 h. The cells were lysed, and the total RNA was analyzed by RT-PCR. PCR amplification of the housekeeping gene, β -actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel, and stained with ethidium bromide. One of three representative experiments is shown. (A) Quantified COX-2 levels are shown as COX-2 / β -actin, and (B) mRNA expression reported as mean SD. The ratio of the RT-PCR products of 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.v

Effects of the CHT on COX-2 protein expression

The effects of the CHT on COX-2 protein expression in RAW 264.7 macrophages were examined by Western blotting. As shown in Fig. 10, 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 g/ml) after 20 h. Treatment with CHT caused dose dependent decreases in LPS-induced COX-2 protein expression.

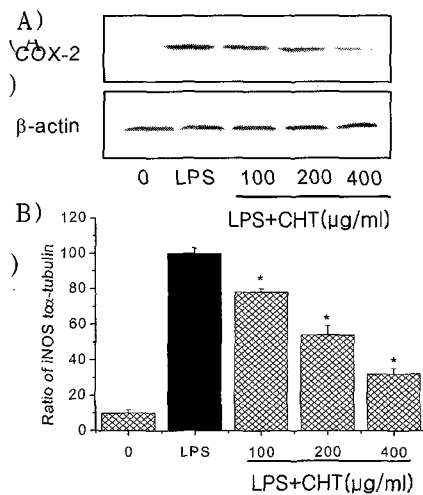


Fig. 10. Effect of CHT on LPS-induced expression of LPS- induced COX-2 protein levels. RAW 264.7 cell lysates were extracted after the treatment with LPS and/or CHT (100, 200, 400 μ g/ml) and western blot analysis was performed. (A) Cell lysates (80 μ g protein) were subjected to 10%

SDS-PAGE, and expression of COX-2 and was determined by Western blotting using specific anti-COX-2. α -tubulin was used as an internal control. (B) Densitometry analysis of COX-2-protein expression. Expression of COX-2 protein was induced by LPS treatment (0.5 μ g/ml) and co-incubation with 100, 200, and 400 μ g/ml of CHT. Mean densitometry units SD of three separate experiments. * P <0.01, significantly different from the LPS.

Effects of the CHT on NF- κ B activation

To further investigate the role of CHT on iNOS and inflammatory cytokines gene expression, the effect of CHT on NF- κ B-dependent gene expression was assessed using the luciferase reporter gene assay. RAW 264.7 cells were transiently transfected with a plasmid containing four copies of the NF- κ B binding sites, and the luciferase activities were measured. A near six fold increase in luciferase activity was observed compared to the unstimulated control cells, when cells were stimulated with LPS. Consistent with NO production and iNOS mRNA measurement, CHT also significantly decreased NF- κ B-dependent luciferase activities in a dose-dependent manner (Fig. 11). These results indicate that the down-regulation of the iNOS and inflammatory cytokine genes by CHT is mediated by the inhibition of NF- κ B transactivation.

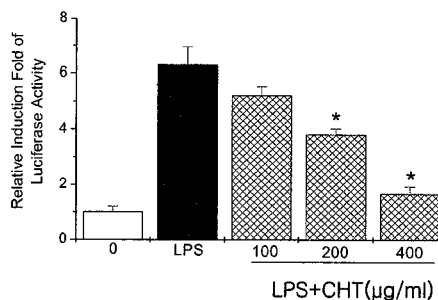


Fig. 11. Effects of CHT on NF- κ B-dependent luciferase gene expression. RAW 264.7 cells were transiently co-transfected with pGL3-4 κ B-Luc and pCMV- β -gal. After 18 h, the cells were treated with LPS (0.5 μ g/ml) and/or CHT (100, 200, and 400 μ g/ml) for 18 h, harvested and their luciferase and β -galactosidase activities determined. Luciferase activities were expressed relative to the mean SD of three independent experiments performed in triplicate. * P <0.01, significantly different from the LPS.

IV. Discussion

In this study, we demonstrated that CHT inhibited the iNOS, COX-2, and proinflammatory cytokines expression from LPS stimulated RAW 264.7 cells. In this study, we demonstrated that CHT inhibited the NO, PGE₂, and proinflammatory cytokines (TNF- α , IL-6, and IL-1 β) production from LPS stimulated RAW 264.7 cells. From this point of view, we suggest that the presence of CHT is responsible for their strong anti-inflammatory properties. These results suggest that the inhibition of NO and proinflammatory cytokines

production by CHT are regulated by the same mechanism, or that TNF- α , which is reduced first, decreases NO secretion via an autocrine or paracrine system. Several cytokines, such as IL-1 β and TNF- α , are potent activators of NO production in macrophages^{9,10}. In addition, TNF- α is the first compound of the TNF- α and NO series to be secreted by macrophages¹¹. Thus, TNF- α is involved in the early phase of the cytokine cascades and reduces the NO production induced by CHT. TNF- α has an important amplifying effect in asthmatic inflammation, and potently stimulates airway epithelial cells to produce cytokines^{12,13}. IL-6 is also released in asthma. There is evidence for increased release of IL-6 from alveolar macrophages from asthmatic patients after allergen challenge and increased basal release, compared with non-asthmatic subjects¹⁴. Another common theme in asthma and its associated inflammation of the airway is the increased presence of the pro-inflammatory cytokine IL-1 β . IL-1 β levels and IL-1 β producing macrophages are increased in asthmatics compared to normal, and IL-1 β levels are increased in asthmatics following human rhinovirus infection⁵. In order to determine whether CHT regulates NO, PGE₂, and proinflammatory cytokines production at the mRNA level, a RT-PCR assay was conducted with LPS as a positive control.

Consistent with the results obtained from the NO and proinflammatory cytokines assays, LPS-inducible iNOS and proinflammatory cytokines mRNA levels were found to be markedly suppressed by CHT treatment. Therefore, we believe that decreased LPS-inducible NO and proinflammatory cytokines production, by CHT is regulated through transcriptional activation. We also found that CHT decreased LPS-inducible NO and proinflammatory cytokines production and iNOS and proinflammatory cytokines mRNA reduction. The biological significance of the effect of CHT on LPS-inducible NO production needs to be determined. CHT also inhibited LPS-induced NF- κ B activation therefore inhibited expression of proinflammatory cytokines.

CHT appears to decrease the protein levels of iNOS, COX-2, and proinflammatory cytokines by reducing the expression of iNOS, COX-2, and proinflammatory cytokines mRNAs. At the mRNA level, the expression of iNOS, COX-2, and proinflammatory cytokines 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. Transcription factors, such as NF- κ B and AP-1, play an important role in the orchestration of the airway

inflammation in asthma¹⁵). The role of NF- κ B should be seen as an amplifying and perpetuating mechanism that will exaggerate the disease-specific inflammatory process. There is evidence for activation of NF- κ B in the bronchial epithelial cells of patient with asthma¹⁶. NF- κ B bind to specific consensus DNA element present on the promoter of target genes initiates the transcription of proinflammatory cytokines including TNF- α , IL-6, and IL-1 β ¹⁷. NF- κ B is a member of the Rel family, and is a common regulatory element in the promoter region of many inflammatory cytokines. In activated macrophages, NF- κ B in synergy with other transcriptional activators plays a central role in coordinating the expression of genes encoding iNOS, TNF- α , and IL-1 β ¹⁸. One of these transcription factors, NF- κ B which is a primary transcription factor activated by LPS, and regulates various genes, is important in immune response and inflammation. CHT also inhibited LPS-induced NF- κ B activation, and inhibited expression of iNOS and COX-2 expression. These results indicate that the down-regulation of the iNOS and inflammatory cytokine genes by CHT is mediated by the inhibition of NF- κ B transactivation. However, the possibility cannot be excluded that CHT, in addition to the suppression of NF- κ B-dependent activity, modulates iNOS and inflammatory cytokines

mRNA levels by influencing the activity of other transcription factors important for NO and inflammatory cytokines induction, such as AP-1¹⁹. Our results indicated that CHT inhibition of LPS-inducible iNOS and proinflammatory cytokines expression in macrophages is mediated through the NF- κ B sites of the iNOS and proinflammatory cytokines genes. However, the precise mechanism by which CHT suppress iNOS and proinflammatory cytokines expression in macrophages remains unknown. It may be that a direct interaction NF- κ B causes this repression. In summary, our results show for the first time, that CHT inhibits LPS activated macrophage-derived NO and proinflammatory cytokines production, and that CHT is able to down-regulate iNOS and proinflammatory cytokines gene expression by inhibiting NF- κ B transactivation in murine macrophages. The beneficial effect of CHT in the treatment of asthma seems to be due to its actions as an anti-inflammatory agent. Further work will be need to isolate the specific ingredient of this herbal mixture responsible for inhibition of NF- κ B activation.

V. Conclusion

In oriental medicine, Cheongpyehwadamtang (CHT) has been used for the cure of

inflammatory diseases in the lung and bronchus such as bronchitis, bronchial asthma, pneumonia and tuberculosis. To evaluate the anti-inflammatory effects of CHT, we investigated the effects of CHT on the lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E₂ (PGE₂) and pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β) production, and on the level of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and proinflammatory cytokines expression in murine macrophage RAW 264.7 cells. CHT alone did not affect NO, PGE₂ or pro-inflammatory cytokines production. In contrast, CHT inhibited LPS-induced NO, PGE₂ and proinflammatory cytokines production, and also inhibited the levels of LPS-induced iNOS and proinflammatory cytokines mRNA in a dose-dependent manner.

Treatment with CHT in RAW 264.7 cells transfected with a reporter construct indicated a reduced level of LPS-induced nuclear factor- κ B (NF- κ B) activity, and effectively lowered NF- κ B binding as measured by transient transfection assay. CHT also inhibited the nuclear factor- κ B (NF- κ B) activation. Taken together, these results suggested that CHT inhibits the production of NO, PGE₂ and pro-inflammatory cytokines in RAW 264.7 cells through blockade of NF- κ B

activation.

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