Journal of Cellular Biochemistry 85:621–628 (2002)

Zinc Suppresses IL-6 Synthesis by Prostaglandin $F_{2\alpha}$ in Osteoblasts: Inhibition of Phospholipase C and Phospholipase D

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Abstract We previously reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induces phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D through heterotrimeric GTP-binding protein, resulting in the activation of protein kinase C (PKC) in osteoblast-like MC3T3-E1 cells and that $PGF_{2\alpha}$ stimulates the synthesis of interleukin-6 (IL-6) via PKC-dependent p44/p42 mitogen-activated protein (MAP) kinase activation. In the present study, we investigated whether zinc affects the $PGF_{2\alpha}$-induced IL-6 synthesis in these cells. Zinc complex of L-carnosine (L-CAZ) dose-dependently suppressed the $PGF_{2\alpha}$-stimulated IL-6 synthesis. In addition, zinc alone reduced the IL-6 synthesis. L-CAZ suppressed the $PGF_{2\alpha}$-induced p44/p42 MAP kinase phosphorylation. However, the p44/p42 MAP kinase phosphorylation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a direct activator of PKC, or NaF, a direct activator of GTP-binding protein, was not affected by L-CAZ. L-CAZ reduced the $PGF_{2\alpha}$-stimulated formation of inositol phosphates and choline. However, L-CAZ did not affect the formation of inositol phosphates or choline induced by NaF. These results strongly suggest that zinc reduces $PGF_{2\alpha}$-induced IL-6 synthesis via suppression of phosphoinositide-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D in osteoblasts. J. Cell. Biochem. 85: 621–628, 2002.

Key words: zinc; prostaglandin $F_{2\alpha}$; interleukin-6; mitogen-activated protein (MAP) kinase; osteoblast

Zinc is a crucial regulator of biological functions in humans and many animals [Vallee and Falchuk, 1993]. It is well known that growth failure and impaired wound healing occur due to the absence of adequate dietary zinc [Vallee and Falchuk, 1993]. In bone metabolism, zinc deficiency leads to bone loss. It has been reported that zinc has a stimulatory effect on bone formation and mineralization in vivo and in vitro [Yamaguchi and Yamaguchi, 1986; Hall et al., 1999]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption, respectively [Nijweide et al., 1986]. In osteoblasts, it has been shown that zinc increases alkaline phosphatase activity and protein concentration in osteoblast-like MC3T3-E1 cells [Hashizume and Yamaguchi, 1994]. In addition, zinc reportedly increases the activity of 1α,25-dihydroxyvitamin D_3-dependent promoters, and the anabolic effect of insulin- like growth factor-I in osteoblasts is enhanced by zinc [Matsui and Yamaguchi, 1995; Lutz et al., 2000]. Accumulating evidence suggests that in bone metabolism, zinc plays an important role as a positive regulator of bone formation. However, the exact mechanism of zinc in osteoblasts has not yet been precisely clarified.

Prostaglandins (PGs) are well known to act as paracrine/autocrine modulators in osteoblasts [Nijweide et al., 1986]. Among them, it has been reported that $PGF_{2\alpha}$, a bone resorptive agent,

Grant sponsor: Ministry of Education, Science, Sports, and Culture of Japan (Grant-in-Aid for Scientific Reseach); Grant numbers: 11838005, 12470015.

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Received 3 January 2002; Accepted 31 January 2002
DOI 10.1002/jcb.10166

stimulates the proliferation of osteoblasts and inhibits the differentiation [Pilbeam et al., 1996]. In previous studies [Miwa et al., 1990; Kozawa et al., 1994], we have shown that $PGF_{2\alpha}$ activates both phosphoinositide-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D via pertussis toxin-sensitive heterotrimeric GTP-binding protein in osteoblast-like MC3T3-E1 cells. It is well recognized that the activation of these two phospholipases forms diacylglycerol, which is well known as a physiological activator of protein kinase C (PKC) [Nishizuka, 1986; Exton, 1999].

Interleukin-6 (IL-6) is a multifunctional cytokine, which regulates a variety of cellular functions such as promotion of B cell differentiation, T-cell activation, and induction of acute-phase proteins [Kishimoto et al., 1995; Lutz et al., 2000]. In bone metabolism, it has been shown that IL-6 acts as a local factor, which induces osteoclast formation and stimulates osteoclast activity to resorb bone [Roodman, 1992; Rifas, 1999]. Bone resorptive agents such as parathyroid hormone, tumour necrosis factor and interleukin-1 have been reported to stimulate IL-6 production and secretion in osteoblasts [Helle et al., 1988; Feyen et al., 1989; Ishimi et al., 1990]. Thus, it is recognized that IL-6 secreted from osteoblasts acts as a downstream effector of diverse bone resorptive agents, resulting in the induction of bone resorption. In a previous study [Kozawa et al., 1997], we have reported that $PGF_{2\alpha}$ stimulates IL-6 synthesis via activation of PKC in osteoblast-like MC3T3-E1 cells. In addition, we have recently shown that p44/p42 mitogen-activated protein (MAP) kinase functions at a point downstream from PKC in the $PGF_{2\alpha}$-induced IL-6 synthesis in these cells [Tokuda et al., 1999]. In the present study, we examined the effect of zinc on the $PGF_{2\alpha}$-induced IL-6 synthesis in osteoblast-like MC3T3- E1 cells and the mechanism of zinc. Here, we show that zinc inhibits $PGF_{2\alpha}$-induced IL-6 synthesis via suppression of phosphoinositide-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D.

MATERIALS AND METHODS

Materials

myo-[^{3}H]inositol (81.5 Ci/mmol) and [*methyl*-^{3}H]choline chloride (85 Ci/mmol) were purchased from Amersham Japan (Tokyo, Japan). Zinc complex of L-carnosine (L-CAZ) was kindly provided from Zeria Pharmaceutical Co. (Tokyo, Japan). Zinc sulfate ($ZnSO_4$) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). $PGF_{2\alpha}$ and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical (St. Louis, MO). Mouse IL-6 ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Phospho-specific p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) and p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) were purchased from New England Bio-Labs, Inc. (Beverly, MA). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. $PGF_{2\alpha}$ was dissolved in ethanol. TPA was dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect the measurement of the assay for IL-6, Western blotting analysis and measurement of the formation of inositol phosphates or choline.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Sudo et al., 1983] were maintained as previously described [Shinoda et al., 1995]. 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% CO2/95% air. The cells were seeded into 35- or 90-mm-diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 24 h. When indicated, the cells were pretreated with L-CAZ or zinc for 20 min.

Assay for IL-6 Synthesis

The cultured cells were stimulated by $PGF_{2\alpha}$ 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 ELISA kit.

Western Blot Analysis of p44/p42 MAP Kinase

The cultured cells were stimulated by $PGF_{2\alpha}$ TPA or NaF in α-MEM for the indicated periods. The cells were washed twice with phosphate-buffered saline (PBS) and then lysed, homogenized, and sonicated in a lysis

buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as the supernatant after centrifugation at 125,000*g* for 10 min at 4°C. Sodium Dodecyl Sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli [1970]] in 10% polyacrylamide gel. Western blot analysis was performed as described previously [Kawamura et al., 1999] by using phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as secondary antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

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) in inositol-free α-MEM 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 (HEPES), 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) at 37°C. The cells were then stimulated by $PGF_{2\alpha}$ or NaF for 30 min. 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). The radioactive inositol phosphates were eluted from the column with 8 ml of 0.1 M formic acid containing 1 M ammonium formate as described previously [Shinoda et al., 1995].

Measurement of the Formation of Choline

To determine phosphatidylcholine-hydrolyzing by phospholipase D activity, the cultured cells were labeled with *methyl*-[^{3}H]choline chloride (2 μCi/dish) for 24 h. The labeled cells were stimulated by $PGF_{2\alpha}$ or NaF for 20 min in 1 ml of the assay buffer. The reaction was terminated by adding 0.75 ml of ice-cold methanol. The dishes were placed on ice for 10 min. The contents were transferred to tubes to which chloroform was added and placed on ice for 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 1 ml Dowex 50-WH^+ column (200–400 mesh) as described previously [Kozawa et al., 1994]. In brief, the phase was diluted to 5 ml of water and applied to the column. Choline was eluted with 10 ml of 1 M HCl.

Determination

The absorbance of ELISA samples was measured at 450 nm with SLT-Labinstruments EAR 340 AT. Absorbance was correlated with concentration through a standard curve. The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

The data were analyzed by ANOVA followed by Bonferroni method for multiple comparisons between pairs, and a $P < 0.05$ was considered significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

RESULTS

Effect of L-CAZ on $PGF_{2\alpha}$-Induced IL-6 Synthesis in MC3T3-E1 Cells

L-CAZ is approved as an anti-ulcer drug of membrane protection type [Matsukura and Tanaka, 2000]. L-carnosine forms stable complexes with metal ions of biological significance, such as zinc [Matsukura and Tanaka, 2000]. In a previous study [Kozawa et al., 1997], we showed that $PGF_{2\alpha}$ stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells. First, we examined the effect of L-CAZ on the IL-6 synthesis induced by $PGF_{2\alpha}$. L-CAZ, which by itself hardly affected IL-6 synthesis, significantly inhibited the IL-6 synthesis stimulated by 10 μM $PGF_{2\alpha}$ (Fig. 1). The inhibitory effect of L-CAZ on the IL-6 syn-thesis was dose dependent in the range between 1 and 100 μM (Fig. 1). The maximum effect of L-CAZ was observed at 100 μM, a dose that caused about 50% reduction in the $PGF_{2\alpha}$-effect.

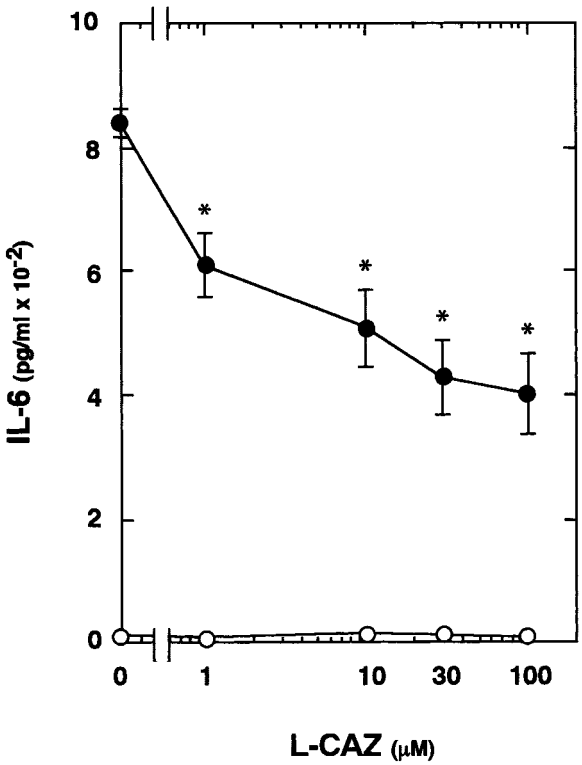

Fig. 1. Effect of L-CAZ on $PGF_{2\alpha}$-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of L-CAZ for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared with the value of $PGF_{2\alpha}$ alone.

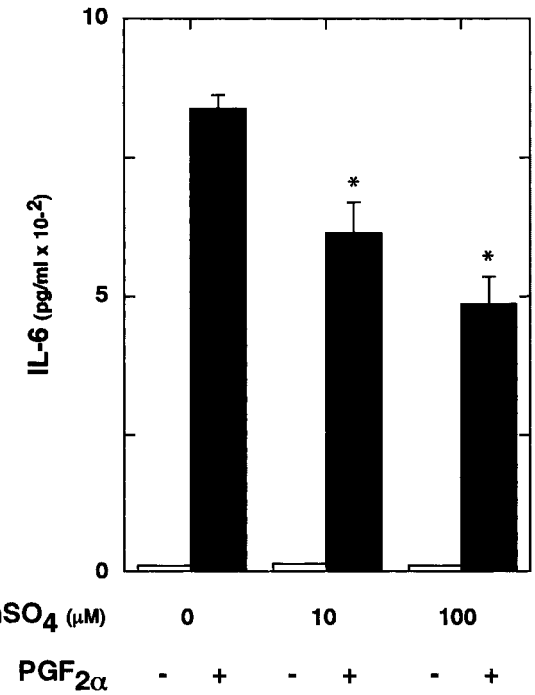

Fig. 2. Effect of $ZnSO_4$ on $PGF_{2\alpha}$-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of $ZnSO_4$ for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ (closed bar) or vehicle (open bar) for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared with the value of $PGF_{2\alpha}$ alone.

Effect of $ZnSO_4$ on $PGF_{2\alpha}$-Induced IL-6 Synthesis in MC3T3-E1 Cells

To confirm whether the effect of L-CAZ on IL-6 synthesis was caused by zinc ion, we next examined the effect of $ZnSO_4$ on the synthesis of IL-6 induced by $PGF_{2\alpha}$. $ZnSO_4$ markedly reduced the $PGF_{2\alpha}$-induced IL-6 synthesis. The inhibitory effect of $ZnSO_4$ was dose dependent in the range between 10 and 100 μM (Fig. 2). The maximum effect of $ZnSO_4$ was observed at 100 μM, a dose that caused about 40% reduction in the effect of $PGF_{2\alpha}$.

Effect of L-CAZ on $PGF_{2\alpha}$-Induced p44/p42 MAP Kinase Phosphorylation in MC3T3-E1 Cells

We have recently reported that $PGF_{2\alpha}$ stimulates p44/p42 MAP kinase activation in osteoblast-like MC3T3-E1 cells and the p44/p42 MAP kinase acts at a point downstream from PKC in the $PGF_{2\alpha}$-induced IL-6 synthesis [Tokuda et al., 1999]. To clarify whether zinc affects the p44/p42 MAP kinase activation stimulated by $PGF_{2\alpha}$ in these cells, we examined the effect of L-CAZ on the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$. L-CAZ significantly suppressed the $PGF_{2\alpha}$-induced the phosphorylation of p44/p42 MAP kinase (Fig. 3A). According to the densitometric analysis, L-CAZ caused about 50% reduction in the effect of $PGF_{2\alpha}$ (Fig. 3B).

Effects of L-CAZ on TPA or NaF-Induced p44/p42 MAP Kinase Phosphorylation in MC3T3-E1 Cells

We next examined the effect of L-CAZ on the phosphorylation of the p44/p42 MAP kinase stimulated by TPA, a direct activator of PKC [Nishizuka, 1986], or NaF, a direct activator of heterotrimeric GTP-binding protein [Gilman, 1987]. L-CAZ had little effect on the p44/p42 MAP kinase phosphorylation induced by TPA (Fig. 4A) or NaF (Fig. 4B). According to the densitometric analysis, L-CAZ did not affect the phosphorylation by TPA (Fig. 4A) or NaF (Fig. 4B).

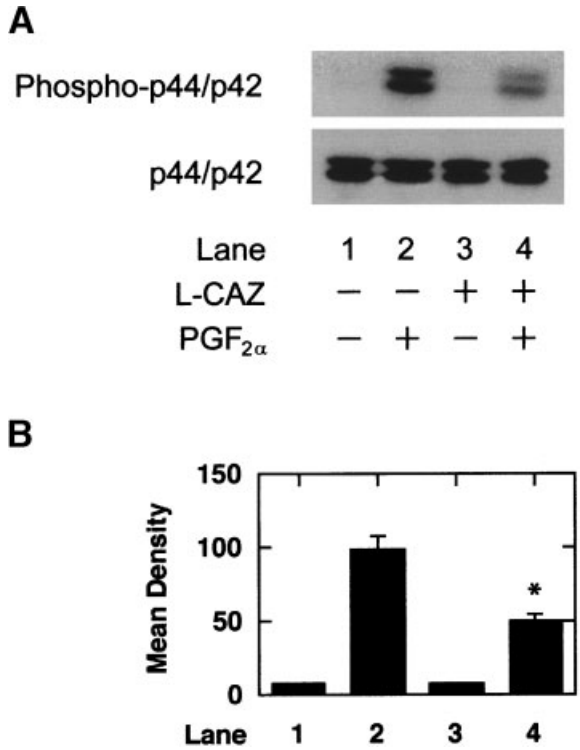

Fig. 3. Effect of L-CAZ on $PGF_{2\alpha}$-induced p44/p42 MAP kinase phosphorylation in MC3T3-E1 cells. **A**: The cultured cells were pretreated with 100 μM L-CAZ or vehicle for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE against phosphospecific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies. **B**: The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced p44/p42 MAP kinase phosphorylation obtained from laser densitometric analysis of three independent experiments. Densitometry results were expressed as mean density. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of $PGF_{2\alpha}$ alone.

Effects of L-CAZ on $PGF_{2\alpha}$- or NaF-Induced Formation of Inositol Phosphates in MC3T3-E1 Cells

We previously reported that $PGF_{2\alpha}$ stimulates phosphoinositide hydrolysis by phospholipase C via pertusis-toxin sensitive heterotrimeric GTP-binding protein in osteoblast-like MC3T3-E1 cells, resulting in the activation of PKC [Miwa et al., 1990]. In order to investigate whether zinc affects the hydrolysis by phospholipase C in MC3T3-E1 cells, we next examined the effect of L-CAZ on the $PGF_{2\alpha}$-induced formation of inositol phosphates. L-CAZ, which alone had little effect on the basal level of inositol phosphates, significantly suppressed the formation of inositol phosphates stimulated by $PGF_{2\alpha}$ in a dose-dependent manner between 10 and 100 μM (Fig. 5). The maximum effect of L-CAZ was observed at 100 μM, a dose that caused a 90% reduction in the effect of $PGF_{2\alpha}$. We next examined the effect of L-CAZ on the NaF-induced formation of inositol phosphates. L-CAZ did not affect the formation of inositol phosphates induced by NaF (Table I).

Effects of L-CAZ on $PGF_{2\alpha}$- or NaF-Induced Formation of Choline in MC3T3-E1 Cells

In a previous study [Kozawa et al., 1994], we have shown the $PGF_{2\alpha}$ induces phosphatidylcholine hydrolysis by phospholipase D independently of phosphoinositide hydrolysis by phospholipase C via pertusis toxin-sensitive heterotrimeric GTP-binding protein in MC3T3-E1 cells. In order to investigate whether zinc affects the hydrolysis by phospholipase D in MC3T3-E1 cells, we next examined the effect of L-CAZ on $PGF_{2\alpha}$-induced formation of choline. L-CAZ significantly suppressed the $PGF_{2\alpha}$-induced formation of choline as well as the formation of inositol phosphates (Fig. 6). On the other hand, L-CAZ had little effect on the NaF-induced choline formation (data not shown).

DISCUSSION

In the present study, we showed that zinc complex of L-carnosine (L-CAZ) significantly inhibited the $PGF_{2\alpha}$-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. To clarify whether the inhibitory effect of L-CAZ on the $PGF_{2\alpha}$-induced IL-6 synthesis is caused by zinc, we next examined the effect of zinc sulfate ($ZnSO_4$) on the IL-6 synthesis stimulated by $PGF_{2\alpha}$ in these cells. $ZnSO_4$ suppressed the $PGF_{2\alpha}$-induced IL-6 synthesis as well as L-CAZ. Therefore, it is most likely that zinc plays an inhibitory role in the $PGF_{2\alpha}$-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

In previous studies [Miwa et al., 1990; Kozawa et al., 1994], we have shown that $PGF_{2\alpha}$ activates phosphoinositide-hydrolyzing phospholipase C and phosphatidylcoline-hydrolyzing phospholipase D in osteoblast-like MC3T3-E1 cells and that a heterotrimeric GTP-binding protein is involved in the $PGF_{2\alpha}$-induced activation of phospholipases. It is well known that the activation of these two phospholipases forms diacylglycerol, a physiological activator of PKC [Nishizuka, 1986]. Additionally, we have reported that PKC functions as a positive regulator in

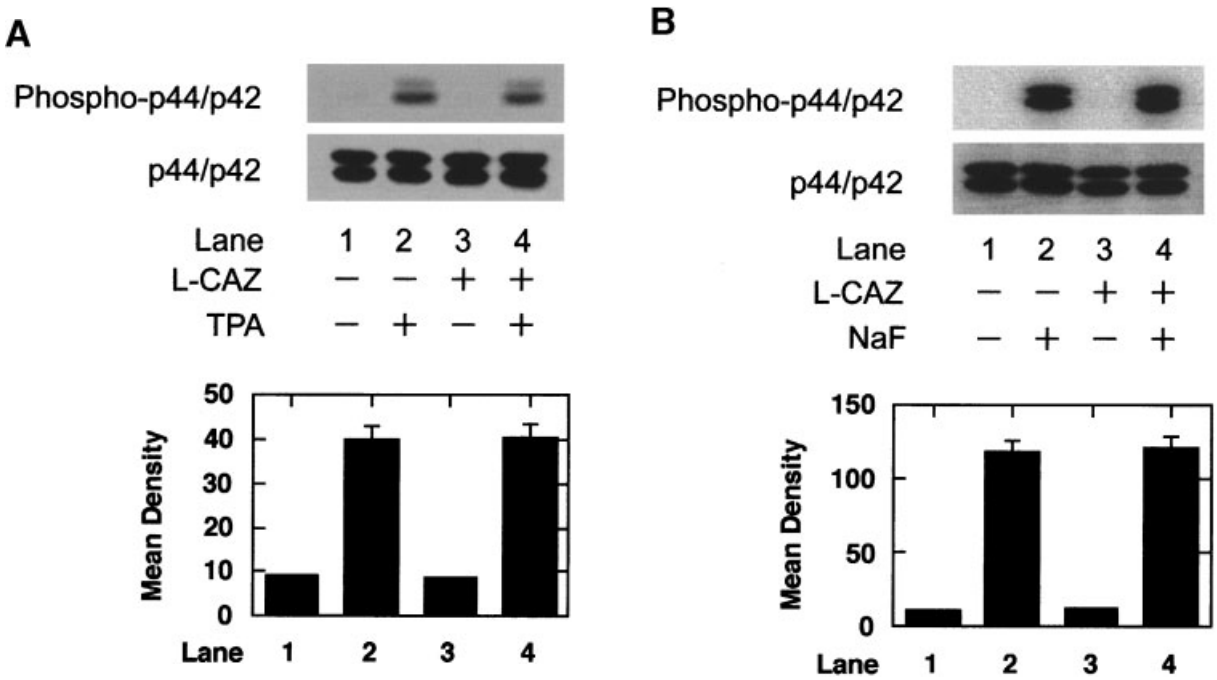

Fig. 4. Effects of L-CAZ on TPA- or NaF-induced p44/p42 MAP kinase phosphorylation in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM L-CAZ or vehicle for 20 min. **A**: The cells were then stimulated by 1 nM TPA or vehicle for 90 min. **B**: The cells were then stimulated by 40 mM NaF or vehicle for 5 mim. The extracts of cells were subjected to SDS–PAGE against phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies. The histogram shows quantitative representations of the levels of TPA- or NaF-induced p44/p42 MAP kinase phosphorylation obtained from laser densitometric analysis of three independent experiments. Densitometry results were expressed as mean density. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

the $PGF_{2\alpha}$-induced IL-6 synthesis in MC3T3-E1 cells and that p44/p42 MAP kinase acts as a downstream effector of PKC in the $PGF_{2\alpha}$-signaling [Kozawa et al., 1997; Tokuda et al., 1999]. Thus, we investigated where the effect of zinc is exerted in the $PGF_{2\alpha}$-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. First, we examined the effect of zinc on the $PGF_{2\alpha}$-induced p44/p42 MAP kinase phosphorylation. L-CAZ significantly reduced the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$. These findings suggest that zinc inhibits the $PGF_{2\alpha}$-induced IL-6 synthesis at a point upstream from p44/p42 MAP kinase in MC3T3-E1 cells. We next investigated whether or not the inhibitory effect of zinc is exerted at a point downstream from PKC in these cells. However, L-CAZ failed to affect the phosphorylation of p44/p42 MAP kinase induced by TPA. Based on these findings, it seems unlikely that zinc suppresses the $PGF_{2\alpha}$-stimulated IL-6 synthesis at a point downstream from PKC.

We next examined the effect of zinc on the $PGF_{2\alpha}$-induced phosphoinositide hydrolysis by phospholipase C in MC3T3-E1 cells. We demonstrated that L-CAZ significantly inhibited the formation of inositol phosphates stimulated by $PGF_{2\alpha}$. However, the formation of inositol phosphates induced by NaF was not affected by L-CAZ. It seems that zinc functions at a point upstream from a heterotrimeric GTP-binding protein in MC3T3-E1 cells. In addition, we found that L-CAZ reduced the $PGF_{2\alpha}$-induced formation of choline, while having no effect on the NaF-induced choline formation. Furthermore, we showed that the NaF-induced p44/p42 MAP kinase phosphorylation was not suppressed by L-CAZ. Thus, these results led us to speculate that zinc suppresses the $PGF_{2\alpha}$-induced IL-6 synthesis at a point upstream from a heterotrimeric GTP-binding protein. Taking our findings into account, it is most likely that zinc reduces the IL-6 synthesis stimulated by $PGF_{2\alpha}$ via suppression of both the activation of phosphoinositide-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D in osteoblast-like MC3T3- E1 cells.

It has been reported that IL-6 secreted from osteoblasts modulates a variety of bone cell function [Helle et al., 1988; Feyen et al., 1989;

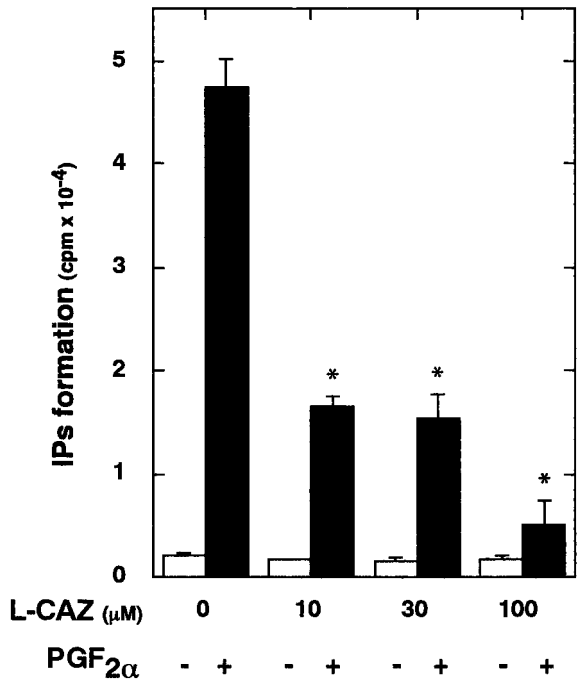

Fig. 5. Effect of L-CAZ on $PGF_{2\alpha}$-induced formation of inositol phosphates in MC3T3-E1 cells. The labeled cells were pretreated with various doses of L-CAZ for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ (closed bar) or vehicle (open bar) for 30 min. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of $PGF_{2\alpha}$ alone.

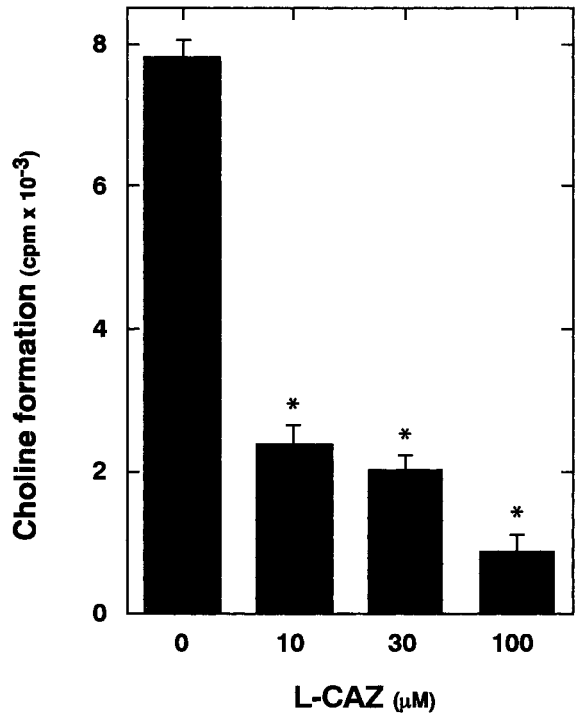

Fig. 6. Effect of L-CAZ on $PGF_{2\alpha}$-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with various doses of L-CAZ for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of $PGF_{2\alpha}$ alone.

Ishimi et al., 1990]. In bone metabolism, IL-6 is recognized to act as an autocrine/paracrine factor, which induces osteoclast formation and stimulates its activity to resorb bone [Rifas, 1999]. It has been shown that zinc acts as a positive regulator in bone formation and mineralization [Yamaguchi and Yamaguchi, 1986; Hall et al., 1999]. In osteoblasts, zinc reportedly stimulates bone protein synthesis [Lutz et al., 2000]. In the present study, we showed that zinc suppresses IL-6 synthesis induced by $PGF_{2\alpha}$ in osteoblast-like MC3T3-E1 cells. Taking these findings into account as a whole, it is probable that the zinc-induced inhibition of IL-6 synthesis in osteoblasts takes part in the stimulatory effect of zinc on bone formation.

In conclusion, our results strongly suggest that zinc inhibits $PGF_{2\alpha}$-induced IL-6 synthesis via suppression of phosphoinositide-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D in osteoblasts.

TABLE I. Effect of L-CAZ on NaF-Induced Formation of Inositol Phosphates in MC3T3-E1 Cells

L-CAZ (100 μM)	NaF (40 mM)	IPs formation (cpm)
−	−	639.3 ± 187.0
−	+	24,504.4 ± 982.5
+	−	544.0 ± 94.9
+	+	26,618.7 ± 1,600.0

The labeled cells were pretreated with 100 μM of L-CAZ or vehicle for 20 min and then stimulated by 40 mM NaF or vehicle for 30 min. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

ACKNOWLEDGMENTS

We thank Masaichi Miwa and Hidenori Kawamura for their skillful technical assistance.

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