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Involvement of p38 Mitogen-Activated Protein Kinase in Basic Fibroblast Growth Factor-Induced Interleukin-6 Synthesis in Osteoblasts

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Abstract We previously showed that basic fibroblast growth factor (bFGF)-induced activation of protein kinase C (PKC) via phosphatidylinositol-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D suppresses interleukin-6 (IL-6) synthesis by bFGF itself in osteoblast-like MC3T3-E1 cells. In the present study, we further investigated the mechanism underlying the bFGF-induced IL-6 synthesis in MC3T3-E1 cells. bFGF time-dependently induced the phosphorylation of p38 mitogen-activated protein (MAP) kinase. SB203580, a specific inhibitor of p38 MAP kinase, suppressed the bFGF-induced IL-6 synthesis dose-dependently. The phosphorylation of p38 MAP kinase by bFGF was suppressed by TMB-8, an inhibitor of intracellular Ca^{2+} mobilization, or the depletion of extracellular Ca^{2+} with EGTA. A23187, a Ca-ionophore, stimulated the phosphorylation of p38 MAP kinase. SB203580 inhibited the A23187-induced synthesis of IL-6. 1-Oleoyl-2-acetyl-sn-glycerol, a synthetic diacylglycerol activating PKC, reduced the bFGF-induced IL-6 synthesis. 12-*O*-Tetradecanoylphorbol-13-acetate, an activator of PKC, attenuated the phosphorylation of p38 MAP kinase by bFGF, but did not affect the A23187-induced phosphorylation. These results strongly suggest that bFGF-induced IL-6 synthesis is mediated via p38 MAP kinase activation in osteoblasts, and that PKC acts at a point upstream from p38 MAP kinase. J. Cell. Biochem. 74:479–485, 1999.

Key words: bFGF; MAP kinase; interleukin-6; osteoblasts

Fibroblast growth factors (FGFs) comprise a family of heparin-binding growth factors [Basilico and Mascatelli, 1992]. Among them, basic FGF (bFGF or FGF-2) is well recognized to play key roles in cell functions of many types of cells [Gospodarowicz et al., 1987; Bikfalvi et al., 1997]. In bone tissues, it has been reported that bFGF is found in bone matrix and that osteoblasts produce bFGF [Globus et al., 1989; Baylink et al., 1993; Hurley et al., 1994]. It is known that bFGF expression is detected during all stages of fracture repair: stage 1, immediate injury response; stage 2, intramembranous ossification; stage 3, chondrogenesis; stage 4, endochondral ossification [Bolander, 1992]. Thus, it is nowadays recognized that bFGF plays an crucial role in bone metabolism and fracture healing. In osteoblasts, it has been reported that bFGF stimulates their proliferation, and reduce alkaline phosphatase activity, osteocalcin mRNA level and type I collagen mRNA level, which are markers of differentiation of osteoblasts [Rodan et al., 1989; Hurley et al., 1993 and 1994].

As for intracellular signaling system of bFGF, it is recognized that there are four structurally related high affinity receptors (FGF receptor 1 to 4) that have an intrinsic protein tyrosine kinase activity, and induce tyrosine autophosphorylation of the receptor [Coughlin et al., 1988; Johnson and Williams, 1993]. We have reported that FGF receptor 1 and 2 are autophosphorylated by bFGF in osteoblast-like MC3T3-E1 cells [Suzuki et al., 1996]. Tyrosine phosphorylation creates high affinity sites for binding of proteins that contain *src* homology 2-domain, resulting in the mediation of its sig-

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naling. We previously showed that bFGF induces both phosphatidylinositol hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D in MC3T3-E1 cells, resulting in the formation of diacylglycerol [Suzuki et al., 1996]. Diacylglycerol is well known to be a physiological activator of protein kinase C (PKC) [Nishizuka, 1986]. In addition, we have recently reported that bFGF stimulates interleukin-6 (IL-6) synthesis and the bFGF-activated PKC limits the synthesis of IL-6 by bFGF itself in these cells [Kozawa et al., 1997]. However, the exact signaling system of bFGF in osteoblasts has not yet been fully clarified.

Mitogen-activated protein (MAP) kinase family plays important roles in intracellular signaling of a variety of agonists [Nishida and Gotoh, 1993]. p38 MAP kinase is a member of MAP kinase superfamily. It is recognized that p38 MAP kinase is activated by cellular stresses such as heat, osmotic stress and UV irradiation [Raingeaud et al., 1995; Jiang et al., 1997]. In addition, recent studies showed that some growth factors such as interleukin-3 and erythropoietin activate p38 MAP kinase in certain cell types [Foltz et al., 1997; Nagata et al., 1997]. However, the exact role of p38 MAP kinase in osteoblasts remains to be clarified. In the present study, we investigated whether bFGF activates p38 MAP kinase in osteoblast-like MC3T3-E1 cells, and the potential involvement of p38 MAP kinase in the signaling for IL-6 synthesis induced by bFGF. We herein show that p38 MAP kinase is involved in the bFGF-induced IL-6 synthesis in MC3T3-E1 cells.

MATERIALS AND METHODS

Mouse IL-6 enzyme immunoassay kit and ECL Western blotting detection system were purchased from Amersham Japan (Tokyo, Japan). bFGF was obtained from Boehringer Mannheim (Tokyo, Japan). SB203580 was obtained from Calbiochem (La Jolla, CA). A23187 and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were obtained from Sigma Chemical Co. (St. Louis, MO). 1-Oleoyl-2-acety-sn-glycerol (OAG) was obtained from Nacalai Tesque Co. (Kyoto, Japan). 8-(N,N-Diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) was purchased from Biomol Research Laboratories Inc. (Plymouth, PA). Phospho-specific p38 MAP kinase antibodies and p38 MAP kinase antibodies were obtained from New England Biolabs, Inc. (Beverly, MA). Other materials and chemicals were obtained from commercial sources. SB203580, A23187, and TPA were dissolved in dimethyl sulfoxide. TMB-8 was dissolved in ethanol. The maximum concentration of dimethyl sulfoxide or ethanol was 0.1%, which did not affect the assay for IL-6 nor the analysis of p38 MAP kinase.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983] were maintained as previously described [Kozawa et al., 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 (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.

Assay for IL-6

The cultured cells were stimulated by various doses of bFGF 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 enzyme immunoassay kit. When indicated, the cells were pretreated with SB203580 or OAG for 20 min.

Analysis of p38 MAP Kinase

The cultured cells were stimulated by bFGF in 1 ml of α-MEM for the indicated periods. The cells were washed twice with 1 ml of 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, 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. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli [Laemmli, 1970] in 10% polyacrylamide gels. Western blotting analysis was performed as described previously [Kato et al., 1996] by using phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies, and peroxidase-labeled antibodies raised in goat against rabbit IgG as second antibodies. Peroxidase activity on the nitrocellulose sheet

was visualized on x-ray film by ECL Western blotting detection system. When indicated, the cells were pretreated with TPA or TMB-8 for 20 min.

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 Student's *t*-test 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 bFGF on p38 MAP Kinase Activation in MC3T3-E1 Cells

We examined the effect of bFGF on the phosphorylation of p38 MAP kinase, in order to investigate whether bFGF activates p38 MAP kinase in MC3T3-E1 cells. bFGF significantly stimulated the phosphorylation of p38 MAP kinase time-dependently for up to 30 min (Fig. 1). The maximum effect of bFGF on the phosphorylation of p38 MAP kinase was observed at 10 min after the stimulation.

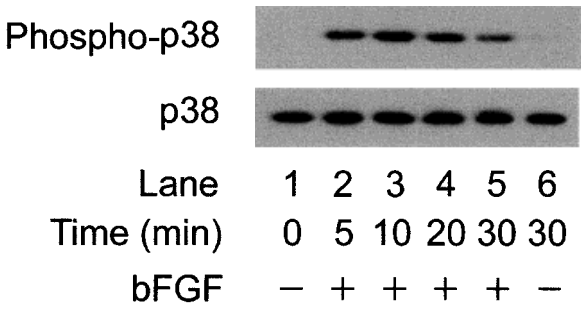

Fig. 1. Effect of bFGF on the phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were stimulated by 30 μM bFGF for 5 min **(lane 2)**, 10 min **(lane 3)**, 20 min **(lane 4)**, 30 min **(lane 5)**, or vehicle for 30 min **(lane 6)**. The extracts of cells were subjected to SDS-polyacrylamide gel electrophoresis with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. **Lane 1:** control cells.

Effect of SB203580 on bFGF-Induced IL-6 Synthesis in MC3T3-E1 Cells

We previously reported that bFGF stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1997]. To investigate whether p38 MAP kinase is involved in the bFGF-stimulated IL-6 synthesis, we examined the effect of SB203580, which is known to be an specific inhibitor of p38 MAP kinase [Lee et al., 1994; Cuenda et al., 1995], on the IL-6 synthesis induced by bFGF. SB203580, which by itself did not affect IL-6 synthesis, significantly suppressed the bFGF-induced IL-6 synthesis (Fig. 2A). The inhibitory effect of SB203580 on the bFGF-induced IL-6 synthesis was dose-dependent in the range between 1 and 30 μM (Fig. 2B). The maximum effect of SB203580 was observed at 30 μM, a dose that caused about 85% reduction in the bFGF-effect.

Effect of Ca^{2+} on the bFGF-Stimulated Phosphorylation of p38 MAP Kinase in MC3T3-E1 Cells

In a previous study [Kozawa et al., 1997], we demonstrated that bFGF-induced IL-6 synthesis is dependent on intracellular Ca^{2+} mobilization in MC3T3-E1 cells. So, to clarify the role of intracellular Ca^{2+} mobilization on the bFGF-induced activation of p38 MAP kinase, the effect of TMB-8, known to inhibit intracellular

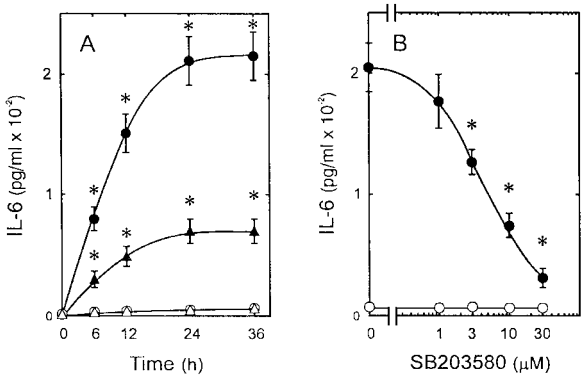

Fig. 2. Effect of SB203580 on the bFGF-induced synthesis of IL-6 in MC3T3-E1 cells. **A:** The cultured cells were pretreated with 10 μM SB203580 (triangle) or vehicle (circle) for 20 min, and then stimulated by 30 μM bFGF (solid circle and triangle) or vehicle (open circle and triangle) for the indicated periods. **B:** The cultured cells were pretreated with various doses of SB203580 for 20 min, and then stimulated by 30 μM bFGF (solid circle) or vehicle (open circle) 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 bFGF alone.

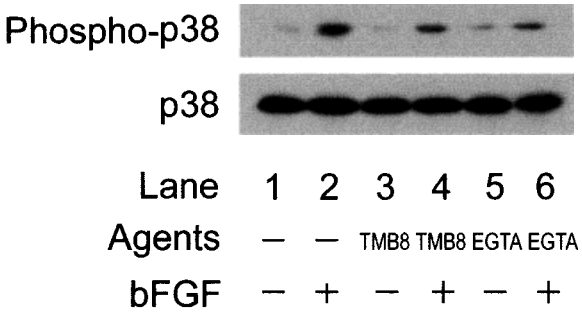

Fig. 3. Effects of TMB-8 and depletion of extracellular Ca^{2+} by EGTA on the bFGF-stimulated phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM TMB-8 or 2.5 mM EGTA for 20 min, and then stimulated by 30 μM bFGF for 10 min. The extracts of cells were subjected to SDS-polyacrylamide gel electrophoresis with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase.

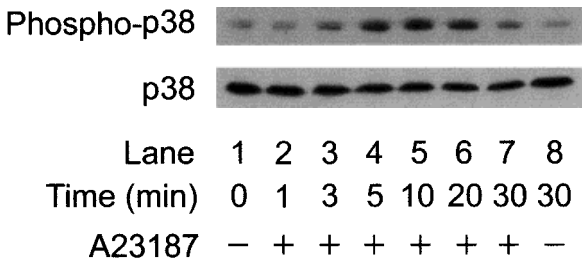

Fig. 4. Effect of A23187 on the phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were stimulated by 1 μM A23187 for 1 min **(lane 2)**, 3 min **(lane 3)**, 5 min **(lane 4)**, 10 min **(lane 5)**, 20 min **(lane 6)**, 30 min **(lane 7)**, or vehicle for 30 min **(lane 8)**. The extracts of cells were subjected to SDS-polyacrylamide gel electrophoresis with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. **Lane 1:** control cells.

Ca^{2+} mobilization [Chiou and Malagodi, 1975; Charo et al., 1976], on the phosphorylation of p38 MAP kinase induced by bFGF was examined. TMB-8 significantly suppressed the phosphorylation of p38 MAP kinase by bFGF (Fig. 3). In addition, the depletion of extracellular Ca^{2+} by EGTA suppressed the phosphorylation of p38 MAP kinase (Fig. 3). A23187, a Ca ionophore, by itself stimulated the phosphorylation of p38 MAP kinase time-dependently (Fig. 4). The maximum effect of A23187 on the p38 MAP kinase phosphorylation was observed at 10 min after the stimulation.

We previously showed that A23187 by itself induces the IL-6 synthesis in MC3T3-E1 cells [Kozawa et al., 1997]. Thus, we next examined the effect of SB203580 on the A23187-induced IL-6 synthesis. The synthesis of IL-6 by A23187 was significantly reduced by SB203580 in MC3T3-E1 cells (Table I). SB203580 (30 μM) caused about 55% reduction in the A23187-effect.

TABLE I. Effect of SB203580 on the A23187-Induced Synthesis of IL-6 in MC3T3-E1 Cells[a]

SB203580	A23187	IL-6 (pg/ml)
–	–	<10
–	+	817 ± 68
+	–	<10
+	+	362 ± 31*

[a]The cultured cells were pretreated with 30 μM SB203580 for 20 min. The cells were then stimulated by 1 μM A23187 or vehicle for 1 h, and subsequently incubated 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 A23187 alone.

Effect of PKC on the bFGF-Stimulated Phosphorylation of p38 MAP Kinase in MC3T3-E1 Cells

We have shown that the PKC activated by bFGF inhibits the IL-6 synthesis by bFGF itself in MC3T3-E1 cells [Kozawa et al., 1997]. Thus, to investigate whether the inhibition of bFGF-induced IL-6 synthesis by PKC is exerted at a point upstream from p38 MAP kinase or not, we examined the effects of TPA, a PKC-activating phorbol ester [Nishizuka, 1986], on the bFGF-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. TPA significantly attenuated the phosphorylation of p38 MAP kinase by bFGF (Fig. 5). In addition, OAG, a synthetic diacylglycerol activating PKC [Nishizuka, 1986], markedly attenuated the bFGF-induced IL-6 synthesis (Table II).

Furthermore, we examined the effect of PKC activation on the A23187-induced phosphorylation of p38 MAP kinase. TPA did not reduce the phosphorylation of p38 MAP kinase by A23187 (Fig. 6). According to the densitometric analysis, the levels of p38 MAP kinase phosphorylation by A23187 with TPA pretreatment was 118% of the levels of A23187 alone.

DISCUSSION

In our previous report [Kozawa et al., 1997], we have demonstrated that bFGF stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells. In the present study, we further investi-

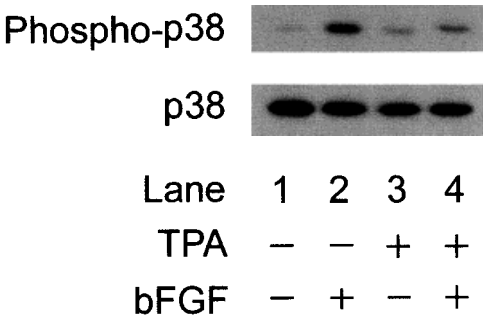

Fig. 5. Effect of TPA on the bFGF-stimulated phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM TPA for 20 min, and then stimulated by 30 μM bFGF for 10 min. The extracts of cells were subjected to SDS-polyacrylamide gel electrophoresis with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase.

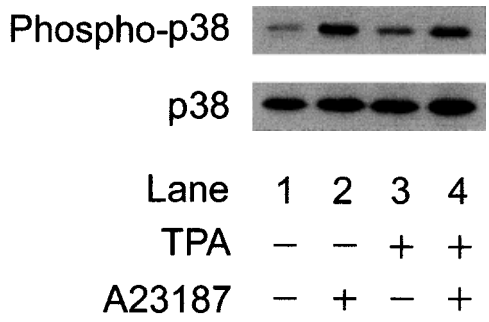

Fig. 6. Effect of TPA on the A23187-stimulated phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM TPA for 20 min, and then stimulated by 1 μM A23187 for 10 min. The extracts of cells were subjected to SDS-polyacrylamide gel electrophoresis with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase.

TABLE II. Effect of OAG on the bFGF-Induced Synthesis of IL-6 in MC3T3-E1 Cells[a]

OAG	bFGF	IL-6 (pg/ml)
−	−	<10
−	+	205 ± 18
+	−	<10
+	+	136 ± 12*

[a]The cultured cells were pretreated with 0.1 mM OAG for 20 min. The cells were then stimulated by 30 μM bFGF 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 bFGF alone.

gated the exact mechanism underlying bFGF-stimulated IL-6 synthesis in these cells. We showed that SB203580 significantly inhibited the bFGF-induced IL-6 synthesis. Thus, it seems that p38 MAP kinase is involved in the IL-6 synthesis by bFGF. In addition, we demonstrated that bFGF stimulated the phosphorylation of p38 MAP kinase in MC3T3-E1 cells. It is well recognized that p38 MAP kinase is activated by phosphorylation on threonine and tyrosine residues by dual-specificity MAP kinase kinase [Raingeaud et al., 1995]. So, it is most likely that bFGF actually activates p38 MAP kinase in MC3T3-E1 cells. Therefore, these findings suggest that p38 MAP kinase activation is required for the bFGF-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

Herein, we showed that TMB-8 and extracellular Ca^{2+} depletion by EGTA inhibited the bFGF-induced phosphorylation of p38 MAP kinase in osteoblast-like MC3TC-E1 cells. Thus, based on our findings, it is probable that intracellular Ca^{2+} mobilization is required for the activation of p38 MAP kinase by bFGF in these cells. We have previously reported that the IL-6 synthesis induced by bFGF is dependent on intracellular Ca^{2+} mobilization in MC3T3-E1 cells [Kozawa et al., 1997]. In the present study, we showed that A23187 alone induced the phosphorylation of p38 MAP kinase. Furthermore, SB203580 significantly reduced the IL-6 synthesis induced by A23187. Therefore, it is most likely that bFGF activates p38 MAP kinase through intracellular Ca^{2+} mobilization, resulting in the IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

We have reported that bFGF-activated PKC through phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D negatively regulates the IL-6 synthesis by bFGF itself in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1997]. In the present study, exogenously activated PKC inhibited both the phosphorylation of p38 MAP kinase and the IL-6 synthesis induced by bFGF. Thus, our findings suggest that the effect of PKC is exerted at a point upstream from p38 MAP kinase in MC3T3-E1 cells. On the contrary, we showed that TPA did not affect the A23187-induced phosphorylation of p38 MAP kinase. Taking our results into account, it is most likely that PKC activation by bFGF has a role in the automodulation of bFGF itself-induced IL-6 synthesis through p38 MAP kinase activation in osteoblast-like MC3T3-E1 cells, and that the effect of PKC is exerted at the point between the receptor and intracellular Ca^{2+} mobilization.

IL-6 is currently recognized to be secreted from osteoblasts and act as an important downstream effector of bone resorbing agents [Helle et al., 1988; Feyen et al., 1989; Ishimi et al., 1990; Littlewood et al., 1991; Franchimont and Canalis, 1995]. The IL-6 synthesis has been shown to be regulated by several transcription factors such as nuclear factor for IL-6 and nuclear factor κB [Bankers-Fulbright et al., 1996]. In addition, it is known that phosphorylated MAP kinase is able to translocate into nucleus and regulate transcription factors, which mediate changes in gene expression [Minden and Karin, 1997]. Thus, these findings make us speculate that pathological bone resorption might result from failure in the regulatory system of p38 MAP kinase in osteoblasts. Further investigation would be required to clarify the exact role of p38 MAP kinase in bone metabolism.

In conclusion, these results strongly suggest that p38 MAP kinase activation is required for bFGF-induced IL-6 synthesis in osteoblasts and that PKC has a role in automodulating the IL-6 synthesis at a point upstream from p38 MAP kinase.

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