 DOI 10.1002/mnfr.200800583 *Mol. Nutr. Food Res.* 2009, *53*, 1386–1395

RESEARCH ARTICLE

Differential inhibitory effects of inotilone on inflammatory mediators, inducible nitric oxide synthase and cyclooxygenase-2, in LPS-stimulated murine macrophage

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The inhibitory effects of inotilone and methylinotilone on the induction of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in murine RAW 264.7 cells activated with LPS were investigated. The results show that both hydroxyl groups on the benzene ring of the inotilone molecule are required for better anti-inflammatory effect. Western blotting and RT-PCR analyses demonstrated that inotilone blocked protein and mRNA expression of iNOS but not COX-2. Instead, inotilone inhibited prostaglandin E_2 production through decreasing the enzyme activity of COX-2. The repression of iNOS but not COX-2 expression may come from the differential effect of inotilone on nuclear factor-κB (NFκB) and CCAAT/enhancer-binding protein beta Treatment with inotilone resulted in the reduction of LPS-induced nuclear translocation of NFκB subunit and the NFκB-dependent transcriptional activity by blocking phosphorylation of inhibitor κB(IκB)α and p65 and subsequent degradation of inhibitor κBα. Inotilone also inhibited LPS-induced activation of PI3K/Akt and extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase. Our results suggest that inotilone may have potential to be developed into an effective anti-inflammatory agent.

Received: December 20, 2008
Revised: February 22, 2009
Accepted: February 28, 2009

Keywords:
CCAAT/enhancer-binding protein beta / Cyclooxygenase-2 (COX-2) / Inducible NO synthase (iNOS) / Inotilone / LPS

1 Introduction

Inflammation and infections lead to the up-regulation of a series of enzymes and signaling proteins in affected tissues and cells. Inducible nitric oxide synthase (iNOS), a member of the NOS protein family, catalyzes the formation of nitric oxide (NO) from L-arginine [1]. Low concentration of NO produced by iNOS are likely to contribute to the anti-microbial activity of macrophages against certain bacterial pathogens. However, high concentration of NO and its derivatives, such as nitrogen dioxide and peroxynitrite, are found to play important roles in inflammation and carcinogenesis [2]. iNOS can be induced by bacterial endotoxic LPS, interferon-γ (IFN-γ), and a variety of pro-inflammatory cytokines [3, 4]. There are a number of binding sites for transcription factors in the region of iNOS promoter, including nuclear factor-κB (NFκB), activator protein-1,

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Abbreviations: C/EBP, CCAAT/enhancer-binding protein; **COX-2,** cyclooxygenase-2; **ERK,** extracellular signal-regulated kinase; **iNOS,** inducible nitric oxide synthase; **IκB,** inhibitor κB; **IKK,** IκB kinases; **MAPK,** mitogen-activated protein kinase; **NO,** nitric oxide; **NF-κB,** nuclear factor-κB; **PGE_2,** prostaglandin E2

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interferon regulatory factor 1, and CCAAT/enhancer-binding protein (C/EBP) [5–8]. Among these transcription factors, NFκB is essential for LPS-activated NO production. NFκB is composed mainly of two proteins: p50 and p65. In resting cells, the NFκB heterodimer is held in the cytosol through interaction with inhibitor κB (IκB) inhibitory proteins [9]. With exposure to proinflammatory stimuli, IκB becomes phosphorylated, ubiquitinated, and undergoes degradation [10]. Thus, the liberated NFκB dimers are translocated to the nucleus, where the transcriptions of immune and inflammatory genes are induced. In addition, many studies imply cytokine in the induction of transcription activity of NFκB through mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase and PI3K/AKT pathways [11–15].

Cyclooxygenase-2 (COX-2), another inflammatory mediator inducible by LPS in macrophages, is a key enzyme catalyzing the conversion of arachidonic acid to prostaglandins (PGEs), especially PGE_2, and it affects cell proliferation, tumor growth [16, 17], invasion, metastasis, and angiogenesis [18]. The promoter region of COX-2 contains several regulatory elements including NFκB, C/EBP, and cAMP response element. It is known that the binding of C/EBPβ and C/EBPδ at C/EBP element are important in LPS-induced COX-2 expression of murine macrophages [19–21].

Mushrooms have attracted a great interest because of their nutritional value and their biopharmaceutical properties for a long time [22]. Recently, a great amount of attention has been given to the *Inonotus species*. Extracts of this species have been reported to exhibit several therapeutic effects, such as anti-inflammatory [23], anti-hyperglycemic and anti-lipid peroxidative [24], anti-cancer [25], and anti-tumor activities [26]. Inotilone, an unusual 5-methyl-3(2H)-furanone derivative, extracted from the *Inonotus species,* has been previously shown to be a potent inflammatory inhibitor [27]. However, the effect of inotilone at the cellular level remains unclear. In the present study, the anti-inflammatory effects of inotilone and its analog, methylinotilone (Fig. 1), were evaluated in LPS-stimulated murine macrophages. The results demonstrated that inotilone, but not methylinotilone, reduced LPS-induced NO production and iNOS expression through inhibiting the activation of NFκB and its upstream kinases, p38, ERK1/2, and PI3K/Akt. On the contrary, inotilone inhibited PGE_2 production by blocking the enzyme activity of COX-2 instead of its protein expression.

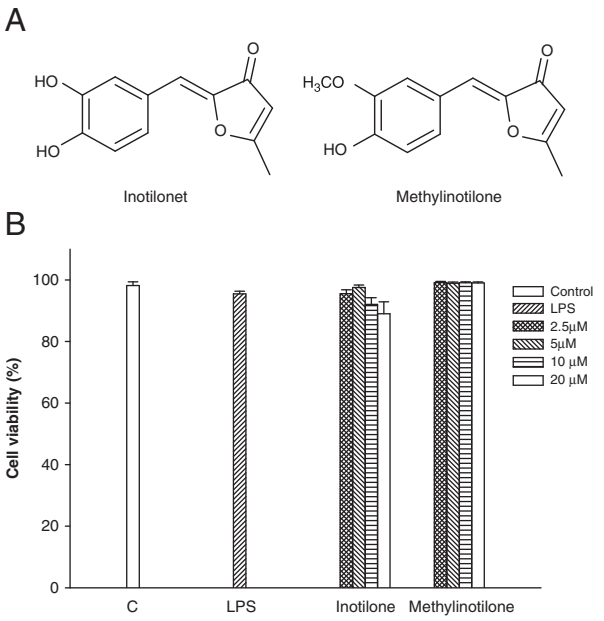

Figure 1. (A) Chemical structure of inotilone and methylinotilone. (B) Cytotoxic effects of inotilone and methylinotilone in RAW 264.7 cells. Cytotoxicity was estimated by trypan blue exclusion using a hemocytometer chamber. The values are expressed as means ± standard error of triplicate tests.

2 Materials and methods

2.1 Materials

LPS (*Escherichia coli* 0127: E8), sufanilamide, naphthylethylenediamine dihydrochloride, and DTT were purchased from Sigma Chemical (St. Louis, MO, USA). Anti-iNOS, anti-COX-2 antibody were purchased from BD Biosciences (San Jose, CA, USA). Inotilone and methylinotilone were synthesized according to the method of Shamshina and Snowden [28].

2.2 Cell culture

RAW 264.7 cells, derived from murine macrophages, were obtained from the American Type Culture Collection (Rockville, MD, USA). RAW 264.7 cells were cultured in DMEM supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin. When the cells reached a density of $2–3 \times 10^6$ cells/mL, the culture media were changed to serum-free DMEM without phenol red; they were activated by incubation in medium containing *E. coli* LPS (100 ng/mL). Various concentrations of test compounds dissolved in dimethylsulfoxide were added together with LPS. Cells were treated with 0.05% DMSO as vehicle control.

2.3 Cytotoxicity assay

The RAW 264.7 cells were cultivated at a density of 2×10^5 cells in a six-well plate. The polyphenols studied were added to the medium 18 h after inoculation. The cells were

harvested after 18 h. Viability was determined by trypan blue exclusion and microscopy examination.

2.4 Nitrite assay

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction [29]. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was measured with an ELISA plate reader (Dynatech MR-7000; Dynatech Labs, Chantilly, VA, USA).

2.5 Determination of PGE_2

The culture medium of control and treated cells was collected, centrifuged and stored at −80°C until tested. The level of PGE_2 released into culture medium was quantified using a specific enzyme immunoassay according to the manufacturer's instructions (Assay Designs, Ann Harbor, MI, USA).

2.6 Western blotting

The stimulated murine macrophage cell line RAW 264.7 cells were washed with PBS and lysed in an ice-cold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 20 mM Tris buffer (pH 7.9), 100 μM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, and one protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA)] on ice for 1 h, followed by centrifugation at 12 000 rpm for 30 min at 4°C. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amount of total cellular protein (50 μg) were resolved by SDS-polyacrylamide minigels and transferred onto immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) as described previously [30]. The membrane was then blocked at room temperature for 1 h with blocking solution (20 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.2% Tween-20, 1% BSA, and 0.1% sodium azide) followed by incubated with the primary antibody overnight at 4°C. The membrane was then washed with 0.2% TPBS (0.2% Tween-20/PBS) and subsequently probed with anti-mouse, anti-rabbit, or anti-goat IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY, USA) and visualized using enhanced chemiluminescence (Amersham). Primary antibodies of specific protein were purchased from various location as listed below: for anti-COX-2 and anti-iNOS from Transduction Laboratories, for anti-C/EBPβ and C/EBPδ from Santa Cruz Biotechnology (Santa Cruz, CA, USA), for anti-IκBα, anti-p65, and anti-phospho (Ser 32)-specific IκBα from New England Biolabs (Ipwich, MA, USA), for anti-β-actin monoclonal antibodies (Oncogene Science, Uniondale, NJ, USA), for anti-phospho-Akt (Ser473), anti-phospho-p65 (Ser536), anti-phospho-p38 (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr202/Tyr204), ERK, p38, and Akt antibodies obtained from Cell Signaling Technology (Beverly, MA, USA) were used to determine the level of phosphorylated proteins.

2.7 Semiquantitative RT-PCR

Total RNA was isolated from mouse macrophage RAW 264.7 cells using Trizol reagent according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA). Changes in the steady-state concentration of mRNA in iNOS, COX-2, and β-actin were assessed by RT-PCR. Total of 2 μg RNA was transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Renfrewshire, UK) in a final volume of 20 μL. RT reactions were performed at 50°C for 50 min and 70°C for 15 min in Gene Cycler thermal cycler (Bio-Rad). The thermal cycle conditions were initiated at 95°C for 1 min, and 30 cycles of amplification (94°C for 30 s, 58°C for 25 s, and 72°C for 1 min), followed by extension at 72°C for 3 min. The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Amplification of β-actin was served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and antisense primer: iNOS, forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (2944–2968), reverse primer 5′-GGCTGTCAGAGAGCCTCGT GGCTTTGG-3′ (3416–3440); COX-2, forward primer 5′-GGAGAGACTATCAAGATAGTGATC-3′ (1094–1117), reverse primer 5′-ATGGTCAGTAGACTTTTACAGCTC-3′ (1931–1954); β-actin, forward primer 5′-ACCAACTGGGAC-GATATGGAGAAGA-3′, reverse primer 5′-TACGACCAGA GGCATACAGGGACAA-3′. Confirmation of the correct amplicons was obtained by direct DNA sequencing of the PCR products.

2.8 Quantitative real-time RT-PCR

A total of 2 μg RNA was transcribed into cDNA using SuperScript II RNAse H– reverse transcriptase (Invitrogen) in a final volume of 20 μL. RT reactions were performed at 42°C for 50 min and 99°C for 5 min in a LightCycler® 1.5 System (Roche). Negative controls were simultaneously performed with all of the components except RT.

In the real-time PCR analysis, specific primers and fluorogenic probes were designed to target the conserved regions of various genes using the Lightcycler (LC) probe design software (Roche), according to the manufacture's guidelines for the design of PCR primers and TaqMan probes. The PCR primers and TaqMan probes used in this

experiment are as follows: iNOS, 5′-ACCCTAAGAGTCA CCAAAATGG-3′ and 5′-CCAGGGATTCTGGAACATTCT-3′; COX-2, 5′-GGGAGTCTGGACATTGTGAA-3′ and 5′-GC ACGTTGATTGTAGGTGGACTGT-3′; β-actin, 5′-CCAACC GTGAAAAGATGACC and 5′-ACCAGAGGCATACAG GGACA.

All TaqMan PCR primers were located in two different exons of each gene to avoid amplification of any contaminating genomic DNA. All PCR reactions were performed using the LightCycler System (Roche Diagnostics, Switzerland) in a total volume of 20 μL containing 1 × Taq polymerase buffer, 5 mmol/L $MgCl_2$, 200 μmol/L deoxynucleotides, 300 nmol/L each primer, 150 nmol/L probe, 1 U Taq polymerase, and 20 ng cDNA. For the negative controls, water was used instead of cDNA. Gene amplification was done in duplicate for each sample. The thermal cycling conditions were 5 min at 94°C, followed by 45 cycles of 94°C for 15 s and at 60°C for 1 min. The relative expression levels of the genes in each sample were calculated with the LightCycler software and normalized with a housekeeping control (β-actin).

2.9 Transient transfection and luciferase assay

The luciferase assay was performed as described by George *et al.* [31] with some modifications. RAW 264.7 cells were seeded in a 60-mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Gibco). The cells were then transfected with the pNFκB-Luc plasmid reporter gene (Stratagene, Jalla, CA, USA) using LipofectAMINETM reagent (Gibo, NRL, Life Technologies). After 24 h of incubation, the medium was replaced with complete medium. After another 24 h, the cells were trypsinized and equal numbers of cells were plated in 12-well tissue culture plates for 6 h. The cells were then incubated with 100 ng/mL LPS and inotilone for 6 h. Luciferase activity was assayed by means of the briteliteTM plus luciferase reporter gene kit (PerkinElmer Life And Analytical Sciences, Boston, MA, USA). Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912 V) in single-photon counting mode for 0.1 min/well.

2.10 Extraction of nucleus and cytosolic protein

Nuclear and cytoplasmic extracts were prepared as previous reported with slight modification. The cells were suspended in hypotonic buffer (10 mM NaH_2PO_4, 10 mM NaF, 5.0 mM EDTA, 1.0 PMSF, 1.0% NP-40, and 5.0 mM $MgCl_2$) for 10 min on ice followed by centrifugation at $4000 \times g$ for 15 min. The supernatants containing cytosolic proteins were collected. The pellet containing nuclei was resuspended in hypertonic buffer (0 mM NaH_2PO_4, 10 mM NaF, 5.0 mM EDTA, 1.0 PMSF, 1.0% NP-40, 5.0 mM $MgCl_2$, and 2% NaCl) for 3 h on ice followed by centrifugation at 12 000 rpm for 30 min. The supernatants containing nucleus proteins were collected for further Western blot analysis.

2.11 Statistical analysis

Data are presented as means ± SE for the indicated number of independently performed experiments. One way Student's *t*-test was used to assess the statistical significance between the LPS- and inotilone plus LPS-treated cells. A p-value < 0.05 was considered statistically significant.

3 Results

3.1 Effects of inotilone and methylinotilone on the production of nitrite and PGE_2 in RAW 264.7 macrophages

To investigate the anti-inflammatory effects of inotilone, the levels of nitrite and prostaglandin in the culture media of RAW 264.7 cells were determined 24 h after co-treatment with 100 ng/mL LPS and various concentrations of inotilone or methylinotilone. As shown in Fig. 2A, inotilone inhibited LPS-induced nitrite production in a dose-dependent manner with IC_{50} at 11.2 μM. However, methylinotilone only slightly inhibited the nitrite production but had no effect on LPS-induced PGE_2 production. The inhibition effect of inotilone can also be seen in PGE_2 production (Fig. 2B). The data indicated that inotilone was more active than methylinotilone. Inhibition of nitrite and PGE_2 production was not toxic, as determined by the trypan blue exclusion assay (Fig. 1B).

As inotilone have been previously reported to inhibit the enzyme activity of COX-2 [27], indirect nitrite assay were also performed to assess the possibility that it inhibits the intrinsic activity of NOS enzyme and as a result of NO reduction. Cells were stimulated with LPS for 12 h and washed with PBS to remove LPS. The cells were then treated with different doses of inotilone and the nitrite in the media was determined after another 12 h. As can be seen in Fig. 3A, inotilone was unable to inhibit the nitrite production in these cells indicating that the inhibition effect is not attributed to influencing the activity of NOS enzyme. Western blot analysis revealed that inotilone did not affect on iNOS protein level in LPS-stimulated RAW 264.7 cells (Fig. 3B).

3.2 Effects of inotilone and methylinotilone on LPS-inducible iNOS and COX-2 expression

Since iNOS and COX-2 are the key enzymes for the production of nitrite and PGE_2, respectively; the effects of

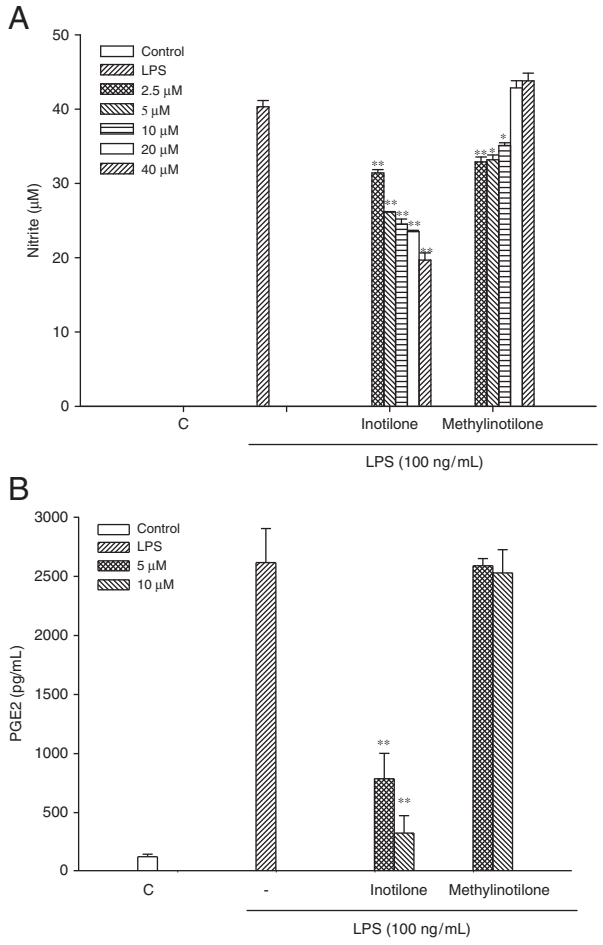

Figure 2. Effects of inotilone and methylinotilone on LPS-induced NO and PGE_2 production in RAW 264.7 macrophage. The cells were treated with 100 ng/mL LPS only or with different concentrations of inotilone or methylinotilone. After incubation for 24 h, 100 μL of culture media were collected for (A) nitrite assay, (B) PGE_2. The values are expressed as means ± SE of triplicate tests. $^{**}p<0.01$ and $^{***}p<0.001$ indicate statistically significant differences from the LPS-treated group.

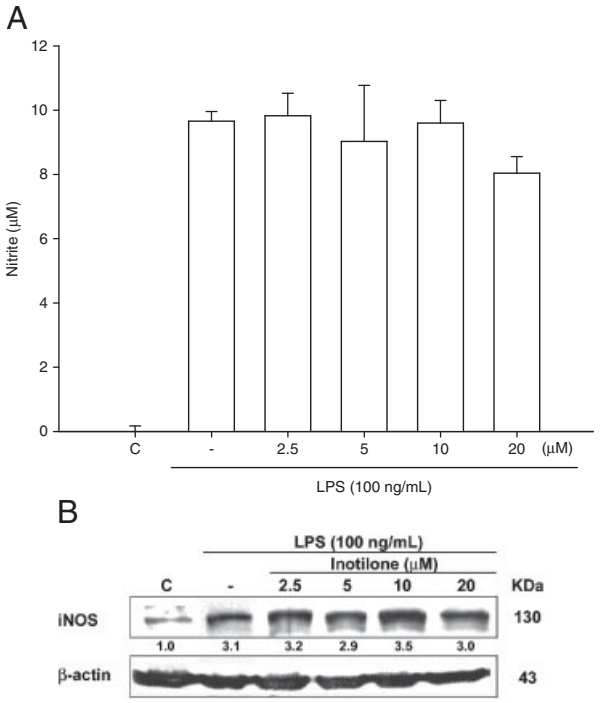

Figure 3. Effect of inotilone on the activity of NOS enzyme in RAW 264.7 cells. (A) The cells were stimulated with 100 ng/mL LPS for 12 h and were washed with PBS to remove LPS before being treated with indicated concentration of inotilone for another 12 h. The culture media were then collected for nitrite assay. (B) The levels of iNOS in lysates were analyzed by Western blotting. β-actin was used as a loading control.

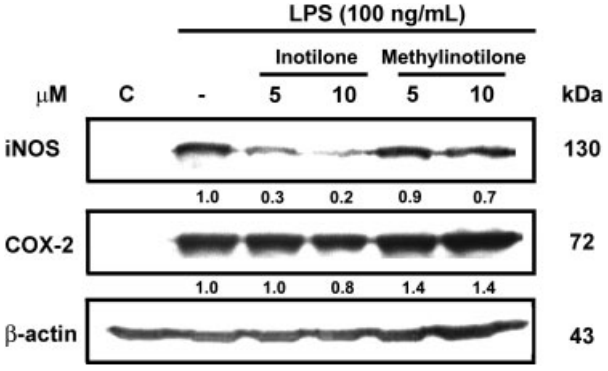

Figure 4. Effects of inotilone and methylinotilone on LPS-induced iNOS and COX-2 protein levels in RAW 264.7 cells. The cells were treated with 100 ng/mL LPS only or with inotilone or methylinotilone (5 and 10 μM) for 24 h. The levels of iNOS or COX-2 in lysates were analyzed by Western blotting. β-actin was used as a loading control.

inotilone and methylinotilone on LPS-induced iNOS and COX-2 protein expressions were studied by Western blotting. As presented in Fig. 4, LPS treatment significantly increased iNOS and COX-2 protein levels, whereas co-treatment with inotilone suppressed the induction of iNOS but not COX-2 in a concentration-dependent manner. On the other hand, methylinotilone showed slighted stimulated LPS-induced COX-2 protein expression but no effect on LPS-induced iNOS protein expression. RT-PCR and real-time PCR analysis were used to assess the effect of inotilone on *iNOS* and *COX-2* mRNA expression. Consistent with Western blotting, LPS-stimulated gene expression of *iNOS* was restrained by inotilone, whereas that of *COX-2* remained the same (Fig. 5A). From the result of real-time PCR, we have found that through inotilone there is a statistically significant suppression of *iNOS* gene expression in a dose-dependent manner in RAW 264.7 cells (Fig. 5B), whereas that of *COX-2* remained the same (Fig. 5C). These data suggest that transcription of LPS-induced *iNOS* and *COX-2* can be regulated differently by inotilone.

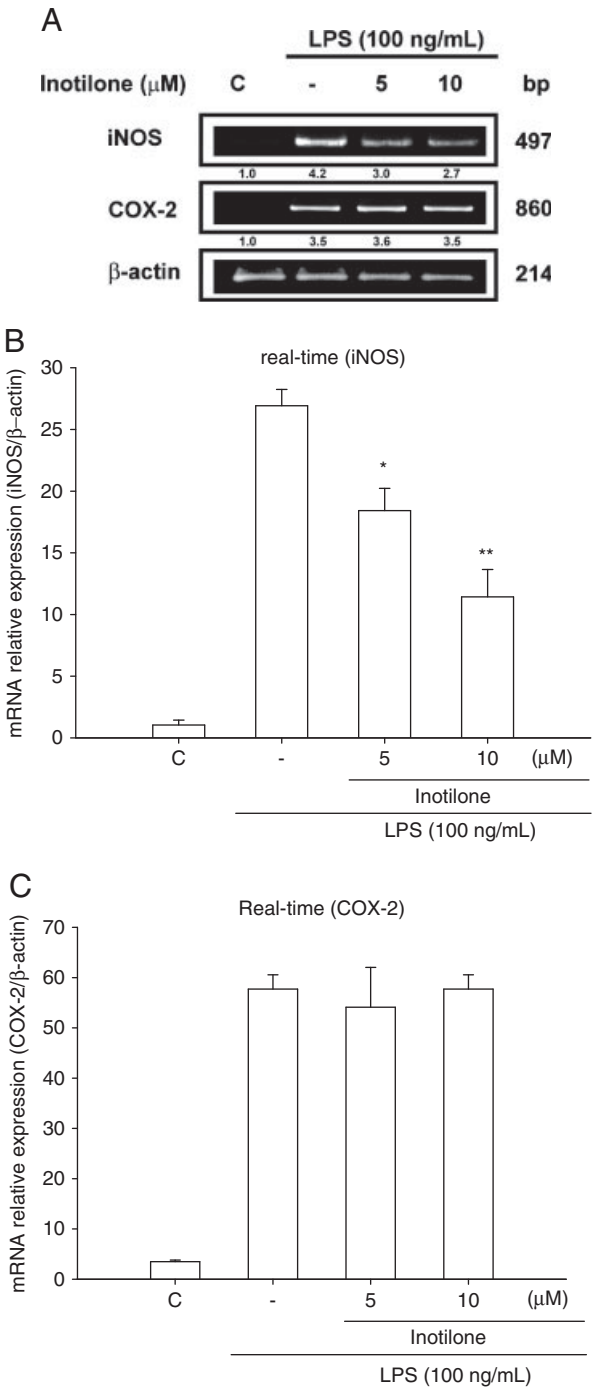

Figure 5. Effects of inotilone on LPS-stimulated *iNOS* and *COX-2* gene expression in RAW 264.7 cells. The cells were treated with 100 ng/mL LPS only or with inotilone (5 and 10 μM) for 5 h and total RNA were isolated. (A) The mRNA expressions of iNOS and COX-2 were determined by semiquantitative RT-PCR. This experiment was repeated three times with similar results. (B) The mRNA levels of *iNOS*, and (C) *COX-2*, were quantified using the LightCycler System and TaqMan probe real-time PCR. Data are mean ± SE. *, $p<0.05$; **$p<0.01$ was *versus* LPS alone.

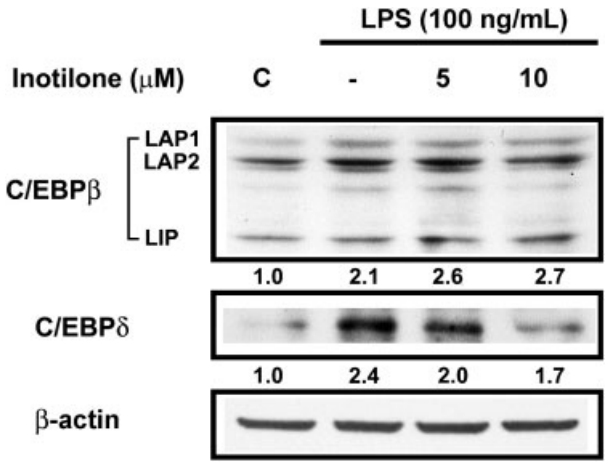

Figure 6. Effects of inotilone on LPS-induced C/EBPβ and C/EBPδ protein levels in RAW 264.7 cells. The cells were treated with 100 ng/mL LPS with or without inotilone (5 or 10 μM) for 30 min. Total cell lysates were then prepared for Western blot analysis.

3.3 Differential effect of inotilone on C/EBP and NFκB in LPS-activated macrophages

The promoter regions of iNOS and COX-2 both contain the binding sites of C/EBP and NFêB. Therefore, to investigate the differential effects of inotilone on iNOS and COX-2 expression, the effect of inotilone on these two transcription factors were determined by Western blotting. As shown in Fig. 6, cells treated with LPS increased the expression of both C/EBPβ and C/EBPδ, whereas concurrent inotilone treatment greatly inhibited the induction of C/EBPδ but not C/EBPβ. As for NFκB, Fig. 7A shows that inotilone inhibited the nuclear translocation of both of the subunits of NFκB: p50 and p65. Furthermore, the phosphorylation of p65 at serine 536 in LPS-mediated induction was also inhibited by inotilone in a dose-dependent manner. Reporter gene assay was also performed to confirm if inotilone inhibited NFκB transcriptional activity. Macrophages transiently transfected with an NFκB-dependent luciferase reporter plasmid were treated with LPS alone or with inotilone. As shown in Fig. 7B, LPS-induced transcriptional activity of NFêB was strongly reduced by inotilone.

3.4 Blockade of inotilone on LPS-induced phosphorylation and degradation of IκBα

LPS-mediated translocation of NFκB to the nucleus is preceded by proteolytic degradation of IκBα. To explore whether inotilone could affect IκBα in macrophages, the phosphorylation and protein level of IκBα were analyzed by immunoblotting in a time-course study. As shown in Fig. 8A, LPS exposure increased the phosphorylation of IκBα protein after 15 min and peaked at 120 min, whereas

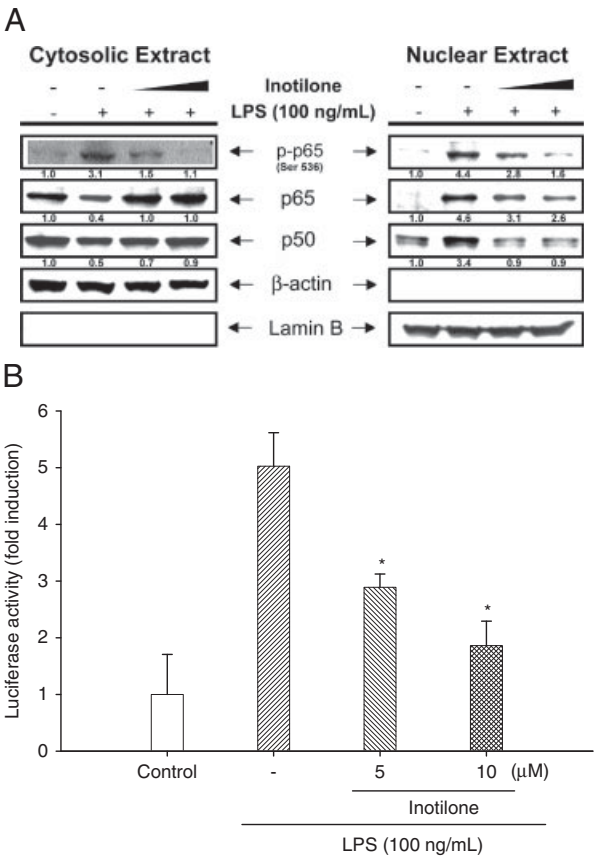

Figure 7. Effects of inotilone on LPS-induced p50 and p65 translocation and NFκB activation in RAW 264.7 cells. (A) The cells were treated with 100 ng/mL LPS alone or with inotilone (5 and 10 μM) for 1 h. Cytosolic and nuclear fractions were prepared and analyzed by Western blotting. (B) The cells were transiently transfected with pNF-κB-Luc reporter gene and then treated with 100 ng/mL LPS in the presence or absence of inotilone (5 and 10 μM) for 6 h. Luciferase activities were determined as described in Section 2. The results are expressed as means ± SE of triplicate tests. $^{*}p < 0.05$ indicates statistically significant differences from the LPS-treated group.

the degradation occurred after 30 min and gradually recovered after 45–120 min. In Fig. 7B, treatment with inotilone effectively attenuated the increased phosphorylation of IκBα and sustained the IκBα protein content. These results suggest that blocking the phorphorylation and the degradation of IκBα protein can prevent the activation and translocation of NFκB to the nucleus and further inhibit the downstream transcriptional activity.

3.5 Effects of inotilone on activation of PI3K/Akt, p38, and p44/42 MAPK kinase

Several signaling cascades have been reported to be involved in the phosphorylation and activation of NFκB [32–34]. To determine whether inotilone can modulate these upstream

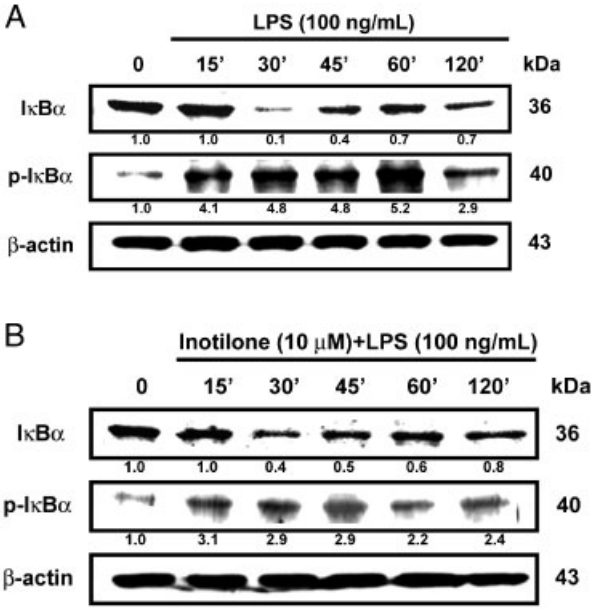

Figure 8. Effects of inotilone on LPS-stimulated phosphorylation and degradation of IκBα in RAW 264.7. The cells were treated with (A) 100 ng/mL LPS alone or (B) co-treatment with LPS and inotilone for different times. Total cell lysates were prepared for Western blot analysis. These experiments were repeated three times with similar results.

signaling pathways, the potential involvement of the PI3K/Akt and MAPK pathways were evaluated. The inhibition effect can be found in PI3K activity and its downstream target, Akt. After cells were treated with inotilone and LPS for 1 h, inotilone treatment decreased the phosphorylation of Akt and PI3K compared with LPS alone. Activation of MAPK requires phosphorylation of threonine and tyrosine residues. Anti-phospho-specific antibody was used in immunoblot analysis of the activation of ERK1/2 and p38 MAPK. When the cells were co-treated with LPS and inotilone for 1 h, inotilone was also found to attenuate the LPS-induced activation of ERK1/2 and p38 MAPK in a dose-dependent manner. These results of immunoblot analyses suggest that inotilone might block the LPS-induced NFκB activation by inhibiting ERK1/2, p38 MAPK, and PI3K/Akt/IκB kinase (IKK) pathway, which interrupts the degradation of IκBα (Fig. 9).

4 Discussion

In this study, the anti-inflammatory effects of inotilone and methylinotilone in LPS-activated murine macrophage were investigated. The results demonstrated that inotilone has an inhibitory effect on LPS-induced NO and PGE_2 production in RAW 264.7 while methylinotilone showed no effect on both of these inflammatory mediators. Several structure–activity relationships studies have indicated that flavonoids with a 4′-OH substitution in the B-ring show

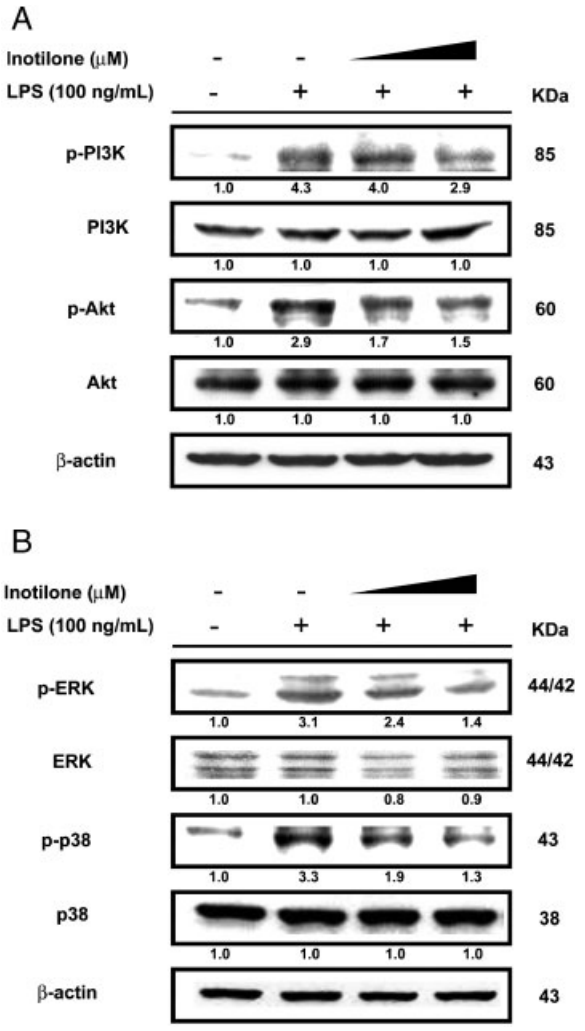

Figure 9. Inhibition of PI3K/Akt, ERK1/2, and p38 MAPK by inotilone in LPS-activated macrophages. The cells were treated with 100 ng/mL LPS with or without inotilone (5 or 10 μM) for 1 h. Total cell lysates were then prepared for Western blot analysis. These experiments were repeated three times with similar results.

better anti-inflammatory effect than 3′-OH-4′-methoxy substitution [35, 36]. We reported previously that 3′,4′-didemethylnobiletin is a more potent anti-inflammatory and anti-tumor agent compared with its parent compound, nobiletin [37]. Therefore, we suggested that inotilone and methylinotilone with similar structure to the B-ring of flavonoid exhibit the same structural related anti-inflammatory effect to flavonoid.

The effects and mechanisms of inotilone on LPS-induced iNOS and COX-2 expression both on protein and gene levels were also explored. Inotilone strongly inhibited iNOS expression in LPS-stimulated macrophages and subsequently inhibited the NO production, whereas it decreased the enzyme activity of COX-2 instead of its expression to reduce PGE_2 production. Although the promoter regions of iNOS and COX-2 contain binding sites for same transcription factors, the transcriptional regulation of LPS-stimulated iNOS and COX-2 can be distinguished [38]. Our data suggest that inotilone repressed iNOS but not COX-2 expression mainly through the differential inhibition of NFêB and C/EBPβ. From the reporter assay reported previously, a mutation in C/EBPβ or NFκB binding sites of COX-2 promoter inhibits LPS-induced reporter activity. Moreover, mutation at NFêB sites shows less inhibitory effect than that of C/EBPβ [21]. Inotilone with the inhibitor effect on NFκB but not C/EBPβ is therefore unable to decrease LPS-induced COX-2 expression. These results further confirmed that a diverse signal pathway indeed occurs in the LPS-induced iNOS and COX-2 transcription activity. Comparing the two transcription factors, C/EBPβ is more dominant in regulating transcription of COX-2 while NFκB is more dominant in iNOS. Based on the different effects of inotilone on *iNOS* and *COX-2* gene expression, inotilone provides an interesting and important evidence to confide the issue of uncoupled transcriptional regulation. However, the detailed mechanism remains to be further elucidated.

CEBPδ and CEBPβ contribute to the regulation of human *COX-2* promoter through the C/EBP and CRE motifs that have been reported. Several pieces of data suggest that the CEBPδ, but not NF-κB, may function on the human *COX-2* reporter in mouse RAW 264.7 cells [39, 40]. However, the details of CEBPδ-participated mouse *COX-2* transcription in RAW 264.7 cells have not been studied. In our case, inotilone inhibits endogenous expression of CEBPδ, but not CEBPβ in mouse RAW 264.7 cells (Fig. 6). This data not only demonstrated that an interesting compound can dissect the CEBPδ and CEBPβ-mediated regulation on mouse *COX-2* gene regulation but also cued a notice for the difference between the transcriptional regulation of human and mouse *COX-2* gene transcription. As mentioned above, NF-κB does not always cooperate with CEBPβ on the *COX-2* gene regulation that can be dissected by chemical compounds including inotilone. It also suggests that the NF-κB and C/EBPs can work independently on *COX-2* promoter. In addition, inotilone attenuates p38 activation but not the protein level of CEBPβ, which suggests that a mechanism of CEBPβ-mediated p38-indpendnt activation of COX-2 gene in RAW 264.7 cells. Taken together, we suggest that the NF-κB plays an important role on *iNOS* transcription, but not *COX-2* transcription by the dissection of inotilone in RAW 264.7 cells. Meanwhile, CEBPβ play a critical role on the maintenance of endogenous *COX-2* transcription in RAW 264.7.

Activation of NFκB by LPS is induced by a cascade of events leading to the activation of IKKs, which in turn phosphorylates IκB and leads to the degradation. NFκB is then translocation to the nucleus and regulates the transcription of target genes. Our results show that inotilone reduces iNOS expression by blocking transcription of its gene, a conclusion supported by the observation that it

reduced the steady state of iNOS mRNA levels, and promoter activity (as assessed by luciferase activity assay). We also found that co-treatment of inotilone blocked the activation of PI3K/Akt, ERK1/2, and p38 MAPK, suggesting that inotilone suppresses LPS-induced NFκB translocation by inhibiting the activation of these intracellular signaling cascades and subsequently decreases the protein level of iNOS.

Because there is a causal relationship between inflammation and cancer, iNOS and COX-2 are considered potential molecular targets for chemoprevention [41]. This study demonstrated that inotilone inhibits LPS-induced NO and PGE_2 production through modulating iNOS expression and COX-2 enzyme activity, respectively. Moreover, the differential effect of inotilone on NFκB and C/EBPβ leads to the inhibition of iNOS but not COX-2 expression. Inotilone as an inhibitor of NFκB signaling pathway in murine macrophage is believed to be accompanied by the suppression of PI3K/Akt/IKK, ERK1/2, and p38 MAPK. Based on these findings, inotilone shows great potential as a novel chemopreventive agent for the treatment of a variety of inflammatory diseases.

This study was supported by the National Science Council of Taiwan under the grant numbers NSC 97-2321-B-022-001 and NSC 95-2313-B-022-003-MY3.

The authors have declared no conflict of interest.

5 References

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