Inhibition of Nitric Oxide and Tumor Necrosis Factor-Alpha by Moutan Cortex in Activated Mouse Peritoneal Macrophages

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Moutan cortex (MC) is one of the most widely used Oriental herbal medicines for treating inflammatory diseases. In this study, the effect of MC on lipopolysaccharide (LPS) and recombinant interferon-gamma (rIFN-γ)-induced production of nitric oxide (NO) and tumor necrosis factor (TNF)-alpha were examined using mouse peritoneal macrophages. MC inhibited the LPS/rIFN-γ-induced expression of inducible nitric oxide synthase (iNOS) and TNF-alpha release. To clarify the mechanism involved, the effect of MC on the activation of nuclear factor (NF)-kappaB was examined. The LPS/rIFN-γ-induced activation of NF-kappaB was almost completely blocked by MC at 0.5 mg/mL. These findings demonstrate that the inhibition of the LPS/rIFN-γ-induced production of NO and TNF-alpha by MC is due to the inhibition of NF-kappaB activation.

Key words  Moutan cortex (MC); nitric oxide; peritoneal macrophage; tumor necrosis factor-α; anti-inflammatory effect

Moutan cortex (MC), the root cortex of Paeonia suffruticosa Andrews (Ranunculaceae), is a Chinese herbal medicine widely used as an analgesic, an antispasmodic, and an anti-inflammatory agent. The drug has long been used in remedies for female diseases such as the menstruation disorder and uteritis. MC is reported to inhibit the secretions of cytokine families. Particularly, binding of NF-κB to specific consensus DNA elements present on the promoters of target genes initiates the transcription of TNF-α, iNOS, cyclo-oxygenase-2 and IL-6. Thus the inhibition of NF-κB may be key to suppression of inflammation effectors such as NO and TNF-α.

The induction of cytokine genes, such as TNF-α, by LPS occurs primarily at the transcriptional level. This involves the action of several transcription factors, including members of the nuclear factor-κB (NF-κB)/rel, C/EBP, Ets, and AP-1 protein families. Particularly, binding of NF-κB to specific consensus DNA elements present on the promoters of target genes initiates the transcription of TNF-α, iNOS, cyclo-oxygenase-2 and IL-6. Thus the inhibition of NF-κB may be key to suppression of inflammation effectors such as NO and TNF-α.

In the present study, we examined the effect of MC on the LPS- and rIFN-γ-induced production of NO and TNF-α, and attempted to clarify the mechanism of action of MC in mouse peritoneal macrophages.

MATERIALS AND METHODS

Reagents  Murine rIFN-γ and OptEIA TNF-α ELISA kit (BD Biosciences, Bedford, MA, U.S.A.); N-(1-naphthyl)-ethylenediamine dihydrochloride, LPS (Escherichia coli serotype 0111:B4) and sodium nitrite (Sigma, St. Louis, MO, U.S.A.); rabbit polyclonal antisera to iNOS and p65 (San- tacruz Biotechnology, California, U.S.A.); protein extraction solution (Intron Biotechnology, Republic of Korea); FBS (Hyclone, Logan, UT, U.S.A.); thioglycolate (Difco Laboratories, Detroit, MI, U.S.A.); 0.2 μm syringe filters, tissue culture plates of 96 and 24 wells, and 100-mm diameter dishes (Corning Inc. NY, U.S.A.); DMEM containing l-arginine (84 mg/l) and other tissue culture reagents (In VitroGen Life Technologies, Rockville, U.S.A.); Male C57BL/6 mice (Sam-taco Bio Korea, Osan, Republic of Korea) were used.

Peritoneal Macrophage Cultures  All experimental animal procedures were conducted with the prior approval of the Kyung Hee University Animal Ethics Committee. Thioglycolate-elicited macrophages were harvested 3—4 d after i.p. injection of 2.5 ml thioglycolate and isolated as reported previously. Using 8 ml of DMEM, peritoneal lavage was per-
formed. Cells were then distributed in DMEM supplemented with 10% heat-inactivated FBS in 24-well tissue culture plates (2.5×10⁵ cells/well), incubated for 3 h at 37°C in an atmosphere of 5% CO₂, washed three times with DMEM to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

**Preparation of MC** We obtained the spray-dried MC extract from Sunten Pharmaceutical Co. (Taipei, Taiwan). One gram of MC powder contained 0.67 g of MC extract and 0.33 g of starch. We dissolved 1 g of MC powder in 0.67 ml of distilled water, centrifuged at 15000 rpm for 10 min, transferred the supernatant to another tube, and filtered the supernatant through a 0.2 μm syringe filter.

**High-Performance Liquid Chromatography (HPLC)** Analysis of MC The spray-dried extract of about 10 mg of MC was accurately weighed, put in a test tube, dissolved in 5 ml of 50% methanol (HPLC reagent, J.T. Baker Co. Ltd., U.S.A.) and filtered using a 0.45 μm syringe filter (PVDF, Waters, U.S.A.). Ten milligrams of Paeonol was used as a standard and was dissolved according to the analysis conditions of standard material. The dissolved standard solution was diluted to 0.1, 0.5, 1.0, 1.5, and 2.0 mg/ml, and then a standard HPLC chromatogram was obtained. The relationship between the concentration and the peak-area was measured by the minimum square method ($R^2$ value). The HPLC apparatus was a Waters Breeze System (717+ Autosampler, 2487 dual λ absorbance detector, 1525 binary HPLC Pump, Waters Co., Milford, U.S.A.), and the associated Waters Breeze System (Version 3.20, Waters Co., Milford, U.S.A.) was used for data acquisition and integration.

**MTS Assay** Cell growth was measured by an MTS assay using the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, U.S.A.). Briefly, 100 μl of the supernatant was added to each well of a 96-well plate. Twenty microliters of the MTS solution was added to each of the 96 wells and incubated at 37°C for 1 h in a humidified (5% CO₂) environment. The absorbance was read at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

**Measurement of Nitrite Concentration** Peritoneal macrophages (2.5×10⁵ cells/well) were treated with various concentrations of MC. The cells were then stimulated with rIFN-γ (20 U/ml) and LPS (10 μg/ml) and incubated for 48 h.

**Assay of TNF-α Release** Peritoneal macrophages (2.5×10⁵ cells/well) were treated with various concentrations of MC. The cells were then stimulated with rIFN-γ (20 U/ml) and LPS (10 μg/ml) and incubated for 24 h. TNF-α release was detected by OptEIA, calibrated according to the manufacturer’s instructions.

**Preparation of Nuclear Extracts** Nuclear extracts were prepared essentially according to Schreiber et al. Briefly, dishes were washed with ice-cold PBS. The dishes were then scraped and cells were transferred to microtubes. Cells were allowed to swell by adding 100 μl lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The tubes were vortexed to disrupt cell membranes. The samples were incubated for 10 min on ice and centrifuged for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 50 μl extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and incubated for 30 min on ice. The samples were centrifuged at 15800 g for 10 min to obtain the supernatant containing nuclear extracts. Extracts were stored at −70°C until use.

**Western Blot Analysis** MC-pretreated peritoneal macrophages (5×10⁵ cells/well) were incubated for 6 h with rIFN-γ (20 U/ml). Cells were then stimulated with LPS (10 μg/ml) for 12 h. Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS-Tween-20 for 1 h at room temperature, and then incubated with anti-iNOS or anti-p65 antibodies. After washing in PBS-Tween-20 three times, the blot was incubated with a secondary antibody for 30 min. The antibody-specific proteins were then visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp., Newark, NJ, U.S.A.).

**Statistical Analysis** Results were expressed as the mean±S.E.M. of at least three independent experiments performed in duplicate. One-way analysis of variance (ANOVA) and Tukey’s test were used for the comparison between groups. The differences were considered to be significant at $p<0.05$.

**RESULTS**

**HPLC Spectrum of MC** Paeonol is one of many active main compounds contained in MC. We performed HPLC fingerprinting to authenticate MC. As shown in Fig. 1, the retention time of paeonol was similar to that of MC, we calculated that 1 g of MC contained 0.46±0.01 mg of paeonol.

**Effect of MC on NO Production in Activated Peritoneal Macrophages** We first investigated the effect of MC on cell viability of peritoneal macrophages with the MTS assay. The highest concentration of MC which did not affect mouse peritoneal macrophage viability was 0.5 mg/ml (Fig. 2). The levels of NO were then examined by co-culturing various concentrations of MC with LPS/rIFN-γ-stimulated peritoneal macrophages. As shown in Fig. 3, production of NO increased with LPS treatment (10 μg/ml) and rIFN-γ (20 U/ml), while MC strongly inhibited the LPS/rIFN-γ-induced production of NO.

**Effect of MC on LPS/rIFN-γ-Activated iNOS Expression** The LPS/rIFN-γ-induced iNOS expression was com-
pletely suppressed by MC at 0.5 mg/ml (Fig. 4). These findings indicate that MC inhibits the LPS/rIFN-\(\gamma\)-induced production of NO through inhibition of iNOS expression.

**Effect of MC on LPS/rIFN-\(\gamma\)-Induced TNF-\(\alpha\) Production** To further investigate the effect of MC on other immunological functions of macrophages, we measured the cytokine production from activated macrophages in the presence of MC. The results indicate that MC significantly inhibited about 47% (\(p<0.005\)) of the TNF-\(\alpha\) production from activated peritoneal macrophages at 0.5 mg/ml (Fig. 5).

**Effect of MC on LPS/rIFN-\(\gamma\)-Induced Activation of NF-\(\kappa\)B** To clarify the mechanism of action of MC in the inhibition of LPS/rIFN-\(\gamma\)-induced production of NO and TNF-\(\alpha\), the effect of MC on LPS/rIFN-\(\gamma\)-induced activation of NF-\(\kappa\)B was examined. Since NF-\(\kappa\)B is a heterodimer of...
p50 and p65, we used the p65 antibody to assess whether MC could affect NF-κB activation. Treatment with LPS/rIFN-γ increased the content of NF-κB in the nuclear extract but decreased in the cytosolic fraction. However, in the presence of MC at 0.5 mg/ml, the content of NF-κB was suppressed in the nuclear extracts but increased in the cytosolic fractions (Fig. 6). These findings indicate that the inhibition of the LPS/rIFN-γ-induced production of NO and TNF-α by MC is mediated through the suppression of the LPS/rIFN-γ-induced translocation of NF-κB in the nuclear.

DISCUSSION

We demonstrated that MC reduces the production of NO and the expression of iNOS protein in LPS/rIFN-γ-treated mouse peritoneal macrophages. In addition, MC inhibited TNF-α secretion in LPS/rIFN-γ-treated mouse peritoneal macrophages. To clarify the mechanism of action of MC in the inhibition of the LPS/rIFN-γ-induced production of NO and TNF-α, the effect of MC on the activation of NF-κB, an essential transcription factor for the expression of iNOS, and TNF-α was examined. Our findings suggest that MC blocks the LPS/rIFN-γ-induced translocation of NF-κB thus inhibiting the LPS/rIFN-γ-induced production of NO and TNF-α.

Our results suggest that the effect of MC was associated not only with iNOS modulation, but also with the cytokine production in activated macrophages. In recent years, overproduction of NO has been correlated with oxidative stress and the pathophysiology of various diseases, including arthritis, septic shock, autoimmune diseases, and chronic inflammation. Inhibition of NO synthesis is, therefore, a potential therapeutic approach for the treatment of these inflammatory diseases. In addition to many synthetic inhibitors of iNOS, natural products which inhibit NO production have been investigated extensively. On the other hand, an excessive amount of TNF-α has also been implicated in the pathogenesis of many chronic inflammatory diseases. Because of its pivotal role in pathogenesis, a significant effort has focused on developing therapeutic drugs that interfere with TNF-α production or action.

MC may be exerting its inhibitory effect on NO and TNF-α production by simply being toxic to peritoneal macrophages. However, cell viability was not significantly altered by MC treatment, indicating that inhibition of NO and cytokine production is not due to a cytotoxic effect of MC. In macrophages, NF-κB (in cooperation with other transcription factors) coordinates the expression of genes encoding iNOS. NF-κB also plays a critical role in the activation of immune cells by up-regulating the expression of many cytokines, including TNF-α. Previous studies have shown that several natural products potently suppress NO and TNF-α production in macrophages by inhibiting NF-κB activation induced by an LPS signal. The amounts of MC which have been used in this study are high concentrations, raising the possibility that the active agent or agents in the MC represent a small component of the total mass. Therefore, further investigation is necessary to clarify unknown constituents which may be more active than MC itself. The studies on the isolation and characterization of the active chemical constituents are in progress.

In conclusion, this study showed that MC suppressed the LPS/rIFN-γ-induced production of NO and TNF-α in peritoneal macrophages through inhibition of NF-κB activation, although the precise mechanism of inhibition of NO and TNF-α production remains to be elucidated. Since NO and TNF-α is a critical transcription factor which regulates the production of various proinflammatory proteins and cytokines in activated macrophages during the process of inflammation, the inhibition of this transcription factor might serve as an effective therapeutic approach for inflammatory diseases.

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REFERENCES

18) Arteel E. G., Kadiiska M. B., Ruyan I., Bradford B. U., Mason R. P.,