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The Naturally Occurring Flavolignan, Deoxypodophyllotoxin, Inhibits Lipopolysaccharide-Induced iNOS Expression through the NF- κ B Activation in RAW264.7 Macrophage Cells

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Deoxypodophyllotoxin (DPT), a naturally occurring flavolignan with anti-inflammatory activity, was isolated from *Anthriscus sylvestris* HOFFM., and we examined its effects on the expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-stimulated, murine macrophage-like RAW264.7 cells. Western blot analysis performed with specific anti-iNOS antibodies showed that a decrease in nitric oxide (NO) was accompanied by a decrease in the iNOS protein level. To clarify the mechanistic basis for DPT's ability to inhibit iNOS induction, we examined the effect of DPT on nuclear factor (NF)- κ B transcriptional activity and DNA binding activity. DPT potently suppressed both reporter gene activity and DNA binding activity. These findings suggest that DPT in RAW264.7 cells abolished LPS-induced iNOS expression by inhibiting the transcription factor, NF- κ B.

Key words deoxypodophyllotoxin; inducible nitric oxide synthase; nuclear factor- κ B; electrophoretic mobility shift analysis; pyrrolidinedithiocarbamate; RAW264.7 cell

The dried root of *Anthriscus sylvestris* (Umbelliferae) is used in Korean traditional medicine as an antipyretic, an analgesic, and a cough remedy. Deoxypodophyllotoxin (DPT; Fig. 1A), widely distributed in higher plants, is a major lignan in this plant. DPT has antiproliferative,¹⁾ antitumor,^{2,3)} and antiviral activities.⁴⁾ Recently, we reported that DPT inhibits the passive cutaneous anaphylaxis (PCA) reaction,⁵⁾ has strong dual cyclooxygenase (COX-2) selective/5-lipoxygenase (LOX) inhibitory activity,⁶⁾ and has anti-asthmatic effects.⁷⁾ However, the underlying mechanisms of its inhibition of nitric oxide (NO) generation have not been elucidated. Therefore, we examined the effect of DPT on NO generation to better understand its anti-inflammatory effects.

NO generation plays an important role in atherosclerosis, inflammation, and carcinogenesis.^{8,9)} NO is generated by NO synthase (NOS), and three isoforms of NOS have been identified: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS).¹⁰⁾ The suppression of NO synthesis might be useful for the treatment of inflammatory diseases. NO produced by constitutive NOS (cNOS) is critical in the maintenance of cellular function, whereas NO produced by iNOS is an important mediator of acute and chronic inflam-

mation.¹¹⁾ If the inflammation is chronic, then healthy host cells may be killed by NO, contributing to inflammatory pathology.¹²⁾

The transcription factor, nuclear factor (NF)- κ B, is activated by a variety of stimuli and regulates diverse gene expression and biological responses. The stimuli include bacterial endotoxin, lipopolysaccharide (LPS), ionizing radiation, and carcinogens that are often associated with inflammatory diseases and tumorigenesis.^{13,14)} The binding of NF- κ B to κ B sites is important for iNOS induction by LPS. NF- κ B exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, I κ B.¹⁵⁾ External stimuli for macrophage activation increase phosphorylation of I κ B α , which causes its degradation, allowing active NF- κ B to translocate to the nucleus and bind its cognate DNA binding sites in the regulatory regions of a variety of genes. p38 and p44/42 mitogen-activated protein kinase (MAPK) are also involved in iNOS expression.¹⁶⁾ The promoter of iNOS contains two consensus NF- κ B binding sites, which mediate LPS-inducibility.¹⁷⁾ The heterodimer p65/p50 is the NF- κ B complex responsible for LPS-induced iNOS expression.

In this study, we investigated the effect of DPT on the LPS-induced NO production and iNOS protein expression, and to clarify its mode of action in RAW264.7 macrophage cells. We found that DPT inhibited NF- κ B activation through the inhibition of I κ B degradation.

MATERIALS AND METHODS

Plant Material DPT was isolated from the dried roots of *A. sylvestris* (Umbelliferae). The chemical structure of the isolated compound was established as deoxypodophyllotoxin (Fig. 1) by comparison of ¹H- and ¹³C-NMR and optical rotation data with those reported previously [mp 166–167 °C, [α]_D²³ –110° (*c*=1.0, CHCl₃)].¹⁸⁾ This compound was at least

Fig. 1. Chemical Structure of DPT Isolated from *Anthriscus sylvestris* HOFFM. (A) and Cytotoxicity in RAW264.7 Cells (B)

Cells were incubated in the presence of 1, 5, 7.5, 10, and 20 nM DPT. Cell viability was assayed by MTT assay. Data represent the mean $\pm$ S.D. of three different samples.

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99.5% pure based on HPLC analysis. The DPT used in this study ran as a single spot on TLC, and a solution was prepared by dissolving pure DPT in dimethyl sulfoxide (DMSO) diluted with DMEM media. The final concentrations of DMSO were adjusted to 0.1% (v/v) in the culture media. Control experiments showed that DMSO at this concentration had no effect on iNOS expression.

Reagents The rabbit polyclonal antibodies specific for I κ B and horseradish peroxidase-labeled donkey anti-rabbit secondary antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). The ECL western blot detection reagent was purchased from Amersham Biosciences, Inc. (Piscataway, NJ, U.S.A.). DMEM, MEM non-essential amino acid solution, and penicillin–streptomycin were obtained from Hyclone (Logan, UT, U.S.A.) and GIBCO-BRL (Carlsbad, CA, U.S.A.). Bacterial lipopolysaccharide (LPS, *Escherichia coli*, serotype 055:B5) was obtained from Difco Laboratories (Detroit, MI, U.S.A.).

Cell Culture RAW264.7 cells, a murine macrophage/monocyte cell line, were obtained from Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 100 μ M MEM non-essential amino acid solution. In all experiments, cells were grown to 80–90% confluence and subjected to no more than 20 cell passages.

Measurement of Cell Viability Cell viability was assessed by MTT assay. RAW264.7 cells plated in 96-well plates (5×10^4 cells/well) were treated with various concentrations of DPT for 20 h. Then MTT (5 mg/ml) was added and incubated for 4 h. The culture medium was removed, and cells were dissolved in 0.04 N HCl/isopropyl alcohol. The optical densities (OD) at 570 nm and 630 nm were measured using a microplate reader.

Measurement of Nitric Oxide RAW264.7 cells (5×10^5 cells) were pre-incubated at 37°C for 12 h in serum-free medium. NO production was monitored by measuring nitrite levels in the culture media using Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid). Absorbance was measured at 570 nm after incubating for 10 min.

SDS-PAGE/Immunoblot Analysis RAW264.7 cells were plated in 6-well plates (1×10^6 cells/well) and treated with LPS for 24 h. The cells were washed and scraped into cold phosphate-buffered saline (PBS) and centrifuged at $500 \times g$ at 4°C. The cell pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin, and 1 μ g/ml leupeptin) and centrifuged to yield whole-cell lysates. The proteins (20 μ g) were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane in 20% methanol, 25 mM Tris, and 192 mM glycine (Schleicher and Schull, Dassel, Germany). The nitrocellulose membrane was then blocked by incubation in TTBS (25 mM Tris–HCl, 150 mM NaCl, and 0.2% Tween 20) containing 5% nonfat milk. Subsequently, the membrane was incubated with either the anti-iNOS or anti-I κ B α antibody for 4 h, washed, and finally incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase. The protein bands were visualized using an enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Transient Transfection and Luciferase Assay

RAW264.7 cells were seeded into 24-well plates (5×10^5 cells/well) and allowed to grow to 50–70% confluence. The pNF- κ B-luciferase plasmid¹⁹ was transfected using Lipofectamine PlusTM reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Cells were pre-treated with various concentrations of DPT for 30 min and were stimulated with 200 ng/ml LPS for 6 h. Then cells were collected and disrupted by sonication in lysis buffer (25 mM Tris–phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol) and the cell lysates were assayed for luciferase activity using a luminometer (Promega) according to the manufacturer's instructions.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA) Nuclear extracts were prepared as described elsewhere.²⁰ Cultured cells were collected by centrifugation, washed, and suspended in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF. After 15 min on ice, the cells were vortexed in the presence of 0.5% Nonidet NP-40. The nuclear pellet was then collected by centrifugation and extracted in a buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF for 15 min at 4°C. Nuclear extract (10–20 μ g) was preincubated at 4°C for 30 min with a 100-fold excess of an unlabeled oligonucleotide (5'-CAGTGGAAATCCCCAGCC-3') spanning the *cis* element of interest. Then the reaction mixture was incubated at 4°C for 20 min in a buffer (25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl, and 2.5% glycerol) with 2 μ g of poly dI/dC and 5 fmol (2×10^4 cpm) of a Klenow end-labeled (³²P-ATP) 30-mer oligonucleotide, which spans the DNA binding site in the iNOS promoter. The reaction mixture was separated at 4°C on a 6% polyacrylamide gel using TBE (89 mM Tris, 89 mM boric acid, and 1 mM EDTA) running buffer. The gel was rinsed with water, dried, and exposed to X-ray film overnight.

RESULTS

Structure of DPT and Cytotoxicity to RAW264.7 Cells

We first measured the cytotoxicity of DPT (Fig. 1A) on RAW264.7 cells by MTT assay. Cell viability was strongly affected by 10 nM DPT (Fig. 1B). Therefore, we chose 5 and 7.5 nM of DPT for the subsequent experiments.

Inhibitory Effect of DPT on NO Production and iNOS Protein Expression in RAW264.7 Cells We first assessed the inhibitory activity of the indicated concentrations of DPT on NO production and iNOS protein expression induced by LPS (200 ng/ml). Maximal NO production was observed at 24 h and maintained at 48 h (data not shown). RAW264.7 cells were preincubated with DPT (5, 7.5 nM) for 30 min and the cells were further incubated for 24 h in LPS-containing medium in the presence or absence of DPT. Levels of iNOS protein expression were determined by Western blot analysis. LPS treatment markedly increased NO production and iNOS protein levels (Figs. 2A, B). DPT (7.5 nM) significantly inhibited the LPS-induced increase in NO and iNOS protein levels.

Effects of DPT on LPS-Induced I κ B Degradation and p65 Nuclear Translocation The nuclear translocation of NF- κ B is preceded by the phosphorylation and degradation of I κ B.²¹ To determine whether DPT inhibited I κ B degrada-

Fig. 2. Effects of DPT on NO Production (A) and iNOS Expression (B) in LPS-Treated RAW264.7 Cells

(A) Cells were pre-incubated in medium containing DPT (5 or 7.5 nM) for 30 min and then treated with LPS 200 (ng/ml) for 24 h. The amount of nitrite in the medium was measured. The data represent the mean $\pm$ S.D. of three different samples. (B) Effects of DPT on LPS-induced iNOS expression. iNOS protein levels were determined by Western blot analysis.

Fig. 3. Effects of DPT on LPS-Induced IκB Degradation in RAW264.7 Cells

Cells were pre-incubated with DPT (5 or 7.5 nM) or PDTC (10^{-4} M) for 30 min and then treated with LPS 200 (ng/ml) for 30 min. The degradation of IκB was assessed by Western blot analysis.

tion, total cell lysates were prepared to analyze IκB levels by Western blot. Cells were pretreated with the indicated concentration of DPT or pyrrolidinedithiocarbamate (PDTC) as a positive control for 30 min and then in the presence or absence of LPS (200 ng/ml) for 30 min. Both PDTC and DPT inhibited IκB degradation, but the inhibitory activity of DPT was weaker than that of PDTC (Fig. 3).

Next, we investigated the translocation of p65, a subunit of NF-κB, from the cytosol to the nucleus using Western blot analysis. A large amount of p65 had translocated into the nucleus at 30 min in the absence of DPT, but DPT inhibited this translocation (Fig. 4A). In addition, LPS stimulation decreased cytosolic p65 levels, but DPT blocked this decrease (Fig. 4B). These results suggest that the inhibition of NF-κB translocation by DPT is due to the prevention of IκB degradation.

DPT Inhibits LPS-Induced NF-κB Activation and Subsequent iNOS Expression NF-κB is activated in cells stimulated with LPS and other inflammatory stimuli, leading to transcriptional activation of responsive genes.^{21,22} Hence, we performed reporter gene analyses using a luciferase plasmid containing the NF-κB binding sequence to determine whether the suppressive effect of DPT on iNOS induction is associated with the inhibition of NF-κB. The NF-κB-luc reporter vector contained four copies of the NF-κB binding site to determine NF-κB-driven transactivation. LPS treatment (6 h), a positive control, increased NF-κB reporter activity 4-fold, and pre-treatment of cells with 5 or 7.5 nM of

Fig. 4. Effects of DPT on LPS-Induced p65 Nuclear Translocation in RAW264.7 Cells

Cells were pretreated with DPT (5 or 7.5 nM) for 30 min and treated with LPS (200 ng/ml) for 30 min. Nuclear extracts (A) and cytosolic extracts (B) were then prepared as described manufacturer's instructions (Panomics AY2002, Redwood City, CA, U.S.A.) and subjected to Western blot analysis.

Fig. 5. Effects of DPT on NF-κB Activity in the iNOS Gene in RAW264.7 Cells

To study the effect of DPT on NF-κB transcriptional activity, cells were transfected with the pNF-κB-Luc plasmid, and reporter gene analysis was performed as described in Materials and Methods. Data represent the mean $\pm$ S.D. of three different samples (significant vs. the LPS-treated group, * $p < 0.05$).

Fig. 6. Effects of DPT on LPS-Induced DNA Binding Activity in RAW264.7 Cells

Cells were pretreated with DPT for 30 min and treated with LPS (200 ng/ml) for 30 min. After incubation, nuclear extracts were prepared from the cells and analyzed by EMSA for the activated NF-κB using radiolabeled oligonucleotide probes, respectively.

DPT significantly inhibited this increase (Fig. 5). These results suggest that DPT inhibits NF-κB activation, which may explain the loss of iNOS induction.

DPT Inhibits the NF-κB Binding Activity in the iNOS Promoter To determine if LPS-induced iNOS expression is associated with an increase in nuclear NF-κB and whether DPT inhibits NF-κB binding activity, EMSA was performed with nuclear extracts of RAW264.7 cells that had been treated with LPS (200 ng/ml). Nuclear extracts were incubated with a ³²P labeled probe for the NF-κB binding site and the mixture was then separated on a 6% non-denaturing poly-

acrylamide gel. LPS-stimulation increased NF- κ B binding activity, and DPT effectively inhibited the increased NF- κ B binding activity (Fig. 6). These results suggest that DPT blocks iNOS expression, at least in part, by decreasing the DNA binding of the transcription factor, NF- κ B.

DISCUSSION

We previously reported on the anti-inflammatory activity of DPT *in vitro* and *in vivo*.^{5–7)} These findings provided a molecular basis for the mode of action of dietary, biochemically-active compounds in preventing inflammatory disease. In this study, we demonstrated that DPT inhibits LPS-induced NO production in RAW264.7 cells. To identify the direct scavenging effect of DPT, we measured the amount of NO remaining in the supernatant shortly after the addition of DPT to the supernatant of LPS-stimulated RAW264.7 cells; however, the amount of preexisting NO in the supernatant was not altered by the presence of DPT (data not shown). These results indicated that the inhibition of NO production in LPS-stimulated RAW264.7 cells by DPT occurred *via* the modulation of iNOS. In fact, DPT inhibited LPS-induced iNOS protein expression in a concentration-dependent manner (Figs. 2A, B). To identify the mechanism of this inhibition, we examined the degradation of I κ B and translocation of NF- κ B. Immunoblot analysis showed that the inhibition of NF- κ B activity by DPT might result from inhibition of I κ B α degradation (Fig. 3). Under unstimulated conditions, NF- κ B is sequestered within the cytosol as an inactive complex bound to I κ B. After stimulation by a various stimuli such as LPS and inflammatory cytokines, I κ B is phosphorylated by the I κ B kinases, IKK α and IKK β and rapid degradation of I κ B subunit by proteasomes.^{23,24)} NF- κ B is then released from I κ B and is translocated into the nucleus. In order to investigate whether DPT prevented the translocation of the subunit of NF- κ B, p65, from the cytosol to nucleus after release from I κ B. Western blot analyses showed that the LPS-stimulated p65 level in the nuclear fraction was strongly blocked by DPT, whereas the decrease of p65 level in the cytosol fraction is blocked by DPT (Figs. 4A, B). In the nucleus, NF- κ B dimers bind to target DNA elements and activate transcription of genes encoding proteins involved in the immune and inflammation responses, as well as with cell growth control.²⁵⁾

Next, we investigated the transcriptional activity of NF- κ B. LPS stimulation can activate a variety of transcription factors, including NF- κ B (p50/p65), AP-1 (c-Fos/c-Jun)²¹⁾ and C/EBP. NF- κ B and AP-1 are essential transcription factors for iNOS gene transcription.^{17,26)} To identify the mechanisms of the effects of DPT on iNOS expression, we investigated the transcriptional regulation of NF- κ B. EMSA using promoter activity studies. Reporter gene analyses using the NF- κ B binding sequence clearly revealed that DPT inhibited LPS-induced NF- κ B activation (Fig. 5) and DNA binding activity (Fig. 6). To further confirm that DPT affects AP-1 activation, we assessed the effect of DPT on the LPS-induced phosphorylation of extracellular signal-regulated kinase

(ERK), p38 MAP kinase (p38), and c-Jun N-terminal kinase (JNK). Interestingly, the phosphorylation of ERK, MAP kinase p38, and JNK in response to LPS was not changed by DPT treatment (data not shown). These results suggest that DPT suppresses NO production and iNOS gene expression through the inhibition of I κ B degradation and NF- κ B activation, without inhibiting MAPK phosphorylation or AP-1 activation.

In the present study, we demonstrated that DPT inhibits LPS-induced production of NO in macrophages by blocking NF- κ B activation. We found that DPT blocks the nuclear translocation of p65 in LPS-treated cells. Because NF- κ B is a critical inflammatory transcription factor, its inhibition by DPT represents a potential therapeutic approach to the treatment of severe inflammatory diseases.

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