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Kamgil-Tang attenuates lipopolysaccharide-induced NF-κB activation in RAW 264.7 cell and acute lung injury in rats

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Abstract We examined the effects of Kamgil-Tang on the process of lipopolysaccharide (LPS)-induced nuclear factor (NF)-κBp65 and inhibitory (I)-κBα alteration in RAW 264.7 cell and acute lung injury in rats.

Immunoblot analysis showed that LPS-induced degradation of I-κBα in RAW 264.7 was inhibited by pretreatment of Kamgil-Tang. The total cells of bronchoalveolar lavage fluid by LPS challenge markedly decreased in the Kamgil-Tang pretreatment rats. Kamgil-Tang pretreatment caused also a decline in neutrophils infiltration into interstitium of the lung. In the alveolar macrophages and neutrophils, decreased NF-κBp65 and inducible nitric oxide synthase and increased I-κBα immunoreaction were detected in Kamgil-Tang pretreated rats compared with LPS alone treated ones.

It may be concluded that Kamgil-Tang attenuates the development of LPS-induced inflammation by reduction of NF-κBp65 activation and neutrophil-mediated acute lung injury. Kamgil-Tang would be useful as a therapeutic agent for endotoxin-induced lung disease.

Key words: Kamgil-Tang, LPS, NF-κBp65, I-κBα, iNOS

Introduction

The administration of lipopolysaccharide (LPS) present in the wall of gram-negative bacteria plays a major role in the release of several pro-inflammatory cytokines and these cytokines induce numerous effects involving fever, septic shock and death [3,18]. LPS induces various cytokines from macrophage and T-cell, which induce expression of inducible nitric oxide synthase (iNOS) mRNA or tumor necrosis factor (TNF)-α through the activation of transcriptional nuclear factor (NF)-κB [1].

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The NF-κB lie dormant in the cytoplasm of unstimulated cells and its activity is negatively regulated by a family of inhibitor protein known as inhibitory (I)-κB [11,15,17,25]. Under the activated conditions, NF-κB is dissociated from I-κB and is translocated into the nucleus where it induces transcriptional up-regulation of various proinflammatory mediators that contribute to the systemic inflammatory response, such as TNF-α and interleukin-8 [2,15].

Kamgil-Tang was recorded in classic Korean medical prescription, Bang-Yak-Hap-Pyeon [13], and has been clinically used for the so-called a disease of respiratory system such as a soar throat, phlegm and pneumonia. Kamgil-Tang has a wide application for the treatment of a disease, especially inflammation, of respiratory tract and lung as a complementary therapeutic agent.

Because LPS stimulation elicits an increase of NF-κB activation with corresponding degradation of its inhibitor I-κB [15,26], the prevention of NF-κB activation may be useful in the therapy of LPS-induced disorders [23].

Thus blocking of NF-κB activation may be an effective strategy in the treatment of LPS-induced lung injury. We postulated that Kamgil-Tang would attenuates lung injury following intraperitoneal challenge with LPS throughout its effect as an inhibitor of NF-κB expression. In the present study, we investigated the effects of Kamgil-Tang, as a complementary therapeutic agent to lung disease, on the alteration of inflammatory proteins such as NF-κBp65, I-κBα and iNOS in the process of LPS-induced lung injury.

Materials and methods

Preparation of aqueous extract

The Kamgil-Tang was prepared as follows : after drying, the two herbs, 14 g of Glycyrrhizae radix (*Glycyrrhiza glabra*) and 6 g of Platycodi radix (*Platycodon grandiflorum*) were extracted with 1 L of distilled water at 100℃ for 1 hour. The extract was filtered through 0.45 ㎛ filter, freeze-dried

and kept at 4℃. The dried extract was dissolved in phosphate buffered saline (PBS) before use.

Reagents

Anti-iNOS rabbit polyclonal antibody was obtained from CALBIOCHEM (San Diego, CA). Rabbit polyclonal antibodies raised against NF-κBp65 and I-κBα and horse radish peroxidase-conjugated anti-rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Avidin-biotin-peroxidase complex kit and substrate kit for peroxidase were purchased from Vector Lab (Burlingame, CA) and LPS (phenol extracted *Salmonella enteritidis*) and other all reagents from Sigma (St. Louis, MO).

Cell culture and LPS and Kamgil-Tang treatment

The RAW 264.7 cells, mouse macrophage cell line, were purchased from the KCLB (Korean Cell Line Bank, Seoul, Korea) and maintained in DMEM (Gibco BRL, Grand Island, NY) supplemented with 1% penicillin-streptomycin (Gibco BRL) and 10% fetal bovine serum (Gibco BRL) at 37℃ in 5% CO_2 enriched air. RAW 264.7 cells (2 X 10^6/dish) were transferred to 100 mm polystyrene culture dish (Falcon, San Jose, CA) and stabilized for 24 hours. Cells were pretreated with 3 mg/ml of Kamgil-Tang for 12 hours. After pretreatment, cells were exposed to 500 ng/ml of LPS for 90 minutes.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Kamgil-Tang and LPS treated cells were harvested with police larva (Falcon), and then washed in cold PBS. The cells were lysed in lysis buffer (250 mM NaCl, 25 mM Tris-Cl pH 7.5, 5 mM EDTA pH 8.0, 1% NP-40, 1 mM PMSF and 5 mM DTT) at 4℃ for 30 minutes. Protein concentrations were quantified using BioRad protein assay kit (BioRad Lab, Hercules, CA), following the procedure described by the manufacturer. 50 mg of proteins were separated by 10% SDS-PAGE. The resulting gels were transferred to nitrocellulose membranes, and then the membranes were blocked with 10% skim milk in PBS-T (0.1% Tween 20 in PBS) for 1 hour at room temperature. After blocking, the membranes were incubated with anti-NF-κBp65 and I-κBα antibodies at 4℃ for overnight. After washing in PBS-T three times, the membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibody and the antibody-specific proteins were visualized by the ECL detection system according to the recommended procedure.

Rat treatment and LPS and Kamgil-Tang administration

Male Sprague-Dawley rats, weighing about 120 g on average, were obtained from Taconic & SamYuk Co. in Korea. Rats were housed under conditioned of 22℃ and 12 hours dark and light cycle, and were fed a commercial diet and allowed tap water *ad libitum* starting 2 weeks before and throughout the study. Rats of the LPS alone and LPS plus Kamgil-Tang group were administrated intraperitoneally 3 times, 24, 8 and 3 hours before LPS challenge, with either PBS or Kamgil-Tang at a concentration of 100 mg/Kg. After pretreatment, rats were challenged intraperitoneally with 6 mg/Kg of LPS and control one with same volume of PBS. Rats were sacrificed at interval 3 and 6 hours after LPS challenge.

Bronchoalveolar lavage fluid and cell counts

For bronchoalveolar lavage fluid, the rats were anestherized with ether and a thoracotomy was performed. The lungs were lavaged with 15 ml of sterile PBS. Cell counts were performed in bronchoalveolar lavage fluid on a hematocytometer.

Histopathology

The lung were fixed in 4% paraformaldehye in PBS for 18 hours and dehydrated in a graded ethanol series. After embedded in paraffin, serial 5 ㎛ thick sections were prepared. For histopathological examinations, hematoxylin-eosin stain and periodic acid Schiffs (PAS) reaction were used.

Immunohistochemistry

After deparaffinized in 58℃ xylene, the sections were exposed for 30 minutes to 0.3% methanolic hydrogen peroxide, followed by washing with PBS. Tissues were then treated with goat normal serum at room temperature for 30 minutes followed by treatment with anti-NF-κBp65, I-κBα and iNOS diluted for 1:500 in moisture chamber for 16 hours at 4℃. After washed by PBS, tissues were incubated with the secondary antisera, biotinylated anti-rabbit Ig G for 30 minutes, followed by washing with PBS. These sections were further incubated in avidin-biotin-peroxidase complex kit for 60 minutes at room temperature. Diaminobenzidine substrate kit for peroxidase was applied. For the controls, treatment with primary and secondary antibodies was omitted.

Results

Effects of Kamgil-Tang on the LPS-induced activation of NF-κBp65 in RAW 264.7.

To examine the effect of Kamgil-Tang on the LPS-induced inflammatory response, we examined alteration of inflammation-related proteins including NF-κBp65 and I-κBα. As a result, protein level of NF-κBp65 was increased by LPS treatment, but Kamgil-Tang-pretreated cells were no effect. Since activation of NF-κBp65 is closely linked to degradation of negative regulator, I-κBα, we analyzed the level of I-κBα. As shown a panel of Fig. 1, LPS-induced deg-

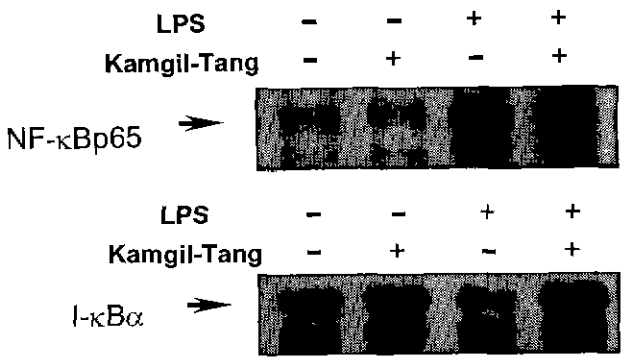

Fig. 1. Inhibitory effect of Kamgil-Tang on the induction of NF-κBp65 and the degradation of I-κBα in RAW 264.7. Cells were pretreated with 3 mg/ml of Kamgil-tang before LPS challenge (500 ng/ml), and then exposed to LPS for 90 minutes. Samples were subjected to SDS-PAGE followed by the Western blot analysis using an NF-κBp65 and I-κBα antibody.

radation of I-κBα was inhibited by pretreatment of Kamgil-Tang. Kamgil-Tang not only inhibits LPS-induced degradation of I-κBα, but also increases the basal level of I-κBα protein.

Cell counts in the bronchoalveolar lavage fluid

Fig. 2 shows the number of total cells in the bronchoalveolar lavage fluid 3 and 6 hours after treatment with LPS. The total cells of bronchoalveolar lavage fluid in the LPS alone treated rats markedly increased compared with control ones. However, Kamgil-Tang pretreatment significantly attenuated, especially 6 hours after LPS challenge, the total cell numbers in the bronchoalveolar lavage fluid.

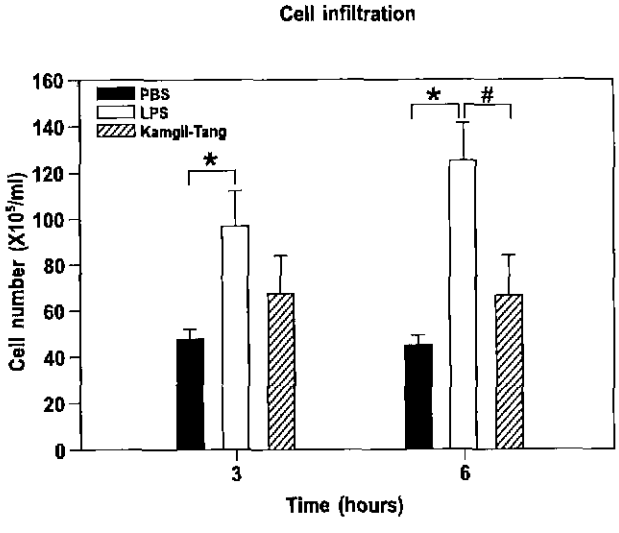

Fig. 2. Total cell of bronchoalveolar lavage in rats treated with intraperitoneal LPS challenge (6 mg/Kg). *p<0.001 for LPS alone treated rats compared with control ones and # p<0.01 for Kamgil-Tang (100 mg/Kg) pretreated rats for compared with LPS alone treated ones.

Histopathology

A mild inflammatory changes was observed in the rat lung from 3 hours after peritoneal LPS challenge. LPS shock caused a rise in neutrophils count and alveolar macrophage was also infiltrated into the interstitium and alveolar space. Although a few of neutrophils were infiltrated into the alveolar space, a severe neutrophilic alveolitis was not detected in LPS challenged rats. The slight decline of alveolar macrophages and neutrophils infiltration was observed in the Kamgil-Tang pretreated rats (Fig. 3).

Immunohistochemistry

The results of the immunohistochemical study on the inflammation-related protein shows Figs. 4-6. NF-κBp65, I-κBα and iNOS expression were mainly detected in the alveolar macrophages and neutrophils in the lung and more intensive expression was observed in the alveolar macrophages. The number of alveolar macrophages and neutrophils showing NF-κBp65, I-κBα and iNOS immunoreaction, especially neutrophils, was increased in the LPS treated rats. Enhanced immunoreaction for NF-κBp65 and iNOS were also observed in rats challenged with LPS. With a slight decline of immunoreactive cells in number, decreased NF-κBp65 and iNOS and increased I-κBα immunoreaction were demonstrated in these cells of Kamgil-Tang pretreated rats.

Discussion

Alveolar macrophages, the key lung cells in host defence, are considered to play a central role in the regulation of the immune response to inhaled pathogens and development of inflammation [11,16]. Under stimulated conditions by LPS challenges, macrophages are a major source of inflammatory cytokines and its cytokine release is predominantly regulated by the transcriptional rates of cytokines gene.

NF-κB is a primary transcription factor for inflammatory cytokines and its activation is also known to produce inflammatory enzymes [12,22]. NF-κB activation at the end of LPS shock indicatives a primed alveolar macrophage [5,10]. Because LPS-induced I-κB degradation is closely related to NF-κB activation, inhibition of NF-κB activation with an inhibitor of I-κB degradation eliminate TNF-α synthesis and the expression of iNOS in the LPS-stimulated cells [9,14].

Therefore we postulated that agents that prevent activation of NF-κB in macrophages could prevent propagation of the inflammatory cascade. When we examined the effect of Kamgil-Tang on alteration of inflammation-related proteins involving NF-κBp65 and I-κBα in LPS treated RAW 264.7 cell, LPS-induced degradation of I-κBα was inhibited by pretreatment of Kamgil-Tang and the basal level

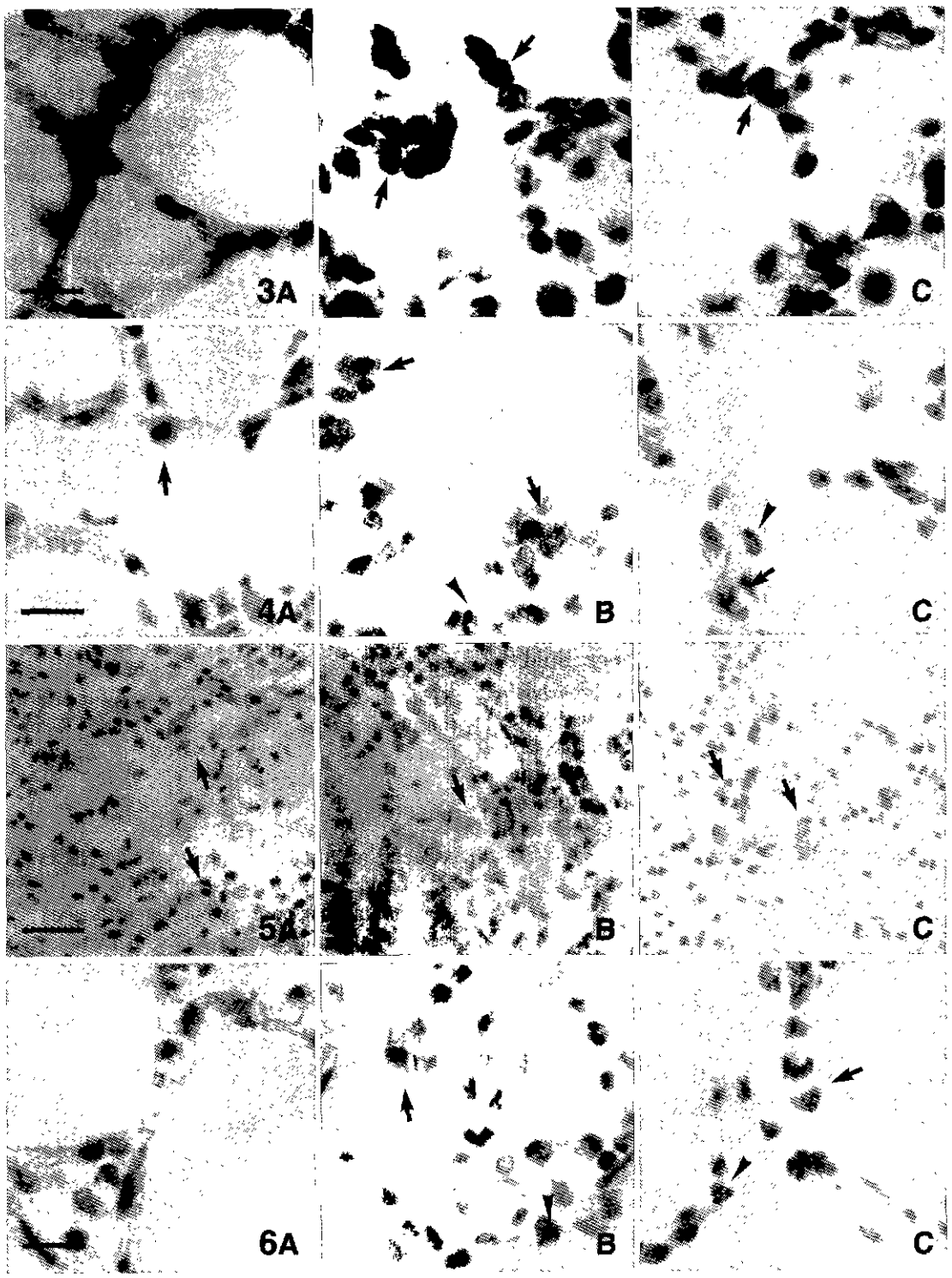

Fig. 3. The PAS reaction in the lung of normal (A), LPS (6 mg/Kg) alone treated (B) and Kamgil-Tang (100 mg/Kg) pretreated rats (C) at 6 hours after LPS challenge Note severe infiltration of neutrophils (arrows) into interstitium in the LPS challenged rats and a decline of neutrophils count in the Kamgil- Tang pretreated rats. Scale bar = 30 μm.

Figs. 4-6. Immunohistochemical localization of NF-κBp65 (Fig. 4), I-κBα (Fig. 5) and iNOS (Fig. 6) in the lung of normal (A), LPS (6 mg/Kg) alone treated (B) and Kamgil-Tang (100 mg/Kg) pretreated rats (C) at 6 hours after LPS challenge. Kamgil-Tang pretreated rats showed a decrease of NF-κBp65 and iNOS immunoreaction in the alveolar macrophage (arrows) and infiltrated neutrophils (arrow heads) and an increase of I-κBα immunoreaction in the macrophage. Scale bar = 30 μm.

of I-κBα protein also increased. It suggests that LPS-induced inflammation was regulated by Kamgil-Tang through increase of the I-κBα level and inhibition of its degradation pathway in mouse macrophage cell line.

With inflammatory cytokines, neutrophils are a principal cellular mediator in the development of acute lung injury. The influx of activated neutrophils into the lungs are thought to be important in the pathogenesis of lung injury [19]. Although several chemotactic factors for neutrophils have been identified, NF-κB is thought to be important in the gene expression of all neutrophilic chemotactic cytokines [4]. Intense neutrophils influx is closely associated with NF-κB activity in the lung tissue [5,7].

So various anti-neutrophilic chemotactic chemokines strategies appear to attenuate acute lung injury. Intraperitoneal LPS injection does not produce significant neutrophilic alveolitis in the first few hours after LPS challenge, but results in systemic inflammation involving inflammatory cells infiltrated in the interstitium and air space of the lung [6,8]. The activation of NF-κB is also associated with the production of inflammatory enzymes iNOS. Enhanced formation of NO by iNOS plays also a critical role in the pathogenesis of LPS-caused acute inflammation as well as dysfunction [20,23].

In the present total cells studies, we observed that Kamgil-Tang pretreatment showed a statistically significant decreases in the total cell of bronchoalveolar lavage fluid. Especially, as for the histopathological studies, Kamgil-Tang pretreatment rats showed a decline count of infiltrated neutrophils into interstitium compared with LPS alone challenged rats. And in the immunohistochemical studies, different immunoreactivity between Kamgil-Tang pretreated rats and LPS alone treated ones were demonstrated in the alveolar macrophages and neutrophils. Decreased immunoreaction for NF-κBp65 and iNOS and increased one for I-κBα were observed in the Kamgil-Tang pretreated rats. Both negative regulation of NF-κB activity in response to specific signals and neutrophils infiltration into the interstitium are very complex process. However, Kamgil-Tang pretreatment inhibited the degradation of I-κBα in mouse macrophage cell line and showed a decrease of NF-κBp65 and iNOS immunoreaction in the alveolar macrophages and neutrophils in the lung tissue.

The extracts of two herbs in Kamgil-Tang, Glycyrrhizae radix and Platycodi radix, has been used as an anti-inflammatory drugs. The major constituents are know to be platycodins in the Platycodi radix and glyderinine in the Glycyrrhizae radix [21,24]. Although the exact role of above two roots has not been clarified, these roots may be partly responsible for the anti-inflammatory effects such as prevention of NF-κB activation.

Kamgil-Tang may act as an inhibitor of NF-κB activation through the inhibition of I-κB degradation and then have an influences on a decline of iNOS production. Decreased intensity of neutrophilic infiltration in the Kamgil-Tang pretreated rats are may due to decreased NF-κB activity in the lung. Therefore it may be concluded that Kamgil-Tang would be useful as a therapeutic agent for inflammatory disease of respiratory system.

References

1. Ando, N., T. Kono, J. Iwamoto, K. Kikuchi-Utsumi, M. Yoneda, H. Karasaki and S. Kasai. 1998. Nitric oxide release from the liver surface to the intraabdominal cavity during acute endotoxemia in rats. *Nitric Oxide.* **2**, 481-488.
2. Baeuerle, P.A. and V.R. Baichwal. 1997. NF-kappa B as a frequent target for immunosuppressive and anti-inflammatory molecules. *Adv. Immunol.* **65**, 111-137.
3. Bellezzo, J.M., R.S. Britton, B.R. Bacon and E.S. Fox. 1996. LPS-mediated NF-kappa beta activation in rat Kupffer cells can be induced independently of CD14. *Am. J. Physiol.* **270**, G956-G961.
4. Blackwell, T.S., T.R. Blackwell and J.W. Christman. 1997. Impaired activation of nuclear factor-kappaB in endotoxin-tolerant rats is associated with down-regulation of chemokine gene expression and inhibition of neutrophilic lung inflammation. *J. Immunol.* **158**, 5934-5940.
5. Blackwell, T.S. and J.W. Christman. 1997. The role of nuclear factor-kappa B in cytokine gene regulation. *Am. J. Respir. Cell Mol. Biol.* **17**, 3-9.
6. Blackwell, T.S., E.P. Holden, T.R. Blackwell, J.E. DeLarco and J.W. Christman. 1994. Cytokine-induced neutrophil chemoattractant mediates neutrophilic alveolitis in rats: Association with nuclear factor kappa B activation. *Am. J. Respir. Cell Mol. Biol.* **11**, 464-472.
7. Blackwell, T.S., L.H. Lancaster, T.R. Blackwell, A. Venkatakrishnan and J.W. Christman. 1999. Differential NF-kappaB activation after intratracheal endotoxin. *Am. J. Physiol.* **277**, L823-830
8. Carter, A.B., M.M. Monick and G.W. Hunninghake. 1998. Lipopolysaccharide-induced NF-kappaB activation and cytokine release in human alveolar macrophages is PKC-independent and TK- and PC-PLC-dependent. *Am. J. Respir. Cell Mol. Biol.* **18**, 384-391.
9. Díaz-Guerra, M.J.M., M. Velasco, P. Martín-Sanz and L. Bosca. 1996. Evidence for common mechanisms in the transcriptional control of type II nitric oxide synthase in isolated hepatocytes: Requirement of NF-κB activation after stimulation with bacterial cell wall products and phorbol esters. *J. Biol. Chem.* **271**, 30114-30120.
10. Fan, J., J.C. Marshall, M. Jimenez, P.N. Shek, J. Zagorski and O.D. Rotstein. 1998. Hemorrhagic shock primes for increased expression of cytokine-induced neutrophil chemoattractant in the lung: role in pulmonary inflammation following lipopolysaccharide. *J. Immunol.* **161**, 440-447.
11. Fels, A.O. and Z.A. Cohn. 1986. The alveolar macrophage. *J. Appl. Physiol.* **60**, 353-369.
12. Hong, K., A. Chu, B.R. Ludviksson, E.L. Berg and R.O Ehrhardt. 1999. IL-12, independently of IFN-gamma, plays a crucial role in the pathogenesis of a murine psoriasis-like skin disorder. *J. Immunol.* **162**, 7480-7491.
13. Hwang, D.Y. 1996. *Bang-Yak-Hap-Pyeon.* pp.240. Nam-San-Dang.

14. Iimuro, Y., B.U. Bradford, S. Yamashina, I. Rusyn, M. Nakagami, N. Enomoto, H. Kono, W. Frey, D. Forman, D. Brenner and R.G. Thurman. 2000. The glutathione precursor L-2-oxothiazolidine-4-carboxylic acid protects against liver injury due to chronic enternal ethanol exposure in the rat. *Hepatology.* **31**, 391-398.
15. Kramer, A.A., K.F. Salhab, A.E. Shafii, J. Norman, L.C. Carey and C. Mendez. 1999. Induction of tolerance to hemorrhagic or endotoxic shock involves activation of NF-κB. *J. Surg. Res.* **83**, 89-94.
16. Li, L., R.F. Hamilton and A. Holian. 1999. Effect of acrolein on human alveolar macrophage NF-κB activity. *Lung Cell. Mol. Physiol.* **21**, L550-557.
17. Mustafa, S.B., B.D. Flickinger and M.S. Olson. 1999. Suppression of lipopolysaccharide-induced nitric oxide synthase expression by platelet-activating factor receptor antagonists in the rat liver and cultured rat Kupffer cells. *Hepatology.* **30**, 1206-1214.
18. Nathan, C.F. and F. Xie. 1994. Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.* **269**, 13725-13728.
19. Nathens, A.B., R. Bitar, C. Davreux, M. Bujard, J.C. Marshall, A.P. Dackiw, R.W. Watson and O.D. Rotstein. 1997. Pyrrolidine dithiocarbamate attenuates endotoxin-induced acute lung injury. *Am. J. Respir. Cell Mol. Biol.* **17**, 608-616.
20. Numata, M., S. Suzuki, N. Miyazawa, A. Miyashita, Y. Nagashima, S. Inoue, T. Kaneko and T. Okubo. 1998. Inhibition of inducible nitirc oxide synthase prevents LPS-induced acute lung injury in dogs. *J. Immunol.* **160**, 3031-3037.
21. Ozaki, Y. 1995. Studies on antiinflammatory effect of Japanese Oriental medicines (kampo medicines) used to treat inflammatory diseases. *Biol. Pharm. Bull.* **18**, 559-62.
22. Rothwarf, D.M. and M. Karin. 1999. The NF-κB activation pathway: A paradigm in information transfer form membrane to nucleus. (available at http://www.stke.org)
23. Ruetten, H. and C. Thiemermann. 1997. Effect of calpain inhibitor I, an inhibitor of the proteolysis of IκB, on the circulatory failure and multiple organ dysfunction caused by endotoxin in the rat. *Br. J. Pharmacol.* **121**, 695-704.
24. Saeki, T., K. Koike, T. Nikaido. 1999. A comparative study on commercial, botanical gardens and wild samples of the roots of *Platycodon grandiflorum* by HPLC analysis. *Planta Med.* **65**, 428-31.
25. Siebenlist, U., G. Franzoso and K. Brown. 1994. Structure, regulation and function of NF-kappa B. *Annu. Rev. Cell Biol.* **10**, 405-455.
26. Yamakawa, T., S. Eguchi, T. Matsumoto, Y. Yamakawa, K. Numaguchi, I. Miyata, C.M. Reynolds, E.D. Motley and T. Inagami. 1999. Intracellular signaling in rat cultured vascular smooth muscle cells: Roles of nuclear factor-κB and p38 mitogen-activated protein kinase on tumor necrosis factor-α production. *Endocrinology.* **140**, 3562-3573.