

Anti-inflammatory effects of diaporisoindole B in LPS-stimulated RAW 264.7 macrophage cells *via* MyD88 activated NF- κ B and MAPKs pathways

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Abstract: Diaporisoindole B (DPB), an isoprenylisoindole alkaloid isolated from the mangrove endophytic fungus *Diaporthe* sp. SYSU-HQ3, has been proved to inhibit the production of nitric oxide (NO) in lipopolysaccharide (LPS)-challenged RAW 264.7 mouse macrophages, showing potent anti-inflammatory effects. In this study, we further investigated the anti-inflammatory effects of DPB and explored the possible mechanisms in LPS-challenged RAW 264.7 mouse macrophages. The results showed that DPB (3.125, 6.2, 12.5 and 25 μ M) could significantly reduce LPS-induced levels of PGE₂, and inhibit the expressions of iNOS and COX-2 in a dose-dependent manner. In addition, DPB also inhibited LPS-induced production of inflammatory cytokines, including TNF- α , IL-1 β , IL-6. Moreover, we further investigated signal transduction mechanisms by which DPB exerted anti-inflammatory effects. DPB could affect LPS-mediated nuclear factor kappa B (NF- κ B) signaling pathway activation *via* down-regulating the upstream myeloid differentiation protein 88 (MyD88) at the protein level. Additionally, DPB also strongly inhibited the phosphorylation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK) and p38. Therefore, DPB might exert anti-inflammatory effects by suppressing NF- κ B activation and MAPKs pathways *via* down-regulating MyD88 in RAW 264.7 cells.

Keywords: Diaporisoindole B; Anti-inflammation; RAW 264.7 macrophage cells; NF- κ B; MAPKs; MyD88

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1. Introduction

Inflammation is a series of protective responses that occur when the body's tissues are damaged (such as various inflammatory factors)^[1,2]. Although inflammation is required by the body to combat bacterial and viral infections, prolonged or excessive inflammation may induce a series of abnormal reactions in the body, causing various diseases, such as diabetes, asthma, and rheumatoid arthritis. In serious cases, symptoms, such as multiple organ dysfunction syndromes (MODS), may arise and pose a threat to the patient's life^[3].

Therefore, continuous research for safe and effective anti-inflammatory drugs is a long-term task.

Macrophages play a central role in inflammatory reactions and can recognize pathogen-associated molecule patterns, such as lipopolysaccharide (LPS)^[4]. LPS can activate macrophages through Toll-like receptors (TLRs) expressed on the cell surface. TLR activation triggers the recruitment of adaptor molecules, such as MyD88^[5]. When MyD88 binds to the death domain of IL-1R-associated kinase (IRAK), IRAK will be phosphorylated, leading to activation and translocation of NF- κ B^[6], which eventually induces inflammation response and produces pro-inflammatory cytokines, including TNF- α , IL-1 β and IL-6, as well as inflammatory mediators, including nitric oxide (NO) and prostaglandin E₂ (PGE₂) produced by inducible NO synthase (iNOS) and cyclooxygenase (COX)-2, respectively^[4]. The MAPKs,

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including ERK1/2, JNK and p38, are also an important downstream pathway^[7,8]. The MAPKs pathway is activated by diverse stimuli, such as inflammatory cytokine, growth factors, and hormones^[9]. Activation of the MAPKs signaling cascades coordinates phosphorylation events that activate transcription factors, together with NF- κ B, exerting major effects on inflammatory events and cell physiology^[10–12].

Mangrove endophytic fungi, as plant mutualists, growing in the intertidal zone of marine coastal environments, are widely recognized as prolific sources of biologically active and structurally unique natural products^[13–15]. Our recent studies have reported that the CH₂Cl₂ extract of a fermentation broth of the fungus *Diaporthe* sp. SYSU-HQ3 leads to the isolation and identification of DPB (Fig. 1), which exerts anti-inflammatory effects in LPS-stimulated RAW 264.7 cells by inhibiting the NO production^[16]. However, the anti-inflammatory mechanism of DPB is unknown. Therefore, we aimed to clarify the anti-inflammatory mechanism of DPB by thoroughly investigating the molecular basis of its anti-inflammatory effects using LPS-challenged RAW 264.7 macrophages.

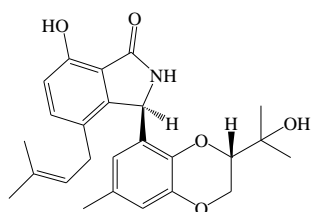


Figure 1. Chemical structure of diaporisoindole B.

2. Materials and methods

2.1. Materials

DPB was isolated from mangrove endophytic fungus *Diaporthe* sp. SYSU-HQ3. A 20-mM solution of DPB

was prepared in 100% dimethyl sulfoxide, stored as small aliquots at -20°C , and then diluted as needed in cell culture medium.

2.2. Cell culture

Murine macrophage RAW 264.7 cells were maintained in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Corning, NY, USA), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37°C in a humidified atmosphere containing 5% CO₂^[17].

2.3. Measurement of PGE2 and pro-inflammatory cytokines

RAW 264.7 cells were seeded in a 12-well plate at a density of 1×10^6 cells per well and incubated for 12 h. Cultured cells were treated with DPB (3.125, 6.25, 12.5 and 25 μM) for 1 h, followed by stimulation with LPS (1 $\mu\text{g/mL}$) for 24 h. Culture medium was collected after centrifugation at 2000 r/min for 10 min and stored at -80°C until further tests. The levels of PGE2, TNF- α , IL-1 β and IL-6 in culture medium were quantitated by enzyme-linked immunosorbent assay (Neobioscience, Shenzhen, China) according to the manufacturer's instructions^[18].

2.4. RNA extraction and real-time RT-PCR

RAW 264.7 cells were seeded in a 12-well plate at a density of 1×10^6 cells per well and incubated for 12 h. Cultured cells were pretreated with DPB (3.125, 6.25, 12.5 and 25 μM) for 1 h, followed by stimulation with LPS (1 $\mu\text{g/mL}$) for 12 h. Total RNA was isolated using Trizol reagent (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions, and reversely transcribed into cDNA using a Superscript III system (Takara Bio Inc., Otsu, Japan). PCR amplification was carried out using PikoReal™ (Thermo Fisher Scientific, MA, USA)

and specific primers^[19,20]. The primers (Generay, Guangzhou, China) were described as follows: mouse iNOS (129 bp), forward primer 5'-CTTGAGCGAG-TTGTGGATTGTC-3' and reverse primer 5'-TAGGT-GAGGGCTTGGCTGAGTG-3'; mouse COX-2 (166 bp), forward primer 5'-TAGTTGCTCATCACCCCACTC-3' and reverse primer 5'-GATCTACCCTCCTCACATCCC-3'; mouse TNF- α (164 bp), forward primer 5'-CTTGT-TGCCTCCTCTTTTGCTTA-3' and reverse primer 5'-CTTTATTTCTCTCAATGACCCGTAG-3'; mouse IL-1 β (105 bp), forward primer 5'-TGTGTTTTTCCTC-CTTGCCTCTGAT-3' and reverse primer 5'-TGCTG-CCTAATGTCCCCTGAAT-3'; mouse IL-6 (104 bp), forward primer 5'-TCACAGAAGGAGTGGCTAAG-GACC-3' and reverse primer 5'-ACGCACTAGGTTT-GCCGAGTAGAT-3'; and glyceral-dehyde 3-phosphate dehydrogenase (GAPDH, 111 bp), forward primer 5'-AAGAAGGTGGTGAAGCAGG-3' and reverse primer 5'-GAAGGTGGAAGAGTGGGAGT-3'.

2.5. Western blotting analysis

RAW 264.7 cells were seeded at a density of 1.5×10^6 cells per well in a 6-well cell culture plate and incubated for 12 h. Cells were pretreated with DPB (3.125, 6.25, 12.5 and 25 μ M) for 1 h, followed by stimulation with LPS (1 μ g/mL) for 24 h. Cells were washed three times with ice-cold phosphate-buffered saline (PBS), resuspended in 200 μ L RIPA lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM PMSF and protease inhibitors cocktails), and incubated for 30 min on ice. The lysate was centrifuged at 12 000 r/min for 15 min at 4 °C, and the supernatant was collected. Protein concentration was determined with BCA Protein Assay Kit (Beyotime, Shanghai, China). The protein complex was subsequently diluted to 20 μ g/ μ L with lysis buffer and 5 \times SDS PAGE sample loading buffer (Sigma, St. Louis, MO, USA), and boiled at 95 °C for

5 min. Protein sample (50 μ g) was separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (8% for iNOS; 10% for COX-2, MyD88, IkB- α , p-IkB- α , NF- κ Bp65, p-NF- κ Bp65, JNK, p-JNK, ERK, p-ERK, p38, p-p38 and β -actin), and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). The transblotted membranes were washed with TBST for 1 min and incubated with 5 % (w/v) skimmed milk in TBST buffer for 1.5 h to block nonspecific binding at room temperature, followed by incubation with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. The membranes were then rinsed three times with TBST for 5 min, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Beyotime, Shanghai, China) for 1 h at room temperature. Blots were again washed three times in TBST. Immunoreactive bands were visualized using a chemiluminescence (ECL) system according to the manufacturer's instructions. Band intensities were quantified using Image J analysis software^[21].

2.6. Data analysis

Results were expressed as means \pm standard deviation (SD). Differences in mean values between groups were analyzed by one-way analysis of variance and Student's *t*-test. Statistical significance was considered at $^{\#}P < 0.05$ compared with the negative group, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the LPS-treated group.

3. Results and discussion

3.1. Inhibitory effect of DPB on LPS-induced expression of iNOS in RAW 264.7 cells

The inflammatory mediator NO is mediated by iNOS, which is almost undetectable under physiological conditions, but during inflammation, a significant increase in iNOS

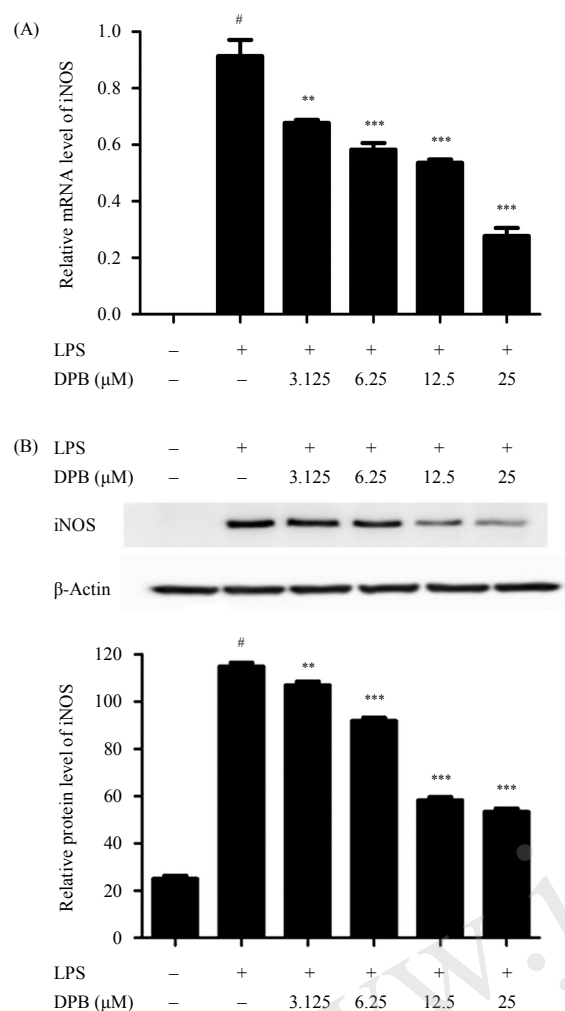


Figure 2. Inhibitory effects of DPB on LPS-induced iNOS mRNA (A) and protein levels (B) in RAW 264.7 cells. Data are means \pm SD of three independent experiments; [#] $P < 0.05$ compared with the negative group, ^{*} $P < 0.05$, ^{**} $P < 0.01$ and ^{***} $P < 0.001$ compared with the LPS-treated group.

acts synergistically with other inflammatory mediators and participates in provoking inflammatory process^[2]. DPB has been proved to inhibit the production of NO in LPS-induced RAW 264.7 macrophage cells^[16]. Here, we examined whether DPB regulated the iNOS expression using RT-PCR and Western blotting analysis. LPS significantly increased the expression of iNOS at both the mRNA and protein levels. However, as shown in Figures 2A and 2B, DPB strongly inhibited the expression of iNOS compared with cells treated with LPS alone in a dose-dependent manner. These data indicated that

DPB inhibited the expression of iNOS at both the mRNA and protein levels, which regulated the production of NO in RAW 264.7 cells.

3.2. Inhibitory effect of DPB on LPS-induced PGE2 production and expression of COX-2 in RAW 264.7 cells

PGE2 is an important inflammatory mediator, which is produced by COX-2. Therefore, its inhibitory effect on the expression of COX-2 has been considered a potential strategy for the development of anti-inflammatory drugs^[22,23]. We evaluated whether DPB regulated PGE2 production and COX-2 expression in RAW 264.7 cells. The quantitative analysis of PGE2 using ELISA showed that the LPS treatment of RAW 264.7 cells promoted the generation and release of PGE2 into the supernatant. Pretreatment with DPB for 1 h significantly inhibited LPS-induced PGE2 production in a dose-dependent manner (Fig. 3A). LPS alone significantly increased the expression of COX-2 at both the mRNA and protein levels. However, pretreatment with DPB for 1 h reduced the expression of LPS-induced COX-2 at the mRNA and protein levels in a dose-dependent manner (Fig. 3B and 3C). Taken together, these data suggested that DPB treatment could reduce the production of PGE2 by inhibiting the expression of COX-2 in LPS-induced RAW 264.7 cells.

3.3. Inhibitory effect of DPB on LPS-induced pro-inflammatory cytokine production in RAW 264.7 cells

Pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, are small secreted proteins that regulate immunity and inflammation. LPS stimulates macrophages to release TNF- α , which induces the release of IL-1 β and IL-6^[24]. TNF- α induces several physiological effects, including inflammation and cytotoxicity. IL-1 β is important for the initiation and enhancement of the

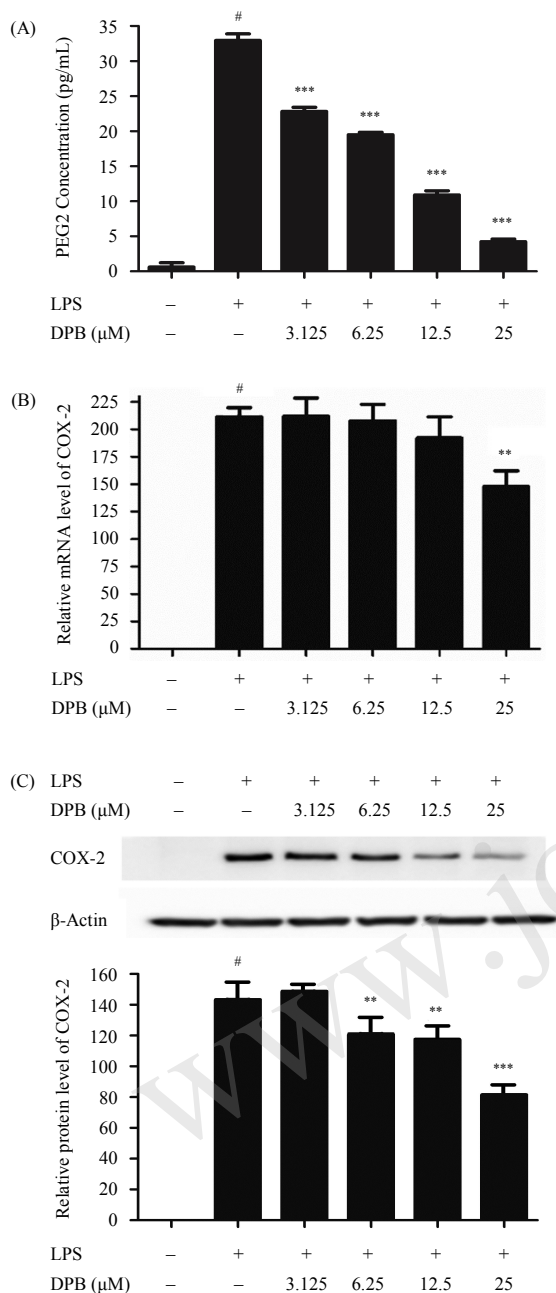


Figure 3. Inhibitory effects of DPB on LPS-induced PGE2 and COX-2 levels in RAW264.7 cells. (A) DPB inhibited LPS-induced PGE2 in RAW264.7 cells. DPB inhibited LPS-induced COX-2 expression at the mRNA (B) and protein (C) levels in RAW 264.7 cells. Data are means \pm SD of three independent experiments; [#] $P < 0.05$ compared with the negative group, ^{*} $P < 0.05$, ^{**} $P < 0.01$ and ^{***} $P < 0.001$ compared with the LPS-treated group.

inflammatory response to microbial infection^[25]. IL-6 is also a pivotal pro-inflammatory cytokine that plays a role in the acute-phase immune response and is regarded as an endogenous mediator of LPS-induced fever^[26]. To

investigate whether DPB affected the expressions of pro-inflammatory cytokines known to play important roles in immune responses, we evaluated the levels of TNF- α , IL-1 β and IL-6 using ELISA and RT-PCR. As shown in Figure 4, LPS stimulation alone markedly increased the levels of TNF- α , IL-1 β and IL-6 compared with the control group, which were significantly inhibited by DPB in a dose-dependent manner (Fig. 4A–C). Under the same conditions, DPB treatment also significantly inhibited the expressions of TNF- α , IL-1 β and IL-6 at the mRNA level in a dose-dependent manner compared with LPS alone (Fig. 4D–F).

3.4. DPB reduces phosphorylation of I κ B- α and NF- κ B p65 in LPS-challenged RAW 264.7 cells

The induction of inflammatory mediators, such as NO and PGE2, and pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, is dependent on the activation of NF- κ B^[27]. Under physiological conditions, NF- κ B is a heterodimer composed of Rel A (p65) and NF- κ B1 (p50), associated with an inhibitory subunit, I κ B, and localizes in the cytoplasm in an inactive form. LPS can activate the NF- κ B pathway by inducing I κ B- α phosphorylation and degradation, causing phosphorylation of dimeric proteins^[28], which induces translocation of NF- κ B into the nucleus^[29], where it binds to target sites to induce the transcription of inflammatory cytokines and trigger the activation of inflammation-related enzymes^[30,31]. Therefore, we evaluated the effect of DPB on the phosphorylation of I κ B- α and NF- κ B p65 in RAW 264.7 cells stimulated with LPS by Western blotting analysis. I κ B- α and NF- κ B p65 were phosphorylated after treatment with LPS for 24 h (Fig. 5). However, pretreatment with DPB for 1 h significantly reduced the LPS-induced phosphorylation of I κ B- α and NF- κ B p65 in RAW264.7 cells. It indicated that DPB could inhibit the activation of NF- κ B by reducing the phosphorylation of I κ B- α and NF- κ B p65.

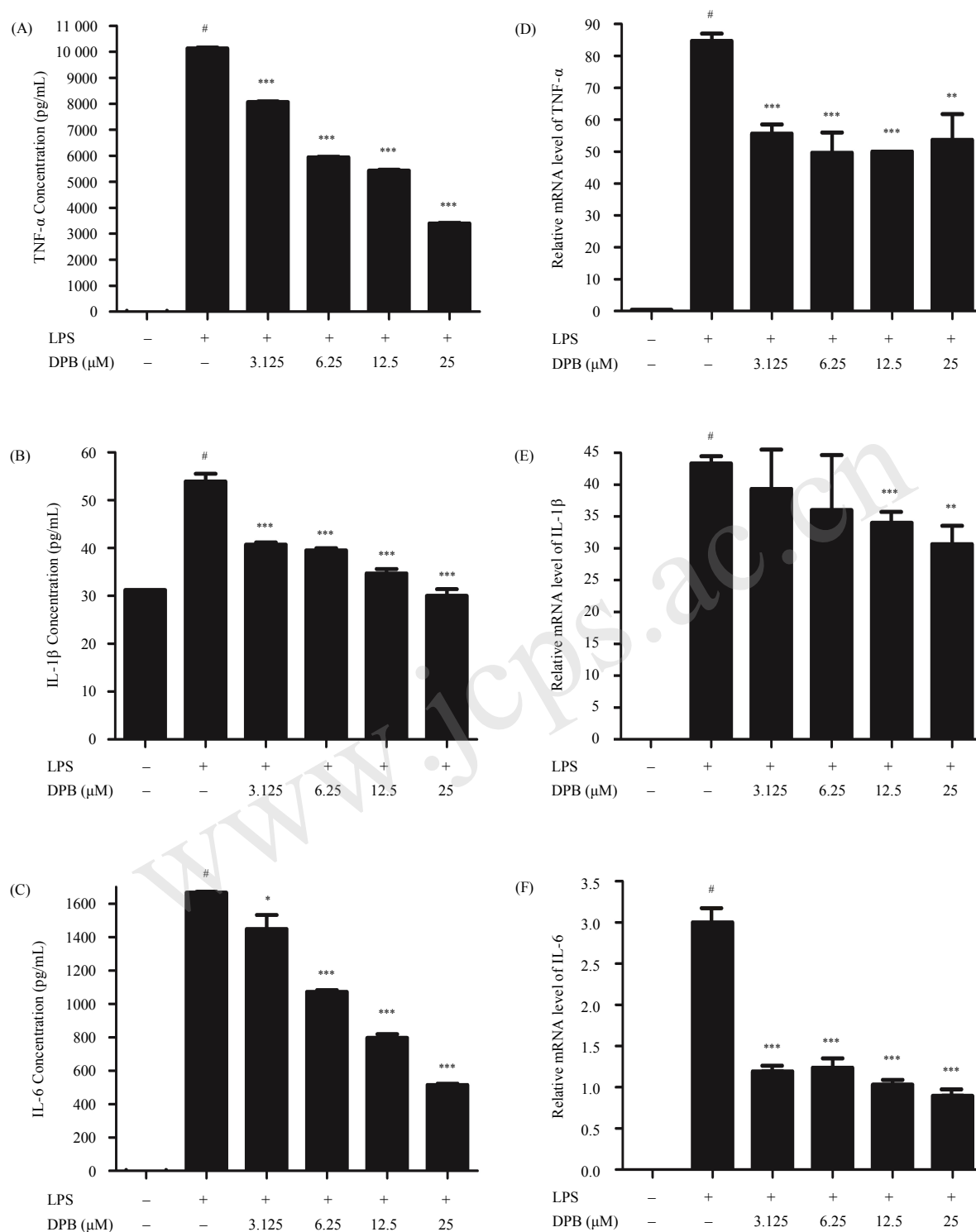


Figure 4. Inhibitory effects of DPB on LPS-induced pro-inflammatory cytokines in RAW 264.7 cells. DPB inhibited LPS-induced pro-inflammatory cytokines TNF- α (A), IL-1 β (B) and IL-6 (C) in RAW 264.7 cells. DPB inhibited LPS-induced pro-inflammatory cytokines TNF- α (D), IL-1 β (E) and IL-6 (F) at the mRNA level in RAW 264.7 cells. Data are means \pm SD of three independent experiments; $^{\#}P < 0.05$ compared with the negative group, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the LPS-treated group.

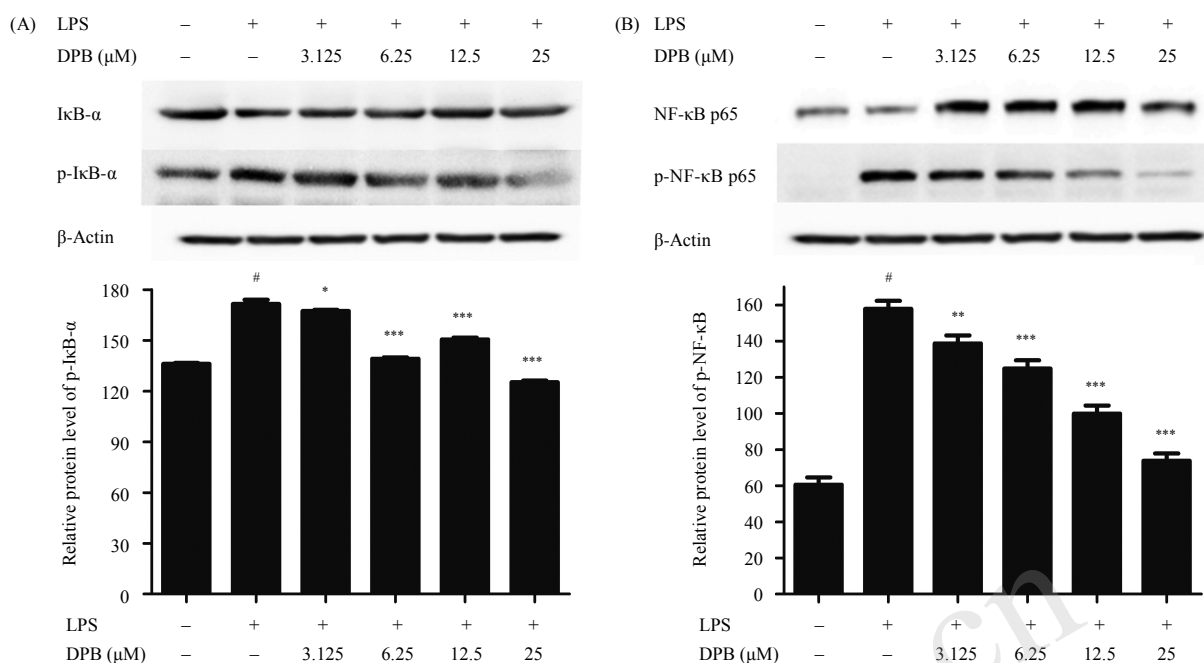


Figure 5. Effects of DPB on LPS-induced IκB-α and NF-κB p65 phosphorylation in RAW 264.7 cells. (A) DPB inhibited LPS-induced phosphorylation of IκB-α. (B) DPB inhibited LPS-induced phosphorylation of NF-κB p65. Quantitative data of IκB-α, p-IκB-α, NF-κB p65 and p-NF-κB p65 were analyzed using Image J software. Data are means ± SD of three independent experiments; [#]*P* < 0.05 compared with the negative group, ^{*}*P* < 0.05, ^{**}*P* < 0.01 and ^{***}*P* < 0.001 compared with the LPS-treated group.

3.5. DPB reduces phosphorylation of MAPK pathways in LPS-challenged RAW 264.7 cells

To fully understand the mechanism by which DPB inhibited LPS-induced production of inflammatory cytokines, we next investigated the effects of DPB on the activation of MAPK pathways. The MAPKs are important serine/threonine signaling kinases, including ERK1/2, JNK and p38. The phosphorylation and activation of ERK1/2, JNK and p38 have been shown to initiate gene expressions of inflammatory mediators in LPS-challenged macrophages. Therefore, inhibition of the phosphorylation of MAPKs will be therapeutically valued^[32]. After cells were stimulated with LPS, the protein levels of phosphorylated and total ERK1/2, JNK and p38 were subsequently measured by Western blotting analysis. LPS induced the phosphorylation of ERK, JNK and p38, while DPB dose-dependently reduced

such increase. Total ERK1/2, JNK and p38 were also examined respectively (Fig. 6), and no regular change in intensity within the cells was observed. These results indicated that DPB inhibited the activation of MAPKs by reducing the phosphorylation of ERK1/2, JNK and p38.

3.6. Suppression of MyD88 by DPB in LPS-challenged RAW 264.7 cells

It is well known that LPS induces NF-κB and inflammatory cytokines through the TLR4-MyD88 signaling pathway^[33]. In this regard, we continued to examine the effects of DPB on MyD88. The results showed that the upregulation of MyD88 at the protein level induced by LPS in RAW 264.7 cell was also inhibited by DPB (Fig. 7), indicating that the anti-inflammatory effects of DPB were tightly regulated by the MyD88 signaling pathway.

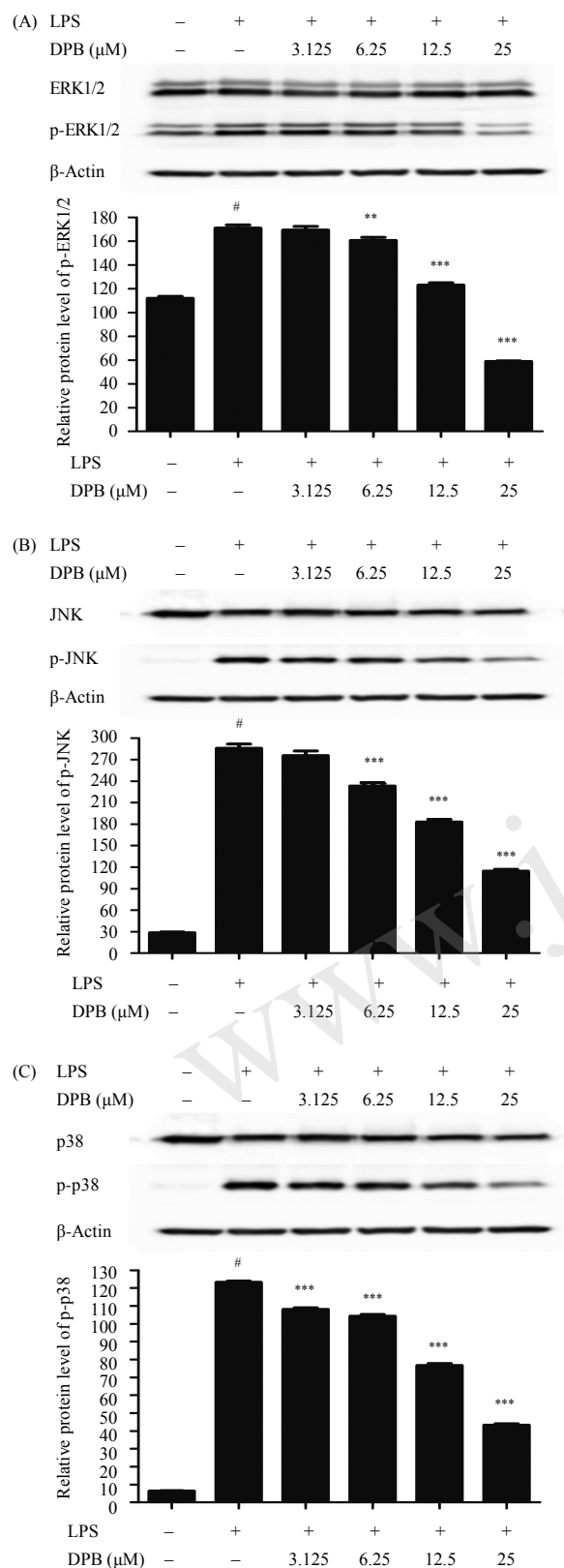


Figure 6. Effects of DPB on LPS-induced phosphorylation of ERK (A), JNK (B), p38 (C) in RAW 264.7 cells. Data are means \pm SD of three independent experiments; [#] $P < 0.05$ compared with the negative group, ^{*} $P < 0.05$, ^{**} $P < 0.01$ and ^{***} $P < 0.001$ compared with the LPS-treated group.

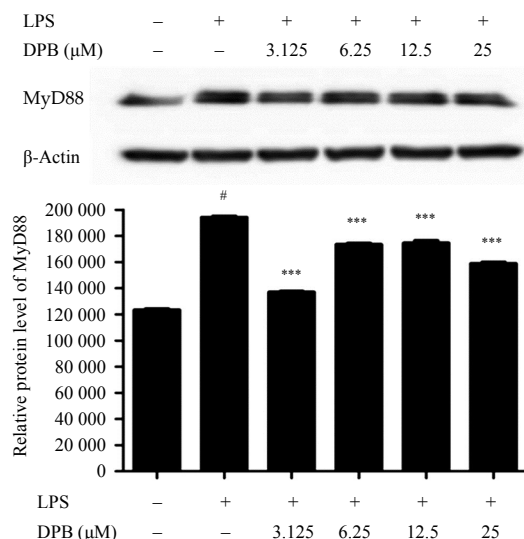


Figure 7. Effects of DPB on LPS-induced upregulation of MyD88 protein in RAW 264.7 cells. Data are means \pm SD of three independent experiments; [#] $P < 0.05$ compared with the negative group, ^{*} $P < 0.05$, ^{**} $P < 0.01$ and ^{***} $P < 0.001$ compared with the LPS-treated group.

4. Conclusions

In conclusion, we demonstrated that DPB inhibited the production of inflammatory mediators, such as PGE₂, and pro-inflammatory cytokines, including TNF- α , IL-1 β and IL-6, in LPS-challenged RAW 264.7 macrophages. Moreover, the underlying mechanism of DPB was more likely to be associated with the inactivation of the NF- κ B and MAPK signaling pathways *via* downgrading the MyD88 protein. These results suggested that DPB was a potential candidate for the development of anti-inflammatory drugs. Finally, although our data validated the anti-inflammatory effects and underlying mechanism of DPB *in vivo*, further studies need to be performed to identify the effects of DPB *in vitro*.

Acknowledgements

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Diaporisoindole B通过抑制MyD88/NF- κ B/MAPKs通路对LPS诱导的RAW 264.7巨噬细胞发挥抗炎作用

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摘要: Diaporisoindole B(DPB)是从红树内生真菌*Diaporthe* sp. SYSU-HQ3的次级代谢产物中分离得到的一种异吲哚类衍生物。前期研究表明DPB可抑制LPS诱导的RAW 264.7巨噬细胞产生NO, 说明其具有潜在的抗炎活性。本文进一步研究了DPB对LPS诱导的RAW 264.7巨噬细胞的抗炎活性及可能的作用机制。DPB(3.125、6.25、12.5和25 μ M)可显著性抑制LPS引起的RAW 264.7小鼠巨噬细胞中PGE₂的水平, 及iNOS和COX-2的表达, 并呈剂量依赖性。DPB也可显著抑制LPS引起的RAW 264.7小鼠巨噬细胞中细胞因子TNF- α , IL-1 β 和IL-6的表达。此外, 我们还研究了DPB发挥抗炎作用的机制。DPB可通过下调上游MyD88 蛋白水平抑制NF- κ B信号通路的激活。此外, DPB还能够抑制MAPKs三个亚型(包括ERK 1/2、JNK和p38)的磷酸化。因此, DPB可能通过下调RAW 264.7巨噬细胞中MyD88蛋白的水平抑制NF- κ B和MAPKs的表达, 从而达到抗炎的作用。

关键词: Diaporisoindole B; 抗炎作用; RAW 264.7巨噬细胞; NF- κ B; MAPKs; MyD88

