The Anti-inflammatory Effects of Methylsulfonylmethane on Lipopolysaccharide-Induced Inflammatory Responses in Murine Macrophages

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Methylsulfonylmethane (MSM), also known as dimethyl sulfoxide and methyl sulfone, is an organic sulfur-containing compound that occurs naturally in a variety of fruits, vegetables, grains, and animals, including humans. In the present study, we demonstrated the anti-inflammatory effects of MSM in lipopolysaccharide (LPS)-stimulated murine macrophages, RAW264.7 cells. MSM significantly inhibited the release of nitric oxide and prostaglandin E2 by alleviating the expression of inducible nitric oxide synthase and cyclooxygenase-2 in LPS-stimulated RAW264.7 cells. Furthermore, the levels of interleukin-6 and tumor necrosis factor-α were decreased by MSM treatment in cell culture supernatants. Further study indicated that the translocation of the p65 subunit of nuclear factor (NF)-κB to the nucleus was inhibited by MSM treatment in LPS-stimulated RAW264.7 cells, in which it helped block degradation of inhibitor of NF-κB. In addition, in vivo studies demonstrated that topical administration of MSM at 500–1250 μg/ear resulted in similar inhibitory activities in 12-O-tetradecanoylphorbol 13-acetate-induced mouse ear edema. Collectively, these results indicate that MSM inhibits LPS-induced release of pro-inflammatory mediators in murine macrophages through downregulation of NF-κB signaling.

Key words methylsulfonylmethane; inflammation; nuclear factor-κB

Inflammation is a beneficial host response to foreign pathogens or tissue injury, and it eventually leads to the restoration of normal tissue structure and function. A normal inflammatory response is self-limiting and involves down-regulation of pro-inflammatory protein expression, increased expression of anti-inflammatory proteins, and reversal of the vascular changes that facilitated the initial immune cell recruitment process. Macrophages play a salient role in the inflammatory response and serve as an essential interface between innate and adaptive immunity. Following activation, macrophages modulate the expression of accessory molecules such as CD14 and toll-like receptor (TLR) 4. Stimulation of TLR4 by lipopolysaccharide (LPS) triggers the recruitment of the cytoplasmic adaptor protein MyD88 and subsequently culminates in the activation of downstream signaling pathways: the transcription factor nuclear factor-κB (NF-κB) pathway. These pathways induce the expression of various inflammatory mediators, including nitric oxide (NO), prostaglandins (PGs), and inflammatory cytokines.

NF-κB, the key molecule in the inflammatory response, is a dimeric transcription factor that is formed by the dimerization of proteins in the Rel family. NF-κB activity is effected through regulation of the expression of genes that encode inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS). NF-κB activity is usually inhibited in the cytoplasm through its association with an endogenous inhibitory protein of the 1κ-B (inhibitor of NF-κB) family. Upon activation, 1κ-B undergoes phosphorylation and degradation, processes that facilitate the movement of NF-κB to the cell nucleus. Once there, it binds to DNA and induces transcription.

There is currently a strong interest in developing new anti-inflammatory agents from natural products. Methylsulfonylmethane (MSM) is found in small amounts in many foods, including unpasteurized milk, grains, meat, eggs, and fish. It is also found in a popular dietary supplement with a molecular weight of 94. Health claims associated with MSM include relief of pain, inflammation, arthritis, allergies, certain parasitic infections, and asthma. In the present study, we investigated the anti-inflammatory effects and underlying mechanisms of action of MSM using LPS-induced inflammatory responses.

MATERIALS AND METHODS

Cell line and Reagents Raw 264.7 murine macrophages were obtained from the Korean Cell Bank (Seoul, Korea). LPS derived from Escherichia coli, 12-O-tetradecanoylphorbol 13-acetate (TPA), dexamethasone and acetone were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin used in this study were obtained from Hyclone (Logan, Utah, U.S.A.). MSM was supplied by AD Biotech, Co., Ltd. (Chuncheon, Korea).

Cell Culture, Cell Viability, and Cytotoxicity Assay Raw 264.7 murine macrophages were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. The effects of MSM on cell viability and cytotoxicity were tested using CellTiter 96® AQueous One Solution Assay of cell proliferation (Promega, Madison, WI, U.S.A.) and CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), respectively. RAW 264.7 cells were plated at a density of 1×104 cells/well in a 96-well flat-bottom plate, and MSM was added to each well at a concen-
titation of 0—10 mg/ml in complete DMEM. After 24 h incubation, viability and cytotoxicity were measured according to the manufacturer’s instructions.

**Measurement of Nitrite and PGE₂** The amount of nitrite and PGE₂ produced by the mouse macrophages was measured in RAW264.7 cell culture supernatant. RAW264.7 cells were plated at a density of 1×10⁵ cells in a 24-well cell culture plate with 500 μl of culture medium and incubated for 18 h. They were then treated with various concentrations (0—10 mg/ml) of MSM in the absence or presence of 500 ng/ml of LPS and incubated for another 24 h. The amount of nitrite was measured using the Griess reagent system (Promega). The amount of PGE₂ produced was measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MI, U.S.A.), according to the manufacturer’s instructions.

**Western Blot Analysis** Cells were washed with ice-cold PBS and scraped, and then whole cell lysates and cytoplasmic and nuclear proteins were extracted using PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, FL, U.S.A.) and a Nuclear Extract Kit (Active Motif, CA, U.S.A.). Twenty micrograms of protein was resolved in a loading buffer for sodium dodecyl sulfate (SDS)—PAGE, electrophoresed on SDS/polyacrylamide gels, and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked in Tris-buffered saline (TBS)-Tween 20 solution containing 5% non-fat dry milk and incubated overnight at 4°C with specific antibodies against iNOS (BD Biosciences, San Jose, CA, U.S.A.), COX-2 (BD Biosciences), Iκ-Bα and interleukin (IL)-6 in the cell culture supernatant were measured using an ELISA kit (eBioscience, San Diego, CA, U.S.A.). RAW264.7 cells were plated in a 24-well cell culture plate at a density of 1×10⁵ cells and incubated for 18 h. They were then treated with 0—10 mg/ml of MSM in the absence or presence of 500 ng/ml of LPS and incubated for another 24 h. The culture supernatant was collected and assayed according to the manufacturer’s instructions.

**Cytokine Assays** The amounts of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured in RAW264.7 cell culture supernatant. RAW264.7 cells were treated with 0—10 mg/ml of MSM for 24 h. Cell viability and cytotoxicity were determined as described in Materials and Methods. The results are reported as means ± S.E.M. for three independent experiments.

**Results**

**MSM Was Not Toxic to Murine Macrophages** Murine RAW264.7 macrophages were chosen for use in an investigation of the anti-inflammatory effects of MSM. We first examined whether MSM has cytotoxicity in RAW264.7 cells. No notable cytotoxicity was observed when the cells were exposed up to 10 mg/ml for 24 h (Fig. 1). Since MSM did not show cytotoxic effects up to 10 mg/ml, we used MSM at a concentration of 0—10 mg/ml for the subsequent experiments.

**MSM Inhibits the Release of NO and PGE₂ by Reducing iNOS and COX-2 Expression in LPS-Stimulated RAW264.7 Cells** We initially determined the concentration of LPS that causes inflammatory reaction, such as production of inflammatory mediators and Iκ-Bα degradation. Both NO- and IL-6 production and Iκ-Bα degradation were induced at concentrations of 10—1000 ng/ml LPS (Fig. 2). Based on these data, we used 500 or 1000 ng/ml of LPS in order to induce inflammatory responses in the rest of the experiments.

In order to assess the anti-inflammatory activities of MSM, RAW264.7 cells were exposed to LPS in the presence or absence of MSM, and the level of nitrite, a stable metabolite of NO, was measured in the medium. As shown in Fig. 3A, MSM inhibited LPS-induced NO production in a dose-dependent manner.

Since PGE₂ is another key inflammatory mediator, we investigated the effects of MSM on PGE₂ production in LPS-stimulated RAW264.7 cells. Similar to the findings related above and control group was treated with TPA only. Mouse ears were excised 6 h after the last application and homogenized in 50 mM Tris–HCl buffer (pH 7.5) with 1 mM EDTA, and their homogenates were incubated on ice for 20 min in the presence of 0.1% Troton X-100. The homogenates were centrifuged at 10000×g for 15 min and supernatant was collected for cytokine measurement. Parts of ear were fixed 10% buffered formalin solution, embedded in paraffin by standard methods and stained with hematoxylin–eosin (H & E).

**Statistical Analysis** Values are expressed as means ± S.E.M. of the results for at least three experiments. One-way analysis of variance (ANOVA) was used for comparison between the control and treatment groups. *p*-values <0.05 were considered statistically significant.

**Fig. 1. Effects of Methylsulfonylmethane (MSM) on Viability and Cytotoxicity in Murine Macrophages** RAW264.7 cells were treated with 0—10 mg/ml of MSM for 24 h. Cell viability and cytotoxicity were determined as described in Materials and Methods. The results are reported as means ± S.E.M. for three independent experiments.
to nitrite accumulation, treatment of RAW264.7 cells with 500 ng/ml of LPS led to a significant increase in PGE$_2$ production. Consistent with the nitrite accumulation, PGE$_2$ production was blocked by MSM treatment (Fig. 3B).

In order to determine the mechanism by which MSM reduces LPS-induced NO and PGE$_2$ production, we studied the effect of MSM on iNOS and COX-2 protein expression in RAW264.7 cells using Western blot analysis. As shown in Fig. 3C, iNOS and COX-2 protein expression were markedly induced in RAW264.7 cells after treatment with 500 ng/ml of LPS for 24 h. This induction was suppressed by MSM treatment in a dose-dependent manner. These results indicate that MSM-induced reduction in the expression of iNOS and COX-2 was responsible for the inhibition of NO and PGE$_2$ production.

**MSM Reduces the Production of Pro-inflammatory Cytokines in LPS-Stimulated RAW264.7 Cells**

We next attempted to examine the potential effects of MSM on the production of the pro-inflammatory cytokines, IL-6 and TNF-α. RAW264.7 cells were incubated with MSM in the presence of 500 ng/ml of LPS for 24 h, and IL-6 and TNF-α levels were evaluated in the culture supernatants. Because of LPS exposure, the IL-6 and TNF-α level had significantly increased to 888.3±53.6 and 12224.0±178.2 pg/ml, respectively. MSM treatment (10 mg/ml) prevented significant increases in IL-6 and TNF-α levels, holding them at 21.9±8.5 and 238.5±41.2 pg/ml, respectively.
and 8528.0 ± 487.1 pg/ml, respectively (Fig. 4).

**MSM Inhibits Degradation of IκBα and Nuclear Translocation of NF-κB p65 in LPS-Stimulated RAW264.7 Cells**  Finally, we examined NF-κB activation to determine the molecular mechanisms by which MSM inhibits LPS-induced inflammatory responses. NF-κB is a major transcription factor involved in the release of proteins that mediate the inflammatory response, and the degradation and phosphorylation of IκB are necessary to release NF-κB from the cytoplasmic NF-κB/IκB complex and to allow its subsequent translocation to the cell nucleus. We evaluated the effect of MSM on NF-κB activation to determine if it is mediated by IκB degradation. We observed that LPS-induced IκB degradation was inhibited after 30 min of exposure to MSM (Fig. 5A).

In order to directly investigate the effect of MSM on nuclear translocation, we determined NF-κB p65 levels among both cytosolic and nuclear proteins. The expression levels of NF-κB p65 protein were decreased in the nuclear fractions of cells when they were exposed to MSM, indicating that MSM inhibits the translocation of NF-κB p65 protein from the cytosol to the nucleus (Fig. 5B). These findings suggest that MSM exerts anti-inflammatory actions by blocking NF-κB signal.

In order to address whether MSM inhibits the activation of NF-κB induced by other stimuli other than LPS, we used recombinant mouse TNF-α as a stimuli for NF-κB activation. MSM also inhibited the degradation of IκBα in TNF-α stimulated RAW264.7 cells (Fig. 5C). This result suggests that MSM blocks NF-κB activation caused by LPS or TNF-α signaling.

**Effect of MSM on TPA-Induced Cutaneous Inflammation**  We assessed the anti-inflammatory activity of MSM in a TPA-induced ear inflammation model. Increased skin thickening is often the first hallmark of skin irritation and local inflammation. Ear edema was measured in the ears prior to and at 6 h following treatments. As shown in Figs. 6A and B, exposure to TPA resulted in marked increases in skin thickness. Topical application of acetone (vehicle) did not alter the skin thickness significantly. However, MSM (500, 1250 µg/ear) or dexametasone significantly inhibited the TPA-induced in-

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Fig. 5. Effects of Methylsulfonylmethane (MSM) on LPS-Induced Degradation of IκBα and Nuclear Translocation of NF-κB-p65 in Murine Macrophages

RAW264.7 cells were treated with 0—10 µg/ml of MSM in the presence of 1 mg/ml of LPS (A, B) or recombinant mouse TNF-α (C) for 30 min. The 50 µg of protein obtained from whole cell lysates, cytosolic and nuclear fractions of each cell were resolved on 10% SDS-PAGE. Western blot analysis was performed as described in Materials and Methods. β-Actin and lamin B were used as loading controls of cytosolic protein and nuclear protein, respectively.

Fig. 6. Effects of Methylsulfonylmethane (MSM) on TPA-Induced Ear Inflammation

Mice were treated with MSM (0, 50, 500, 1250 µg/ear) or dexametasone (50 µg/ear) with topical application of acetone (vehicle) or TPA in acetone. Ear thickness was measured at 6 h after TPA treatment (A). Representative micrographs of H&E-stained mouse ear cross-sections in TPA-induced ear inflammation model. Ears were harvested 6 h post-treatment with acetone vehicle, TPA plus MSM or dexametasone (B). Sections shown are representative of observations from five animals in each group (200× magnification). Serum and ear homogenates were taken 6 h after TPA treatment and examined for the production of the IL-6 using ELISA (C). The results are reported as means±S.E.M. for five mice per group. Significant differences from mice treated with TPA in the absence of MSM are indicated as follows: **p<0.01, ***p<0.001.
creases in ear thickness, indicating the therapeutic effect of this extract.

In addition, topical application of TPA caused a dramatic increase in the production of IL-6 in plasma and ear homogenates by 6 h after challenge. In contrast, treatment with TPA plus MSM (1250 μg/ear) or dexamethasone reduced IL-6 levels significantly (Fig. 6C).

DISCUSSION

It has been reported that MSM has positive effects on a variety of conditions, including osteoarthritis, allergic rhinitis, and cancer. However, studies addressing the working mechanisms of MSM are limited. In the present study, we aimed to examine the anti-inflammatory effects of MSM and to elucidate its underlying mechanisms using murine macrophage cells and mouse ear inflammation induced by TPA.

We found that MSM dramatically inhibits LPS-induced increases in NO and PGE2 production through suppression of iNOS and COX-2 expression (Fig. 3). We also found that MSM strongly inhibits IL-6 and TNF-α production in LPS-stimulated murine macrophages (Fig. 4). The expression of inflammatory mediators such as NO, PGE2, IL-6, and TNF-α is regulated by the key transcription factor, NF-κB. NKxB is maintained in a latent form in the cytoplasm, where it is in complex with the inhibitory Iκ-B proteins. The interaction of NF-κB with Iκ-B masks the nuclear localization signal. Phosphorylation of Iκ-B by Iκ-B kinase leads to ubiquitination of the protein and its subsequent degradation. NF-κB is then free to translocate to the nucleus, where it binds to DNA and induces activation of a wide variety of target genes related to the inflammatory response, including iNOS, COX-2, and various cytokines. We showed that MSM blocks LPS-induced degradation of Iκ-Bα and nuclear translocation of NF-κB p65 in murine macrophages (Fig. 5). These results indicate that MSM exerts at least some of its anti-inflammatory actions through inhibition of NF-κB signal.

IL-6 is a typical pleiotropic cytokine, which plays an important role in the homeostasis of the immune and hematopoietic systems, in addition to its physiological effects upon the nervous and endocrine systems and bone metabolism. However, IL-6 production is rapidly increased in acute inflammatory responses associated with infection, injury, trauma, and other stresses. As such, a dysregulated, high-level production of IL-6 could induce an undesirable inflammatory state. With this study we showed that MSM not only inhibits IL-6 production in LPS-stimulated macrophage cells, but also reduces plasma or local levels of IL-6 in TPA-induced inflammation mice (Figs. 4A, 6C). In this study, the inhibitory effect of MSM on IL-6 production was dreadfully strong in comparison with others. Although the detail mechanism by which MSM selectively reduces IL-6 production is not clearly known and remains to be determined, it is well known that activator protein (AP)-1, interferon regulatory factor (IRF)-1, CCAAT/enhancer binding protein (C/EBP) β are also involved in IL-6 production. MSM might inhibit the activation of not only NF-κB but also these transcription activation pathways.

It is very important whether MSM directly inhibits Iκ-Bα degradation and the nuclear translocation of p65. The nuclear translocation and DNA binding of NF-κB is preceded by the degradation of Iκ-Bα. Our study indicates that MSM inhibited LPS-induced inflammatory responses via blocking the degradation of Iκ-Bα and the subsequent nuclear translocation of NF-κB p65. Previous studies have shown that the phosphorylation of Iκ-B is regulated by α and β isoforms of Iκ-B kinase complex (IKK). Although we did not observe the effect of MSM on IKK activity, MSM surely inhibited Iκ-Bα degradation. It might represent that MSM inhibits IKK activity, Iκ-Bα degradation and nuclear translocation of NF-κB p65 in LPS-stimulated RAW264.7 cells.

MSM is used orally and topically. Although the optimum dosage has not been clearly defined, the suggested oral therapeutic doses are 4—6 g/d. There is limited formal safety data, and no long-term assessment has been performed. However, MSM is rapidly excreted from the body and animal toxicity studies of MSM have shown only minor adverse effects with doses of 1.5 g/kg and 2.0 g/kg of MSM for 90 d. This represents a human dose of 30—42 g/d, which is equivalent to 5—7 times the proposed maximum recommended human dose of 6 g/d. Another study confirmed that MSM has no toxic effects on either pregnant rats or their fetuses. Based on these previous results and the present data, we believe MSM is relatively safe and effective for the treatment of inflammatory diseases.

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REFERENCES