Anti-inflammatory Activities of Cheongpyehwadam-tang

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In oriental medicine, Cheongpyehwadam-tang (CHT) has long been used for the cure of inflammatory diseases in the lung and bronchus such as bronchitis, bronchial asthma, pneumonia and tuberculosis. It's use is currently further extended for the treatment of allergic asthma.To investigate the anti-inflammatory effects of CHT, we investigated the effects of CHT on the lipopolysaccharide (LPS)-induced nitric oxide (NO) and pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) production, and on the level of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines expression in murine macrophage RAW 264.7 cells. CHT alone did not affect NO or pro-inflammatory cytokines production. In contrast, CHT inhibited LPS-induced NO and proinflammatory cytokines and the levels of LPS-induced iNOS and proinflammatory cytokine mRNA in a dose-dependent manner. CHT also inhibited the nuclear factor-kappa B (NF-κB) activation. Taken together, these results suggested that CHT inhibits the production of NO and pro-inflammatory cytokines in RAW 264.7 cells through blockade of NF- κB activation.

Key words : Cheongpyehwadam-tang(CHT), macrophages, iNOS, inflammatory cytokines, NF-κB

Introduction

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 morning[5]. 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 cells[4]. Many of the effector cells, including mast cells in asthma, produce a variety of cytokines[11]. The concentrations of tumor necrosis factor (TNF)-α and interleukin (IL)-6 has been reported to be significantly high in bronchial asthma patients[22]. Another common theme in asthma and its associated inflammation of the airway is the increased presence of the pro-inflammatory cytokine IL-1β[6].

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· Received : 2005/07/30 · Revised : 2005/09/01 · Accepted : 2005/10/04

Nitric oxide (NO) is produced from L-arginine by nitric 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[21]. Molecular cloning and sequencing analyses have revealed the existence of at least three main types of NOS isoforms. Both neuronal NOS and endothelial NOS are constitutively expressed[23], whereas inducible NOS (iNOS) is expressed in response to interferon-γ, lipopolysaccharide (LPS) and a variety of pro-inflammatory cytokines[17]. Following exposure to LPS or cytokines, iNOS can be induced in various cells, such as macrophages, Kupffer cells, smooth muscle cells, and hepatocytes. 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 circulatory shock, inflammation and carcinogenesis[18]. 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[17]. 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[2].

In the present study, we investigated the effect of CHT on NO and pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) production and its molecular mechanism in macrophages. is composed of *Puerariae Radix, Bupleuri Radix, Pinelliae Rhizoma, Citri Pericarpium, Poria, Perillae Folium, Armeniacae 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, 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. Recent study further shows that it is effective for the treatment of asthma.Here, we provide evidence to support to CHT-induced down-regulation of LPS-induced iNOS and pro-inflammatory cytokines expression in macrophages and that suppression is mediated through the NF-κB inactivation of these genes.

Materials and Methods

1. Materials.

CHT used in this study was obtained from Daejeon University Hospital of Oriental medicine, and purified before the experimental use. 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, RPMI 1640, fetal bovine serum, 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 enzymeimmunosorbent assay (ELISA) kit for IL-1β, IL-6, and TNF-α from R&D Systems (Minneapolis, MN, USA). All otherchemicals were of the highest commercial grade available.

2. 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 RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin at 37℃ in a 5% CO_2 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.

3. Nitrite assay.

RAW 264.7 cells (5 X 10^5cells/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 as described previously[13].

4. Immunoassay

For the cytokine immunoassay, the RAW 264.7 cells (2 X 10^5cells/ml) were cultured in 24-well plates. The supernatants were removed at set times and the level of TNF-α, IL-1β, and IL-6 production was measured using a sandwich ELISA as described previously[14].

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

The RAW 264.7 cells (2 X 10^5 cells/ml) were cultured with either CHT or LPS at for 6 h. Total cellular RNA was isolated by the acidic phenol extraction procedure of Chomczynski and Sacchi[8]. The methods used for cDNA synthesis, semiquantitative RT-PCR for IL-1β, IL-6, TNF-α, and β-actin mRNA, and the analysis of the results are described elsewhere[14]. The PCR reactions were electrophoresed through a 2.5% agarose gel and visualized with ethidium bromide staining and UV irradiation.

6. 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 18 h later using the LipofectAMINE Plus according to the manufacturer's protocol. Briefly, atransfection mixture containing 0.5μg of pGL3-4κB-Luc and 0.2μg 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 -galactosidase activities were determined using a method described elsewhere [Kim et al., 2005]. The luciferase activity was normalized with respect to the -galactosidase activity and is expressed relative to the activity of the control.

7. 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. Effect of the CHT on NO production

We decided 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. However, CHT alone did not affect NO production, though CHT inhibited LPS-induced NO production in a dose-dependent manner in RAW 264.7 cells (Fig. 1). 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 (> 95% cell viability, Fig. 1).

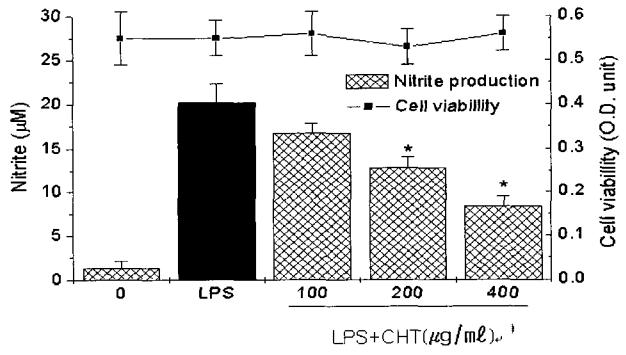

Fig. 1. Effects of CHT on NO production in macrophages. RAW 264.7 cells (5×10^5 cells/ml) were treated with CHT in the presence of LPS (0.5㎍/ml). The medium was harvested 24 h later and assayed for nitrite production. The cell viability was evaluated with the MTT assay (solid line connecting solid squares). The results are presented as a percentage of the control value obtained from non-treated cells. Values are expressed as mean ± SD of three individual experiments, performed in triplicate. *P < 0.01, significantly different from the LPS.

2. Effects of the CHT on macrophage-related proinflammatory cytokines production

We examined the inhibitory effect of CHT on the LPS-induced production of pro-inflammatiroy cytokines (TNF-α, IL-6, IL-1β) from RAW 264.7 cells. Culture supernatants were assayed for each cytokine levels by ELISA method. CHT dose-dependently inhibited the production of TNF-α, IL-6, and IL-1β in LPS-stimulated RAW 264.7 cells. CHT treatment at 400 g/ml blocked TNF-α, IL-6, and IL-1β production by 41.86 ± 2.26%, 55.33 ± 3.65%, and 61.11 ±2.54% as compared with no treatment of CHT ($P < 0.001$), respectively (Table 1).

Table 1. Effects of CHT on LPS-stimulated TNF-α, IL-6, and IL-1β production in RAW 264.7 cells.

Treatment[a]	TNF-α (ng/ml)	IL-1β(ng/ml)	IL-6 (ng/ml)
Control	0.55 ± 0.17[b]	0.28 ± 0.01[b]	0.31 ± 0.03[b]
LPS (0.1 g/ml)	11.58 ± 1.17	4.88 ± 0.65	3.85 ± 0.13
LPS plus CHT (100 g/ml)	10.22 ± 0.05*	4.21 ± 0.33*	3.57 ± 0.24
LPS plus CHT (200 g/ml)	7.12 ± 1.21*	3.72 ± 0.40*	2.95 ± 0.37*
LPS plus CHT (400 g/ml)	4.85 ± 1.30*	2.70 ± 0.23*	2.37 ± 0.27*

[a]The RAW 264.7 cells ($5X\ 10^5$ cells/ml) were pretreated with CHT for 30 min and the challenged with CHT for either 6 h (TNF-α) or 12 h (IL-6 and IL-1β). The amount of TNF-α, IL-6, and IL-1β released to the culture medium were 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.

3. Effects of the CHT on the gene expression of iNOS and pro inflammatory cytokines

In order to determine whether CHT regulates NO and inflammatory 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 inflammatory cytokines production assays, LPS-inducible iNOS, TNF-α, IL-1β and IL-6 mRNA levels were found to be markedly suppressed by CHT treatment (Fig. 2). The control -actin was constitutively expressed and was unaffected by the CHT treatment. Therefore, a decrease in the iNOS, TNF-α, IL-1 β and IL-6 levels by CHT is believed to be regulated by the transcriptional activation.

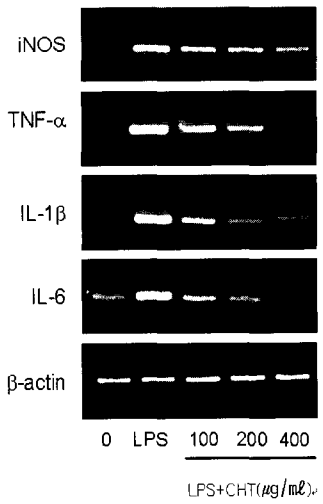

Fig. 2. Effects of CHT on iNOS, TNF-α, IL-1β, and IL-6 mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were treated with LPS (0.5 ㎍/ml) and/or CHT (100, 200, 400 ㎍/ml) for 6 h. The cells were lysed and the total RNA was analyzed by RT-PCR. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide. One of three represe tative experiments is shown. The ratio of the RT-PCR products of iNOS TNF-α, IL-1β, or 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.

4. Effects of the CHT on NF-κB activation

To further investigate the role of CHT on iNOS and

inflammatory cytokines gene expression, the effect of CHT on NF-κB-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-κB 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- κB-dependent luciferase activities ina dose-dependent manner(Fig. 3). These results indicate that the down-regulation of the iNOS and inflammatory cytokine genes by CHTis mediated by the inhibition of NF-κB transactivation.

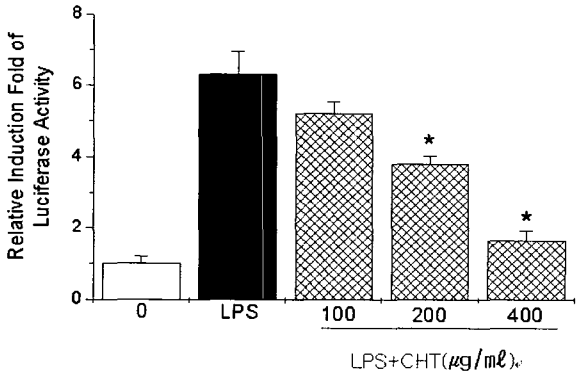

Fig. 3. Effects of CHT on NF-κB-dependent luciferase gene expression. RAW 264.7 cells were transiently co-transfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, the cells were treated with LPS (0.5 ㎍/ml) and/or CHT (100, 200, 400 ㎍/ml) for 18 h, harvested and their luciferase and β -galactosidase activities determined. Luciferase activities were expressed relative to the control. Each bar shows the mean ± SD of three independent experiments performed in triplicate. *P<0.01, significantly different from the LPS.

Discussion

In this study, we demonstrated that CHT inhibited the NO and proinflammatory cytokines (TNF-α, IL-6, and IL-1β) production from LPS stimulated RAW 264.7 cells. These results suggest that the inhibition of NOand proinflammatory cytokines production by CHTare 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-1β and TNF-α, are potent activators of NO production in macrophages[17]. In addition, TNF-α is the first compound of the TNF-αand NO series to be secreted by macrophages[20]. Thus, TNF-α is involved in the early phase of the cytokine cascades and reduces the NO production induced by CHT. TNF-α many have an important amplifying effect in asthmatic inflammation and potently stimulates airway epithelial cells to produced cytokines[15]. 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 subjects[7]. Another common theme in asthma and its associated inflammation of the airway is the increased presence of the pro-inflammatory cytokine IL-1β. IL-1β levels and IL-1β producing macrophages are increased in asthmatics compared to normal, and IL-1β levels are increased in asthmatics following human rhinovirus infection[6].

In order to determine whether CHT regulates NO and proinflammatory cytokines productionat 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 cytokinesassays, 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 CHTis 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-κB activation therefore inhibited expression of proinflammatory cytokines. 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[12]. NF-κB 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-1β[9]. NF-κB 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-κB in synergy with other transcriptional activators plays a central role in coordinating the expression of genes encoding iNOS, TNF-α, and IL-1β[10]. Consistent with NO production and iNOS mRNA measurement, CHT also significantly decreased NF-κB-dependent luciferase activities in a dose-dependent manner. These results indicate that the down-regulation of the iNOS and inflammatory cytokinegenes by CHT is mediated by the inhibition of NF-κB transactivation. However, the possibility cannot be excluded that CHT, in addition to the suppression of NF-κB-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-1[19]. Our results indicated that CHT inhibition of LPS-inducible iNOS and proinflammatory cytokines expression in macrophages is mediated through the NF-κB 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-κB causes this repression. In summary, our results show for the first time, that CHTinhibits 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-κB activation.

Acknowledgments

This work was supported by RIC(R) grants from Traditional and Bio-Medical Research Center, Daejeon University(RRC04700, 2005) by ITEP.

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