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# Sphingosine Modulates Interleukin-6 Synthesis in Osteoblasts

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**Abstract** We previously reported that prostaglandin (PG)$E_1$ and $PGF_{2\alpha}$ induce the synthesis of interleukin-6 (IL-6) via activation of protein kinase (PK)A and PKC, respectively, in osteoblast-like MC3T3-E1 cells. In addition, we have shown that basic fibroblast growth factor (bFGF) elicits IL-6 synthesis through intracellular $Ca^{2+}$ mobilization in these cells and that tumor necrosis factor-α (TNF) induces IL-6 synthesis through sphingosine 1-phosphate produced by sphingomyelin hydrolysis. In the present study, among sphingomyelin metabolites, we examined the effect of sphingosine on IL-6 synthesis induced by various agonists in MC3T3-E1 cells. Sphingosine inhibited the IL-6 synthesis induced by $PGF_{2\alpha}$ or 12-*O*-tetradecanoylphorbol-13-acetate, an activator of PKC. Sphingosine suppressed the $PGE_1$-induced IL-6 synthesis. The IL-6 synthesis induced by cholera toxin, forskolin, or dibutyryl cAMP was inhibited by sphingosine. Sphingosine inhibited the IL-6 synthesis induced by bFGF or A23187. However, sphingosine did not affect the IL-6 synthesis induced by interleukin-1. On the contrary, sphingosine enhanced the TNF-induced IL-6 synthesis. DL-threo-Dihydrosphingosine, an inhibitor of sphingosine kinase, reduced the enhancement by sphingosine as well as the TNF-effect. These results indicate that sphingosine modulates the IL-6 synthesis stimulated by various agonists in osteoblasts. J. Cell. Biochem. 70:338–345. 

**Key words:** sphingosine; interleukin-6; osteoblast

Interleukin-6 (IL-6) is a pleiotropic cytokine that plays critical roles in the regulation of the immune system [Kishimoto, 1989; Van Snick, 1990; Bauer and Herrmann, 1991]. In bone metabolism, it is well known that IL-6 is produced in osteoblasts and secreted and that IL-6 induces osteoclast formation and stimulates bone resorption [Ishimi et al., 1990; Roodman, 1992, 1993]. Bone resorptive agents such as parathyroid hormone, tumor necrosis factor-α (TNF), interleukin-1 (IL-1), and platelet-derived growth factor have been shown to stimulate IL-6 synthesis in cultured osteoblasts [Thomson et al., 1986, 1987; Löwik et al., 1989; Franchimont and Canalis, 1995]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts [Nijweide et al., 1986]. The former cells are responsible for bone formation and the latter for bone resorption. According to current understanding that the receptors of bone resorptive agents are found in osteoblasts, osteoblasts are recognized to have important roles also in the regulation of bone resorption [Nijweide et al., 1986]. Thus accumulating evidence indicates that IL-6 secreted from osteoblasts is an important downstream effector of bone resorptive agents. We previously showed that prostaglandin $E_1$ ($PGE_1$) induces IL-6 synthesis via activation of protein kinase A in osteoblast-like MC3T3-E1 cells [Watanabe-Tomita et al., 1997]. In addition, we have reported that the activation of protein kinase C is involved in the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced IL-6 synthesis in these cells [Kozawa et al., 1997a]. Moreover, we have shown that basic fibroblast growth factor (bFGF) stimulates IL-6 synthesis through intracellular $Ca^{2+}$ mobilization from extracellular space in these cells [Kozawa et al., 1997b]. However, the regulatory mechanism of IL-6 synthesis and the secretion in osteoblasts has not yet been fully elucidated.

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Accumulating evidence suggests that sphingomyelin hydrolysis by sphingomyelinase plays important roles in a variety of cellular functions and the sphingomyelin metabolites mediate biological effects as second messengers [Hannun, 1994; Spiegel and Merrill, 1996]. It is well recognized that sphingomyelinase catalyzes sphingomyelin hydrolysis, resulting in the formation of ceramide [Spiegel and Merrill, 1996]. It is subsequently metabolized to sphingosine and sphingosine 1-phosphate, a phosphorylated product of sphingosine by sphingosine kinase. Ceramide has been reported to induce apoptosis in several cells, whereas sphingosine and sphingosine 1-phosphate are mitogenic. We have recently shown that TNF stimulates sphingomyelin turnover in osteoblast-like MC3T3-E1 cells and that sphingosine 1-phosphate acts as a second messenger for TNF-induced IL-6 synthesis [Kozawa et al., 1997c]. However, ceramide or sphingosine alone did not affect IL-6 synthesis in these cells.

In the present study, among sphingomyelin metabolites, we examined the effect of sphingosine on the IL-6 synthesis induced by various agonists in osteoblast-like MC3T3-E1 cells. Herein, we demonstrate that sphingosine modulates the IL-6 synthesis in these cells.

## MATERIALS AND METHODS

Mouse IL-6 enzyme immunoassay kit was purchased from Amersham Japan (Tokyo, Japan). Sphingosine, $PGF_{2\alpha}$, $PGE_1$, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cholera toxin, forskolin, dibutyryl cAMP ($Bt_2cAMP$), bFGF, and A23187 were purchased from Sigma Chemical Co. (St. Louis, MO). DL-threo-Dihydrosphingosine (DHS) was obtained from Biomol Research Laboratories (Plymouth, PA). IL-1 and TNF were obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. $PGF_{2\alpha}$, $PGE_1$, forskolin, and DHS were dissolved in ethanol. TPA was dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide in the culture medium was 0.2%, and this did not affect the assay for IL-6.

### Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983] were maintained as previously described [Kozawa et al., 1994]. In brief, 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 ($5 \times 10^4$) 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 48 h.

### Assay for IL-6

The cultured cells were pretreated with various doses of sphingosine for 20 min and then stimulated by $PGF_{2\alpha}$, $PGE_1$, TPA, cholera toxin, forskolin, $Bt_2cAMP$, bFGF, IL-1, or TNF for 48 h in 1 ml of α-MEM containing 0.3% FCS. When stimulated by A23187, the medium was exchanged for 1 ml of α-MEM containing 0.3% FCS after 1 h stimulation, and the cells were subsequently incubated for 48 h. The reaction was terminated by collecting the medium, and IL-6 in the medium was then determined with an enzyme immunoassay kit. When indicated, the cells were pretreated with DHS for 20 min prior to the sphingosine treatment.

### Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with SLT-Labinstruments EAR 340 AT. Absorbance was correlated with concentration through a standard curve.

### Statistical Analysis

Each experiment was repeated three times with similar results. The data were analyzed by Student's *t*-test and a $P < 0.05$ was considered significant. All data are presented as the mean ± SD of triplicate determinations.

## RESULTS

### Effects of Sphingosine on IL-6 Synthesis Induced by $PGF_{2\alpha}$, or TPA in MC3T3-E1 Cells

We have shown that $PGF_{2\alpha}$ stimulates the synthesis of IL-6 via activation of protein kinase C in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1997a]. So, we first examined the effect of sphingosine on $PGF_{2\alpha}$-induced IL-6 synthesis in these cells. Sphingosine, which by itself had little effect on IL-6 synthesis as previously described [Kozawa et al., 1997c], significantly suppressed the $PGF_{2\alpha}$-induced IL-6 synthesis (Fig. 1). The inhibitory effect of

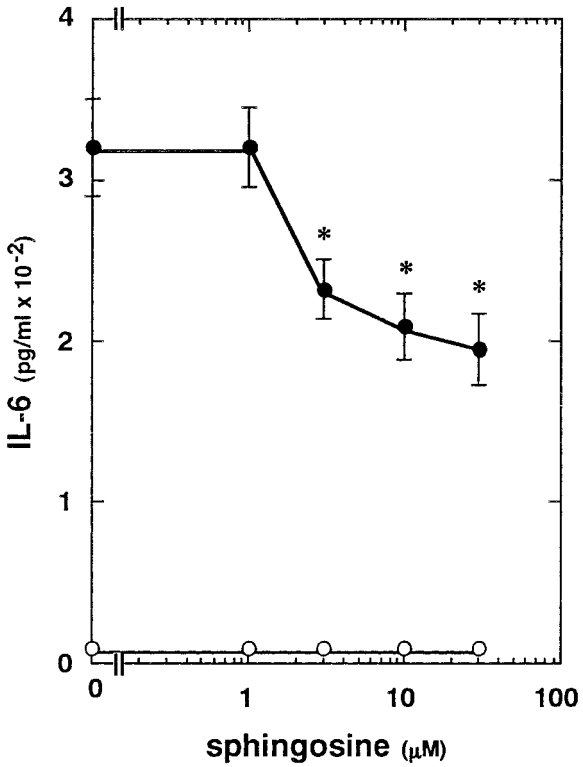


**Fig. 1.** Effect of sphingosine of $PGF_{2\alpha}$-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of sphingosine for 20 min and then stimulated by 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times. *$P < .0.05$ vs. value of $PGF_{2\alpha}$ alone.

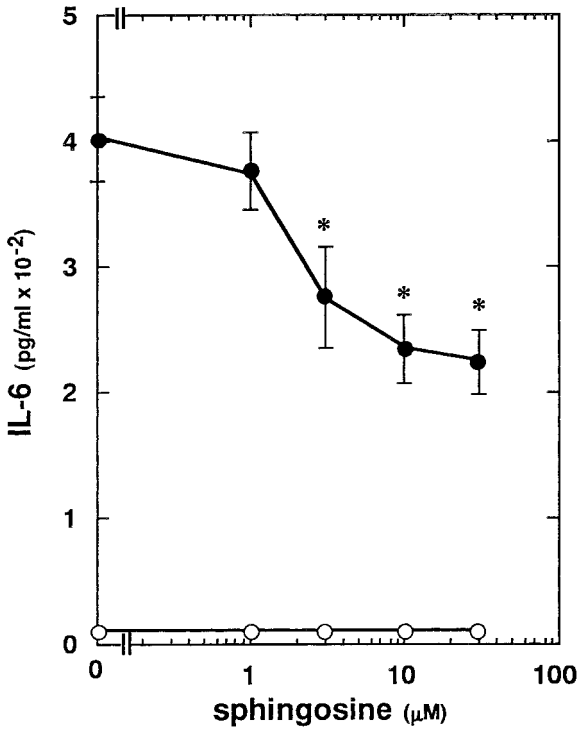


**Fig. 2.** Effect of sphingosine on TPA-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of sphingosine for 20 min and then stimulated by 0.1 μM TPA (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. value of TPA alone.

sphingosine on the IL-6 synthesis was dose-dependent in the range between 1 μM and 30 μM. The maximum effect on the $PGF_{2\alpha}$-induced IL-6 synthesis was observed at 30 μM, and the IL-6 synthesis was suppressed to ~60% of control. In addition, sphingosine markedly inhibited the synthesis of IL-6 stimulated by TPA, an activator of protein kinase C [Nishizuka, 1986], as well as that by $PGF_{2\alpha}$ (Fig. 2). The inhibitory effect of sphingosine was dose-dependent in the range between 1 μM and 30 μM. Sphingosine (30 μM) reduced the IL-6 synthesis by TPA to ~55% of control.

### Effect of Sphingosine on $PGE_1$-Induced IL-6 Synthesis in MC3T3-E1 Cells

We have demonstrated that the IL-6 synthesis is mediated through activation of protein kinase A in MC3T3-E1 cells [Watanabe-Tomita et al., 1997]. We next examined the effect of sphingosine on $PGE_1$-induced IL-6 synthesis in these cells. Sphingosine significantly suppressed the $PGE_1$-induced IL-6 synthesis in a dose-dependent manner in the range between 1 μM and 30 μM (Fig. 3). The maximum effect of sphingosine was observed at 30 μM, a dose that caused ~40% reduction in the effect of $PGE_1$.

### Effects of Sphingosine on IL-6 Synthesis Induced by Cholera Toxin or Forskolin in MC3T3-E1 Cells

To clarify whether or not the inhibitory effect of sphingosine on the $PGE_1$-induced IL-6 synthesis is exerted at a point upstream from Gs, we examined the effect of sphingosine on cholera toxin-induced IL-6 synthesis in MC3T3-E1 cells. Cholera toxin (1 μg/ml), a direct activator of Gs [Gilman, 1987], -induced IL-6 synthesis was significantly inhibited by 30 μM sphingosine (Table I). In addition, we examined the effect of sphingosine on forskolin, a direct activator of adenylate cyclase [Seamon and Daly, 1981], -induced IL-6 synthesis. Sphingosine also had an inhibitory effect on the forskolin-induced IL-6 synthesis (data not shown).

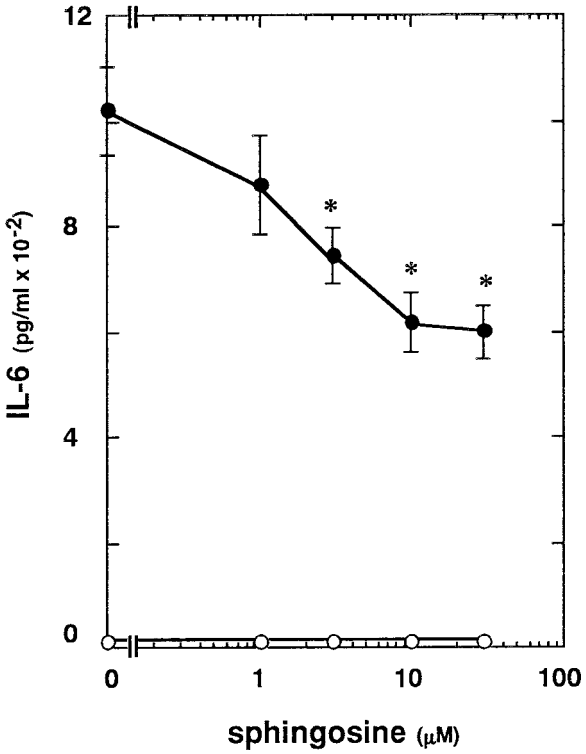


**Fig. 3.** Effect of sphingosine on $PGE_1$-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of sphingosine for 20 min and then stimulated by 10 μM $PGE_1$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. value of $PGE_1$ alone.

**TABLE I. Effect of Sphingosine on Cholera Toxin-induced IL-6 Synthesis in MC3T3-E1 Cells**[a]

| | IL-6 (pg/ml) |
|---|---|
| Control | <10 |
| Sphingosine | <10 |
| Cholera toxin | 487 ± 38* |
| Sphingosine + cholera toxin | 252 ± 20** |

[a]Cultured cells were pretreated with 30 μM sphingosine or vehicle for 20 min and then stimulated by 1 μg/ml cholera toxin or vehicle for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.
*$P < 0.05$ vs. control value; **$P < 0.05$ vs. value of cholera toxin alone.

### Effect of Sphingosine on $Bt_2$cAMP-Induced IL-6 Synthesis in MC3T3-E1 Cells

Furthermore, we examined the effect of sphingosine on the $Bt_2$cAMP-stimulated IL-6 synthesis in MC3T3-E1 cells. Sphingosine markedly inhibited the IL-6 synthesis induced by 3 mM $Bt_2$cAMP (Fig. 4). The inhibitory effect of sphingosine was dose-dependent in the range between 1 μM and 30 μM. The maximum effect of sphingosine was observed at 30 μM, a dose that caused ~60% reduction in the IL-6 synthesis induced by $Bt_2$cAMP.

### Effects of Sphingosine on bFGF- or A23187-Induced IL-6 Synthesis in MC3T3-E1 Cells

We have previously reported that bFGF stimulates IL-6 synthesis through intracellular $Ca^{2+}$ mobilization from extracellular space in MC3T3-E1 cells and that A23187, a Ca-ionophore, alone stimulates IL-6 synthesis [Kozawa et al., 1997b]. Thus we tested the effect of sphingosine on the bFGF-induced IL-6 synthesis. Sphingosine (30 μM) significantly reduced the IL-6 synthesis stimulated by 30 ng/ml bFGF (Table II). We next examined the effect of sphingosine on the A23187-stimulated IL-6 synthesis. Sphingosine significantly inhibited the

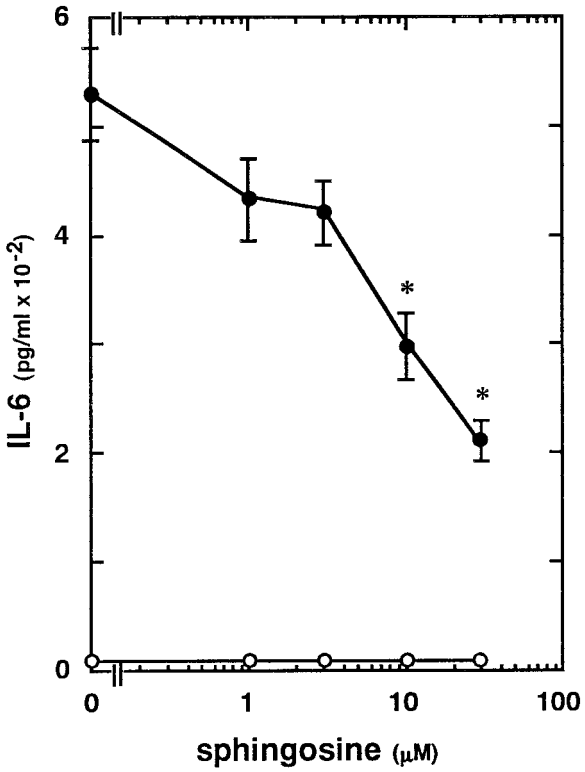


**Fig. 4.** Effect of sphingosine on $Bt_2$cAMP-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of sphingosine for 20 min and then stimulated by 3 mM $Bt_2$cAMP (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. value of $Bt_2$cAMP alone.

**TABLE II. Effect of Sphingosine on bFGF-induced IL-6 Synthesis in MC3T3-E1 Cells**[a]

| | IL-6 (pg/ml) |
|---|---|
| Control | <10 |
| Sphingosine | <10 |
| bFGF | 185 ± 17* |
| Sphingosine + bFGF | 119 ± 10** |

[a]Cultured cells were pretreated with 30 μM sphingosine or vehicle for 20 min and then stimulated by 30 ng/ml bFGF or vehicle for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.
*$P < 0.05$ vs. control value; **$P < 0.05$ vs. value of bFGF alone.

A23187 (1 μM)-induced IL-6 synthesis (Fig. 5). The inhibitory effect of sphingosine was dose-dependent in the range between 1 μM and 30 μM. The maximum effect of sphingosine was observed at 30 μM, a dose that led ~45% reduction in the A23187-effect.

### Effect of Sphingosine on IL-1-Induced IL-6 Synthesis in MC3T3-E1 Cells

It has been reported that IL-6 synthesis is stimulated by IL-1 in osteoblasts [Thomson et al., 1986]. However, the detailed signaling mechanism underlying IL-1-induced IL-6 synthesis in osteoblasts is not known. We also found that IL-1 significantly induces the synthesis of IL-6 in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1997d]. We examined the effect of sphingosine on IL-1-induced IL-6 synthesis in these cells. Sphingosine hardly affected the synthesis of IL-6 induced by IL-1 (30 ng/ml) in the range between 1 μM and 30 μM (Fig. 6).

### Effect of Sphingosine on TNF-Induced IL-6 Synthesis in MC3T3-E1 Cells

In a recent study [Kozawa et al., 1997c], we have shown that TNF stimulates sphingomyelin hydrolysis in MC3T3-E1 cells, and among sphingomyelin metabolites, sphingosine 1-phosphate acts as a second messenger for TNF-induced IL-6 synthesis. We next examined the effect of sphingosine on TNF-induced synthesis of IL-6. Sphingosine significantly amplified the TNF-induced synthesis of IL-6 (Fig. 7). The enhancement effect of sphingosine was dose-dependent in the range between 1 μM and 30 μM. Moreover, DHS, an inhibitor of sphingosine kinase, suppressed not only TNF-induced

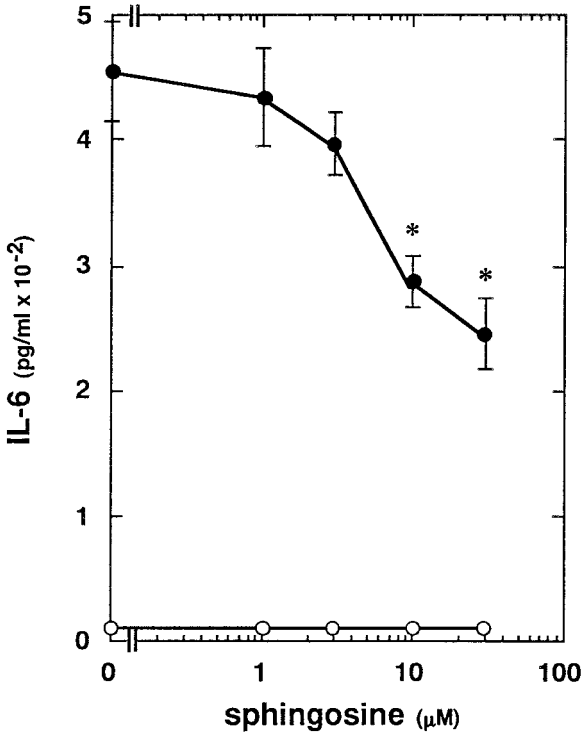


**Fig. 5.** Effect of sphingosine on A23187-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of sphingosine for 20 min and then stimulated by 1 μM A23187 (●) or vehicle (○) for 1 h. After the medium was exchanged, the cells were then incubated for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. value of A23187 alone.

IL-6 synthesis, but also the enhancement effect of sphingosine on the IL-6 synthesis (Table III).

## DISCUSSION

Sphingomyelin metabolites are now recognized to be potent intracellular messengers in a variety of cells [Hannun, 1994; Spiegel and Merrill, 1996]. We previously showed that sphingosine 1-phosphate by itself stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1997c]; on the contrary, ceramide or sphingosine alone did not affect IL-6 synthesis. In the present study, we demonstrated that sphingosine significantly inhibited the $PGF_{2\alpha}$-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. In previous studies [Miwa et al., 1990; Kozawa et al., 1994, 1997a], we have reported that $PGF_{2\alpha}$ activates protein kinase C through both phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D and then stimulates IL-6 synthesis via the activation of

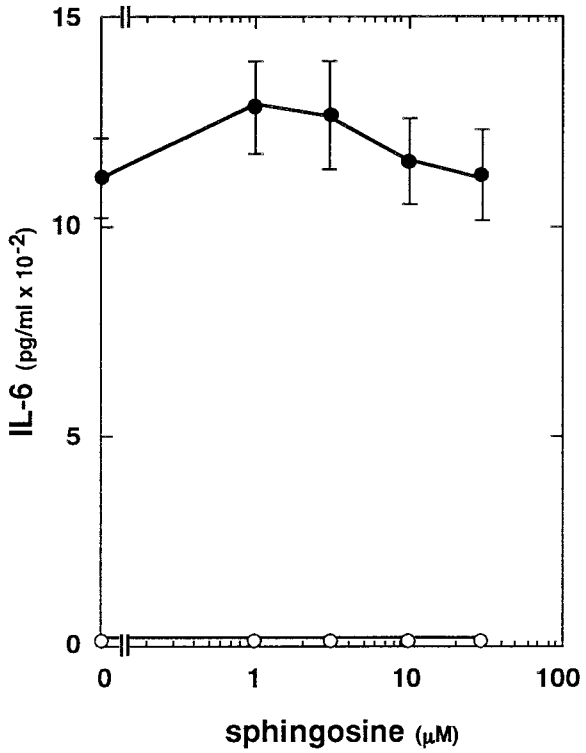


**Fig. 6.** Effect of sphingosine on IL-1-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of sphingosine for 20 min and then stimulated by 30 ng/ml IL-1 (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.

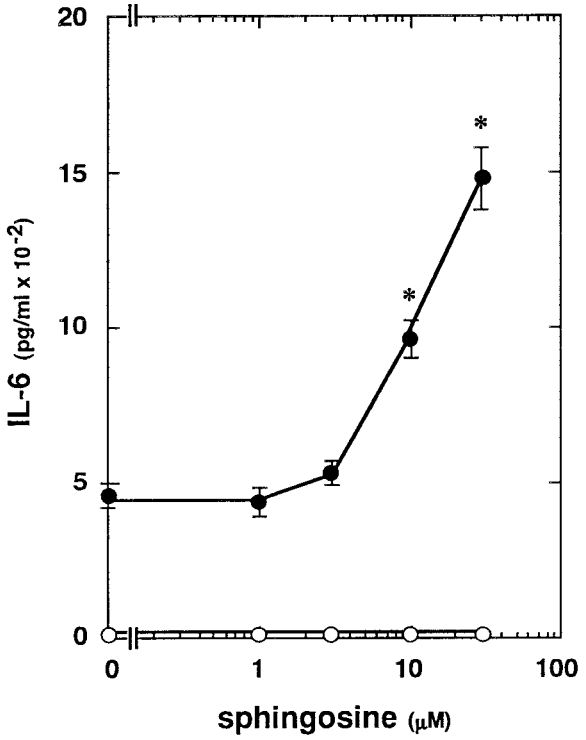


**Fig. 7.** Effect of sphingosine on TNF-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of sphingosine for 20 min and then stimulated by 30 ng/ml TNF (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. value of TNF alone.

protein kinase C in these cells. We showed here that TPA-induced synthesis of IL-6 was also reduced by sphingosine. TPA is well known to be a direct activator of protein kinase C [Nishizuka, 1986]. Therefore, our results suggest that the inhibitory effect of sphingosine on $PGF_{2\alpha}$-induced IL-6 synthesis is exerted at a point downstream from protein kinase C in osteoblast-like MC3T3-E1 cells.

We next demonstrated that sphingosine suppressed the $PGE_1$-induced synthesis of IL-6 in MC3T3-E1 cells. In previous studies [Ito et al., 1996; Watanabe-Tomita et al., 1997], we have shown that $PGE_1$ stimulates IL-6 synthesis via cAMP production without affecting phosphoinositide hydrolysis by phospholipase C in these cells. It is well known that Gs functions as an intermediary in transmembrane signaling from the receptor to adenylate cyclase [Gilman, 1987]. cAMP is produced from ATP by adenylate cyclase and then activates protein kinase A. In the present study, sphingosine inhibited the IL-6 synthesis stimulated by cholera toxin. It is recognized that cholera toxin ADP-ribosylates the α-subunit of Gs and results in continuous activation of Gs [Gilman, 1987]. Next, we found that the IL-6 synthesis induced by forskolin

**TABLE III. Effect of DHS on Enhancement by Sphingosine of TNF-induced IL-6 Synthesis in MC3T3-E1 Cells**[a]

| DHS | Sphingosine | TNF | IL-6 (pg/ml) |
|---|---|---|---|
| − | − | − | <10 |
| − | − | + | 480 ± 33* |
| − | + | − | <10 |
| − | + | + | 914 ± 67** |
| + | − | − | <10 |
| + | − | + | 358 ± 29** |
| + | + | − | <10 |
| + | + | + | 649 ± 55*** |

[a]Cultured cells were pretreated with 5 μM DHS or vehicle for 20 min and subsequently treated with 10 μM sphingosine or vehicle for 20 min. The cells were then stimulated by 30 ng/ml TNF or vehicle for 48 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.
*$P < 0.05$ vs. control value; **$P < 0.05$ vs. value of TNF alone; ***$P < 0.05$ vs. value of TNF with sphingosine.

was reduced by sphingosine. Forskolin is well recognized directly to activate adenylate cyclase [Seamon and Daly, 1981]. Thus these results suggest that the inhibitory effect of sphingosine is exerted at a point downstream from adenylate cyclase in MC3T3-E1 cells. In addition, we showed that sphingosine inhibited the $Bt_2$cAMP-induced IL-6 synthesis. Therefore, based on our findings, it is most likely that sphingosine inhibits the $PGE_1$-induced IL-6 synthesis at a point downstream from cAMP in osteoblast-like MC3T3-E1 cells.

We showed here that sphingosine reduced bFGF-induced IL-6 synthesis in MC3T3-E1 cells. We previously reported that bFGF elicits IL-6 synthesis, which depends on intracellular $Ca^{2+}$ mobilization mainly from extracellular space [Kozawa et al., 1997b]. Moreover, herein, A23187-induced IL-6 synthesis was also suppressed by sphingosine. So, it is probable that the inhibitory effect of sphingosine on bFGF-induced IL-6 synthesis is exerted at a point downstream from $Ca^{2+}$ mobilization in osteoblast-like MC3T3-E1 cells.

Additionally, IL-1-induced IL-6 synthesis was not affected by sphingosine in MC3T3-E1 cells. The exact mechanism of IL-1-induced IL-6 synthesis in osteoblasts has not yet been clarified [Thomson et al., 1986]. Currently, accumulating evidence suggests that several signal transduction pathways such as mitogen-activated protein kinase, nuclear factor for IL-6, and nuclear factor-κB are involved in IL-1-induced IL-6 gene expression [Banker-Fullbright et al., 1996]. Thus, it seems unlikely that sphingosine might affect the stimulative pathways of IL-1-induced IL-6 synthesis. In addition, it is probable that the inhibition by sphingosine of IL-6 synthesis induced by $PGF_{2\alpha}$, $PGE_1$ or bFGF are not toxic but specific effects.

We have recently reported that TNF induces sphingomyelin turnover in MC3T3-E1 cells and that among sphingomyelin metabolites, sphingosine 1-phosphate acts as a second messenger for TNF-induced IL-6 synthesis [Kozawa et al., 1997c]. The activation of sphingomyelinase hydrolyzes sphingomyelin to form ceramide, which is degraded into sphingosine. Sphingosine 1-phosphate is a phosphorylated product of sphingosine by sphingosine kinase. We demonstrated here that TNF-induced IL-6 synthesis was markedly amplified by sphingosine, which alone had no effect on IL-6 synthesis in MC3T3-E1 cells, and that DHS reduced the enhancement by sphingosine of the TNF-induced IL-6 synthesis as well as the IL-6 synthesis induced by TNF alone. DHS is known to be a useful tool for studying the involvement of sphingosine kinase [Sheela Rani et al., 1997]. It seems that TNF activates sphingosine kinase, as well as sphingomyelinase as previously described [Kozawa et al., 1997c]. Thus our present results suggest that sphingosine alone does not activate sphingosine kinase in MC3T3-E1 cells, but is phosphorylated by TNF-activated sphingosine kinase, resulting in a large amount of sphingosine 1-phosphate formation. Therefore, it is most likely that when TNF and sphingosine are added together, sphingosine 1-phosphate is produced not only from intrinsic sphingosine, a product of sphingomyelin turnover by TNF-activated sphingomyelinase, but also from extracellularly added sphingosine, resulting in the IL-6 synthesis enhancement in osteoblast-like MC3T3-E1 cells.

In conclusion, these results strongly suggest that sphingosine modulates IL-6 synthesis by various agonists in osteoblasts.

## ACKNOWLEDGMENTS

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