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Extracellular Sphingomyelinase Induces Interleukin-6 Synthesis in Osteoblasts

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Abstract In osteoblast-like MC3T3-E1 cells, we have recently reported that sphingosine 1-phosphate among sphingomyelin metabolites acts as a second messenger for tumor necrosis factor-α (TNF)–induced interleukin-6 (IL-6) synthesis. In the present study, we investigated the effect of extracellular sphingomyelinase on IL-6 synthesis in MC3T3-E1 cells. Sphingomyelinase stimulated IL-6 synthesis in a time-dependent manner for up to 24 h. This stimulative effect was dose dependent in the range between 1 and 300 mU/ml. Calphostin C, a highly and potent inhibitor of protein kinase C, enhanced sphingomyelinase-induced IL-6 synthesis. DL-Threo-dihydrosphingosine, an inhibitor of sphingosine kinase, significantly inhibited the IL-6 synthesis induced by sphingomyelinase. Sphingomyelinase markedly elicited sphingomyelin hydrolysis. In addition, the effect of a combination of sphingomyelinase and TNF on IL-6 synthesis was synergistic. These results strongly suggest that extracellular sphingomyelinase induces sphingomyelin hydrolysis in osteoblasts, resulting in IL-6 synthesis, and that protein kinase C acts as a negative controller of the IL-6 synthesis. J. Cell. Biochem. 72:262–268, 1999.

Key words: sphingomyelinase; interleukin-6; protein kinase C; osteoblast

Bone metabolism is maintained by two types of functional cells, osteoblasts and osteoclasts [Nijweide et al., 1986]. The former are responsible for bone formation, and the latter are responsible for bone resorption. The formation of bone structures and bone remodeling results from coupling bone resorption by activated osteoclasts with subsequent deposition of new matrix by osteoblasts. During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which are locally proliferate and differentiate into osteoblasts, migrate into the resorption lacuna. Thus, it is currently recognized that the activity of osteoblasts, osteoclasts, and endothelial cells is closely coordinated and regulates bone remodeling [Erlebacher et al., 1995]. These functional cells are thought to influence one another via humoral factors as well as by direct cell-to-cell contact. It has been reported that the receptors of endothelin, produced by endothelial cells [Masaki, 1993; Pollock et al., 1995], exist on cultured osteoblasts [Takuwa et al., 1989; Sakurai et al., 1992; Semler et al., 1995], and that endothelin-1 modulates cell functions of osteoblasts through activating intracellular signaling systems such as phospholipase C and Ca^{2+} mobilization [Takuwa et al., 1989; 1990; Tatrai et al., 1992a,b], suggesting the intercellular communications between endothelial cells and osteoblasts via secretion of bioactive substances. We have recently reported that endothelin-1 induces interleukin-6 (IL-6) synthesis in osteoblast-like MC3T3-E1 cells [Matsuno et al., 1998]. However, the intercellular signaling mechanism among endothelial cells, osteoblasts, and osteoclasts has not yet been fully clarified.

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It is widely accepted that IL-6 is produced and secreted in a variety of cells and that diverse cell functions are regulated by IL-6 [Akira et al., 1990; van Snick, 1995]. In bone cells, it has been reported that bone resorption and osteoclast formation are promoted by IL-6 [Ishimi et al., 1990; Roodman, 1992]. Bone resorptive agents such as interleukin-1 (IL-1), parathyroid hormone, tumor necrosis factor-α (TNF) and platelet-derived growth factor (PDGF) have been reported to stimulate the production and the secretion of IL-6 in cultured osteoblasts [Helle et al., 1988; Feyen et al., 1989; Ishimi et al., 1990; Franchimont and Canalis, 1995]. Therefore, it is nowadays recognized that osteoblast-secreted IL-6 plays a crucial role in bone metabolism as a downstream effector of bone resorptive agents. As for the regulation of IL-6 synthesis by osteoblasts, it has been reported that PDGF stimulates IL-6 synthesis through the activation of protein kinase C (PKC) in primary cultured osteoblasts [Franchimont and Canalis, 1995]. We have recently reported that PKC activation mediates the stimulatory effects of prostaglandin $F_{2\alpha}$ or endothelin-1 on IL-6 synthesis in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1997a; Matsuno et al., 1998]. On the contrary, we have shown that the IL-6 synthesis induced by basic fibroblast growth factor, prostaglandin E_2, thrombin, IL-1, or TNF is negatively regulated by PKC in these cells [Kozawa et al., 1997b–e, 1998a]. Thus, it is proposed that PKC plays critical roles in the regulation of IL-6 synthesis in osteoblasts.

Sphingomyelin hydrolysis by sphingomyelinase is now recognized to play important roles in diverse cellular functions, and the sphingomyelin metabolites act as second messengers mediating biological effects of some kinds of physiological agonists [Hannun, 1994; Spiegel and Merrill, 1996]. Sphingomyelinase induces hydrolysis of membrane sphingomyelin, resulting in the formation of ceramide and phosphocholine. Ceramide is subsequently metabolized to sphingosine, which is further degraded into sphingosine 1-phosphate by sphingosine kinase. It has been reported that sphingosine and sphingosine 1-phosphate show mitogenic effects in several cells, whereas ceramide functions as a second messenger inducing apoptosis. We have recently shown that sphingosine 1-phosphate acts as a second messenger for TNF-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells and that activation of p42/p44 mitogen-activated protein kinase functions at a point downstream from sphingosine 1-phosphate [Kozawa et al., 1997e,f]. Recently, it has been reported that cultured endothelial cells secrete sphingomyelinase [Marathe et al., 1998], suggesting that sphingomyelinase plays a physiological role as an intercellular messenger from endothelial cells. In bone cells, it has recently been reported that extracellular sphingomyelinase causes inhibition of F-actin ring in and bone resorption by mature osteoclasts isolated from rabbit long bones [Takeda et al., 1998]. However, the role of extracellular sphingomyelinase in osteoblasts functions has not yet been clarified.

In the present study, we investigated the effect of extracellular sphingomyelinase on IL-6 synthesis and its regulatory mechanism in osteoblast-like MC3T3-E1 cells. Herein, we show that extracellular sphingomyelinase induces IL-6 synthesis in osteoblasts and that PKC acts as a negative regulator of the IL-6 synthesis.

MATERIALS AND METHODS

Sphingomyelinase, calphostin C, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). TNF was purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). DL-Threo-dihydrosphingosine (DHS) was purchased from BIOMOL Research Laboratories, Inc. (Plymouth, PA). Mouse IL-6 enzyme immunoassay (EIA) kit and [*methyl*-^{3}H]choline chloride (85 Ci/mmol) were purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Calphostin C and TPA were dissolved in dimethyl sulfoxide. DHS was dissolved in ethanol. The maximum concentration of dimethyl sulfoxide or ethanol was 0.1%, which 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., 1997a]. In brief, the cells (5×10^4) were seeded into 35-mm-diameter dishes in 2 ml of α-minimum essential medium (α-MEM) containing 10% fetal calf serum (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. When indicated, the cells were pretreated with 0.1 μM TPA for 24 h as previously reported [Sakai et al., 1992].

Assay for IL-6

The cultured cells were stimulated by sphingomyelinase or TNF in 1 ml of α-MEM containing 0.3% FCS for 24 h. The conditioned medium was collected, and IL-6 in the medium was measured by an IL-6 EIA kit. When indicated, the cells were pretreated with calphostin C or DHS for 20 min.

Assay for Sphingomyelin Turnover

Sphingomyelin levels were measured as described by Okazaki et al. [1989]. In brief, the cultured cells were labeled with [*methyl*-^{3}H]choline chloride (2 μCi/dish) for 24 h. The labeled cells were stimulated by sphingomyelinase in an assay buffer (5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1 mM $CaCl_2$) containing 0.01% bovine serum albumin at 37°C for the indicated periods. The lipids were extracted by the method of Bligh and Dyer [Bligh and Dyer, 1959]. The samples were dried down, dissolved in chloroform, and then applied on Gel 60A TLC plate. To identify sphingomyelin, TLC plate was developed in chloroform:methanol:acetic acid:water (50:30:8:5). The sphingomyelin spot was scraped.

Determinations

The absorbance of EIA samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). The radioactivity of ^{3}H-labeled samples was determined with a Beckman LS-6500IC liquid scintillation spectrometer (Palo Alto, CA).

Statistical Analysis

The data were analyzed by Student's *t*-test, and $P < .05$ was considered significant. All data are presented as the mean ±SD of triplicate determinations.

RESULTS

Effect of Sphingomyelinase on IL-6 Synthesis in MC3T3-E1 Cells

We first examined the effect of extracellular sphingomyelinase on IL-6 synthesis in osteoblast-like MC3T3-E1 cells. Sphingomyelinase

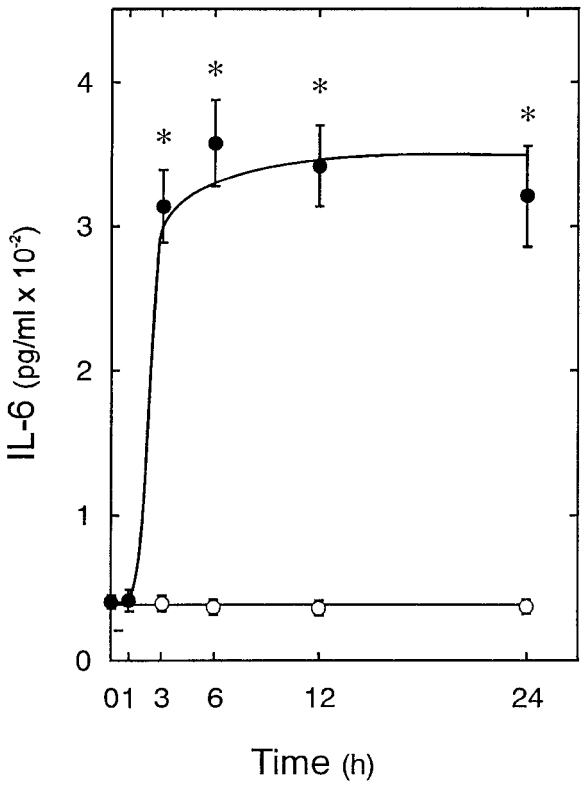

Fig. 1. Effect of sphingomyelinase on IL-6 synthesis in MC3T3-E1 cells: time-course study. The cultured cells were stimulated by 300 mU/ml (solid circles) or vehicle (open circles) for the indicated periods. Each value represents the mean ±SD of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *$P < .05$, compared to the control value.

(300 mU/ml) significantly stimulated IL-6 synthesis in a time-dependent manner for up to 24 h (Fig. 1). The stimulative effect of sphingomyelinase was dose dependent in the range between 1 and 300 mU/ml (Fig. 2). The maximum effect of sphingomyelinase was observed at 300 mU/ml.

Effect of Calphostin C on Sphingomyelinase-Induced IL-6 Synthesis in MC3T3-E1 Cells

We have recently reported that PKC positively or negatively regulates IL-6 synthesis depending upon the intracellular signaling systems of each agonists in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1997a–e, 1998a; Matsuno et al., 1998]. To investigate the role of PKC in the regulation of sphingomyelinase-induced IL-6 synthesis, the effect of calphostin C, a highly potent and specific inhibitor of PKC [Kobayashi et al., 1989], was examined. Calphostin C, which alone did not affect the level of

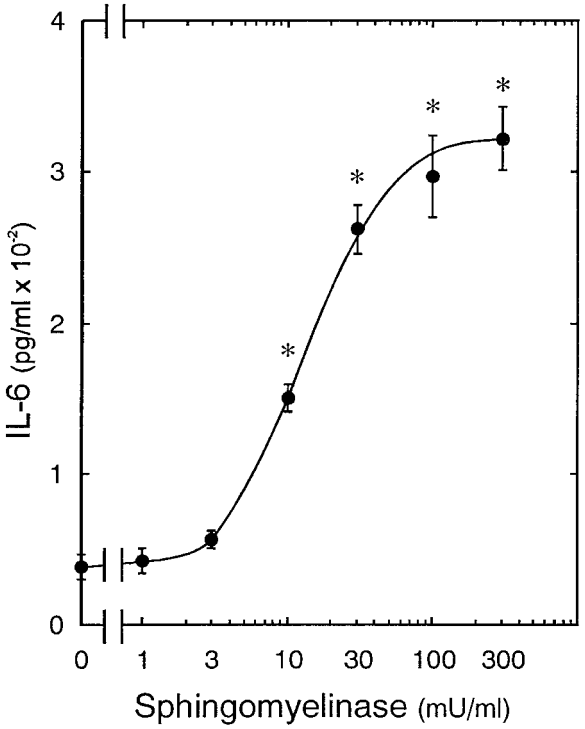

Fig. 2. Dose-dependent effect of sphingomyelinase on IL-6 synthesis in MC3T3-E1 cells. The cultured cells were stimulated by various doses of sphingomyelinase for 24 h. Each value represents the mean ±SD of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *P < .05, compared to the control value.

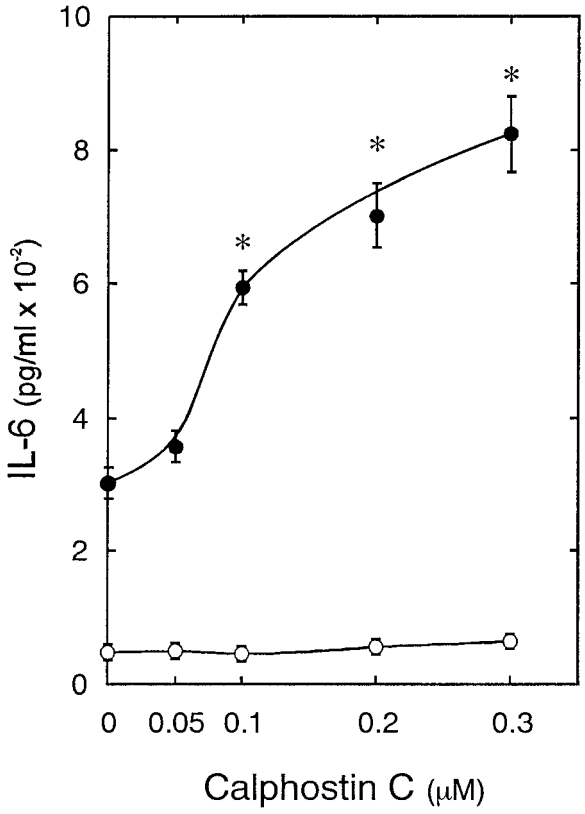

Fig. 3. Effect of calphostin C on sphingomyelinase-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of calphostin C for 20 min and then stimulated by 300 mU/ml sphingomyelinase (solid circles) or vehicle (open circles) for 24 h. Each value represents the mean ± SD of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *P < .05, compared to the value of sphingomyelinase alone.

IL-6, significantly enhanced the sphingomyelinase-induced IL-6 synthesis (Fig. 3). The effect of calphostin C was dose dependent in the range between 50 nM and 0.3 μM.

Effect of Downregulation of PKC on Synthesis of IL-6 Induced by Sphingomyelinase in MC3T3-E1 Cells

The long-term pretreatment of 0.1 μM TPA (24 h) has been shown to downregulate PKC in osteoblast-like MC3T3-E1 cells [Sakai et al., 1992]. We have also reported that approximately 70% reduction of the binding capacity of phorbol-12,13-dibutyrate, a PKC-activating phorbol ester [Nishizuka, 1986], occurs in PKC downregulated MC3T3-E1 cells compared to the capacity in intact cells [Kozawa et al., 1997a]. To further clarify the role of PKC in the regulation of sphingomyelinase-induced IL-6 synthesis, we next examined the effect of TPA (0.1 μM) long-term pretreatment on the synthesis of IL-6 induced by sphingomyelinase. The effect of sphingomyelinase on IL-6 synthesis was significantly enhanced in the PKC downregulated cells compared with that in the cells without TPA pretreatment (Table I).

Effect of DHS on Sphingomyelinase-Induced IL-6 Synthesis in MC3T3-E1 Cells

Sphingomyelinase induces hydrolysis of membrane sphingomyelin, resulting in the formation of ceramide and phosphocholine. Ceramide is subsequently metabolized to sphingosine, which is further degraded into sphingosine 1-phosphate by sphingosine kinase [Hannun, 1994]. In a recent study [Kozawa et al., 1997e], we have shown that, among sphingomyelin metabolites, sphingosine 1-phosphate acts as a second messenger for TNF-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We first tested the effect of sphingomyelinase on sphingomyelin levels in these cells and found that

TABLE I. Effect of PKC Downregulation on the Sphingomyelinase-Induced IL-6 Synthesis in MC3T3-E1 Cells[a]

TPA pretreatment (0.1 μM)	Sphingomyelinase (300 mU/ml)	IL-6 (pg/ml)
−	−	<10
−	+	323 ± 40
+	−	<10
+	+	1,056 ± 98*

[a]The cultured cells were pretreated with 0.1 μM TPA or vehicle for 24 h and then stimulated by 300 mU/ml sphingomyelinase or vehicle for 24 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.
*P < .05 vs. the value of sphingomyelinase alone.

TABLE II. Effect of Sphingomyelinase on Sphingomyelin Turnover in MC3T3-E1 Cells[a]

Time (min)	Sphingomyelin (% of control)
0	100 ± 3.1
10	8.1 ± 1.0*
30	7.1 ± 0.7*
60	5.0 ± 0.3*

[a]The cultured cells were stimulated by 300 mU/ml sphingomyelinase for the indicated periods. The data are shown as percent of control (in the absence of sphingomyelinase). Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.
*P < .05 vs. the value of control.

sphingomyelinase (300 mU/ml) significantly decreased the levels to less than 10% of control 10 min after the stimulation (Table II). This stimulative effect of sphingomyelinase on sphingomyelin turnover was sustained up to 60 min. We next examined the effect of DHS, an inhibitor of sphingosine kinase [Sheela Rani et al., 1997], on the synthesis of IL-6 induced by sphingomyelinase in these cells. DHS, which by itself did not affect IL-6 synthesis, markedly inhibited sphingomyelinase-induced IL-6 synthesis in a dose-dependent manner in the range between 1 and 10 μM (Fig. 4). The maximum inhibitory effect of DHS was observed at 10 μM, a dose that caused approximately 70% reduction in the sphingomyelinase effect.

Effect of a Combination of Sphingomyelinase and TNF on IL-6 Synthesis in MC3T3-E1 Cells

Furthermore, we examined the combination effect of sphingomyelinase and TNF on the synthesis of IL-6 in osteoblast-like MC3T3-E1 cells. As previously reported [Kozawa et al., 1997e], TNF (30 ng/ml) significantly stimulated IL-6 synthesis (Table III). The synthesis of IL-6 stimulated by a combination of sphingomyelinase and TNF was synergistic (Table III).

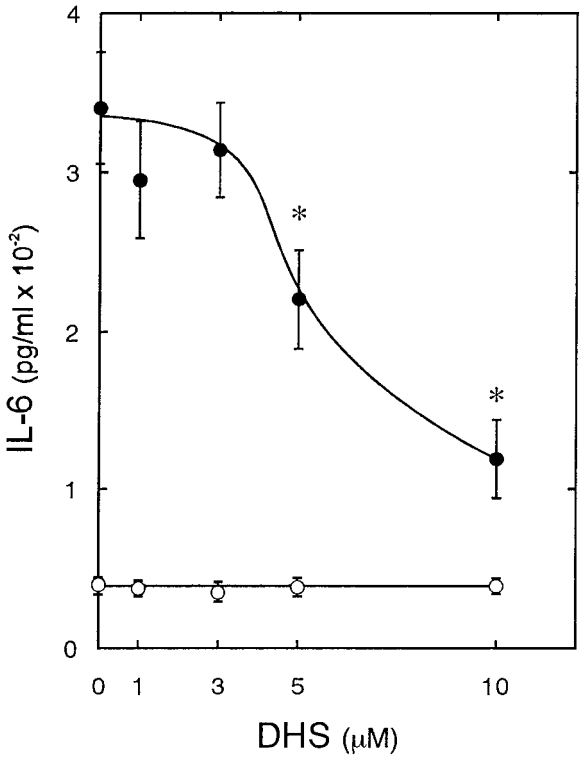

Fig. 4. Effect of DHS on sphingomyelinase-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of DHS for 20 min and then stimulated by 300 mU/ml sphingomyelinase (solid circles) or vehicle (open circles) for 24 h. Each value represents the mean ± SD of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *P < .05, compared to the value of sphingomyelinase alone.

TABLE III. Effect of a Combination of Sphingomyelinase and TNF on the Synthesis of IL-6 in MC3T3-E1 Cells[a]

	IL-6 (pg/ml)
Control	<10
Sphingomyelinase	333 ± 32*
TNF	631 ± 50*
Sphingomyelinase + TNF	5,256 ± 421**

[a]The cultured cells were stimulated by 300 mU/ml sphingomyelinase, 30 ng/ml TNF, or their combination for 24 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.
*$P < .05$ vs. control value.
**$P < .05$ vs. the value of sphingomyelinase alone.

DISCUSSION

In the present study, we showed that extracellular sphingomyelinase induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. This is probably the first report showing the effect of extracellular sphingomyelinase on the function of osteoblasts as far as we know. In addition, we demonstrated that calphostin C enhanced the IL-6 synthesis stimulated by sphingomyelinase. Thus, it seems that PKC regulates extracellular sphingomyelinase-induced IL-6 synthesis in these cells. We demonstrated here that the stimulative effect of sphingomyelinase on IL-6 synthesis was markedly amplified in the PKC downregulated cells compared with that in intact cells. These observations suggest that PKC acts as a negative controller of the IL-6 synthesis induced by extracellular sphingomyelinase in osteoblast-like cells.

Sphingomyelin is hydrolyzed by sphingomyelinase, resulting in the formation of ceramide, which is further metabolized to sphingosine and sphingosine 1-phosphate. Herein, we showed that extracellular sphingomyelinase potently and continuously elicited sphingomyelin hydrolysis in osteoblast-like MC3T3-E1 cells. So, it is probable that the stimulatory effect of sphingomyelinase on IL-6 synthesis is mediated by the sphingomyelin metabolites. We have recently reported that sphingosine 1-phosphate acts as a second messenger for TNF-induced IL-6 synthesis through activation of p42/p44 mitogen-activated protein kinase in these cells [Kozawa et al., 1997e,f]. Thus, we next examined the effect of DHS on the IL-6 synthesis induced by extracellular sphingomyelinase, and demonstrated that DHS inhibited the stimulatory effect of sphingomyelinase. Thus, it is most likely that the action of sphingosine kinase is required for sphingomyelinase-induced IL-6 synthesis, in turn, the production of sphingosine 1-phosphate from sphingosine plays a key role in the IL-6 synthesis induced by extracellular sphingomyelinase in osteoblast-like cells. In addition, we showed here that the effect of a combination of sphingomyelinase and TNF was synergistic in osteoblast-like MC3T3-E1 cells. We have recently reported that sphingosine enhances the IL-6 synthesis stimulated by TNF in these cells [Kozawa et al., 1998b]. As described above, sphingomyelinase potently and continuously induced sphingomyelin turnover; namely, it was able to supply a large mount of sphingosine. So, it is probable that the synergistic effect of sphingomyelinase and TNF on IL-6 synthesis results from the enhancement of TNF-induced IL-6 synthesis by sphingosine, a metabolite of sphingomyelinase-induced sphingomyelin hydrolysis.

In a recent report [Marathe et al., 1998], it has been demonstrated that sphingomyelinase is secreted from vascular endothelial cells, which cooperate with the functional bone cells, osteoblasts and osteoclasts, in bone remodeling [Erlebacher et al., 1995]. From our present results, it is probable that extracellular sphingomyelinase induces IL-6 synthesis in a similar fashion to TNF in osteoblasts. Taking our present findings into account, it is most likely that sphingomyelinase plays an important role as a intercellular messenger in bone remodeling from capillary endothelial cells to osteoblasts, stimulating the synthesis of IL-6, which is known to promote osteoclastic bone resorption. In addition, our findings strongly suggest the involvement of endothelial cells in bone resorption and bone remodeling.

In conclusion, our present findings strongly suggest that extracellular sphingomyelinase induces IL-6 synthesis in osteoblasts and that PKC acts as a negative controller of the IL-6 synthesis.

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