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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.
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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 000g 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.

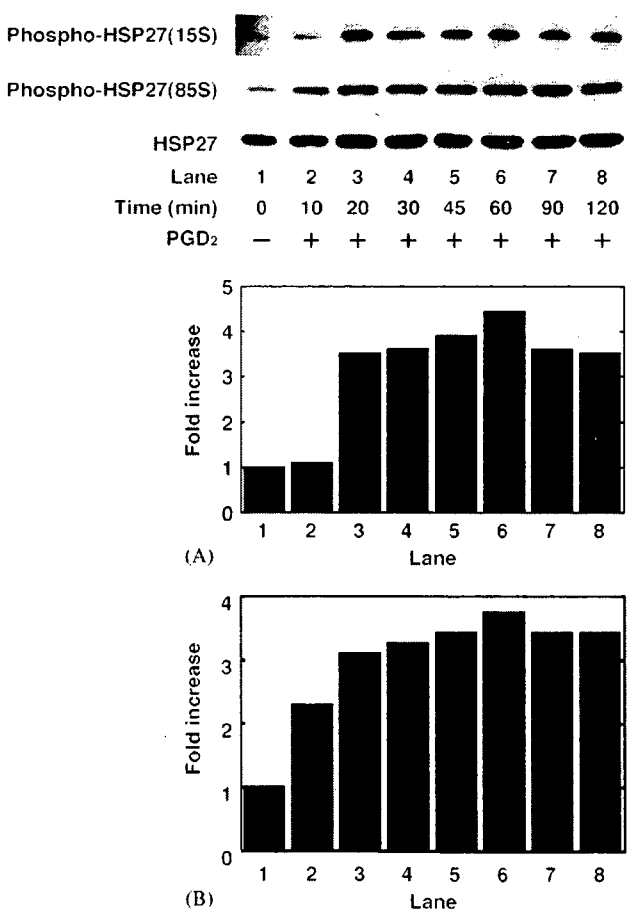

Fig. 1. Effect of PGD_2 on the phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. The cultured cells were stimulated by 10 μM PGD_2 for the indicated periods. The extracts of cells were subjected to SDS-PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and 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

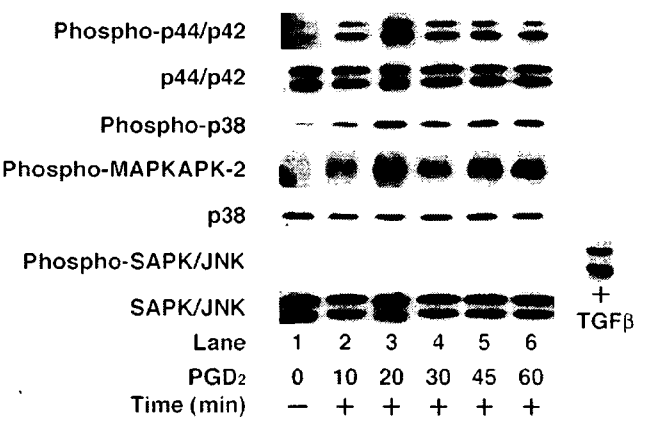

Fig. 2. Effects of PGD_2 on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, MAPKAP kinase 2, SAPK/JNK in MC3T3-E1 cells exposed to heat shock. The cultured cells were stimulated by 10 μM PGD_2 for the indicated periods. The extracts of cells were subjected to SDS-PAGE using antibodies against 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).

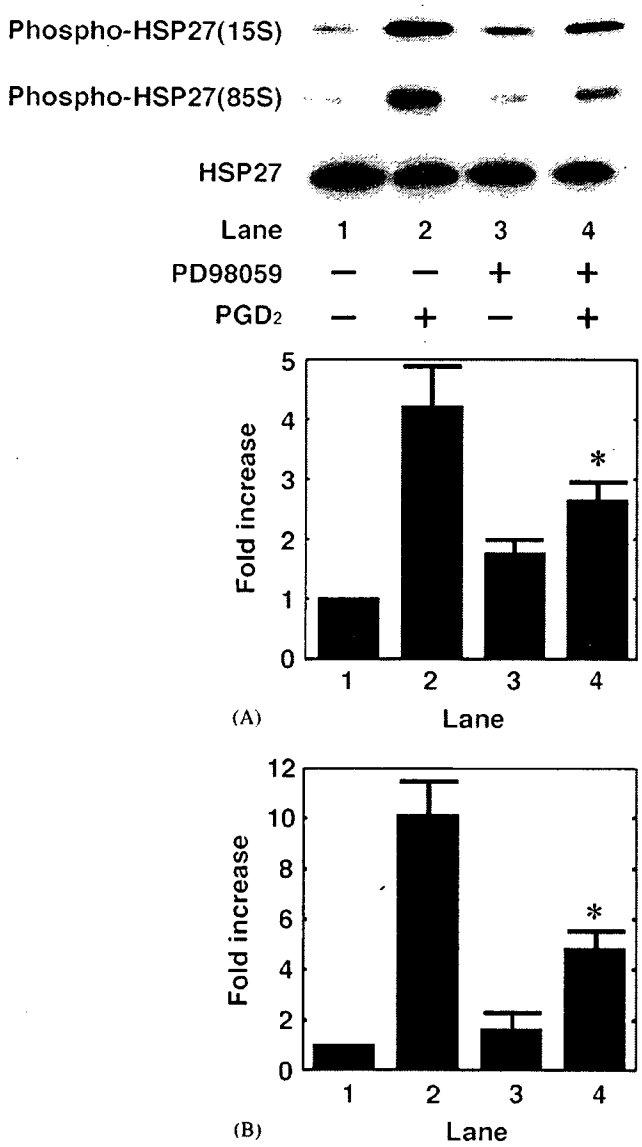

Fig. 3. Effect of PD98059 on the PGD_2-induced phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. The cultured cells were pretreated with 50 μM PD98059 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE using 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.

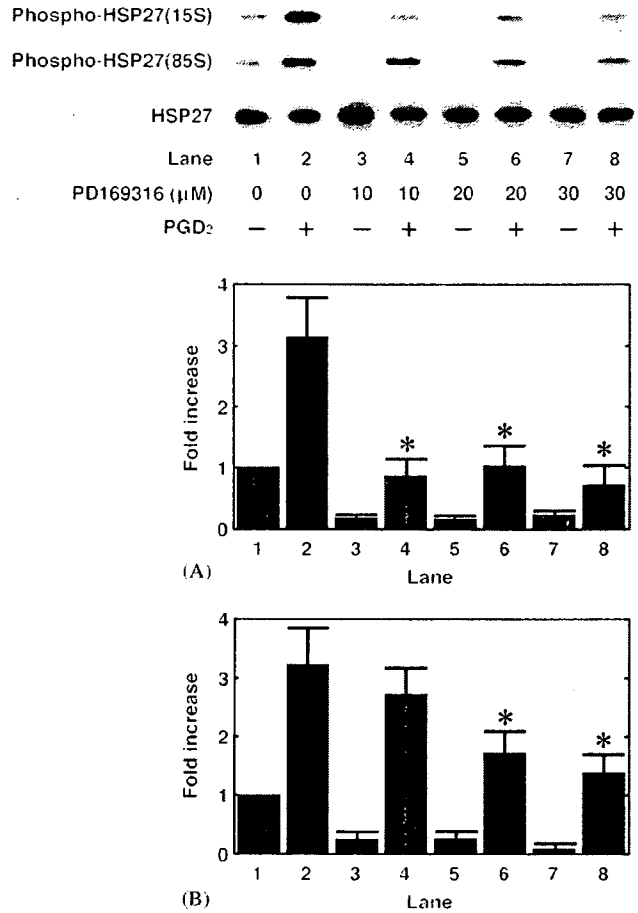

Fig. 4. Effect of PD169316 on the PGD_2-induced phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. The cultured cells were pretreated with various doses of PD169316 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The extracts of cells were subjected to 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

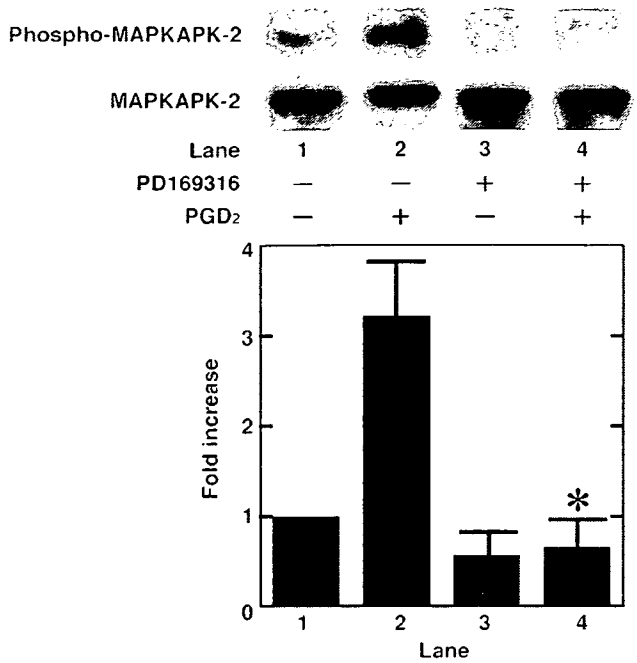

Fig. 5. Effect of PD169316 on the PGD_2-induced phosphorylation of MAPKAP kinase 2 in MC3T3-E1 cells exposed to heat shock. The cultured cells were pretreated with 10 μM PD169316 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE 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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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, platelet-derived growth factor and cytokines [12]. Akt regulates biological functions such as gene expression, survival and oncogenesis [12]. Akt is a downstream target of phosphatidylinositol 3-kinase (PI3-kinase) [13,14]. Akt containing a pleckstrin homology domain is recruited to the plasma membrane by the lipid product of phosphatidylinositol 3-kinase and activated. As for osteoblasts, insulin-like growth factor-I and platelet-derived growth factor induce translocation of Akt to the nucleus [15]. Recently, Akt is reportedly activated by cyclic stretch or androgen [16,17]. We have recently shown that Akt plays an important role in insulin-like growth factor-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells [18]. However, the correlation between TNF-α and PI3-kinase/Akt in osteoblasts has not yet been clarified.

In the present study, we have investigated whether PI3-kinase/Akt signaling pathway plays a role in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We show here that TNF-α-stimulated IL-6 synthesis through the PI3-kinase/Akt pathway in addition to p44/p42 MAP kinase in these cells.

Materials and Methods

Materials

TNF-α was obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). IL-6 ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Akt inhibitor [1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate], wortmannin, LY294002, PD98059 and rapamycin were obtained from Calbiochem. Co. (La Jolla, CA). Phospho-specific Akt antibodies, Akt antibodies, phosphor-specific p44/p42 MAP kinase and p44/p42 MAP kinase were purchased from New England BioLabs, 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, LY294002, PD98059 and rapamycin were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect assay for IL-6 activity or Western blotting analysis.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [19] were maintained as previously described [20]. 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 diameter dishes 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 hours.

Freshly isolated osteoblasts were obtained from the calvaria of new-born (1 or 2-day-old) balb/c mice as previously described [21]. They were seeded into 90 mm diameter dishes (25×104 cells) in α-MEM containing 10% FCS. The medium was changed every 3 days until the cells had reached confluence at about the 5the day. Then, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 hours.

Assay for IL-6

The cultured cells were stimulated by TNF-α in 1 ml of α-MEM containing 0.3%. FCS, and then incubated for the indicated periods. The conditioned medium was collected, and IL-6 in the medium was then measured by an IL-6 ELISA kit. Unless otherwise indicated, the cells were pretreated with Akt inhibitor, wortmannin, LY294002, PD98059 or rapamycin, for 60 minutes.

Analysis of Western blotting

The cultured cells were stimulated by TNF-α 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 according to Laemmli [22] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [23] by using phospho-specific Akt antibodies, Akt antibodies, phosphor-specific p44/p42 MAP kinase, or p44/p42 MAP kinase with peroxidase-labeled antibodies raised in goat against rabbit IgG as second antibodies. Peroxidase activity on PVDG membrane was visualized on X-ray film by means of the ECL Western blotting detection system. Unless otherwise indicated, the cells were pretreated with Akt inhibitor, wortmannin, LY294002 or PD98059 for 60 minutes.

Determinations

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).

cDNA Microarray

The cultured cells were washed twice with PBS, and total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Using the obtained total RNAs, cDNA microarray analysis (GeneChip® Expression Analysis; AFFYMETRIX) was performed once by KURABO Industries (Osaka, Japan) with the authorization of AFFYMETRIX Japan K.K. (Tokyo, Japan). The arrays were scanned and the array image was analyzed with GeneChip® operating software (AFFYMETRIX).

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 statistically significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effects of TNF-α on the phosphorylation of Akt in MC3T3-E1 cells

To investigate whether TNF-α activates Akt in osteoblast-like MC3T3-E1 cells, we examined the effects of TNF-α on the phosphorylation of Akt. TNF-α significantly induced the phosphorylation of Akt in a time dependent manner (Fig. **1**). The phosphorylation of Akt reached its peak at 15 min, and decreased thereafter.

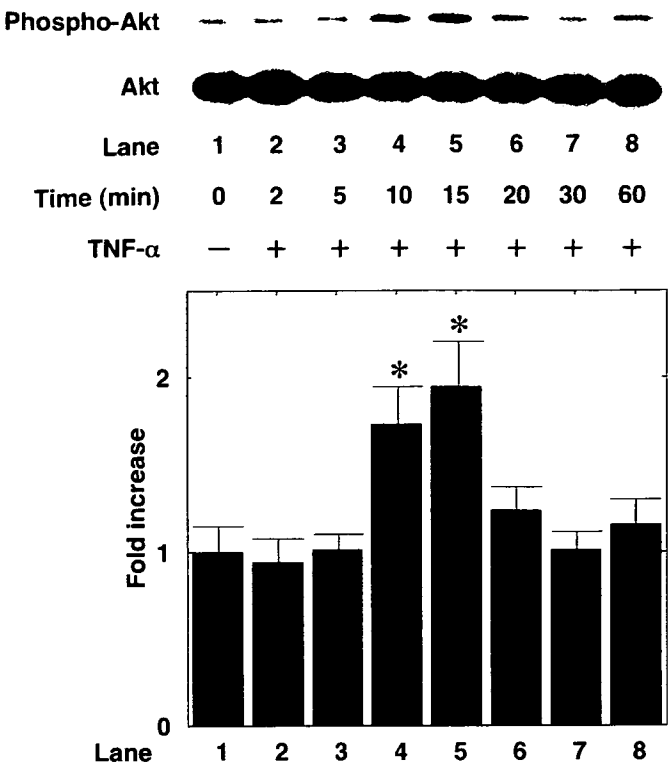

Fig. 1 Effect of TNF-α on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated with 30 ng/ml TNF-α for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of TNF-α-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of control.

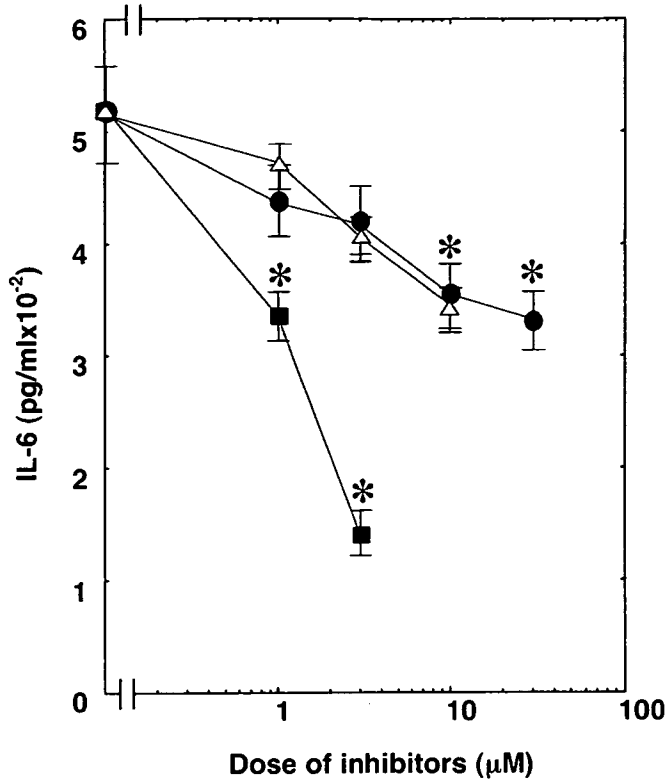

Fig. 2 Effect of LY294002, wortmannin, or Akt inhibitor on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Akt inhibitor (●), LY294002 (■), or wortmannin (△) for 60 min, and then stimulated by 30 ng/ml TNF-α for 48 h. In the absence of TNF-α all IL-6 levels were approximately 25 pg/ml and did not vary with any inhibitor. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of TNF-α alone.

Effect of Akt inhibitor, LY294002, or wortmannin on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells

We next examined the effect of Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate [24], on the TNF-α-stimulated IL-6 synthesis in order to investigate whether Akt is involved in the IL-6 synthesis in MC3T3-E1 cells. Akt inhibitor, which alone did not affect the basal levels of IL-6, significantly suppressed the TNF-α-induced IL-6 synthesis. The inhibitory effect of Akt inhibitor on the IL-6 synthesis was partial and dose dependent in the range between 1 and 30 μM (Fig. **2**). The maximum inhibitory effect of Akt inhibitor at 30 μM caused about 40% reduction in the TNF-α-effect. It is generally known that Akt acts as a downstream effector of PI3-kinase [13,14]. We next examined the effect of LY294002, a specific inhibitor of PI3-kinase [25], on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. LY294002 significantly reduced the synthesis of IL-6 stimulated by TNF-α in a dose-dependent manner between 1 and 3 μM (Fig. **2**). Furthermore, the effect of wortmannin, another inhibitor of PI3-kinase [26], on the IL-6 synthesis was examined. The TNF-α-stimulated synthesis of IL-6 was markedly reduced by wortmannin in a dose-dependent manner between 1 and 10 μM (Fig. **2**).

Effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary culture of osteoblasts

We investigated the effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary culture of osteoblasts. Akt-inhibitor also significantly suppressed the TNF-α-induced IL-6 synthesis (Fig. **3**). The inhibitory effect of Akt inhibitor on the IL-6 synthesis was partial and dose dependent in the range between 1 and 30 μM (Fig. **3**). The maximum inhibitory effect of Akt inhibitor at 30 μM caused about 25% reduction in the TNF-α-effect.

Effects of LY294002 or wortmannin on the phosphorylation of Akt induced by TNF-α in MC3T3-E1 cells

We found that the phosphorylation of Akt induced by TNF-α was markedly attenuated by LY294002 (Fig. **4A**). According to the densitometric analysis, LY294002 (10 μM) caused almost complete reduction of the TNF-α-effect on the Akt phosphorylation. In addition, wortmannin significantly reduced the Akt phosphorylation induced by TNF-α (Fig. **4B**).

Effects of Akt inhibitor, wortmannin or LY294002 on the phosphorylation of p44/p42 MAP kinase induced by TNF-α, and effect of PD98059 on the TNF-α-induced Akt phosphorylation in MC3T3-E1 cells

We have previously shown that p44/p42 MAP kinase plays a part in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-

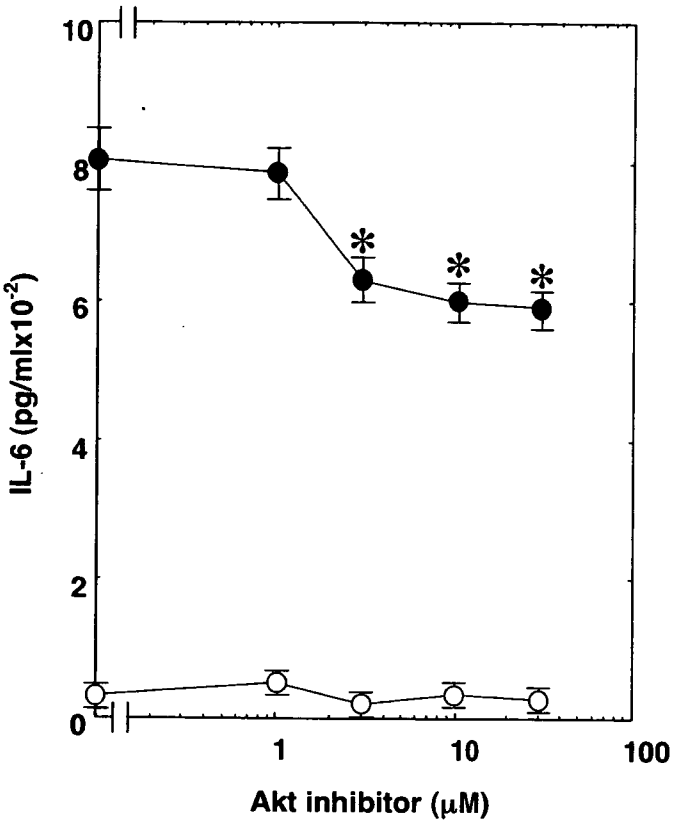

Fig. 3 Effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary osteoblasts. The cultured cells were pretreated with various doses Akt inhibitor for 60 min, and then stimulated by 30 ng/ml TNF-α (●) or vehicle (○) for 48 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of TNF-α alone.

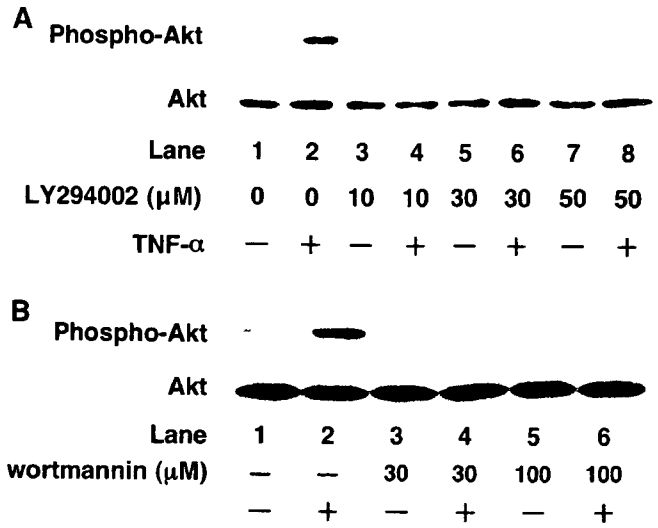

Fig. 4 Effect of LY294002 or wortmannin on the TNF-α-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 (**A**) or wortmannin (**B**) for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. Similar results were obtained with two additional and different cell preparations.

E1 cells [10,11], which led us to investigate the relationship between p44/p42 MAP kinase and Akt in these cells. Akt inhibitor had little effect on the TNF-α-induced phosphorylation of p44/p42 MAP kinase (Fig. **5A**). In addition, the TNF-α-induced phosphorylation of p44/p42 MAP kinase was slightly suppressed by wortmannin (Fig. **5B**) or LY294002 (data not shown). These suppressive effects were not statistically significant. Furthermore, PD98059, a specific inhibitor of MEK, upstream kinase that activates p44/p42 MAP kinase [27] failed to affect the TNF-α-induced phosphorylation of Akt (Fig. **5C**).

Effects of rapamycin on TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells

It is well known that mammalian target of rapamycin (mTOR) is a downstream target of Akt. We examined the effect of rapamycin on TNF-α-stimulated IL-6 synthesis in these cells. We found that rapamycin markedly increased the TNF-α-induced IL-6 release (25 ± 4 pg/ml for control; 45 ± 9 pg/ml for 10 μM rapamycin; 584 ± 63 pg/ml for 30 ng/ml TNF-α alone; and 1.062 ± 96 pg/ml for 30 ng/ml TNF-α with 10 μM rapamycin, as measured during the stimulation for 48 h).

Effect of TNF-α on mRNA expression of IL-6 receptor and gp130

We next investigated the effect of TNF-α on mRNA expression of IL-6 receptor and gp130 by cDNA microarray. Approximately 45,000 genes were screened. As a result, 1,372 genes showed a greater than 2-fold change in expression level after TNF-α stimulation. The genes of neither IL-6 receptor nor gp130 were included in them. On the other hand, the gene of IL-6 was truly included. It is unlikely that TNF-α affects the IL-6 effect via up-regulation of the receptor or the signal transducer in osteoblasts.

Combined effect of Akt inhibitor and PD98059 on TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells

We have shown that the suppressive effect of PD98059 (50 μM) on TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells was partial [10]. We further examined the effect of a combination of Akt inhibitor and PD98059 on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. Combining Akt inhibitor and PD98059 significantly suppressed the TNF-α-stimulated IL-6 synthesis in an additive manner (Table **1**). We have previously confirmed that PD98059 at the concentration used in this experiment is not toxic but specific to MEK1/2 [28].

Discussion

In the present study, we have demonstrated that TNF-α induces the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells depending upon time. Akt mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions such as proliferation and cell survival in a variety of cells [12]. Akt is also activated by phosphorylation of threonine and serine residues [13,14]. Taking these results into account, it is most likely that TNF-α activates Akt in osteoblast-like MC3T3-E1 cells.

We investigated whether Akt is involved in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. Herein, we

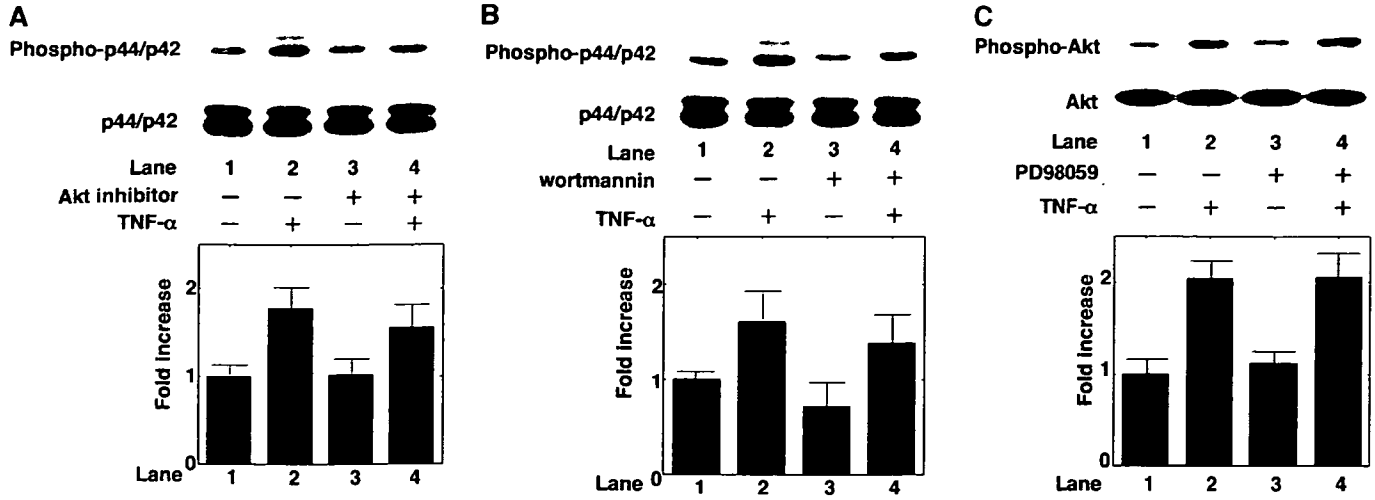

Fig. 5 Effect of Akt inhibitor or wortmannin on the TNF-α-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. Effect of PD98059 on the TNF-α-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 50 nM Akt inhibitor (**A**) or wortmannin (**B**) for 60 min, and then stimulated by 30 ng/ml TNF-α 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. (**C**) The cultured cells were pretreated with 50 μM of PD98059 for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 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 TNF-α-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean-SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

Table 1 Effect of a combination of Akt inhibitor and PD98059 on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells

Akt inhibitor	*PD98059*	*TNF-α*	*IL-6 (pg/ml)*
–	–	–	23 ± 4
–	–	+	506 ± 30*
–	+	–	25 ± 3
–	+	+	388 ± 18**
+	–	–	23 ± 5
+	–	+	359 ± 12**
+	+	–	25 ± 4
+	+	+	257 ± 20***

The cultured cells were pretreated with 30 μM Akt inhibitor, 50 μM PD98059 or vehicle for 60 min, and then stimulated by 30 ng/ml TNF-α 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 to the control. **$p < 0.05$, compared to the value of TNF-α alone. ***$p < 0.05$, compared to the value of TNF-α with Akt inhibitor or PD98059.

show that a specific inhibitor of Akt [24], significantly suppressed the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. We also confirmed that Akt-inhibitor significantly suppressed the TNF-α-induced IL-6 synthesis also in primary culture of osteoblasts. Thus, it is probable that the activation of Akt is involved in TNF-α-stimulated IL-6 synthesis in osteoblasts. While Akt is a downstream target of PI3-kinase [12–14], we next examined the effects of PI3-kinase inhibitors on the TNF-α-stimulated synthesis of IL-6 in MC3T3-E1 cells. We found that wortmannin [26], significantly reduced the IL-6 synthesis stimulated by TNF-α and markedly attenuated the TNF-α-induced Akt phosphorylation. These findings suggest that PI3-kinase is implicated in TNF-α-stimulated IL-6 synthesis through Akt in MC3T3-E1 cells. We have shown earlier that the IL-6 synthesis induced by TNF-α was significantly suppressed by LY294002 [25]. LY294002 dose dependently reduced the TNF-α-stimulated phosphorylation of Akt. Our results suggest that Akt plays a crucial role as a positive regulator in TNF-α-stimulated IL-6 synthesis at a point downstream from PI3-kinase in osteoblast-like MC3T3-E1 cells.

We have previously reported that the activation of p44/p42 MAP kinase is involved in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells [10,11]. Hence, we investigated to clarify the relationship between p44/p42 MAP kinase and Akt in these cells. We found that Akt inhibitor- and wortmannin-caused minor reductions in TNF-α-induced phosphorylation of p44/p42 MAP kinase did occur in MC3T3-E1 cells, and a specific MEK inhibitor, PD98059 [27] failed to affect the TNF-α-induced phosphorylation of Akt. These results indicate that some cross talk may exist between the PI3-kinase/Akt and the p44/p42 MAP kinase systems, although mostly these are independent. It is not at all unexpected in that Akt will affect many things downstream which may indirectly affect the p44/p42 MAP kinase system. As for the involvement of mTOR, we found that rapamycin markedly increased the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. It is unlikely that TNF-α induces IL-6 synthesis through the PI3-kinase/Akt-dependent activation of mTOR. TNF-α is known to increase the expression of IL-6 genes through activation of nuclear factor-κB (NFκB) in osteoblast-like cells [29]. TNFR2 reportedly facilitates PI3-kinase-dependent NFκB activation [30]. It is possible that the inhibition of PI3-kinase/Akt pathway suppress TNF-α-induced IL-6 synthesis through NFκB inhibition. In addition, from cDNA microarray results, TNF-α truly induced the up-regulation of IL-6 mRNA expression, but influenced the mRNA levels of neither IL-6 receptor nor gp130 in MC3T3-E1 cells. It is unlikely that TNF-α affects the IL-6 effect via up-regulation of the receptor or the signal transducer in osteoblasts.

Additionally, we have shown that the inhibitory effect of PD98059 on the TNF-α-stimulated IL-6 synthesis was partial [10]. In this study, the TNF-α-stimulated IL-6 synthesis was partially reduced by Akt inhibitor (30 μM). We found that a combination of Akt inhibitor and PD98059 additively suppressed TNF-α-stimulated IL-6 synthesis. Based on our findings as a whole, it is most likely that TNF-α stimulates the synthesis of IL-6 via Akt and p44/p42 MAP kinase, and maybe mainly independent of each other in osteoblast-like MC3T3-E1 cells.

The PI3-kinase/Akt pathway plays a pivotal role in several cellular functions, such as proliferation and cell survival in a variety of cells [12]. Our present findings suggest that the PI3-kinase/Akt pathway in osteoblasts has an important role in the regulatory mechanism of the TNF-α-induced production of IL-6 in bone metabolism. IL-6 are potent bone resorptive agent and induces osteoclast formation [1, 4]. Therefore, our present results lead us to speculate that TNF-α-activated PI3-kinase/Akt signaling acts as a positive regulator directing toward bone resorption. It is possible that the PI3-kinase/Akt pathway in osteoblasts might be considered as a molecular target of bone resorption concurrent with various bone diseases. Both TNF-α and IL-6 are well recognized as inflammatory cytokines which play crucial roles in the process of acute and chronic inflammatory diseases. Our present findings might suggest that PI3-kinase/Akt pathway is involved in the process of pathological bone resorption especially in inflammatory bone diseases. In addition, *in vivo* and *in vitro* models of postmenopausal osteoporosis reportedly demonstrate that estrogen deficiency leads to an increase in the adaptive immune function that culminates in an increased production of TNF-α by activated T cells [31]. The signaling molecules contributed to the TNF-α-induced IL-6 synthesis in osteoblasts might be considerable as therapeutic targets of postmenopausal osteoporosis. Further investigation is required to clarify the exact role of PI3-kinase/Akt in osteoblast cell function and bone metabolism.

In conclusion, our results strongly suggest that TNF-α stimulated IL-6 synthesis via PI3-kinase/Akt in addition to p44/p42 MAP kinase in osteoblasts.

Acknowledgements

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Modulation by the Steroid/Thyroid Hormone Superfamily of TGF-β-Stimulated VEGF Release From Vascular Smooth Muscle Cells

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Abstract We previously reported that transforming growth factor-β (TGF-β) stimulates the release of vascular endothelial growth factor (VEGF) from aortic smooth muscle A10 cells via activation of p38 mitogen-activated protein (MAP) kinase. In the present study, we investigated whether nuclear hormone receptor superfamily members affect TGF-β-stimulated VEGF release from A10 cells. Retinoic acid or 1,25-dihydroxyvitamin D_3 enhanced TGF-β-induced VEGF release in a concentration-dependent manner, whereas dexamethasone or corticosterone suppressed TGF-β-induced VEGF release. 1,25-Dihydroxyvitamin D_3 and TGF-β stimulated phosphorylation of p38 MAP kinase in an additive manner. SB203580, an inhibitor of p38 MAP kinase, decreased the VEGF release induced by TGF-β or 1,25-dihydroxyvitamin D_3. However, retinoic acid, dexamethasone, or corticosterone did not affect phosphorylation of p38 MAP kinase. These results indicate that retinoic acid, 1,25-dihydroxyvitamin D_3, and glucocorticoids affect TGF-β-stimulated VEGF release from aortic smooth muscle cells. The stimulatory effect of 1,25-dihydroxyvitamin D_3 occurs, in part, via modification of TGF-β-induced activation of p38 MAP kinase. J. Cell. Biochem. 99: 187–195, 2006.
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Key words: steroid/thyroid hormone superfamily; TGF-β; VEGF; p38 MAP kinase; vascular smooth muscle cells

Regulation of vascular smooth muscle cell proliferation and differentiation is critical for vasculogenesis, angiogenesis, and the maintenance of homeostasis in mature vessel walls [Hungerford and Little, 1999; Ross, 1999]. Proliferation and differentiation of these cells are central importance in the pathogenesis of atherosclerosis, hypertension, and restenosis after procedural revascularization [Hungerford and Little, 1999; Ross, 1999]. Vascular smooth muscle cells and vascular endothelial cells interact with each other; for example, vasoactive agents produced by aortic smooth muscle cells, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), and interleukin (IL)-1β, affect endothelial cell function [Hungerford and Little, 1999; Ross, 1999]. VEGF is a heparin-binding angiogenic growth factor that is highly specific for endothelial cells; VEGF binds to tyrosine kinase receptors expressed almost exclusively in endothelial cells and stimulates endothelial cell proliferation, migration, and inhibition of apoptosis [Gospodarowicz et al., 1989; Neufeld et al., 1999]. VEGF also induces angiogenesis, increases blood vessel permeability, and plays a central

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role in the regulation of vasculogenesis [Neufeld et al., 1999]. Deregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis and contributes to the development of diseases characterized by abnormal angiogenesis [Neufeld et al., 1999]. Vascular smooth muscle cells are the predominant source of VEGF [Tischer et al., 1991]. Platelet-derived growth factor (PDGF) BB, TGF-β, hypoxia, endothelin, and bFGF induce VEGF production in these cells [Brogi et al., 1994; Stavri et al., 1995; Pedram et al., 1997]. However, the mechanisms underlying VEGF synthesis in vascular smooth muscle cells have not been fully elucidated.

The TGF-β superfamily comprises a large number of structurally related polypeptide growth factors, each capable of regulating an array of cellular processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and death [Massagué, 1998; Miyazono et al., 2000]. In regard to the vascular system, TGF-β plays a pivotal role in promoting alterations in vessel structure [Massagué, 1998]. Recent evidence suggests that alterations in the local abundance of TGF-β in the arterial wall promote vascular cell transdifferentiation, vascular wall remodeling, arterial lesion growth, and lesion regression associated with apoptosis [Schulick et al., 1998]. In these processes, TGF-β stimulates VEGF synthesis in vascular smooth muscle cells and exerts angiogenic effects [Brogi et al., 1994; Stavri et al., 1995]. We previously reported that TGF-β stimulates the release of VEGF from aortic smooth muscle A10 cells at least in part via p38 mitogen-activated protein (MAP) kinase [Yamamoto et al., 2001].

Recently, the nuclear hormone receptor superfamily has been shown to modulate vascular tone and vascular smooth muscle cell proliferation and differentiation [Kornel, 1993; Miano and Berk, 2000; Mizuma et al., 2001; Dubey et al., 2002; Rebsamen et al., 2002]. This superfamily includes receptors for retinoids, vitamin D, steroid hormones, and thyroid hormone [Evans, 1988; Carlberg, 1995; Miano and Berk, 2000]. These receptors are ligand-activated transcription factors that bind discrete *cis* elements within the regulatory regions of a growing list of target genes [Evans, 1988; Carlberg, 1995; Miano and Berk, 2000]. We previously reported that among nuclear hormone receptor superfamily members, 1,25-dihydroxyvitamin D_3 or retinoic acid alone stimulates the release of VEGF from A10 cells [Yamamoto et al., 2002; Tanabe et al., 2004]. In the present study, we investigated the effect of nuclear hormone receptor superfamily members on TGF-β-induced VEGF release from A10 cells.

MATERIALS AND METHODS

Materials

A mouse VEGF enzyme-linked immunosorbent assay (ELISA) kit (recognizing both 120- and 164-amino acid forms of murine VEGF) and active TGF-β were purchased from R&D Systems (Minneapolis, MN). All-*trans* retinoic acid (retinoic acid), 9α-fluoro-16α-methylprednisolone (dexamethasone), 4-pregnene-11β, 21-diol-3, 20-dione (corticosterone), 4-pregnene-3,20-dione (progesterone), 4-androsten-17β-ol-3-one (testosterone), 1,3,5[10]-estratriene-3,17β-diol (estradiol), and 3,3′,5-triiodo-L-thyronine (T_3) were obtained from Sigma-Aldrich (St. Louis, MO). 1α,25-Dihydroxyvitamin D_3 (vitamin D_3) and SB203580 were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Antibodies against phospho-p38 MAP kinase and p38 MAP kinase were from New England BioLabs, Inc. (Beverly, MA). The ECL Western blot detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Retinoic acid, vitamin D_3, dexamethasone, corticosterone, progesterone, testosterone, and estradiol were dissolved in ethanol. T_3 was dissolved in 0.1 N NaOH. SB203580 was dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect VEGF immunoassay or Western blot results.

Cell Culture

A10 cells derived from fetal rat aortic smooth muscle [Kimes and Brandt, 1976] were obtained from American Type Culture Collection (Manassas, VA). Cells were seeded into 35-mm (1×10^5 cells) or 90-mm (5×10^5 cells) diameter dishes and maintained at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal calf serum. After 5 days, the medium was replaced with serum-free DMEM. Cells were used for experiments 48 h thereafter.

VEGF Assay

Cultured cells were pretreated with various agents belonging to the nuclear hormone receptor superfamily members in serum-free DMEM for 9 h and were then stimulated with TGF-β for the indicated periods. When indicated, the cells were pretreated with SB203580 for 60 min prior to stimulation with vitamin D_3. The conditioned medium was then collected, and the VEGF in the medium was measured with a VEGF ELISA kit that recognizes rat VEGF (according to the manufacturer), as described previously [Seko et al., 1999].

Western Blot Analysis of p38 MAP Kinase

Cultured cells were pretreated with retinoic acid, vitamin D_3, dexamethasone, or corticosterone in serum-free DMEM for 9 h and were then stimulated with TGF-β for 45 min. When indicated, the cells were pretreated with SB203580 for 60 min prior to stimulation with vitamin D_3. Stimulated cells were rinsed twice with phosphate-buffered saline, then lysed, homogenized, and sonicated in 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 analysis of p38 MAP kinase by Western blotting. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the Laemmli method in 10% polyacrylamide gels [Laemmli, 1970]. Western blot analysis was performed as described previously [Yamamoto et al., 2001, 2002] with phospho-specific p38 MAP kinase antibody or p38 MAP kinase antibody with peroxidase-labeled goat anti-rabbit IgG as a secondary antibody. Peroxidase activity was visualized on X-ray film by ECL.

Other Methods

The absorbance of ELISA samples at 450 nm was measured with a Multiskan JX ELISA reader (Thermo Labsystems, Helsinki, Finland). Absorbance was correlated with concentration by means of a standard curve. Densitometric analysis was performed with Molecular Analyst Software for Macintosh (BioRad, Hercules, CA).

Statistical Analysis

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

RESULTS

Effect of Retinoic Acid or Vitamin D_3 on TGF-β-Induced VEGF Release

We previously reported increased VEGF release by A10 cells after 12 h or more of TGF-β stimulation, and the effect of TGF-β was significant at concentrations greater than 1 ng/ml [Yamamoto et al., 2001]. In addition, retinoic acid alone significantly stimulates VEGF release in time-and concentration-dependent manners [Tanabe et al., 2004]. In the present study, retinoic acid and TGF-β additively induced VEGF release in a time-dependent manner up to 60 h (Fig. 1A), the effect being significant after 24 h or more of stimulation. The additive effect of retinoic acid on TGF-β-stimulated VEGF release was concentration-dependent from 0.1 nM to 0.1 μM (Fig. 1B). The effect was significant at concentrations greater than 10 nM.

We previously reported that vitamin D_3 alone at concentrations greater than 10 pM stimulates VEGF release from A10 cells [Yamamoto et al., 2002]. Vitamin D_3 and TGF-β synergistically induced VEGF release in a time-dependent manner up to 60 h (Fig. 1C), the effect being significant after 12 h or more of stimulation. The synergistic effect of vitamin D_3 was concentration-dependent from 0.1 to 10 nM (Fig. 1D). The effect was significant at concentrations greater than 0.1 nM.

Effect of Dexamethasone or Corticosterone on TGF-β-Induced VEGF Release

Dexamethasone alone had little effect on the basal level of VEGF release but significantly decreased TGF-β-induced VEGF release in a time-dependent manner up to 60 h (Fig. 2A), the effect being significant after 12 h or more of stimulation. The inhibitory effect of dexamethasone was concentration-dependent from 0.1 to 10 nM (Fig. 2B). The effect was significant at concentrations greater than 1 nM.

Corticosterone, another glucocorticoid, which alone did not affect the basal level of VEGF release, significantly decreased TGF-β-stimulated VEGF release. The inhibitory effect of corticosterone was concentration-dependent

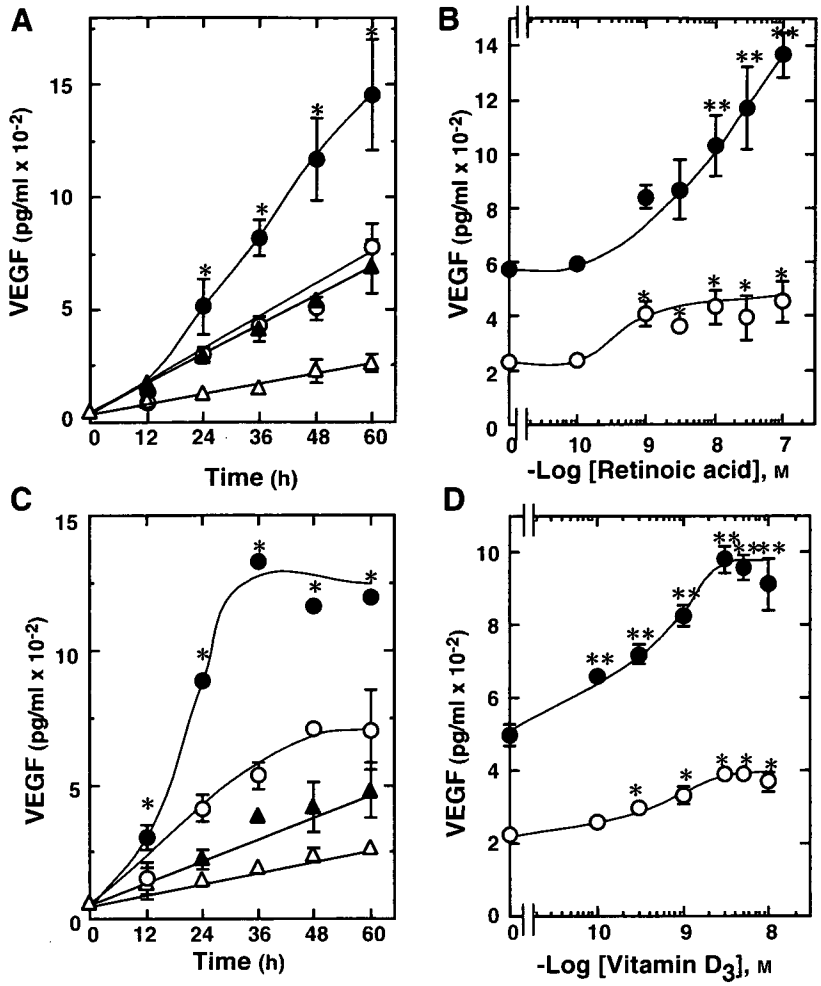

Fig. 1. Effects of retinoic acid or vitamin D_3 on the TGF-β-induced vascular endothelial growth factor (VEGF) release from A10 cells. **A**: Time-course of VEGF release after TGF-β-stimulation. Cultured cells were treated by 0.1 μM retinoic acid (●, ▲) or vehicle (○, △) for 9 h, and then stimulated by 5 ng/ml TGF-β (circles) or vehicle (triangles) for the indicated periods. $^*P < 0.05$ compared with TGF-β or retinoic acid alone. **B**: Dose-dependent effect of retinoic acid on the TGF-β-induced VEGF release. Cultured cells were treated by various doses of retinoic acid for 9 h, and then stimulated by 5 ng/ml TGF-β (●) or vehicle (○) for 48 h. $^*P < 0.05$ compared with vehicle alone. $^{**}P < 0.05$ compared with TGF-β or retinoic acid alone. **C**: Time-course of VEGF release after TGF-β-stimulation. Cultured cells were treated by 10 nM vitamin D_3 (●, ▲) or vehicle (○, △) for 9 h, and then stimulated by 5 ng/ml TGF-β (circles) or vehicle (triangles) for the indicated periods. $^*P < 0.05$ compared with TGF-β or vitamin D_3 alone. **D**: Dose-dependent effect of vitamin D_3 on the TGF-β-induced VEGF release. Cultured cells were treated by various doses of vitamin D_3 for 9 h, and then stimulated by 5 ng/ml TGF-β (●) or vehicle (○) for 48 h. $^*P < 0.05$ compared with vehicle alone. $^{**}P < 0.05$ compared with TGF-β or vitamin D_3 alone. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

from 0.1 to 10 nM (Fig. 2B). The effect was significant at 10 nM.

Effect of Sex Hormones or T_3 on TGF-β-Induced VEGF Release

Other hormones of the steroid/thyroid hormone superfamily, including the sex hormones progesterone, testosterone, and estradiol and T_3, had no affect on TGF-β-induced VEGF release from A10 cells (Fig. 2C,D).

Effect of Retinoic Acid, Vitamin D_3, Dexamethasone, or Corticosterone on TGF-β-Induced Phosphorylation of p38 MAP Kinase

We previously reported that TGF-β stimulates the release of VEGF from A10 cells at least in part via activation of p38 MAP kinase, with a maximum effect at 5 ng/ml TGF-β [Yamamoto et al., 2001]. Therefore, we investigated whether retinoic acid, vitamin D_3, dexamethasone, or

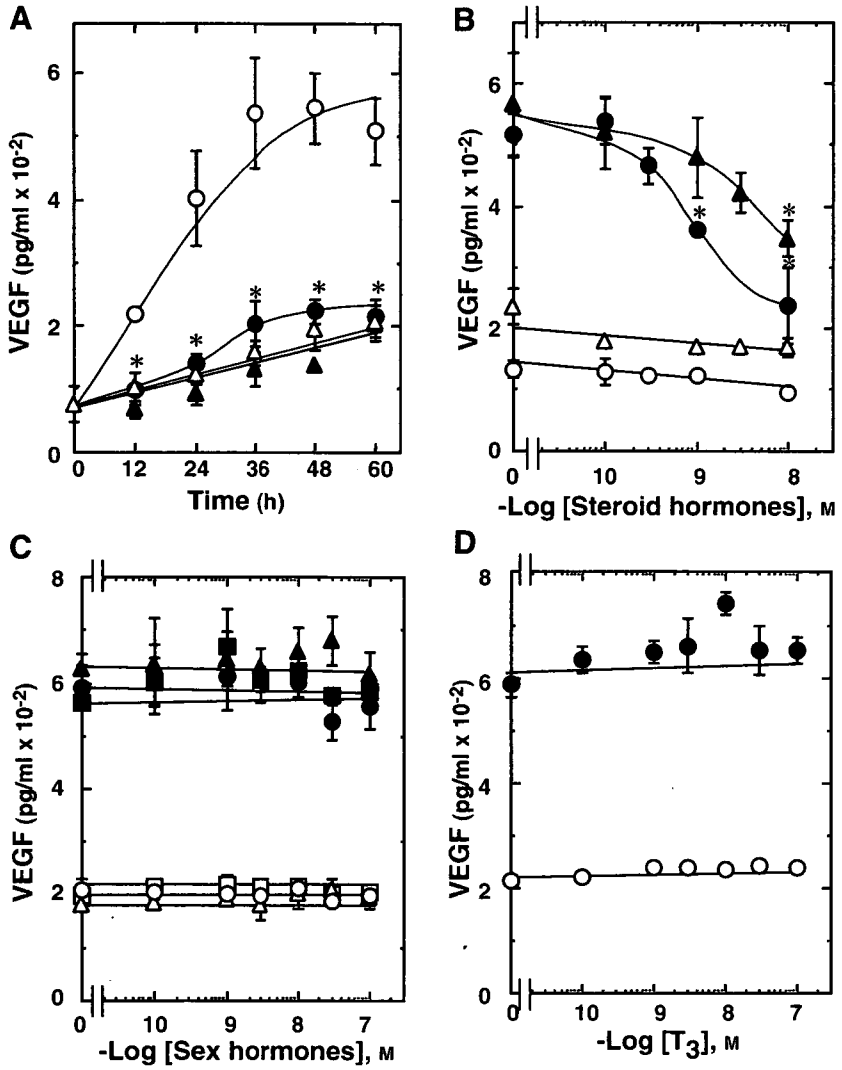

Fig. 2. Effects of dexamethasone, corticosterone, progesterone, testosterone, estradiol, or T_3 on the TGF-β-induced VEGF release from A10 cells. **A**: Time-course of VEGF release after TGF-β-stimulation. Cultured cells were treated by 10 nM dexamethasone (●, ▲) or vehicle (○, △) for 9 h, and then stimulated by 5 ng/ml TGF-β (circles) or vehicle (triangles) for the indicated periods. **B**: Dose-dependent effects of dexamethasone or corticosterone on the TGF-β-induced VEGF release. Cultured cells were treated by various doses of dexamethasone (circles) or corticosterone (triangles) for 9 h, and then stimulated by 5 ng/ml TGF-β (●, ▲) or vehicle (○, △) for 48 h. $^*P < 0.05$ compared with TGF-β alone. **C**: Cultured cells were treated by various doses of progesterone (circles), testosterone (triangles), or estradiol (squares) for 9 h, and then stimulated by 5 ng/ml TGF-β (closed symbols) or vehicle (open symbols) for 48 h. **D**: Cultured cells were treated by various doses of T_3 for 9 h, and then stimulated by 5 ng/ml TGF-β (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

corticosterone affects TGF-β (5 ng/ml)-induced p38 MAP kinase phosphorylation in these cells. Retinoic acid (0.1 μM), which by itself had little effect on p38 MAP kinase phosphorylation, had no affect on TGF-β-induced phosphorylation of p38 MAP kinase (Fig. 3A). We previously showed that vitamin D_3 markedly induces p38 MAP kinase phosphorylation, with a maximum effect at 10 nM vitamin D_3 [Yamamoto et al., 2002]. TGF-β and vitamin D_3 (10 nM) induced phosphorylation of p38 MAP kinase in an additive manner (Fig. 3B). Dexamethasone (10 nM), which by itself had little effect on p38 MAP kinase phosphorylation, had no affect on TGF-β-induced phosphorylation (Fig. 3C). In addition, corticosterone (10 nM) had no effect on TGF-β-induced phosphorylation of p38 MAP kinase (data not shown).

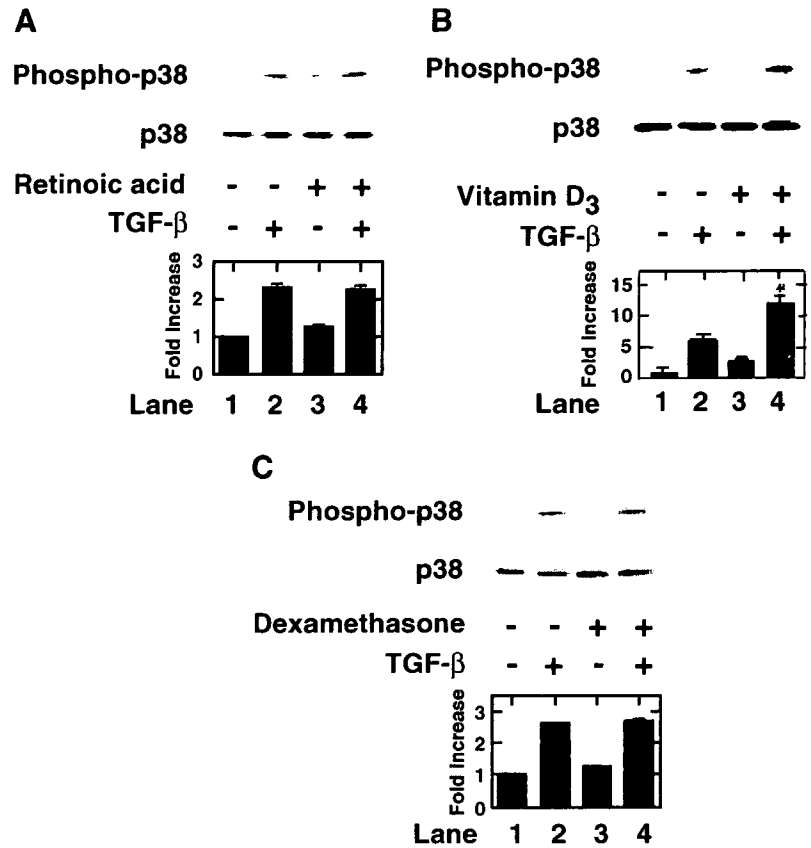

Fig. 3. Effects of retinoic acid, vitamin D_3, or dexamethasone on TGF-β-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase in A10 cells. Cultured cells were treated with 0.1 μM retinoic acid (**A**), 10 nM vitamin D_3 (**B**), 10 nM dexamethasone (**C**), or vehicle for 9 h, and then stimulated by 5 ng/ml TGF-β or vehicle for 45 min. Cell lysates were subjected to SDS–PAGE followed by Western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the phosphorylation level of p38 MAP kinase obtained from laser densitometric analysis. $^*P < 0.05$ compared with the value in TGF-β alone. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

Effect of SB203580 on TGF-β and Vitamin D_3-induced VEGF Release and Phosphorylation of p38 MAP Kinase

We examined the effect of SB203580, an inhibitor of p38 MAP kinase [Cuenda et al., 1995], on VEGF release and p38 MAP kinase phosphorylation induced by TGF-β in A10 cells in the absence or presence of vitamin D_3. SB203580 alone had little effect on the basal level of VEGF release but inhibited VEGF release induced by TGF-β or vitamin D_3. The enhancement of VEGF release induced by TGF-β plus vitamin D_3 was also inhibited by SB203580 (Table I). However, SB203580 alone had no affect on the basal level of phosphorylation of p38 MAP kinase and also had little effect on phosphorylation induced by TGF-β plus vitamin D_3 (Fig. 4).

TABLE I. Effects of SB203580 on Vitamin D_3 or/and TGF-β-induced Vascular Endothelial Growth Factor (VEGF) Release From A10 Cells

SB203580 (30 μM)	Vitamin D_3(10 nM)	TGF-β (5 ng/ml)	VEGF (pg/ml)
−	−	−	185 ± 11
−	−	+	583 ± 47*
−	+	−	467 ± 35*
−	+	+	1174 ± 88*
+	−	−	180 ± 10
+	−	+	298 ± 28**
+	+	−	302 ± 25**
+	+	+	496 ± 38**

Cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min and then stimulated by 10 nM vitamin D_3 for 9 h and/or 5 ng/ml TGF-β for 48 h. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.
$^*P < 0.05$ compared with vehicle alone.
$^{**}P < 0.05$ compared with vitamin D_3 and/or TGF-β.

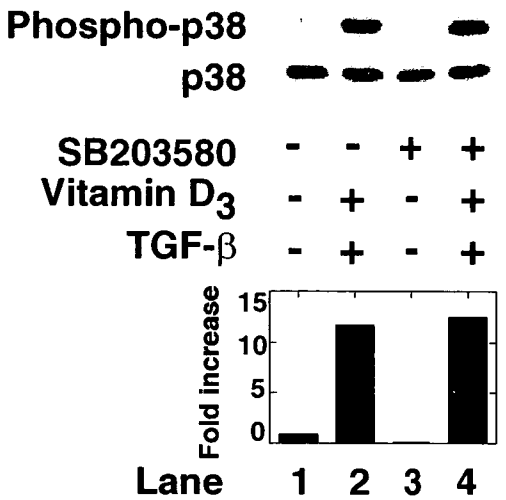

Fig. 4. Effect of SB203580 on a combination with TGF-β and vitamin D_3-induced phosphorylation of p38 MAP kinase in A10 cells. Cultured cells were treated with 30 μM SB203580 or vehicle for 60 min and then stimulated by 10 nM vitamin D_3 for 9 h and 5 ng/ml TGF-β for 45 min. Cell lysates were subjected to SDS–PAGE followed by Western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the phosphorylation level of p38 MAP kinase obtained from laser densitometric analysis. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

DISCUSSION

TGF-β family members are multifunctional agonists whose effects depend on the state of responsiveness of target cells [Massagué, 1998]. Alterations in the local abundance of TGF-β in the arterial wall promote vascular cell transdifferentiation, vascular wall remodeling, arterial lesion growth, and lesion regression associated with apoptosis [Schulick et al., 1998]. One possible explanation for the angiogenic effect of TGF-β is that TGF-β is an indirect mitogen that acts via the production of other angiogenic factors. TGF-β promotes VEGF synthesis in vascular smooth muscle cells and exerts angiogenic effects [Brogi et al., 1994; Stavri et al., 1995]. We previously reported that TGF-β stimulates the release of VEGF from aortic smooth muscle A10 cells via activation of the p38 MAP kinase pathway [Yamamoto et al., 2001].

In the present study, both retinoic acid and vitamin D_3, which alone stimulate VEGF release from A10 cells [Yamamoto et al., 2002; Tanabe et al., 2004], induced VEGF release with TGF-β in an additive manner. Retinoic acid or vitamin D_3 modulate growth and differentiation of vascular smooth muscle cells [Miano and Berk, 2000; Neuville et al., 2000; Rebsamen et al., 2002]. In vitro, it has been shown that in the absence of growth factor, retinoic acid stimulates vascular smooth muscle cell proliferation but attenuates growth factor-stimulated proliferation [Miano and Berk, 2000]. In addition, retinoic acid decreases the size of neointimal masses, elicits favorable remodeling, and increases lumen diameter and area of injured arteries in vivo [Neuville et al., 2000]. In vascular endothelial cells, retinoic acid enhances the expression of the TGF-β receptor, potentiates TGF-β-induced inhibition of fibrinolytic activity and cell proliferation, and modulates endothelial cell growth and morphology [Kojima and Rifkin, 1993; Yoshizawa et al., 1998; Miano and Berk, 2000]. VEGF also induces endothelial cell proliferation [Neufeld et al., 1999]. Thus, it is likely that retinoic acid, along with TGF-β, modulates endothelial cell growth. With respect to vitamin D_3, it plays an important role in regulatory calcium homeostasis, cell differentiation, and proliferation [Kato, 2000]. In the vascular system, vitamin D_3 contributes to the development of hypertension, induces vascular calcification, and modulates vascular smooth muscle cell growth [Rebsamen et al., 2002]. Vitamin D_3 also inhibits VEGF-induced angiogenesis by inhibiting vascular endothelial cell proliferation [Mantell et al., 2000]. Our results suggest that vitamin D_3 modulates endothelial cell function by working with TGF-β to stimulate VEGF release.

We also showed that dexamethasone and corticosterone inhibited TGF-β-induced VEGF release in A10 cells. Glucocorticoids inhibit angiogenesis [Auerbach and Auerbach, 1994], and dexamethasone, hydrocortisone, or cortisone inhibit PDGF-induced VEGF gene expression and VEGF release in vascular smooth muscle cells [Nauck et al., 1998]. Our present study is consistent with this report. Thus, it is likely that dexamethasone and corticosterone inhibit angiogenesis, at least in part, by inhibiting TGF-β-induced VEGF release from vascular smooth muscle cells.

We also investigated whether the stimulatory and inhibitory effects of retinoic acid, vitamin D_3, dexamethasone, or corticosterone on VEGF release are mediated via TGF-β-induced p38 MAP kinase activation in A10 cells. Retinoic