Lucidone Inhibits iNOS and COX-2 Expression in LPS-Induced RAW 264.7 Murine Macrophage Cells via NF-κB and MAPKs Signaling Pathways

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Key words
- *Lindera erythrocarpa*
- Lauraceae
- lucidone
- anti-inflammatory activity
- NF-κB
- MAPKs

Abstract

The anti-inflammatory mechanism of lucidone isolated from the fruits of *Lindera erythrocarpa* Makino was investigated. Our data indicate that lucidone significantly inhibits the production of NO and PGE_2 autacoids in LPS-induced RAW 264.7 murine macrophage cells. Moreover, it also notably decreased the secretion of tumor necrosis factor-alpha (TNF-α). Consistent with these observations, the mRNA and protein expression levels of iNOS and COX-2 were also inhibited by lucidone in a dose-dependent manner. Lucidone also reduced the translocation of NF-κB induced by LPS, which is associated with the prevention of the degradation of I-κB, and subsequently decreased p65/p50 protein levels in the nucleus. Lucidone also inhibited NF-κB activation by impairing the binding of NF-κB to its *cis*-acting element. In addition, lucidone inhibited JNK and p38MAPKs signals, which are the most significant signals involved in NO, PGE_2 and TNF-α production; NF-κB/AP-1 activation was also inhibited by lucidone. Taken together, the anti-inflammatory activity of lucidone might be caused by the inhibition of iNOS and COX-2 expressions through downregulation of NF-κB and AP-1 binding.

Abbreviations

AP-1:	activator protein-1
ATF-2:	activating transcription factor-2
COX-2:	cyclooxygenase-2
DMEM:	Dulbecco's modified Eagle's medium
EMSA:	electrophoretic mobility shift assay
FBS:	fetal bovine serum
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
IKK:	Iκ-B kinase
iNOS:	inducible nitric oxide synthase
Iκ-B:	inhibitor of nuclear factor kappa-B
JNK:	c-JUN N-terminal kinase
LPS:	lipopolysaccharide
MAPK:	mitogen activated protein kinase
MKK:	mitogen kinase kinase
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide
NF-κB:	nuclear factor kappa-B
NO:	nitric oxide
PGE_2:	prostaglandin E_2
SAPK:	stress-activated protein kinase
TNF-α:	tumor necrosis factor alpha

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction

Natural products have served as an important source of drugs since ancient times, and now more than 50% of the pharmaceuticals used today are derived from natural products [1]. The prevention and treatment of inflammatory conditions is an important application for natural products [2]. Inflammation is a central feature of many pathological conditions and is mediated by a variety of soluble factors and cellular signaling events. For example, NF-κB-dependent gene expression plays an important role in inflammatory responses and increases the expression of genes encoding cytokines and receptors involved in pro-inflammatory enzyme pathways such as iNOS and COX-2 [3].

In response to extracellular stimuli, for instance, the bacterial product LPS, TNF-α or other inflammatory mediators such as the transcription factors NF-κB and AP-1 are often activated, which subsequently stimulates the transcription of a number of genes involved in inflammation, resulting in increasing levels of the inflammatory

enzymes cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), and of specific cytokines [4]. The transcription factor NF-κB forms a cytoplasmic complex with its inhibitors, the I-κBs, under normal physiological conditions. Once the Iκ-Bs become phosphorylated, NF-κB is released and translocated to the nucleus where its target genes are then activated [5]. Two I-κB kinases, viz IKK-α and IKK-β, are involved in the signal-induced phosphorylation of I-κB; Iκ-Bα degradation results in rapid changes in NF-κB activation, whereas Iκ-Bβ degradation is associated with prolonged NF-κB activation [6]. NF-κB activation mediates the expression of a number of rapid response genes involved in the inflammatory response to injury, including iNOS and COX-2 [7]. Recently, many studies have demonstrated the role of phytochemicals in anti-inflammatory activity through downregulation of the NF-κB pathway [8]. In addition, activator protein-1 (AP-1), another early transcriptional factor, is also involved in the pro-inflammatory response, either alone or by coupling with NF-κB [9]. Lee and his coworkers found that a member of the mitogen-activated protein kinase (MAPK) family, i.e., p38 kinase, which acts as a specific target for a novel class of cytokine-suppressive anti-inflammatory drugs (CSAIDs), plays a key role in this regulation [10].

Lindera erythrocarpa Makino (Lauraceae) is an evergreen tree, the fruits of which are used as a folk medicine with analgestic, digestive, diuretic, antidotal and antibacterial properties [11–14]. It has been reported that cyclopentenediones from the methanolic extract of the fruits of *L. erythrocarpa* inhibit farnesyl protein transferase [15]. The anti-inflammatory activity of *L. erythrocarpa* fruits has been preliminarily evaluated. Four anti-inflammatory cyclopentenediones were identified by bioactivity-guided fractionation, among which lucidone (**Fig. 1S**, Supporting Information) was the strongest inhibitor of NO production. Meanwhile, lucidone was also a potent anti-inflammatory agent in a croton oil-induced mouse ear edema assay [16]. To further understand the anti-inflammation mechanism involved, the effects of lucidone on activation of the NF-κB pathway as well as on downstream mediators of inflammation, viz. iNOS, COX-2 and TNF-α, were investigated in this study.

Materials and Methods

Plant materials and reagents

Lucidone was prepared according to the protocol described previously [16], to a purity above 99% according to HPLC and ^{1}H-NMR analysis. DMEM and other cell culture reagents including FBS were purchased from GIBCO BRL Life Technologies (Invitrogen). LPS, MTT, curcumin (purity: 96%) and Griess reagent were purchased from Sigma-Aldrich. The antibodies used in this study are listed in **Table 1S**, Supporting Information. All other chemicals and solvents used in this study were of reagent or HPLC grade.

Cell culture and sample treatment

RAW 264.7 cells purchased from ATCC were cultured at 37 °C in DMEM supplemented with 10% FBS, 4.5 g/L glucose, 4 mM glutamine, penicillin (100 units/mL), and streptomycin (100 µg/mL) in a humidified atmosphere in a 5% CO_2 incubator as recommended by ATCC. The cells were incubated with lucidone at different concentrations [1 (3.9), 5 (19.5), and 10 (39.1) µg/mL (µM)] and curcumin [10 (27.1) µg/mL (µM)] and stimulated with LPS (1 µg/mL) for 1 to 20 h.

Griess nitrite assay and determination of prostaglandin E_2 production

The concentration of NO in culture supernatants was determined as nitrite, a major stable product of NO, by the Griess reagent assay, and cell viabilities were determined using the MTT assay as described previously [16]. PGE_2 production from endogeneous arachidonic acid was measured in cell culture supernatants with an ELISA kit according to the supplier's instructions (EIA; Cayman Chemical).

Determination of TNF-α production

Levels of TNF-α production were measured in cell culture supernatants with a mouse TNF-α ELISA kit (Biosource) according to the manufacturer's recommendation. The macrophage cells were treated with the indicated concentrations of lucidone and curcumin in the presence or absence of LPS (1 µg/mL) for 20 h. The medium was collected and assayed for TNF-α using an ELISA kit (EIA; Cayman Chemical) quantified with an ELISA reader (µ-Quant; Bio-Tek Instruments).

Preparation of whole cell, cytosolic and nuclear extracts

Whole cell protein extraction was carried out with the Mammalian Protein Extraction Reagent (Cayman Chemicals) as described previously [16]. The cytosolic and nuclear proteins were extracted using a Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology), and protein levels were measured with the Bradford method (Bio-Rad).

RNA extraction and RT-PCR

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) were performed as described previously [4]. In brief, total RNA was extracted from macrophages using PureLink Micro-to-Midi Total RNA purification system (Invitrogen). For cDNA preparation, total RNA (5 µg) was incubated at 37 °C for 90 min with a first-strand cDNA synthesis kit (Invitrogen). The cDNAs were incubated with appropriate PCR primers as summarized in **Table 2S**, Supporting Information. The PCR products were separated on 2% agarose gels, and digitally imaged after staining with ethidium bromide.

Quantitative real-time PCR

Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) analyses of iNOS, COX-2 and GAPDH mRNA were performed using a Bio-Rad iCycler iQ as described previously [17]. The melting point, optimal conditions and the specificity of the reaction were first determined using a standard procedure. The working stock solution of SYBR Green was 1 : 100 (Bio-Rad). Quantitative PCR was carried out in 96-well plates with 10 µM forward and reverse primers, and the working solution SYBR Green, using a customer PCR master mix, with the following conditions: 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, 55 °C for 45 s, 72 °C for 30 s. The housekeeping gene *GADPH* was used as an internal control. The sequences of the primers are shown in **Table 2S**, Supporting Information. The copy number of each transcript was calculated relative to the *GADPH* copy number.

Western blot analysis

Western blot analyses were performed as described previously [16]. Immunoblotting was performed with appropriate antibodies using the Enhanced Chemi-Luminescence (ECL) Western Blotting Reagent (Immobilon; Millipore) and images were visualized

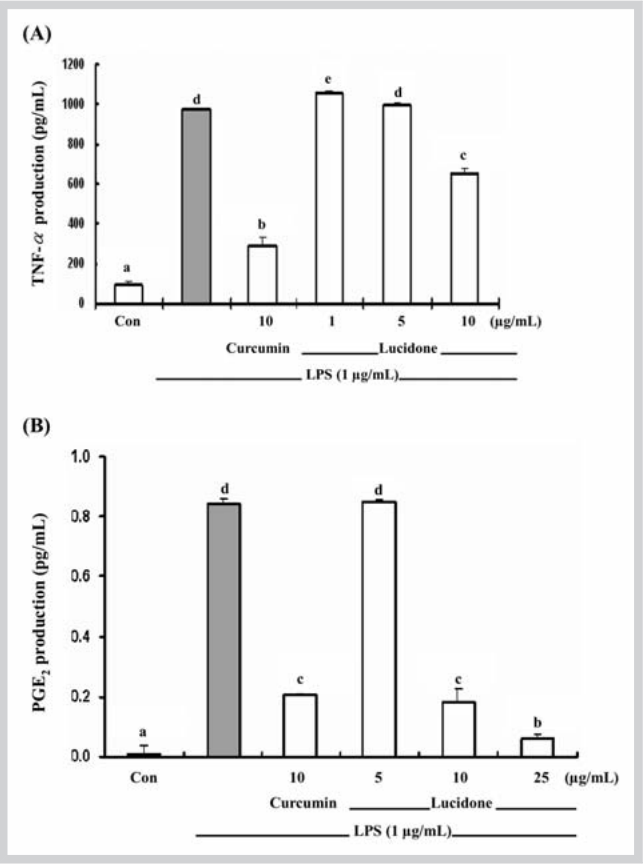

Fig. 1 Effects of lucidone on TNF-α and PGE_2 production in LPS-induced RAW 264.7 macrophages. **A** The cells were treated with 1 μg/mL of LPS alone or LPS with indicated concentrations of lucidone for 20 h. At the end of the incubation 50 μL of medium was removed to measure TNF-α levels. **B** Aspirin-pretreated RAW 264.7 cells were treated with the indicated concentrations of lucidone and curcumin for 1 h and then stimulated with LPS for 16 h. PGE_2 level in culture medium was measured as described in the Materials and Methods section. Control values were obtained in the absence of LPS. Each value represents the mean ± S. E. M. of three independent experiments. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

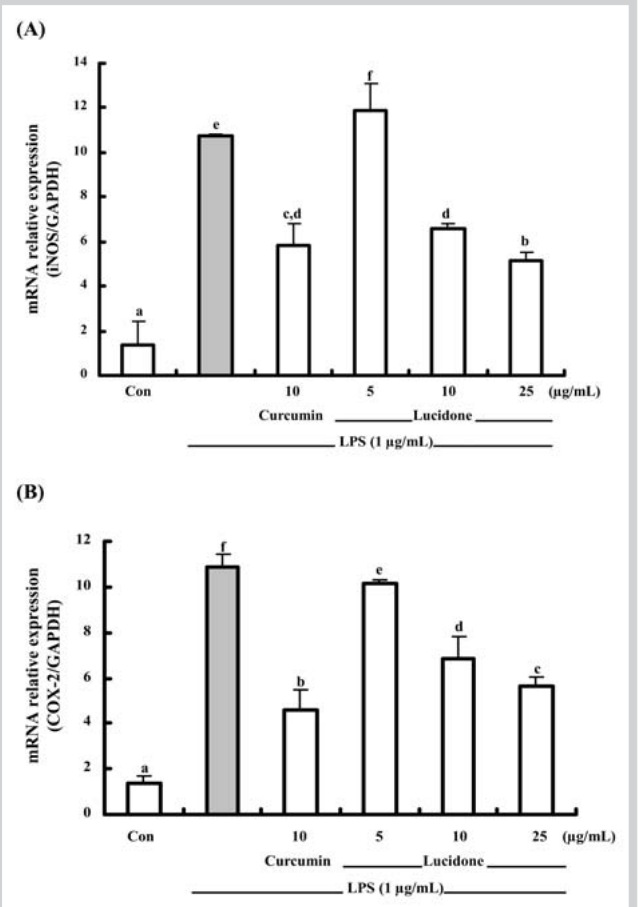

Fig. 2 Real time RT-PCR analysis of iNOS and COX-2 mRNA expression in RAW 264.7 cells. **A** The cells were treated with 1 μg/mL of LPS or with the indicated concentrations of lucidone and curcumin for 8 h, and total RNA was subjected to RT-PCR. The RT products were labeled with SYBR Green dye. Relative iNOS (**A**) and COX-2 (**B**) mRNA expression ($2^{-\Delta Ct}$) was determined by real-time PCR and calculated by subtracting the C_t value for iNOS and COX-2 from GAPDH mRNA. $\Delta Ct = C_{tiNOS\ or\ COX\text{-}2} - C_{tGAPDH}$. Each value represents the mean ± S. E. M. of three independent experiments. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

with a VL Chemi-Smart 3000 (Viogene Biotek). β-Actin was used as an internal control.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously [4]. In brief, RAW 264.7 macrophages (2.5×10^6) were plated in a 6-cm dish, treated with various concentrations of lucidone (1, 5, 10 μg/mL), and stimulated with LPS for 2 h, when nuclear extracts were then prepared as described above. The oligonucleotide probes (listed in **Table 2S**, Supporting Information) were synthesized by Tri-I Biotech, and then annealed with TE buffer. Nuclear extract (20 μg) was incubated with 20 ng of double-standard NF-κB/AP-1 oligonucleotides for 30 min at room temperature with 5 μL of binding buffer. The DNA protein complex was separated on a 6% native polyacrylamide gel. The complex was visualized using a LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology) and luminescence intensity quantified on a VL Chemi-Smart 3000 (Viogene Biotek).

Statistical analysis

The results were expressed as the mean standard error of the mean of three independent expriments. The data were analyzed statistically by one-way ANOVA and different group means were compared by Duncan's multiple range test. The software pack SPSS for windows, version 10.0, SPSS Inc. was used for analysis of data. $P < 0.05$ was considered significant in all cases.

Supporting information

Information on antibodies and oligonucleotides, the chemical structure of lucidone and data on the effects of lucidone on NO production and cell viability in LPS-induced RAW 264.7 macrophages, on RT-PCR analysis of the expression of iNOS and COX-2 mRNA and on the effects of lucidone on iNOS and COX-2 protein levels in LPS-induced macrophages are available as Supporting Information.

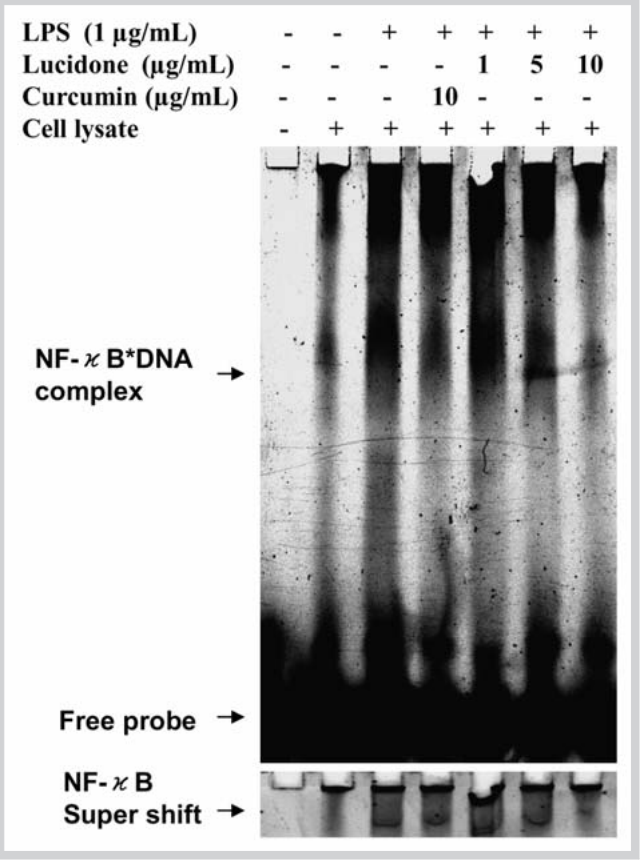

Fig. 3 Effect of lucidone on NF-κB DNA binding activity in LPS induced macrophages. Nuclear fractions were prepared from control cells, or from 2 h LPS (1 µg/mL)-stimulated cells with or without pretreatment with increasing concentrations of lucidone. Extracts were assayed for NF-κB binding by EMSA as described in the Materials and Methods section.

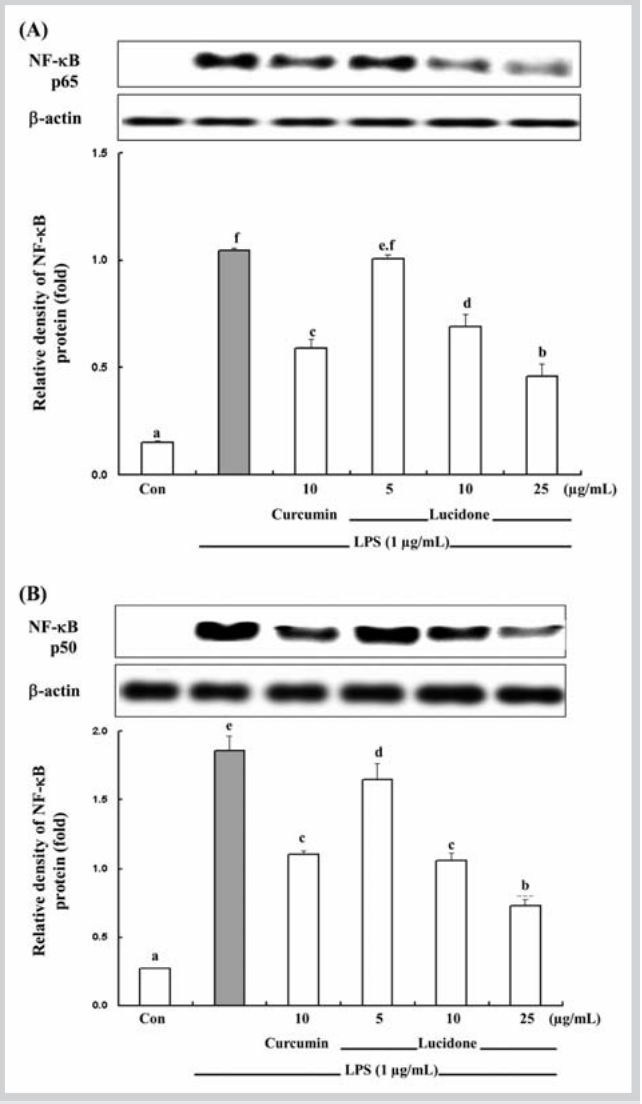

Fig. 4 Effect of lucidone on NF-κB nuclear translocation in LPS-induced macrophages. The cells were treated with or without 1 µg/mL of LPS and with the indicated concentrations of lucidone and curcumin for 1 h, when levels of p65 and of the p50 subunits of NF-κB in nuclear extracts were determined by Western blotting. Histograms show the relative intensity of p65 (**A**) and p50 (**B**) normalized to β-actin. Each value represents the mean ± S. E. M. of three independent experiments. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

Results and Discussion

Our previous study showed that lucidone inhibited LPS-induced NO production in RAW 264.7 cells in a dose-dependent manner [16]. In our present study, we found that the IC_{50} of lucidone for inhibition of NO production was 2.77 µg/mL (**Fig. 2SA**, Supporting Information). In macrophages incubated with lucidone (10 µg/mL), then challenged with 1 µg/mL LPS for various time periods, lucidone also inhibited NO production (**Fig. 2SB**, Supporting Information). In line with the Griess assay of NO production, the tested cells were healthy and viable at these concentrations of lucidone and LPS (**Figs. 2SC** and **2SD**, Supporting Information).

We investigated the effect of lucidone on production of inflammatory cytokines. In LPS-stimulated RAW 264.7 cells, lucidone treatment led to a concentration-dependent inhibition of TNF-α (**Fig. 1A**) and PGE_2 production (**Fig. 1B**). At 10 µg/mL, lucidone dramatically reduced PGE_2 production from 846 pg/mL to 154 pg/mL, and at 25 µg/mL PGE_2 levels decreased even further to 57 pg/mL.

Since lucidone was found to significantly inhibit LPS-induced NO, TNF-α and PGE_2 production, we reasoned that the inhibition of LPS-induced pro-inflammatory molecules could be due to suppression of iNOS and COX-2 at the transcriptional level. As shown in **Fig. 3SA** Supporting Information, unstimulated RAW 264.7 cells expressed low or undetectable levels of iNOS mRNA. In contrast, strong iNOS mRNA expression was induced after the cells had been incubated with 1 µg/mL LPS. Lucidone suppressed LPS-induced iNOS and COX-2 mRNA expression in a dose-dependent manner (**Fig. 3SB**, Supporting Information). Similar results were obtained from real-time PCR analysis of specific iNOS and COX-2 mRNA in cell extracts (**Figs. 2A** and **B**). Lucidone also significantly suppressed LPS-induced iNOS and COX-2 (**Fig. 4SA**, Supporting Information), but not COX-1 (**Fig. 4SB**, Supporting Information) protein expression. These results confirm the target specificity of lucidone.

We hypothesized that the inhibition of iNOS and COX-2 expression by lucidone could be due to a reduction of nuclear translocation and DNA binding ability of NF-κB. The DNA binding activity of nuclear NF-κB was examined with the EMSA assay. As shown

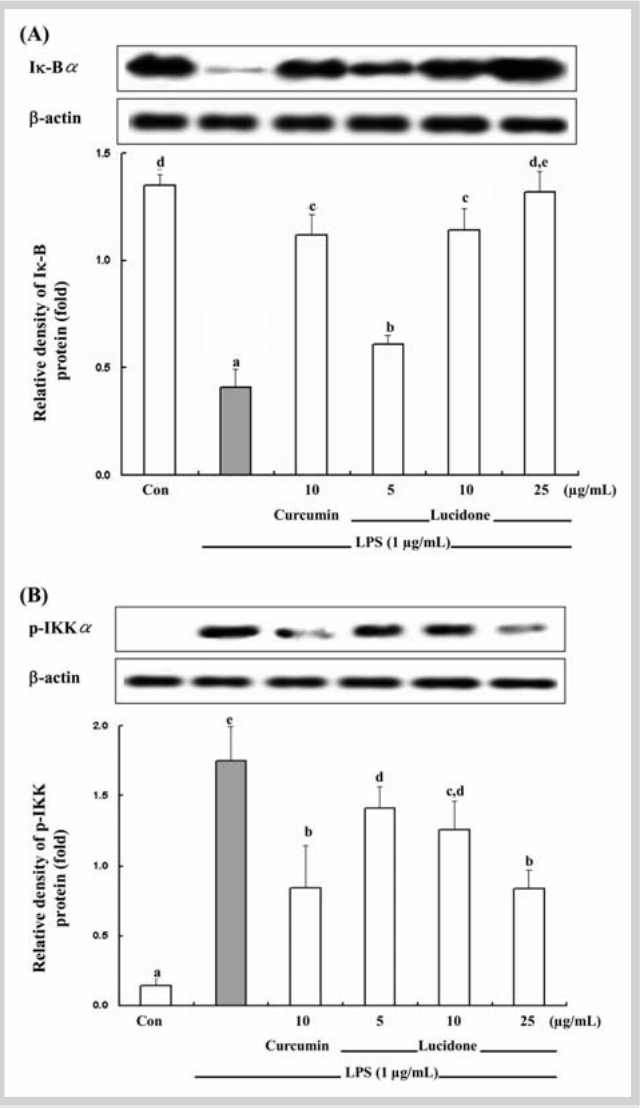

Fig. 5 Effects of lucidone on Iκ-B and phosphorylated IKK protein stability in LPS-induced macrophages. The cells were treated with or without 1 μg/mL of LPS and with the indicated concentrations of lucidone and curcumin for 1 h, when levels of Iκ-B and p-IKK in the cytoplasmic fraction were determined by Western blotting. Histograms show the relative intensity of Iκ-B (**A**) and p-IKK (**B**) normalized to β-actin. Each value represents the mean ± S. E. M. of three independent experiments. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

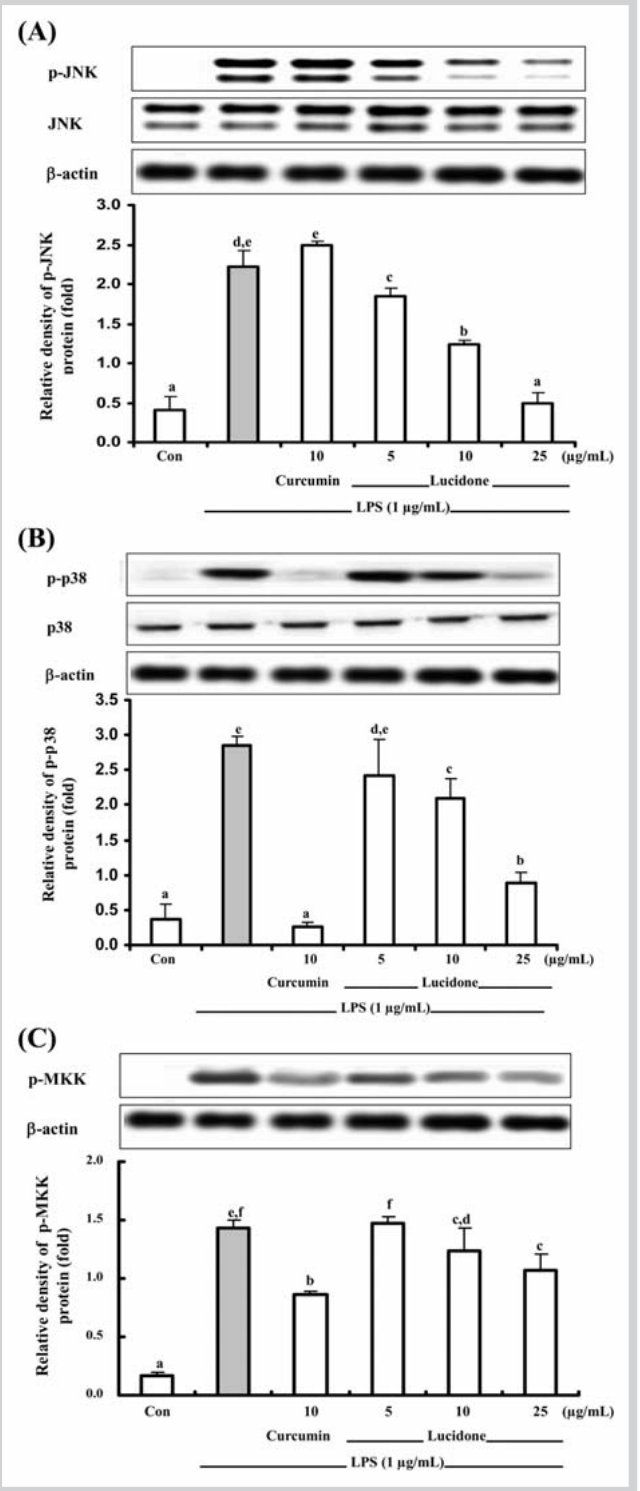

Fig. 6 Effect of lucidone on phosphorylation of MAPKs in LPS-induced macrophages. The cells were treated with or without 1 μg/mL of LPS and with the indicated concentrations of lucidone for 16 h. The phosphorylation of MAPKs was detected by immunoblotting using antibodies against the corresponding activated forms of MAPKs (phosphorylated MAPK). Histograms show the relative intensity of p-JNK/JNK (**A**) p-p38MAPK/p38MAPK (**B**) and p-MKK (**C**) normalized to β-actin. Each value represents the mean ± S. E. M. of three independent experiments. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

in ▶ **Fig. 3A**, nuclear NF-κB DNA binding activity was hardly detectable in unstimulated cells, but LPS stimulation resulted in a significant increase. Lucidone strongly inhibited LPS-induced NF-κB binding activity. NF-κB translocation to the nucleus has been shown to be required for NF-κB-dependent transcription following LPS stimulation. In agreement with this, the levels of NF-κB subunits p65 and p50 in nuclear extracts were enhanced in the presence of LPS (1 μg/mL) compared to non-stimulated cells. The nuclear localization of p65 (▶ **Fig. 4A**) and p50 (▶ **Fig. 4B**) was decreased in a dose-dependent manner by different concentrations of lucidone.

The poor nuclear translocation and DNA binding of NF-κB induced by lucidone could be due to its decreased activation through stabilization of Iκ-B. As shown in ▶ **Fig. 5A**, lucidone

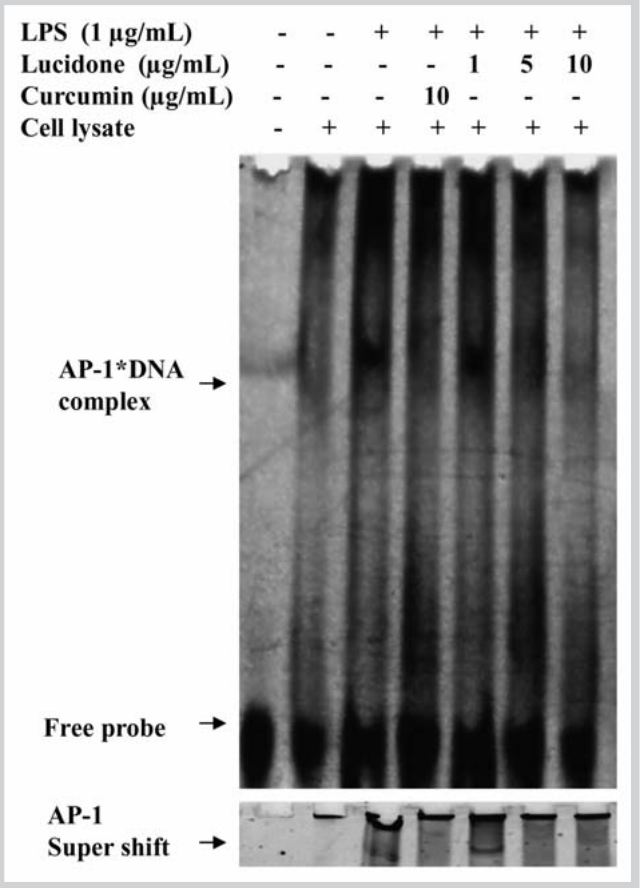

Fig. 7 Effect of lucidone on AP-1 DNA binding activity in LPS induced macrophages. The cells were treated with or without 1 µg/mL of LPS and with the indicated concentrations of lucidone for 16 h. The nuclear extract was incubated with AP-1 probes, and DNA binding was determined by EMSA assay as described in the Materials and Methods section.

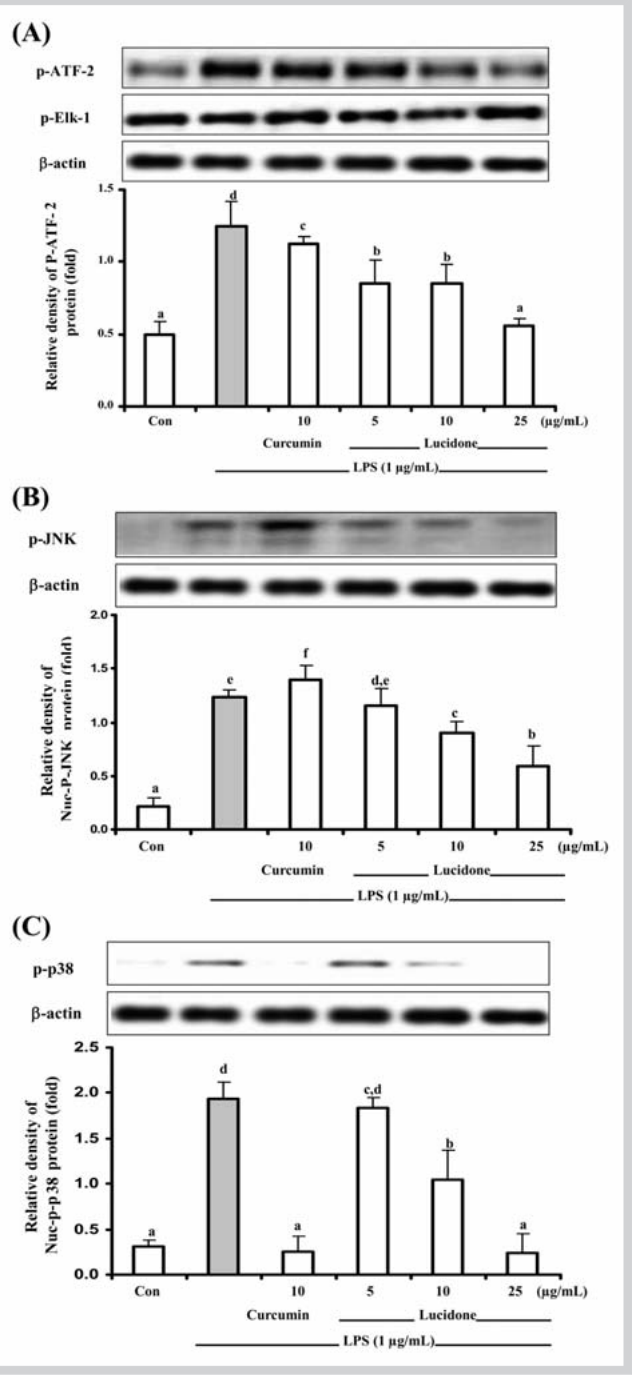

Fig. 8 Effect of lucidone on ATF-2 activation and MAPKs translocation in LPS-induced macrophages. The levels of p-ATF-2, p-Elk-1, p-JNK and p-p38MAPK in the nuclear extract were determined by Western blotting as described in the Material and Methods section. Histograms show the relative intensity of p-ATF-2 and p-Elk-1 (**A**) p-JNK (**B**) and p-p38MAPK (**C**) normalized to β-actin. Each value represents the mean ± S. E. M. of three independent experiments. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

caused a dose-dependent enhancement of Iκ-B protein stability. In addition, the level of phosphorylated IKK in the cyotosolic extract was decreased by lucidone in a dose-dependent manner, compared to non-stimulated cells and cells stimulated with LPS alone (**Fig. 5B**).

A separate transcription factor, AP-1, represents an alternative pathway for macrophage pro-inflammatory cytokine production. The MAPK cascade regulates both NF-κB and AP-1-associated gene transcription through several cross-amplifying phosphorylation kinases, specifically p38 and JNK/SAPK [8]. As shown in **Fig. 6**, lucidone suppressed LPS-induced activation of phosphorylated JNK/SAPK (**A**), p38MAPK (**B**) and MKK (**C**), again in a concentration-dependent manner. These results indicate that phosphorylated MAPK protein stability was inhibited by lucidone pretreatment. Interestingly, curcumin does not attenuate phosphorylation of JNK/SAPK. The activation of these kinases in the proximal MAPK cascade modulates AP-1 activation. We hypothesize that inhibition of MAPK signaling cascades by lucidone could be due to poor ATF-2 (part of the AP-1 complex) activation and DNA binding ability of AP-1 through the nuclear translocation of phosphorylated MAPKs. As shown in **Fig. 7**, nuclear AP-1 DNA binding activity was significantly inhibited by lucidone, compared with non-stimulated cells and cells stimulated with LPS alone.

We further evaluated the effect of lucidone on LPS-induced translocation of MAPKs and activation of AP-1 in RAW 264.7 cells. Lucidone significantly suppressed LPS-induced ATF-2 activity in a dose-dependent manner (**Fig. 8A**). Pretreatment of cells with lucidone did not alter LPS-induced Elk-1 expression. These re-

sults suggest that lucidone inhibits the activation of ATF-2, which might be associated with the blocking of LPS-inducible iNOS and COX-2 expression. Since LPS-stimulated activation of ATF-2 is correlated with translocation of phosphorylated JNK/SAPK and p38MAPKs, the effects of lucidone on phosphorylated JNK1/2 (**Fig. 8B**) and p38MAPK (**Fig. 8C**) expression in nuclear fraction was examined to clarify the inhibitory action of lucidone. By comparison, the well-known anti-inflammatory phytocompound curcumin does not alter phosphorylated JNK/SAPK proteins. Interestingly, lucidone attenuates phosphorylated JNK/SAPK proteins [20]. Taken together, these results indicate that lucidone inhibits both LPS-induced ATF-2 (AP-1) activation and phosphorylated JNK1/2 and p38 MAPKs translocation.

In summary, this study provides more evidence that lucidone inhibits the production of pro-inflammatory cytokines NO, TNF-α, and PGE_2 in LPS-stimulated macrophages. This anti-inflammatory effect occurs by downregulation of iNOS and COX-2 expression via suppression of the activation of the pro-inflammatory transcription factors NF-κB and AP-1. This suggests, therefore, a novel mechanism of anti-inflammatory action for this phytocompound. However, further *in vivo* investigation of this activity is necessary to elaborate the mechanisms and permit full exploitation of its promise, specifically the role of altered signal transduction.

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