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Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase expression by a novel compound, mercaptopyrazine, through suppression of nuclear factor-kappaB binding to DNA

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Abstract

Macrophage cells in response to cytokines and endotoxins produced a large amount of nitric oxide (NO) by expression of inducible nitric oxide synthase (iNOS), resulting in acute or chronic inflammatory disorders including septic hypotension and atherosclerosis. In the present study, we investigated the effect and the mechanism of mercaptopyrazine (MP) in the induction of iNOS and NO production as a culminating factor for several inflammatory disorders. Pretreatment of MP alleviated the mortality of endotoxemic mice receiving a lethal bolus of lipopolysaccharide (LPS), which was associated with the reduced levels of serum nitrite/nitrate and IL-1β. In RAW264.7 mouse macrophage cells, MP (300 μM) inhibited both protein and mRNA levels of iNOS stimulated by LPS/interferon-γ (IFNγ) up to 50%. The nuclear factor-kappa B (NF-κB)-driven transactivation was also suppressed by MP to the same degree. Treatment of MP reduced the binding of NF-κB to the oligonucleotides containing NF-κB consensus sequence, while it did not affect the translocation of NF-κB to nuclear. Suppression of NF-κB activity by MP was completely reversed by a reducing agent, dithiothreitol, implying that MP might oxidize the sulfhydryl group(s) of DNA binding domain of NF-κB. In conclusion, MP would be one of interesting candidates or chemical moieties of iNOS expression inhibitor via specific suppression of NF-κB binding to DNA, and be useful as a chemopreventive agent or a therapeutic against iNOS-associated inflammatory diseases.

Keywords: Mercaptopyrazine; iNOS inhibitor; LPS; NF-κB activation; DNA binding; Inflammation

Nitric oxide (NO), a radical produced from L-arginine via NO synthase (NOS), plays a dual role as both a beneficial and a detrimental molecule in the process of inflammation. NO produced by constitutive NOS (cNOS) is critical in maintaining cellular function, whereas NO produced by inducible NOS (iNOS) is an important mediator of acute or chronic inflammation [1,2]. The iNOS-mediated high output production of NO contributes to the killing of virally infected cells, tumor cells, and some pathogens partly because it inactivates their mitochondrial respiratory chain enzymes. If the inflammation becomes chronic, then even healthy host cells also may be killed by NO, contributing to inflammatory pathologies [3].

The iNOS expression is induced by the activated NF-κB since iNOS gene contains functional nuclear factor-κB (NF-κB) binding sites in its promoter [4,5]. The transcription factor NF-κB is activated by a variety of stimuli and regulates diverse gene expressions and biological responses. The stimuli include bacterial endotoxin, lipopolysaccharide (LPS), ionizing radiation, and carcinogens

Abbreviations: 2-AP, 2-allylthiopyrazine; MP, 2-mercaptopyrazine; DTBP, 2,2′-dithiobispyrazine; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOx, nitrite/nitrate; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; PDTC, pyrolidine dithiocarbamate; PPAR-γ, peroxisome proliferator activated receptor-γ; PPRE, PPAR responsive element; DTT, dithiothreitol

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that are often associated with inflammatory diseases or tumorigenesis. Recent observations support that NF-κB activation also may be important in pathogenesis of chronic diseases such as atherosclerosis [6] and diabetes [7]. That is, an activated NF-κB was found in atherosclerotic lesion [8] and several types of cells from diabetic animal models [9]. Moreover, the activated NF-κB induces transcription of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin-1β [10,11] as well as iNOS. The cytokines secreted from macrophages, bind to their receptors on macrophage itself (autocrine) or on other cells (paracrine) to further activate NF-κB in the cells, consequently aggravating the diseases. It is interesting to note that the elevated level of TNF-α was also reported to associate with the development of insulin resistance, possibly with diabetes [12].

Persistent and excessive NO production by iNOS relaxes the vascular smooth muscle and decreases its responsiveness to vasoconstrictive agents such as norepinephrine, resulting in septic hypotension [13]. Since the deleterious production of NO is resulted from the induction of the iNOS gene, the most effective way to repress NO production would be the suppression of iNOS gene expression. For treatment of septic shock, however, only competitive inhibitors for L-arginine, a substrate of NOS, such as L-N^G-monomethyl arginine (L-NMMA) [14], nitro-L-arginine methyl ester (L-NAME) [15], and N^G-nitro-L-arginine (L-NLA) [16], are available. These inhibitors act non-selectively on all forms of NOS including beneficial endothelial constitutive NOS (ecNOS), which may result in increased organ damage and mortality. Therefore, a selective inhibition of iNOS transcription via NF-κB inactivation would be more desirable to treat the patients with septicemia. Moreover, a weak and chronic suppression of NF-κB would also be beneficial to prevent the chronic disease development, if we understand the chronic diseases like atherosclerosis as inflammatory disease in the respect that the inflammatory environment increases the production of NO by iNOS in human atherosclerotic lesion in macrophages and foam cells, contributing to vascular injury [17,18].

We previously reported that the mortality of mice receiving a lethal dose of LPS was decreased by 2-(allylthio)pyrazine (2-AP), an experimental chemopreventive compound and an analog of diallyl sulfide [13]. As an effort to screen new therapeutic agents against septic shock or chronic inflammatory disorder caused by iNOS induction, we investigated analogs of diallyl sulfide as an inhibitor of iNOS induction. We report here that 2-mercaptopyrazine (MP; Fig. 1), which is a synthetic precursor and one of metabolites of 2-AP, inhibits LPS-induced iNOS expression in mice and macrophages. Furthermore, MP interfered the NF-κB binding to iNOS promoter through –SH blockage of NF-κB, but had no effect on the nuclear translocation of NF-κB. To our knowledge, it is the first time to identify a NF-κB inhibitor, which blocks the step of DNA binding specifically.

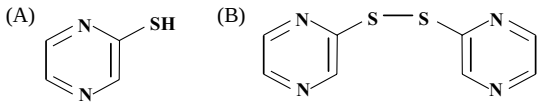

Fig. 1. Chemical structures of (A) 2-mercaptopyrazine (pyrazine-2-thiol) and (B) 2,2′-dithiobispyrazine.

1. Materials and methods

1.1. Synthesis of 2-mercaptopyrazine and 2,2′-dithiobispyrazine (DTBP)

To a solution of sodium ethoxide, prepared from sodium (6.9 g, 0.3 mol) and ethanol (150 ml), *N*,*N*-dimethylformamide (150 ml) was added. After removing the ethanol by distillation, the residual solution was saturated with hydrogen sulfide. The deep green solution was heated with 2-chloropyrazine (17.25 g, 150 mmol) at 100 °C for 3 h, and solvent was then removed under reduced pressure. The residue was dissolved in water, then acidified with acetic acid to give yellow precipitates, which were extracted with 2N NaOH (75 ml). After filtration, acidification of the solution gave MP (15 g, 88%), m.p. 209–214 °C. A solution of iodine (1.3 g) in potassium iodide (2.5 g) and water (10 ml) was added drop-wise to a solution of 2-mercaptopyrazine (0.55 g) in 2N NaOH (5 ml). After refrigeration, the crystalline precipitate (0.3 g) was filtered off and identified as DTBP by its proton NMR spectra and m.p. 106–108 °C.

1.2. Animal treatment and sample preparation

Animal studies were conducted in accordance with the institutional guidelines for care and use of laboratory animals. Male ICR mice at 6 weeks of age (25–30 g) were supplied from DaiHan Experimental Animal Center and maintained under 12-h light/12-h dark cycles in an air-conditioned room with commercially available rat chow (Purina, Korea) and water available ad libitum. LPS (20 mg/kg, *Escherichia coli*, serotype 0127B8, Sigma Co.) was intra-peritoneally injected, as dissolved in sterile saline (10 ml/kg body weight). MP was orally administered as a suspension of olive oil (100 or 200 mg/kg body weight/day) for 3 days prior to LPS injection. Control mice received olive oil as a vehicle. Blood was collected from retroorbital sinuses of mice at the indicated time point after LPS injection. Animals were sacrificed under light anesthesia with diethyl ether and left lung was isolated and homogenized for the detection of iNOS.

1.3. Cell culture and transfections

The macrophage cell line RAW264.7 (ATCC No. TIB-71) was cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin,

in 5% CO_2 at 37 °C. RAW264.7 cells (2×10^5 cells/well) in 6-well plate were pretreated with either MP or pyrolidine dithiocarbamate (PDTC) for 2 h at the indicated final concentrations (up to 500 μM) prior to the stimulation with LPS (100 ng/ml, *E. coli*, serotype 0127B8, Sigma Co.) and IFNγ (100 U/ml, recombinant murine, GIBCO-BRL) for 24 h. The cells were harvested with lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, and 1% Triton X-100) for western analysis. To measure nitrite/nitrate contents in conditioned media, the cells (10^4 cells/well) were cultured in 96-well plate as described above except using phenol red-free MEM.

For transfections, RAW264.7 cells were grown in 6-well plates with DMEM supplemented with 10% FBS and transfected for 5 h with either pNF-κB-luc (1 μg) or piNOS-luc (1 μg) together with pcDNA3.1-LacZ (Invitrogen) by calcium phosphate co-precipitation method [19,20]. To inhibit the lysosomal degradation of the DNA constructs, the cells were transfected in the presence of 50 μM chloroquine in complete media and shocked with 10% DMSO in DMEM for 5 min after the transfection. Then, the cells were washed twice with PBS and incubated in DMEM with 10% FBS for 16 h. Treatment of either MP or PDTC was followed at the indicated concentrations for 2 h before the stimulation with LPS (100 ng/ml) plus IFNγ (100 U/ml). In some experiments, cells were treated with dithiothreitol (DTT, 100 mM) for 2 h prior to MP treatment. Luciferase activity was measured from the harvested cells using Luciferase Assay Kit (Promega, Madison, WI) and luminometer (Berthold, German). The transfection efficiencies were normalized by the β-galactosidase activity. All data are the mean ± S.D. of three independent measurements of duplicate.

1.4. Plasmids

The −973/+82 fragment of murine iNOS promoter from piNOS973-CAT [5] was subcloned into *Bam*HI/*Hin*dIII sites of pGL2-basic luciferase vector (Promega Co.). The cloned promoter was sequenced to ensure the fidelity of the resulting constructs. The pNF-κB-luc was the luciferase reporter construct containing three upstream NF-κB binding elements as previously described [19,20].

1.5. Western blot analysis

Lung homogenates from the mice were prepared in 0.1 M Tris acetate buffer (pH 7.4) containing 0.1 M potassium chloride and 1 mM EDTA by centrifugation at 10,000 × *g* for 10 min and the supernatants were collected and stored at −70 °C until use. Cell lysate proteins (25 μg) or lung homogenates (100 μg) were analyzed by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot using rabbit anti-murine iNOS polyclonal antibody (Transduction Lab). Equivalent loading of protein was verified by anti-β-actin monoclonal antibody (Sigma Co.). In order to analyze the translocation of NF-κB, nuclei from the treated cells were isolated as described [21]. The nuclear proteins (20 μg) were subjected onto 10% SDS–PAGE and Western blot analysis was performed using antibodies against either p65 or p50 subunit of NF-κB (Transduction Lab, Lexington, KY) [22]. HRP-conjugated goat anti-rabbit IgG or alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) was used as a secondary antibody and the nitrocellulose paper was developed using enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

1.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from cell monolayers was isolated using guanidinium isothiocyanate and phenol [20]. Total cDNA synthesized from 2 μg of total RNA was amplified for 30 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 2 min. The oligonucleotide primer set (5′-ATG TCC GAA GCA AAC ATC AC-3′ and 5′-TAA TGT CCA GGA AGT AGG TG-3′) was used for amplification of a 450 bp fragment of mouse iNOS. The reaction products from the PCR were examined by 1% agarose gel electrophoresis. Band intensities were quantified by densitometer and normalized by comparison to the RT-PCR product of glyceraldehyde-3-phosphate dehydrogenase mRNA [20].

1.7. Enzyme-linked immunosorbent assay (ELISA)

The IL-1β level in the serum of mice was measured by ELISA Kit (Endogen) for murine IL-1β using rabbit anti-mouse IL-1β antibody and a biotinylated secondary antibody according to the manufacturer's instruction.

1.8. Electrophoretic mobility shift assays (EMSA)

EMSA was performed using the end-labeled NF-κB probe of double-stranded synthetic oligonucleotides containing NF-κB motif of iNOS promoter (−92 to −65nt, 5′-tcgaCCA ACT GGG GAC TCT CCC TTT GGG AAC A-3′ and 5′-tcgaTG TTC CCA AAG GGA GAG TCC CCA GTC CCC AGT TGG-3′) and nuclear extract isolated from the cells (2×10^5) as described [21]. Briefly, the nuclear extracts without DTT were prepared from LPS/IFNγ-stimulated RAW264.7 cells and treated with MP (0–500 μM) for 30 min at room temperature. The end-labeled annealed oligonucleotides (50 ng, 5×10^4 dpm) were incubated for 30 min at 4 °C with the nuclear extracts (10 μg) in the binding buffer (10 mM Tris–HCl, pH 7.8, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 1 μg/ml poly dI.dC) in the absence of DTT. The oligonucleotide–protein complexes were separated onto 6% polyacrylamide gel. One hundred-fold excess amount of the cold annealed oligonucleotides

were added in binding reaction to prove if the shifted bands binds specifically to NF-κB oligonucleotides. For antibody supershift/inhibition assays, 5 μg of rabbit anti-p65 polyclonal antibodies (Santa Cruz Co.) were incubated with the protein extract on ice for 30 min prior to binding reaction. The dried gel was analyzed by exposure to X-OMAT AR film (Kodak, Rochester) at −70 °C.

1.9. Nitrite/nitrate determination

RAW264.7 cells were grown on 96-well plates in phenol red-free MEM and the nitrite/nitrate (NOx) contents of the conditioned media were analyzed by NO analyzer (Antek). In the case of serum sample, 1 μl of serum was used for the analysis.

1.10. Statistical analysis

Student's *t* test was used to determine the statistical differences between various experimental and control groups. *P* value <0.05 was considered as significant.

2. Results

2.1. Pretreatment of MP alleviates the LPS-induced mortality

We studied whether MP contains the in vivo protective effects against systemic inflammatory toxicity by LPS. ICR mice that were pretreated with MP for 3 days (100 and 200 mg/kg/day, p.o.) were exposed to lethal doses of LPS (55 mg/kg). Cumulative proportions of mice surviving after lethal dose of LPS are shown in Fig. 2. MP pretreatment of mice prior to LPS injection elevated the survival rate by 70–80% from 40% in LPS alone when examined at 24 h after LPS injection. At 72 h after LPS injection, survival rate of MP pretreated mice (200 mg/kg) was 80% while that of untreated mice was less than 10%. It is possible that MP is a potential chemical moiety that may regulate immune responses in animal. It is interesting to note that the MP-treated mice without LPS injection do not show any signs of toxicity. Instead, we observed protective effects of MP at 10 mg/kg on chemical-induced hepatotoxicity, suggesting a possible use of MP as a chemopreventive small chemical (data not shown).

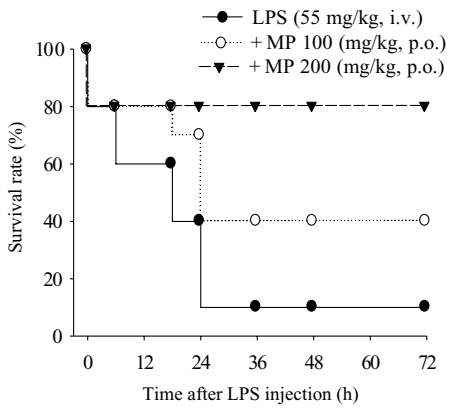

Fig. 2. MP protects the increase in mortality induced by LPS. MP (100 or 200 mg/kg) was orally administered for 3 days prior to LPS injection. LPS was intravenously injected at the dose of 55 mg/kg. (●) LPS-treated group; (○) LPS-treated + MP (100 mg/kg) group; (▼) LPS-treated + MP (200 mg/kg) group, $n = 10$.

2.2. MP inhibits the LPS-induced increase of serum NO and iNOS protein expression in mice

We assessed the effect of MP on the production of NO in serum of mice treated with LPS. A single dose of LPS treatment (20 mg/kg, i.p.) increased nitrite/nitrate (NOx) production over the basal level from 6 h (400 μM) and peaked at 24 h (1200 μM). Pretreatment of MP (200 mg/kg/3 days, p.o.) reduced the serum NOx concentration below 400 μM even at 24 h (Fig. 3A). Inhibition of LPS-induced NOx production by MP was achieved in a dose-dependent manner. IC_{50} of MP was approximately 100 mg/kg (Fig. 3B). These results suggest that the mechanistic basis for the protective effect of MP against LPS toxicity results from the inhibition of the LPS-induced NO production. To test whether MP affects the transcription of iNOS gene, we further determined the expression level of iNOS protein in lung homogenates of the mice using Western blot analysis. Lung homogenates were chosen for iNOS protein analysis since lung had been suggested as a major organ for iNOS production resulting in LPS-induced septic hypotension. Fig. 3C showed that a single dose of LPS (20 mg/kg, i.p.) strongly increased iNOS protein levels at 6 h and 12 h, and returned to control level at 24 h (Fig. 3C). Treatment of mice with MP (200 mg/kg for 3 days) blocked the LPS-induced iNOS protein expression approximately 50% at both 6 h and 12 h post LPS stimulation (Fig. 3D).

2.3. MP inhibits IL-1β secretion into serum

IL-1β is one of principal mediators of the responses to LPS and is involved in the inflammatory process [11,23]. To determine whether IL-1β plays a role in inhibitory effects of MP on the LPS-induced responses, the concentrations of plasma IL-1β of mice were assessed using ELISA. As shown in Fig. 4, the elevation of plasma IL-1β up to 463 pg/ml of plasma at 2 h after LPS treatment was prevented by the three consecutive treatments of MP (200 mg/kg, p.o. per day) in a concentration-dependent manner.

2.4. MP inhibits the NO production by suppression of iNOS expression in macrophages

Since macrophages are the major cells that produce NO by iNOS, the mouse macrophage cell RAW264.7 was

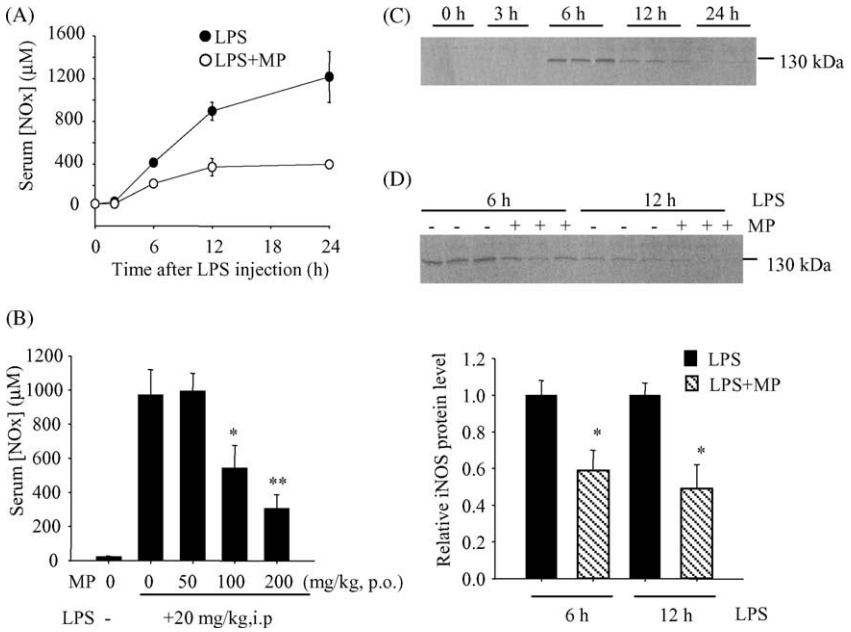

Fig. 3. MP inhibits the increase in serum NOx and lung iNOS expression in mice treated with LPS. MP (200 mg/kg) was orally administered for 3 days prior to i.p. injection of LPS (20 mg/kg). Serum NOx level was determined using NO analyzer with or without treatment of MP. (A) Time-dependent effects of MP on the level of serum NOx ((●) LPS-treated group; (○) LPS-treated + MP group) and (B) dose-dependent effects of MP on the level of serum NOx. The iNOS expression was monitored by Western blot analysis using anti-mouse iNOS antibody. (C) Time-dependent changes in the expression of iNOS protein in lung homogenate of LPS-treated mice. (D) Effect of MP on pulmonary iNOS protein levels in LPS-treated mice. Time denotes the time after LPS injection. The graph shows the result of densitometric scanning of immunoblot. Values are mean ± S.E.M. Asterisk denote values are significantly different from the LPS-treated group, $^{*}P < 0.05$ and $^{**}P < 0.01$, $n = 3$.

introduced for further study. When RAW264.7 cells were pretreated with MP (100 μM) for 2 h prior to LPS + IFNγ stimulation, the NO content in the conditioned media was decreased as expected to the same level with PDTC (100 μM), a known NF-κB inhibitor (Fig. 5A). Treatment of MP also leads to a significant decrease of both iNOS protein (Fig. 5B) and mRNA levels (Fig. 5C) in a dose-dependent manner. At a concentration of 300 μM, MP decreased the expression of iNOS protein up to 95% and IC_{50} was approximately 100 μM. The mRNA level of LPS/IFNγ-stimulated iNOS was suppressed 26 and 44% by MP at concentrations of 100 and 300 μM, respectively. Similarly, PDTC decreased the iNOS mRNA levels by 20–40%.

2.5. MP inhibits the NF-κB-driven transcriptional activity

The iNOS promoter contains two NF-κB binding sites, which mainly regulate the transcription of iNOS mRNA [5]. To study if MP blocks iNOS expression via suppression of NF-κB activity, the effects of MP were determined on the luciferase activity of the pNF-κB-luc-transfected RAW264.7 cells. This NFκB-luc reporter vector contains three copies of NF-κB-binding sites in E-selectin promoter and utilized for determining the NF-κB-driven transactivation [19]. MP inhibits the NF-κB transcriptional activity in a dose-dependent manner from 10 to 300 μM (Fig. 6A).

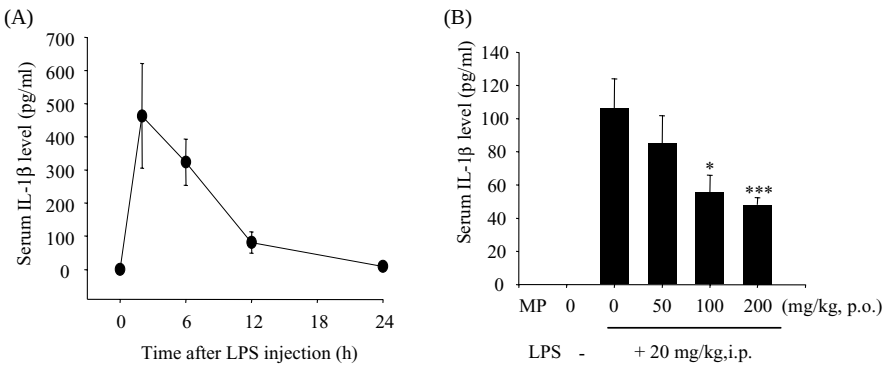

Fig. 4. Dose-dependent decrease of LPS-induced serum IL-1β by MP. (A) Time-course of serum IL-1β level in LPS-treated mice. (B) Dose-dependent effect of MP on LPS-induced serum IL-1β increase in LPS-treated mice. MP was orally administered for 3 days prior to LPS injection (20 mg/kg, i.p.). Blood was collected 2 h after LPS treatment. Values are mean ± S.E.M. Asterisk denote values are significantly different from LPS-treated group, $^{*}P < 0.05$ and $^{***}P < 0.001$, $n = 5$–6.

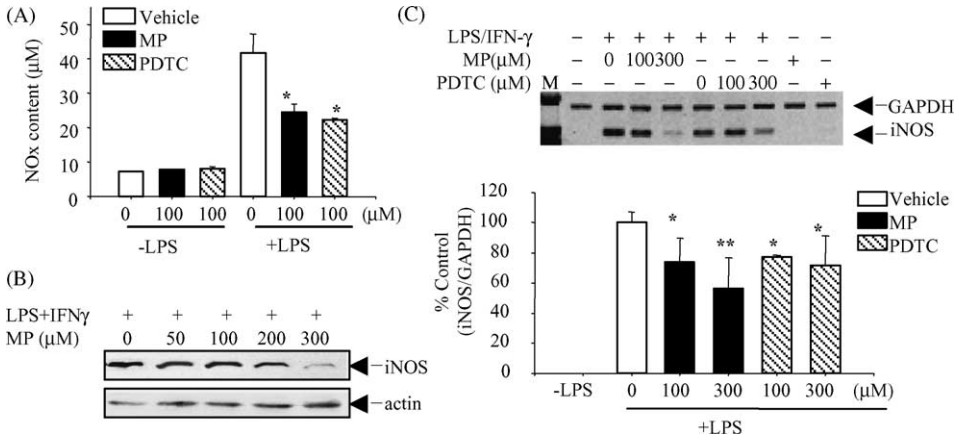

Fig. 5. MP inhibits the production of NO by iNOS. RAW264.7 cells (2×10^5 cells/well) in 6-well plate were pretreated with either MP or PDTC at the indicated final concentrations for 2 h. In the absence or presence of 24 h incubation with LPS (100 ng/ml) + IFNγ (100 ng/ml), (A) the nitrite contents in the conditioned media were determined using NO analyzer and (B) the expression of iNOS protein in total cell extracts (25 μg) was analyzed by western blot. (C) After 4 h of incubation instead of 24 h with LPS + IFNγ, total RNA was isolated to assess the level of iNOS mRNA by RT-PCR and 1% agarose gel. M denotes molecular weight marker. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized for the normalization of data. Band intensities of the agarose gel were quantified with a densitometer. All data are mean ± S.D. of three independent measurements of duplicates. Asterisk denote values are significantly different from the LPS + IFNγ-treated control. $^{*}P < 0.05$; $^{**}P < 0.01$.

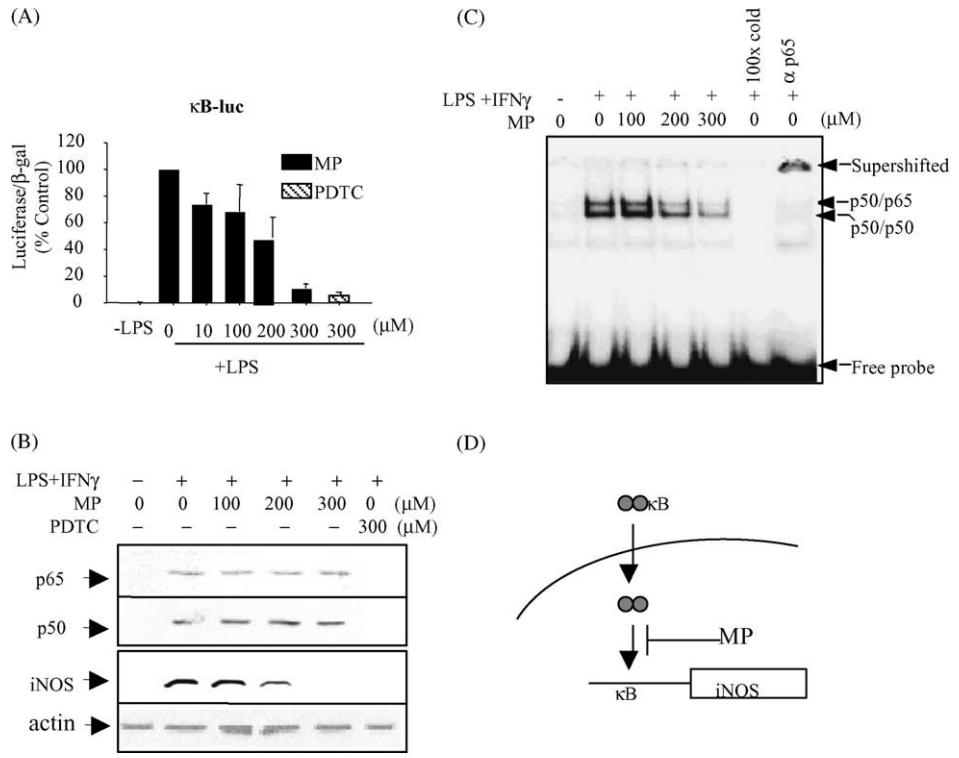

Fig. 6. MP suppresses the activation of NF-κB via inhibition of DNA binding. (A) RAW264.7 cells were transfected with a reporter gene κB-luc and *LacZ* expression vector. The transfected cells were treated with MP or PDTC as indicated for 2 h, stimulated with LPS + IFNγ for 24 h and harvested for luciferase assay. Normalized luciferase expressions ($n = 6$) were calculated relative to the *LacZ* expressions and the results were expressed as percent control over the value obtained with the LPS + IFNγ. (B) RAW264.7 cells were pretreated with MP as (A), and nuclei were prepared. Nuclear proteins (20 μg) were separated onto 10% SDS–PAGE and analyzed by Western blot using p65 or p50 antibodies. Protein expression of iNOS and β-actin in total cell lysates (25 μg) were analyzed by Western blot for comparison. (C) The nuclear extracts were prepared from LPS-stimulated RAW264.7 cells and treated with MP for 30 min at room temperature as indicated. The MP-treated nuclear extracts were subjected onto the NF-κB EMSA assay in the absence of DTT as described in Section 2. NF-κB binding activities to DNA were decreased as MP increased. The shifted bands were abolished by incubating with 100-fold excess amount of cold probes. The p65 antibody supershifted the NF-κB complex. (D) The inhibition mode of iNOS or κB-dependent expression by MP is schematically presented.

The NF-κB-dependent transactivation could be blocked by ligands for peroxisome proliferator activated receptor-γ (PPAR-γ) [20]. We tested if MP might be a PPAR-γ ligand using the PPAR responsive element (PPRE)-driven transactivation assay in RAW264.7 cells [19]. MP did not activate the PPRE-dependent luciferase activity implying that MP was not a PPAR-γ ligand (data not shown).

2.6. MP does not inhibit the nuclear translocation of NF-κB protein

Most known NF-κB inhibitors, including PDTC, prevent NF-κB activation by a mechanism that involves inhibition of I-κB degradation followed by IKK-induced phosphorylation [24–26]. Since we also hypothesized that MP might block the NF-κB translocation into nuclei in the similar manner of PDTC, the content of p65 and p50 subunit of NF-κB in the nuclei were quantified by western analysis. Surprisingly, the protein levels of p65 and p50 in nuclei were not altered in spite of MP treatments at the same concentrations that suppress the transactivation of NF-κB and iNOS expression (Fig. 6B). In cytosol, p65 and p50 proteins were not detected at all after the treatment (data not shown). This result showed that MP did not affect the translocation of NF-κB into the nucleus as well as I-κB degradation, which was distinct from PDTC or other NF-κB inhibitors.

2.7. MP prevents the binding of NF-κB to DNA

Although the most common mechanism of NF-κB inactivation is the blockade of nuclear translocation of NF-κB, MP did not inhibit this step. Then, it would be possible for MP to reduce the DNA binding affinity onto NF-κB binding site of the promoter. To test whether MP interferes with binding activity of NF-κB protein to the NF-κB binding sites, electrophoretic mobility shift analysis of isolated nuclear extract was performed. As shown in Fig. 6C, the MP treatment inhibited the binding of NF-κB protein to the oligonucleotides containing the NF-κB consensus sequence of iNOS promoter in a dose-dependent manner.

2.8. MP may interact with sulfhydryl groups of NF-κB subunits to suppress its activity

In order to determine if the sulfhydryl functional group of MP reacted with the cysteine residues in DNA-binding domains of p65 or p50 of NF-κB subunits [27,28], RAW 264.7 cells were treated with 100 μM DTT for 2 h prior to the treatment of MP (100 μM for 2 h) and then exposed to LPS. DTT did not alter the NF-κB-driven transactivation of NF-κB-luc by LPS; instead it reversed the action of MP (data not shown). To confirm if the effect of MP and/or DTT on NF-κB would be reproduced in iNOS promoter-luc system, we constructed iNOS promoter region from −973 to +82 into pGL2-basic luciferase reporter and performed transient transfection followed by luciferase assay as similarly as pNF-κB-luc experiments. As expected, DTT pretreatment also reversed the MP-mediated suppression of iNOS promoter activity (Fig. 7A). It is very possible that DTT treatment maintains the reduced status of NF-κB subunits as an active form. Then MP might oxidize the sulfhydryl group of NF-κB by formation of disulfide bond with MP. To check if the –SH group of MP is a reactive site for NF-κB inactivation, we synthesized DTBP (Fig. 1B), a MP analogue of which two pyrazine moieties from two MPs were connected via disulfide bond. DTBP did not inhibit the

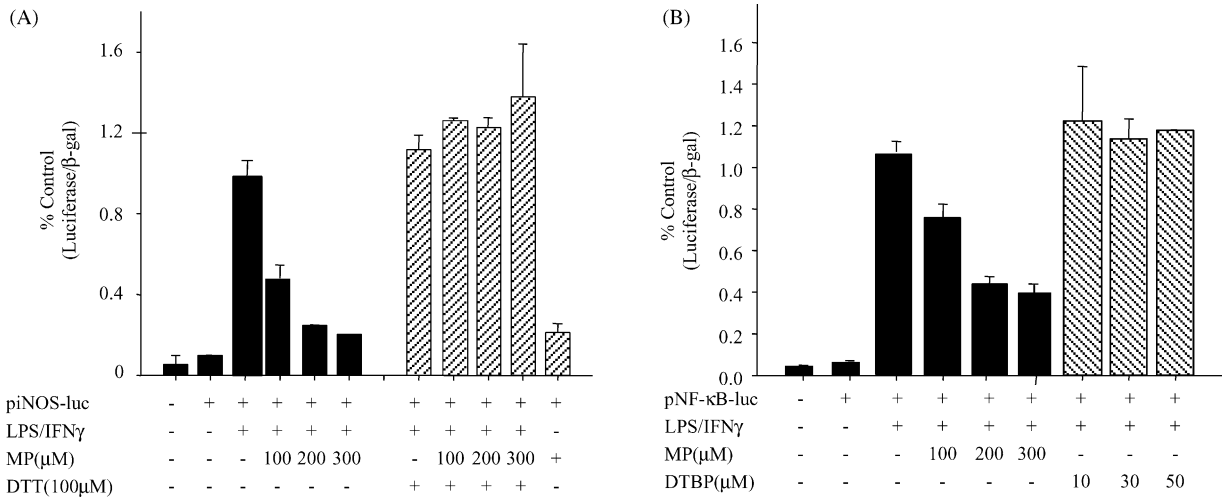

Fig. 7. MP suppresses the iNOS promoter- and NF-κB-driven transactivation via modification of –SH group of NF-κB subunits. RAW264.7 cells were transfected with a reporter gene (A) iNOS-promoter-luc (piNOS-luc), (B) pNF-κB-luc) together with *LacZ* expression vector. The transfected cells were treated with MP or DTBP for 2 h as indicated, stimulated with LPS + IFNγ for 24 h, and harvested for luciferase assay. DTT (100 μM) was pretreated for 2 h prior to MP treatment. Normalized luciferase expressions ($n = 6$) were calculated relative to the *LacZ* expressions and the results were expressed as percent control over the value obtained with the LPS + IFNγ.

NF-κB-driven transactivation by LPS at 50 μM concentration (Fig 7B). This concentration of DTBP is equivalent to 100 μM MP because 100 μM MP can be produced from reduction of 50 μM DTBP. It should be noted that DS-MS was toxic to the cells at higher than 50 μM. It was concluded that MP inhibited the LPS-induced expression of iNOS and/or NF-κB activation in macrophages by suppression of NF-κB binding to DNA (Fig. 6D). MP might achieve the suppression via modification of the –SH groups of NF-κB subunits.

3. Discussion

In this study we demonstrated that MP, a chemically synthesized sulfhydryl group-containing small molecule, inhibited the NO production by suppression of iNOS protein expression in vivo and in vitro. MP blocked the iNOS transcription by interfering with NF-κB binding to the iNOS promoter DNA in a dose-dependent manner. MP did not affect the signaling pathway to the nuclear translocation of NF-κB, which was distinct from other known inhibitors.

The promoter of iNOS contains two consensus sequences for the binding of NF-κB, which mediates LPS-inducibility [4]. The heterodimer of p65/p50 has been reported as a responsible NF-κB complex for LPS-induced iNOS expression. When the activated NF-κB binds to NF-κB-responsive elements on promoters of target genes in nuclei, –SH functional group in DNA binding domains of p50 or p65 seemed to be in reduced state. Accumulating reports support that the cellular redox status can modify the function of NF-κB [29,30]. Oxidative, nitrosative, and –SH modifying agents such as iodoacetamide or *N*-ethylmaleimide can inhibit the DNA binding activity of NF-κB. Pineda-Molina et al. [31] actually reported that *S*-glutathionylation or sulfenic acid formation of cysteine residue 62 of the NF-κB p50 subunit inhibited the DNA binding using MALDI-TOF mass spectroscopy. The report strongly suggests that modification of –SH groups of NF-κB subunits may be a powerful tool for interfering NF-κB.

Several similar situations like NF-κB were also reported recently. Cysteine sulfinic acid formation in active site of peroxiredoxin by oxidation totally blocked its peroxidase activity [32]. Oxidation of a cysteine residue in phosphotyrosine phosphatase 1B (PTP1B) active site could irreversibly inactivate the enzyme activity [33]. The reduced and oxidized states of cysteines on the active (or binding) site of the proteins may provide an important cyclic (possibly reversible) modification/regulation strategy to –SH containing proteins. By this reason, NF-κB could act as a sensor for redox status in cells. Although MP might affect the interaction of –SH of NF-κB subunit with DNA, it is not clear if MP directly modifies the –SH of NF-κB subunits, or if it alters the intracellular redox indicator like glutathiones (GSH/GSSG ratio) or peroxiredoxins to change DNA binding. We have to investigate further by monitoring the direct interaction between MP and NF-κB.

There are several ways to inhibit the NF-κB-dependent gene transcription. One of them is to inhibit the signaling pathways leading to I-κB degradation. The oxygen radical scavenger PDTC blocks the release of I-κB from NF-κB. Requirement of zinc ion for PDTC-induced NF-κB inactivation implies that zinc may be an important regulator for NF-κB activity [34]. In our study, however, MP inhibited NF-κB activity even under serum-free condition, suggesting that zinc ion is not required for DNA binding activity. Anti-inflammatory drugs like salicylates inhibited I-κBα phosphorylation and degradation [35] and glucocorticoid dexamethasone increased the expression of I-κBα, followed by repressing the nuclear NF-κB p65 level [36]. Recently, Kitazawa et al. [37] reported that a cystine derivative, *N*,*N*'-diacetyl-L-cystine demethylester prevented the activated NF-κB from binding DNA, without influencing the I-κB degradation. This cystine derivative enhanced the intracellular GSSG sufficiently enough to shift the redox balance to the oxidized state. But DTBP in our study did not suppress the NF-κB binding to DNA although its structure is similar to the cystine derivative in the aspect that two MP molecules are connected via disulfide bond. Thus, the inhibitory mechanism of MP may be different from the cystine derivative. On the other hand, avicins, a family of triterpenoid saponins were reported to inhibit the activation of NF-κB by blocking both its localization and DNA binding ability [38]. Like MP, the treatment of DTT reversed the avicin-induced NF-κB activation. Since avicin does not contain sulfhydryl in its structure, two ester groups of avicin were suggested to react with cysteine of NF-κB.

Excessive proinflammatory cytokine and NO production by activated macrophages play a critical role in septic hypotension and other chronic inflammatory diseases such as atherosclerosis and neurodegenerative disorders. Rosselet et al. [39] reported that selective iNOS inhibition is better than norepinephrine in the treatment of endotoxic shock in rats. Cho et al. [40] showed that *N*-acetyl-*O*-methyldopamine, a neuroprotectant for neurodegenerative diseases, down-regulates the genes involved in macroglial activation including TNF-α, IL-1β, and iNOS. These reports support an idea that repression of the proinflammatory cytokines and iNOS gene expression in macrophages or macrophage-like cells may provide new therapeutic or chemopreventive strategies for chronic degenerative diseases or endotoxin-induced septic shock.

One of the major challenges of chemoprevention is the development of new effective drugs that have little or no effect on normal cells and tissues. From our in vivo study, we demonstrated the pretreatment of MP significantly reduced the LPS-induced mortality in mice. The present result provides a possibility for MP to develop as a new therapeutic or chemopreventive agent against chronic and

acute inflammatory disorders in which iNOS induction involves in their pathogenesis.

Acknowledgments

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