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Biochemical and Biophysical Research Communications 313 (2004) 171–177
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Costunolide inhibits interleukin-1β expression by down-regulation of AP-1 and MAPK activity in LPS-stimulated RAW 264.7 cells☆

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Received 22 September 2003

Abstract

Costunolide, a sesquiterpene lactone isolated from the root of *Saussurea lappa* Clarke, is known to have a variety of biological activities, including anti-carcinogenic and anti-fungal activities. Here, we demonstrated the inhibitory effect of costunolide on the protein and mRNA expression of interleukin-1β (IL-1β) in LPS-stimulated RAW 264.7 cells. We also showed that costunolide suppressed the transcriptional activity of the IL-1β promoter. Moreover, costunolide inhibited the activity of AP-1 transcription factor, and the phosphorylation of MAPKs, including SAPK/JNK and p38 MAP kinase. The inhibitory effect of costunolide on AP-1 activity was also confirmed by an electrophoretic mobility shift assay. Additionally, specific inhibitors of SAPK/JNK and p38 MAP kinase, SP600125 and SB203580, also suppressed LPS-induced increase in IL-1β gene expression and AP-1 DNA binding. Taken together, these results demonstrate that costunolide inhibits IL-1β gene expression by blocking the activation of MAPKs and DNA binding of AP-1 in LPS-stimulated RAW 264.7 cells.

Keywords: Costunolide; Interleukin-1β; Inflammation; AP-1; MAP kinase

Sesquiterpene lactones constitute a large family of more than 5000 compounds mainly isolated from members of the Compositae and the Magnoliaceae [1–3], and possess a wide spectrum of biological activities, including anti-neoplastic and anti-inflammatory activities [4,5]. Costunolide, a well-known sesquiterpene lactone present in many medicinal plants, exerts an anti-carcinogenic activity [6], an anti-viral activity [7], and an anti-fungal activity [1,8]. Immunosuppressive activities of costunolide were also reported previously. Taniguchi et al. [9] reported that costunolide inhibits the killing activity of cytotoxic T lymphocytes by preventing tyrosine phosphorylation in response to the crosslinking of T cell receptors. Moreover, costunolide was reported to have an inhibitory effect on nitric oxide production and NF-κB/Rel transcriptional activity [10–12].

The function of macrophages, which play an important role in host defense and inflammation, is mediated by a variety of cytokines and inflammatory mediators, such as IL-1β, TNF-α, nitric oxide, and prostagladins. IL-1β is a multifunctional pro-inflammatory cytokine produced mainly by cells of the monocyte/macrophage lineage. The transcriptional regulation of IL-1β expression is tightly controlled events, and several transcription factors, such as AP-1, NF-κB, CREB, and NF-IL6, are known to be involved in the transcriptional regulation of IL-1β gene expression [13–16]. AP-1, a well-known regulator of cell survival and death, is implicated in the regulation of gene expression of a variety of cytokines, including IL-1β. Serkkola and Hurme [13] reported that the synergism between protein-kinase C and cAMP-dependent pathway in the expression of the IL-1β gene is mediated via the AP-1 enhancer activity in THP-1, a human monocytic cell line. Hurme and Matikainen [17] also showed that the potentiation of phobol ester-induced IL-1β expression by okadaic acid is mediated by the

☆ *Abbreviations:* IL-1β, interleukin-1β; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinases; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal protein kinase; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-polymerase chain reaction.

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doi:10.1016/j.bbrc.2003.11.109

up-regulation of AP-1. Moreover, it is reported that synthetic lipopeptides derived from *Mycoplasma fermentans* increase IL-1β and TNF-α expression by induction of MAPKs and AP-1 activities in RAW 264.7, a murine macrophage cell line [18]. These reports suggest the involvement of AP-1 activity in the regulation of IL-1β expression in cells of the monocyte/macrophage lineage. AP-1 activity is induced by various factors, such as growth factors, cytokines, hormones, bacterial and viral infections, and various stresses. Signals initiated by these factors activate MAPK signaling cascades to activate AP-1 activity (reviewed in [19]).

The objective of the present study was to investigate the effect of costunolide on gene expression of IL-1β, an important immune regulator, and to reveal the mode of action of costunolide on the effect. Our study suggests that costunolide suppresses IL-1β gene expression possibly by inhibiting AP-1 activity and MAPK activation.

Materials and methods

Chemicals, animals, and cell culture. All reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. Costunolide was dissolved in dimethyl sulfoxide and freshly diluted in culture media for all experiments. RAW 264.7 cells (ATCC TIB71) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in 5% CO_2 humidified air.

Enzyme-linked immunosorbent assay. RAW 264.7 cells were plated at 5×10^5 cells/ml and stimulated with LPS (300 ng/ml) in the presence or absence of costunolide (0.1, 0.3, 1 or 3 μM) for 24 h. Cells were lysed and the concentration of total IL-1β was determined by sandwich immunoassays using a protocol supplied by R&D Systems (Minneapolis, MN).

RT-PCR. The expressions of the mRNA transcripts of IL-1β (forward primer: 5′-TGCAGAGTTCCTACATGGTCAACCC-3′, reverse primer: 5′-GTGCTGCCTAATGTCCCCTTGAATC-3′) and β-actin (forward primer: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, reverse primer: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′) were evaluated by RT-PCR as described previously with slight modifications. Briefly, total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH) as described previously. Equal amounts of RNA were reverse transcribed into cDNA using oligo$(dT)_{15}$ primers. Samples were heated to 94 °C for 5 min and cycled 30 times at 94 °C for 30 s, and 54 °C for 30 s, and 72 °C for 45 s, and this was followed by an additional extension step at 72 °C for 5 min. PCR products were electrophoresed in a 1.5% agarose gel and followed by ethidium bromide staining and photography.

Transient transfection and CAT reporter gene assay. p$(AP\text{-}1)_3$-CAT plasmid has been described previously [20]. To construct pIL-1β(−1809/−2679)-CAT containing an upstream promoter region of IL-1β gene, the 870 bp fragment from −1809 to −2679 site in the promoter region of IL-1β gene was amplified from mouse genomic DNA by using PCR (forward primer: 5′-GCAGATCTGCTCGTGCCTGTAA TCTC-3′, reverse primer: 5′-GCAGATCTAACCAGGGGACAGAG AAG-3′). PCR-amplified products were purified using the PCR Prep DNA purification system (Promega, Madison, WI) and inserted into pCAT-Promoter (Promega) upstream of the CAT gene to form the construct pIL-1β(−1809/−2679)-CAT. The subcloned 870-bp fragment was confirmed by nucleotide sequence analysis using the Sanger dideoxynucleotide protocol. Transient transfection was performed using the DEAE-dextran method with slight modifications [21]. After transfection, cells were plated at 5×10^5 cells/ml and incubated for 24 h. The transfectants were treated with costunolide (0.1, 0.3, 1 or 3 μM) 1 h before the treatment of LPS (300 ng/ml), harvested 24 h after LPS treatment, and lysed. The CAT enzyme expression levels were determined using a CAT enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

EMSA. Nuclear extracts were prepared as described previously. The protein content of the nuclear extracts was determined using a Bio-Rad protein assay kit according to the manufacturer's instructions. The oligonucleotide sequence for NF-κB/Rel was 5′-GATC TCAGAGGGGACTTTCCGAGAGA-3′. Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP. Nuclear extracts (5 μg) were incubated with 2 μg poly(dI–dC) and a ^{32}P-labeled DNA probe, and DNA binding activity was analyzed using a 4.8% polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography. The specificity of binding was examined by competition with an unlabeled oligonucleotide.

Western immunoblot analysis. Whole-cell lysates (20 μg) were separated by 10% SDS–polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were pre-incubated for 1 h at room temperature in Tris-buffered saline (TBS), pH 7.6, containing 0.05% Tween 20 and 3% fatty acid-free bovine serum albumin. The nitrocellulose membranes were then incubated with specific antibodies against phosphorylated p44/42 (ERK), p44/42, phosphorylated SAPK/JNK, SAPK/JNK, phosphorylated p38, or p38 (Cell Signaling Technology, Beverly, MA). Immunoreactive bands were then detected by incubating with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham International).

Statistical analysis. The mean ± SD was determined for each treatment group in each experiment. Significance was determined by either Dunnett's two-tailed *t* test for comparison between two groups or by analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons.

Results

Inhibition of IL-1β production by costunolide in LPS-stimulated RAW 264.7 cells

As described previously, IL-1β is mainly produced by monocytes/macrophages and plays an important role in regulating a variety of biological functions. Here, we examined the effect of costunolide on the production of IL-1β in LPS-stimulated RAW 264.7, a murine macrophage cell line. Treatment of RAW 264.7 cells with LPS (300 ng/ml) caused a substantial increase in the production of IL-1β (Fig. 1). However, the LPS-induced production of IL-1β was inhibited by costunolide in a dose-dependent manner (Fig. 1). The inhibition of IL-1β production by low doses of costunolide (0.1 and 0.3 μM) was relatively mild, but treatment of 1 and 3 μM of costunolide caused 70% and 95% inhibition of IL-1β production, respectively. The concentration and duration of costunolide treatment used in this study had no significant effect on the viability of RAW 264.7 cells (data not shown).

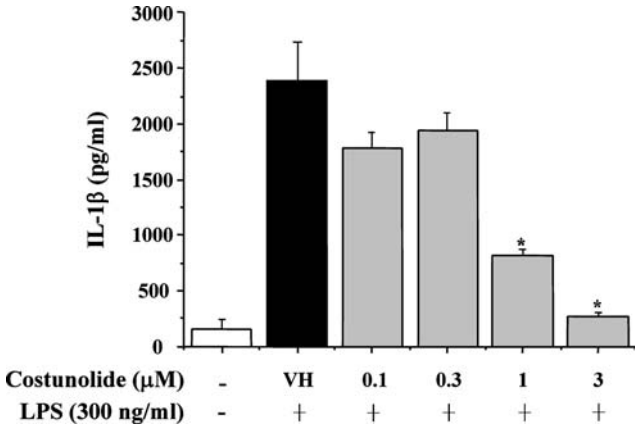

Fig. 1. Inhibition of IL-1β production by costunolide in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with vehicle (DMSO) or indicated concentrations of costunolide for 1 h before being incubated with LPS (300 ng/ml) for 24 h. Cells were lysed and analyzed for IL-1β production as described in Materials and methods. Each column shows the mean ± SD of triplicate determinations. Significance was determined using Student's t test versus the control group ($*p < 0.01$). The result presented is a representative of three independent experiments.

Inhibitory effect of costunolide on mRNA expression of IL-1β in LPS-stimulated RAW 264.7 cells

The effect of costunolide on mRNA expression of IL-1β was examined by RT-PCR. As shown in Fig. 2, the mRNA expression of IL-1β was not detectable in unstimulated cells, but substantially increased 6 h after LPS (300 ng/ml) treatment. However, costunolide inhibited IL-1β mRNA expression in LPS-stimulated RAW 264.7 cells in a concentration-dependent manner (Fig. 2). Consistent with the previous result (Fig. 1), only high doses of costunolide (1 and 3 μM) caused a marked inhibition of the mRNA expression of IL-1β. The mRNA expression of β-actin was not affected by either LPS or costunolide treatment (Fig. 2).

Effect of costunolide on transcriptional activity of IL-1β promoter (−1809/−2679) in LPS-stimulated RAW 264.7 cells

To investigate the effect of costunolide on transcriptional activation of IL-1β promoter region, we performed a transient transfection and reporter gene assay. Costunolide is a well-known inhibitor of NF-κB/Rel. To examine the effect of costunolide on other transcription factors, we made a reporter gene construct containing the partial promoter region of IL-1β from −1809 to −2679, which includes binding sites for AP-1, CREB, and NF-IL6, but not for NF-κB. Transfection of cells with pIL-1β (−1809/−2679)-CAT and treatment with LPS (300 ng/ml) caused 9.3-fold induction of transcriptional activity (Fig. 3). Costunolide inhibited LPS-induced transcriptional activity of pIL-1β (−1809/−2679)-CAT in a

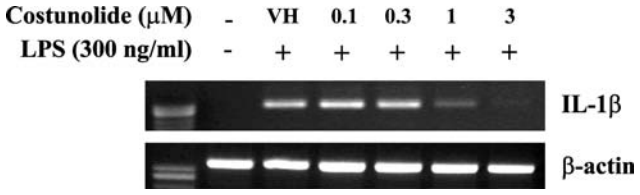

Fig. 2. Inhibition of IL-1β mRNA expression by costunolide in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with vehicle (DMSO) or indicated concentrations of costunolide for 1 h before being incubated with LPS (300 ng/ml) for 6 h. Total RNAs were isolated and IL-1β mRNA expression was determined by RT-PCR. One of two representative experiments is shown.

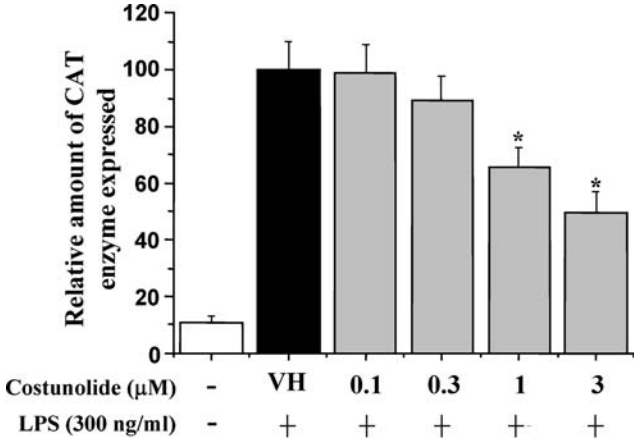

Fig. 3. Inhibitory effect of costunolide on IL-1β promoter activity in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were transiently transfected with pIL-1β (−1809/−2679)-CAT and the expression of CAT enzyme was analyzed by ELISA as described in Materials and methods. Each column shows the mean ± SD of triplicate determinations. Significance was determined using Student's t test versus the control group ($*p < 0.01$). The result presented is a representative of three independent experiments.

dose-dependent manner (Fig. 3). This result suggests that the inhibitory effect of costunolide on IL-1β gene expression is mediated, at least in part, by blocking the transcription activation of the IL-1β promoter.

Inhibition of AP-1 transcriptional activity and DNA binding by costunolide in LPS-stimulated RAW 264.7 cells

As discussed earlier, the transcription factor AP-1 is a regulator of cellular proliferation, transformation, and death [19] and is also known to be important for the gene expression of IL-1β [13]. Therefore, we examined the effect of costunolide on the activity of AP-1. AP-1-dependent transcriptional activity of the reporter gene construct was markedly enhanced by LPS (300 ng/ml), but costunolide significantly suppressed LPS-induced AP-1 activity (Fig. 4). To further confirm the inhibitory effect of costunolide on AP-1 activity, we examined the

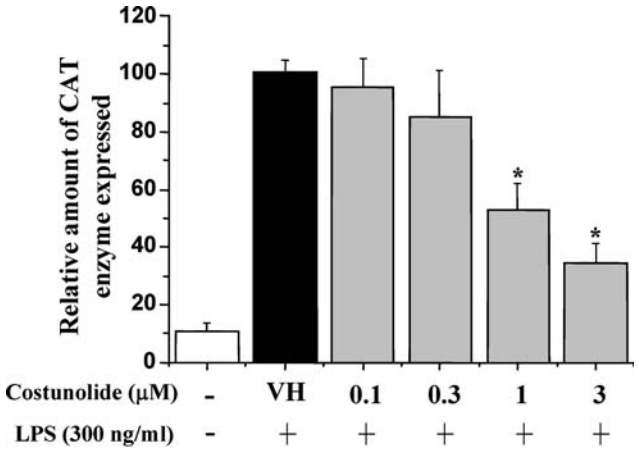

Fig. 4. Inhibitory effect of costunolide on AP-1 activation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were transiently transfected with $p(AP\text{-}1)_3$-CAT and the expression of CAT enzyme was analyzed by ELISA as described in Materials and methods. Each column shows the mean ± SD of triplicate determinations. Significance was determined using Student's t test versus the control group ($^*p < 0.01$). The result presented is a representative of three independent experiments.

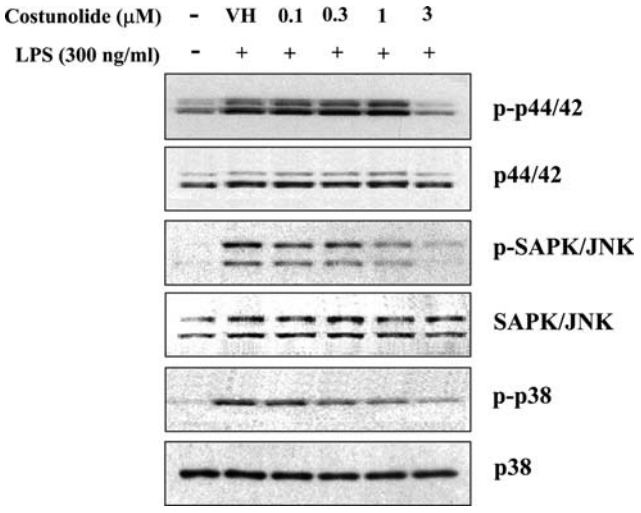

Fig. 6. Effect of costunolde on LPS-induced phosphorylation of ERK, JNK, and p38 MAP kinase in RAW 264.7 cells. RAW 264.7 cells were treated with vehicle (DMSO) or indicated concentrations of costunolide for 1 h before being incubated with LPS (300 ng/ml) for 15 min. The whole-cell lysates were analyzed by Western immunoblot analysis. The results presented are representative of three independent experiments.

effect of costunolide on LPS-induced DNA binding of AP-1 in RAW 264.7 cells using electrophoretic mobility shift assay. AP-1 DNA binding was also induced by LPS (300 ng/ml) and costunolide inhibited LPS-induced DNA binding of AP-1 in a dose-dependent manner (Fig. 5). These results suggest that AP-1 may be involved

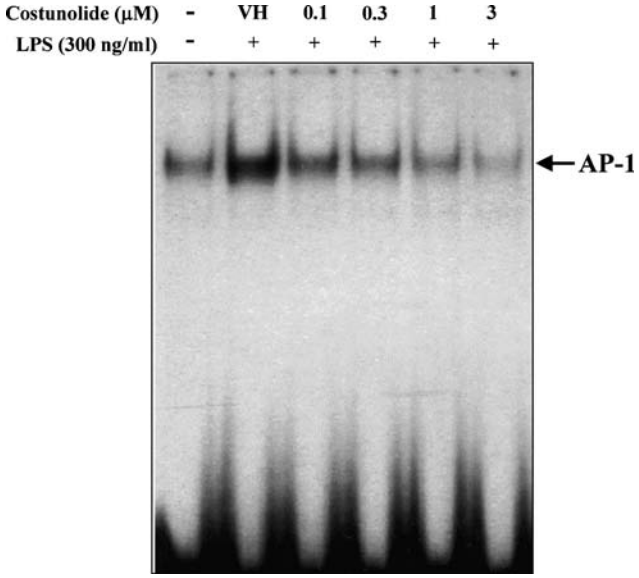

Fig. 5. Effect of costunolide on DNA binding activity of AP-1 in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with vehicle (DMSO) or indicated concentrations of costunolide for 1 h before being incubated with LPS (300 ng/ml) for 3 h. Nuclear extracts were then prepared and AP-1 DNA binding was determined by electrophoretic mobility shift assay. The result presented is a representative of three independent experiments.

in the inhibitory activity of costunolide on the gene expression of IL-1β.

Inhibition of the phosphorylation of MAPKs by costunolide in LPS-stimulated RAW 264.7 cells

To further investigate whether the inhibitory effect of costunolide on AP-1 activity is mediated by the modulation of MAPK activation, we examined the effect of costunolide on MAPK activation. As shown in Fig. 6, costunolide suppressed LPS-induced activation of SAPK/JNK and p38 MAP kinase in a dose-dependent manner. Consistent with the previous results (Figs. 1–3), 1 and 3 μM of costunolide markedly inhibited SAPK/JNK and p38 MAP kinase activation, suggesting the involvement of these MAPKs in the inhibitory effect of costunolide on AP-1 activity. LPS-induced activation of ERK was able to be inhibited only by a high dose of costunolide (3 μM) (Fig. 6). The amount of non-phosphorylated form of MAPKs was unaffected by either LPS or costunolide treatment.

Effect of specfic inhibitors of MAPKs on IL-1β production, IL-1β mRNA expression and AP-1 DNA binding

To further investigate the involvement of MAPKs in the regulation of IL-1β gene expression and AP-1 activity in our system, we examined the effect of specific inhibitors of MAPKs on IL-1β production, IL-1β mRNA expression, and AP-1 DNA binding. Specific inhibitors of SAPK/JNK and p38 MAP kinase, SP600125 and SB203580, potently inhibited the LPS-induced IL-1β gene expression in RAW 264.7 cells,

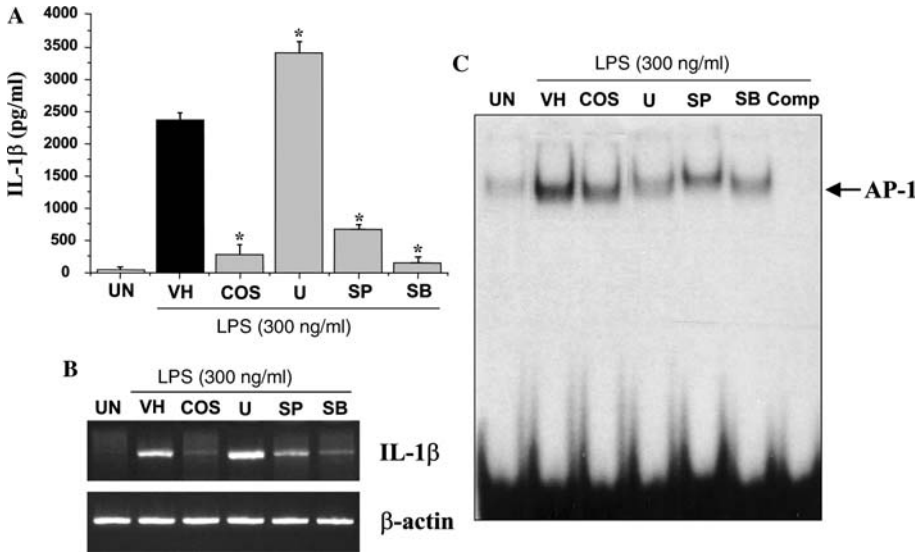

Fig. 7. Effect of MAPK inhibitors on IL-1β production, IL-1β mRNA expression, and AP-1 DNA binding. RAW 264.7 cells were treated with vehicle (DMSO), costunolde (3 μM), U0126 (30 μM), SP600125 (10 μM) or SB203580 (30 μM) for 1 h before being incubated with LPS (300 ng/ml). Cell lysates, total RNAs, and nuclear extracts were prepared and assayed as described in Materials and methods. The results presented are representative of more than two independent experiments.

whereas U0126, a specific inhibitor of ERK, potentiated IL-1β gene expression in LPS-stimulated RAW 264.7 cells (Figs. 7A and B). However, the LPS-induced DNA binding of AP-1 was inhibited by all of 3 MAPK inhibitors (Fig. 7C). These results suggest that MAPKs are involved in the regulation of IL-1β gene expression and AP-1 activity in our system.

Discussion

There have been many reports demonstrating an anti-inflammatory effect of sesquiterpene lactones, and several lines of evidence suggest that blocking the NF-κB/Rel activity is a possible mechanism responsible for this effect [22–24]. Recently, costunolide was also reported to inhibit iNOS gene expression by blocking NF-κB/Rel activation [10,12,25]. In the present study, we demonstrated that costunolide exerts its anti-inflammatory effect by blocking the activation of AP-1 and the phosphorylation of SAPK/JNK and p38 MAP kinase in LPS-stimulated RAW 264.7 cells.

AP-1 activity is induced by a variety of stimuli, including growth factors, cytokines, and bacterial infections, and the induction of AP-1 activity is known to be mediated by MAPK signaling cascade. Serum and growth factors induce AP-1 by activating ERK subgroup of MAPKs [26]. The induction of AP-1 by pro-inflammatory cytokines is mostly mediated by the SAPK/JNK and p38 MAP kinase [27]. Stimulation of macrophages by LPS activates all of 3 MAPK pathways, and the activation of these signaling pathways in turn activates a variety of transcription factors, such as AP-1 and NF-κB/Rel [28]. These reports suggest that MAPK signaling pathways are crucial for the regulation of AP-1 activity. Here, we also showed that all of 3 MPAKs play an important role in the regulation of AP-1 activity in RAW 264.7 cells using specific inhibitors of MAPKs. Furthermore, we demonstrated that costunolide inhibited LPS-induced transcriptional activity of AP-1 and phosphorylation of SAPK/JNK, and p38 MAP kinase. Considering the importance of AP-1 and MAPKs in the regulation of IL-1β gene expression, these results suggest that the inhibition of IL-1β gene expression by costunolide is mediated via the inhibition of AP-1, SAPK/JNK, and p38 MAP kinase activity. To our knowledge, the current data are the first demonstration of the inhibitory effect of costunolide on the activity of AP-1 and MAPKs. A number of reports demonstrated that NF-κB/Rel activity is regulated by MAPKs (reviewed in [29]). We also previously showed that SB203580 blocked the LPS-mediated induction of NF-κB/Rel in RAW 264.7 cells, suggesting the importance of p38 MAP kinase in the NF-κB/Rel activity in our system [30]. Therefore, our results also suggest a possible mechanism responsible for the well-known inhibitory effect of costunolide on NF-κB/Rel activity.

A number of sesquiterpene lactones have been reported to have anti-inflammatory effects. Parthenolide, one of the best known anti-inflammatory sesquiterpene lactones, has been reported to inhibit expressions of several inflammatory mediators, including iNOS, TNF-α, IL-1β, IL-8, and IL-12 [23,31]. The inhibitory effect of parthenolide on IL-4 gene expression in peripheral blood T cells was also reported [32]. Moreover, yomogin, another sesquiterpene lactone isolated from

medicinal plant *Artemisia princes Pampan*, was reported to inhibit iNOS gene expression in LPS-stimulated macrophages [33]. There have been several reports describing the mechanism responsible for the anti-inflammatory effects of sesquiterpene lactones. Hall and coworkers reported that α-methylene-γ-butyrolactone moiety of sesquiterpene lactones is required for their anti-inflammatory activity [34]. Recent studies also demonstrated that sesquiterpene lactones with the α-methylene-γ-butyrolactone moiety exert an inhibitory effect on iNOS gene expression by blocking NF-κB/Rel activation [10,35]. Moreover, modified parthenolides, which lack the α-methylene-γ-butyrolactone moiety, were reported to lose their anti-inflammatory properties [24]. The fact that costunolide also possesses the α-methylene-γ-butyrolactone moiety might provide a possible explanation for its anti-inflammatory properties. However, the relationship between the inhibitory effect of costunolide on AP-1 and MAPK activity and its α-methylene-γ-butyrolactone moiety needs to be further studied.

In conclusion, the present study demonstrates the inhibitory effect of costunolide on IL-1β gene expression in macrophages. Our results also suggest that the effect of costunolide on IL-1β gene expression is mediated by the inhibition of the activity of AP-1 and the phosphorylation of SAPK/JNK and p38. The current data provide a new insight into a mechanism responsible for the anti-inflammatory effect of costunolide.

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