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Inhibition of LPS-induced NO Production and NF-κB Activation by a Sesquiterpene from *Saussurea lappa*

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To elucidate the molecular mechanisms for the suppression of LPS-induced nitric oxide (NO) production by a dehydrocostus lactone (DL) from *Saussurea lappa*, we examined the preventive effect of this compound on NF-κB activation in LPS-treated RAW 264.7 macrophages and U937 human monocytic cells. The results suggest that the suppression of NO production is mediated by the inhibitory action on the i-NOS gene expression through the inactivation of NF-κB and this sesquiterpene lactone can act as a pharmacological inhibitor of the NF-κB activation.

Key words: Dehydrocostus lactone, Sesquiterpene lactone, *Saussurea lappa*, Nitric oxide, Inducible nitric oxide synthase, NF-κB

INTRODUCTION

In the process of pathogenic inflammatory disease, excessive production of nitric oxide (NO) by inducible nitric oxide synthase (i-NOS) plays a crucial role in induction of septic shock (Moncada *et al.*,1991; Nathan *et al.*, 1992). It is well known that the i-NOS gene expression is regulated by several transcription factors. Mutagenesis studies of the i-NOS promoter has shown that NF-κB functions as a transcriptional regulator of the i-NOS gene induced by LPS and cytokines such as TNF -α and INF-γ (MacMicking *et al.*, 1995; Xie *et al.*, 1994 ; Kim *et al.*, 1997). NF-κB is composed of a homo- and hetero dimer of Rel family DNA-binding subunits (frequently p65/p50), and these proteins are sequestered by inhibitory protein IkB in the cytoplasm. Upon the variety of stimuli including LPS, inflammatory cytokines, phorbol esters and UV irradiation, the IκB protein can be post-translationally phosphorylated and degraded. These events allow NF-κB to enter the nucleus, binds to its target sequences and then induces transcription of the target genes involved in inflammatory reactions. (reviewed by Baeuerle and Baltimore, 1996 ; Palombella *et al.*, 1994). Recently Lee *et al.* (1999) reported that a sesquiterpene lactone from *S. lappa* effectively inhibited TNF-α and i-NOS expression in LPS-activated macrophages. In this report, we examined whether the DL can exert an inhibitory effect on the i-NOS gene expression through the suppression of activity of NF-κB.

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MATERIALS AND METHODS

Chemicals

Dehydrocostus lactone (DL) from *Saussurea lappa* was extracted and purified as described by previous report (Lee *et al.*, 1999). Pyrrolidine dithiocarbamate (PDTC), lipopolysaccharide (LPS, *Escherichia coli*, 0127:B8), bovine serum albumine, sodium nitrite, sulfanilamide and naphthylene diamine were obtained from Sigma Chemical Co. (St. Louis, MO) (Fig. 1).

Cell culture

The macrophage cell line, RAW 264.7 (American Type

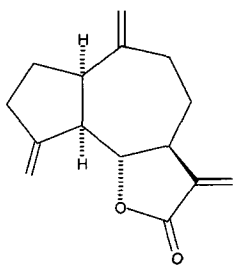

Fig. 1. The structure of dehydrocostus lactone from *Saussurea lappa*

Culture Collection) were grown in DMED with 10 % fetal bovine serum (Gibco BRL, Detroit, MI), penicilline (100 U/ml), streptomycin (100 μg/ml) in 5% CO_2 humidified incubator at 37°C and were split twice a week. The human promonocytic cell line U937 were grown in RPMI 1640 under the same condition.

NO Assay

NO from macrophages was assessed by determining NO_2^- concentration in the culture supernatant. Samples (100 ml) of culture media were incubated with 150 ml of Griess reagent (1% sulfanilamide, 0.1% naphthylene diamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well plate (Green *et al.*, 1982). Absorbance at 540 nm were read using an ELISA plate reader (Dynatech, U.S.A.). Standard calibration curves were prepared using sodium nitrite as a standard.

Plasmids

The NF-κB dependent chloramphenicol acetyltransferase (CAT) reporter plasmid, J16 contains two copies of synthetic κB sites in the reverse direction directly upstream of a truncated c-*fos* promoter (Gross *et al.*, 1985). A control plasmid J32 contains critical mutated sequences in binding sites. Plasmids (Grosschedle *et al.*, 1985) containing the wild type (J16) or mutant sequences (J32) placed in front of the CAT structural gene were kindly given by Dr. S. Kim (IMBG, Seoul National University).

Transfection and CAT assay

For transient transfection RAW 264.7 macrophages were transfected by the Lipofectamine (Gibco BRL) using the method of manufacturer's manual. CAT activity was determined according to a standard method (Gorman *et al.*, 1982). Equal amount of protein were incubated with acetyl coenzyme A (1 mM) (Sigma Co., St. Louis, MO) and [^{14}C] chloramphenicol at 37°C for 2 h. Acetylated products were separated by silica gel thin layer chromatography (Merck, Germany) with $CHCl_3$-MeOH (95:5 v/v) as eluent. The amounts of converted acetylation were quantified by phosphoimager (Fuji, BAS system).

Preparation of total protein extraction

Cells (1-2 × 10^6) were harvested, washed twice in cold PBS and resuspended in 100 ml of lysis buffer (20 mM Hepes, pH 7.9, 0.35M NaCl, 20% Glycerol, 1% NP-40, 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM PMSF) for 10 min on ice. Samples were centrifuged at 14,240 g, for 10 min at 4°C. Supernatants were collected, dialyzed to buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 0.1M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) overnight and stored at -70°C until use.

Table I. The inhibition of NO production by dehydrocostus lactone or PDTC in LPS-activated RAW 264.7 cells.

Conc. (μM)	Inhibition (%)[a]	
	dehydrocostus lactone	PDTC
0.1	4	-
0.5	17	-
1	-	43
2.5	44	-
5	79	49
10	93	63
15	100	-
25	-	84
IC50	3.0 μM	5.4 μM

[a] Inhibition (%) was calculated as the inhibition of NO production compared with LPS control
Inhibition (%) = [(LPS Sample)/(LPS Media)] × 100
Results were expressed as mean of three experiments.

Electrophoretic mobility shift assay (EMSA)

Equal amounts (15 μg) of supernatant were incubated with 2 mg of poly (dI-dC), 2 mg of bovin serum albumin, 3 mM GTP and 0.2 ng of [^{32}P] labeled oligonucleotide in 10 × binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA pH 7.5, Glycerol) to make a final volume of 20 μl. The free and oligonucleotide bound proteins were separated by the electrophoresis on 4% polyacrylamide gel. The specificity of the retarded complexes was confirmed by competition assay with 500 fold excess of cold wild type (W) or mutated (M) oligonucleotides or AP-1 oligonucleotide (Sen *et al.*, 1986). The gel was dried and exposed to an X-ray film (Amersham Hyperfilm). The following oligonucleotides were used for NF-κB binding: NF-κB consensus : 5'AGTTGAGGGGACTTTCCCAGGC 3', mutant sequences : 5' ACTTCACCCCACTTTCCCACCC 3'

RESULTS AND DISCUSSION

RAW 264.7 cells were stimulated with 1 μg/ml of LPS and the production of NO was increased by the enzymatic reaction of i-NOS. DL and PDTC inhibited the production of NO in dose dependent manner when they were added to the culture media 2 hr before the onset of cell stimulation (Table I). The concentration required for inhibiting the production of NO by 50% (IC_{50} value) was calculated on the basis of concentration of nitrite released into the culture media. The IC_{50} values of DL and PDTC were 3.0 μM and 5.4 μM, respectively.

PDTC is a well-known specific NF-κB inhibitor which blocked the transcription of proinflammatory genes

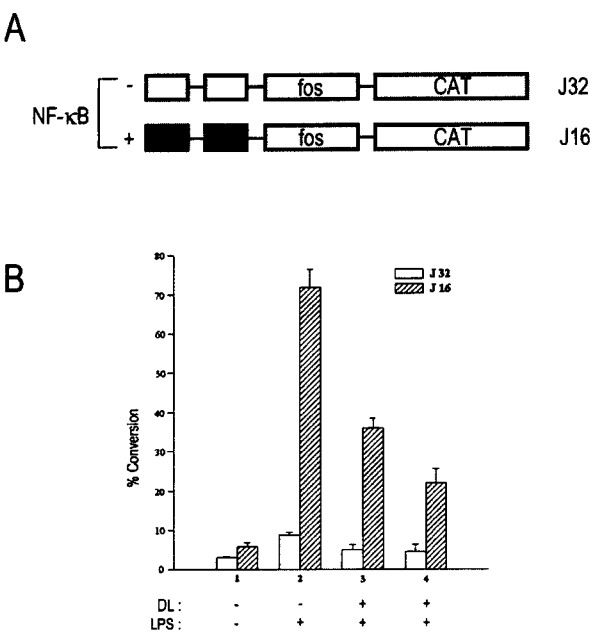

Fig. 2. Inhibitory effect of dehydrocostus lactone (DL) on the LPS-induced CAT activity. (A) Reporter plasmid. J 16 contains 2 copies of synthetic NF-kB binding sites (TCGACA G**GGGACTTTC**CGAGAGGC) in the reverse direction directly upstream of a truncated *c-fos* promoter. The sequence corresponding to that found in immunoglobulin k light chain gene intron. J 32 contains 2 copies of mutant NF-kB binding sites (TCGACAG**AATTCACTTTC**CGAGAGGC).
(B) RAW 264.7 cells were transiently transfected with either 8 mg of J16 (wild type κB) or J 32 (mutant). Following transfection, cells were pretreated with DL (5 mM; 3, 10 mM; 4), for 2 h and then exposed to LPS (1 mg/ml) for 22 h. Data are expressed as the mean±SD (n=3). Students t-test: $P<0.05$

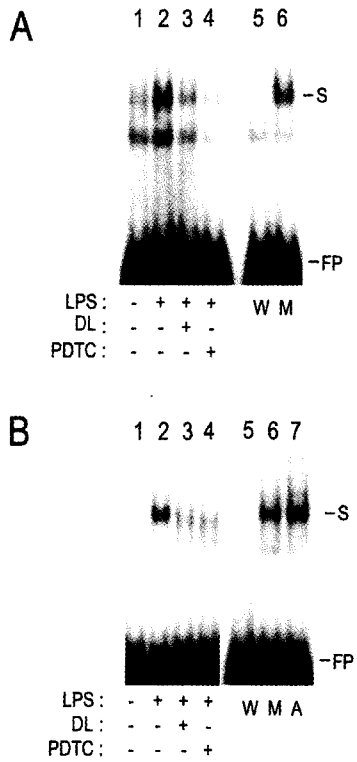

Fig. 3. Inhibition of LPS-induced NF-kB binding activity by DL. (A) EMSA with [^{32} P]-labeled NF-κB oligonucleotides. After preincubation with DL (10 mM, lane 3) and PDTC (50 mM, lane 4), total protein extracts (15 mg) were prepared from RAW 264.7 cells treated with LPS (1 mg/ml) for 6 h. Specificity of NF-κB was verified by competition assays by incubating the protein samples used in lane 2 with unlabeled wild-type (W) or mutated (M) oligonucleotide. S: specific DNA-protein complex, FP: free probe.
(B) Total protein extract from U937 cells were incubated with [^{32}P] labeled NF-κB oligonucleotides under same condition as panel (A). Specificity of NF-κB was verified by competition assays by incubating the protein samples used in lane 2 with unlabeled wild-type (W) or mutated (M) oligonucleotide, AP-1 oligonucleotide (A). S: specific DNA-protein complex, FP: free probe.

including i-NOS through the inhibition of the NF-κB activation. (Munoz. *et al.*, 1996 ; Xie *et al.*, 1994). The gene for i-NOS has a NF-κB binding motif in the promoter region and the transcriptional activity of NF-κB has been shown to be involved in the induction of i-NOS by LPS (Xie *et al.*, 1994). To examine the NF-κB activities related in DLs suppressive effect of NO production, we performed the transient transfecion with a NF-κB inducible CAT reporter plasmid. Transfection of these plasmids into macrophages showed high levels of CAT activity after LPS-treatment. However, preincubation of DL for 2 h before LPS stimulation resulted in a dramatic decrease in the CAT activity of the transfected cells (Fig. 2). LPS and DL had no significant effect on CAT expression from the NF-κB mutant reporter plasmid. These results indicate that DL can efficiently inhibit NF-κB driven transcription. To further elucidate the molecular aspects of the NF-κB inhibition by DL, we tested DNA binding activity of NF-κB in electrophoretic mobility shift assay. After treatment of RAW 264.7 and U937 cell with or without 10 μM DL for 2 h followed LPS activation, total protein extracts were prepared and assayed for binding with double stranded NF-κB consensus oligonucleotides. LPS activation of these cells significantly increased the amounts of the retarded DNA-protein complexes con-sistent with other report (Legrand-Poels *et al.*, 1997). The binding of NF-κB with DNA was completely inhibited by 10 μM DL and 50 μM PDTC in LPS-activated cells (Fig. 3, A). These complexes were specific for NF-κB because they were effectively competed by unlabeled wild type κB sequence. These complexes were not competed by neither a mutant oligonucleotide which is identical to the wild type probe

except for six base change nor AP-1 oligonucleotide including unrelated sequence with NF-κB binding sites. We also observed the same results (Fig 3, B) in U937 human premonocyte. Whereas DL did not influence the activity of AP-1 transcription factor, which is also implicated in the mechanism of stimulation of various cell type (Angel *et al.*, 1991). Treatment of DL did not influence an electrophoretically retarded DNA-protein complex with same protein extract and AP-1 consensus oligonucleo-tides (data not shown). These results clearly indicated that DL has a specific inhibitory effect on NF-κB dependent gene expression presumably by suppression of the expression or DNA binding activity of NF-κB. As the target genes of NF-κB are immunologically importance (Tanos *et al.*, 1995), inhibitors of NF-κB have a potential of therapeutic purpose for chronic inflam-mation and/or acute situation like septic shock, which are associated with massive activation of NF-κB (Murphy *et al.*, 1998). The NF-κB inhibitors are categorized into three groups depending on action mechanisms. One group including PDTC, curcumine, acetyl salicylic acid and N-acetyl-L-cystein, exerts their action by scavenging reactive oxygen intermediates (Frantz *et al.*, 1995 ; Schreck *et al.*, 1992; Sigh *et al.*, 1995). Another group like glucocorticoid exerts the inhibitory effect only in nucleus by impairing the transcription activity of DNA-bound NF-κB (De Bosschner *et al.*, 1997). The third group affects the function of the upstream signal molecules, which results in interfering with the induced degradation of IκB (Hehner *et al.*, 1998). It seems unlikely that DL has same action mechanism as PDTC which have antioxidant activity. DL did not interfere with already bound NF-κB-DNA complex (preliminary data). Several sesquiterpene lactones have the structure which can form covalent bond with biological functional group of proteins (Schmidt *et al.*, 1997), and Lyss *et al.* (1997; 1998) reported that a sesquiterpene lactone alkylates cystein residue of NF-κB p65 subunit. We may postulate that DL has also alkylating activity with a-methylen γ-lactone group essential for this activity. The exact mechanism of DL inhibition of NF-κB activation in LPS-activated macrophages is needed to be further studied.

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