



4,7-Dimethoxy-5-methyl-1,3-benzodioxole from *Antrodia camphorata* inhibits LPS-induced inflammation via suppression of NF- κ B and induction HO-1 in RAW264.7 cells



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ABSTRACT

Several benzenoid compounds have been isolated from *Antrodia camphorata* are known to have excellent anti-inflammatory activity. In this study, we investigated the anti-inflammatory potential of 4,7-dimethoxy-5-methyl-1,3-benzodioxole (DMB), one of the major benzenoid compounds isolated from the mycelia of *A. camphorata*. DMB significantly decreased the LPS-induced production of pro-inflammatory molecules, such as nitric oxide (NO), interleukin-1 β (IL-1 β), and tumor necrosis factor- α (TNF- α) in RAW264.7 cells. In addition, DMB suppressed the protein levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in a dose dependent manner. Moreover, DMB significantly suppressed LPS-induced nuclear translocation of nuclear factor- κ B (NF- κ B), and this inhibition was found to be associated with decreases in the phosphorylation and degradation of its inhibitor, inhibitory κ B- α (I κ B- α). Moreover, we found that DMB markedly inhibited the protein expression level of Toll-like receptor 4 (TLR4). Furthermore, treatment with DMB significantly increased hemoxygenase-1 (HO-1) expression in RAW264.7 cells, which is further confirmed by hemin, a HO-1 enhancer, significantly attenuated the LPS-induced pro-inflammatory molecules and iNOS and TLR4 protein levels. Taken together, the present study suggests that DMB may have therapeutic potential for the treatment of inflammatory diseases.

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1. Introduction

Macrophages represent a major component of the innate immune system, and play an important role in inflammatory disease. Activated macrophages release pro-inflammatory molecules, such as nitric oxide (NO), prostaglandin E₂ (PGE₂), tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β). These molecules are involved in the pathogenesis of many inflammation-associated human diseases, such as acute or chronic inflammation, sepsis, septic shock, and cancer [1–3]. Therefore, inhibition of these pro-inflammatory molecules may be an effective strategy for the treatment of inflammatory diseases.

Antrodia camphorata is a medicinal mushroom used as traditional Chinese medicine in Taiwan for treating liver diseases, food and drug intoxication, diarrhea, abdominal pain, hypertension, allergies, skin itching, and tumorigenic diseases [4]. Besides, many studies have shown that *A. camphorata* possesses a wide range of biological activities, including anti-cancer, antioxidative, anti-inflammatory, hepatoprotective, and immunomodulatory effects [5–9]. *A. camphorata* is rich in

benzenoids, terpenoids, and polysaccharides [10]. Several studies have indicated that compounds found in *A. camphorata* are effective in relief of symptoms from inflammatory disease [11–13]. However, the other effects of this potentially beneficial compound have not been investigated.

4,7-Dimethoxy-5-methyl-1,3-benzodioxole (DMB) is one of benzenoid compounds isolated from the fruiting bodies and mycelia of *A. camphorata*. Many reports have been shown that benzenoid compounds from *A. camphorata* are excellent anti-inflammatory agents [10,14]. Previous studies have shown that DMB exhibits anti-proliferative, anti-tumor, and anti-inflammatory effects through inhibition of superoxide generation in human neutrophils [15–17]. However, there was no study have been reported to investigate the detail molecular mechanism involved in the anti-inflammatory effect of DMB.

In this study, lipopolysaccharides (LPS)-induced inflammation in RAW264.7 cells *in vitro* was used to investigating the anti-inflammatory effect of DMB. Anti-inflammatory effect of DMB was firstly examined and estimated as its inhibition against the production of NO, TNF- α , and IL-1 β in LPS-induced RAW264.7 cells. The effects of DMB on inflammatory protein expression were also investigated in order to clarify that DMB could control the production of these inflammatory molecules at protein levels.

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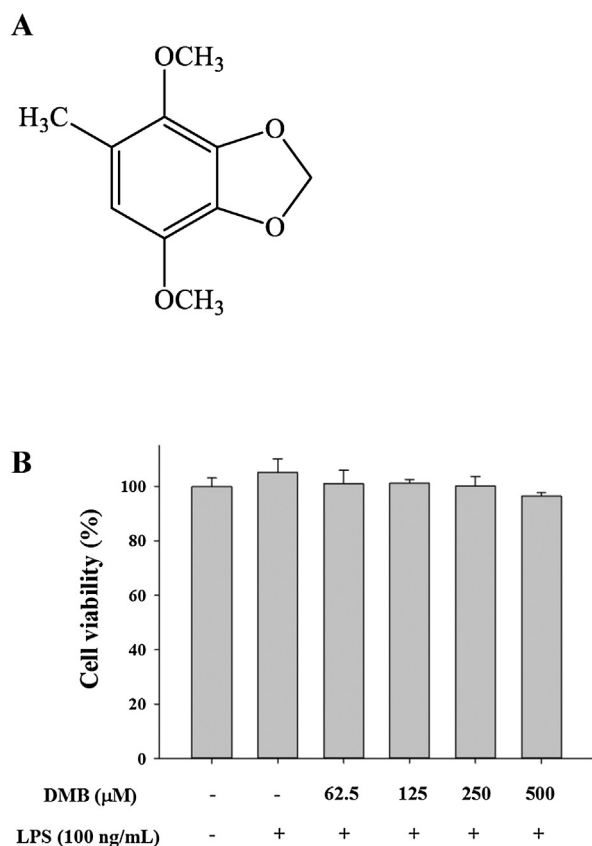


Fig. 1. (A) Chemical structures of 4,7-dimethoxy-5-methyl-1,3-benzodioxole from mycelia of *A. camphorata*; (B) cytotoxicity of DMB in LPS-stimulated RAW264.7 cells. Cells were treated with DMB at 62.5, 125, 250 and 500 μM for 24 h, and cell viability was assayed by the MTT assay. Data are expressed as the means ± S.D. of three independent experiments.

2. Materials and methods

2.1. Chemicals and reagents

Lipopolysaccharide (LPS), methyl thiazolyl tetrazolium (MTT), and hemin were purchased from Sigma Chemical Co. (Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and L-glutamine were purchased from Gibco (Grand Island, NY, USA). Antibody against iNOS, HO-1, IκB-α, p-IκB-α, NF-κB, and β-actin were obtained from Abcam (Cambridge, UK, USA). Antibody against COX-2 was purchased from Millipore (Billerica, MA, USA). Anti-lamin B antibody was purchased from Gene Tex (San Antonio, TX, USA). Antibody against TLR4 was obtained from Cell Signaling Technology (Danvers, MA, USA).

2.2. Isolation of DMB from *A. camphorata*

The mycelia of *A. camphorata* were freeze-dried and powdered. The dried material (5 kg) was extracted with methanol (10 L, four times) then filtered. The filtrate was concentrated by evaporation under reduced pressure to yield the methanol extract (2.8 kg, yield: 56%). The extract was suspended in distilled water and the aqueous suspension was partitioned with *n*-hexane, ethyl acetate, and *n*-butanol, respectively. The ethyl acetate layer was evaporated to dry and the residue was chromatographed on silica gel with *n*-hexane:ethyl acetate (7:1) to elute a crude fraction that included DMB. This fraction was purified by Sephadex LH-20 column using methanol as the eluent to obtain 4.79 g of DMB (Fig. 1A). The structure of DMB was identified by detailed analysis of 1D-NMR spectroscopic data: ^1H NMR (500 MHz, CDCl_3): δ_{H} 2.15 (3H, s, CH_3), 3.82 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 5.91 (2H, s,

$\text{O}-\text{CH}_2-\text{O}$), and 6.28 (1H, s, aromatic proton). ^{13}C NMR (125 MHz, CDCl_3): δ_{C} 15.92 (CH_3), 56.85 (OCH_3), 59.94 (OCH_3), 101.44 ($\text{O}-\text{CH}_2-\text{O}$), 108.72, 123.66, 134.63, 136.51, 138.64, 138.83. EI-MS $[\text{M}]^+$ m/z : 196 calcd. for $\text{C}_{10}\text{H}_{12}\text{O}_4$. The result was confirmed by comparison with the previously published literature [18].

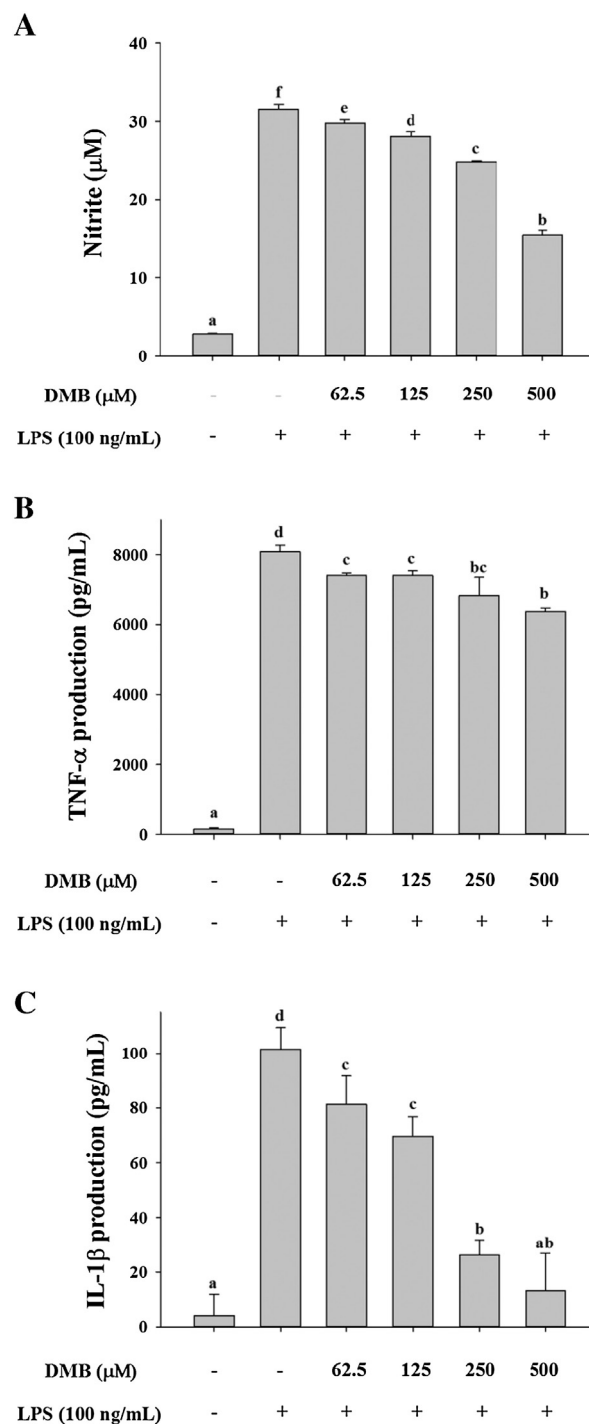


Fig. 2. Effect of DMB on (A) NO, (B) TNF-α, and (C) IL-1β production in LPS-stimulated RAW264.7 cells. Cells were incubated with or without LPS (100 ng/mL) in the presence of various doses (62.5, 125, 250 and 500 μM) of DMB for 24 h. Values are expressed as mean ± S.D. of three replicates. Means with different letters represent significantly different ($p < 0.05$) by Scheffé's test.

2.3. Cell culture

The murine macrophage cell line RAW264.7 (BCRC No. 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM), which contain 4 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and supplemented with 10% fetal bovine serum at 37 °C in a humidified incubator containing 5% CO₂.

2.4. Cell viability assay

RAW264.7 cells (5×10^4 cells/well) were cultured in 96-well plate in DMEM containing 10% FBS for 24 h to become nearly confluent. Then cells were cultured with increasing concentrations of DMB (62.5–500 μM) in the presence of 100 ng/mL LPS for 24 h. After that, cells were incubated with 100 μL of 0.5 mg/mL MTT for 4 h at 37 °C. After incubation, the colored formazan crystals formed in culture plate was dissolved in 0.04 N HCl/isopropanol. The optical densities (OD) were measured at 570 nm using a microplate reader (Molecular Devices, USA). The viability of RAW264.7 cells in each well was presented as compared with percentage of untreated control cells.

2.5. Measurement of nitric oxide

NO production was indirectly assessed by measuring the nitrite levels in the culture media using Griess reagent assay. Briefly, RAW264.7 cells were seeded at a density of 5×10^4 cells/well in 96-well plates for 24 h. After incubation, the cells were treated with DMB (62.5, 125, 250 and 500 μM) in the presence of LPS (100 ng/mL) for 24 h. The culture supernatant was collected for nitrite assay. Each of 100 μL of culture media was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% phosphoric acid) and incubated at room temperature for 5 min, the absorbance was measured at 540 nm with a microplate reader (Molecular Devices). Fresh culture media were used as blanks and the nitrite levels were determined by using a standard curve obtained from sodium nitrite.

2.6. Measurement of TNF-α and IL-1β

Macrophages were seeded at 5×10^4 cells/well in 96-well plates. Cells were incubated with DMB (62.5, 125, 250 and 500 μM) in the presence of LPS (100 ng/mL) for 24 h. Cell culture supernatants were centrifuged at $5000 \times g$ for 3 min at 4 °C to remove insoluble material. Secreted IL-1β and TNF-α were measured in cell culture supernatants

using commercially-available ELISA kits (BioLegend, San Diego, CA) following the instructions provided by the manufacturers. The absorbance (450 nm) for each sample was analyzed using microplate reader and was interpolated with a standard curve. Results of three independent experiments were used for statistical analysis.

2.7. Protein extraction and Western blot analysis

RAW264.7 cells were seeded at a density of 5×10^6 cells/dish in 10 cm dish and then with different concentrations of DMB (62.5, 125, 250 and 500 μM) and 100 ng/mL of LPS for 24 h to measure the protein expression levels. The cells were harvested and lysed by RIPA buffer (Thermo Fisher Scientific, Waltham, MA) for 20 min on ice, and the lysates were centrifuged at $10,000 \times g$ for 15 min at 4 °C. Nuclear and cytosolic extracts of the cells were prepared by Nuclear Extraction Kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA, USA). After quantification of protein concentration, equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly-vinylidene difluoride PVDF membrane (Immobilon, Millipore, Bedford, MA, USA). Membranes were blocked for 1 h at room temperature with 5% nonfat milk in PBS-Tween 20 (0.1%) and incubated with target antibodies overnight at 4 °C. After incubation, the membranes were washed and incubated with the corresponding secondary antibodies for 2 h at room temperature. Blots were developed on membranes using Pierce™ ECL Western Blotting Substrate (Thermo Scientific Hudson, USA). The protein bands were detected and quantified by measuring the relative intensity compared to control, using Kodak Molecular Imaging Software (Version 4.0.5, Eastman Kodak Company, Rochester, NY) and represented in the relative intensities.

2.8. Immunofluorescence staining

RAW264.7 cells (1×10^4 cells/well) were cultured on cover slips in 24-well plate. After DMB treatment, cells were fixed with methanol for 10 min at 4 °C. The cells were treated with 0.1% Triton X-100 for 15 min then blocked with 3% (w/v) bovine serum albumin in PBS for 2 h. The samples were incubated with primary antibody at 4 °C overnight, followed by DyLight™ 488-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA), and diluted (1:300) for 2 h at room temperature. The nucleus was stained with 100 ng/mL of 4',6-diamidino-2-phenylindole (DAPI) for 10 min in the dark. The cell imaging was obtained by confocal laser scanning microscope (Leica, TCS SP2, Germany).

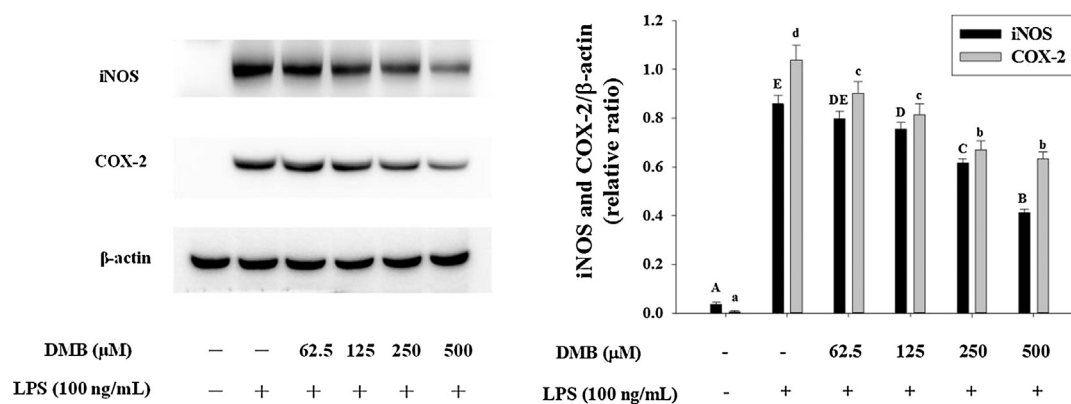


Fig. 3. Effects of DMB on iNOS and COX-2 protein expression in LPS-induced RAW264.7 cells. Cells were incubated with or without LPS (100 ng/mL) in the presence of various concentrations (62.5, 125, 250 and 500 μM) of DMB for 24 h. Values are expressed as mean \pm S.D. of three replicates. Means with different letters represent significantly different ($p < 0.05$) by Scheffé's test.

2.9. Data processing and statistical analysis

All data were expressed as mean ± standard deviation (S.D.) of three replicates. Data were analyzed using SPSS version 20.0 statistical software (IBM Corp., Armonk, NY) via one-way ANOVA followed by Scheffé’s multiple range tests. The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of DMB on cell viability of RAW264.7 cells

Prior to the anti-inflammatory investigation, we first examined the cytotoxic effect of DMB on RAW264.7 cells using the MTT colorimetric assay. As shown in Fig. 1B, treatment of RAW264.7 cells with DMB at 62.5, 125, 250 and 500 μM for 24 h did not affect cell viability compared to control group (absence of LPS and DMB). Therefore, DMB concentrations ranging from 62.5 to 500 μM were selected for subsequent experiments.

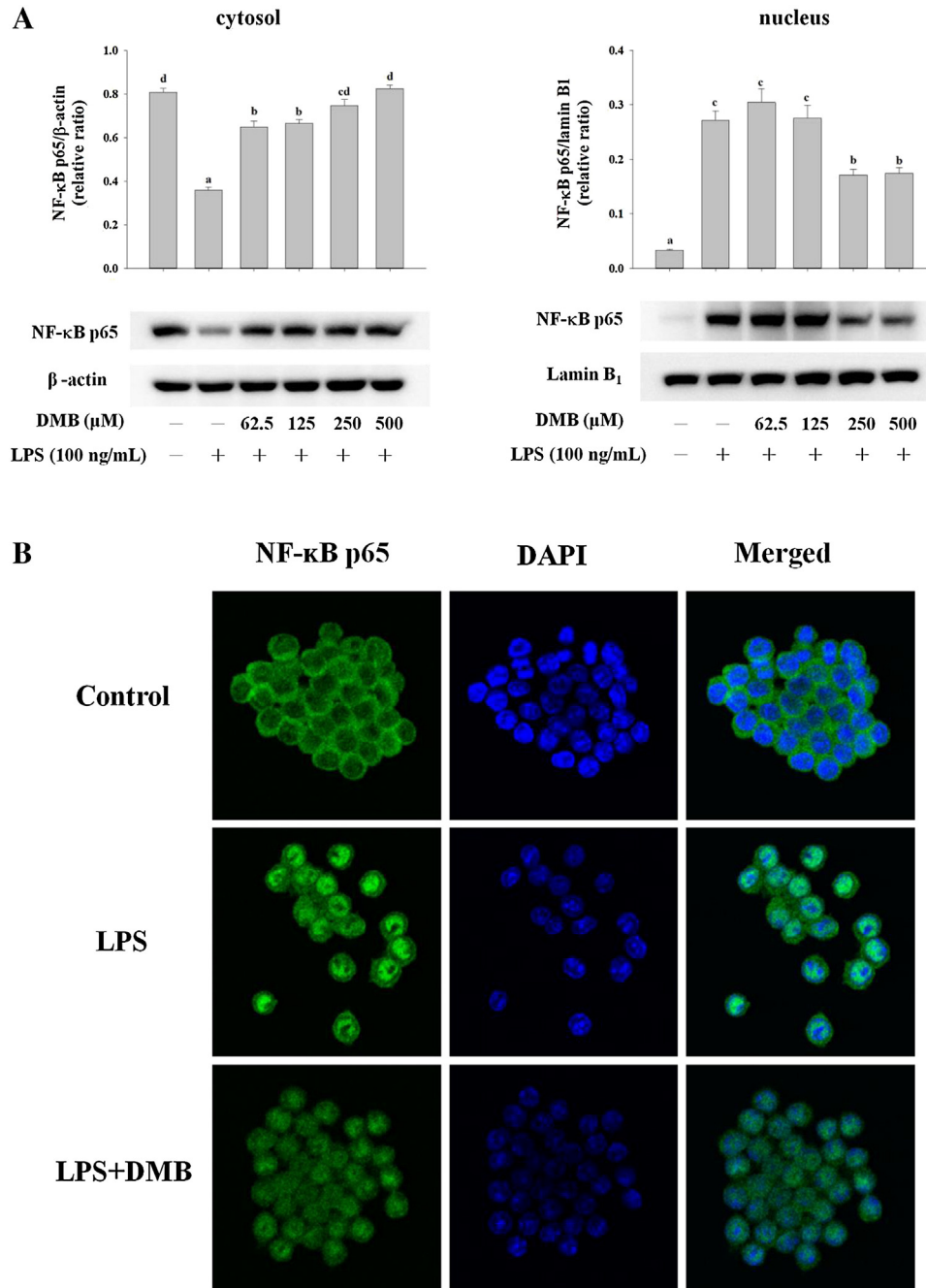


Fig. 4. Effects of DMB on NF- κB p65 nuclear translocation in LPS-induced RAW264.7 cells. (A) RAW264.7 cells were treated with DMB (62.5, 125, 250 and 500 μM) and LPS (100 ng/mL) for 30 min, the protein levels of NF- κB p65 in the cytosol and nucleus were analyzed by Western blot analysis. The values under each lane indicate relative intensity of the band normalized to β -actin (for total protein normalization) or lamin B₁ (for nuclear protein normalization) respectively. Values are expressed as mean ± S.D. of three replicates. Means with different letters represent significantly different ($p < 0.05$) by Scheffé’s test. (B) Immunofluorescence staining of NF- κB . Cells were incubated with or without LPS (100 ng/mL) in the presence of DMB (100 $\mu\text{g}/\text{mL}$) for 30 min. NF- κB p65 was detected by DyLight 488-labeled immunostaining (green); nuclei were stained by DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. DMB inhibits the production of NO, TNF- α , and IL-1 β on LPS-induced RAW264.7 cells

To determine the effect of DMB on NO production, RAW264.7 cells were incubated with LPS (100 ng/mL) in the presence or absence of various concentrations of DMB (62.5, 125, 250 and 500 μ M) for 24 h. Nitrite levels in culture media was determined by Griess reagent assay. As shown in Fig. 2A, treatment with DMB significantly inhibited the LPS-induced NO production in RAW264.7 cells in a dose-dependent manner. More precisely, compared with control (2.8 μ M), LPS treatment markedly increased NO production to 31.5 μ M, whereas co-treatment with DMB significantly decreased NO production to 29.8, 28.2, 24.8, and 15.5 μ M by 62.5, 125, 250 and 500 μ M, respectively.

Pro-inflammatory cytokines are molecules that increase inflammatory response. Therefore, next we examined whether DMB inhibit LPS-stimulated production of pro-inflammatory cytokines in RAW264.7 cells. As shown in Fig. 2B, stimulation of cells with LPS for 24 h exhibited a dramatic increase in TNF- α production from basal level (157.9 \pm 37 pg/mL) to 8080.7 \pm 201.5 pg/mL. The increased TNF- α levels were slightly inhibited by DMB. In other hand, LPS-treatment markedly increased IL-1 β level from 4.2 pg/mL to 101.5 pg/mL, whereas treatment with DMB significantly decreased IL-1 β levels to 81.2, 69.6, 26.3, and 13.2 pg/mL by 62.5, 125, 250 and 500 μ M, respectively (Fig. 2C). These results indicate that DMB has ability to suppress LPS-induced secretion of pro-inflammatory molecules in macrophage cells.

3.3. DMB inhibits the expression of iNOS and COX-2 in LPS-induced RAW264.7 cells

iNOS and COX-2 are responsible for the catalysis of NO and PGE₂, respectively. It is well know that LPS strongly up-regulates iNOS and COX-2 levels in RAW264.7 cells [19]. Therefore, next we determined whether the inhibitory effect of DMB on NO production was related to the modulation of iNOS and COX-2 expression. Western blot analysis shows that protein levels of iNOS and COX-2 were markedly increased after exposure to LPS, however the increased iNOS and COX-2 levels were significantly inhibited by DMB in a dose-dependent manner Fig. 3. Histogram shows the iNOS protein levels were decreased to 28.2% and 52.0% by DMB at 250 and 500 μ M in LPS-stimulated cells, respectively. The COX-2 protein level was decreased to 35.4% and 39.0% by DMB at 250 and 500 μ M in LPS-stimulated cells, respectively compared with that of LPS-alone group (100%). In addition, the expression levels of iNOS

and COX-2 protein were close to baseline in un-stimulated RAW 264.7 cells. The detection of β -actin was also performed in the same blot as an internal control. These results suggest that DMB suppresses the productions of NO by inhibiting the expressions of iNOS and COX-2 in LPS-stimulated RAW264.7 cells.

3.4. DMB suppressed LPS-induced nuclear translocation of NF- κ B p65 and phosphorylation of I κ B- α

Since the expressions of pro-inflammatory proteins and cytokines, such as iNOS, COX-2, TNF- α , and IL-1 β , are known to be modulated by NF- κ B [20]. We next examined whether DMB modulates LPS-stimulated nuclear translocation of NF- κ B, RAW264.7 cells were treated with DMB (62.5, 125, 250 and 500 μ M) in the presence or absence of LPS (100 ng/mL) for 30 min. Localization of NF- κ B was determined by nuclear and cytosolic extracts using Western blot analysis. As shown in Fig. 4A, LPS-alone group markedly increased the translocation of NF- κ B to the nucleus. However, treatment of DMB (250 and 500 μ M) significantly reduced the nuclear translocation of NF- κ B. To further confirm these results, immunofluorescence was performed to examine NF- κ B localization in RAW264.7 cells. As shown in Fig. 4B, compared to control cells, LPS treatment markedly increased nuclear export of NF- κ B as evidenced by that DyLight labeled NF- κ B p65 was predominantly found in the nucleus. However, treatment with DMB at 500 μ M for 30 min significantly decreased the amount of NF- κ B in the nucleus and increased the protein levels in the cytosol in LPS-stimulated cells.

In unstimulated cells, NF- κ B is localized in the cytosol due to its binding with I κ B- α . However, when the cells are activated by LPS, I κ B- α is phosphorylated by I κ B- α kinase (IKK- α) and goes to proteasomal degradation. As a result, NF- κ B is released from its association with I κ B- α , the unbound NF- κ B then translocated into the nucleus and transcribe number of its target genes [21]. To further explore whether DMB regulates the NF- κ B pathway, we examine the effect of DMB on I κ B- α phosphorylation and degradation in LPS-stimulated RAW264.7 cells. The cytosolic fractions were prepared and assayed for I κ B- α phosphorylation and degradation by Western blot analysis. The results showed that DMB significantly reduced phosphorylation and degradation of I κ B- α in a dose-dependent manner (Fig. 5). These findings suggest that DMB suppressed LPS-induced nuclear translocation of NF- κ B through the inhibition of phosphorylation and degradation of I κ B α in macrophage cells.

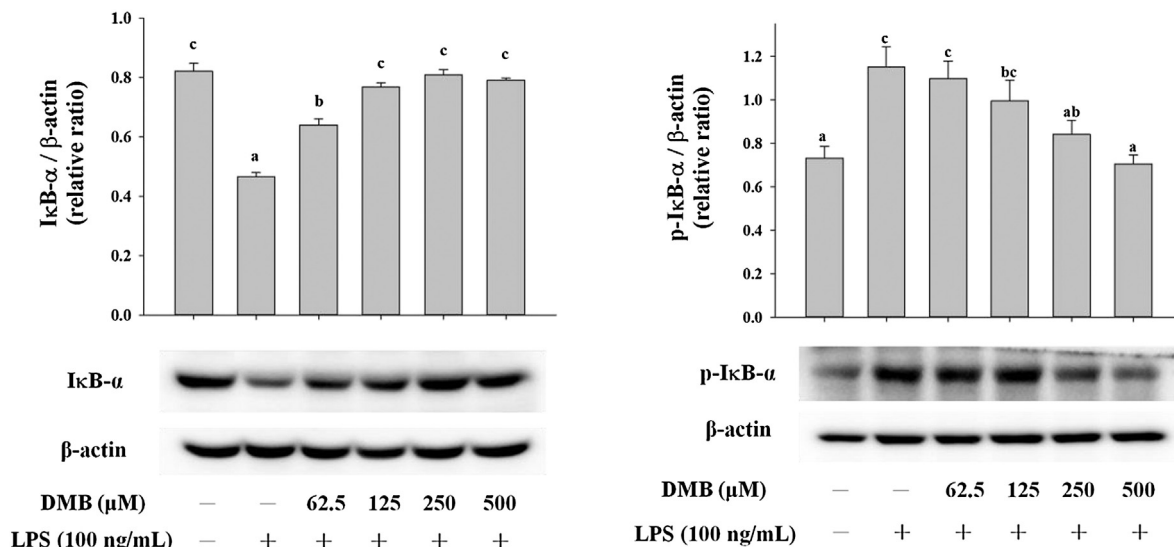


Fig. 5. Effect of DMB on phosphorylation and degradation of I κ B- α in LPS-stimulated RAW264.7 cells. Cells were treated with DMB (62.5, 125, 250 and 500 μ M) and LPS (100 ng/mL) for 30 min. The protein expression levels of p-I κ B- α and I κ B- α were examined by Western blot analysis. Values are expressed as mean \pm S.D. of three replicates. Means with different letters represent significantly different ($p < 0.05$) by Scheffé's test.

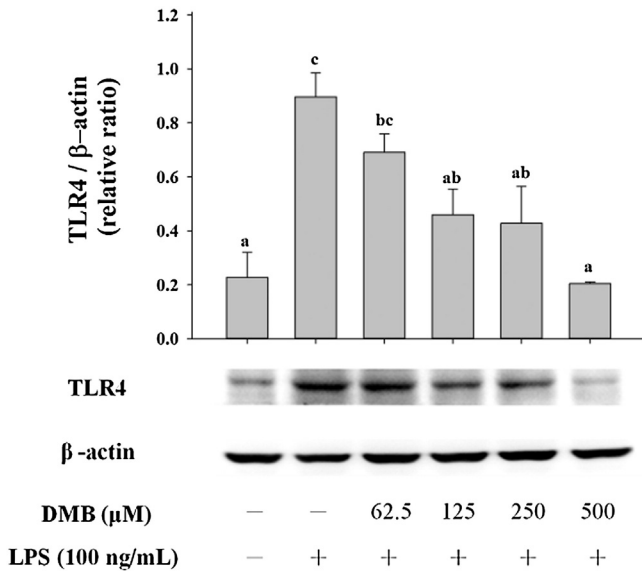
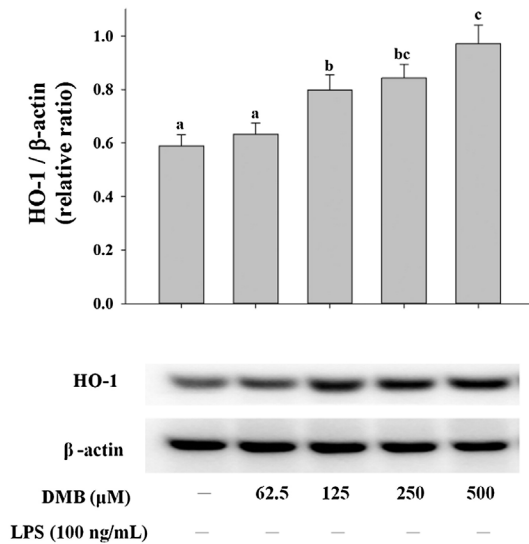


Fig. 6. Effect of DMB on TLR4 protein expression in LPS-stimulated RAW264.7 cells. Cells were treated with DMB (62.5, 125, 250 and 500 μM) and LPS (100 ng/mL) for 24 h. The protein expression levels were examined by Western blot analysis. Values are expressed as mean ± S.D. of three replicates. Means with different letters represent significantly different ($p < 0.05$) by Scheffé's test.

3.5. DMB down-regulates LPS-induced TLR4 expression in RAW264.7 cells

Activation of TLR4 signaling by LPS is associated with secretion of pro-inflammatory cytokines [22]. Therefore, next we examined whether the inhibition of pro-inflammatory molecules by DMB is associated with down-regulation of TLR4, the TLR4 expression level was determined by Western blot analysis. As shown in Fig. 6, treatment with LPS markedly increased the protein expression level of TLR4, whereas co-treatment with DMB significantly as well as dose-dependently inhibited the LPS-induced up-regulation of TLR4 in RAW264.7 cells. This data indicated that DMB inhibits the LPS-induced secretion of pro-inflammatory molecules through the down-regulation of TLR4.



3.6. DMB induces HO-1 expression in RAW264.7 cells

HO-1 is an inducible and rate-limiting enzyme plays a major role in cellular anti-oxidant defense and also inhibits inflammation and cellular apoptosis [23]. Therefore, we examined whether the DMB induce HO-1 expression in RAW264.7 cells. The RAW264.7 cells were treated with various concentrations of DMB (62.5, 125, 250 and 500 μM) for 24 h and the protein level of HO-1 was determined by Western blot analysis. As shown in Fig. 7, compared with untreated control cells, treatment with DMB (62.5, 125, 250 and 500 μM) significantly increased HO-1 protein expression by 1.1-fold, 1.4-fold, 1.4-fold, and 1.6-fold in RAW264.7 cells, respectively. We also assessed HO-1 protein levels in RAW264.7 cells incubated with DMB in the presence of LPS. Compared with LPS-alone group, DMB also markedly induced protein levels in LPS-induced cells.

3.7. The anti-inflammatory effect of DMB is associated with induction of HO-1 in RAW264.7 cells

To assess the involvement of HO in modulating DMB-mediated anti-inflammatory effects, cells were treated with DMB and hemin (a HO-1 enhancer). As shown in Fig. 8A, hemin treatment (20 μM) significantly inhibited LPS-induced iNOS expression, but no effect on COX-2 protein levels. In addition, hemin exhibited a significant inhibitory effect on production of NO, TNF-α, and IL-1β in LPS-induced RAW264.7 cells (Fig. 8B). We further investigated whether HO-1 act as a negative regulator of TLR4, cells were incubated with hemin alone or combination with DMB for 24 h and the protein expression levels of TLR4 and HO-1 were determined by immunoblotting. As illustrated in Fig. 8C, treatment with hemin or DMB significantly reduced the TLR4 and increased HO-1. However, a remarkable decrease in TLR4 and increase in HO-1 were observed in a combination treatment. These results strongly suggest that the anti-inflammatory effect of DMB is associated with induction of HO-1 in RAW264.7 cells.

4. Discussion

Long-term expression of pro-inflammatory genes (such as iNOS and COX-2) leads to chronic inflammation that is responsible for many diseases including cardiovascular disease and cancer [24,25]. NO is one of pro-inflammatory molecules, it plays important roles in inflammatory responses [26]. Overproduction of NO has been associated with

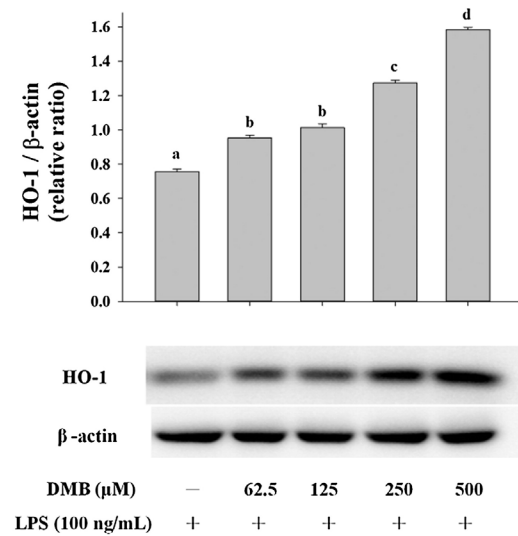


Fig. 7. Effect of DMB on the expression of HO-1 in RAW264.7 cells. Cells were incubated with various concentrations of DMB (62.5, 125, 250 and 500 μM) in the presence or absence of LPS (100 ng/mL) for 24 h. The protein expression levels of HO-1 were examined by immunoblotting. Values are expressed as mean ± S.D. of three replicates. Means with different letters represent significantly different ($p < 0.05$) by Scheffé's test.

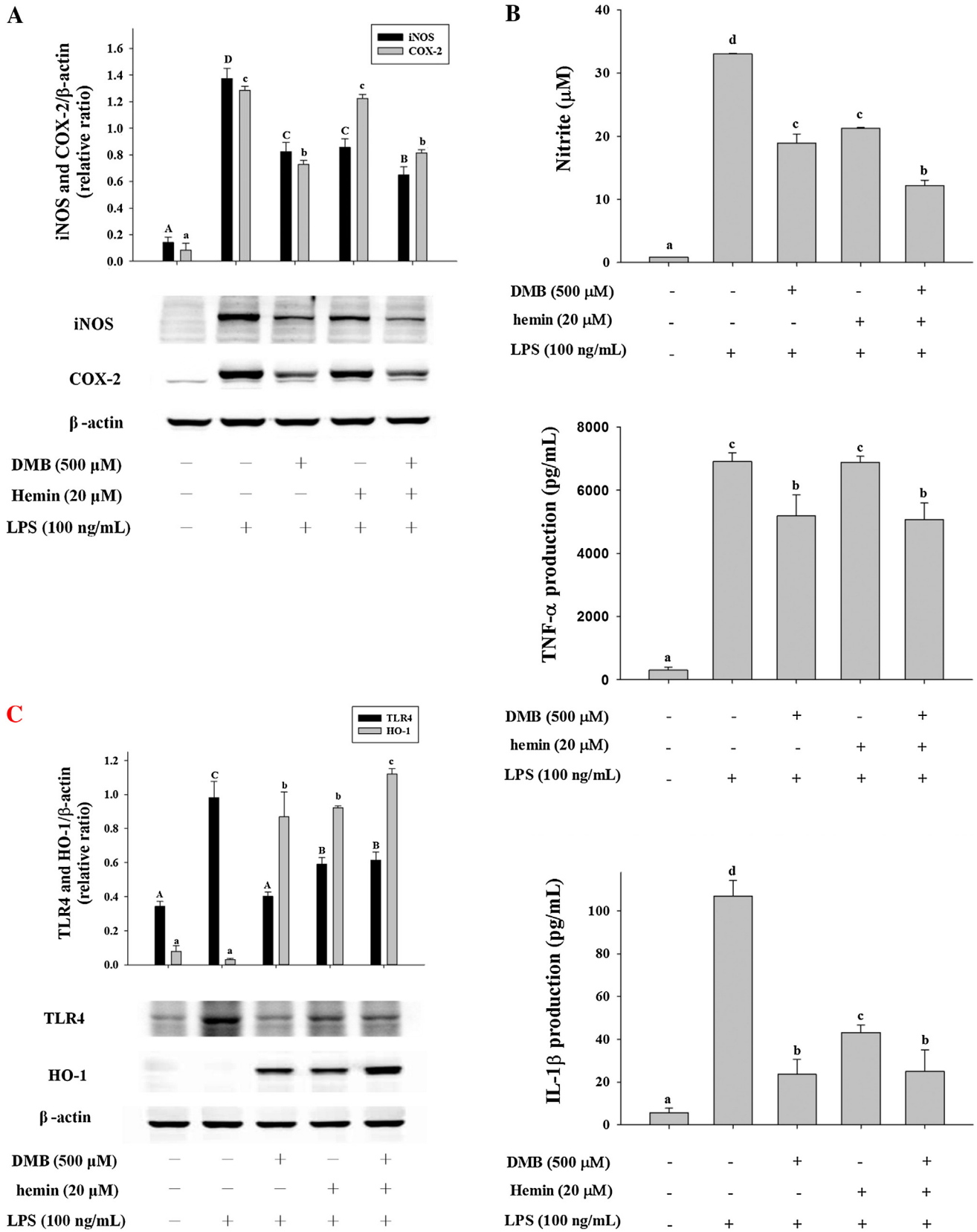


Fig. 8. Effect of HO-1 mediates the expression of iNOS, COX-2, and TLR4 (A, and C) and production of NO, TNF-α, and IL-1β (B) in LPS-stimulated RAW264.7 cells. Cells were incubated with DMB (500 μM) in presence or absence of hemin (20 μM), a HO-1 inducer, and stimulated with or without LPS (100 ng/mL) for 24 h. Values are expressed as mean ± S.D. of three replicates. Means with different letters represent significantly different ($p < 0.05$) by Scheffé's test.

persistent inflammation and tissue destruction [27]. Therefore, modulation of NO is considered one of strategies to against these diseases. Another important enzyme, COX-2, it is an inducible enzyme catalyzing the conversion of arachidonic acid to prostaglandins. Many studies have suggested that increased levels of prostaglandins and cyclooxygenase activity may promote inflammatory pain [28]. In the present study, we found that NO production and iNOS and COX-2 protein expression were decreased by DMB treatment in LPS-stimulated RAW264.7 cells.

TNF- α and IL-1 β are major secreted cytokines that mediate and regulate inflammatory diseases [29]. LPS stimulated macrophages to release pro-inflammatory cytokines. TNF- α plays a major role in the cascade of pro-inflammatory cytokines and the subsequent inflammatory process [30]. IL-1 β is implicated in the pathophysiological changes that occur during different disease states, such as rheumatoid arthritis, pain, inflammatory bowel disease, vascular disease, multiple sclerosis, Alzheimer's disease and stroke [29]. In the present study, we found that TNF- α and IL-1 β were highly stimulated by LPS and were inhibited by DMB (Fig. 2B and 2C). In particular, IL-1 β production was more strongly inhibited by DMB than TNF- α .

A number of reports suggest that transcription factor NF- κ B is involved in the regulation of inflammation-associated genes expression, such as iNOS, COX-2, TNF- α , and IL-1 β [31]. Therefore, the modulation of pro-inflammatory enzyme expression and cytokines production by DMB prompted us to examine the effect of DMB on this transcription factor activity. Under normal conditions, NF- κ B is inactive state tightly bound to the inhibitory protein of I κ B- α in the cytoplasm. However, LPS leads to phosphorylation and degradation of I κ B. As a result, NF- κ B is released from its inhibition by I κ B, and activated NF- κ B is then translocated into the nucleus. This study indicated that DMB inhibited the nuclear translocation of NF- κ B p65 protein *via* suppressing I κ B- α degradation following phosphorylation of I κ B- α , which provides strong evidence that DMB suppressed the LPS-induced activation of NF- κ B signaling probably resulting in the reduced productions of pro-inflammatory molecules. TLR4 is the major pattern recognition receptor in LPS-induced inflammation, which involved in the expression of pro-inflammatory gene and secretion of pro-inflammatory molecules by activation of NF- κ B [32]. In this study, we found that DMB down-regulates the protein expression level of TLR4, which further leads the suppressive effect of NF- κ B signaling. This data providing a positive feedback that DMB inhibits TLR4 expression and breaking the relay of NF- κ B activities.

Several evidences demonstrating that the induction of HO-1 can prevent or mitigate the symptoms associated with inflammation [33,34]. Therefore, targeted induction of HO-1 may be beneficial for treatment of inflammatory diseases. In this study, we explored anti-inflammatory action of DMB with up-regulation of HO-1 in RAW 264.7 cells. We clearly demonstrated that DMB significantly increased HO-1 expression in present and absent LPS-induced RAW264.7 cells (Fig. 6). HO-1 is an inducible enzyme that catalyzes heme into three products: carbon oxide (CO), biliverdin/bilirubin, and free ion (Fe²⁺) [35]. Number of studies has indicated that the anti-inflammatory properties of HO-1 are not only mediated through the degradation of free heme, but also through the production of CO and bilirubin [36,37]. CO has been demonstrated to play an important role in the down-regulation of proinflammatory mediators in LPS-stimulated macrophages through inhibition of NF- κ B activation [36,38]. In addition, previous reports show that induction of HO-1 act a negative regulator of TLR4 [39–41]. Hemin is an effective inducer of HO-1 mRNA expression and activated HO-1 enzyme activity in mammalian cells [42]. To address whether the HO-1 pathway mediates the inflammatory effect of DMB, we treated RAW264.7 cells with hemin in present and absent LPS treatment. In this study, we found that hemin also exhibited inhibitory effect on iNOS, NO, TNF- α , IL-1 β and TLR4 in LPS-induced RAW264.7 cells (Fig. 8). These results suggest that DMB-induced HO-1 expression is partially responsible for the resulting anti-inflammatory effect of this

compound. However, the protein expression of COX-2 was not changed significantly by 20 μ M hemin treatment in LPS-induced RAW264.7 cells. Thus, we could infer that DMB-induced HO-1 expression may be a key mechanism for the resulting anti-inflammatory effect of this compound.

Several studies have demonstrated that *A. camphorata* exerts its anti-inflammatory activity *in vivo* and *in vitro* [43–45]. However, few studies have demonstrated that extracts of *A. camphorata* inhibit LPS-stimulated inflammation *via* down-regulation of NF- κ B signaling pathway [46,47]. In the present study, we also confirmed that DMB could suppress inflammation through the suppression of NF- κ B activation and promote HO-1 expression in LPS-stimulated RAW264.7 cells. DMB was only found in *A. camphorata*, and it had high content in fruiting bodies and mycelia of *A. camphorata* [17,48,49].

In addition, previous studies confirm that number of compounds from *A. camphorata*, such as dehydrosulfurenic acid, dehydroeburicoic acid and zhankuic acid have potent anti-inflammatory activity. Moreover, in the present study we found that DMB is one of most abundant component of *A. camphorata* [48,49]. These results imply that, the anti-inflammatory activity of *A. camphorata* may be attributed to DMB. Furthermore, our results showed that DMB has the anti-inflammatory property *in vitro*, suggesting that it may be a potential compound for the treatment of inflammatory disorders.

5. Conclusions

In the present study, we demonstrate that DMB exerts anti-inflammatory effects *via* suppressing the production of pro-inflammatory molecules through the down-regulation of NF- κ B and TLR4 signaling pathway in LPS-stimulated RAW264.7 cells. Treatment with DMB increase the expression of HO-1 then subsequently inhibits the pro-inflammatory molecules NO, TNF- α , and IL-1 β production and iNOS protein expression in LPS-induced RAW264.7 cells. In addition, induction of HO-1 plays an important role in the regulation of TLR4 expression in LPS-induced RAW264.7 cells. Therefore, DMB may be an important regulator for the management of inflammatory disorders. These findings provide a particle description of mechanism underlying the anti-inflammatory effect of DMB.

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