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Dioscoreanone Suppresses LPS-Induced Nitric Oxide Production and Inflammatory Cytokine Expression in RAW 264.7 Macrophages by NF-κB and ERK1/2 Signaling Transduction

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ABSTRACT

Dioscoreanone, a 1,4-phenanthraquinone isolated from an ethanolic extract of the rhizome of *Dioscorea membranacea*, Pierre ex Prain & Burkill, a plant which has been used to treat inflammation and cancer in Thai Traditional Medicine. In this study, the mechanisms of dioscoreanone on LPS-induced NO production and cytokine expression through the activation of NF-κB and ERK1/2 are demonstrated in RAW 264.7 cells. Through measurement with Griess reagent, dioscoreanone was found to reduce NO levels with an IC_{50} value of 2.50 ± 0.64 μM, due to the significant suppression of LPS-induced iNOS mRNA expression, as well as IL-1β and IL-6 levels at a concentration of 6 μM. At the signal transduction level, using the pNFκB-Luciferase reporter system, dioscoreanone significantly inhibited NF-κB transcriptional activity, which resulted from the prevention of IκBα degradation. In addition, dioscoreanone in the range of 1.2–5 μM significantly enhanced LPS-induced ERK1/2 activation and dioscoreanone alone induced the activation of ERK1/2 proteins in a concentration- and time-dependent response. The activation of ERK1/2 proteins by dioscoreanone was due to both an arylating reaction, which was suppressed by *N*-acetyl cysteine, and a redox cycling reaction of NQOR, which was inhibited by dicoumarol. In conclusion, the mechanisms of dioscoreanone on the inhibition of NO production and mRNA expression of iNOS, IL-1β, and IL-6 were due to both the inhibition of NF-κB activation and the activation of ERK1/2 proteins. The activation of dioscoreanone may in turn inhibit the binding of NF-κB to pro-inflammatory gene promoters in LPS-induced RAW264.7 macrophage cells. J. Cell. Biochem. 113: 3427–3435, 2012.

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

Macrophage accumulation and the associated release of mediators have been shown to participate in inflammation [Laskin and Pendino, 1995]. The up-regulation of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, and IFN-γ in turn enhances the production of toxic reactive nitrogen species (RNS). Nitric oxide (NO) is one of the critical RNSs continuously produced from 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 [Roberts 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].

Quinone compounds have a number of biological activities which can be exploited for anti-cancer effects [Klotz et al., 2002; Abdelmohsen et al., 2003; Sandur et al., 2006] and to reduce inflammation [Shin et al., 2006]. Dioscoreanone, a 1,4 phenanthraquinone is found in the dried rhizome of *Dioscorea membranacea*, Pierre ex Prain & Burkill [Itharat et al., 2003]. This plant is

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included in 2,449 Thai traditional medicine recipes for the treatment of bone and joint diseases, cancer-related inflammation and other diseases such as lymphopathy, dermopathy, venereal diseases, and leprosy [Itharat, 2002]. Previously, dioscoreanone has been reported to have a cytotoxic effect against cancer cell lines [Itharat et al., 2003], the inhibition of β-hexosaminidase, TNF-α, and IL-4 production from an RBL-2H3 cell line [Tewtrakul and Itharat, 2006] and the inhibition of HIV1 protease and integrase activity [Tewtrakul et al., 2006]. Recently, the inhibitory effect of dioscoreanone on NO and TNF-α production was shown in LPS-induced RAW 264.7 macrophage cells [Tewtrakul and Itharat, 2007].

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, especially myeloid differentiation protein (MyD88) [West et al., 2006]. MyD88 associates with two serine/threonine 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 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 genes 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] while, Sp1 transcription factor, which is induced by the activation of MAPK pathway, participates in the up-regulation of IL-10, the well-known anti-inflammatory cytokine [Chanteux et al., 2007].

Based on previous data, we hypothesized that dioscoreanone may exert an anti-inflammatory activity through the inhibition of inflammatory mediators production at the transcriptional level. In this study, we evaluated the effects of dioscoreanone on the activation of the NF-κB and ERK1/2 MAPK signaling pathways, which correlate with induced 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), penicillin–streptomycin (10,000 U/ml) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY) and BIOCHROM AG (Berlin, Germany), respectively. Sulfanilamide, *o*-phosphoric acid (H_2PO_4), *N*-(1-napthyl)-ethylenediamine dihydrochloride, and sodium nitrite ($NaNO_2$) were obtained from Merck (NJ). LPS from *Escherichia coli* serotype O111: B4, butylated hydroxytoluene (BHT), L-ascorbic acid, Trolox, 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). Path Detect 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-IκBα, anti-p-ERK, anti-ERK1, anti-ERK2, and anti-mouse and anti-rabit IgG were purchased from Santa Cruz biotechnology (Santa Cruz, CA). Anti-actin antibody was obtained from Sigma.

ISOLATION OF DIOSCOREANONE FROM THE ETHANOL EXTRACT OF *D. MEMBRANACEA*

Dioscoreanone was extracted from rhizomes of *D. membranacea* Pierre ex Prain & Burkill 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 [SKP A062041305], 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 dioscoreanone [Tewtrakul and Itharat, 2007]. The structure of the pure compound (Fig. 1) was confirmed by comparing it with previously reported ^{1}H- and ^{13}C-NMR spectral data [Itharat et al., 2003]. The purified dioscoreanone was dissolved in DMSO (5 mM). In all experiments DMSO never exceeded 0.2%.

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 dioscoreanone at various concentrations [50,10, 5, 2.5, 1.2, 0.6 μg/ml] 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 dihydrochloride (0.2%, w/v) was

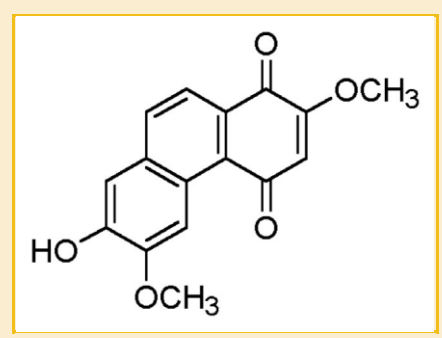

Fig. 1. The chemical structure of dioscoreanone (DN) isolated from the rhizomes of *Dioscorea membranacea* Pierre ex Prain & Burkill.

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 0–100 μM $NaNO_2$.

MEASUREMENT OF CELL VIABILITY BY MTT ASSAY

The MTT assay was performed to determine cell viability. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide 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. Protected from light, 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 dioscoreanone 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 six-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 dioscoreanone 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, 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 cDNAs obtained 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 successively 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).

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 for a transfection period of 24 h. After that, the cell culture medium was removed and replaced with fresh medium containing various concentrations of dioscoreanone for 30 min, followed by co-incubation with 2.5 μg/ml of LPS for 6 h. The total cell proteins were then extracted and luciferase activity was measured using luciferase assay kits and following the manufacturer's protocol. The chemiluminescence light intensity was measured using a Tropix TR 717 Luminometer Plate Reader (Applied Biosystems, Foster City, CA).

TABLE I. Primer Sequences for Amplification

Targets	Primer sequence	Product (bp)	T_m (°C)
β-Actin			
F-primer	5′-GACTACCTCATGAAGATCCT-3′	510	45
R-primer	5′-CCA CAT CTG CTG GAAGGTGG-3′		
iNOS			
F-primer	5′-ATGTCCGAAGCAAACATCAC-3′	450	45
R-primer	5′-TAATGTCCAGGAAGTAGGTG-3′		
IL-1β			
F-primer	5′-AAGCTCTCCACCTCAATGGA-3′	301	45
R-primer	5′-TGCTTGTGAGGTGCTGATGT-3′		
IL-6			
F-primer	5′-TTCCATCCAGTTGCCTTCTTGG-3′	360	45
R-primer	5′-CTTCATGTACTCCAGGTAG-3′		
IL-10			
F-primer	5′-GTGAAGACTTTCTTTCAAACAAAG-3′	273	45
R-primer	5′-CTGCTCCACTGCCTTGCTCTTATT-3′		

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

The RAW 264.7 cells (1×10^7 cells/dish) were cultured in 10-cm dishes and allowed to adhere for 24 h. After treatment with dioscoreanone, 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) 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 using the Bradford method standardized with BSA. Sample aliquots (100 μg of total proteins/lane) were separated on 12% SDS–polyacrylamide gel 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 dried 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-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. The membranes were then washed three times for 10 min each time with 1% milk TTBS. Finally, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary 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 dioscoreanone 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 were analyzed by ANOVA (LSD and Turkey test in SPSS program), and a P-value < 0.05 was considered significant.

RESULTS

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

Murine macrophages RAW 264.7 in resting state released 0.85 ± 0.27 μM of NO into culture media during incubation for 24 h, whereas upon exposure to LPS the cells markedly increased NO production to 10.29 ± 0.32 μM (in the presence of DMSO as a vehicle). An half an hour of pre-treatment with dioscoreanone (DN)-inhibited LPS-induced NO production in a dose-dependent manner, with IC_{50} values of 2.50 ± 0.64 μM (Fig. 2A). No significant difference in NO level was found between resting RAW 264.7 cells and the cells treated with 10 μM of DN. At the concentration tested, DN did not show any cytotoxicity to RAW 264.7 cells in the MTT and trypan blue exclusion assays, indicating that any inhibitory effects of this compound on NO production were not due to their non-specific cell toxicity (Fig. 2B,C). To investigate whether the inhibition action of DN on NO production was reversible, DN (2.5 μM) was added to the RAW 264.7 cells at various time points, from 3 h pre-treatment to 18 h post-treatment, and then the level of NO was determined 24 h after stimulation with LPS. After the 24-h period of LPS exposure, a significant inhibitory effect of DN was

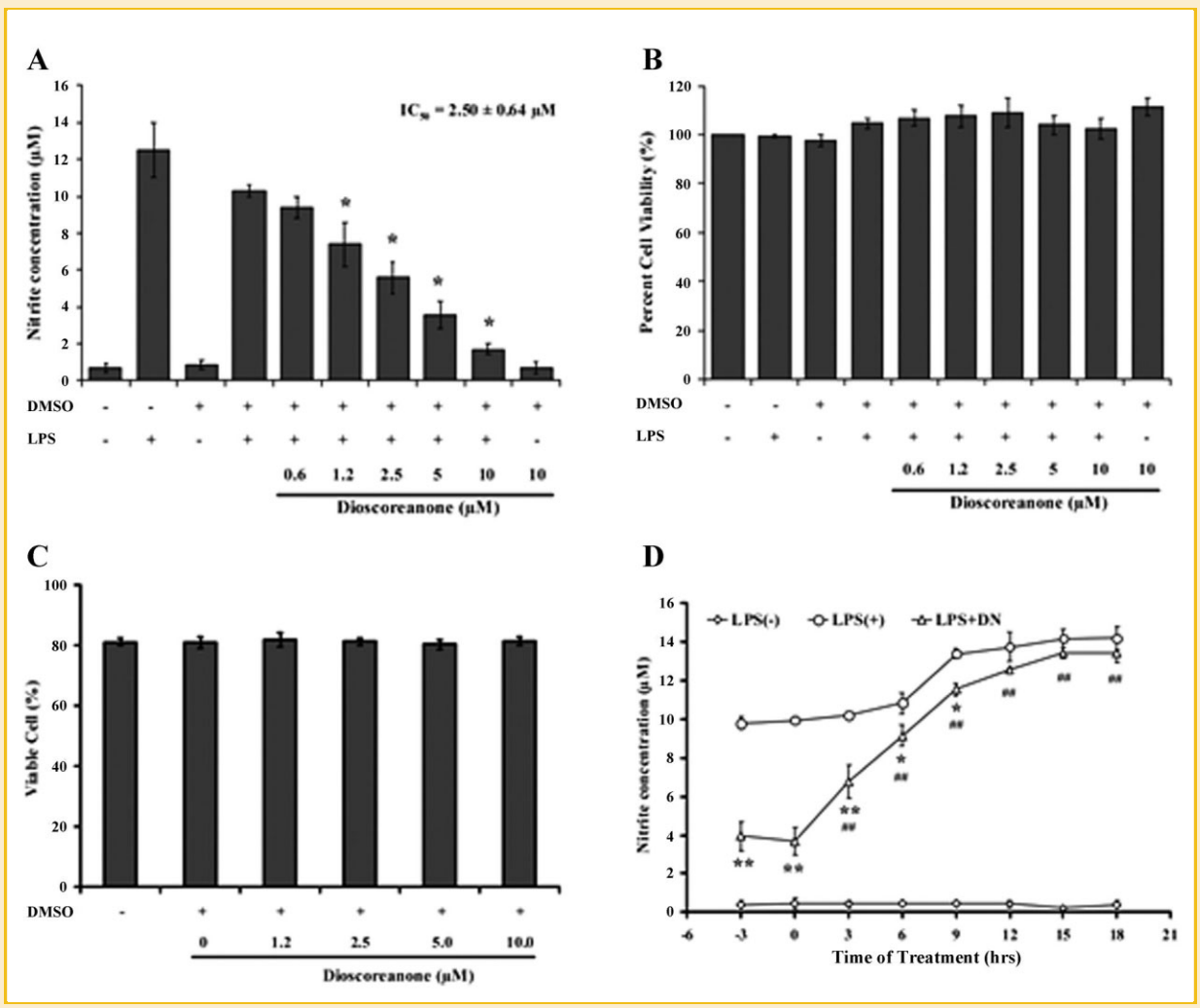

Fig. 2. The effects of dioscoreanone (DN) on the LPS-induced NO production in RAW 264.7 cells. A: RAW 264.7 cells (1×10^5 cells/well) were treated with various concentrations of DN for 30 min prior to stimulation with 2.5 μg/ml LPS for 24 h. NO in the culture medium was measured as described in Materials 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 DN were trypsinized and determined by trypan blue exclusion assay. D: DN at 2.5 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 and $\#\#P < 0.01$ compared with the treatment of DN at time zero.

found at all the times tested up to 9 h [asterisk (*) in Fig. 2D, $P < 0.05$] and the inhibitory effect was significantly reversed when DN was added more than 3 h after LPS stimulation [hash (#) in Fig. 2D, $P < 0.05$].

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

To investigate whether DN could affect NO production due to the suppression of iNOS, semi-quantitative RT-PCR was carried out with LPS-stimulated RAW 264.7 murine macrophages. Figure 3 shows that iNOS mRNA transcription was slightly detectable in resting RAW 264.7 cells, but markedly increased (at least 1.5-fold from basal level) upon exposure to LPS alone. DN at dose 5.0 μM significantly attenuated LPS-induced expression of iNOS transcription with 89.21 ± 15.64% inhibition. In addition, the exposure of LPS to RAW 264.7 macrophages also stimulated the transcription of inflammatory cytokines including IL-1β, IL-6, and IL-10. Figure 3 shows that DN at 5.0 μM suppressed the transcription of IL-1β and IL-6 with 43.00 ± 0.82 and 85.08 ± 2.60% inhibition, respectively. In contrast, no difference was found in the inhibitory effect of DN on IL-10 mRNA expression. DN alone did not alter mRNA levels of iNOS, IL-1β, IL-6, and IL-10 (data not shown).

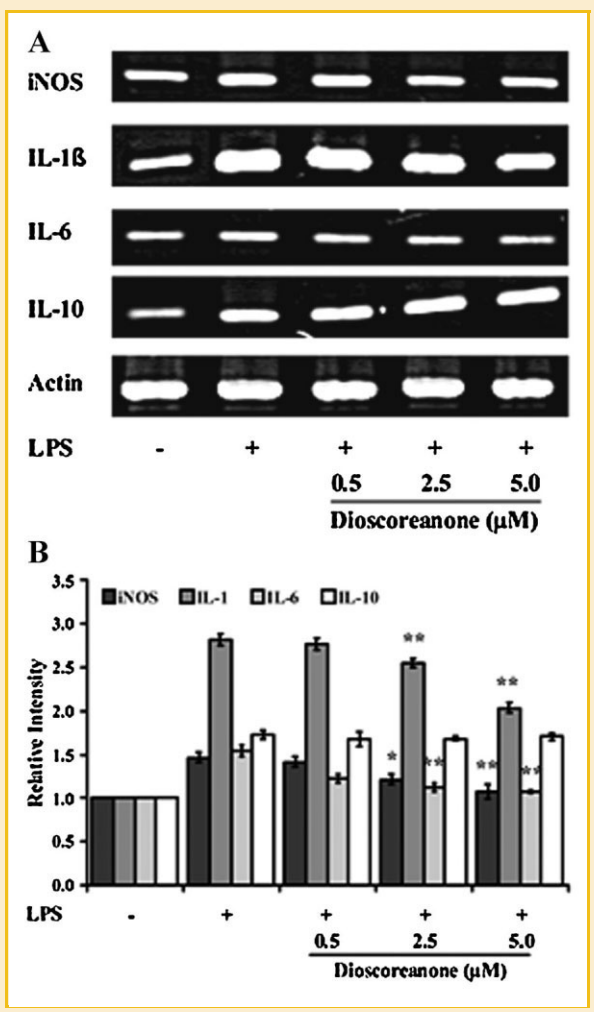

Fig. 3. The effects of dioscoreanone (DN) on LPS-induced mRNA expression of iNOS and inflammatory cytokines in RAW 264.7 cells. A: RAW 264.7 cells (2×10^6 cells/well in six-well plates) were pre-treated with various concentrations of DN for 30 min prior to stimulation with 2.5 μg/ml LPS for 9 h. Total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

THE EFFECT OF DIOSCOREANONE ON NF-κB-DRIVEN LUCIFERASE ACTIVITY IN pNFκB-LUC-TRANSFECTED RAW 264.7 CELLS

NF-κB activation is essential for the expression of various pro-inflammatory genes, including the iNOS, IL-1β, and IL-6 genes, which contain several binding sites for NF-κB in their promoter region [Siebenlist et al., 1994]. Since DN-inhibited transcriptional activation of iNOS, IL-1β, and IL-6 gene induced by LPS, we decided to analyze the effect of DN on LPS-induced NF-κB transcriptional activity. RAW 264.7 murine macrophages were transfected with pNFκB-Luc construct encoding five repeated NF-κB-binding consensus sequences (5′-TGGGGACTTTCCGC-3′) fused to the *Photinus pyralis* (firefly) Luciferase gene as a reporter. Upon exposure to LPS alone for 6 h, the transfected RAW 264.7 cells increased luciferase expression 7.29 ± 0.51-fold over the basal level (Fig. 4A), indicating that cellular NF-κB is transcriptionally functional. DN at doses of 1.2, 2.5, and 5.0 μM significantly inhibited LPS-induced Luciferase activity in a dose-dependent manner, with 24%, 24%, and 41% inhibition, respectively.

THE EFFECT OF DIOSCOREANONE ON THE ACTIVATION OF IκBα PROTEIN IN LPS-INDUCED RAW 264.7 CELLS

The phenomenon of NF-κB transcription activity is proceeded by the phosphorylation and proteolytic degradation of IκBα. We next determined whether DN could affect LPs-induced IκBα degradation, using Western immunoblot analysis with total cell extract of RAW 264.7 murine macrophages. Upon exposure to LPS alone, IκBα was dramatically degraded within 20 min, and then recovered to the normal level at 30 min after LPS stimulation (data not show). DN at doses of 1.2–5.0 μM significantly inhibited IκBα degradation and reversed it to the basal level (Fig. 4B).

THE EFFECT OF DIOSCOREANONE ON THE ACTIVATION OF ERK1/2 PROTEIN IN LPS-INDUCED RAW 264.7 CELLS

DN-inhibited IL-1β and IL-6 mRNA expression but did not suppress IL-10 [Fig. 3A]. The exposure to LPS of macrophages also stimulates the activation of JNK and p38 MAPKs, leading to the expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [West et al., 2006]. In contrast, the activation of ERK proteins leads to the inhibition of NF-κB-binding activity [Xiao et al., 2002] and the expression of anti-inflammatory cytokine IL-10 [Chanteux et al., 2007]. We proposed that DN may affect the activation of ERK1/2 pathway. So the effect of DN on LPS-induced phosphorylation of ERK proteins was evaluated by Western blot analysis in RAW 264.7 macrophages. The phosphorylation of ERK proteins (p-ERK1/2) was slightly detected at resting state of RAW 264.7 cells. Upon exposure to LPS, p-ERK1/2 protein levels were increased dramatically in a time-dependent response. The maximal level of p-ERK1/2 was found at 20 min and declined at 50 min after LPS stimulation (data

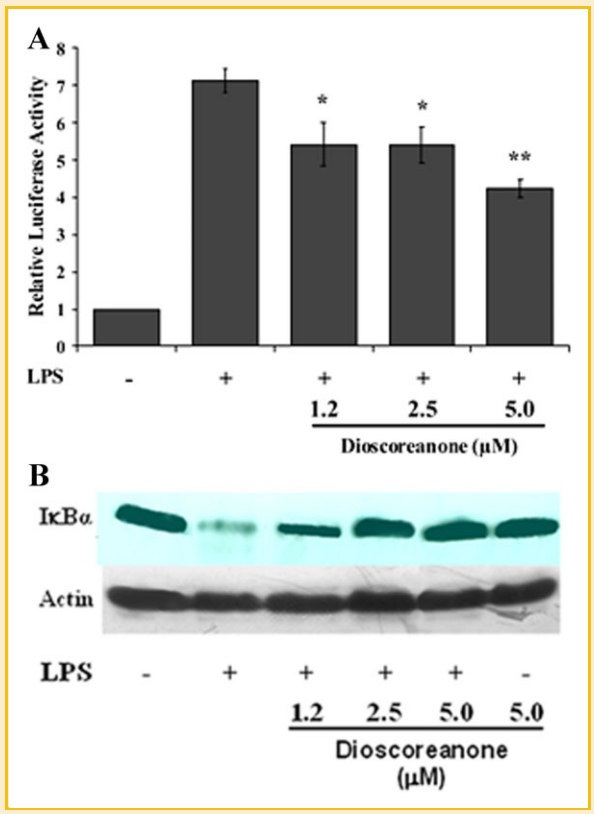

Fig. 4. The effects of dioscoreanone (DN) on LPS-induced NF-κB activation in RAW 264.7 cells. A: RAW 264.7 cells (1×10^5 cells/well in 48-well plates) were transfected with pNFκB-Luc plasmid for 24 h and then pre-incubated with DN for 30 min followed by stimulation with 2.5 μg/ml LPS. After 6 h of activation, total cells were extracted and Luciferase activity determined as described in Materials and Methods Section. The relative light emission was expressed as mean ± SEM derived from three independent experiments. $^*P < 0.05$ (B) RAW 264.7 cells (10×10^6 cells/well in 10-cm dishes) were pre-incubated with DN (1.2–5.0 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. The degradation of IκBα was determined using IκBα primary antibody. These results represent one of three independent experiments with similar results.

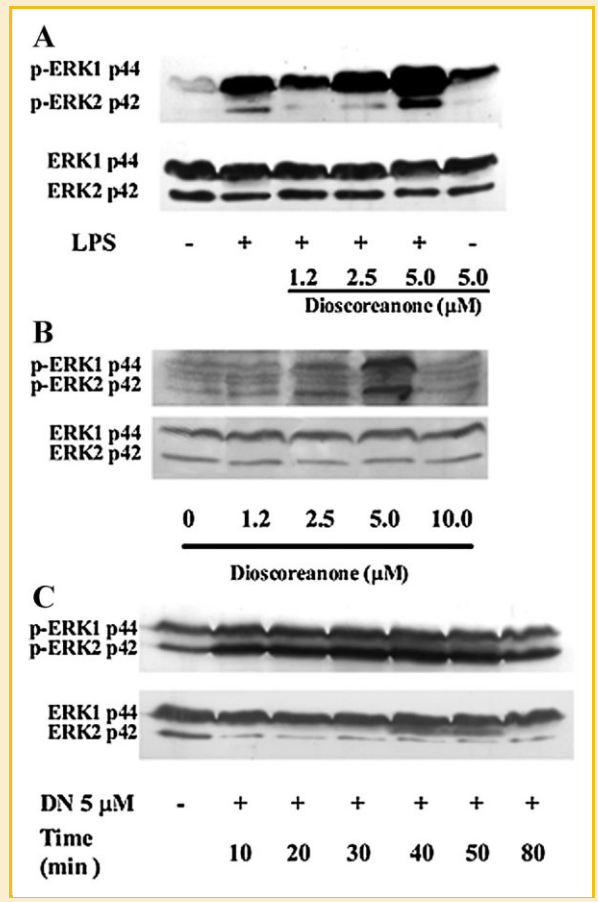

Fig. 5. The effects of dioscoreanone (DN) on LPS-induced ERK1/2 MAPK activation pathways in RAW 264.7 cells. A: RAW 264.7 cells (10×10^6 cells/well in 10-cm dishes) were pre-incubated with DN (1.2–5.0 μM) for 30 min followed by stimulation with LPS (2.5 μg/ml). After 20 min of activation, total cell lysates were extracted and processed SDS–PAGE analysis. The activation of ERK1/2 proteins was evaluated using p-ERK1/2 and ERK1/2 primary antibody. RAW 264.7 cells were treated with DN alone to determine (B) a concentration-dependent response and (C) a time-course response on ERK1/2 activation using Western blot. These results represent one of three independent experiments with similar results.

not show). After 20 min of LPS exposure, pre-treatment of DN slightly inhibited p-ERK1/2 level at dose 1.2 μM without significant but the activation of ERK1/2 proteins was increased in dose-response effect at doses of 2.5 and 5.0 μM (Fig. 5A). The induction of p-ERK1/2 level was also represented by the pre-treatment of DN alone at a dose of 5.0 μM as shown in lane 6 of Figure 5A.

THE EFFECT OF DIOSCOREANONE ON THE ACTIVATION OF ERK1/2 PROTEINS IN RAW 264.7 MURINE MACROPHAGES

The result described immediately above showed that DN alone could induce the activation of ERK1/2 protein. So, the dose- and time-effects of DN on p-ERK1/2 protein level were evaluated using immunoblot analysis. As shown in Figure 5B, ERK1/2 proteins were significantly activated by 1.2–5.0 μM of DN in a dose-dependent response. The maximal level of p-ERK1/2 proteins was found when RAW 264.7 cells were treated with 5.0 μM of DN. The stimulation activity of DN on ERK1/2 proteins declined at 10 μM, but this result was not affected by the alteration of total ERK1/2 proteins. In addition, the time-dependent activation response of DN at 5.0 μM is shown in Figure 5C. After exposure to DN at dose of 5.0 μM for 10 min, the phosphorylation level of ERK1/2 proteins significantly increased. The maximal levels of DN-induced ERK1/2 activation were maintained from 20 to 40 min and then declined at 80 min. The time-dependent response of DN-induced ERK1/2 activation was not due to the significant change of ERK1/2 proteins.

THE MECHANISM OF DIOSCOREANONE ON THE ACTIVATION OF ERK1/2 PROTEINS IN RAW 264.7 MURINE MACROPHAGES

Quinone compounds can induce the activation of MAPK signaling pathway by either a redox cycling reaction, which is inhibited by dicoumaral (Dic) [Klotz et al., 2002], or arylation of a sulfhydryl

group, which is inhibited by *N*-acetyl cysteine (NAC) [Abdelmohsen et al., 2003]. To test for the involvement of either arylation or redox cycling reaction in the activation of ERK1/2, RAW 264.7 macrophages were pre-treated with NAC or Dic for 15 min and then stimulated with 5.0 μM of DN. As shown in Figure 6A, NAC or Dic alone did not affect the activation of ERK1/2 proteins (lanes 2 and 3 vs. lane 1). In the presence of DN (5.0 μM) alone, the level of p-ERK1/2 proteins was significantly increased (lane 4 compared to lane 1). The DN-induced ERK1/2 activation was inhibited by Dic as shown in lane 5 (vs. lane 4) of Figure 6A, whereas NAC dominantly inhibited DN-induced ERK1 activation and slightly inhibited ERK2 activation (lane 6 compare to lane 4).

The catalyzing groove of MAPK phosphatase consists cysteine as an active site [Farooq and Zhou, 2004], which can be arylated with quinone compounds [Abdelmohsen et al., 2003], leading to accumulation of the active ERK1/2 proteins in cytoplasm. Previous results suggested that the activation of ERK1/2 proteins by DN was effected by the arylation of the sulfhydryl group. To confirm this evidence, the time-course effect of LPS in the presence of DN was determined. Figure 6B shows that ERK1/2 phosphorylation was observed at 10 min and declined at 50 min after LPS stimulation (lanes 2–4). Pre-treatment with DN for 30 min increased the level of p-ERK1/2 from 10 to 20 min with a decrease 50 min after LPS stimulation (Fig. 6B, lanes 5–7). At 50 min of LPS activation, the phosphorylated form of ERK1/2 in the presence of DN pre-treatment had more intensity than LPS alone (Fig. 6B, lane 7 vs. lane 4). When RAW 264.7 cells were co-cultured with DN and LPS for 20 min, the level of p-ERK1/2 protein was more than LPS alone or DN alone at the same time (lane 8 compared with lane 3 and lane 6).

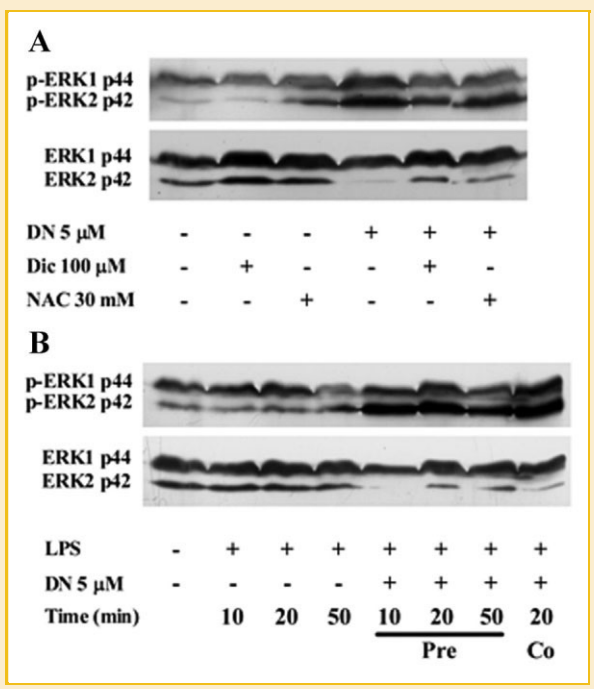

Fig. 6. The mechanism of dioscoreanone on the activation of ERK1/2 proteins. A: RAW 264.7 macrophages (10×10^6 cells/well) were seeded in 10-cm dishes and allowed to adhere for 24 h. The adhered cells were pre-treated with either *N*-acetyl cysteine (NAC) or Dicoumarol (Dic) for 15 min prior to stimulation with DN for 20 min and then the total proteins were collected. B: RAW 264.7 cells were treated with LPS in the absence of DN, 30-min pre-treatment (Pre) with DN or co-incubation (Co) with DN. The total proteins were collected for the indicated times. The activation of ERK1/2 protein was analyzed using Western blot with anti-p-ERK1/2 and anti-ERK1/2 primary antibody. These results represent one of three independent experiments with similar results.

DISCUSSION AND CONCLUSION

In the present study, DN (1–4 phenantraquinone, Fig. 1) was found to have dose-dependent inhibitory effects on LPS-induced NO production in murine macrophages RAW 264.7 (Fig. 2A), these were not due to the cytotoxic action of DN (Fig. 2B,C). The inhibitory effects of DN on NO production were reversed after 3 h of LPS activation (Fig. 2D), indicating that DN affected the signal transduction. Using semi-quantitative PCR, DN-attenuated LPS-induced expression of iNOS, IL-1β, and IL-6 mRNA (Fig. 3). Previously, DN has been reported to have an inhibitory effect on NO and TNF-α production in RAW 264.7 cells [Tewtrakul and Itharat, 2007]. These data suggested that DN-inhibited NO production at the signaling pathway, leading to reduce the transcription of inflammatory genes.

In LPS-induced macrophages, the transcription of inflammatory mediators such as iNOS, IL-1β, IL-6, and IL-12 is mediated by the regulation of the NF-κB signaling pathway [Li and Verma, 2002]. NF-κB is a ubiquitous protein transcription factor which induces a variety of gene participations in the inflammatory processes [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 IκB Kinase (IKKs), then ubiquitinated and further degraded by proteasome [Karin and Ben-Neriah, 2000]. After that, an active form of NF-κB protein is released and translocated to activate the transcription of target genes in the nucleus [Baldwin, 1996]. In this report, we found that DN-inhibited NF-κB binding activity determined using reporter assay (Fig. 4A). This is due to the inhibition of IκBα degradation, which was determined using Western blot (Fig. 4B). In addition, treatment with DN did not alter the levels of p-IκB proteins, which were present at basal levels (data not shown), implying that the inhibitory effect of DN 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].

Additionally, the mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases which are part of the signal transduction pathways, and connect the extracellular signal of LPS to intracellular response in macrophages [Lee and Kim, 2007]. p38 MAPK and JNK are members of the MAPK family that regulate inflammatory proteins as well as immune responses and expression of various pro-inflammatory mediators, for example, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and iNOS [Hommes et al., 2003]. In contrast, ERK1/2 MAPK members

were suggested to exert anti-inflammatory signaling in macrophages [Watters et al., 2002] by inducing the expression of IL-10 [Chanteux et al., 2007] and activation of MAPK phosphatase 1 (MKP-1) which interfere with NF-κB-binding activity [Chen et al., 2002; Xiao et al., 2002]. In this study, DN-inhibited iNOS, IL-1β, and IL-6 but did not have an effect on IL-10 expression. The up-regulation of LPS-induced ERK1/2 activation was enhanced by DN in dose-dependent manner (Fig. 5A). The results implied that LPS-induced NF-κB-binding activity may be inhibited via the activation of ERK1/2 proteins and DN maintains the expression of IL-10, the anti-inflammatory cytokine.

Using Western blot analysis, DN alone induced the activation of ERK1/2 proteins in the absence of LPS in dose- and time-response (Fig. 5B,C). These phenomenon were not due to the LPS contamination that was rule out since DN alone did not increase NO production (Fig. 2A) and all determined mRNA (data not shown). This mechanism of DN may involve the quinone group which is part of its structure. Quinone compounds such as menadione, *p*-benzoquinone, and dimethoxy napthoquinone have been reported to induce ERK1/2 activation by two pathways. The first one depends on the arylation of the nucleophilic thiol group, leading to the inactivation of protein phosphatase and an increase in intracellular oxidative stress by reducing the intracellular anti-oxidant glutathione (GSH) [Abdelmohsen et al., 2003]. The second pathway, also called redox cycling, is elicited by NAD(P)H: quinone oxidoreductase (NQOR) and consequently produces the superoxide anion which in turn activates the phosphorylation of ERK1/2 proteins [Klotz et al., 2002]. When NAC and Dic were tested as inhibitors of arylation and redox cycling reactions in the presence of DN, the activation of ERK1 protein was inhibited by both compounds, whereas the activation of ERK2 protein was only suppressed by NAC (Fig. 6A). These data suggest that the mechanism of DN on the activation of ERK1 was mediated by the stress induction elicited through the activity of NQOR and arylation with sulfhydryl group. In contrast, the activation of ERK2 by DN was mediated through redox cycling reaction alone. In addition, the decline phase of LPS-induced ERK1/2 activation was delayed with pre-treatment with DN, as shown in Figure 6B. These data confirmed the ability of DN to conjugate with the sulfhydryl group of amino acids, such as cysteine, in the catalytic grove of phosphatase [Farooq and Zhou, 2004], leading to inhibition of phosphatase activity [Abdelmohsen et al., 2003] and accumulate the active ERK1/2 proteins.

In conclusion, the present study demonstrates the inhibitory effect of dioscoreanone at both transcription and signal transduction level. Dioscoreanone exerts a significant anti-inflammatory effect through the reduction of NO production, which is due to the suppression of iNOS expression, as well as suppression of pro-inflammatory mediators, including IL-1β and IL-6, at the transcriptional level. At the signal transduction level, dioscoreanone inhibits IκBα degradation and enhances ERK1/2 activation, which suppresses LPS-stimulated NF-κB-binding activity. The mechanisms of dioscoreanone are stress induction, through both the redox cycling reaction of NQOR and the arylation of the sulfhydryl group, leading to reduced cellular antioxidant levels and inhibition of MAPK phosphatase.

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