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Methylene Blue Attenuates iNOS Induction Through Suppression of Transcriptional Factor Binding Amid iNOS mRNA Transcription

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ABSTRACT

Inducible nitric oxide synthase (iNOS) critically contributes to the development of endotoxin-mediated inflammation. It can be induced by cytokines or endotoxins via distinct signaling pathways. Lipopolysaccharide (LPS) triggers iNOS expression through activation of the inhibitor of κB-α (IκB-α)-nuclear factor κB (NF-κB) cascade, whereas interferon-γ (IFN-γ) acts primarily through Janus kinase (JAK)-signal transducer and activator of transcription 1 (STAT1). Methylene blue (MB), an agent used clinically to treat numerous ailments, has been shown to reduce NO accumulation through suppression of iNOS activity. But it remains unclear whether MB affects iNOS induction. This knowledge gap is addressed in the present study using cultured cells and endotoxemic mice. With mouse macrophages, MB treatment prevented the LPS- and/or IFN-γ-stimulated iNOS protein expression. Real-time PCR experiments showed that iNOS mRNA transcription was robustly blocked by MB treatment. The inhibitory effect of MB on iNOS expression was confirmed in vivo in endotoxemic mice. Further analysis showed that MB had no significant effect on IκB-α degradation and NF-κB or STAT1 phosphorylation in LPS/IFN-γ-stimulated cells. The nuclear transport of active NF-κB or STAT1 was also not affected by MB treatment. But MB treatment markedly reduced the binding of NF-κB and STAT1 to their DNA elements. Chromatin immunoprecipitation assays confirmed that MB reduced NF-κB and STAT1 bindings to iNOS promoter inside the cell. These studies show that MB attenuates transcriptional factor binding amid iNOS mRNA transcription, providing further insight into the molecular mechanism of MB in disease therapy. J. Cell. Biochem. 116: 1730–1740, 2015.

KEY WORDS: METHYLENE BLUE; INDUCIBLE NITRIC OXIDE SYNTHASE; INHIBITOR OF κB-α; NUCLEAR FACTOR κB; SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 1; LIPOPOLYSACCHARIDE

Nitric oxide (NO) is an important signaling molecule in cells. Its first identified role was vasodilation [Moncada et al., 1991]. Subsequent studies have shown that NO participates in the transmission of neuronal and cardiovascular signals [Chow et al., 2012; Haldar and Stamler, 2013]. This evidence established NO as a fundamental signaling molecule in physiological conditions. In addition to the signaling role, NO also participates in host defense and inflammatory response through killing bacteria, tumor cells, and viruses [Frese et al., 2002; Bryan et al., 2009; Cardnell and Mikkelsen, 2011; Esquivel-Solís et al., 2013]. On the other hand, excessive NO induces self-damage in a diversity of inflammatory disorders. For example, accumulated NO has been shown to damage mitochondrial functions and induce cellular apoptosis [Dodson et al., 2013; Rocha et al., 2014]. High levels of NO cause cell injury and perpetual hypotension in severe sepsis [Su et al., 2007]. In addition, the constant production of NO renders the vasculature refractory to typical therapies for septic shock such as epinephrine treatment and volume supplementation [Su et al., 2010]. Thus, suppression of NO formation would be beneficial to improve vascular sensitivity to vasoconstrictors.

NO is synthesized by a family of NO synthases (NOS) consisting of three isoforms, including inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS) [O′Connor and O′Brien, 2009]. Unlike the constitutively expressed eNOS and nNOS, little iNOS is

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detected in quiescent cells. But in activated cells, iNOS protein can be markedly induced by various stimuli such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ) [Kleinert et al., 2004]. LPS binds with Toll-like receptors leading to iNOS gene transcription through the classical inhibitor of κB kinase (IKK)-inhibitor of κB α (IκB-α)-nuclear factor κB (NF-κB) signaling pathway [Hayden and Ghosh, 2004]. In this signaling pathway, IκB-α is degraded by the ubiquitin-proteasome system. Then the free and active NF-κB is transferred into the nucleus, where it initiates iNOS gene transcription. Distinct from the mechanism of LPS, IFN-γ triggers iNOS gene expression primarily through the Janus kinase (JAK)-signal transducer and activator of transcription 1 (STAT1) signaling pathway [Dell'Albani et al., 2001; Kleinert et al., 2004]. IFN-γ binds its receptor leading to JAK activation. JAK phosphorylates and activates transcriptional factor STAT1, which then initiates iNOS gene transcription by binding to the GAS site of the iNOS promoter [Levy and Darnell, 2002; Nathan, 1997; Ganster et al., 2005]. Regulation of NO action may be accomplished by control of iNOS induction.

Methylene blue (MB) is a diamino phenothiazine that has been in clinical use for approximately 100 years to treat a variety of ailments [Donati and Preiser, 2006]. For example, it is used to treat malaria [Krafts et al., 2012] and congenital-induced methemoglobinemia [Hall et al., 1986]. Protection of neurons from ischemic-reperfusion injury [Miclescu et al., 2010] and enhancement of β-oxidation of long-chain fatty acids [Visarius et al., 1997] have also been observed. Administering MB in vivo appears to improve central nervous system dysfunction [Sontag et al., 2012]. Further, MB has the ability to restore normal arterial pressure in rabbits or rats with severe sepsis [Keaney et al., 1994; Jeroukhimov et al., 2001] and increase mean arterial pressure (MAP) in a prospective trial that included 20 patients with septic shock [Park et al., 2005]. Thus, it is considered to be an effective agent for the therapy of septic shock. As for the therapeutic mechanism of action of MB in sepsis, previous studies showed that it may be associated with the attenuation of NO signals. This possibility is supported by the following evidence: (1) MB blocks the signal transduction of NO through deactivating soluble guanylyl cyclase (sGC), a cGMP-producing enzyme that can be triggered by NO in vitro and in vivo [Wang et al., 1995; Sobey and Faraci, 1997]; (2) MB attenuates endotoxin-induced NO production and hypotension through direct inhibition of iNOS enzymatic activity [Lomniczi et al., 2000]. In the present study, the effect of MB on NO signals through regulation of iNOS protein expression was investigated. Results demonstrate that MB prevents iNOS expression and NO production through attenuation of iNOS gene transcriptional factor function in both cultured cells and endotoxemic mice. This finding may provide further insight into the molecular mechanism of MB in disease therapy.

MATERIALS AND METHODS

MATERIALS

MB was purchased from Calbiochem (San Diego, CA). LPS was the product of Sigma (St. Louis, MO). IFN-γ was purchased from PeproTech (Rocky Hill, NJ). Antibodies against Histone H2A, glyceral-dehyde-3-phosphate dehydrogenase (GAPDH), iNOS, IκB-α, p-NF-κB p65 (Ser536), NF-κB p65, p-STAT1 (Tyr701), and STAT1 were purchased from Cell Signaling Technology (Beverly, MA). Protein A/G PLUS-agarose was the product of Santa Cruz Biotechnology (Santa Cruz, CA). Other related agents were purchased from commercial suppliers. MB was dissolved in di-H_2O. Prepared MB stock solutions were protected from light and stored at −20°C.

ANIMALS AND EXPERIMENTAL PROTOCOL

The use of C57BL/6 male mice was approved by the University Animal Ethics Committee of Nantong University (Permit Number: 2110836). Six to eight weeks old mice were randomly divided into four groups. 25 mg/kg body weight (BW) of LPS was selected to induce inflammation according to previous studies. In saline and LPS-treated groups, mice were injected intraperitoneally with 100 μl of saline and 25 mg/kg LPS in 100 μl of saline. In MB alone-treated groups, mice were administered with a single dose of MB 5 mg/kg. In MB and LPS-co-treated groups, mice were administered with a single dose of MB 5 mg/kg 1 h before LPS injection. After that, the liver, lung, and heart were immediately excised and each portion was snap-frozen in liquid nitrogen and stored at −80°C. Additional portions of these organs were stored in RNA stabilization reagent, RNAlater (Qiagen GmbH, Hilden, Germany), for RNA extraction.

CELL CULTURE

RAW 264.7 cells were purchased from ATCC (Manassas, VA) and grown in DMEM/F12 with 10% fetal bovine serum (FBS) (Gibco). The medium was replaced every 3 days. All cells were grown in 37°C incubator containing 95% air and 5% CO_2. After treatment, cell supernatants were collected and frozen at −80°C for NO detection. Peritoneal macrophages were harvested 4 days after intraperitoneal injection of 3% thioglycollate (Sigma). Macrophages were washed and plated in 24-well plates at 0.5×10^6 cells per well. After incubation for 2 h at 37°C, the wells were washed three times to remove all non-adherent cells. Finally, the culture medium was replaced with RPMI supplemented with 10% FBS, sodium pyruvate, non-essential amino acid, penicillin G (100 IU/ml), and streptomycin (100 μg/ml).

CELL VIABILITY ASSAY

Cell viability was measured using MTT Cell Proliferation and Cytotoxicity Assay Kit (Bi Yuntian Biological Technology Institution, Shanghai, China). Briefly, methylthiazolyldiphenyl tetrazolium bromide (5 mg/ml) was dissolved in prepared MTT-dissolved solutions and kept at −20°C. After washing with PBS, the cells in plates were added 20 μl of MTT solutions and kept at 37°C for 4 h. The blue crystals were dissolved in formazan-dissolved solutions. The absorbance was read at 570 nm.

NO AND CYTOKINE DETECTION

Total nitrite levels were measured with a Griess reagent kit (Invitrogen). The reaction consisted of 20 μl of Griess Reagent, 150 μl of serum or cell supernatants, and 130 μl of de-ionized water. After incubation of the mixture for 30 min at room temperature, nitrite levels were measured at 548 nm using an M2 spectrophotometric microplate reader (Molecular Devices). TNF-α levels were determined

using cytokine specific BD OptEIA enzyme-linked immunosorbent assay kits (BD Biosciences Pharmingen, San Diego, CA).

REAL-TIME PCR

At the end of each treatment, total RNA was isolated from cells or tissues using the RNeasy mini kit according to the manufacturer's instructions (Qiagen, GmbH). First-strand cDNA was generated by reverse transcription of total RNA using the RT system (Promega, Madison, WI). Real-time PCR reactions were conducted with Faststart SYBR Green Master Mix (Roche Molecular Biochemicals) according to our previous studies [Huang et al., 2013a]. Briefly, 2 μl of diluted cDNA, 0.5 μM primers, 2 μM $MgCl_2$, and 1× FastStart SYBR Green Master mix were employed. The primers were quoted as follows: iNOS 5′-CTC ACT GGG ACA GCACAG AA-3′ (F), 5′-TGG TCA AAC TCT TGG GGT TC-3′ (R); 18S rRNA 5′-GTA ACC CGT TGA ACC CCA TT-3′ (F), 5′-CCA TCC AAT CGG TAG TAG CG-3′ (R). PCR products were detected by monitoring the fluorescence increase of double-stranded DNA-binding dye SYBR Green during amplification. The expression levels of target genes were normalized to the house-keeping gene (18S rRNA). The fold-changes in the target gene expression between experimental groups were expressed as a ratio. Relative gene expression was calculated by the comparative cycle threshold (Ct) method. Melt-curve analysis and agarose gel electrophoresis were used to examine the authenticity of the PCR products.

NF-κB AND STAT1 BINDING ASSAYS

The nuclei were extracted from cells by firstly incubating them in hypotonic buffer (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2{\cdot}6H_2O$) at 4°C for 20 min. After homogenization, cell homogenates were spun at 3,000*g* for 5 min. The supernatants were collected for Western blot. The pellets were recovered, extensively washed, and re-suspended in the nuclear extraction buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate, protease inhibitors). The NF-κB and STAT1 binding activity of nuclear extracts were measured with the TransFactor NF-κB colorimetric kit (Clontech, Mountain View) and the DuoSet mouse active STAT1 binding kit (RD Systems, Minneapolis) respectively according to the manufacturer's instruction.

WESTERN BLOT

To extract the total proteins, tissues or cells were lysed on ice for 30 min in lyses buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 20 mM NaF, 3 mM Na_3VO_4, 1 mM PMSF, with 1% (v/v) NP-40, and protease inhibitor cocktail) [Huang et al., 2013b]. The lysates were centrifuged at 12,000*g* for 15 min, and the supernatants were recovered. After denaturation, different proteins were separated on 10% SDS/PAGE gels and then transferred to nitrocellulose membranes by using a transfer cell system (Bio-Rad, CA). After being blocked with 5% non-fat dried milk powder/Tris-buffered saline Tween-20 for 1 h, membranes were probed with 1:500 primary antibodies against iNOS, IκB-α, p-NF-κB, NF-κB, p-STAT-1, STAT-1, and Histone H2A or 1:10,000 primary antibody against GAPDH overnight at 4°C. Primary antibodies were then removed by washing the membranes three times in TBST, and incubated for further 2 h at room temperature with IRDye 680-labled secondary antibodies (1:3,000–1:5,000). Immunoblots were visualized by scanning using Odyssey CLx Western blot detection system. The Nuclear Protein Extraction Kit was used to separate proteins in the cytoplasm and nucleus according to supplier's recommendations (Bi Yuntian Biological Technology Institution, Shanghai, China). Separated proteins were normalized to GAPDH or Histone-H2A. The band density was quantified using Image J software.

CHROMATIN IMMUNOPRECIPITATION (ChIP)

The ChIP experiment was performed as described previously [Luo et al., 2011]. RAW 264.7 cells were treated with LPS (1 μg/ml) or IFN-γ (20 ng/ml) for 1 h in the absence or presence of MB. One percent of formaldehyde was added to the culture medium, and after incubation on the rocker for 10 min at room temperature, cells were rinsed twice with PBS and lysed for 15 min at 4°C. After sonication, the lysate was used as DNA input control. The remaining lysates were diluted 10-fold with ChIP dilution buffer followed by incubation with NF-κB p65 or p-STAT-1 antibody overnight at 4°C. Immumoprecipitated complexes were collected using protein A/G Plus-agarose beads. The precipitates were extensively washed and then incubated in the elution buffer (1% SDS and 0.1 M $NaHCO_3$) at room temperature for 15 min. Cross-linking of protein-DNA complex was reversed at 65°C for 4 h. DNA was extracted with the Qiagen PCR purification kit. ChIP assays addressing NF-κB used the PCR primers 5′-CAAGCCAGGGTATGTGGTTT-3′ (F) and 5′-GCAGCAGCCATCAGGTATTT-3′ (R). ChIP assays for activated STAT1 binding to its IFN-γ-regulated transcription factor STAT1 (GAS) site on the iNOS promoter used primers 5′-ACACGAGGCTGAGCTGACTT-3′ (F) and 5′-CACACATGGCATGGAATTTT-3′ (R). The resulting products were separated by 2% agarose gel electrophoresis.

STATISTICAL ANALYSIS

Data were expressed as means ± SE. One-way analysis of variance (ANOVA) followed by subsequent post hoc analysis was used for the statistical analysis by employing SPSS 11.0 software. Differences were considered significant at $P < 0.05$ or $P < 0.01$.

RESULTS

MB SUPPRESSES iNOS INDUCTION IN LPS-STIMULATED MACROPHAGES

To determine the role and effective period of MB on iNOS induction, dose- and time-dependent effects of MB on iNOS protein expression in mouse macrophages (RAW 264.7 cells) were investigated. Cells were pretreated with MB for 30 min at concentrations ranging between 0.1 and 1 μM when 1 μg/ml of LPS and 20 ng/ml of IFN-γ (24 h) were applied to induce iNOS protein expression. As shown in Figure 1A, MB significantly reduced the increase in iNOS protein expression in LPS/IFN-γ-stimulated cells. Peak inhibition was observed at the concentration of 1 μM. For this reason, 1 μM of MB was selected for the following experiments. A time-dependent response curve showed that pretreatment of cells with MB (1 μM, 30 min) almost completely blocked iNOS protein expression at 8, 16, and 24 h time points (Fig. 1B). Cell viability of macrophages was not affected by MB administration at 0.1–5 μM (Fig. 1C). Parallel to the

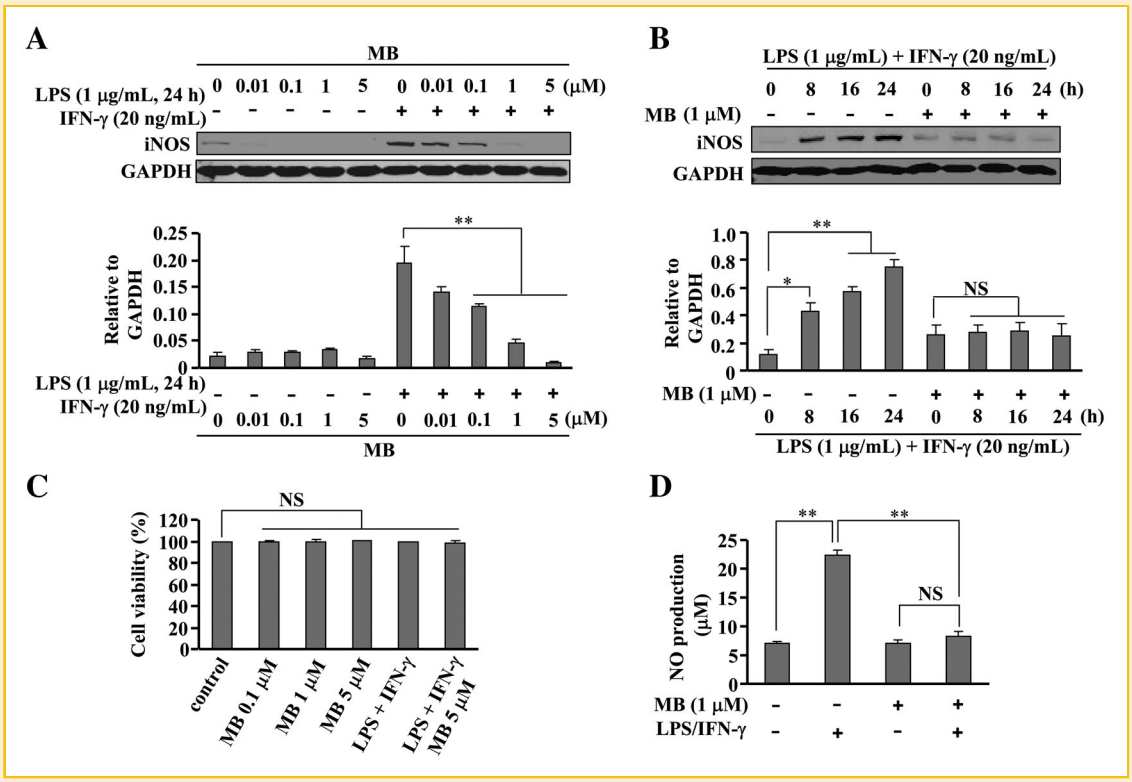

Fig. 1. MB functionally attenuates iNOS induction in cells exposed to LPS/IFN-γ. (A) Upper: Representative images showing that MB pretreatment (30 min) inhibited iNOS protein expression in cells stimulated by LPS/IFN-γ; Lower: quantitative analysis of iNOS protein expression in cells upon MB and/or LPS/IFN-γ treatment (n = 3, $^{**}P < 0.01$ vs. LPS/IFN-γ alone-treated group). (B) Upper: Representative images showing the time-dependent (8, 16, 24 h) effect of MB on LPS/IFN-γ-induced iNOS protein expression; Lower: a time-course analysis of iNOS protein expression upon MB incubation (n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control). C: Quantitative analysis of the cell viability of macrophages after MB and/or LPS/IFN-γ treatment (n = 8). D: Quantitative analysis of NO content in MB- and/or LPS/IFN-γ-treated cells (n = 3, $^{**}P < 0.01$ vs. control or LPS/IFN-γ alone-treated group). All data were shown as mean ± SE. NS, no significance.

effect of MB on iNOS protein, the level of NO released in the medium was also significantly reduced in MB-treated cells (Fig. 1D).

LPS and IFN-γ each can stimulate iNOS gene expression through distinct signaling pathways [Dell'Albani et al., 2001; Hayden and Ghosh, 2004; Kleinert et al., 2004]. Thus, the role of MB in iNOS protein expression induced by LPS and IFN-γ were each examined. As shown in Figure 2, MB treatment (1 μM) robustly reduced the LPS-induced increase in iNOS protein expression in both RAW 264.7 cells (Fig. 2A,B) and primary macrophages (Fig. 2C,D). The absence of iNOS protein could be due to the deficiency of either gene transcription or protein translation. To distinguish these possibilities, iNOS mRNA formation was measured in the absence or presence of MB using real-time PCR. As shown in Figure 2E, pretreatment of primary macrophages with MB (1 μM, 30 min) significantly reduced iNOS mRNA formation. These data demonstrate that MB blocks the LPS-induced iNOS gene transcription in mouse macrophages.

In RAW 264.7 cells and primary macrophages stimulated by IFN-γ, iNOS protein expression was also abolished by MB treatment (1 μM) (Fig. 3A–D). Real-time PCR data showed that iNOS mRNA formation was markedly reduced by MB in IFN-γ-stimulated primary macrophages (Fig. 3E). Hence, MB also prevents IFN-γ-stimulated iNOS gene transcription in mouse macrophages.

MB SUPPRESSES iNOS EXPRESSION IN ENDOTOXEMIC MICE IN VIVO

The above studies revealed the inhibitory role of MB in iNOS protein and gene expression in cultured cells under inflammatory stimuli. To establish the relevance of these biological findings in disease, the effect of MB on iNOS expression in vivo was investigated in endotoxemic mice. While no iNOS was detectable in the mice in the sham group, injecting LPS into mice induced iNOS protein and mRNA expression in vital organs such as lung (Fig. 4A–C), liver (Fig. 4D–F), and heart (Fig. 4G–I). Consistent with the results observed in cultured cells, pretreating mice with MB markedly prevented iNOS protein and mRNA expression in endotoxemic mice (Fig. 4). These results showed that MB attenuates iNOS induction in endotoxemic mice in vivo.

MB DOES NOT SUPPRESS IκB-α-NF-κB SIGNALS IN LPS-STIMULATED CELLS

To explore the mechanism underlying the effect of MB treatment on iNOS gene transcription, the effect of MB on the activation of IκB-α-NF-κB signals was measured in RAW 264.7 cells. As shown in Figure 5A,B, LPS induced a dramatic increase in IκB-α degradation, and MB pretreatment (1 μM, 30 min) did not alter this degradation. It has been reported that the full activation of NF-κB needs an increase in NF-κB phosphorylation. Therefore, the effect of MB treatment on

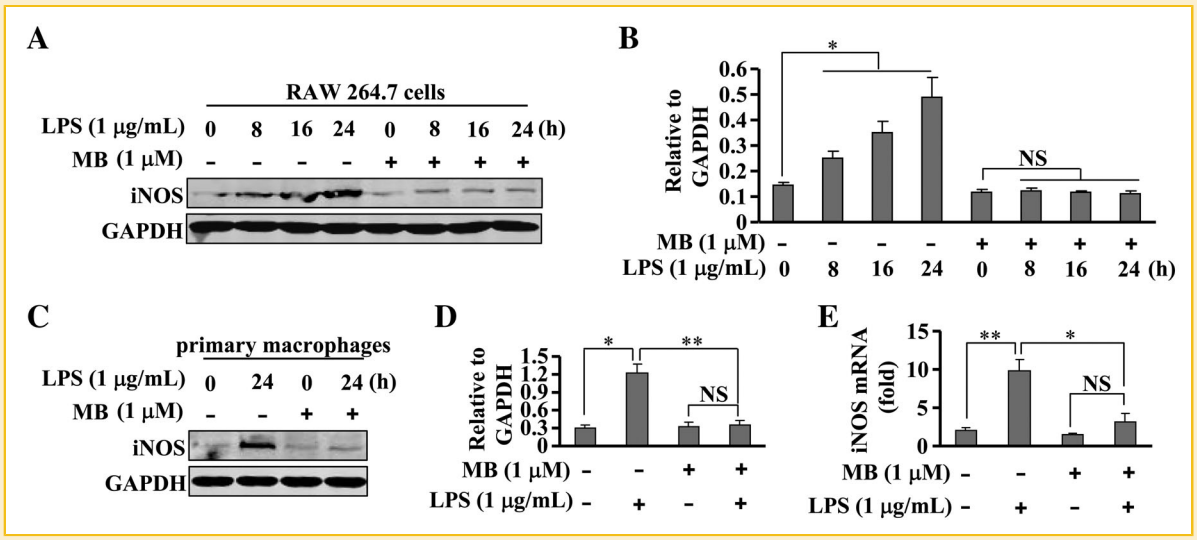

Fig. 2. MB attenuates LPS-induced iNOS expression. A: Effect of MB pretreatment (30 min, 1 μM) on iNOS protein expression in LPS-stimulated RAW 264.7 cells. B: Quantitative analysis of the effect MB on iNOS protein expression in LPS-stimulated cells ($n = 3$, $^{*}P < 0.05$ vs. control). C: Effect of MB pretreatment on iNOS protein expression in LPS-stimulated primary macrophages. D: Quantitative analysis of the effect MB on iNOS protein expression in LPS-stimulated primary macrophages ($n = 3$, $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. LPS alone-treated group). E: Quantitative analysis of the mRNA level of iNOS in primary macrophages upon LPS treatment in the absence or presence of MB ($n = 3$, $^{*}P < 0.05$ vs. LPS alone-treated group, $^{**}P < 0.01$ vs. control). All data were shown as mean ± SE. NS, no significance.

NF-κB phosphorylation was investigated in LPS-stimulated cells. In accordance with the influence of MB on IκB-α degradation, MB treatment did not affect NF-κB phosphorylation in LPS-stimulated cells (Fig. 5A,C). NF-κB must enter nuclei in order to initiate gene transcription. Thus, the change in NF-κB level in the cytoplasm and nucleus of cells stimulated by LPS was analyzed. As shown in Figure 5D–F, NF-κB was present predominantly in the cytoplasm of unstimulated cells. LPS treatment resulted in NF-κB nuclear translocation, and this translocation was not inhibited by MB treatment (Fig. 5D–F).

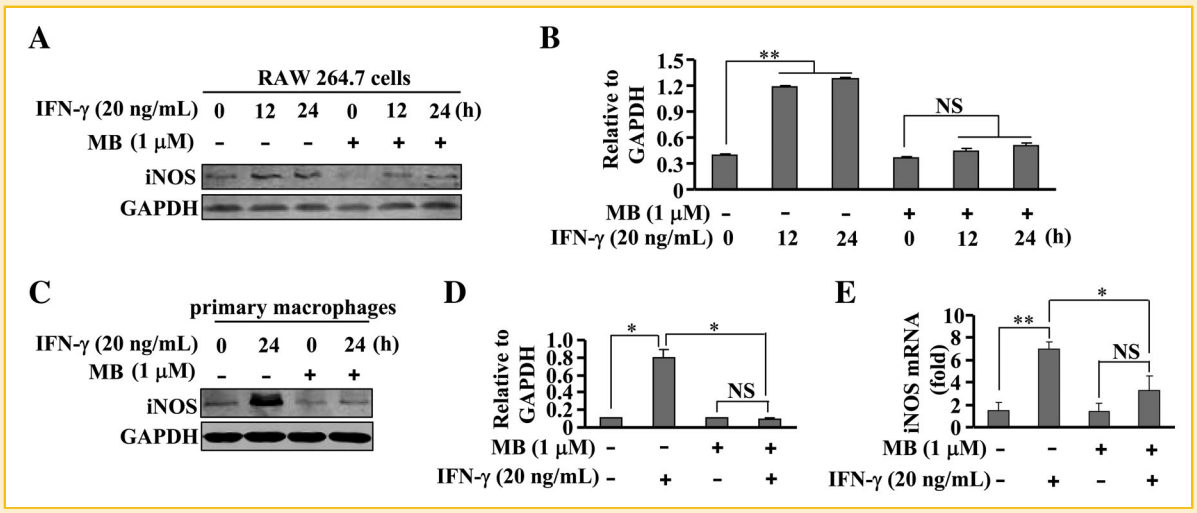

Fig. 3. MB attenuates IFN-γ-induced iNOS expression. A: Effect of MB pretreatment (30 min, 1 μM) on iNOS protein expression in IFN-γ-stimulated RAW 264.7 cells. B: Quantitative analysis of the effect MB on iNOS protein expression in IFN-γ-stimulated cells ($n = 3$, $^{**}P < 0.01$ vs. control). C: Effect of MB pretreatment on iNOS protein expression in IFN-γ-stimulated primary macrophages. D: Quantitative analysis of the effect MB on iNOS protein expression in IFN-γ-stimulated primary macrophages ($n = 3$, $^{*}P < 0.05$ vs. control or IFN-γ alone-treated group). E: Quantitative analysis of the mRNA level of iNOS in primary macrophages upon IFN-γ treatment in the absence or presence of MB ($n = 3$, $^{*}P < 0.05$ vs. IFN-γ alone-treated group, $^{**}P < 0.01$ vs. control). All data were shown as mean ± SE. NS, no significance.

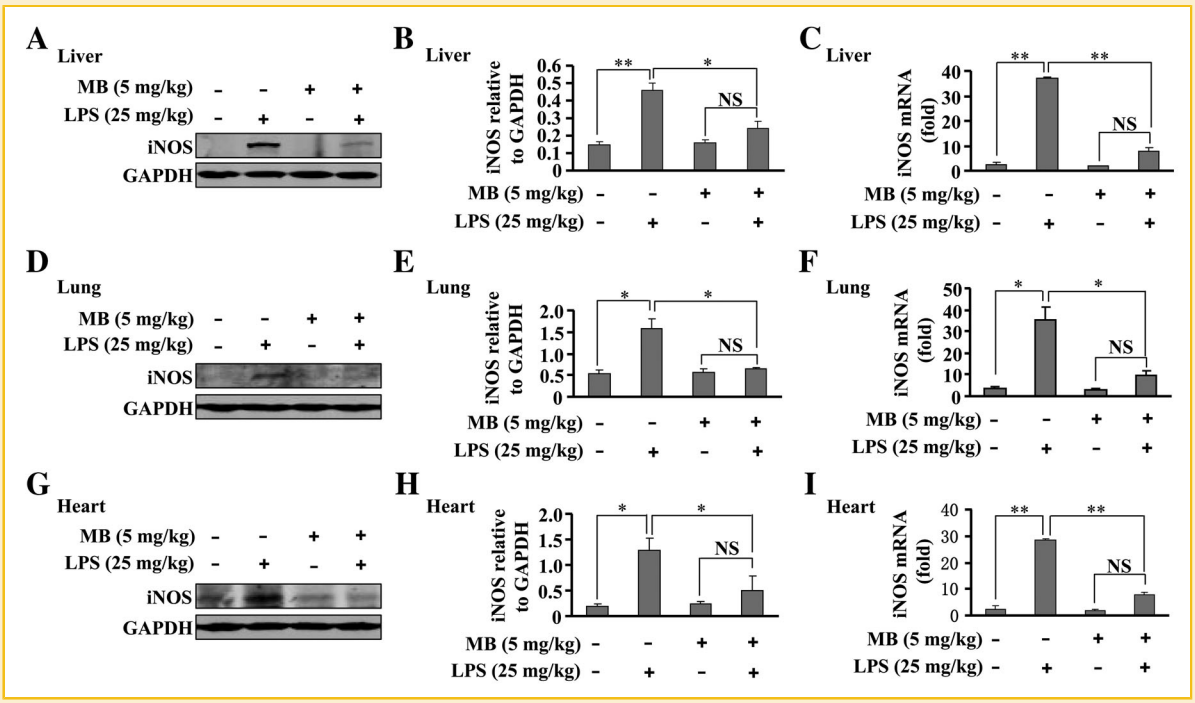

Fig. 4. Effects of MB on iNOS expression in endotoxemia. Mice were divided into sham (saline), endotoxemia (LPS 25 mg/kg), MB alone treatment (MB 5 mg/kg), and LPS/MB treatment (LPS 25 mg/kg and MB 5 mg/kg) groups. Mice were sacrificed 20 h after injections and iNOS protein and mRNA levels were measured in livers (A–C), lungs (D–F), and hearts (G–I). As shown, MB treatment significantly suppressed both iNOS protein and mRNA expressions in all the vital organs of endotoxemic mice ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control or LPS alone-treated group). All data were shown as mean ± SE. NS, no significance.

MB HAD NO SIGNIFICANT EFFECT ON STAT-1 ACTIVATION IN IFN-γ-STIMULATED CELLS

Next, the effect of MB on STAT1 activation in the process of iNOS expression was studied in IFN-γ-stimulated RAW 264.7 cells. As expected, IFN-γ activated STAT1 by triggering the phosphorylation of its Tyr701 residue (Fig. 6A,B). MB treatment affected neither the magnitude, nor the time course of STAT1 activation after IFN-γ treatment. Short-term exposure to MB also had no significant effect on cytosolic STAT1 expression (Fig. 6A,B). These data demonstrated that MB does not block the STAT1 activation process amid IFN-γ-stimulated iNOS expression.

To initiate gene transcription, STAT1 also needs to enter into the nucleus. To determine whether MB treatment affects nuclear transport of STAT1, alterations of the STAT1 level in cytosols and nuclei were monitored in cells stimulated in the absence and presence of MB. As shown in Figure 6C–E, STAT1 resided in the cytosol of resting cells. IFN-γ stimulation resulted in STAT1 nuclear translocation, and this process was not altered by MB (Fig. 6C–E). Thus, MB treatment had no effect on nuclear transport of STAT1 in IFN-γ-stimulated cells.

MB ATTENUATES THE DNA BINDING ACTIVITY OF NF-κB AND STAT1 IN LPS- OR IFN-γ-STIMULATED MOUSE MACROPHAGES

Since MB treatment did not appear to affect IκB-α-NF-κB and JAK-STAT1 signals, the next investigation evaluated the possibility that MB may suppress iNOS transcription by directly interfering with NF-κB and STAT1 binding to their DNA elements in promoters. To explore this possibility, RAW 264.7 cells were stimulated with LPS or IFN-γ to activate NF-κB or STAT1 first. Then, the binding of active NF-κB and STAT1 with labeled DNA oligos corresponding to their promoters was monitored in the absence and presence of MB. As shown in Figure 7A, LPS stimulated a dramatic increase in NF-κB binding activity in nuclei, and this was blocked by MB (1 μM). A similar blocking effect of MB was also seen on STAT1-binding activity in the nucleus of IFN-γ-stimulated cells (Fig. 7B). Since the TNF-α promoter also possesses a binding site for NF-κB, it was determined whether MB interferes with TNF-α production in LPS-stimulated cells. MB induced a significant reduction in TNF-α content in LPS-treated cells (Fig. 7C). These data suggest that MB attenuates the binding of both active NF-κB and STAT1 with the DNA elements in their promoters during iNOS induction. To prove that MB indeed attenuates the binding of active NF-κB and STAT1 to the iNOS promoter, ChIP assays were performed on iNOS promoters in LPS- or IFN-γ-stimulated RAW 264.7 cells in the absence or presence of MB (1 μM). It is known that there is no NF-κB and STAT1 binding to the iNOS promoter in un-stimulated cells. As shown in Figure 7D, LPS-elicited NF-κB binding to the iNOS promoter was robustly blocked by MB treatment. Similarly, the binding of STAT1 to the iNOS promoter was also abrogated by MB treatment in IFN-γ-stimulated cells (Fig. 7E). Taken together, these results indicated that MB inhibits the bindings of active NF-κB and STAT1 to iNOS promoters.

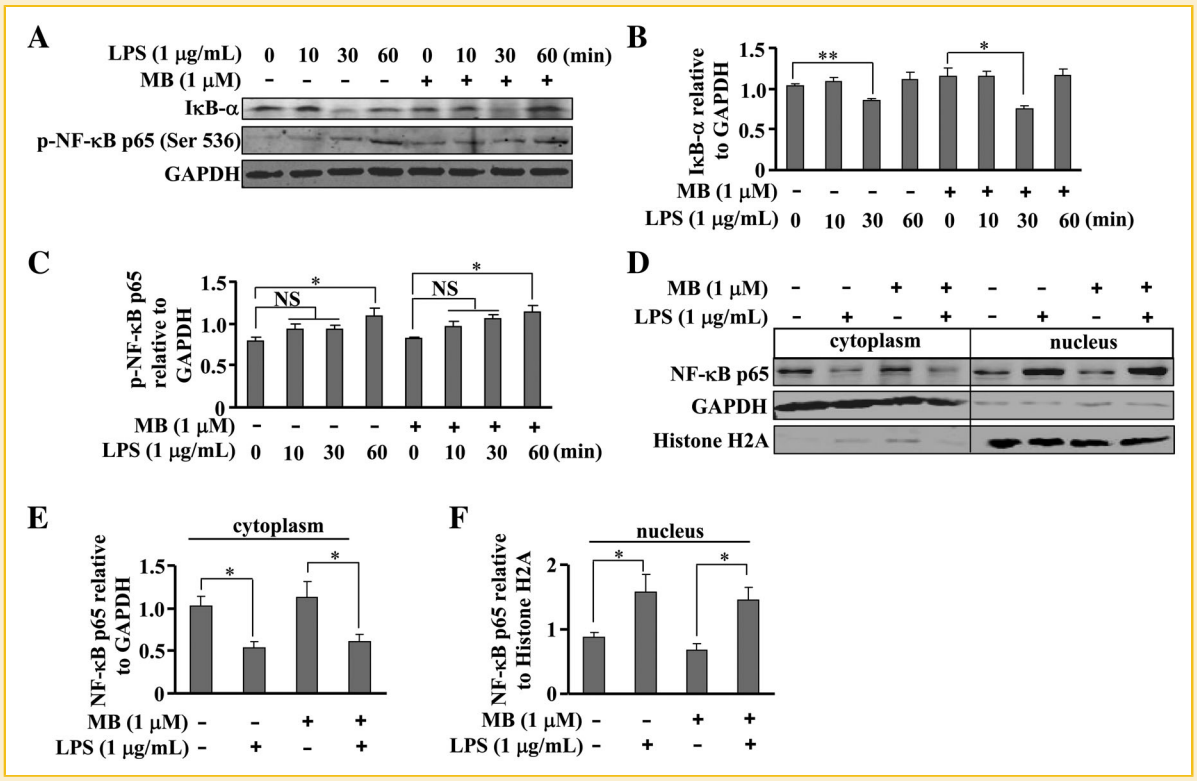

Fig. 5. Effects of MB on LPS-initiated IκB-α degradation, NF-κB phosphorylation, and NF-κB nuclear translocation. A: As shown, LPS-induced increases in IκB-α degradation and NF-κB phosphorylation level were not affected by MB in RAW 264.7 cells (n = 3). B,C: Quantitative analysis of the effects of MB on IκB-α degradation (B) and NF-κB phosphorylation (C) in LPS-treated cells (n = 3, $^{*}P < 0.05$ vs. control or MB alone-treated group, $^{**}P < 0.01$ vs. control). D: Representative images showing the effect of MB on NF-κB nuclear translocation. Cells were stimulated with LPS for 30 min and the levels of NF-κB in the cytoplasm and nucleus were detected. In MB-interfered groups, cells were incubated with MB for 30 min prior to LPS stimulation. E,F: Quantitative analysis of NF-κB expression in the cytoplasm (E) and nucleus (F) after MB administration (n = 3, $^{*}P < 0.05$ vs. control or MB alone-treated group). All data were shown as mean ± SE. NS, no significance.

DISCUSSION

The main finding of this study is the identification of the effect and mechanism of MB on iNOS induction. In traditional applications, MB is used to treat numerous human disorders such as malaria, methemoglobinemia, septic shock, vasoplegic syndrome after cardiac surgery, and cyanide poisoning [Hall et al., 1986; van Heijst et al., 1987; Keaney et al., 1994; Krafts et al., 2012]. Later, MB was found to improve memory consolidation at low doses in animal brains [Miclescu et al., 2010]. MB has also been shown to protect neurons from ischemic or neurodegenerative injuries [Miclescu et al., 2010; Stack et al., 2014]. The initial evidence implicating MB in the regulation of NO signals was from studies on sGC and iNOS activity regulation [Wang et al., 1995; Sobey and Faraci, 1997; Lomniczi et al., 2000]. As a cGMP producing enzyme, sGC can be triggered by NO in vitro and in vivo [Wang et al., 1995; Sobey and Faraci, 1997]. Suppression of sGC activity by MB blocks the transduction of NO signals, resulting in reversal of hypotension in clinical patients with septic shock. Unlike sGC, iNOS is a direct source of NO. Inhibition of iNOS activity by MB attenuates NO production as well as tissue injury and hypotension, in endotoxemic mice [Wang et al., 1995; Rezzani et al., 2001].

Besides iNOS activity, the content of NO is also dependent on the abundance of iNOS protein. Whether MB affects iNOS protein expression remains obscure. In the present study, MB prevented iNOS protein expression in cells stimulated by LPS and IFN-γ. Mechanistic studies indicated that the loss of iNOS protein in MB-treated cells was due to lack of iNOS mRNA transcription. The fact that MB treatment prevented iNOS expression by both LPS and IFN-γ underscored the importance of MB in iNOS induction. Indeed, in vivo animal experiments confirmed the inhibitory role of MB in iNOS expression in LPS-stimulated liver, lung, and heart. Taken together, these data establish an inhibitory role of MB in iNOS induction in both cell culture and animal disease in vivo, providing another explanation for the MB-mediated regulation of NO-associated disorders such as vasoplegic syndrome and septic shock. Exploration of the in-depth mechanism of MB on iNOS expression may be critical to further understand therapeutic and side effects of MB in clinical practice.

Blockade of a gene transcription in stimulated cells is often due to one or multiple interruptions in the signal propagation from the stimuli to the corresponding transcriptional factors [Levy and Darnell, 2002; Hayden and Ghosh, 2004]. However, MB treatment, while blocking iNOS gene transcription, had no significant effect on either IκB-α degradation, NF-κB phosphorylation, and nuclear

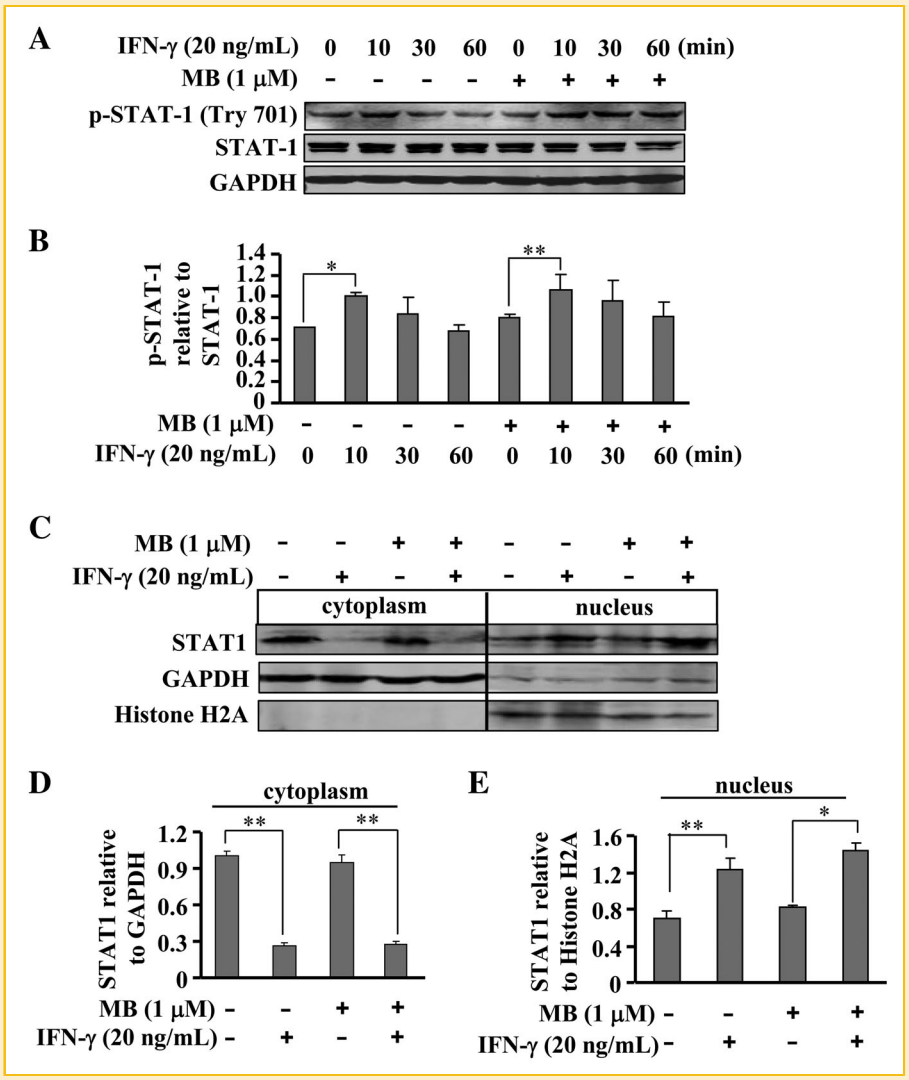

Fig. 6. MB fails to inhibit STAT-1 phosphorylation and nuclear translocation in IFN-γ-stimulated cells. A: As shown, MB treatment did not attenuate the increase in STAT1 phosphorylation level in IFN-γ-stimulated RAW 264.7 cells (n = 3). B: Quantitative analysis of the effect of MB on STAT-1 phosphorylation in IFN-γ-treated cells (n = 3, $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. or MB alone-treated group). C: Representative images showing the effect of MB on STAT-1 nuclear translocation. Cells were stimulated with IFN-γ for 30 min and the levels of STAT-1 in the cytoplasm and nucleus were detected. In MB-interfered groups, cells were incubated with MB for 30 min prior to IFN-γ stimulation. D,E: Quantitative analysis of STAT-1 expression in the cytoplasm (D) and nucleus (E) after MB administration (n = 3, $^{*}P < 0.05$ vs. MB alone-treated group, $^{**}P < 0.01$ vs. control or MB alone-treated group). All data were shown as mean ± SE.

translocation in LPS-stimulated cells, or STAT1 phosphorylation and nuclear translocation in IFN-γ-stimulated cells. These findings suggest that MB may interfere with signal events downstream of the nuclear translocation of NF-κB and STAT1. A significant blockage of the binding of active NF-κB and STAT1 to their DNA elements was observed, as well as the production of TNF-α in MB-treated cells. Further studies with ChIP assay showed that MB robustly prevented active NF-κB and STAT1 from binding to the iNOS promoter. These studies indicate that MB may directly interrupt the binding of NF-κB and STAT1 to the promoters in the onset of iNOS gene transcription. This may provide a plausible explanation to why iNOS gene transcription was halted, despite that activation of IκB-α-NF-κB and STAT1 signals was not perturbed in MB-treated cells. How exactly MB affects the bindings of NF-κB and STAT1 to their DNA elements in the iNOS promoter remains to be determined.

It is worth mentioning that besides sGC and iNOS, MB also targets the other molecules, such as adenosine 5′-monophosphate-activated protein kinase (AMPK) and NF-E2-related factor 2 (Nrf2). Activation of AMPK by MB promotes mitochondrial respiration [Shin et al., 2014] and autophagy processes [Xie et al., 2013]. Since activation of AMPK has also been shown to reduce pro-inflammatory cytokine production in microglia and macrophages [Giri et al., 2004; Meares et al., 2013; O′Neill and Hardie, 2013], it is likely that MB may affect iNOS induction through AMPK activation. However, the reported mechanism of AMPK activation on pro-inflammatory cytokines fails to support this hypothesis. AMPK activation inhibited the

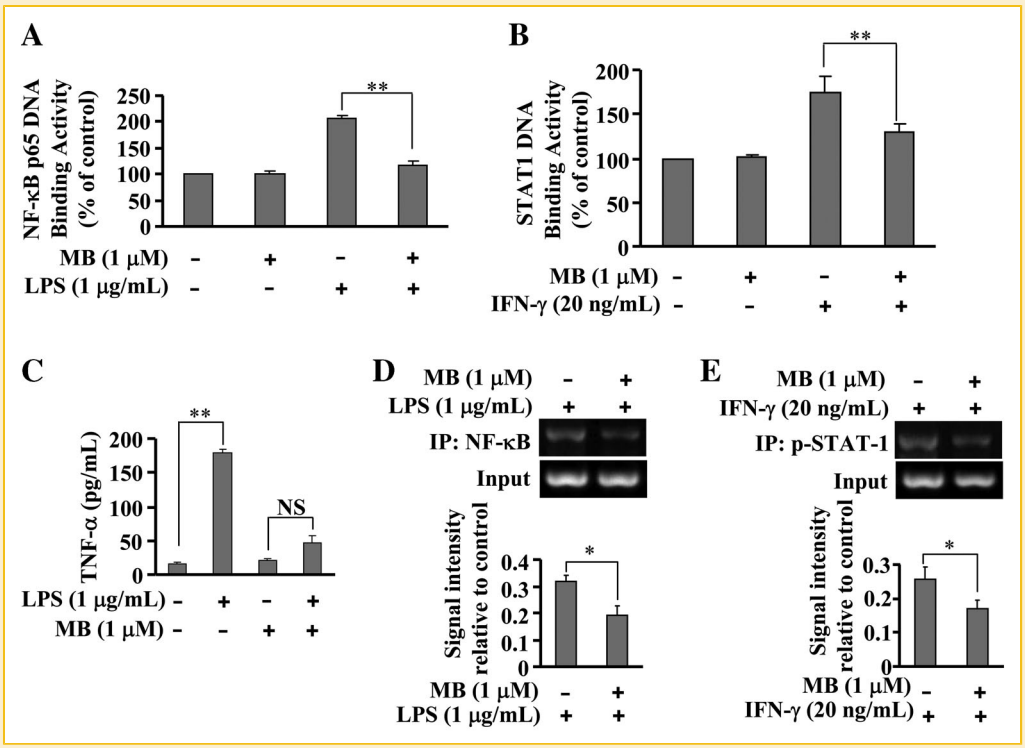

Fig. 7. MB attenuates the binding of active NF-κB and STAT-1 to their DNA elements in iNOS promoters. A: Quantitative analysis of the effect of MB on NF-κB binding to its DNA element. RAW 264.7 cells were stimulated with LPS and the binding activity of NF-κB p65 was measured in the absence or presence of MB ($n = 5$, $^{**}P < 0.01$ vs. LPS alone-treated group). B: Quantitative analysis of the effect of MB on p-STAT-1 binding to its DNA element. Cells were stimulated with IFN-γ and the binding activity of p-STAT-1 was measured in the absence or presence of MB ($n = 5$, $^{**}P < 0.01$ vs. LPS alone-treated group). C: Quantitative analysis of TNF-α content in MB- and/or LPS-treated cells ($n = 6$, $^{**}P < 0.01$ vs. control). (D) Upper: representative ChIP data showing that MB markedly reduced NF-κB binding to the iNOS promoter in LPS-stimulated cells. The chromatin was immunoprecipitated using the anti-p65 antibody; Lower: Quantitative analysis of the effect of MB on the binding of NF-κB to the iNOS promoter in LPS-stimulated cells ($n = 3$, $^{*}P < 0.05$ vs. control). (E) Upper: representative ChIP data showing that MB markedly reduced p-STAT1 binding to the iNOS promoter in IFN-γ-stimulated cells. Lower: Quantitative analysis of the effect of MB on the binding of p-STAT1 to the iNOS promoter in IFN-γ-stimulated cells ($n = 3$, $^{*}P < 0.05$ vs. control). All data were shown as mean ± SE. NS, no significance.

inflammatory response through blockage of IκB-α degradation and NF-κB or STAT1 nuclear translocation [Meares et al., 2013; O'Neill and Hardie, 2013], while MB had no significant effect on either IκB-α-NF-κB activation in LPS-stimulated cells or STAT1 activation in IFN-γ-stimulated cells. Therefore, AMPK does not appear to mediate the effect of MB on iNOS induction, at least in the current experimental systems. Nrf2 is another important anti-inflammatory molecule in the cell [Petri et al., 2012]. Upon stimulation with stress factors, Nrf2 dissociates from its binding protein Keap1, and is free to translocate to the nucleus [Niture et al., 2010]. Nuclear Nrf2 suppresses inflammatory responses through activation of antioxidant response elements (ARE) and induction of anti-oxidants such as quinone oxidoreductase-1 (NOQ1) and heme oxygenase-1 (HO-1) [Petri et al., 2012]. Recently, MB was found to up-regulate Nrf2/HO-1 protein expression in the P301S transgenic mice expressing P301S mutations in the gene encoding the human microtubule-associated protein tau (MAPT) [Stack et al., 2014]. This suggests that Nrf2/HO-1 signals may meditate the function of MB in disease therapies. Intriguingly, the authors observed a robust decrease in iNOS protein expression in MB-treated P301S mice [Stack et al., 2014]. Considering the importance of HO-1 in regulation of iNOS induction, the effect of MB on iNOS induction could be mediated by HO-1. But the following considerations deny this speculation: (1) some previous studies have reported that HO-1 prevents iNOS induction through inhibition of IκB-α degradation and NF-κB nuclear translocation [Ashino et al., 2008; Bang et al., 2010]; (2) direct correlation between the change in HO-1 and iNOS expression in MB-treated P301S mice has not been constructed [Stack et al., 2014]. In general, whether the anti-inflammatory molecule AMPK or Nrf2 mediates the preventative effect of MB on NF-κB and STAT1 binding to their DNA elements in iNOS promoters remains an open question. More studies need to be done to investigate these issues.

In conclusion, our findings identify an inhibitory role of MB in iNOS gene transcription through interruption of NF-κB and STAT1 association with their DNA elements in iNOS promoters in inflammatory settings. This may shed light on a new molecular basis of MB in disease therapy.

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