Activation of mitogen-activated protein kinase is involved in sphingosine 1-phosphate-stimulated interleukin-6 synthesis in osteoblasts

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Abstract We previously showed that sphingosine 1-phosphate (SPP) acts as a second messenger for tumor necrosis factor α-induced interleukin-6 (IL-6) synthesis in osteoblast-like MC3T3-E1 cells. In the present study, we further investigated the mechanism of IL-6 synthesis induced by SPP in MC3T3-E1 cells. SPP significantly induced p42/p44 mitogen-activated protein (MAP) kinase activity. PD98059, an inhibitor of MAP kinase kinase, suppressed SPP-induced IL-6 synthesis as well as SPP-induced MAP kinase activation. The patterns of both inhibitions were similar. TMB-8, an inhibitor of Ca^{2+} mobilization from intracellular Ca^{2+} stores, significantly suppressed the SPP-induced IL-6 synthesis. These results strongly suggest that SPP-induced IL-6 synthesis is mediated via p42/p44 MAP kinase activation in osteoblast-like cells and that the SPP-induced IL-6 synthesis is dependent on intracellular Ca^{2+} mobilization.

Key words: Mitogen-activated protein kinase; Sphingosine 1-phosphate; Interleukin-6

1. Introduction

Recently, sphingolipid and its metabolites have been implicated in diverse cellular functions [1,2]. Breakdown of sphingomyelin through sphingomyelinase results in the formation of ceramide, which is subsequently metabolized to sphingosine and sphingosine 1-phosphate (SPP) [1,2]. Ceramide has been reported to induce apoptosis in several cells, whereas sphingosine and SPP are mitogenic. Accumulating evidence indicates that these sphingosine metabolites act as second messengers and mediate several biological effects induced by extracellular agonists such as tumor necrosis factor-α (TNF) [3–5]. In bone cells, it has been reported that not ceramide but sphingosine and SPP induce a transient elevation in intracellular free Ca^{2+} from intracellular stores in osteoblast-like MC3T3-E1 cells [6]. However, the physiological roles of sphingosine derivatives have not yet been clarified. We have recently reported that TNF, a potent bone resorptive agent [7], induces sphingomyelin turnover in osteoblast-like MC3T3-E1 cells and SPP acts as a second messenger for TNF-induced IL-6 synthesis [8]. We also showed that TNF autoregulates IL-6 synthesis due to protein kinase C activation via phosphatidylcholine-specific phospholipase C [8].

Mitogen-activated protein (MAP) kinases play important roles in intracellular signaling of a variety of agonists [9]. In osteoblasts, it has been reported that MAP kinases are activated by several agonists such as basic fibroblast growth factor and estrogen [10,11]. In the present study, we investigated the potential involvement of MAP kinase in signaling for IL-6 synthesis induced by SPP in osteoblast-like MC3T3-E1 cells. We here show that p42/p44 MAP kinase activation is involved in SPP-induced IL-6 synthesis in these cells and that the IL-6 synthesis is dependent on intracellular Ca^{2+} mobilization.

2. Materials and methods

2.1. Materials

Mouse IL-6 enzyme immunoassay kit and p42/p44 MAP kinase enzyme assay system were purchased from Amersham Japan (Tokyo, Japan). SPP was obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). PD98059 was obtained from Calbiochem (La Jolla, CA, USA). 8-(*N*,*N*-Diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) was purchased from Research Biochemicals Inc. (Natick, MA, USA). Other materials and chemicals were obtained from commercial sources. PD98059 was dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for IL-6 nor the measurement of p42/p44 MAP kinase activity.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [12,13] were maintained as previously described [14]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells (5×10^4) were seeded into 35-mm diameter dishes in 2 ml of α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 2 ml of α-MEM containing 0.3% FCS. The cells were used for experiments after 24 h.

2.3. Assay for IL-6

The cultured cells were stimulated with various doses of SPP in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. The conditioned medium was collected, and IL-6 in the medium was measured by an IL-6 enzyme immunoassay kit. When indicated, the cells were pretreated with PD98059 or TMB-8 for 20 min.

2.4. Measurement of p42/p44 MAP kinase activity

The cultured cells were stimulated with SPP in 1 ml of an assay buffer [5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, 5.5 mM glucose] containing 0.01% bovine serum albumin for the indicated periods. The cells were washed twice with 1 ml of phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM sodium orthovanadate. The cytosolic fraction was collected as the supernatant after centrifugation at 125 000×*g* for 10 min at 4°C. MAP kinase activity of the cell extracts was then assayed with the use of the p42/p44 MAP kinase enzyme assay system.

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Abbreviations: SPP, sphingosine 1-phosphate; TNF, tumor necrosis factor-α; IL-6, interleukin-6; MAP kinase, mitogen-activated protein kinase; α-MEM, α-minimum essential medium; FCS, fetal calf serum; MEK, MAP kinase kinase

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2.5. Determination

The radioactivity of ^{32}P-labeled samples was determined with a Beckman LS-6500IC liquid scintillation spectrometer.

2.6. Statistical analysis

The data were analyzed by Student's *t*-test and $P < 0.05$ was considered significant. All data are presented as the mean ± S.D. of triplicate determinations.

3. Results

3.1. Effect of SPP on P42/P44 MAP kinase activation in MC3T3-E1 cells

We examined the effect of SPP on p42/p44 MAP kinase activation in MC3T3-E1 cells. SPP significantly activated p42/p44 MAP kinase time-dependently up to 30 min (Fig. 1A). The stimulative effect of SPP on the MAP kinase activation was dose-dependent in the range between 1 and 30 μM (Fig. 1B). In addition, we investigated the effect of PD98059, an inhibitor of MAP kinase kinase (MEK) [15,16], on the SPP-induced activation of p42/p44 MAP kinase. PD98059 suppressed the p42/p44 MAP kinase activation by SPP dose-dependently in the range between 0.1 and 30 μM (Fig. 2).

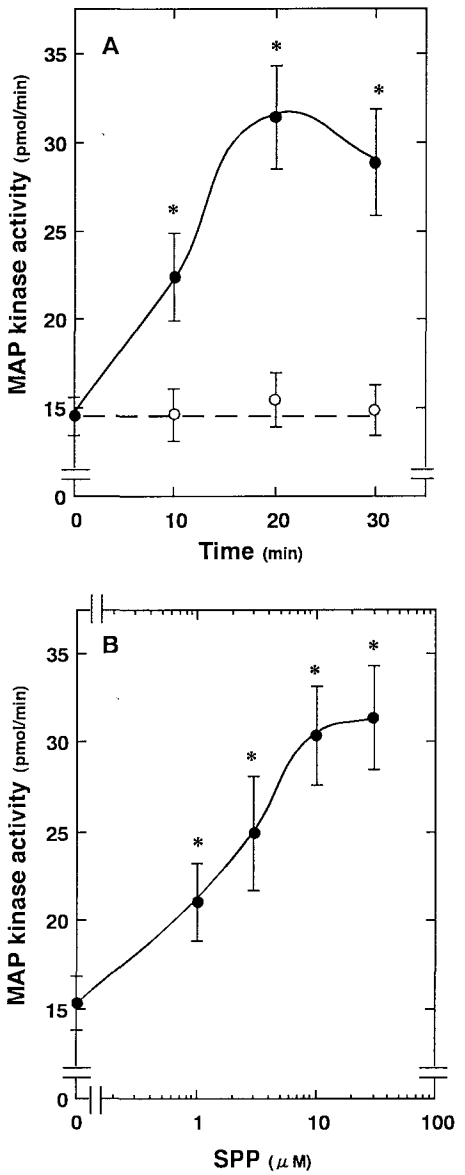

Fig. 1. Effect of SPP on p42/p44 MAP kinase activity in MC3T3-E1 cells. A: The cultured cells were stimulated with 30 μM SPP (●) or vehicle (○) for the indicated periods. B: The cultured cells were stimulated with various doses of SPP for 20 min. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of control.

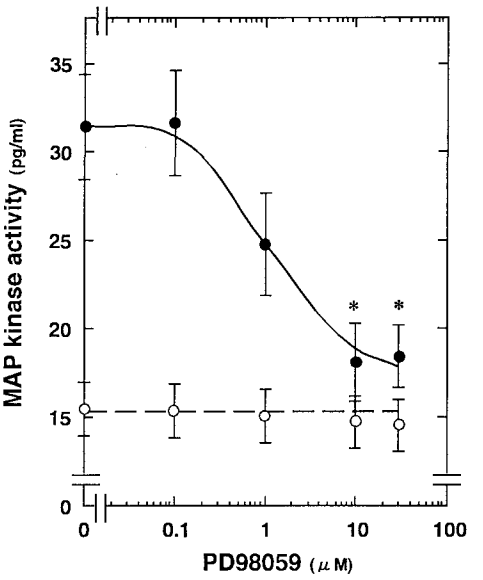

Fig. 2. Effect of PD98059 on the SPP-induced activation of p42/p44 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD98059 for 20 min, and then stimulated with 30 μM SPP (●) or vehicle (○) for 20 min. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of SPP alone.

3.2. Effect of PD98059 on SPP-induced IL-6 synthesis in MC3T3-E1 cells

We previously showed that IL-6 synthesis is stimulated not by ceramide or sphingosine but by SPP in osteoblast-like MC3T3-E1 cells [8]. In order to investigate whether p42/p44 MAP kinase is involved in SPP-stimulated IL-6 synthesis, we examined the effect of MEK inhibitor on the IL-6 synthesis induced by SPP. PD98059 significantly suppressed the SPP-induced IL-6 synthesis (Fig. 3). The inhibitory effect of PD98059 on IL-6 synthesis was dose-dependent in the range between 0.1 and 30 μM.

Table 1
Effect of TMB-8 on the SPP-induced IL-6 synthesis in MC3T3-E1 cells

TMB-8 (30 μM)	SPP (30 μM)	IL-6 (pg/ml)
−	−	< 10
−	+	229 ± 29
+	−	< 10
+	+	88 ± 11*

The cultured cells were pretreated with 30 μM TMB-8 or vehicle for 20 min, and then stimulated with 30 μM SPP or vehicle for 48 h. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of SPP alone.

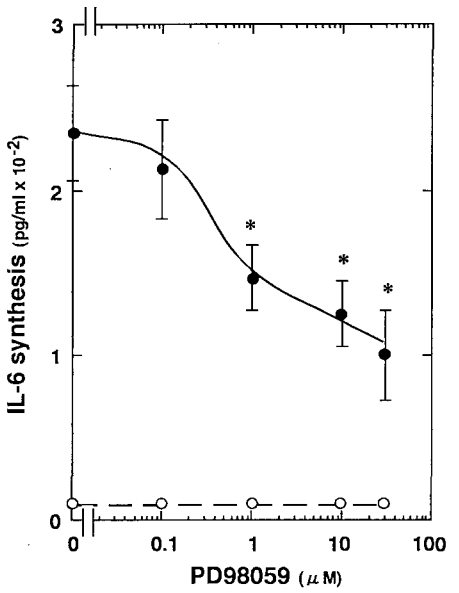

Fig. 3. Effect of PD098059 on the SPP-induced synthesis of IL-6 in MC3T3-E1 cells. The cultured cels were pretreated with various doses of PD98059 for 20 min, and then stimulated with 30 μM SPP (●) or vehicle (○) for 48 h. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*P < 0.05$, compared to the value of SPP alone.

3.3. *Effect of TMB-8 on SPP-induced IL-6 synthesis and p42/p44 MAP kinase activation in MC3T3-E1 cells*

It has been reported that SPP mobilizes free Ca^{2+} from intracellular stores in osteoblast-like MC3T3-E1 cells [7]. Thus, we next examined the effect of TMB-8, an inhibitor of Ca^{2+} mobilization from intracellular stores [17], on the SPP-induced IL-6 synthesis. TMB-8, which alone had little effect on IL-6 synthesis, inhibited the IL-6 synthesis stimulated by SPP (Table 1). In contrast, the SPP-induced p42/p44 MAP kinase activation was not affected by TMB-8 (SPP alone, 32.2 ± 3.0 pmol/min; SPP with 30 μM TMB-8, 31.1 ± 2.9 pmol/min, as measured during 20 min stimulation after 20 min pretreatment of TMB-8 or vehicle).

4. Discussion

In our previous report [8], we demonstrated that TNF induces sphingomyelin hydrolysis in osteoblast-like MC3T3-E1 cells, and that SPP acts as an intracellular second messenger in TNF-induced IL-6 synthesis. In the present study, we further investigated the exact mechanism underlying SPP-stimulated IL-6 synthesis in these cells. We showed that SPP markedly stimulated p42/p44 MAP kinase in MC3T3-E1 cells and that an MEK inhibitor suppressed SPP-induced synthesis of IL-6 as well as SPP-activated p42/p44 MAP kinase. The dose response for the inhibition of IL-6 synthesis by the MEK inhibitor closely correlated with the concentration required for the p42/p44 MAP kinase activation. Therefore, these findings suggest that p42/p44 MAP kinase activation is involved in the SPP-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

We next demonstrated that TMB-8 suppressed the SPP-induced IL-6 synthesis without affecting p42/p44 MAP kinase activation induced by SPP. It has been reported that SPP induces Ca^{2+} mobilization from intracellular stores in several cells [18,19]. In osteoblast-like MC3T3-E1 cells [7], SPP has been shown to mobilize Ca^{2+} from intracellular stores. Taking these findings into account, it is most likely that intracellular Ca^{2+} mobilization by SPP has an important role at a point downstream from p42/p44 MAP kinase in the SPP-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

In conclusion, these results strongly suggest that in IL-6 synthesis, p42/p44 MAP kinase may act at a point downstream from SPP in osteoblast-like cells and that the SPP-induced IL-6 synthesis is dependent on intracellular Ca^{2+} mobilization.

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