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Effects of sphondin, isolated from *Heracleum laciniatum*, on IL–1β-induced cyclooxygenase-2 expression in human pulmonary epithelial cells

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Abstract

Recently, under large-scale screening experiments, we found that sphondin, a furanocoumarin derivative isolated from *Heracleum laciniatum*, possessed an inhibitory effect on IL–1β-induced increase in the level of COX–2 protein and PGE_2 release in A549 cells. Accordingly, we examined in the present study the action mechanism of sphondin on the inhibition of IL–1β-induced COX–2 protein expression and PGE_2 release in a human pulmonary epithelial cell line (A549). Pretreatment of cells with sphondin (10–50 μM) concentration-dependently attenuated IL–1β-induced COX–2 protein expression and PGE_2 release. The IL–1β-induced increase in COX–2 mRNA expression was also attenuated by sphondin (50 μM). The selective COX–2 inhibitor, NS–398 (0.01–1 μM), inhibited the activity of the COX–2 enzyme in a concentration-dependent manner, while sphondin (10–50 μM) had no effect. Sphondin (50 μM) did not affect the IL–1β-induced activations of p44/42 MAPK, p38 MAPK, and JNK. Treatment of cells with sphondin (50 μM) or the NF-κB inhibitor, PDTC (50 μM) partially inhibited IL–1β-induced degradation of IκB-α in the cytosol and translocation of p65 NF-κB from the cytosol to the nucleus. Furthermore, IL–1β-induced NF-κB-specific DNA-protein complex formation in the nucleus was partially inhibited by sphondin (50 μM) or PDTC (50 μM). Taken together, we demonstrate that sphondin inhibits IL–1β-induced PGE_2 release in A549 cells; this inhibition is mediated by suppressing of COX–2 expression, rather than by inhibiting COX–2 enzyme activity. The inhibitory mechanism of sphondin on IL–1β-

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induced COX−2 expression may be, at least in part, through suppression of NF-κB activity. We conclude that sphondin may have the therapeutic potential as an anti-inflammatory drug on airway inflammation.

Keywords: Sphondin; *Heracleum laciniatum*; Cyclooxygenase-2; Nuclear factor-κB

Introduction

Prostaglandins (PGs) are lipid mediators that are involved in many normal physiological processes, and are implicated in many pathophysiological processes such as inflammation, edema, fever, hyperalgesia, colonic cancer, and Alzheimer's disease [1]. Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to PGH_2, the precursor of a wide group of biologically active mediators such as PGE_2, prostacyclin, and thromboxane A_2 [2]. There are two COX isozymes that have been identified in humans and which bear 60% homology: COX−1 and COX−2 [2,3]. COX−1 is generally thought to produce prostaglandins, which serve to maintain cellular homeostasis, and is known to be expressed constitutively in many cell types including endothelial cells, platelets, and gastric mucosa [4]. COX−2, on the other hand, is induced by many pro-inflammatory stimuli, including cytokines [5] and bacterial lipopolysaccharide (LPS) [6] in cells *in vitro* and at the site of inflammation *in vivo* [7]. COX−2 is thought to be the isoform responsible for the production of pro-inflammatory prostanoids in various models of inflammation [8]. As COX is a target for non-steroidal anti-inflammatory drugs (NSAIDs), this pathway is pharmacologically important [1]. Furthermore the use of isoform-selective COX inhibitors has revealed that many anti-inflammatory benefits of NSAIDs are derived from COX−2 inhibition while many undesirable side effects result from COX−1 inhibition [1,9]. Despite the clinical usefulness of NSAIDs, currently the most-effective drugs in the treatment of chronic inflammatory diseases, such as asthma, are corticosteroids [10]. These down-regulate various inflammatory processes, including PG synthesis, via repression of pro-inflammatory genes such as COX−2 [10,11].

Epithelial cells have an active role in inflammation by producing multiple mediators. It has been demonstrated that treatment of airway epithelial cells with pro-inflammatory cytokines, such as IL−1β, caused COX−2 expression and PGE_2 release [12]. This response has also been observed in human pulmonary epithelial cells (A549) [11,12]. Medicinal plants have been used as traditional remedies in oriental countries over hundreds of years. *Heracleum laciniatum* is a well known oriental crude drug employed as sedative, hypnotic, analgesic, and anti-inflammatory agents [13]. Induction of photocontact

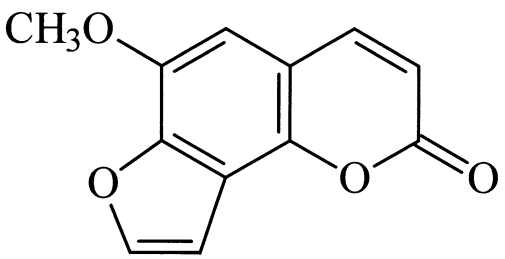

Fig. 1. Chemical structure of sphondin.

allergy to *Heracleum laciniatum* has also been demonstrated [14]. Recently, under large-scale screening experiments, we found that sphondin (Fig. 1), a furanocoumarin derivative isolated from *Heracleum laciniatum* [15], possessed an inhibitory effect on IL−1β-induced COX−2 protein expression and PGE_2 release in A549 cells. Previous studies have demonstrated that sphondin inhibited LPS-induced inducible nitric oxide synthase expression in RAW 264.7 macrophages [16]. Sphondin also effectively inhibited coumarin 7-hydroxylase activity in mouse microsomes [17]. In the present study, we investigated the inhibitory mechanism of sphondin on IL−1β-induced COX−2 protein expression and PGE_2 release in A549 cells.

Materials and Methods

Materials

Sphondin was isolated from *Heracleum laciniatum* according to the methods of Kavli et al. (1983) [15], and provided by Prof. K.Y. Yen (Graduate Institute of Pharmacognosy Science, Taipei Medical University, Taipei, Taiwan). Sphondin was dissolved in dimethylsulfoxide (DMSO) at a final concentration of DMSO, which did not exceed 0.05% and had no effect on the basal COX−2 expression and PGE_2 release. Pyrrolidine dithiocarbamate (PDTC), Trizma base, dithiothreitol (DTT), glycerol, phenylmethylsulphonyl fluoride (PMSF), pepstatin A, leupeptin, sodium dodecyl sulfate (SDS), dimethylsulfoxide (DMSO), and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were purchased from Sigma Chem. (St. Louis, MO, U.S.A.). NS-398 and PD 98059 were purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). If drugs were dissolved in dimethylsulfoxide (DMSO), the final concentration of DMSO did not exceed 0.05%. Penicillin/streptomycin, fetal calf serum (FCS), and Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 were purchased from Life Technologies (Gaithersburg, MD, U.S.A.). PGE_2 enzyme immunoassay kit and human COX−2 cDNA probe were obtained from Cayman Chem. (Ann Arbor, MI, U.S.A.). Antibodies specific for COX−2 and p65 NF-κB were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Antibodies specific for phospho-p44/42 mitogen-activated protein kinase (MAPK), p44/42 MAPK, p38 MAPK, IκB-α, and α-tubulin were purchased from Santa Cruz Biochemicals (Santz Cruz, CA, U.S.A.). Antibodies specific for phospho-p38 MAPK, phospho-c-jun NH_2 terminal kinase (JNK), and JNK were purchased from New England Biolabs, (Beverly, MA, U.S.A.). Anti-mouse IgG-conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories (West Grove, PA, U.S.A.). A digoxigenin (DIG) gel shift kit, 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Boehringer Mannheim (Mannheim, Germany). Protein assay reagents were purchased from Bio-Rad (Hercules, CA, U.S.A.).

Cell culture

A549 cells, a human pulmonary epithelial carcinoma cell line with type II alveolar epithelial cell differentiation, were obtained from American Type Culture Collection and grown in DMEM/Ham's F-12 nutrient mixture containing 10% FCS and penicillin/streptomycin (50 U/ml) in a humidified 37 °C incubator. After reaching confluence, cells were disaggregated in a trypsin solution, washed in DMEM/Ham's F-12 supplemented with 10% FCS, centrifuged at 125 × *g* for 5 min, and then subcultured according to standard protocols.

Measurements of PGE_2 release and the activity of the COX−2 enzyme

A549 cells were cultured in 12-well culture plates. For experiments designed to examine the effects of sphondin on the release of PGE_2 due to endogeneous arachidonic acid, cells were treated with vehicle (0.05% DMSO, control), IL−1β (0.1 ng/ml), or pretreated with sphondin (10-50 μM) or PDTC (50 μM) for 30 min followed by IL−1β, and incubated in a humidified incubator at 37 °C for 24 h. After incubation, the medium was removed and stored at -80 °C until assay. PGE_2 in the medium was assayed by using the PGE_2 enzyme immunoassay kit according to the procedures described by the manufacturer. In experiments designed to measure the effects of sphondin on the activity of the COX−2 enzyme, cells were treated with IL−1β (0.1 ng/ml) for 24 h, after which cells were washed with phosphate-buffered saline (PBS, pH 7.4), and fresh medium was added. Cells were then treated with vehicle (0.05% DMSO, control), NS-398 (0.01-1 μM), or sphondin (10-50 μM) for 30 min followed by arachidonic acid (30 μM), and incubated in a humidified incubator at 37 °C for 30 min. After incubation, the medium was removed for PGE_2 enzyme immunoassay.

Measurement of cell viability

The viability of A549 cells treated with sphondin was determined by assaying the ability of mitochondrial dehydrogenase to convert the soluble 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) into insoluble purple formazan through cleavage of the tetrazolium ring [18]. A549 cells were cultured in 12-well culture plates. Cells were treated with vehicle (0.05% DMSO, control) or sphondin (10-50 μM) for 24 h, and incubated in a humidified incubator at 37 °C. After incubation, the cells were washed with PBS, resuspended in medium with MTT (5 mg/ml), and incubated in a humidified incubator at 37 °C for 4 h. The medium was then removed, and the converted purple dye was dissolved in DMSO. Absorbance of converted dye was measured at 550 nm using a microplate reader.

Protein preparation and Western blotting

Western blotting analysis was performed as described previously [19]. Briefly, A549 cells were cultured in 10-cm petri dishes. After reaching confluence, cells were treated with vehicle (0.05% DMSO, control), IL−1β (0.1 ng/ml), or pretreated with sphondin (10-50 μM) for 30 min followed by IL−1β, and incubated in a humidified incubator at 37 °C for 24 h (for COX−2 and α-tubulin) or 10 min (for phospho-p44/42 MAPK, p44/42 MAPK, phospho-p38 MAPK, p38 MAPK, phospho-JNK, and JNK). After incubation, cells were washed with PBS (pH 7.4), incubated with extraction buffer (10 mM Tris [pH 7.0], 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5% NP-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin) with gentle shaking, and then centrifuged at 12,500 × *g* for 30 min. The cell extract was then boiled in a ratio of 1:1 with sample buffer (100 mM Tris [pH 6.8], 20% glycerol, 4% SDS, and 0.2% bromophenol blue). Electrophoresis was performed using 10% SDS-polyacrylamide gels (2 h, 110 V, 40 mA, 30 μg of protein per lane). Separated proteins were transferred to PVDF membranes (2 h, 40 V); nonspecific IgGs were blocked with 5% fat-free milk powder, and incubated for 2 h with specific antibodies for COX−2, α-tubulin, phospho-p44/42 MAPK, p44/42 MAPK, phospho-p38 MAPK, p38 MAPK, phospho-JNK, or JNK. The blot (for COX−2, α-tubulin, phospho-p44/42 MAPK, and p44/42 MAPK) was then incubated with anti-mouse or anti-rabbit IgG linked to alkaline phosphatase for 2 h. Subsequently, the membrane was developed with NBT/BCIP as a substrate. The blot (for phospho-p38

MAPK, p38 MAPK, phospho-JNK, and JNK) was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 2 h. After incubation, the immunoreactive band was detected with ECL-detecting reagents and developed with Hyperfilm-ECL. Quantitative data were obtained using a computing densitometer with Image-Pro plus software (Media Cybernetics, MD).

Northern Blotting

A549 cells were cultured in 10-cm petri dishes. After reaching confluence, cells were treated with vehicle (0.05% DMSO, control), IL−1β (0.1 ng/ml), or pretreated with sphondin (50 μM) for 30 min followed by IL−1β, and incubated in a humidified incubator at 37 °C for 4 h. Total RNA was isolated by acid guanidinium thiocyanate-phenol-chlorform extraction [20]. Total RNA (20 μg) was denatured with formaldehyde/formamide and incubated at 65 °C for 10 min, size-fractioned on 0.8% formaldehyde-containing agarose gel, and transferred onto Nylon membrane in 20X standard saline citrate (3 M sodium chloride and 0.3 M sodium citrate, pH7.0). The blotted membrane was hybridized with human COX−2 cDNA probe (Cayman Chem. MI, U.S.A.), which was labeled with ^{32}P by using a Random Primer Labelling kit (Amersham). After hybridization, the membrane was washed, dried and autoradiographed with X-ray film (Kodak, NY, U.S.A.). After hybridization with COX−2-specific probe, the blot was stripped and reprobed with a probe for human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA as a control.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

A549 cells were cultured in 10-cm culture petri dishes. After reaching confluence, cells were incubated with vehicle (0.05% DMSO, control), IL−1β (0.1 ng/ml), or pretreated with sphondin (50 μM) or PDTC (50 μM) for 30 min followed by IL−1β, and then incubated in a humidified incubator at 37 °C for 30 min. The cytosolic and nuclear protein fractions were then separated as described previously [21]. Briefly, cells were washed with ice-cold PBS, and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.5 mM DTT, 10 mM aprotinin, 10 mM leupeptin, and 20 mM PMSF) for 15 min on ice, and vortexed for 10 s. Nuclei were pelleted by centrifugation at 15,000 × *g* for 1 min. Supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypertonic buffer (20 mM HEPES [pH 7.6], 25% glycerol, 1.5 mM $MgCl_2$, 4 mM EDTA, 0.05 mM DTT, 10 mM aprotinin, 10 mM leupeptin, and 20 mM PMSF) for 30 min on ice. Supernatants containing nuclear proteins were collected by centrifugation at 15,000 × *g* for 2 min and then stored at − 70 °C. In studies of p65 NF-κB translocation, the nuclear extracts were used; only cytosolic extracts were used to study IκB-α degradation. The extracts were subjected to SDS-PAGE using a 10% running gel, and Western blotting analysis was performed as described above.

An electrophoretic mobility shift assay (EMSA) was performed using a DIG gel shift kit. Briefly, a double-stranded oligonucleotide probe containing NF-κB sequences (5′-AGTTGAGGGGACTTTCC-CAGGC-3′; Promega) was purchased and end labeled with DIG using terminal transferase. The nuclear extract (5-10 μg) was incubated with 4 ng of a DIG-labeled NF-κB probe in 10 μl binding buffer containing 10 μg poly(dI-dc), 1 μg poly L-lysine, 100 mM HEPES [pH 7.6], 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, 1% (w/v) Tween 20, and 150 mM KCl at 25 °C for 15 min. DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on a 6% polyacrylamide gel. The gel was then transferred to a nylon membrane. The gel was incubated with 0.1% milk in TBST at

room temperature for 30 min, and then with anti-DIG linked to alkaline phosphatase for 30 min. The immunoreactive band was finally detected with the chemiluminescent substrate CSPD (disodium3-[4-methoxyspiro{1,2-dioxetane-3,2′-[5-chloro]tricyclo[3.3.1.13,7]decan}-4-yl] phenyl phosphate) and exposed to X-ray film. Quantitative data were obtained using a computing densitometer with Image-Pro plus software (Media Cybernetics).

Statistical analysis

Results shown are the mean ± s.e.mean from three to four independent experiments. One-way analysis of variance (ANOVA) followed by, when appropriate, Bonferroni's multiple range test was used to determine the statistical significance of the difference between means. A *P*-value of less than 0.05 was taken to be statistically significant.

Results

Effects of sphondin on IL−1β-induced PGE_2 release, COX−2 protein expression, and COX−2 mRNA expression

Basal levels of PGE_2 released from A549 cells were low (5.2 ± 0.8 ng/ml, $n = 4$). However, incubation of A549 cells with IL−1β (0.1 ng/ml) for 24 h resulted in a marked release of PGE_2 (22.0 ± 2.7 ng/ml, $n = 4$). Pretreatment of cells with sphondin (10-50 μM) for 30 min attenuated IL−1β-induced PGE_2 release in a concentration-dependent manner (Fig. 2). It has been demonstrated that PDTC, which

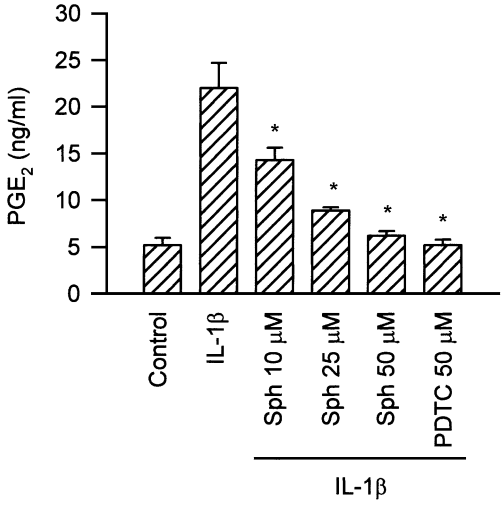

Fig. 2. Effects of sphondin and PDTC on IL−1β-induced PGE_2 release in A549 cells. Cells were pretreated with various concentrations of sphondin or PDTC (50 μM) for 30 min before incubation with IL−1β (0.1 ng/ml) for 24 h. The medium was then removed, and the release of PGE_2 was measured. Results are expressed as the mean ± s.e.mean (n = 4). * $P < 0.05$ compared with the IL−1β-treated group. Sph, sphondin.

inhibits NF-κB activation [22], markedly inhibited the IL−1β-induced COX−2 protein expression and PGE_2 release in A549 cells [23]. In this study, we also found that treatment of the cells with PDTC (50 μM) for 30 min almost completely inhibited IL−1β-induced PGE_2 release (Fig. 2). Stimulation of A549 cells with IL−1β (0.1 ng/ml) for 24 h caused the expression of COX−2 protein. When cells were pretreated for 30 min with sphondin (10-50 μM), the IL−1β-induced COX−2 protein expression was concentration-dependently inhibited by sphondin (Fig. 3). PDTC (50 μM) also markedly attenuated the IL−1β-induced COX−2 protein expression (Fig. 3). In order to investigate whether the suppression of COX−2 protein expression was due to a decrease in COX−2 mRNA level, Northern blot analysis for total mRNA extracted from A549 cells was carried out. Treatment of A549 cells with IL−1β (0.1 ng/ml) for 4 h caused a marked expression of COX−2 mRNA. When cells were pretreated for 30 min with sphondin (50 μM), the IL−1β-induced COX−2 mRNA expression was inhibited (Fig. 4A, B). Treatment of A549 cells with sphondin (10, 25, and 50 μM) for 24 h did not affect the cell viability (98.4% ± 1.6%, 96.7% ± 2.1%, and 97.7% ± 1.4% of the control, respectively, $n = 4$).

Effects of sphondin on the activity of the COX−2 enzyme

The activity of the COX−2 enzyme was quantified by providing cells with exogeneous arachidonic acid, the substrate for COX, and measuring its conversion into PGE_2. Incubation of A549 cells with

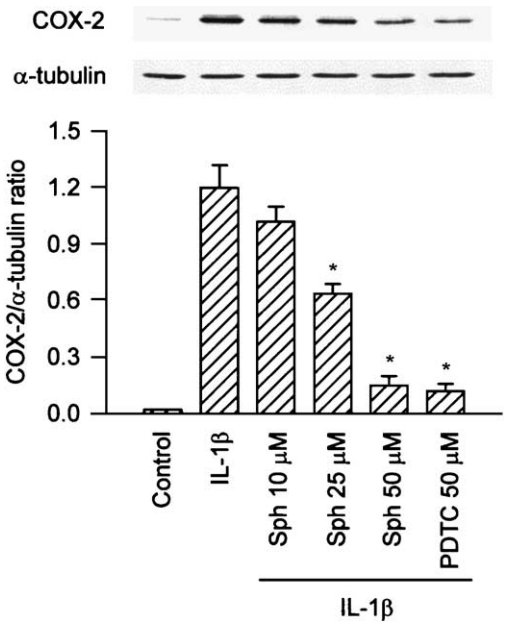

Fig. 3. Effects of sphondin and PDTC on IL−1β-induced COX−2COX−2 protein expression in A549 cells. Cells were pretreated with various concentrations of sphondin or PDTC (50 μM) for 30 min before incubation with IL−1β (0.1 ng/ml) for 24 h, and then prepared for immunodetection using COX−2- or α-tubulin-specific antibody as described in *Methods*. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. The extents of COX−2 and α-tubulin protein expression were quantitated using a densitometer with Image-Pro plus software. The relative level was calculated as the ratio of COX−2 to α-tubulin protein levels. Results are expressed as the mean ± s.e.mean ($n = 4$). * $P < 0.05$ compared with the IL−1β-treated group. Sph, sphondin.

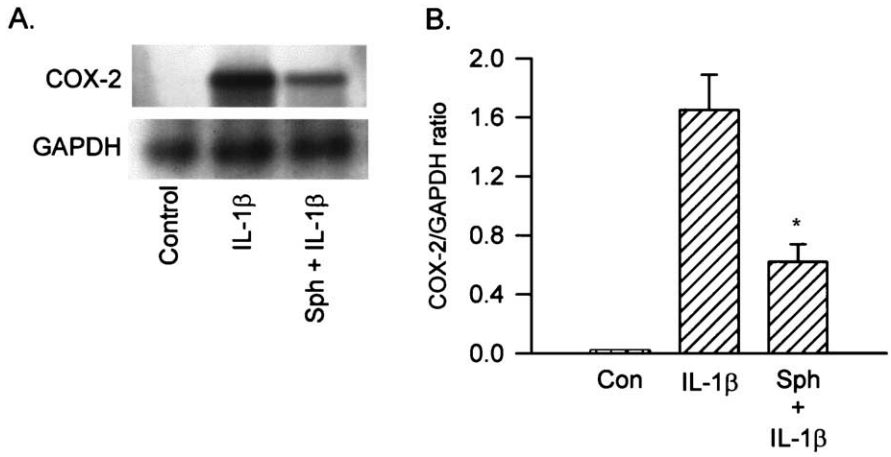

Fig. 4. Effects of sphondin on IL−1β-induced COX−2 mRNA expression in A549 cells. In (A), cells were pretreated with sphondin (50 μM) for 30 min before incubation with IL−1β (0.1 ng/ml) for 4 h. Total RNA was extracted from treated cells and assayed for COX−2 mRNA expression by Northern blot analysis. Blots were hydridized to ^{32}P-labelled COX−2 probe as described in *Methods*. Equal loading in each lane is demonstrated by similar intensities of glyceraldehydes-3-phosphate dehydrogenase (GAPDH). In (B), the extents of COX−2 and GAPDH mRNA expression were quantitated using a densitometer with Image-Pro plus software. The relative level was calculated as the ratio of COX−2 to GAPDH mRNA levels. Results are expressed as the mean ± s.e.mean (n = 3). * $P < 0.05$ compared with the IL−1β-treated group. Sph, sphondin.

IL−1β (0.1 ng/ml) for 24 h resulted in a marked increase in the activity of the COX−2 enzyme (measured in the presence of 30 μM exogeneous arachidonic acid for 30 min). Treatment of cells with the selective COX−2 inhibitor, NS-398 (0.01-1 μM), concentration-dependently inhibited the

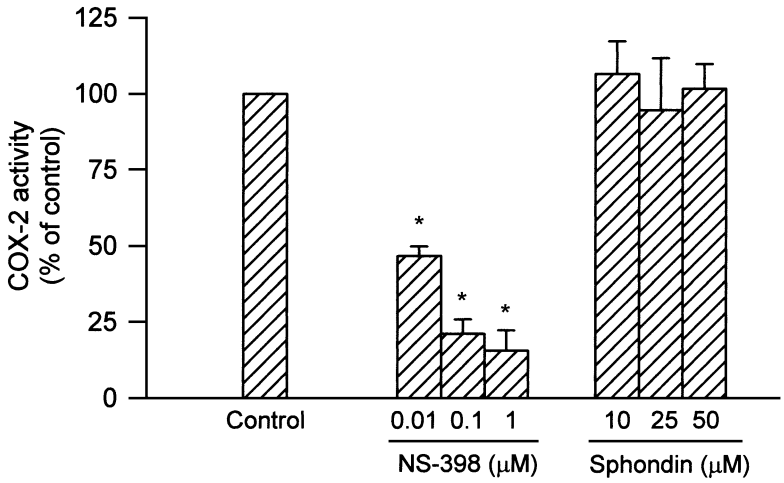

Fig. 5. Effects of NS-398 and sphondin on increases in activity of the COX−2 enzyme caused by IL−1β in A549 cells. Cells were treated with vehicle or IL−1β (0.1 ng/ml) for 24 h, after which cells were washed and fresh medium was added. Cells were then treated with various concentrations of NS-398 or sphondin for 30 min followed by arachidonic acid (30 μM), and incubated at 37 °C for 30 min. After incubation, the medium was removed for PGE_2 enzyme immunoassay. Results are expressed as the mean ± s.e.mean (n = 3). * $P < 0.05$ compared with the control group.

IL−1β-induced increase in the enzyme activity of COX−2, while sphondin (10-50 μM) did not affect it (Fig. 5).

Effects of sphondin on IL−1β-induced p44/42 MAPK, p38 MAPK, and JNK activation

The activations of p44/42 and p38 MAPK have been demonstrated to be involved in the IL−1β-induced COX−2 protein expression and PGE_2 release in pulmonary epithelial cells [24]. It has also been demonstrated that activation of JNK is involved in IL−1β-induced COX−2 protein expression and PGE_2 release in rat renal mesangial cells [25]. Since activation of MAPKs requires phosphorylation at the threonine and tyrosine residues, immunoblot analysis was performed to examine MAPK phosphorylation using antibodies specific for phospho-p44/42 MAPK, phospho-p38 MAPK, and phospho-JNK. Treatment of A549 cells with IL−1β (0.1 ng/ml) for 10 min resulted in marked activation of p44/42 MAPK. When cells were pretreated for 30 min with sphondin (50 μM) or the MEK inhibitor, PD 98059 (30 μM), the IL−1β-induced activation of p44/42 MAPK was completely inhibited by PD 98059, while sphondin had no effect (Fig. 6A, B). None of these treatments had any effect on the protein level of p44/42 MAPK (Fig. 6A). Treatment of A549 cells with IL−1β (0.1 ng/ml)

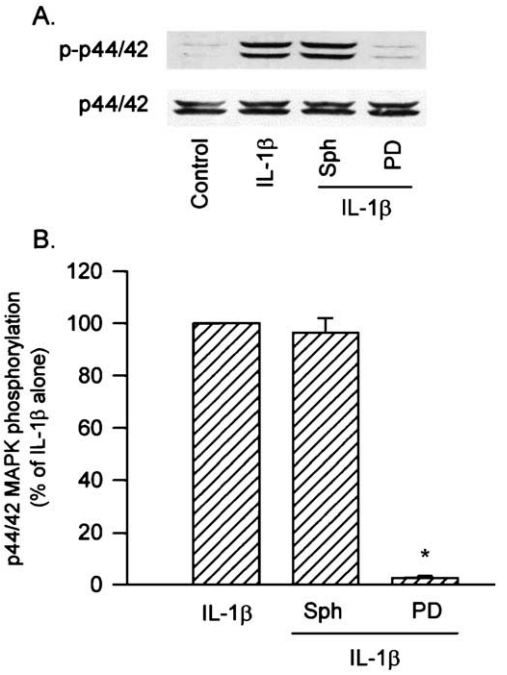

Fig. 6. Effects of sphondin and PD 98059 on IL−1β-induced activation of p44/42 MAPK in A549 cells. In (A), cells were pretreated with sphondin (50 μM) or PD 98059 (30 μM) for 30 min before incubation with IL−1β (0.1 ng/ml) for 10 min. Whole cell lysates were prepared and subjected to Western blotting using antibodies specific for phospho-p44/42 MAPK (p-p44/42) or p44/42 MAPK (p44/42) as described in *Methods*. In (B), the extent of p44/42 MAPK activation was quantitated using a desitometer with Image-Pro plus software. Results are expressed as the mean ± s.e.mean ($n = 4$). * $P < 0.05$ as compared with the IL−1β-treated group. Sph, sphondin; PD, PD 98059.

for 10 min resulted in marked activations of p38 MAPK and JNK. When cells were pretreated for 30 min with sphondin (50 μM), the IL−1β-induced activations of p44/42 MAPK and JNK were not affected by sphondin (Fig. 7A–D). None of these treatments had any effect on the protein levels of p38 MAPK and JNK (Fig. 7A, B).

Effects of sphondin on IL−1β-induced NF-κB activation

Previous studies have demonstrated that treatment of A549 cells with IL−1β results in marked translocation of p65 NF-κB from cytosol to the nucleus as well as the degradation of IκB-α in the cytosol [23]. To determine whether the inhibitory action of sphondin is due to its effect on degradation of

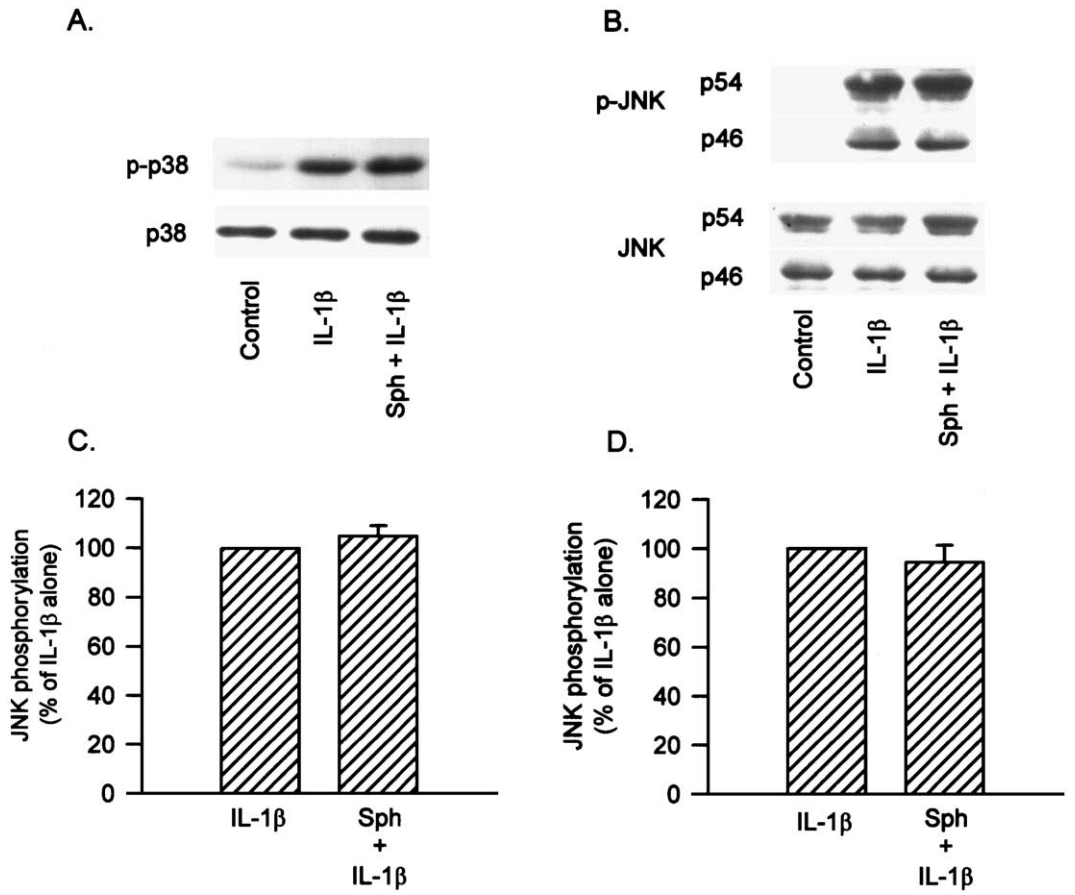

Fig. 7. Effects of sphondin on IL−1β-induced activations of p38 MAPK and JNK in A549 cells. In (A) and (B), cells were pretreated with sphondin (50 μM) for 30 min before incubation with IL−1β (0.1 ng/ml) for 10 min. Whole cell lysates were prepared and subjected to Western blotting using antibodies specific for phospho-p38 MAPK (p-p38), p38 MAPK (p38), phospho-JNK (p-JNK), or JNK as described in *Methods*. In (C) and (D), the extent of activations of p38 MAPK and JNK were quantitated using a desitometer with Image-Pro plus software. Results are expressed as the mean ± s.e.mean (n = 3). * $P<$ 0.05 as compared with the IL−1β-treated group. Sph, sphondin.

IκB-α and translocation of p65 NF-κB, immunoblot analysis of IκB-α and p65 NF-κB in the cytosol and nucleus, respectively, were performed. Stimulation of cells with IL−1β (0.1 ng/ml) for 30 min resulted in marked translocation of p65 NF-κB from cytosol to the nucleus as well as degradation of IκB-α in the cytosol. After pretreatment of cells for 30 min with sphondin (50 μM) or PDTC (50 μM), IL−1β-induced degradation of IκB-α (Fig. 8A, B) and translocation of p65 NF-κB (Fig. 8A, C) were partially inhibited by sphondin or PDTC. To investigate whether sphondin inhibits the activation of NF-κB, analysis of NF-κB-specific DNA-protein binding activity by EMSA was performed. In nuclear extracts of unstimulated cells, a slight intensity in NF-κB-specific DNA-protein complex formation was observed. Stimulation of cells with IL−1β (0.1 ng/ml) for 30 min resulted in marked activation of NF-κB-specific DNA-protein complex formation. When cells were pretreated for 30 min with sphondin

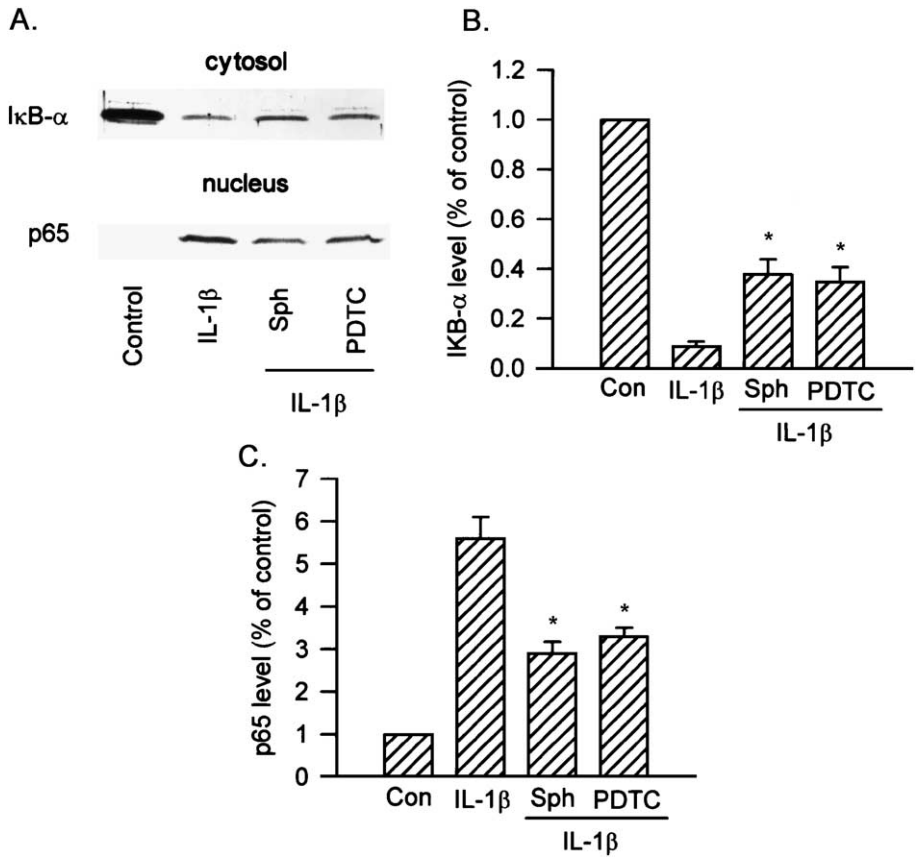

Fig. 8. Effects of sphondin and PDTC on degradation of IκB-α and translocation of p65 NF-κB caused by IL−1β in A549 cells. In (A), cells were pretreated with sphondin (50 μM) or PDTC (50 μM) for 30 min before incubation with IL−1β (0.1 ng/ml) for 30 min, and then the subcellular (cytosol and nucleus) fractions were prepared. Cytosolic levels of IκB-α and nuclear levels of p65 NF-κB were immunodetected with IκB-α- or p65 NF-κB-specific antibody, respectively, as described in *Methods*. In (B) and (C), the extents of p65 NF-κB and IκB-α protein levels were quantitated using a desitometer with Image-Pro plus software. Results are expressed as the mean ± s.e.mean ($n = 3$). * $P < 0.05$ as compared with the IL−1β-treated group. Sph, sphondin.

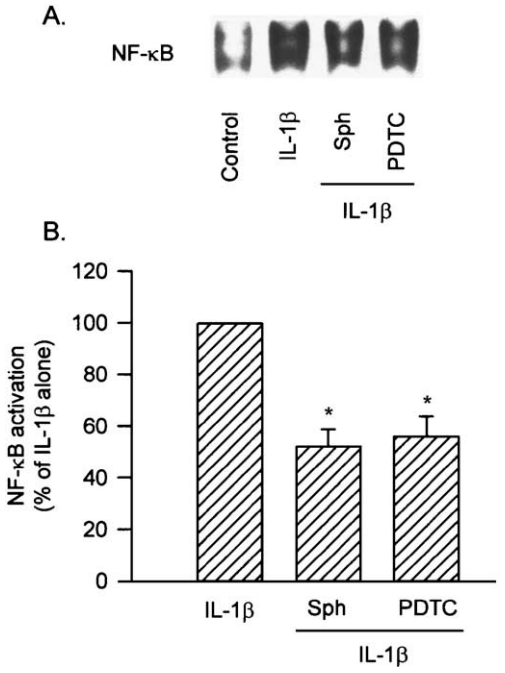

Fig. 9. Effects of sphondin and PDTC on IL−1β-induced NF-κB-specific DNA-protein complex formation in nuclear extracts of A549 cells. In (A), cells were pretreated with sphondin (50 μM) or PDTC (50 μM) for 30 min before incubation with IL−1β (0.1 ng/ml) for 30 min. Nuclear extracts were prepared for determination of NF-κB-specific DNA-protein binding activity by EMSA as described in *Methods*. In (B), the extent of NF-κB activation was quantitated using a desitometer with Image-Pro plus software. Results are expressed as the mean ± s.e.mean ($n = 3$). * $P < 0.05$ as compared with the IL−1β-treated group. Sph, sphondin.

(50 μM) or PDTC (50 μM), the IL−1IL−1β-induced activation of NF-κB-specific DNA-protein complex formation was partially inhibited by sphondin or PDTC (Fig. 9A, B).

Discussion

In the present study, we found that sphondin suppressed IL−1β-induced COX−2 expression and PGE_2 release. Furthermore, sphondin did not affect the activity of the COX−2 enzyme. These results suggest that the inhibitory effects of sphondin on IL−1β-induced PGE_2 release may be through suppression of COX−2 expression, but not the activity of the COX−2 enzyme. Furthermore, we also found that sphondin (50 μM) markedly inhibited the IL−1β-induced increase in COX−2 mRNA expression. This result suggests that the inhibition of COX−2 gene expression might be involved in the inhibitory effect of sphondin on IL−1β-induced COX−2 protein expression. The activations of p44/42 and p38 MAPK has been demonstrated to be involved in the IL−1β-induced COX−2 protein expression and PGE_2 release in pulmonary epithelial cells [24]. It has also been demonstrated that activation of JNK is involved in IL−1β-induced COX−2 protein expression and PGE_2 release in rat renal mesangial cells [25]. In this study, we found that sphondin did not inhibit IL−1β-induced activations of p44/42 MAPK,

p38 MAPK and JNK, suggesting that the inhibitory effect of sphondin on IL−1IL−1β-induced COX−2 protein expression might not be mediated through suppression of the activations of p44/42 MAPK, p38 MAPK and JNK.

The promoter region of the COX−2 gene contains a TATA box and binding sites for several transcription factors including NF-κB, the nuclear factor for interleukin-6 expression (NF-IL-6) and the cyclic AMP response element binding protein [26,27]. Previous studies have also demonstrated that transcription factor NF-κB is involved in IL−1β-induced COX−2 protein expression in A549 cells [23,28]. Therefore, it is possible that suppression of IL−1β-mediated COX−2 expression by sphondin may be mediated by suppression of NF-κB activity. The transcription factor NF-κB is constitutively present in cells as a heterodimer, consisting of a p50 DNA-binding subunit and a p65 trans-activating subunit. NF-κB is normally held in cytoplasm in an inactivated state by the inhibitor protein, IκB-α. After activation, the cytosolic NF-κB/IκB-α complex dissociates, and free NF-κB is translocated to the nucleus where it activates the responsive gene [29,30]. Our present study demonstrats that IL−1β-induced degradation of IκB-α and translocation of p65 NF-κB are partially inhibited by sphondin and the NF-κB inhibitor, PDTC. Furthermore, we found that the IL−1β-induced activation of NF-κB-specific DNA-protein complex formation is partially inhibited by sphondin and PDTC. These results indicate that the inhibitory action of sphondin on IL−1β-mediated COX−2 expression may be mediated, at least in part, by suppression of the activity of this transcription factor. This agrees with more-complex regulation of COX−2 expression in which other transcription factors, such as nuclear factor-interleukin-6 or the cAMP response element, may be involved [26,27].

In conclusion, we have demonstrated that sphondin may inhibit IL−1β-induced COX−2 expression and PGE_2 release in A549 cells. These inhibitory effects of sphondin on IL−1β-induced PGE_2 release may occur through suppression of COX−2 expression, rather than inhibition of COX−2 enzyme activity. The action mechanism of sphondin on the inhibition of IL−1β-induced COX−2 expression may be, at least in part, through inhibiting NF-κB activation. Thus, sphondin may have therapeutic potential as an anti-inflammatory drug on airway inflammation.

Acknowledgements

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