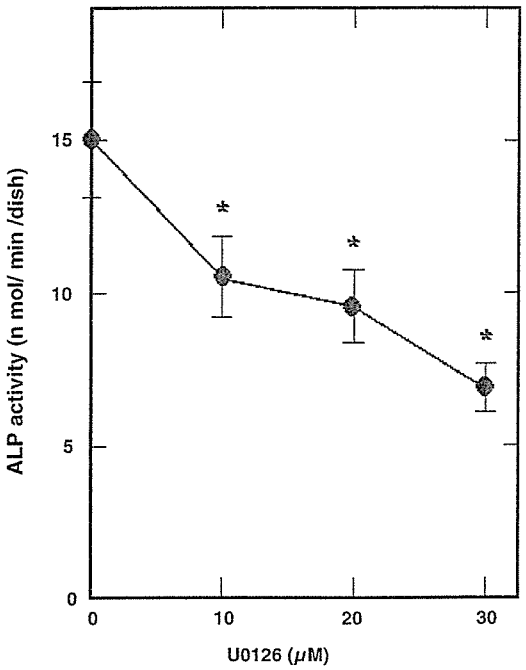

Fig. 4. Dose-dependent effect of U0126 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. The cultured cells were pretreated with various doses of U0126 for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 48 h. Values for IGF-I-unstimulated cells were subtracted to produce each data point. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of IGF-I alone.

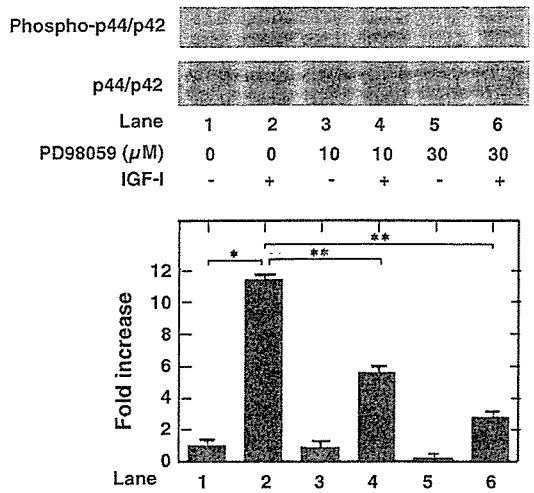

Fig. 5. Effect of PD98059 on the IGF-I-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD98059 for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of the levels of IGF-I-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of control; $^{**}p < 0.05$, compared to the value of IGF-I alone.

activity stimulated by IGF-I as well as PD98059 (Fig. 4). The maximum inhibitory effect of U0126 at 30 μM caused about 55% reduction in the IGF-I-effect.

3.4. Effects of PD98059 and U0126 on the phosphorylation of p44/p42 MAP kinase induced by IGF-I in MC3T3-E1 cells

We found that PD98059 truly suppressed the phosphorylation of p44/p42 MAP kinase induced by IGF-I in a dose-dependent manner (Fig. 5). According to the densitometric analysis, PD98059 at 30 μM caused about 75% reduction of the IGF-I-effect on the p44/p42 MAP kinase phosphorylation. Furthermore, U0126 markedly inhibited the IGF-I-induced phosphorylation of p44/p42 MAP kinase (data not shown). According to the densitometric analysis, U0126 caused almost complete reduction of the IGF-I-effect on the activity.

3.5. Effects of SB203580 and PD169316 on the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells

To investigate whether p38 MAP kinase is involved in the IGF-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells, we examined the effects of SB203580 and PD169316, highly specific inhibitors of p38 MAP kinase (Cuenda et al., 1995; Kummer et al., 1997), on the alkaline phosphatase activity. However, SB203580 or PD169316 hardly affected the IGF-I-induced alkaline phosphatase activity (15.5 ± 1.6 nmol/min/dish for IGF-I alone; 16.0 ± 1.7 nmol/min/dish for IGF-I with 30 μM SB203580; and 15.1 ± 1.4 nmol/min/dish for IGF-I with 30 μM PD169316, as measured during the stimulation for 48 h, values for IGF-I-unstimulated cells were subtracted to produce each data point).

3.6. Effect of PD169316 on the IGF-I-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells

We found that PD169316 markedly inhibited the phosphorylation of p38 MAP kinase induced by IGF-I (Fig. 6). According to the densitometric analysis, PD169316 caused about 40% reduction of the IGF-I-effect on the p38 MAP kinase phosphorylation.

3.7. Effect of LY294002 or wortmannin on the IGF-I-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells

We have recently reported that in MC3T3-E1 cells, IGF-I-induced alkaline phosphatase is PI3K/Akt dependent (Noda et al., 2005). To test the interaction between PI3K/Akt and p44/p42 MAPK pathway, we examined the effect of PI3K/Akt blockade on the IGF-I-induced p44/p42 MAPK phosphorylation in MC3T3-E1 cells. LY294002 (Vlahos et al., 1994), a specific inhibitor for PI3K/Akt pathway have no significant effect on the IGF-I-induced p44/p42 MAPK phosphorylations in MC3T3-E1 cells (Fig. 7). Wortmannin (Arcaro and Wymann, 1993), an another inhibitor for PI3K/Akt pathway, also had no significant

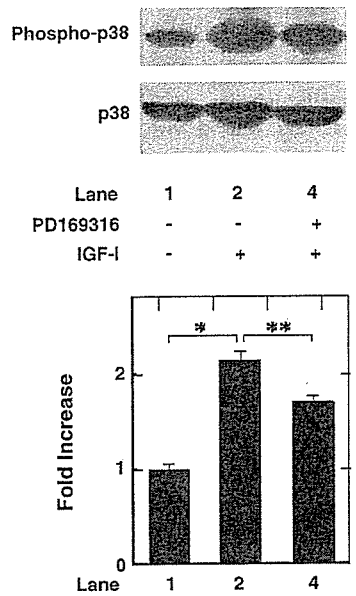

Fig. 6. Effect of PD169316 on the IGF-I-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD169316 or vehicle for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of IGF-I-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p < 0.05$, compared to the value of control; $^{**}p < 0.05$, compared to the value of IGF-I alone.

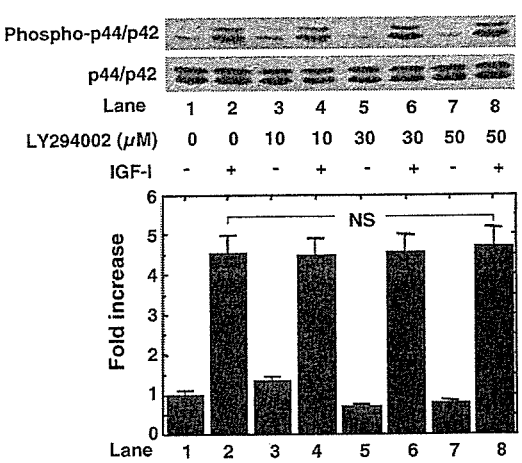

Fig. 7. Effect of LY294002 on the IGF-I-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 for 60 min, and then stimulated by 0.1 μM IGF-I or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of the levels of IGF-I-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

effect (data not shown). As shown in our previous study, the inhibitor concentrations used were sufficient to block the IGF-I-induced ALP activation in these cells (~50 μM for LY294002 and ~30 nM for wortmannin).

4. Discussion

In the present study, we demonstrated that IGF-I induces the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase but not SAPK/JNK in osteoblast-like MC3T3-E1 cells. It is currently understood that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions including proliferation, differentiation, and cell death in a variety of cells (Widmann et al., 1999). Three major MAP kinase, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK, are known as central elements used by mammalian cells to transducer the diverse messages (Widmann et al., 1999). It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase (Raingeaud et al., 1995; Widmann et al., 1999). Therefore, taking these results into account, it is most likely that IGF-I induces both the activation of p44/p42 MAP kinase and p38 MAP kinase without activating SAPK/JNK in osteoblast-like MC3T3-E1 cells.

We next investigated the involvement of p44/p42 MAP kinase in the IGF-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells. Herein, we showed that PD98059 (Alessi et al., 1995) and U0126 (Favata et al., 1998) significantly suppressed the IGF-I-induced alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. In addition, we found that these inhibitors truly attenuated the phosphorylation of p44/p42 MAP kinase induced by IGF-I, suggesting that the activation of p44/p42 MAP kinase is involved in IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. Therefore, our findings indicate that p44/p42 MAP kinase participates at least in part in the IGF-I-stimulated alkaline phosphatase activity.

In order to clarify whether p38 MAP kinase plays a role in the IGF-I-stimulated alkaline phosphatase activity, we examined the effect of specific inhibitors of p38 MAP kinase such as SB203580 (Cuenda et al., 1995) and PD169316 (Kummer et al., 1997) on the alkaline phosphatase activity. However, either SB203580 or PD169316 failed to affect the IGF-I-induced activity of alkaline phosphatase. We found that PD169316 truly reduced the IGF-I-induced phosphorylation of p38 MAP kinase. Based on these results, it seems unlikely that p38 MAP kinase is involved in IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells.

We have recently reported that in MC3T3-E1 cells, IGF-I-induced alkaline phosphatase is PI3K/Akt dependent (Noda et al., 2005). To confirm the interaction between PI3K/Akt pathway and p44/p42 MAP kinase pathway, we examined the effect of PI3K/Akt pathway blockade on the IGF-I-induced p44/p42 MAP kinase phosphorylation in MC3T3-E1 cells, and found that LY294002 (Vlahos et al., 1994) and wortmannin (Arcaro and Wymann, 1993) had no significant effect on the IGF-I-induced p44/p42 MAP kinase phosphorylation in MC3T3-E1 cells. It is likely that these two pathways independently regulate IGF-I-

induced ALP induction, or at least, PI3K/Akt pathway does not exert its effect at a point upstream of p44/p42 MAP kinase in IGF-I-induced ALP activation.

It is well known that alkaline phosphatase is a biochemical marker of bone formation (Robinson et al., 1973; Seibel, 2000). During progression from an immature progenitor cell to a mature osteoblast resulting in mineralization, expression of alkaline phosphatase occurs (Aubin and Liu, 1996). Thus, it is generally recognized that alkaline phosphatase plays an important role in mineralization although the precise function of the enzyme is not clarified. It has been recently reported that PD98059 blocks IGF-I-induced expression of core binding factor α1 in osteoblast-like MC3T3-E1 cells, suggesting involvement of p44/p42 MAP kinase in the expression (Kummer et al., 1997). Expression of core binding factor α1 is the earliest osteoblast-specific event identified in the osteoblast differentiation pathway and critical for osteoblastogenesis (Komori et al., 1997). We here showed that p44/p42 MAP kinase plays a role in the IGF-I-induced alkaline phosphatase activity in MC3T3-E1 cells. It is most likely that p44/p42 MAP kinase activation has an important role in IGF-I-induced bone formation. In response to various physiological agonists, such as estrogen and parathyroid hormone, the expression of IGF-I is known to be increased in osteoblasts (Conover and Rosen, 2002), suggesting that IGF-I is an autocrine/paracrine regulator of osteoblast functions. Thus, it is likely that IGF-I-stimulated p44/p42 MAP kinase plays a crucial role in the regulation of bone metabolism under the physiological conditions. In addition, we investigated the effects of IGF-I treatment on the several known osteoblast differentiation markers (osteocalcin, osteopontin, Runx2 and collagen α1(I)) in osteoblast-like MC3T3-E1 cells. We found that IGF-I had no significant effect on up-regulation of these mRNA expressions at a dose that significantly induces ALP activation. It seems unlikely that the involvement of IGF-I signaling is common in differentially expressed osteoblast lineage markers. Mineralization defects have reportedly seen in ALP knockout mouse (Fedde et al., 1999). Taking these findings into account, as a whole, it is possible that the blockade of IGF-I signaling including p44/p42 MAP kinase might result in the impairment of mineralization. Further investigations would be required to clarify the details.

In conclusion, these results strongly suggest that p44/p42 MAP kinase among the MAP kinase superfamily plays a crucial role in the IGF-I-stimulated alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells.

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Phosphatidylinositol 3-Kinase/Akt Auto-Regulates PDGF-BB-Stimulated Interleukin-6 Synthesis in Osteoblasts

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Abstract It has been reported that platelet-derived growth factor (PDGF)-BB stimulates the synthesis of interleukin (IL)-6 in osteoblasts. In the present study, we investigated whether the phosphatidylinositol 3-kinase (PI3K)/Akt is involved in the PDGF-BB-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. PDGF-BB markedly induced the phosphorylation of Akt and GSK-3β. Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-O-octadecylcarbonate, significantly amplified the synthesis of IL-6 by PDGF-BB. The PDGF-BB-induced GSK-3β phosphorylation was suppressed by the Akt inhibitor. The IL-6 synthesis stimulated by PDGF-BB was markedly enhanced by LY294002 and wortmannin, inhibitors of PI3K. Wortmannin and LY294002 suppressed the PDGF-BB-induced phosphorylation of Akt and GSK-3β. Taken together, these results strongly suggest that PI3K/Akt negatively regulates the PDGF-BB-stimulated IL-6 synthesis in osteoblasts. J. Cell. Biochem. 99: 1564–1571, 2006. © 2006 Wiley-Liss, Inc.

Key words: platelet-derived growth factor (PDGF); interleukin-6 (IL-6); phosphatidylinositol 3-kinase; Akt; osteoblast

It is generally known that platelet-derived growth factor (PDGF) is a mitogenic factor, which mainly acts on connective tissue cells [Heldin and Westermark, 1999; Heldin et al., 2002]. PDGF occurs as five different isoforms [Heldin et al., 2002]. PDGF isoforms were originally isolated from platelets, but have been shown to be produced and released from a variety of cell types including osteosarcoma [Heldin et al., 1986; Heldin and Westermark, 1999]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [Nijweide et al., 1986]. As for stimulation of biological activities in bone cells, PDGF-BB is a potent stimulator and induces osteoblast proliferation and collagen synthesis [Canalis et al., 1992]. It is recognized that PDGF, released during platelet aggregation, has a pivotal role in fracture healing as a systemic factor and that PDGF also regulates bone remodeling as a local factor [Canalis et al., 1992]. PDGF receptor has an intrinsic protein tyrosine kinase activity and associates with SH-2 domain-containing substrates such as phospholipase C and phosphatidylinositol 3-kinase (PI3K) [Heldin and Westermark, 1999]. We have previously reported that PDGF-BB activates phosphatidylcholine-hydrolyzing phospholipase D via tyrosine kinase activation, resulting in protein kinase C activation in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1995]. However, the exact role of PDGF in osteoblasts is not precisely known.

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Interleukin-6 (IL-6) is a multifunctional cytokine that has important physiological effects on a wide range of functions, such as promoting B cell differentiation, T cell activation, and inducing acute phase proteins [Akira et al., 1993; Heymann and Rousselle, 2000; Kwan Tat et al., 2004]. As for bone metabolism, IL-6 has been shown to stimulate bone resorption and promote osteoclast formation [Ishimi et al., 1990; Roodman, 1992; Heymann and Rousselle, 2000; Kwan Tat et al., 2004]. It has been reported that bone resorptive agents such as tumor necrosis factor-α (TNF-α) and IL-1 stimulate IL-6 synthesis in osteoblasts [Helle et al., 1988; Ishimi et al., 1990; Littlewood et al., 1991]. Thus, accumulating evidence indicates that IL-6 secreted from osteoblasts plays a key role as a downstream effector of bone resorptive agents. It has been shown that PDGF-BB induces the transcription of IL-6 through the activator protein-1 complex and activating transcription factor-2 in primary cultured rat osteoblasts [Franchimont et al., 1999]. However, the exact mechanism underlying PDGF-BB-stimulated IL-6 synthesis in osteoblasts has not yet been precisely clarified.

It is currently known that Akt, also called protein kinase B, is a serine/threonine protein kinase that plays crucial roles in mediating intracellular signaling of variety of agonists including insulin-like growth factor-I, PDGF, and cytokines [Coffer et al., 1998]. It has been shown that Akt regulates biological functions, such as gene expression, survival, and oncogenesis [Coffer et al., 1998]. Accumulating evidence suggests that PI3K functions at a point upstream from Akt [Chan et al., 1999; Cantley, 2002]. Akt containing a pleckstrin homology domain is recruited to the plasma membrane by the lipid product of PI3K and activated. As for osteoblasts, it has been reported that TNF-α and PDGF induce translocation of Akt to the nucleus [Borgatti et al., 2000]. In addition, Akt is reportedly activated by cyclic stretch [Danciu et al., 2003] or androgen [Kang et al., 2004]. However, the exact role of Akt in PDGF-BB-effect on osteoblasts has not yet been clarified.

In the present study, we investigated whether PI3K/Akt is involved in the PDGF-BB-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We here show that PI3-kinase/Akt activated by PDGF-BB plays an inhibitory role in the IL-6 synthesis in these cells.

MATERIALS AND METHODS

Materials

PDGF-BB and mouse IL-6 enzyme immunoassay kit were purchased from R&D Systems, Inc. (Minneapolis, MN). Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate), LY294002 and wortmannin were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3β antibodies, and GSK-3β antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Akt inhibitor, wortmannin or LY294002 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for IL-6 or Western blot analysis.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Sudo et al., 1983] were maintained as previously described [Kozawa et al., 1992]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm 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 48 h. The plating density of the cells was about 8×10^5 cells/dish for 35-mm diameter dish, or 7×10^6 cells/dish for 90-mm diameter dish. The media were changed again when the various inhibitors and/or PDGF-BB were subsequently added.

IL-6 Assay

The cultured cells were stimulated by various dose of PDGF-BB in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pre-treated with Akt inhibitor, wortmannin or LY294002 for 60 min. The conditioned medium was collected at the end of the incubation, and the IL-6 concentration was measured by ELISA kit.

Western Blot Analysis

The cultured cells were stimulated by PDGF-BB in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline 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 a supernatant after centrifugation at 125,000*g* for 10 min at 4°C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [1970] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [Kato et al., 1996] by using phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3β antibodies, or GSK-3β antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

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

RESULTS

Effect of PDGF-BB on the Phosphorylation of Akt in MC3T3-E1 Cells

We examined the effect of PDGF-BB on the phosphorylation of Akt in order to investigate whether PDGF-BB activates Akt in osteoblast-like MC3T3-E1 cells. PDGF-BB time-dependently stimulated the phosphorylation of Akt up to 120 min (Fig. 1). The maximum effect of PDGF-BB on the phosphorylation of Akt was observed at 20 min after the stimulation.

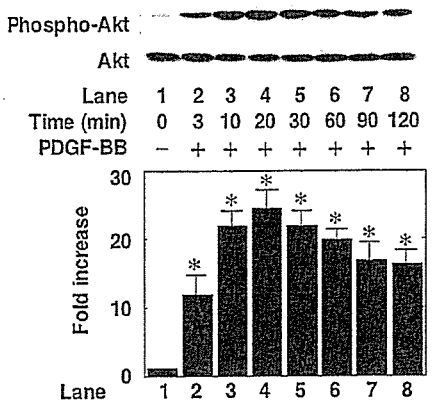

Fig. 1. Effect of PDGF-BB on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated by 50 ng/ml PDGF-BB for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of control.

Effect of Akt Inhibitor on the IL-6 Synthesis by PDGF-BB in MC3T3-E1 Cells

It has been reported that PDGF-BB induces IL-6 transcription in osteoblasts from fetal rat calvariae [Franchimont et al., 1999]. We found that PDGF-BB stimulated IL-6 synthesis in a dose-dependent manner between 10 ng/ml and 70 ng/ml in osteoblast-like MC3T3-E1 cells (data not shown). In order to clarify the involvement of Akt pathway in the PDGF-BB-stimulated IL-6 synthesis in these cells, we first examined the effect of Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate [Hu et al., 2000], on the IL-6 synthesis. The Akt inhibitor, which by itself hardly affected the IL-6 levels, significantly enhanced the PDGF-BB-stimulated synthesis of IL-6 (Fig. 2). The amplifying effect of the Akt inhibitor on the IL-6 synthesis was dose-dependent between 1 μM and 3 μM (Fig. 2). The Akt inhibitor at 10 μM caused about 100% enhancement in the PDGF-BB-effect.

Effect of Akt Inhibitor on the Phosphorylation of GSK-3β Induced by PDGF-BB in MC3T3-E1 Cells

It is generally known that GSK-3β is a critical downstream target molecule of the Akt, and its

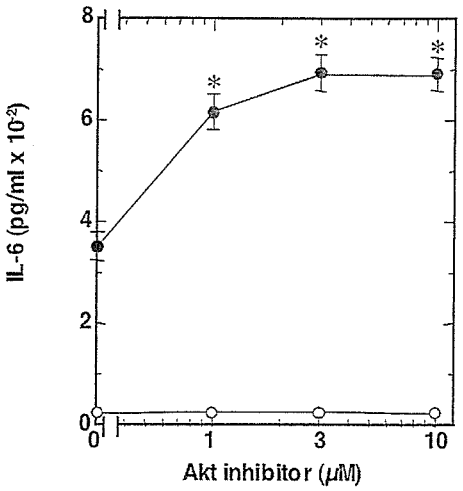

Fig. 2. Effect of Akt inhibitor on the PDGF-BB-stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pre-treated with various doses of Akt inhibitor for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 24 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

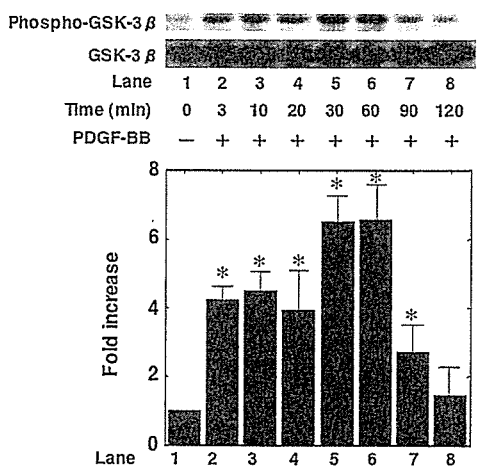

Fig. 3. Effect of PDGF-BB on the phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were stimulated by 50 ng/ml PDGF-BB for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of control.

activity can be inhibited by Akt-mediated phosphorylation of GSK-3β at Ser9 [Cross et al., 1995; Srivastava and Pandey, 1998]. We found that PDGF-BB truly induced the Akt-mediated phosphorylation of GSK-3β in a time-dependent manner in MC3T3-E1 cells (Fig. 3). The maximum effect of PDGF-BB on the phosphorylation of GSK-3β was observed at 60 min after the stimulation. We next examined the effect of Akt inhibitor on the phosphorylation of GSK-3β induced by PDGF-BB in these cells. Akt inhibitor markedly reduced the PDGF-BB-induced phosphorylation of GSK-3β (Fig. 4).

Effects of LY294002 or Wortmannin on the PDGF-BB-Induced Phosphorylation of Akt in MC3T3-E1 Cells

To clarify whether or not PI3K functions at a point upstream from Akt in MC3T3-E1 cells, we examined the effect of LY294002, a specific inhibitor of PI3-kinase [Vlahos et al., 1994], on the Akt phosphorylation induced by PDGF-BB.

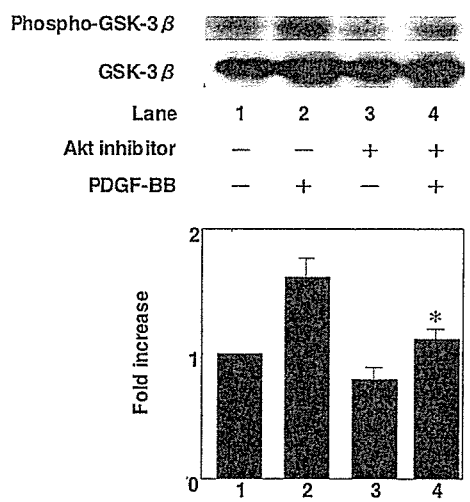

Fig. 4. Effect of Akt inhibitor on the PDGF-BB-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pre-treated with 50 μM Akt inhibitor for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 60 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

LY294002 suppressed the PDGF-BB-induced phosphorylation of Akt (Fig. 5). The inhibitory effect of LY294002 was dose-dependent between 10 μM and 50 μM. In addition, the PDGF-BB-induced phosphorylation of Akt was markedly attenuated by wortmannin, another PI3-kinase inhibitor [Arcaro and Wymann, 1993], (Fig. 6).

Effects of LY294002 or Wortmannin on the PDGF-BB-Stimulated IL-6 Synthesis and Phosphorylation of GSK-3β in MC3T3-E1 Cells

LY294002, which by itself hardly affected the IL-6 levels, significantly amplified the PDGF-BB-stimulated synthesis of IL-6 (Fig. 7). The amplifying effect of LY294002 was dose-dependent between 1 μM and 10 μM. The LY294002 at 10 μM caused about 80% enhancement in the PDGF-BB effect. Wortmannin, as well as LY294002, also enhanced the PDGF-BB-stimulated synthesis of IL-6 without affecting IL-6 synthesis alone (data not shown). In addition, we found that the phosphorylation of GSK-3β

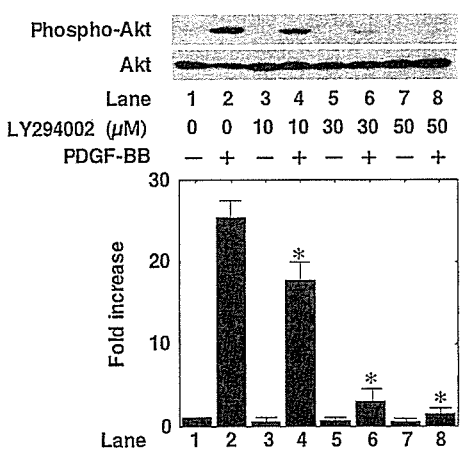

Fig. 5. Effect of LY294002 on the PDGF-BB-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pre-treated with various doses of LY294002 for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

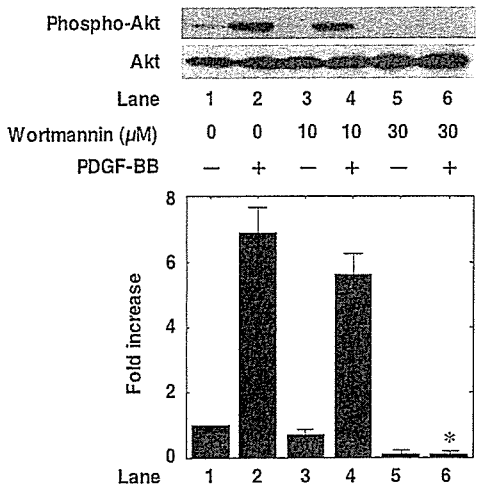

Fig. 6. Effect of wortmannin on the PDGF-BB-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pre-treated with various doses of wortmannin for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

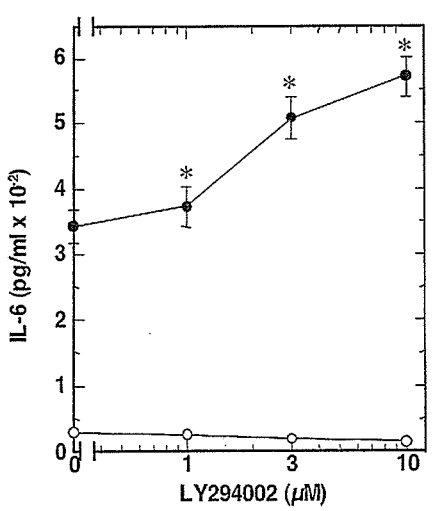

Fig. 7. Effect of LY294002 on the PDGF-BB-stimulated IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pre-treated with various doses of LY294002 for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 24 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

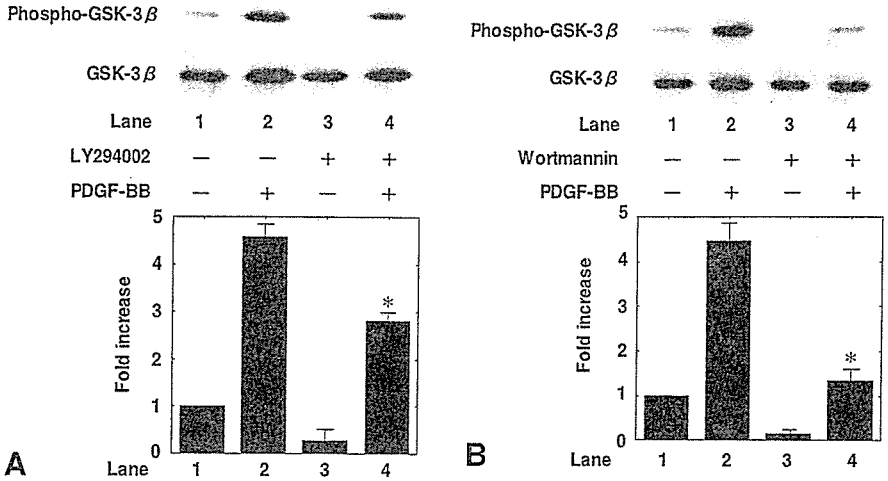

Fig. 8. Effects of LY294002 or wortmannin on the PDGF-BB-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pre-treated with 30 μM of LY294002 (**A**), 30 μM wortmannin (**B**) or vehicle for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 60 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. The phosphorylation levels were expressed as the fold increase to the basal levels presented as **lane 1**. The values were calculated as the average values of lane 1 to be 1.0. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P < 0.05$, compared to the value of PDGF-BB alone.

induced by PDGF-BB was significantly reduced by LY294002 or wortmannin (Fig. 8A,B).

DISCUSSION

In the present study, we found that PDGF-BB time-dependently induced the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells. It has been reported that Akt is activated by PDGF-BB in osteoblastic cells [Chaudhary and Hruska, 2001]. In addition, we demonstrated that PI3K inhibitors, such as LY294002 [Vlahos et al., 1994] and wortmannin [Arcaro and Wymann, 1993] attenuated the PDGF-BB-induced phosphorylation of Akt in MC3T3-E1 cells. It has been shown that Akt is activated by phosphorylation of threonine and serine residues [Coffer et al., 1998; Chan et al., 1999]. The Akt signaling pathway is currently recognized to play a critical role in mediating survival signals in a wide range of cell types [Chan et al., 1999]. Therefore, these results suggest that PDGF-BB activates Akt via PI3K in osteoblast-like MC3T3-E1 cells.

We investigated whether or not PI3K/Akt functions in the PDGF-BB-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. First, Akt inhibitor [Hu et al., 2000] significantly enhanced the PDGF-BB-stimulated synthesis of IL-6. It is generally known that GSK-3β is a downstream target of Akt and phosphorylated by Akt [Cross et al., 1995; Srivastava and Pandey, 1998]. We confirmed that the PDGF-BB-induced phosphorylation of GSK-3β was truly reduced by the Akt inhibitor. These results suggest that the activated Akt has an inhibitory effect on IL-6 release by PDGF-BB in osteoblast-like MC3T3-E1 cells. In addition, we found that wortmannin [Arcaro and Wymann, 1993] and LY294002 [Vlahos et al., 1994] markedly amplified the PDGF-BB-induced IL-6 synthesis. Furthermore, the PDGF-BB-induced phosphorylation of GSK-3β was suppressed by wortmannin or LY294002. Therefore, taking our results into account as a whole, it is most likely that PDGF-BB activates the PI3K/Akt pathway, resulting in the reduction of IL-6 synthesis. It is probable that the PI3K/Akt signaling pathway activated by PDGF-BB limits the PDGF-BB-stimulated IL-6 synthesis. To the best of our knowledge, our present finding is probably the first report to show that the activation of PI3K/Akt leads to the negative-feedback regulation of IL-6 synthesis in osteoblasts.

The PI3K/Akt pathway is recognized to play a crucial role in several cellular functions, such as proliferation and cell survival in a variety of cells [Coffer et al., 1998]. Our present results indicate that the PI3K/Akt pathway in

osteoblasts has an important role in the regulatory mechanism of the production of IL-6 in bone metabolism. As for the PDGF-induced IL-6 production, it has been reported that three isoforms of PDGF including PDGF-BB induces the expression of IL-6 gene, resulting in the proliferation of human fibroblasts, vascular smooth muscle cells, and mesangial cells [Roth et al., 1995]. In addition, hypoxia-induced IL-6 production associated with the cell proliferation is reportedly mediated by PDGF in human lung-derived fibroblasts and vascular smooth muscle cells [Tamm et al., 1998]. However, the role of PI3K/Akt pathway in the PDGF-induced IL-6 production has not yet been clarified. As far as we know, this is probably the first report showing the role of PI3K/Akt as a negative regulator in the IL-6 production elicited by PDGF-BB. It is well recognized that IL-6 is a potent bone resorptive agent and induces osteoclast formation [Kwan Tat et al., 2004]. Therefore, our present results lead us to speculate that PDGF-BB-activated PI3K/Akt signaling acts as a negative regulator against bone resorption. It is possible that the PI3/Akt pathway in osteoblasts might be considered as a new candidate as a molecular target of bone resorption concurrent with various bone diseases. The physiological significance of the auto-regulation by PI3K/Akt of PDGF-BB effect remains still unclear. Further investigation is necessary to clarify the exact role of PI3K/Akt in osteoblasts.

In conclusion, our results strongly suggest that PI3K/Akt plays a crucial role in the regulation of PDGF-BB-stimulated IL-6 synthesis in osteoblasts, and may serve as a negative-feedback mechanism to avoid the over-synthesis of IL-6.

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Prostaglandin D_2 induces the phosphorylation of HSP27 in osteoblasts: Function of the MAP kinase superfamily

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Abstract

We previously reported that prostaglandin D_2 (PGD_2) stimulates the induction of heat shock protein 27 (HSP27) in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether PGD_2 stimulates the phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. In the cultured MC3T3-E1 cells, PGD_2 markedly stimulated the phosphorylation of HSP27 at Ser-15 and Ser-85 in a time-dependent manner. Among the mitogen-activated protein (MAP) kinase superfamily, p44/p42 MAP kinase and p38 MAP kinase were phosphorylated by PGD_2 which had little effect on the phosphorylation of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK). The PGD_2-induced phosphorylation of HSP27 was attenuated by PD169316, an inhibitor of p38 MAP kinase or PD98059, a MEK inhibitor. SP600125, a SAPK/JNK inhibitor did not affect the HSP27 phosphorylation. In addition, PD169316 suppressed the PGD_2-induced phosphorylation of MAPKAP kinase 2. These results strongly suggest that PGD_2 stimulates HSP27 phosphorylation via p44/p42 MAP kinase and p38 MAP kinase but not SAPK/JNK in osteoblasts.

1. Introduction

Prostaglandins (PGs) act as local modulators in bone metabolism and play important roles in the regulation [1,2]. It is well recognized that bone metabolism is strictly regulated by osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [1]. Among PGs, prostaglandin D_2 (PGD_2) is generally known as a potent regulator of osteoblastic functions [3,4]. As an element of the intracellular signaling system of PGD_2 in osteoblasts, we have previously reported that PGD_2 activates protein kinase C via phosphoinositide-hydrolyzing phospholipase C in osteoblast-like MC3T3-E1 cells, resulting in the stimulation of proliferation [5]. In addition, we showed that PGD_2 stimulates the synthesis of interleukin-6 through a calcium-dependent manner in these cells [6]. It has recently been reported that PGD_2 produced in human osteoblasts acts as an autacoid to suppress both osteoprotegerin synthesis and RANKL expression in these cells, leading to an anabolic response in bone [7]. These findings made us to speculate that PGD_2 plays a crucial role in bone metabolism. However, the mechanism by which PGD_2 modulates osteoblast functions is not fully clarified.

It is well known that cells produce heat shock proteins (HSPs), when exposed to biological stresses such as heat stress and chemical stress [8]. HSPs are classified into high-molecular-weight HSPs and low-molecular-weight

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HSPs based on apparent molecular sizes. It is recognized that the high-molecular-weight HSPs such as HSP90 and HSP70 act as molecular chaperones in protein folding, oligomerization and translocation [8]. Low-molecular-weight HSPs with molecular masses from 10 to 30 kDa, such as HSP27 and αB-crystallin have high homology in amino acid sequences [8]. Though the functions of the low-molecular-weight HSPs are known less than those of the high-molecular-weight HSPs, it is recognized that they may have chaperoning functions like the high-molecular-weight HSPs [8]. In a previous study [9], we have shown that low-molecular-weight HSPs are present in various tissues and cells, especially in skeletal muscle and smooth muscle cells. The HSPs are present in significant amounts even in several unstressed cells including myocytes where they may have essential functions [8]. It is recognized that HSP27 activity is regulated by post-translational modifications such as phosphorylation [8,10]. It has been shown that HSP27 is phosphorylated at two sites (Ser-15 and Ser-85) of serine in mouse and rats [8]. In previous studies [11,12], we have shown that PGD_2 stimulates the induction of HSP27 via three mitogen-activated protein (MAP) kinases, p44/p42 MAP kinase, p38 MAP kinase and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) in osteoblast-like MC3T3-E1 cells. However, the details of HSP27 phosphorylation in osteoblasts are not known.

In the present study, we investigated whether PGD_2 stimulates the phosphorylation of HSP27 in osteoblast-like MC3T3-E1 cells exposed to heat shock, and the mechanism behind the phosphorylation. We here report that PGD_2 stimulates the phosphorylation of HSP27 via p38 MAP kinase and p44/p42 MAP kinase in these cells.

2. Materials and methods

2.1. Materials

PGD_2 was purchased from Sigma (St. Louis, MO). PD98059, PD169316 and SP600125 were obtained from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific MAPKAP kinase 2 antibodies and MAPKAP kinase 2 antibodies were obtained from New England BioLabs (Beverly, MA). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. PD98059, PD169316 or SP600125 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells, which have been derived from newborn mouse calvaria [13], were maintained as previously described [14]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 90-mm diameter dishes (5×10^5 cells/dish) in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS, and the dishes were exposed to heat shock for 30 min at 43 °C. The cells were used for experiments after cultured at 37 °C over night in a humidified atmosphere of 5% CO_2/95% air. When indicated, the cells were pretreated with PD98059, PD169316 or SP600125 for 60 min.

2.3. Western blot analysis

Cultured cells were stimulated by PGD_2 in serum-free α-MEM for the indicated periods. Cells were washed twice with phosphate-buffered saline and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125 000*g* for 10 min at 4 °C. The supernatant was used for the analysis of HSP27, each MAP kinase or MAKAP kinase 2 by Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli in 10% polyacrylamide gel [15]. Western blot analysis was performed as described previously [16,17], using HSP27 antibodies, phospho-specific HSP27 antibodies, each of the MAP kinase antibodies or MAPKAP kinase 2 antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on PVDF membrane was visualized on X-ray film by means of the ECL Western blotting detection system. Densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad, Hercules, CA).

2.4. Immunochemical reagents

Antibodies specifically recognizing phosphorylated serine residues at Ser-15 and Ser-85 in HSP27 were prepared as previously described [18]. HSP27 was purified from skeletal muscle as previously described [9].

2.5. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs. $P<0.05$ was considered significant. The data are presented as the means ±SD of triplicate determinations from three independent experiments.

3. Results

3.1. Effect of PGD_2 on the phosphorylation of HSP27 in heat shock-exposed MC3T3-E1 cells

PGD_2 markedly stimulated the phosphorylation of HSP27 at Ser-15 (15S) and Ser-85 (85S) in HSP27 in a time dependent manner in the cultured osteoblast-like MC3T3-E1 cells exposed to heat shock (Fig. 1). PGD_2 did not affect the levels of HSP27 up to 120 min in these cells.

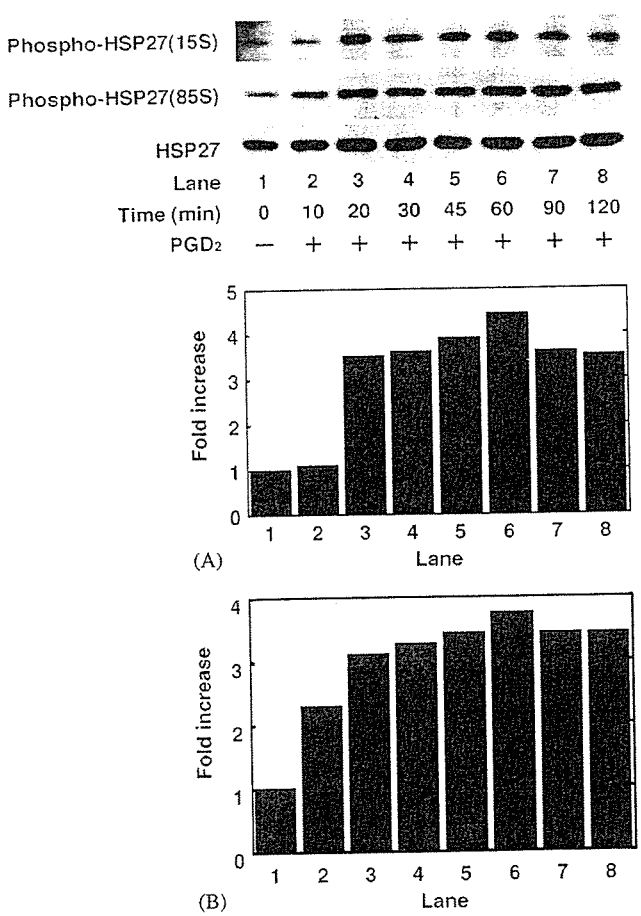

Fig. 1. Effect of PGD_2 on the phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. The cultured cells were stimulated by 10 μM PGD_2 for the indicated periods. The extracts of cells were subjected to SDS-PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and HSP27. The histogram shows quantitative representations of the phosphorylation of HSP27 ((A) Ser-15; (B) Ser-85) obtained from laser densitometric analysis. Similar results were obtained in two other cell preparations.

3.2. Effect of PGD_2 on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in heat shock-exposed MC3T3-E1 cells

It is recognized that the MAP kinase superfamily is involve in the phosphorylation of HSP27 [8]. We next examined the effect of PGD_2 on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK in the MC3T3-E1 cells exposed to heat shock in order to investigate whether PGD_2 activates the MAP kinases. PGD_2 time dependently induced the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase (Fig. 2). On the other hand, PGD_2 did not elicit the phosphorylation of SAPK/JNK (Fig. 2). We showed the phosphorylation of SAPK/JNK by TGFβ as a positive control as previously reported [19].

3.3. Effects of PD98059, PD169316 or SP600125 on the PGD_2-induced phosphorylation of HSP27 in heat shock-exposed MC3T3-E1 cells

In order to clarify whether the MAP kinase superfamily is involved in the PGD_2-induced phosphorylation of HSP27 in the MC3T3-E1 cells, we next examined the effect of PD98059, a specific inhibitor of upstream kinase that activates p44/p42 MAP kinase [20], on the HSP27 phosphorylation. PD98059 markedly suppressed

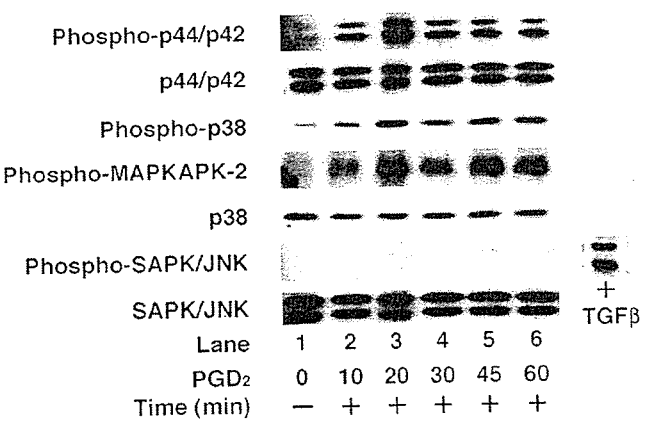

Fig. 2. Effects of PGD_2 on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, MAPKAP kinase 2, SAPK/JNK in MC3T3-E1 cells exposed to heat shock. The cultured cells were stimulated by 10 μM PGD_2 for the indicated periods. The extracts of cells were subjected to SDS-PAGE using antibodies against phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies or phospho-specific MAPKAP kinase 2 antibodies. Right blot shows the positive control for the TGFβ-induced phosphorylation of SAPK/JNK. Similar results were obtained in two other cell preparations.

the PGD_2-induced phosphorylation of HSP27 at Ser-15 and Ser-85 (Fig. 3).

We examined the effect of PD169316, a specific inhibitor of p38 MAP kinase [21], on the PGD_2-induced phosphorylation of HSP27. PD169316 significantly suppressed the phosphorylation of HSP27 stimulated by PGD_2 at Ser-15 and Ser-85 (Fig. 4). The inhibitory effect of PD169316 on the HSP27 phosphorylation at two sites was dose dependent in the range between 10 and 30 μM. In addition, SB203580, another specific inhibitor of p38 MAP kinase [22], had an inhibitory effect on the HSP27 phosphorylation as well as PD169316 (data not shown). Furthermore, we found that SP600125, a highly specific inhibitor of SAPK/JNK [23], did not affect the PGD_2-induced phosphorylation of HSP27 at Ser-15 and Ser-85 (data not shown).

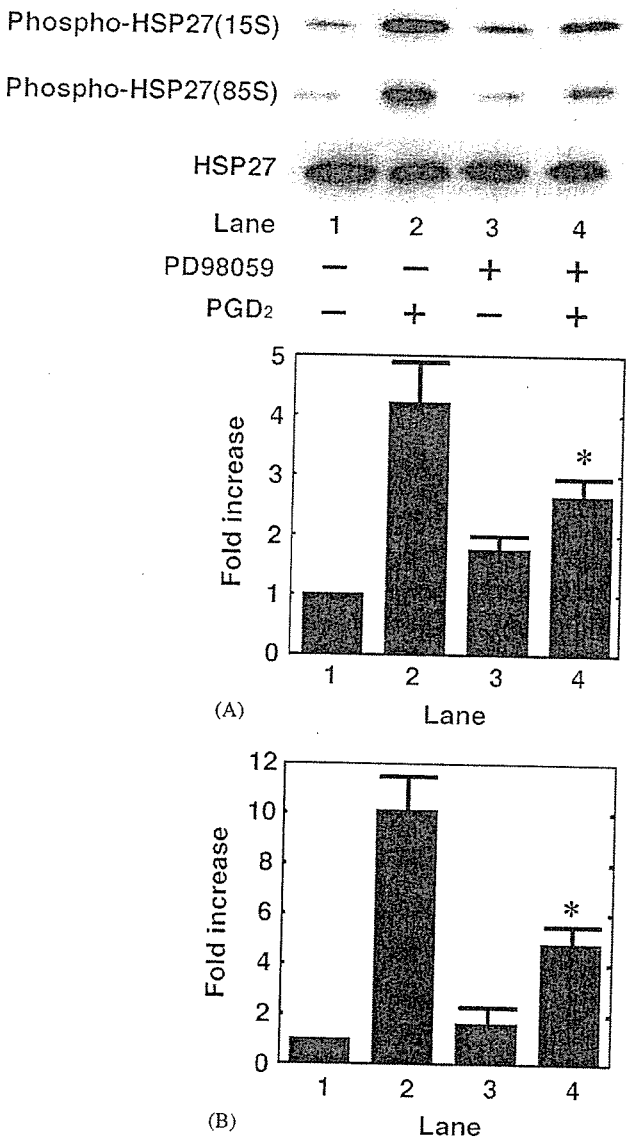

Fig. 3. Effect of PD98059 on the PGD_2-induced phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. The cultured cells were pretreated with 50 μM PD98059 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and HSP27. The histogram shows quantitative representations of the phosphorylation of HSP27 ((A) Ser-15; (B) Ser-85) obtained from laser densitometric analysis. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained in two other cell preparations. $^*P<0.05$ compared with the value of PGD_2 alone.

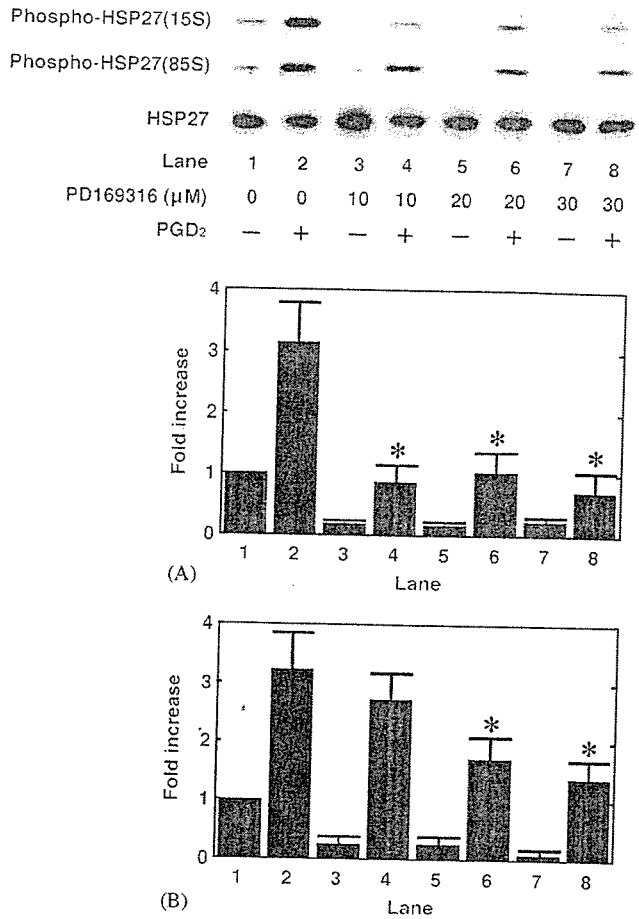

Fig. 4. Effect of PD169316 on the PGD_2-induced phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. The cultured cells were pretreated with various doses of PD169316 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and HSP27. The histogram shows quantitative representations of the phosphorylation of HSP27 ((A) Ser-15; (B) Ser-85) obtained from laser densitometric analysis. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained in two other cell preparations. $^*P<0.05$ compared with the value of PGD_2 alone.

3.4. *Effect of PGD_2 on the phosphorylation of MAPKAP kinase 2 and effect of PD169316 on the phosphorylation of MAPKAP kinase 2 in heat shock-exposed MC3T3-E1 cells*

It has been shown that MAPKAP kinase 2 acts at a point downstream of p38 MAP kinase in the HSP27

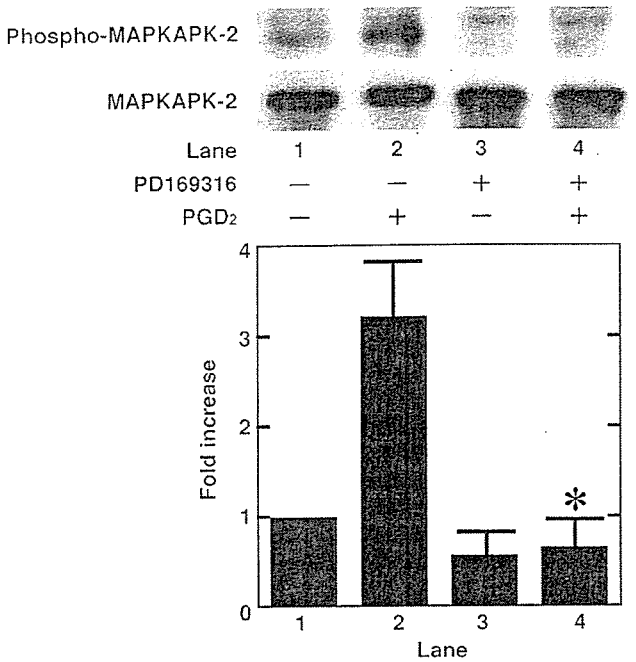

Fig. 5. Effect of PD169316 on the PGD_2-induced phosphorylation of MAPKAP kinase 2 in MC3T3-E1 cells exposed to heat shock. The cultured cells were pretreated with 10 μM PD169316 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE using antibodies against phosphorylated MAPKAP kinase 2 or MAPKAP kinase 2. The histogram shows quantitative representations of the phosphorylation of MAPKAP kinase 2 obtained from laser densitometric analysis. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained in two other cell preparations. $*P<0.05$ compared with the value of PGD_2 alone.

phosphorylation [8]. Thus, we next examined the effect of PGD_2 on the phosphorylation of MAPKAP kinase 2 in the MC3T3-E1 cells exposed to heat shock in order to investigate whether PGD_2 activates MAPKAP kinase 2. PGD_2 induced the phosphorylation of MAPKAP kinase 2 (Fig. 2). In addition, the PGD_2-induced phosphorylation of MAPKAP kinase 2 was markedly suppressed by PD169316 (Fig. 5).

4. Discussion

In the present study, we first investigated whether PGD_2 phosphorylates HSP27 in osteoblast-like MC3T3-E1 cells exposed to heat shock. In the cultured MC3T3-E1 cells, PGD_2 truly stimulated the phosphorylation of HSP27 at two sites. We next examined the mechanism behind the PGD_2-induced phosphorylation in these MC3T3-E1 cells. It has been shown that the activation of the MAP kinase superfamily is involved in HSP27 phosphorylation [8]. Herein, we showed that PGD_2 induced the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase while having little effect on the SAPK/JNK phosphorylation in the osteoblast-like MC3T3-E1 cells exposed to heat shock. It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase [24,25]. Thus, it is most likely that PGD_2 activates p44/p42 MAP kinase and p38 MAP kinase without affecting SAPK/JNK in the MC3T3-E1 cells exposed to heat shock.

Therefore, we next investigated whether two MAP kinases are involved in the PGD_2-induced HSP27 phosphorylation. PD98059, a specific MEK inhibitor [20], markedly suppressed the PGD_2-induced phosphorylation of HSP27 (Ser-15 and Ser-85). We found that the PGD_2-induced p44/p42 MAP kinase was significantly reduced by PD98059. Therefore, these results suggest that p44/p42 MAP kinase is involved in the PGD_2-induced HSP27 phosphorylation in the MC3T3-E1 cells exposed to heat shock. In addition, we showed that PD169316, a specific inhibitor of p38 MAP kinase [21], suppressed the PGD_2-induced HSP27 phosphorylations at two sites. Furthermore, we found that SB203580, another inhibitor of p38 MAP kinase [22], reduced the HSP27 phosphorylation by PGD_2 and that MAPKAP kinase 2 functions at a site downstream from p38 MAP kinase in the HSP27 phosphorylation. Based on these findings, it is most likely that PGD_2 stimulates HSP27 phosphorylation at two sites (Ser-15 and Ser-85) via p38 MAP kinase as well as p44/p42 MAP kinase in osteoblasts. On the other hand, we found that the PGD_2-induced phosphorylation of HSP27 at Ser-15 and Ser-85 was not affected by SP600125, a highly specific inhibitor of SAPK/JNK [23], which was consistent with our findings that PGD_2 had little effect on the SAPK/JNK phosphorylation. Therefore, it seems unlikely that SAPK/JNK mediates the PGD_2-induced HSP27 phosphorylation in osteoblasts.

It is well recognized that HSP27 is present at two forms, an aggregated form and a dissociated small form in unstressed cells [8]. It has been shown that HSP27 is constitutively expressed at high levels in various tissues and cells, especially in skeletal muscle cells and smooth muscle cells [8]. Post-translational modifications such as phosphorylation and oligomelization are crucial regulators of its functions [8]. In a previous study [26], we have reported that HSP27 is dissociated concomitantly with the phosphorylation of the aggregated form of HSP27 and that dephosphorylation of the dissociated HSP27 causes aggregation. In addition, we have shown that conversion of the non-phosphorylated, aggregated form of HSP27 to the phosphorylated, dissociated form results in decreased tolerance to heat stress [26]. It has been reported that HSP27 is related with cellular dynamics, such as modulation of actin filament and stability, growth, and secretion in several types of cells [27–29]. Taking our findings into account, it is probable that PGD_2 directly modulates osteoblast functions

through the induction of HSP27 phosphorylation. However, the physiological significance of HSP27 in bone metabolism has not yet been precisely clarified. Further investigations are necessary to clarify the exact roles of non-phosphorylated- and phosphorylated-HSP27 in osteoblasts.

In conclusion, these results strongly suggest that PGD_2 induces the phosphorylation of HSP27 via p44/p42 MAP kinase and p38 MAP kinase in osteoblasts.

Acknowledgments

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Phosphatidylinositol 3-Kinase/Akt Plays a Part in Tumor Necrosis Factor-α-induced Interleukin-6 Synthesis in Osteoblasts

Abstract

We previously showed that tumor necrosis factor-α (TNF-α) stimulates synthesis of interleukin-6 (IL-6), a potent bone resorptive agent, via p44/p42 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether phosphatidylinositol 3-kinase (PI3-kinase)/protein kinase B (Akt) is involved in TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. TNF-α induced the phosphorylation of Akt depending upon time. Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate, significantly suppressed the TNF-α-stimulated IL-6 synthesis, but the inhibitory effect was partial. The phosphorylation of Akt induced by TNF-α was markedly attenuated by LY294002 and wortmannin, inhibitors of PI3-kinase. Wortmannin and LY294002 significantly reduce the TNF-α-induced IL-6 synthesis. On the contrary, the suppressive effects of Akt inhibitor, wortmannin or LY294002 on TNF-α-induced phosphorylation of p44/p42 MAP kinase were minor. PD98059, a specific inhibitor of MEK, had little effect on the TNF-α-induced phosphorylation of Akt. A combination of Akt inhibitor and PD98059 suppressed the TNF-α-induced IL-6 synthesis in an additive manner. These results strongly suggest that PI3-kinase/Akt plays a role in the TNF-α-stimulated IL-6 synthesis mainly independent of p44/p42 MAP kinase in osteoblasts.

Key words
TNF-α · IL-6 · Akt · phosphatidylinositol 3-kinase · osteoblast

Introduction

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine responsible for inflammation, infection and cancer [1,2]. TNF-α induces numerous physiological effects on a variety of cells [1,2]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [3]. It is well-recognized that TNF-α is one of the most potent osteoclastogenic factors [2]. Bone resorption is mediated by the increased local production of inflammatory cytokines such as TNF-α and interleukin (IL)-1. In osteoblasts [4–6], it has been reported that bone resorptive agents such as TNF-α and IL-1 stimulate the synthesis of IL-6, which is a pleiotropic cytokine that has important physiological effects on a wide range of functions such as promoting B cell differentiation, T cell activation and inducing acute phase proteins [1,7,8]. As for bone metabolism, IL-6 has been shown to stimulate bone resorption and induce osteoclast formation [1,4,8,9]. Thus, accumulating evidences indicate that IL-6 secreted from osteoblasts plays a key role as a downstream effector of bone resorptive agents. In previous studies [10,11], we have shown that TNF-α induces the activation of p44/p42 mitogen-activated protein (MAP) kinase, a member of the MAP kinase superfamily, through sphingomyelin turnover, resulting in the IL-6 synthesis in osteoblast-like MC3T3-E1 cells. However, the exact mechanism of TNF-α behind the IL-6 synthesis in osteoblasts remains to be elucidated.

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