Short Communication

***Ephedrae herba* Decreases Lipopolysaccharide-Induced Cyclooxygenase-2 Protein Expression and NF- κ B-Dependent Transcription in C6 Rat Glioma Cells**

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Abstract. We studied the effects of *Mao* (*Ephedrae herba*) on lipopolysaccharide (LPS)-induced expression of inducible cyclooxygenase (COX-2) in C6 rat glioma cells. Western blot analysis showed that *Mao* inhibited LPS-induced expression of COX-2 protein, but not that of constitutive cyclooxygenase. Reporter gene assays showed that *Mao* reduced LPS-inducible NF- κ B-dependent transcription that plays a crucial role in induction of COX-2 gene expression. This crude drug decreased LPS-induced I κ B α degradation in a concentration-dependent manner without affecting LPS-induced I κ B phosphorylation. These results suggest that *Mao* prevents LPS-induced NF- κ B-dependent transcription by inhibiting I κ B degradation and suppresses an increase in COX-2 protein expression in C6 cells.

Keywords: *Ephedrae herba*, COX-2 gene expression, NF- κ B-dependent transcription

Prostaglandins (PGs), arachidonic acid (AA) metabolites of the cyclooxygenase (COX) pathway, are major mediators in the regulation of inflammation and immune function (1). The interplay between PGE₂ and other local factors, including inflammatory cytokines, is likely to influence the outcome of inflammatory and immune responses in the central nervous system (CNS). It has been therefore suggested that clinical control of AA metabolism, particularly PGE₂ production, appears beneficial to patients with inflammatory conditions (2).

COX is the rate-limiting enzyme in PG production. This enzyme exists as two isoforms, namely constitutive COX (COX-1) and inducible COX (COX-2). These isoforms originate from two distinct genes, but are structurally conserved (1, 3). COX-1 serves as a constitutive enzyme responsible for PG synthesis that is essential for fluid and electrolyte homeostasis, gastric acid secretion, and platelet aggregation, while COX-2 is induced by inflammatory stimuli, and growth factors and glucocorticoids (4).

Activation of nuclear factor- κ B (NF- κ B) has been

recently reported to actually participate in the transcriptional activation of COX-2 gene induced by interleukin-1 (5), tumor necrosis factor- α (6), and lipopolysaccharide (LPS) (7). Furthermore the LPS-induced activation of the COX-2 gene has been shown to be mediated by inhibitor κ B (I κ B) kinase (IKK), which specifically catalyzes I κ B phosphorylation followed by its degradation and the subsequent NF- κ B nuclear translocation, leading to a stimulation of the *cis*-acting κ B element-mediated transcription (8).

Mao (*Ephedrae herba*) has been used for many years as one component of various traditional Chinese medicines effective for the treatment of multiple symptoms of cold and allergy (9). Consistently, in the earlier study, we have revealed that this crude drug inhibits IgE-mediated histamine release from rat basophilic leukemia (RBL-2H3) cells (10). Here we provide the first evidence that *Mao* decreases expression of COX-2 protein and NF- κ B-dependent transcription in C6 glioma cells which is well known to be a useful model system for studying PG production in the astrocytes.

Ephedra herb employed in the present study was imported from China. *Mao* was prepared as the hot-water extract from *Ephedra herb* as described previously

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(10) and kindly supplied from Kotaro Pharmaceutical Co., Ltd. (Osaka). The yield of *Mao* extract from the plant was 17.2%, and the total content of ephedrine, including *l*-ephedrine and *d*-pseudoephedrine (the ratio of the former to the latter was 2:1), of the extract was approximately 4%. Fetal bovine serum, horse serum, and Ham's F-10 medium were purchased from Cell Laboratory (Cleveland, OH, USA), Dainippon Pharmaceutical Co., Ltd. (Osaka), and Invitrogen (Carlsbad, CA, USA), respectively. Anti-COX-1 and anti-COX-2 antibodies, anti-14-3-3 β antibody, anti-I κ B α and anti-I κ B β antibodies, and anti-phospho-I κ B α antibody were obtained from Cayman (Ann Arbor, MI, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Cell Signaling Technology (Beverly, MA, USA), respectively. EndoFree Plasmid Maxi Kit, and phRG-TK vector and Dual-Luciferase[®] Reporter Assay System were purchased from QIAGEN K.K. (Tokyo) and Promega (Madison, WI, USA), respectively.

C6 cells were grown in F-10 medium containing 15% horse serum, 2.5% fetal bovine serum in a 37°C humidified incubator in an atmosphere of 5% CO₂ – 95% air (11). Under this culture condition, the pH of the culture medium was approximately 7.4.

For immunoblotting, C6 cells were seeded onto 35-mm dishes at the density of 2.0×10^5 cells per dish. Two days after seeding, cells were treated with or without *Mao* for 3 h. Thereafter cells were incubated with or without 1 μ g/mg LPS for an additional 6 h. Western blot analyses were conducted as described previously (11): Cells were lysed by addition of SDS-PAGE sample buffer. The protein samples were subjected to SDS-PAGE and then transferred electrically onto polyvinylidene difluoride membranes (Immobilon P; Millipore Corporation, Bedford, MA, USA). The blots were incubated with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 at 25°C for 1 h and incubated with anti-COX-1, anti-COX-2, anti-I κ B α , anti-I κ B β , anti-phospho-I κ B α , or anti-14-3-3 β -antibodies at 4°C overnight. The blots were washed several times and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated affinity-purified secondary antibodies in Tris-buffered saline/Tween 20 containing 5% skim milk at 25°C for 1 h. Immunoreactive signals were visualized by incubation of the blots with chemiluminescence assay reagents, followed by exposing them to Hyperfilm ECL (Amersham Biosciences,).

For the transcription activity assay, C6 cells were plated onto 48-well plates at the density of 1.2×10^4 cells per well. After culture for 48 h, cells were transfected with 0.19 μ g of firefly luciferase reporter plasmids of pNF- κ B₅-Luc firefly luciferase reporter construct containing five repeated NF- κ B-responsive elements. Cells

were co-transfected with 0.01 μ g of phRG-TK plasmid to normalize transfection efficiency as described previously (11). Thirteen hours later, cells were preincubated in the presence or absence of *Mao* for 3 h and incubated with or without 1 μ g/ml LPS for 8 h. Cells were harvested to determine each luciferase activity using the Dual-Luciferase[®] Reporter gene assay System (Promega).

The data are expressed as the means $\pm$ S.E.M., and a significant difference ($P < 0.05$) was analyzed by analysis of variance (ANOVA).

COX is a key enzyme for prostaglandin production because this enzyme is involved in the rate-limiting step in the conversion of AA to prostaglandins (12). We examined whether *Mao* affected the expressions of COX-1 and COX-2 proteins in C6 cells treated with LPS by Western blot analysis. In untreated cells, expressions of COX-1 and COX-2 proteins were detected. When treated with LPS, C6 cells showed an increase in expressions of COX-2 but not COX-1 proteins (Fig. 1). *Mao* was observed to inhibit the LPS-induced increase in expression of COX-2 protein in a concentration-dependent manner, although *Mao* exhibited no effects on expressions of COX-1 protein (Fig. 1). Also *Mao* had little effect on the cell viability at the concentrations ranging from 50 to 400 μ g/ml (data not shown).

Activation of NF- κ B participates in the induction of expression of COX-2 mRNA by LPS (13). Therefore, to examine whether *Mao* actually inhibits NF- κ B-dependent transcription activity in LPS-induced C6 cells, we next conducted transient transfection of C6 cells with pNF κ B-Luc, an NF- κ B-dependent luciferase reporter plasmid, and assayed the luciferase activity in C6 cells treated with vehicle or *Mao* in the absence or presence of LPS. This crude drug was found to inhibit the LPS-induced NF- κ B-dependent transcription and exhibited a concentration dependent inhibition. On the other hand, *Mao* has no effect on the basal NF- κ B-dependent transcription (Fig. 2). We further analyzed effects of

Fig. 1. Concentration-dependent inhibitory effects of *Mao* on the LPS-induced expression of COX protein in C6 rat glioma cells. Cells were plated at a density of 2×10^5 cells/dish and cultured for 48 h. After pretreatment with *Mao* at the indicated concentrations for 3 h, cells were treated with 1 μ g/ml LPS for 6 h and then harvested to analyze the expression of COX proteins. Western blot analysis was performed using anti-COX-1 and anti-COX-2-antibodies. Similar results were obtained from at least two independent experiments.

Fig. 2. Effects of *Mao* on LPS-induced NF- κ B-dependent transcription in C6 rat glioma cells. After 48-h culture, cells were transfected with 0.19 μ g of pNF- κ B-Luc reporter plasmid and 0.01 μ g of phRG-TK plasmid. Cells were pretreated with or without *Mao* at the indicated concentrations for 3 h and were treated with or without 1 μ g/ml LPS for 8 h. Cells were harvested to determine luciferase activity. Luciferase activity was determined as described under the experimental procedures and the relative transcription activity was calculated as the ratio of firefly luciferase activity to the *Renilla* luciferase one. Each column represents the mean $\pm$ S.E.M. of relative NF- κ B-mediated transcription activity ($n=4$). ** $P<0.01$, compared with the value upon treatment with LPS in the absence of *Mao*.

Mao on the phosphorylation and degradation of $\text{I}\kappa\text{B}\alpha$ in C6 cells treated with LPS by Western blotting, since the LPS-inducible phosphorylation of $\text{I}\kappa\text{B}$ led to its degradation which results in the activation of NF- κ B-dependent transcription (8, 11). In C6 cells, both $\text{I}\kappa\text{B}\alpha$ and $\text{I}\kappa\text{B}\beta$ proteins were expressed and appeared to be degraded following treatment with LPS. It was shown that *Mao* inhibited LPS-induced degradation of $\text{I}\kappa\text{B}\alpha$ but not that of $\text{I}\kappa\text{B}\beta$ in a concentration-dependent manner with no effects on LPS-induced phosphorylation of $\text{I}\kappa\text{B}\alpha$ (Fig. 3: A and B).

Mao has been widely used as a component of various traditional Chinese medicines in East Asia, China, and Japan for many years, and we reported that it can exert an anti-allergic action (10). In the present study, it was for the first time found that *Mao* suppressed LPS-induced increase in COX-2 protein expression without affecting the cell viability in C6 rat glioma cells. We also demonstrated that this crude drug inhibited the LPS-induced stimulation of NF- κ B-dependent transcription by preventing LPS-induced degradation of $\text{I}\kappa\text{B}\alpha$ without any effects on LPS-induced phosphorylation of $\text{I}\kappa\text{B}\alpha$ in C6 cells. These findings suggested that *Mao* inhibits LPS-induced augmentation of COX-2 gene expression accompanied by prevention of an $\text{I}\kappa\text{B}$ /NF- κ B-dependent intracellular signaling pathway.

COX-2 gene expression is induced as an immediate-early gene in certain types of cells in response to a wide

Fig. 3. Effects of *Mao* on the LPS-induced degradation and phosphorylation of $\text{I}\kappa\text{B}$ in C6 rat glioma cells. Cells were plated at a density of 2×10^5 cells/dish and cultured for 48 h. After pretreatment with *Mao* at the indicated concentrations for 3 h, cells were treated with 1 μ g/ml LPS for 30 min and harvested to analyze the degradation of $\text{I}\kappa\text{B}\alpha$ and $\text{I}\kappa\text{B}\beta$ proteins. Blots were probed with anti- $\text{I}\kappa\text{B}\alpha$ or anti- $\text{I}\kappa\text{B}\beta$ -antibody and then reprobbed with anti-14-3-3- β antibody to verify that each lane contained equal amount of proteins. Similar results were obtained from at least two independent experiments.

variety of inflammatory stimuli (4, 12). It has been demonstrated that LPS induces COX-2 gene expression via NF- κ B-mediated transcription in U937 cells (7) and C6 cells (11). Furthermore, we found that LPS induced increase in the protein expression of COX-2 but not COX-1 in C6 cells as described previously (11) and that *Mao* inhibited the LPS-induced increase in expression of COX-2 protein in a concentration-dependent manner. In addition, *l*-ephedrine, a major component of *Mao*, exhibited no effects on the LPS-induced increase in expression of COX-2 protein in C6 cells (unpublished observations), raising the possibility that *l*-ephedrine may not be responsible for inhibition of LPS-induced augmentation of expression of COX-2 protein by *Mao*. It was also shown that this crude drug did tend to reduce the LPS-induced augmentation of the luciferase activity in C6 cells transfected with pHES2 (-327/+59)-Luc, a human COX-2 reporter gene containing the NF- κ B-responsive element (11), at 200 μ g/ml (unpublished observations). The most important finding of the present study is that *Mao* has the pharmacological activity to prevent stimulation of NF- κ B, a central mediator of inflammation by LPS, by inhibiting LPS-induced degradation of $\text{I}\kappa\text{B}$, although the inhibitory mechanism of LPS-induced degradation of $\text{I}\kappa\text{B}$ remains to be defined. It is therefore possible that *Mao* inhibits an $\text{I}\kappa\text{B}$ /NF- κ B-dependent signaling probably by prevention of a ubiquitination step of $\text{I}\kappa\text{B}\alpha$ or a protein degradation step after

the ubiquitination to decrease the LPS-induced augmentation of COX-2 gene expression in vivo.

In conclusion, we found that *Mao* suppressed LPS-induced expression of COX-2 protein and activation of an $\text{I}\kappa\text{B}$ /NF- κB -dependent signaling pathway in C6 cells. These findings suggest the possible anti-inflammatory action of *Mao* in vivo.

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