ARTICLE
Journal of Cellular Biochemistry 109:1057–1063 (2010)

Journal of Cellular Biochemistry

Dioscorealide B Suppresses LPS-Induced Nitric Oxide Production and Inflammatory Cytokine Expression in RAW 264.7 Macrophages: The Inhibition of NF-κB and ERK1/2 Activation

Poonsit Hiransai,[1*] Suvina Ratanachaiyavong,[1] Arunporn Itharat,[2] Potchanapond Graidist,[1,3] Prasit Ruengrairatanaroj,[4] and Juntipa Purintrapiban[1]

[1] *Faculty of Medicine, Department of Biomedical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90110, Thailand*

[2] *Faculty of Medicine, Applied Thai Traditional Medicine Center, Thammasat University, Klongluang, Pathumtani 12121, Thailand*

[3] *Tumor Biology Research Unit, Faculty of Medicine, Prince of Songkla University, Hat-Yai, Songkhla 90110, Thailand*

[4] *Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat-Yai, Songkhla 90110, Thailand*

ABSTRACT

Dioscorealide B (DB), a naphthofuranoxepin has been purified from an ethanolic extract of the rhizome of *Dioscorea membranacea* Pierre ex Prain & Burkill which has been used to treat inflammation and cancer in Thai Traditional Medicine. Previously, DB has been reported to have anti-inflammatory activities through reducing nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production in lipopolysaccharides (LPS)-induced RAW 264.7 macrophage cells. In this study, the mechanisms of DB on LPS-induced NO production and cytokine expression through the activation of nuclear factor-κB (NF-κB) and ERK1/2 are demonstrated in RAW 264.7 cells. Through measurement with Griess's reagent, DB reduced NO level with an IC_{50} value of $2.85 \pm 0.62\,\mu M$ that was due to the significant suppression of LPS-induced iNOS mRNA expression as well as IL-1β, IL-6, and IL-10 mRNA at a concentration of 6 μM. At the signal transduction level, DB significantly inhibited NF-κB binding activity, as determined using pNFκB-Luciferase reporter system, which action resulted from the prevention of IκBα degradation. In addition, DB in the range of 1.5–6 μM significantly suppressed the activation of the ERK1/2 protein. In conclusion, the molecular mechanisms of DB on the inhibition of NO production and mRNA expression of iNOS, IL-1β, IL-6, and IL-10 were due to the inhibition of the upstream kinases activation, which further alleviated the NF-κB and MAPK/ERK signaling pathway in LPS-induced RAW264.7 macrophage cells. J. Cell. Biochem. 109: 1057–1063, 2010.

KEY WORDS: NAPHTHOFURANOXEPIN; *DIOSCOREA MEMBRANACEA*; NITRIC OXIDE PRODUCTION; INFLAMMATORY CYTOKINES; RAW 264.7 CELLS

Macrophage accumulation and the associated release of inflammatory mediators have been shown to participate in the patho-physiogenesis of xenobiotic-induced tissue damage [Laskin and Pendino, 1995]. The up-regulation of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-12, and IFN-γ in turn enhance the production of toxic agents such as reactive nitrogen species (RNS). Nitric oxide (NO) is one of the critical RNSs continuously produced from the inducible nitric oxide synthase (iNOS) by macrophages in the presence of lipopolysaccharides (LPS) and pro-inflammatory cytokines [Nathan and Xie, 1994]. The excessive production of NO and pro-inflammatory cytokines are involved in the pathogenesis of many human diseases such as rheumatoid arthritis [Robert and McColl, 2004], inflammatory bowel disease [Wang and Fu, 2005], asthma [Ricciardolo et al., 2004], and endotoxin-induced multiple organ failure [Carrigan et al., 2004].

Dioscorea membranacea Pierre ex Prain & Burkill, popularly knows as Hua-Khao-Yen, is a member of the Dioscoreaceae or Yam

Grant sponsor: Commission on Higher Education, Bangkok, Thailand; Grant sponsor: The Graduate School, Prince of Songkla University, Songkhla, Thailand.

*Correspondence to: Poonsit Hiransai, Faculty of Medicine, Department of Biomedical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90110, Thailand. E-mail: webspider_13@hotmail.com

Received 20 October 2009; Accepted 15 January 2010 • DOI 10.1002/jcb.22535 • © 2010 Wiley-Liss, Inc.
Published online 11 March 2010 in Wiley InterScience (www.interscience.wiley.com).

family. In Thailand, the plant named Hua-Khao-Yen were included in the multi-medicinal plants formulae for more than 2,449 recipes, based on selective interviews with 23 folk medicinal doctors. These recipes are used in traditional medicine in the treatment of bone and joint diseases (26.1%), cancer-related inflammation (43.5%), and the other diseases such as lymphopathy, dermopathy, venereal diseases, and leprosy. Dioscorealide B (DB), the naphthofuranoxepin isolated from this plant, is the active component which was found to have a cytotoxic effect against cancer cell lines [Itharat et al., 2003] and inhibited the production of β-hexosaminidase, TNF-α, and IL-4 from an RBL-2H3 cell line [Tewtrakul et al., 2006]. Recently, the inhibitory effect of DB on NO and TNF-α production was shown in LPS-induced RAW 264.7 macrophage cells [Tewtrakul and Itharat, 2007]. Base on these data, we hypothesized that DB may exert an anti-inflammatory activity through the inhibition of inflammatory mediator production at the transcriptional level.

In macrophages, LPS-induced production of inflammatory mediators depends on the triggering of the Toll-like receptor 4 (TLR4)-CD14 complex [Takeda et al., 2003]. The binding of LPS to TLR4-CD14 complex recruits downstream-associated proteins such as MyD88, TIRAP, TRAM, and TRIF [West et al., 2006]. Then, MyD88 associates with two IL-1R-associated kinases (IRAK-1 and IRAK-4), leading to the sequential activation of TNF receptor-associated factor 6 (TRAF6) which further regulates TGF-β-activated kinase-1 (TAK1). Active TAK1 stimulates downstream signaling pathways such as the IκB kinases (IKKs) and mitogen-activated protein kinases (MAPKs) pathways [Lee and Kim, 2007]. These two signaling pathways in turn activate a variety of transcription factors which coordinate the induction of many gene encoding inflammatory mediators. For example, nuclear factor-κB (NF-κB) transcription factor is required for the expression of pro-inflammatory mediators such as iNOS, TNF-α, IL-1β, and IL-6 [Siebenlist et al., 1994]. In addition, LPS can induce the up-regulation of IL-10, the well-known anti-inflammatory cytokine through the activation of the p38, JNK, and ERK1/2 MAPK pathway leading to the activation of the Sp1 transcription factor [Chanteux et al., 2007]. In this study, we evaluated the effects of DB on the activation of the NF-κB and ERK1/2 MAPK signaling pathways which correlate to induce NO production and expression of inflammatory mediators including iNOS, IL-1β, IL-6, and IL-10 in an LPS-induced RAW 264.7 murine macrophage cell line.

MATERIALS AND METHODS

MATERIALS AND EQUIPMENTS

Dulbecco's Modified Eagle Medium (DMEM) and penicillin-streptomycin (10,000 U/ml) were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtain from Biochrom AG (Berlin, Germany). Sulfanilamide, *o*-phosphoric acid (H_2PO_4), *N*-(1-napthyl)-ethylenediamine dihydrochloride, and sodium nitrite ($NaNO_2$) were obtained from Merck (NJ, USA). LPS from *E. coli* serotype O111: B4, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). TRIZOL reagent, M-MLV reverse Transcriptase, RNasOUT, and *Taq* DNA Polymerase were obtained from Invitrogen (Carlsbad, CA). pNFκB-Luc plasmid and Luciferase assay kits were obtained from Stratagene (La Jolla, CA). Fugene 6 transfection reagent was purchased from Roche Applied Science (Mannheim, Germany). For Western blot analysis, RIPA lysis buffer, anti-p-IκBα, anti-IκBα, anti-p-ERK, anti-ERK1, anti-ERK2, and anti-mouse and anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin antibody was obtained from Sigma.

ISOLATION OF DIOSCOREALIDE B FROM THE ETHANOLIC EXTRACT OF *DIOSCOREA MEMBRANACEA*

DB was extracted from rhizomes of *D. membranacea* Pierre ex Prain & Burkill which were collected from Chumporn province, Thailand. This plant was identified by Assoc. Prof. Dr. Arunporn Itharat, and voucher specimens are deposited at the herbarium of the Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The plant material was prepared as previously described [Itharat et al., 2004]. The dried powdered rhizomes of this plant were extracted using ethanol, and then purified using a previous protocol to obtain DB [Itharat et al., 2003]. The structure of the pure compound (Fig. 1) was elucidated by comparing it with previously reported ^{1}H- and ^{13}C-NMR spectral data [Itharat et al., 2003].

CELL LINE AND CELL CULTURE

The murine macrophage cell line RAW 264.7 (TIB-71) was obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in DMEM supplemented with 10% FBS, 100 U/ml of penicillin and streptomycin at 37°C with 5% CO_2.

MEASUREMENT OF NITRITE IN CULTURE MEDIA

RAW 264.7 cells (1×10^5 cells/well) were seeded in a 96-well flat bottom plate for 24 h at 37°C with 5% CO_2. The culture medium was removed and replaced with fresh medium containing DB at various concentrations for 30 min prior to challenging with 2.5 μg/ml of LPS for the specified times. The nitrite concentration was measured in the culture supernatant after 24 h co-incubation. Briefly, 100 μl of each supernatant was incubated in triplicate with 50 μl of 2% (w/v) sulfanilamide in 10% (v/v) *o*-phosphoric acid for 15 min at room temperature. Then, 50 μl of *N*-(1-napthyl)-ethylenediamine

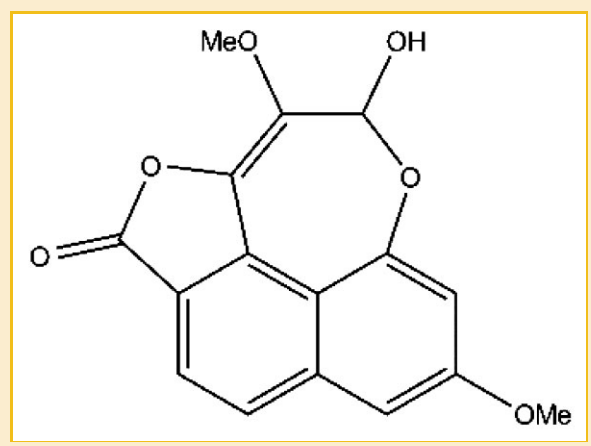

Fig. 1. The chemical structure of Dioscorealide B isolated from the rhizomes of *Dioscorea membranacea* Pierre ex Prain & Burkill.

dihydrochloride (0.2%, w/v) was added and left to incubate for a further 15 min at room temperature. The absorbance at 570 nm was determined by a microplate reader. The quantification of nitrite was standardized with $NaNO_2$ at 0–100 μM concentrations.

MEASUREMENT OF CELL VIABILITY BY MTT ASSAY

An MTT assay was performed to determine cell viability. MTT was dissolved in PBS at 5 mg/ml as a stock solution and sterilized using 0.2 μm filter. After the experimental period, RAW 264.7 cells were washed twice with PBS and incubated with 100 μl of MTT solution (0.5 mg/ml in medium) at 37°C for 1 h. Under light protection, the resulting dark blue crystals of formazan were dissolved with 200 μl of dimethyl sulfoxide (DMSO) at room temperature for 30 min and read at 570 nm on a microplate reader.

CELL VIABILITY USING TRYPAN BLUE EXCLUSION ASSAY

RAW 264.7 cells (2.5×10^5 cells/well) were seeded in a 24-well plate for 24 h at 37°C with 5% CO_2. The medium was removed and replaced with fresh medium containing various concentrations of DB and then incubated for 24 h. After that, RAW 264.7 cells were harvested by trypsinization. The viable cell and death cell numbers were assessed by 0.4% trypan blue staining solution and counted by hemacytometer under an inverted microscope. The percentage of viable cells was calculated by following equation

$$\% \text{ Viable cell} = \frac{\text{Viable cells}}{\text{Viable cells} + \text{Death cells}} \times 100$$

REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION

RAW 264.7 cells (2×10^6 cells/well) were seeded in a 6-well plate for 24 h at 37°C with 5% CO_2. The medium was removed and replaced with fresh medium containing various concentrations of DB for 30 min followed by stimulation with 2.5 μg/ml of LPS and incubated further for the specified times. The cells were then harvested and washed twice with cold PBS and the total RNA was isolated with a TRIZOL reagent according to the supplier's instructions. Equal amounts of isolated RNA (2.0 μg) aliquots from each sample were reverse transcribed with M-MLV reverse transcriptase at 37°C for 50 min and finalized at 72°C for 15 min. The obtaining cDNAs were amplified with *Taq* polymerase using the appropriate oligonucleotide primers complementary to iNOS, IL-1β, IL-6, IL-10, and β-Actin, as shown in Table I. The PCR was carried out in a thermal cycler (BIO-RAD, Hercules, CA) under the following conditions: initial denaturation at 94°C for 3 min; 35 cycles each at 94°C for 45 s, 45°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 10 min. Equal volumes of PCR products were fractionated on 1.8% agarose in 0.5× TBE gels containing 0.5 μg/ml ethidium bromide. The gel images were captured and the intensity of the PCR products analyzed by an automated gel doc system (Bioimagine System, Syngene, USA).

NF-κB-DRIVEN LUCIFERASE REPORTER ASSAY

RAW 264.7 cells (1×10^5 cells/well) were seeded in a 48-well plate and allowed to adhere for 24 h. The cells were then transfected with pNFκB-Luc plasmid using 0.2 μg of plasmid per 0.3 μl of Fugene6 transfection reagent for each well and then incubated a transfection period of 24 h. After that, the cell culture mediums were removed and replaced with fresh mediums containing various concentrations of DB for 30 min, followed by co-incubation with 2.5 μg/ml of LPS for 6 h. Then the total cell proteins were extracted and the Luciferase activity was measured using Luciferase assay kits following the manufacturer's protocol. The chemiluminescence light intensity was measured using a Tropix TR 717 Luminometer Plate Reader (Applied Biosystems, Foster City, CA).

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

The RAW 264.7 cells (1×10^7 cells) were cultured in 10 cm-dishes and allowed to adhere for 24 h. After treatment with DB, the cells were washed twice with cold PBS and lysed in RIPA lysis buffer (1× TBS, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1% PMSF, 1% sodium orthovanadate, and 1% protease inhibitor cocktail) following the manufacturer's instructions. The cell lysates were rocked for 1 h at 4°C followed by a 15 min centrifugation at 10,000*g*. The protein concentration of cell lysates was determined by the Bradford method standardized with BSA. Sample aliquots (100 μg of total proteins/lane) were separated on 12% SDS–polyacrylamide gels electrophoresis (80 V 30 min for the stacking gel and 150 V 90 min for the separating gel) and transferred to a nitrocellulose membrane (35 V overnight and finalized at 100 V 30 min.). The membranes were subsequently incubated for 1 h at room temperature with 5% non-fat dry milk in TTBS buffer (0.1% (v/v) Tween-20 in Tris-buffered saline) to block non-specific binding and incubated with an appropriate primary antibody (one of anti-p-IκBα (1:100), anti-IκBα (1:100), anti-p-ERK1/2 (1:500), anti-ERK1/2 (1:1,000) or anti-actin (1:2,000)) in 1% milk TTBS at 4°C overnight. Then the membranes were washed three times with 1% milk TTBS for 10 min each time. Finally, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary

TABLE I. Primer Sequences for Amplification and Size of PCR Products

Gene	Primer sequence	Accession no.	Product (bp)
iNOS	Forward: 5′-ATGTCCGAAGCAAACATCAC-3′ Reverse: 5′-TAATGTCCAGGAAGTAGGTG-3′	NM 010927	450
IL-1β	Forward: 5′-AAGCTCTCCACCTCAATGGA-3′ Reverse: 5′-TGCTTGTGAGGTGCTGATGT-3′	NM 008361	301
IL-6	Forward: 5′-TTCCATCCAGTTGCCTTCTTGG-3′ Reverse: 5′-CTTCATGTACTCCAGGTAG-3′	NM 031168	360
IL-10	Forward: 5′-GTGAAGACTTTCTTTCAAACAAAG-3′ Reverse: 5′-CTGCTCCACTGCCTTGCTCTTATT-3′	NM 010548	273
β-Actin	Forward: 5′-GACTACCTCATGAAGATCCT-3′ Reverse: 5′-CCA CAT CTG CTG GAA GGT GG-3′	NM 007393	510

antibody (1:2,000) and washed again three times with TTBS. Immunoreactive protein bands were visualized on CL-XPosure film using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

STATISTICAL ANALYSIS

All assays were carried out in triplicate with a minimum of three independent experiments and results were expressed as the mean ± SEM. The 50% inhibitory concentration (IC_{50}) of DB on NO production was obtained by linear regression analysis of concentration–response curve plotting between percentage of inhibition and sample concentration of three independent assays. Comparison data was analyzed by pair-Student's *t*-test, and a *P*-value <0.05 was considered significant.

RESULTS

THE EFFECT OF DIOSCOREALIDE B ON LPS-INDUCED NO PRODUCTION IN RAW 264.7 CELLS

Pre-incubation of RAW 264.7 cells with DB at concentrations ranging from 0 to 16 μM prior to the activation with 2.5 μg/ml of LPS significantly suppressed the production of NO when compared to LPS activation, with IC_{50} values of 2.85 ± 0.62. As shown in Figure 2, NO productions at basal levels were 0.14 ± 0.09 and 0.30 ± 0.21 μM in the absence and presence of 0.2% DMSO, respectively. Upon stimulation with 2.5 μg/ml of LPS, NO production rose significantly to the levels of 9.27 ± 1.69 and 7.57 ± 1.30 μM in the absence and presence of 0.2% DMSO, respectively. In addition, DB exhibited LPS-induced NO suppression in a concentration-dependent manner (Fig. 2A). The suppression

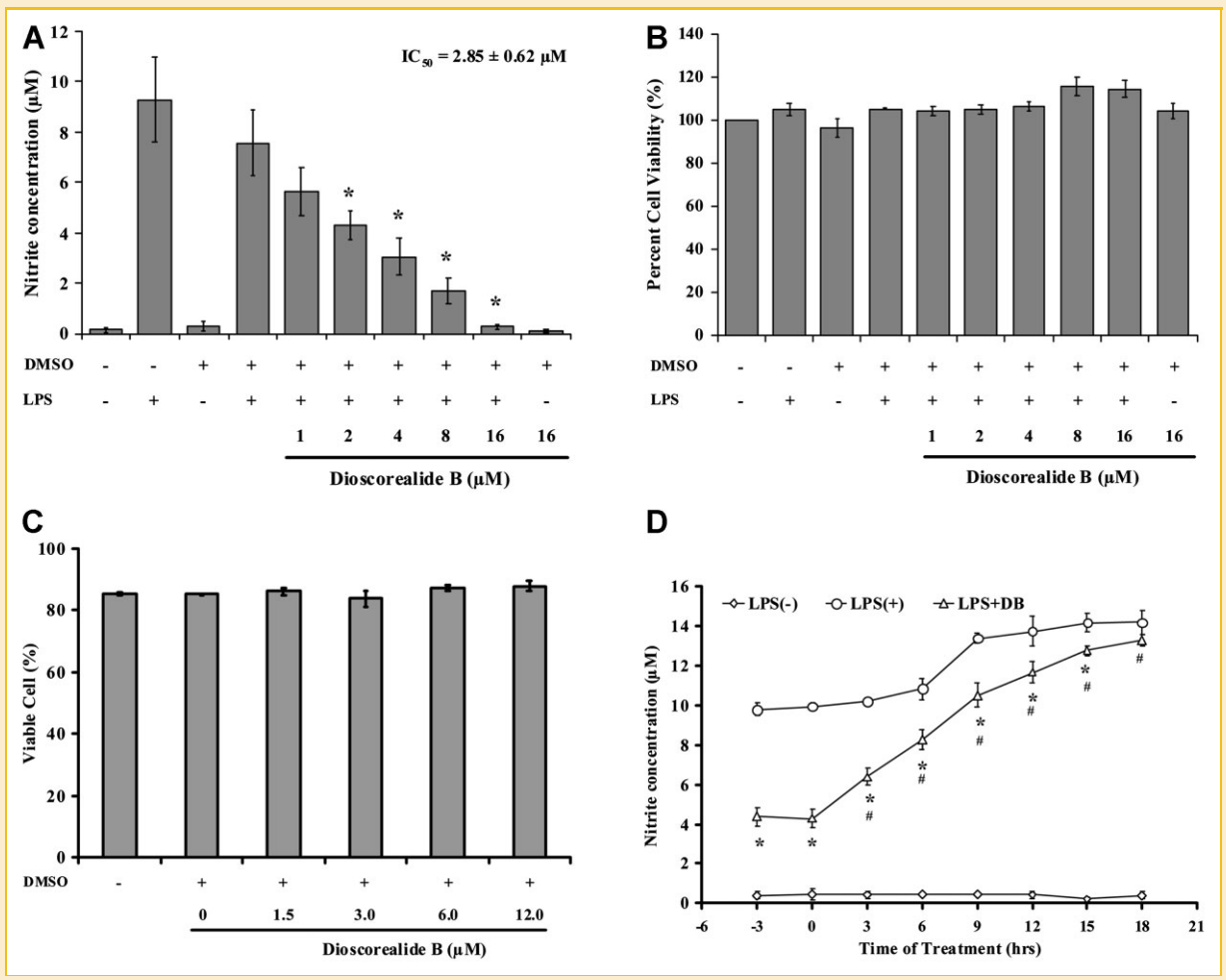

Fig. 2. The effects of Dioscorealide B on the LPS-induced NO production in RAW 264.7 cells. A: RAW 264.7 cells (1×10^5 cells/well) were treated with various concentrations of Dioscorealide B for 30 min prior to stimulation with 2.5 μg/ml LPS for 24 h. NO in the culture medium was measured as described in the Materials and Methods Section. B: The viability of the remaining cells after removal of supernatants was determined by MTT assay. C: The percentage of viable RAW 264.7 cells that were treated with various concentrations of DB, were trypsinized and determined by trypan blue exclusion assay. D: DB at 3.0 μM was added in sequential time-points before and after LPS (2.5 μg/ml) activation. Accumulated nitrite production was measured at 24 h after LPS stimulation. The values were expressed as mean ± SEM from four independent experiments performed in triplicate. $^{*}P<0.05$ compared with the LPS stimulation alone and $^{\#}P<0.05$ compared with the treatment of DB at time zero.

was not due to chemically induced cytotoxicity at any dosages below 16 μM that was determined by MTT and trypan blue exclusion assay (Fig. 2B,C). The time-course suppressive effect of DB on LPS-induced NO production is demonstrated in Figure 2D. DB at 3.0 μM significantly inhibited LPS-induced NO production in RAW 264.7 cells when added either 3 h before or at time zero up to 15 h after LPS stimulation (Fig. 2D, $^{*}P < 0.05$). The inhibitory effect significantly reversed when DB was added more than 3 h after LPS stimulation (Fig. 2D, $^{\#}P < 0.05$).

THE EFFECT OF DIOSCOREALIDE B ON THE mRNA EXPRESSION OF iNOS AND INFLAMMATORY CYTOKINES IN RAW 264.7 CELLS

RAW 264.7 macrophage cells constitutively expressed the mRNA of inflammatory mediators including iNOS, IL-1β, IL-6, and IL10 at low levels. In the presence of 2.5 μg/ml LPS, all mRNA levels increased after 3 h of activation, significantly increased at 9 h, and decreased after 12 h (data not shown). Using optimal conditions at 9 h of LPS stimulation, the mRNA levels of iNOS, IL-1β, IL-6, and IL10 mRNA were, respectively, increased 2.15 ± 0.09-, 2.56 ± 0.06-, 3.01 ± 0.06-, and 1.76 ± 0.06-fold compared with baseline (Fig. 3A, lane 2 compared to lane 1). Pre-treatment of DB ranging 0.6–6.0 μM reduced mRNA levels of all inflammatory mediators in a concentration-dependent manner (Fig. 3A, lanes 3–5). In addition, the iNOS, IL-1β, IL-6, and IL-10 mRNA expressions were significantly suppressed by 6 μM of DB with 75.23 ± 7.02, 71.18 ± 2.54, 97.08 ± 3.01, and 71.75 ± 4.09 percentages of inhibition ($P < 0.05$), respectively (Fig. 3B).

THE EFFECT OF DIOSCOREALIDE B ON NF-κB-DRIVEN LUCIFERASE ACTIVITY IN pNFκB-Luc-TRANSFECTED RAW 264.7 CELLS

RAW 264.7 macrophage cells were transfected with pNFκB-Luc containing five repeated NF-κB binding consensus sequences (5′-TGGGGACTTTCCGC-3′), which in turn activated the expression of firefly Luciferase in the presence of active NF-κB. After a 24-h transfection period, adding LPS (2.5 μg/ml) induced the expression of Luciferase, which enzyme activity increased at 3 h, was significantly detected at 6 h, and decreased after 12 h of LPS activation (data not shown). In the absence of DB, LPS induced the up-regulation of Luciferase activity to 7.29 ± 0.51 times compared with basal levels. In Figure 4, pre-treatment for 30 min prior to LPS activation with 1.5–6.0 μM of DB significantly reduced Luciferase activity with 28% and 45% suppressions at 3.0 and 6.0 μM, respectively ($P < 0.05$).

THE EFFECT OF DIOSCOREALIDE B ON THE ACTIVATION OF IκBα AND ERK1/2 PROTEIN IN LPS-INDUCED RAW 264.7 CELLS

LPS-induced TLR4 activation by turn stimulates the degradation of the inhibitor of NF-κB (IκBα) proteins and then releases the active form of NF-κB proteins. At the same time, the extracellular signal-regulated kinases1 and 2 (ERK1/2) are phosphorylated by the up-stream MAPKinase proteins and then further stimulate Sp1 transcription factor. In our study, the IκBα proteins underwent degradation at 15 min and completely degraded at 20 min in the presence of LPS (2.5 μg/ml). The IκBα proteins returned to normal levels at 30 min. Also, phosphorylated ERK1/2 (p-ERK1/2) proteins

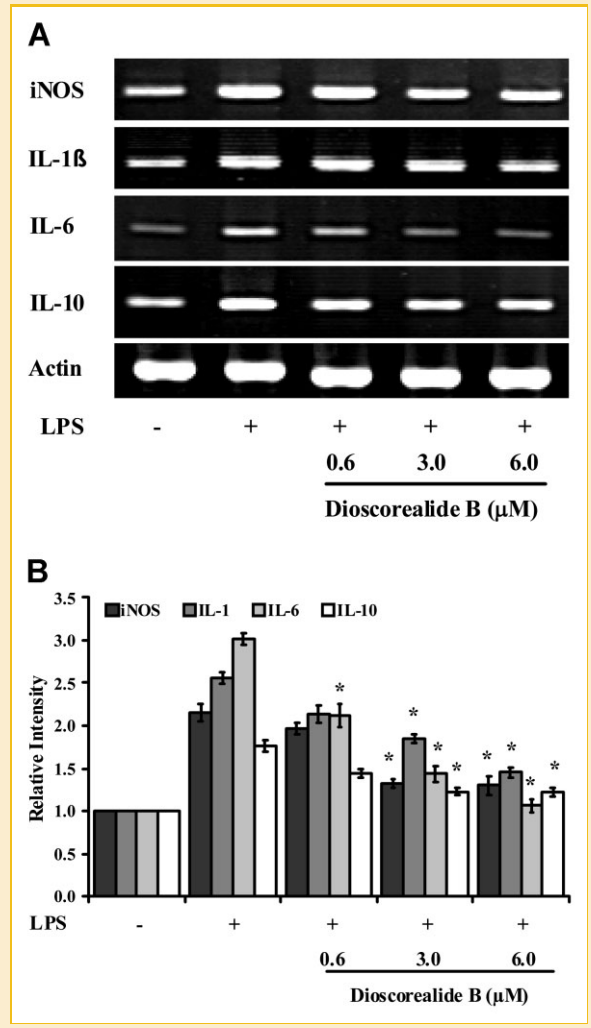

Fig. 3. The effects of Dioscorealide B on LPS-induced mRNA expression of iNOS and inflammatory cytokines in RAW 264.7 cells. A: RAW 264.7 cells (2×10^6 cells/well in 6-well plates) were pre-treated with various concentrations of Dioscorealide B for 30 min prior to stimulation with 2.5 μg/ml LPS for 9 h. Total RNA was isolated for RT-PCR as described in the Materials and Methods Section. B: The band intensity was expressed as mean ± SEM derived from three independent experiments. $^{*}P < 0.05$.

were detected at 10 min and maintained a significant level to 20 min. The levels of p-ERK1/2 proteins decreased after 50 min of LPS stimulation, while the levels of phosphorylated-IκBα (p-IκBα) proteins did not significantly change during the 10–50 min of LPS activation.

Pre-treatment of RAW 264.7 cells with 1.5–6.0 μM of DB for 30 min inhibited LPS-induced degradation of IκBα in a concentration-dependent way (Fig. 5A, lanes 3–5 compared to lane 2). DB at a concentration of 6.0 μM significantly blocked LPS-induced IκBα degradation as shown in lane 5 compared to lane 2 of Figure 5A. In Figure 5B, the level of p-ERK1/2 proteins was significantly suppressed by DB (lanes 3–5 compared to lane 2) and the suppressive effect of DB was not due to the alteration of ERK1/2 total proteins.

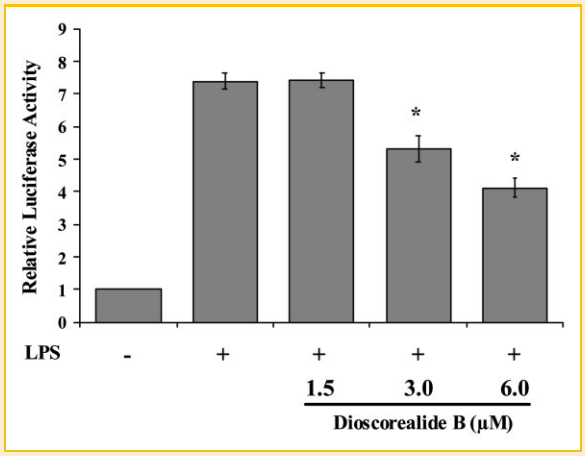

Fig. 4. The effects of Dioscorealide B on LPS-induced NF-κB-driven Luciferase activity in RAW 264.7 cells. RAW 264.7 cells (1×10^5 cells/well in 48-well plates) were transfected with pNFκB-Luc plasmid for 24 h and then pre-incubated with Dioscorealide B for 30 min followed by stimulation with 2.5 μg/ml LPS. After 6 h of activation, total cells were extracted and Luciferase activity determined as described in the Materials and Methods Section. The relative light emission was expressed as mean ± SEM derived from three independent experiments. $^*P < 0.05$.

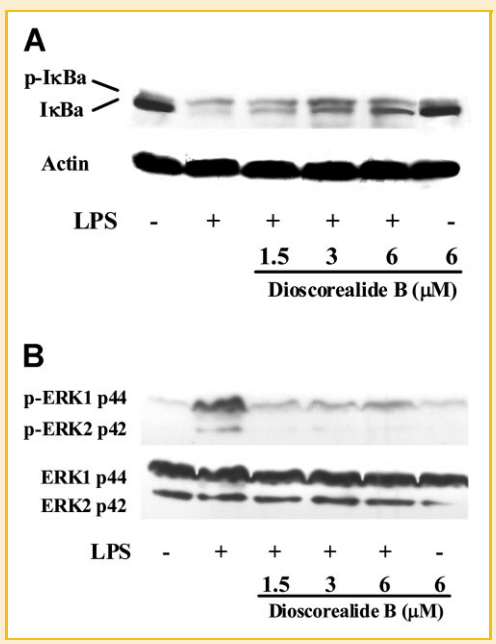

Fig. 5. The effects of Dioscorealide B on LPS-induced IκB and ERK1/2 MAPK activation pathways in RAW 264.7 cells. RAW 264.7 cells (10×10^6 cells/well in 10-cm dishes) were pre-incubated with Dioscorealide B (1.5–6.0 μM) for 30 min followed by stimulation with LPS (2.5 μg/ml). After 20 min of activation, total cell lysates were extracted and processed SDS–PAGE analysis. A: The degradation of IκBα was determined using p-IκBα and IκBα primary antibody. B: The activation of ERK1/2 proteins was evaluated using p-ERK1/2 and ERK1/2 primary antibody. These results represent one of three independent experiments with similar results.

DISCUSSION AND CONCLUSION

Macrophage activation has been reported as associated with a significant proportion of LPS-induced inflammations. During this process, active macrophages produce cytotoxic-inflammatory mediators, including reactive oxygen and nitrogen intermediates, hydrolytic enzymes, lipid mediators, and inflammatory cytokines [Laskin and Pendino, 1995]. In the present study, pre-treatment with DB was found to reduce NO accumulation in the culture medium of LPS-induced RAW 264.7 macrophage cells and this reduction was not due to the cytotoxic response (Fig. 2A–C). The inhibitory effect of DB was reduced when RAW 264.7 cells were treated after 3 h of LPS-stimulation (Fig. 2D). Previously, DB has been reported to have an inhibitory effect on NO and TNF-α production in RAW 264.7 cells [Tewtrakul and Itharat, 2007]. Therefore, we propose the explanation that the inhibition of DB has an effect at the transcriptional level.

In LPS-induced macrophages, NO is mainly produced from iNOS [Knowles and Moncada, 1994], which is synthesized using the NF-κB regulation pathway, similar to TNF-α and other pro-inflammatory cytokines such as IL-1β, IL-6, and IL-12 [Li and Verma, 2002]. To evaluate the mechanism of DB on gene expression, iNOS, IL-1β, and IL-6 mRNA were determined using RT-PCR. Our results show that LPS-induced iNOS, IL-1β, and IL-6 mRNA expression were significantly suppressed by DB (Fig. 3). DB also suppressed the expression of IL-10, which is known as a potent anti-inflammatory cytokine. The expression of IL-10 is under the control of the Sp1 transcription factor that is regulated by MAPK pathway [Chanteux et al., 2007]. Thus, our data indicate that DB inhibits activation of both the NF-κB pathway and the MAPK pathway.

NF-κB is a ubiquitous protein transcription factor which induces a variety of gene participations in the inflammatory processes [Li and Verma, 2002; Richmond, 2002]. Normally, NF-κB is present in the inactive form coupled with an IκB protein [Whiteside and Israel, 1997]. In the activation processes induced by LPS and other cytokines, IκB proteins are phosphorylated by IKKs, then ubiquitinated and further degraded by proteasome [Karin and Ben-Neriah, 2000]. After that, an active form of NF-κB proteins are released and translocated to activate the transcription of target genes in the nucleus [Baldwin, 1996]. In this report, we found that DB reduced the NF-κB-driven Luciferase activity in the LPS-induced pNFκB-Luc-transfected RAW 264.7 cells (Fig. 4). These results were due to the inhibition of IκBα degradation, which was determined using Western blot (Fig. 5A). In addition, treatment with DB did not alter the level of p-IκB proteins, which were present at basal levels (Fig. 5A, upper band). This result implies that the inhibitory effect of DB affects the upstream signaling pathway of IκBα activation, such as the IKK proteins, because the level of p-IκBα should increase in the presence of a downstream inhibitor such as a proteasome inhibitor [Takada et al., 2003].

The MAPK pathways include ERK1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK. These signaling proteins in turn activate a variety of transcription factors, such as Sp1, which coordinate the induction of gene-encoding IL-10, the potent anti-inflammatory cytokine [Chanteux et al., 2007]. In this study, active ERK1/2

proteins (p-ERK1/2) were significantly inhibited by DB (Fig. 5B). This result suggests that the reduction of IL-10 mRNA is a consequence of the inhibition of ERK1/2 activation by DB. Although the effective anti-inflammatory cytokine IL-10 is also inhibited by DB, a recent study has reported that the IL-10 receptor is dysfunctional during chronic inflammation [Avdiushko et al., 2001].

Focusing on the stimulation of macrophages, LPS induces the activation of a protein cascade through the TLR4-CD14 complex [Takeda et al., 2003], which regulates the downstream signaling transduction to TAK1 [West et al., 2006]. At this point, TAK1 activates the cascade protein in both the IKKs and MAPK signaling pathways [Lee and Kim, 2007]. Our results show that DB inhibits both the IKKs and ERK1/2 activation pathways. These data lead to the hypothesis that the inhibitory effect of DB may have an effect at the upstream signaling pathway on the activation of IKKs and MEK1/2 proteins such as the TAK1 protein. As well, the point at which natural compounds may act as non-specific kinase inhibitors, such as Quercetin [Davies et al., 2000], is interesting, an area we recommend for further investigation.

In conclusion, the present study demonstrates that DB exerts significant anti-inflammation through the reduction of NO production through the suppression of iNOS expression as well as pro-inflammatory mediators, including IL-1β and IL-6, at the transcriptional level. The inhibitory effects of DB interfere the activation of NF-κB transcription factor by the inhibition of IκBα phosphorylation. In addition, DB also inhibits the activation of the ERK/MAPK pathway, which participates in the expression of IL-10. In conclusion, our studies provide the support of anti-inflammatory effects of *D. membranacea* (Peirre ex Prain & Burkill) or Hua-Kao-Yen commonly prescribed in Thai Traditional Medicine.

ACKNOWLEDGMENTS

The University Development Commission (UDC) scholarship granted to P.H. and the financial support to the Ph.D. program in Biomedical Sciences, PSU (during 2002–2006) from the Commission of Higher Education (CHE), Thailand are kindly acknowledged.

REFERENCES

Avdiushko R, Hongo D, Lake-Bullock H, Kaplan A, Cohen D. 2001. IL-10 receptor dysfunction in macrophages during chronic inflammation. J Leukoc Biol 70:624–632.

Baldwin AS, Jr. 1996. The NF-kappa B and I kappa B proteins: New discoveries and insights. Annu Rev Immunol 14:649–683.

Carrigan SD, Scott G, Tabrizian M. 2004. Toward resolving the challenges of sepsis diagnosis. Clin Chem 50:1301–1314.

Chanteux H, Guisset AC, Pilette C, Sibille Y. 2007. LPS induces IL-10 production by human alveolar macrophages via MAPKinases- and Sp1-dependent mechanisms. Respir Res 8:71.

Davies SP, Reddy H, Caivano M, Cohen P. 2000. Specificity and mechanism of action of some commonly use protein kinase inhibitors. Biochem J 351: 95–105.

Itharat A, Plubrukran A, Kongsaeree P, Buti T, Keawpradub N, Houghton PJ. 2003. Dioscorealides and dioscoreanone, novel cytotoxic napthofuranoxepins, and 1, 4- phenanthraquinone from *Dioscorea membranacea*, Pierre. Org Lett 5:2879–2882.

Itharat A, Houghton PJ, Eno-Amooquave E, Burke P, Sampson J, Raman A. 2004. In vitro cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. J Ethnopharmacol 90:33–38.

Karin M, Ben-Neriah Y. 2000. Phosphorylation meets ubiquitination: The control of NF-kappa B activity. Annu Rev Immunol 18:621–663.

Knowles RG, Moncada S. 1994. Nitric oxide synthases in mammals. Biochem J 298(Pt2): 249–258.

Laskin DL, Pendino KJ. 1995. Macrophages and inflammatory mediators in tissue injury. Annu Rev Pharmacol Toxicol 35:655–677.

Lee Ms, Kim YJ. 2007. Signaling pathways downstream of pattern-recognition receptors and their cross talk. Annu Rev Biochem 76:447–480.

Li Q, Verma IM. 2002. NFkappa B regulation in the immune system. Nat Rev Immunol 2:725–734.

Nathan C, Xie QW. 1994. Regulation of biosynthesis of nitric oxide. J Biol Chem 269:13725–13728.

Ricciardolo FL, Sterk PJ, Gaston B, Folkerts B. 2004. Nitric oxide in health and disease of the respiratory system. Physiol Rev 84:731–765.

Richmond A. 2002. NF-kappa B, chemokine gene transcription and tumour growth. Nat Rev Immunol 2:664–674.

Robert L, McColl GJ. 2004. Tumour necrosis factor inhibitors: Risks and benefits in patients with rheumatoid arthritis. Intern Med J 34:687–693.

Siebenlist U, Franzoso G, Brown K. 1994. Structure, regulation and function of NF-kappa B. Annu Rev Cell Biol 10:405–455.

Takada Y, Mukhopadhyay A, Kundu GC, Mahabeleshwar GH, Singh S, Aggarwal BB. 2003. Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: Evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase. J Biol Chem 278:24233–24241.

Takeda K, Kaisho T, Akira S. 2003. Toll-like receptors. Annu Rev Immunol 21:335–376.

Tewtrakul S, Itharat A. 2007. Nitric oxide inhibitory substances from the rhizomes of *Dioscorea membranacea*. J Ethnopharmacol 109:412–416.

Tewtrakul S, Itharat A, Rattansuwan P. 2006. Anti-HIV-1 protease- and HIV-1 integrase activities of Thai medicinal plants known as Hua-Khao-Yen. J Ethnopharmacol 105:312–315.

Wang J, Fu YX. 2005. Tumor necrosis factor family members and inflammatory bowel disease. Immunol Rev 204:144–155.

West AP, Koblansky AA, Ghosh S. 2006. Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol 22:409–437.

Whiteside ST, Israel A. 1997. I kappa B proteins: Structure, function and regulation. Semin Cancer Biol 8:75–82.