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Protein Kinase C Activation by Interleukin (IL)-1 Limits IL-1-Induced IL-6 Synthesis in Osteoblast-Like Cells: Involvement of Phosphatidylcholine-Specific Phospholipase C

Osamu Kozawa,[1]* Atsushi Suzuki,[2] Haruhiko Tokuda,[3] Takehiro Kaida,[1] and Toshihiko Uematsu[1]

[1]Department of Pharmacology, Gifu University School of Medicine, Gifu, Japan
[2]First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya, Japan
[3]Department of Internal Medicine, Chubu National Hospital: National Institute for Longevity Sciences, Obu, Japan

Abstract We investigated the regulatory mechanism of interleukin-6 (IL-6) synthesis induced by interleukin-1 (IL-1) in osteoblast-like MC3T3-E1 cells. IL-1 stimulated the secretion of IL-6 in a dose-dependent manner in the range between 0.1 and 100 ng/ml. Staurosporine and calphostin C, inhibitors of protein kinase C (PKC), significantly enhanced the IL-1-induced secretion of IL-6. The stimulative effect of IL-1 was markedly amplified in PKC down-regulated MC3T3-E1 cells. IL-1 produced diacylglycerol in MC3T3-E1 cells. IL-1 had little effect on the formation of inositol phosphates and choline. On the contrary, IL-1 significantly stimulated the formation of phosphocholine dose-dependently. D-609, an inhibitor of phosphatidylcholine-specific phospholipase C, suppressed the IL-1-induced diacylglycerol production. The IL-1-induced IL-6 secretion was significantly enhanced by D-609. These results indicate that IL-1 activates PKC via phosphatidylcholine-specific phospholipase C in osteoblast-like cells, and the PKC activation then limits IL-6 synthesis induced by IL-1 itself. J. Cell. Biochem. 67:103–111, 1997.

Key words: interleukin-1; interleukin-6; protein kinase C; phosphatidylcholine; phospholipase C; osteoblast

Interleukin-1 (IL-1) is a multifunctional cytokine responsible for inflammation, infection, and cancer, and IL-1 induces numerous physiological effects on a wide variety of cells [Wilder, 1995; Dinarello, 1996]. In bone, it is well known that IL-1 is a potent bone resorptive agent [Nijweide et al., 1986; Ishimi et al., 1990]. Bone metabolism is maintained by two types of functional bone cells, osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively [Nijweide et al., 1986]. Accumulating evidence indicates that osteoblasts, rather than osteoclasts, posses receptors for many bone resorptive agents [Nijweide et al., 1986]. Osteoclast activity has been reported to be coupled through cytokine (such as IL-1 and tumor necrosis factor-α) stimulation of osteoblasts and the subsequent production of secondary peptide, which activates osteoclasts [Thomson et al., 1986, 1987]. As for intracellular signaling of IL-1, IL-1 has been shown to catalyze phosphatidylcholine hydrolysis via activation of phosphatidylcholine-specific phospholipase C [Galella et al., 1992; Schutze et al., 1994; Lozano et al., 1994], resulting in the production of phosphocholine and diacylglycerol, which is generally recognized to be a physiological activator of protein kinase C (PKC) [Nishizuka, 1986]. However, the precise intracellular signaling system of IL-1 in osteoblasts and its role in bone metabolism have not yet been fully clarified.

It is well known that interleukin-6 (IL-6) is a pleiotropic multifunctional cytokine that regulates diverse cell functions such as promotion of B cell differentiation and T cell activation, and induction of acute phase proteins [Akira et al., 1990a; van Snick, 1990]. It is recognized that IL-6 is produced and secreted in a variety of cells. As for bone metabolism, it has been reported that IL-6 stimulates bone resorption and

*Correspondence to: Osamu Kozawa, Department of Pharmacology, Gifu University School of Medicine, Gifu 500, Japan.

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induces osteoclast formation [Ishimi et al., 1990; Roodman, 1992]. Bone resorptive agents such as IL-1, parathyroid hormone, tumor necrosis factor-α, and platelet-derived growth factor have been reported to stimulate IL-6 production and its secretion in cultured osteoblasts [Helle et al., 1988; Feyen et al., 1989; Ishimi et al., 1990; Franchimont and Canalis, 1995]. Thus, accumulating evidence suggests that IL-6 secreted from osteoblasts plays an important role in bone resorption as a downstream effector of a variety of bone resorptive agents.

In the present study, we investigated the regulatory mechanism of IL-6 synthesis induced by IL-1 in osteoblast-like MC3T3-E1 cells. We here show that IL-1-induced PKC activation via phosphatidylcholine-specific phospholipase C limits IL-6 synthesis induced by IL-1 itself in osteoblast-like cells.

METHODS

Materials

myo-[^{3}H]Inositol (90 Ci/mmol), [*methyl*-^{3}H]choline chloride (85 Ci/mmol), and mouse IL-6 enzyme immunoassay (EIA) kit were purchased from Amersham Japan (Tokyo, Japan). IL-1 and potassium tricyclo(5,2,1,0)-decyl-[9(8)-xanthogenate] (D-609) were obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Staurosporine, calphostin C, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and NaF were purchased from Sigma Chemical Co. (St. Louis, MO). *dl*-Propranolol hydrochloride (propranolol) was purchased from Wako Pure Chemical Co. (Osaka, Japan). Other materials and chemicals were obtained from commercial sources. Staurosporine, calphostin C, TPA, and propranolol were dissolved in dimethylsulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for IL-6 nor the measurement of diacylglycerol formation.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983] were generously provided by Dr. M. Kumegawa (Meikai University, Sakado, Japan) and maintained 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 48 h. In the experiments for the measurement of inositol phosphates, the medium was exchanged for 2 ml of inositol-free α-MEM containing 0.3% FCS.

Assay for IL-6

The cultured cells were stimulated by IL-1 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 EIA kit. When indicated, the cells were pretreated with staurosporine, calphostin C, or D-609 for 20 min.

Measurement of the Formation of Inositol Phosphates

To determine phosphoinositide-hydrolyzing phospholipase C activity, the cultured cells were labeled with *myo*-[^{3}H]inositol (3 μCi/dish) for 48 h. The labeled cells were preincubated with 10 mM LiCl for 10 min in 1 ml of 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 (BSA). The cells were then stimulated by IL-1 or NaF at 37°C. The reaction was terminated by adding 1 ml of 30% trichloroacetic acid. The acid supernatant was treated with diethyl ether to remove the acid and neutralized with 0.1 M NaOH. The supernatant was applied to a 1-ml Dowex AG1-X8 column (100–200 mesh, formate form) as described by Berridge et al. [Berridge et al., 1983, 1984] with a minor modification [Suzuki et al., 1994]. The radioactive inositol phosphates were eluted from the column with 8 ml of 0.1 M formic acid containing 1 M ammonium formate.

Measurement of the Formation of Water-Soluble Choline Metabolites

To determine phosphatidylcholine-hydrolyzing phospholipase C and phospholipase D activities, the cultured cells were labeled with [*methyl*-^{3}H]choline chloride (2 μCi/dish) for 24 h. The labeled cells were stimulated by IL-1 in the assay buffer containing 0.01% BSA for the indicated periods. The reaction was terminated by adding 0.75 ml of ice-cold methanol. The dishes were placed on ice for 10 min. The con-

tents were transferred to tubes to which chloroform was added and placed on ice for a further 60 min. Chloroform and water were then added for a final chloroform-methanol-water ratio of 1:1:0.9. The tubes were centrifuged at 14,000*g* for 5 min and the upper aqueous methanolic phase was taken for analysis of the water-soluble choline-containing metabolites. The methanolic phase was separated on a 1 ml Dowex 50-WH^+ column (200–400 mesh) as described by Cook and Wakelam [Cook and Wakelam, 1989] with a minor modification [Kozawa et al., 1994]. In brief, the phase was diluted to 5 ml with water and applied to the column. Glycerophosphocholine was removed by 4 ml of water. Phosphocholine was then eluted with 10 ml of water, and choline was eluted with 10 ml of 1 M HCl.

Measurement of Diacylglycerol Formation

The cultured cells were incubated in the assay buffer containing 0.01% BSA at 37°C for 20 min, and then stimulated by IL-1 for 20 min. The reaction was terminated by adding 0.75 ml of ice-cold methanol, and the lipids were extracted by the previously described method [Bligh and Dyer, 1959; Suzuki et al., 1996]. Diacylglycerol was quantitated using *sn*-1,2-diacylglycerol assay reagents system (Amersham) essentially according to the procedure of Preiss et al. [Preiss et al., 1986]. The radioactive spot corresponding to phosphatidic acid was analyzed by BAS2000 (Fuji, Japan) equipped with imaging plates [Amemiya and Miyahara, 1988]. When indicated, the cells were pretreated with D-609 or propranolol for 20 min.

Determination

The radioactivity of ^{3}H-labeled samples was determined with a Beckman LS-6500IC liquid scintillation spectrometer (Beckman, Palo Alto, CA).

Statistical Analysis

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

RESULTS

Effect of IL-1 on IL-6 Secretion in MC3T3-E1 Cells

IL-1 (30 ng/ml) significantly induced the secretion of IL-6 in a time-dependent manner up to 48 h in MC3T3-E1 cells (Fig. 1). The stimulative effect of IL-1 was dose-dependent in the range between 0.1 and 100 ng/ml (Fig. 2). The maximum effect of IL-1 on the secretion of IL-6 was observed at 100 ng/ml.

Effect of Staurosporine or Calphostin C on Secretion of IL-6 Induced by IL-1 in MC3T3-E1 Cells

Staurosporine (10 nM), an inhibitor of protein kinases [Tamaoki et al., 1986], which by itself did not affect IL-6 secretion, enhanced the IL-1-induced IL-6 secretion in MC3T3-E1 cells (Fig. 3A). We also examined the effect of calphostin C, a highly potent and specific inhibitor of PKC [Kobayashi et al., 1989], on the IL-1-induced IL-6 secretion in MC3T3-E1 cells. Calphostin C, which alone had little effect on IL-6 secretion, significantly enhanced the IL-1-induced IL-6 secretion (Fig. 3B). The stimulative effect of calphostin C on the IL-1-induced IL-6 secretion was dose-dependent in the range between 0.03 and 0.3 μM.

Effect of Down-Regulation of PKC on Secretion of IL-6 Induced by IL-1 in MC3T3-E1 Cells

It has been shown that 24-h pretreatment of TPA (0.1 μM) down-regulates PKC in osteoblast-like MC3T3-E1 cells [Sakai et al., 1992]. We also found that the binding capacity of phorbol-12,13-dibutylate, a PKC-activating phorbol ester [Nishizuka, 1986], in MC3T3-E1 cells with 24-h pretreatment of 0.1 μM TPA is reduced to approximately 30% of the capacity in intact cells (data not shown). We next examined the effect of TPA (0.1 μM) long-term pretreatment on the IL-1-induced IL-6 secretion in these cells. The effect of IL-1 on IL-6 secretion was significantly enhanced in the PKC down-regulated MC3T3-E1 cells compared to that in the cells without TPA-pretreatment (Table I).

Effect of IL-1 on Formation of Inositol Phosphates in MC3T3-E1 Cells

To clarify the effect of IL-1 on phosphatidylinositol-specific phospholipase C, we examined whether IL-1 affects the formation of inositol phosphates in MC3T3-E1 cells. However, IL-1 had no effect on the formation of inositol phos-

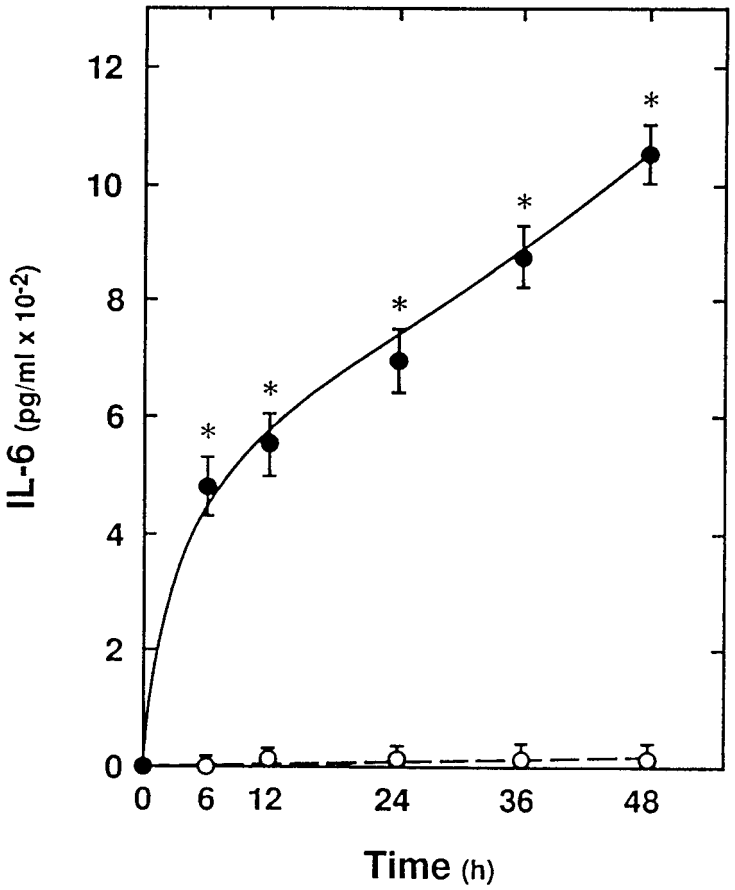

Fig. 1. Effect of IL-1 on IL-6 secretion in MC3T3-E1 cells. The cultured cells were stimulated by 30 ng/ml IL-1 for the indicated periods. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of control.

phates (control, 2,045 ± 99 cpm; 30 ng/ml IL-1, 2,111 ± 106 cpm; each value represents the mean ± SD of triplicate determinations, as measured after 30 min stimulation). On the contrary, NaF, known to be an activator of heterotrimeric GTP-binding proteins [Gilman, 1987], significantly stimulated the formation of inositol phosphates in these cells (control, 2,122 ± 101 cpm; 40 mM NaF, 30,300 ± 887 cpm, each value represents the mean ± SE of triplicate independent cell preparations, as measured after 30 min stimulation).

Effect of IL-1 on Formation of Water-Soluble Choline Metabolites in MC3T3-E1 Cells

IL-1 significantly stimulated the formation of phosphocholine in MC3T3-E1 cells (Fig. 4). However, IL-1 did not affect the formation of choline (control, 4,055 ± 158 cpm; 100 ng/ml IL-1, 4,182 ± 191 cpm, each value represents the mean ± SE of triplicate independent cell preparations, as measured after 30 min stimulation). The stimulative effect of IL-1 on the formation of phosphocholine was dose-dependent in the range between 1 and 100 ng/ml (Fig. 4). The maximum effect of IL-1 on the phosphocholine formation was observed at 100 ng/ml.

Effect of IL-1 on Production of Diacylglycerol in MC3T3-E1 Cells

IL-1 stimulated the production of diacylglycerol in MC3T3-E1 cells (Table II). The stimulative effect of IL-1 on diacylglycerol production was dose-dependent in the range between 1 and 100 ng/ml, and the maximum effect of IL-1 was observed at 100 ng/ml (data not shown). D-609, which is known to be a specific inhibitor of phosphatidylcholine-specific phospholipase C [Schutze et al., 1992], significantly inhibited the production of diacylglycerol induced by IL-1 (Table II). On the contrary, propranolol, an inhibitor of phosphatidic acid phosphohydrolase,

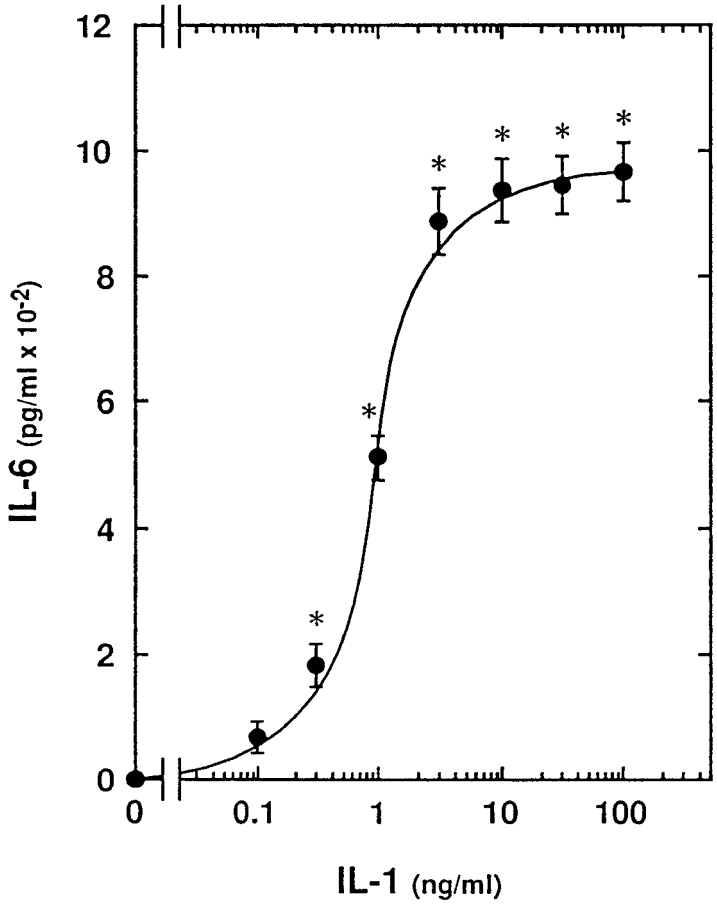

Fig. 2. Dose-dependent effect of IL-1 on IL-6 secretion in MC3T3-E1 cells. The cultured cells were stimulated by various doses of IL-1 for 48 h. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of control.

which catalyzes phosphatidic acid into diacylglycerol [Pappu and Hauser, 1983], had little effect on the IL-1-induced diacylglycerol production (Table II).

Effect of D-609 on IL-1-Induced Secretion of IL-6 in MC3T3-E1 Cells

To clarify the role of phosphatidylcholine-specific phospholipase C in the IL-1-induced secretion of IL-6, we examined the effect of D-609 on the IL-6 secretion in MC3T3-E1 cells. D-609, which alone had little effect on IL-6 secretion, significantly enhanced the secretion of IL-6 induced by IL-1 (Fig. 5). The stimulative effect of D-609 on the IL-1-induced IL-6 secretion was dose-dependent in the range between 0.1 and 3 ng/ml.

DISCUSSION

In the present study, we showed that IL-1 induced the production of diacylglycerol. So, it is probable that IL-1 activates PKC in osteoblast-like MC3T3-E1 cells. In addition, we demonstrated that staurosporine and calphostin C enhanced the IL-1-induced secretion of IL-6. Thus, our findings suggest that the IL-1-induced secretion of IL-6 is inhibited by PKC, which is activated by IL-1 itself in MC3T3-E1 cells. Furthermore, the stimulative effect of IL-1 on IL-6 secretion was markedly amplified in the PKC down-regulated cells compared to that in intact cells. Therefore, it is most likely that PKC activated by IL-1 limits the overstimulation of IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

We next investigated the exact mechanism behind the IL-1-induced activation of PKC. We first showed that IL-1 had no effect on the formation of inositol phosphates. On the contrary, NaF significantly induced the formation of inositol phosphates. It is well known that phosphatidylinositol is hydrolyzed by phospho-

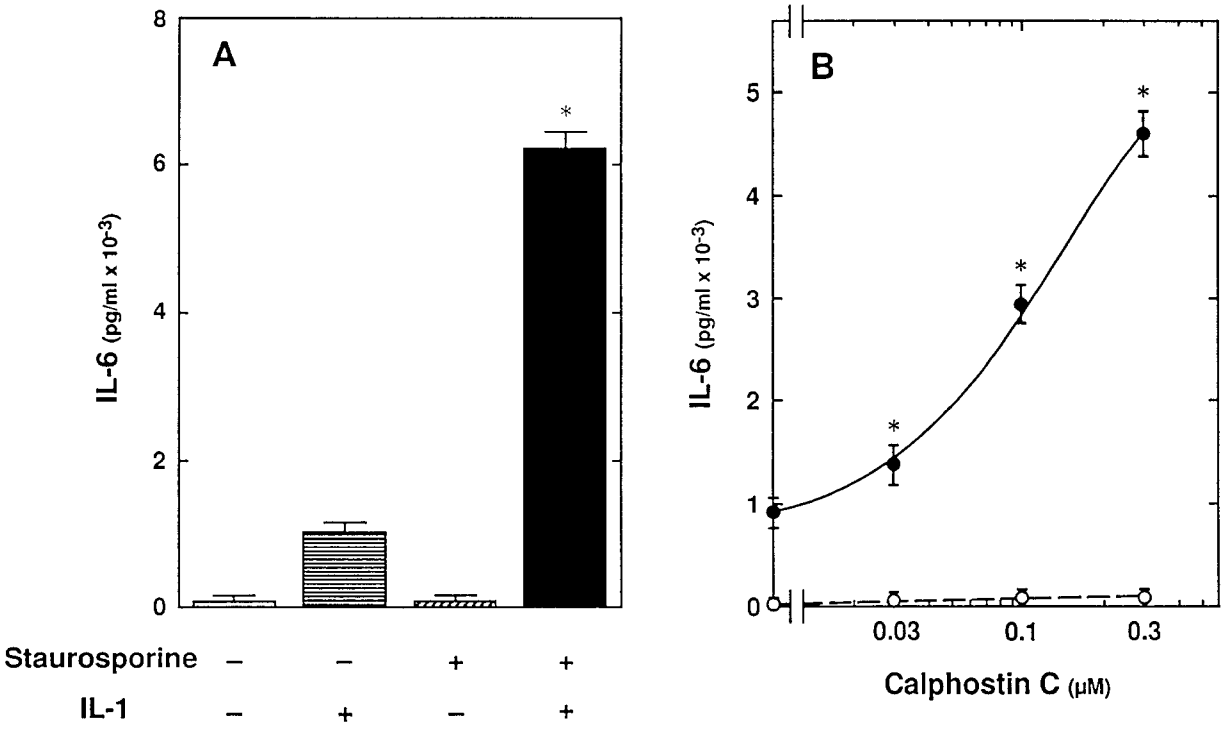

Fig. 3. Effect of staurosporine or calphostin C on the IL-1-induced secretion of IL-6 in MC3T3-E1 cells. The cultured cells were pretreated with 10 nM staurosporine (**A**) or various doses of calphostin C (**B**) for 20 min, and then stimulated by 30 ng/ml IL-1 for 48 h. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of IL-1 alone.

TABLE I. Effect of PKC Down-Regulation on IL-1-Induced Secretion of IL-6 in MC3T3-E1 Cells†

TPA-pretreatment (0.1 μM)	IL-1 (30 ng/ml)	IL-6 (pg/ml)
−	−	<10
−	+	830 ± 55
+	−	<10
+	+	1,440 ± 90*

†The cultured cells were pretreated with 0.1 μM TPA for 24 h, and then stimulated by 30 ng/ml IL-1 for 48 h. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations.
*$P < 0.05$, compared to the value of IL-1 alone.

lipase C, resulting in the formation of inositol phosphates and diacylglycerol and that heterotrimeric GTP-binding protein(s) is coupled to phosphatidylinositol-specific phospholipase C [Gilman, 1987; Berridge, 1993]. So, it seems unlikely that IL-1 activates phosphatidylinositol-specific phospholipase C. Our finding suggests that IL-1 does not activate PKC through phosphoinositide hydrolysis in osteoblast-like MC3T3-E1 cells. In addition, we demonstrated that IL-1 did not affect the formation of choline, and that propranolol had no effect on the diacylglycerol production induced by IL-1. It is recognized that phospholipase D hydrolyzes phosphatidylcholine to yield phosphatidic acid and choline [Exton, 1990; Billah and Anthes, 1990]. Phosphatidic acid is subsequently degraded into diacylglycerol by phosphatidic acid phosphohydrolase. So, from these findings, it is unlikely that IL-1 induces phosphatidylcholine hydrolysis by phospholipase D in MC3T3-E1 cells.

Phosphatidylcholine is also hydrolyzed by phospholipase C, resulting in the formation of diacylglycerol and phosphocholine [Exton, 1990; Billah and Anthes, 1990]. We showed that IL-1 significantly stimulated the formation of phosphocholine. Thus, it seems that IL-1 simulates not phosphatidylcholine-specific phospholipase D but phosphatidylcholine-specific phospholipase C. The effect of IL-1 on IL-6 secretion was more potent than that on the formation of phosphocholine. These findings suggest that IL-1 promotes IL-6 synthesis at a lower dose and modulates the synthesis of IL-6 by IL-1-induced PKC activation at a higher dose. In addition, we demonstrated that D-609 significantly suppressed the diacylglycerol production by IL-1. Therefore, these results suggest that IL-1

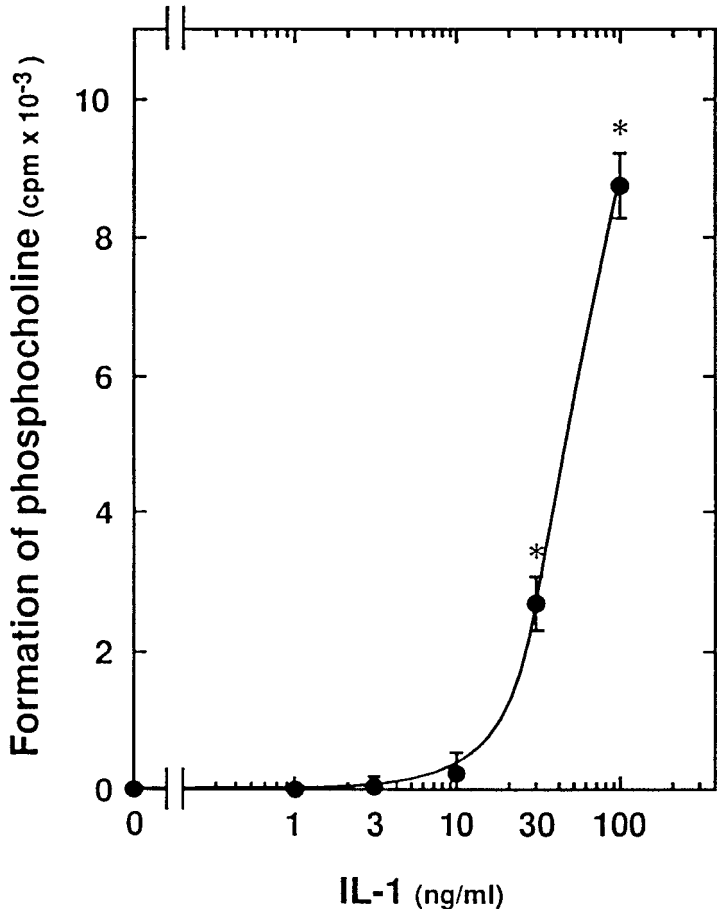

Fig. 4. Effect of IL-1 on the formation of phosphocholine in MC3T3-E1 cells. The labeled cells were stimulated by various doses of IL-1 for 20 min, and then the formation of phosphocholine was determined. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of control.

TABLE II. Effect of D-609 or Propranolol on IL-1-Induced Production of Diacylglycerol in MC3T3-E1 Cells†

Pretreatment	IL-1 (30 ng/ml)	Diacylglycerol (pmol/dish)
−	+	1,123 ± 127
D-609	+	148 ± 23*
Propranolol	+	1,215 ± 177

†The cultured cells were pretreated with 30 ng/ml D-609, 300 μM propranolol, or vehicle for 20 min, and then stimulated by 30 ng/ml IL-1 for 20 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations.
*$P < 0.05$, compared to the value of IL-1 alone.

activates PKC via stimulating phosphatidylcholine-specific phospholipase C in osteoblast-like MC3T3-E1 cells. Furthermore, we found that D-609 markedly enhanced the IL-1-induced IL-6 secretion. Taking our findings into account as a whole, it is most likely that IL-1 itself antagonizes IL-6 synthesis due to PKC activation through phosphatidylcholine-hydrolyzing phospholipase C in osteoblast-like MC3T3-E1 cells. The precise signal transduction pathway mediating IL-6 synthesis by IL-1 remains to be detailed, and is likely to be cell type specific [Gross et al., 1993; Norris et al., 1994]. However, it has been reported that PKC-induced phosphorylation of NFIL-6, a transcriptional factor whose activation has been implicated in upregulation of IL-6 promoter activity [Akira et al., 1990b], inhibits NFIL-6 function at the level of its transcription [Mahoney et al., 1992]. Thus, it is possible that NFIL-6 phosphorylation by IL-1-activated PKC is involved in the limitation of IL-6 synthesis in MC3T3-E1 cells. Further investigation would be required to clarify the details.

In conclusion, these results strongly suggest that IL-1 activates PKC via phosphatidylcho-

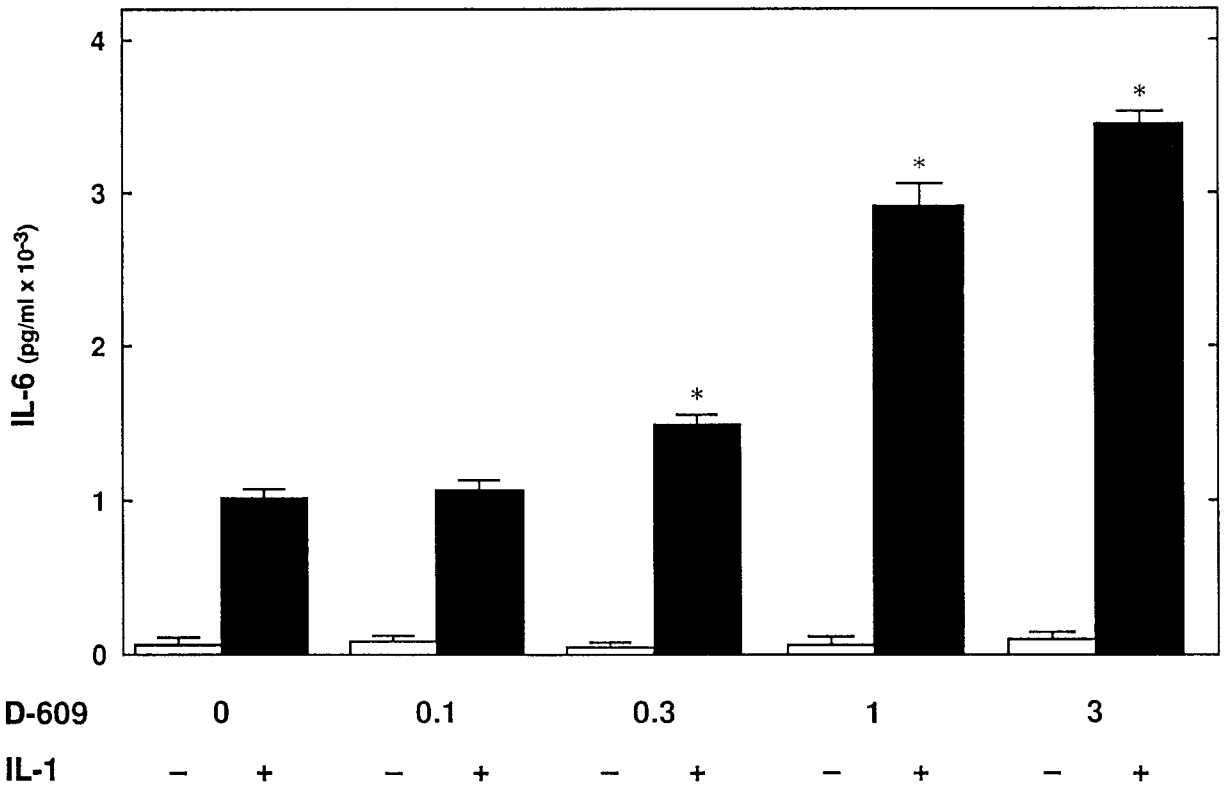

Fig. 5. Effect of D-609 on the IL-1-induced secretion of IL-6 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of D-609 for 20 min, and then stimulated by 30 ng/ml IL-1 for 20 min. Each value represents the mean ± SE of triplicate independent cell preparations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of IL-1 alone.

line-specific phospholipase C in osteoblast-like cells, and the PKC activation then limits IL-6 synthesis induced by IL-1 itself.

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