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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 125 000*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.

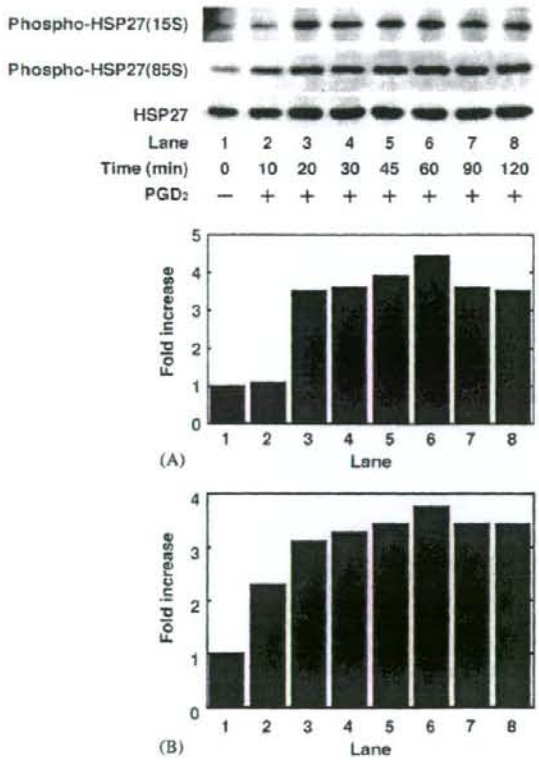

Fig. 1. Effect of PGD_2 on the phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. The cultured cells were stimulated by 10 μM PGD_2 for the indicated periods. The extracts of cells were subjected to SDS-PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and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

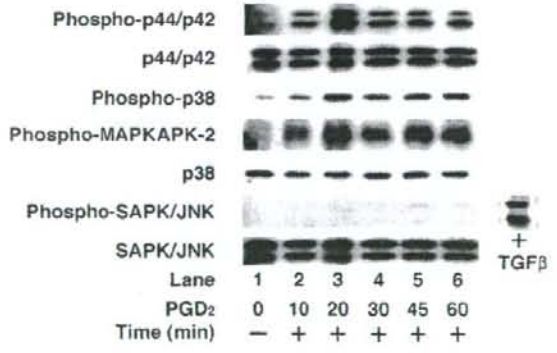

Fig. 2. Effects of PGD_2 on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, MAPKAP kinase 2, SAPK/JNK in MC3T3-E1 cells exposed to heat shock. The cultured cells were stimulated by 10 μM PGD_2 for the indicated periods. The extracts of cells were subjected to SDS-PAGE using antibodies against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

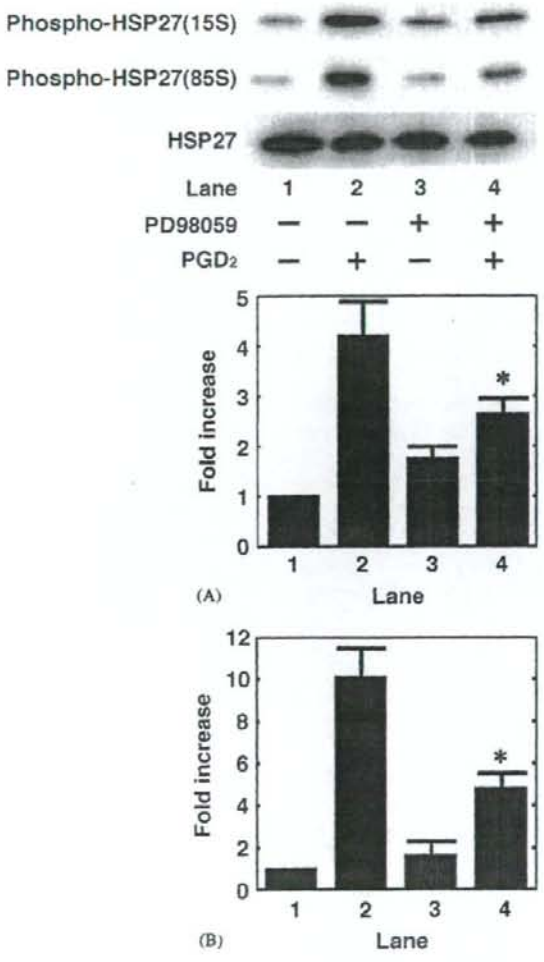

Fig. 3. Effect of PD98059 on the PGD_2-induced phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. The cultured cells were pretreated with 50 μM PD98059 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE using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

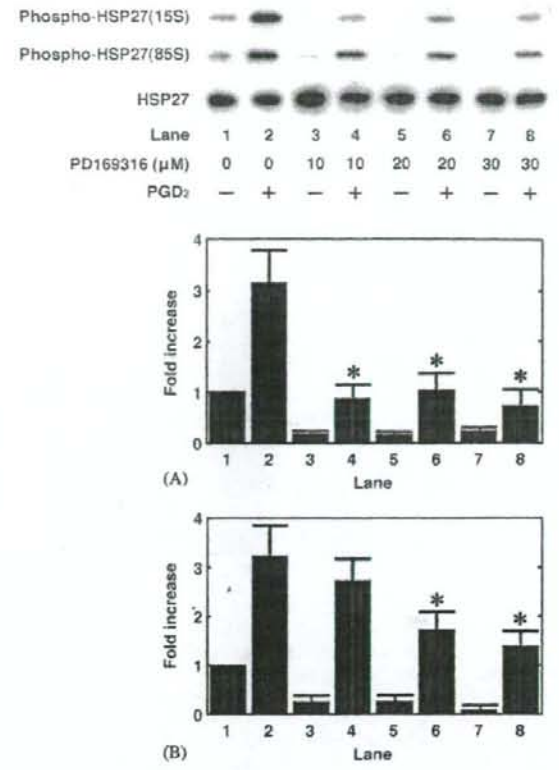

Fig. 4. Effect of PD169316 on the PGD_2-induced phosphorylation of HSP27 in MC3T3-E1 cells exposed to heat shock. The cultured cells were pretreated with various doses of PD169316 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The extracts of cells were subjected to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

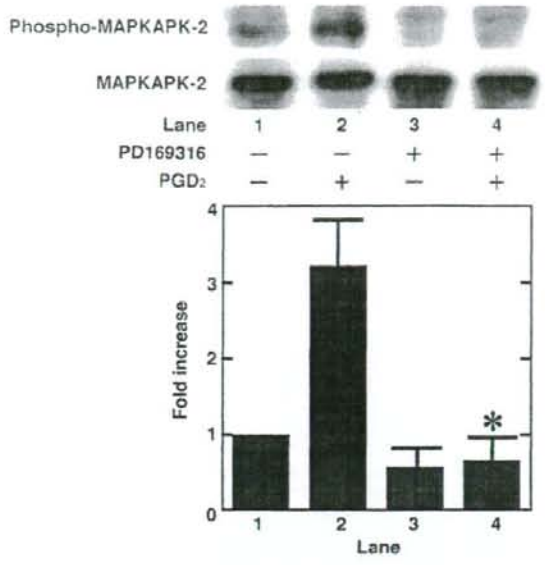

Fig. 5. Effect of PD169316 on the PGD_2-induced phosphorylation of MAPKAP kinase 2 in MC3T3-E1 cells exposed to heat shock. The cultured cells were pretreated with 10 μM PD169316 or vehicle for 60 min, and then stimulated by 10 μM PGD_2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE 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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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.

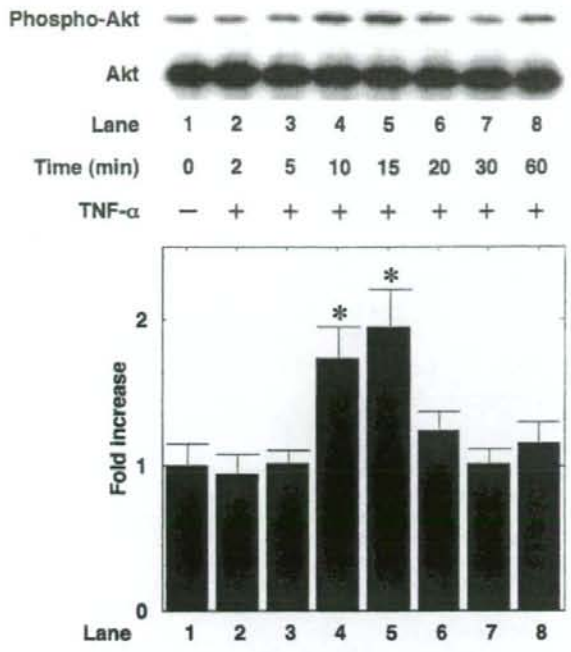

Fig. 1 Effect of TNF-α on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated with 30 ng/ml TNF-α for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The 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.

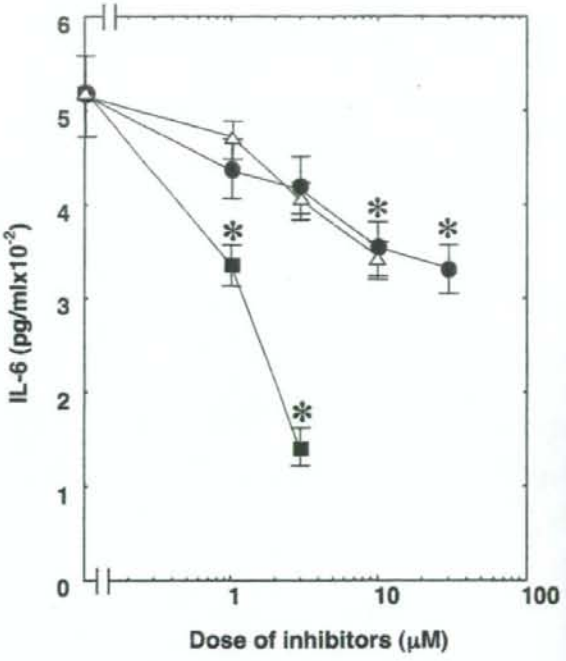

Fig. 2 Effect of LY294002, wortmannin, or Akt inhibitor on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of Akt inhibitor (●), LY294002 (■), or wortmannin (△) for 60 min, and then stimulated by 30 ng/ml TNF-α for 48 h. In the absence of TNF-α 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-

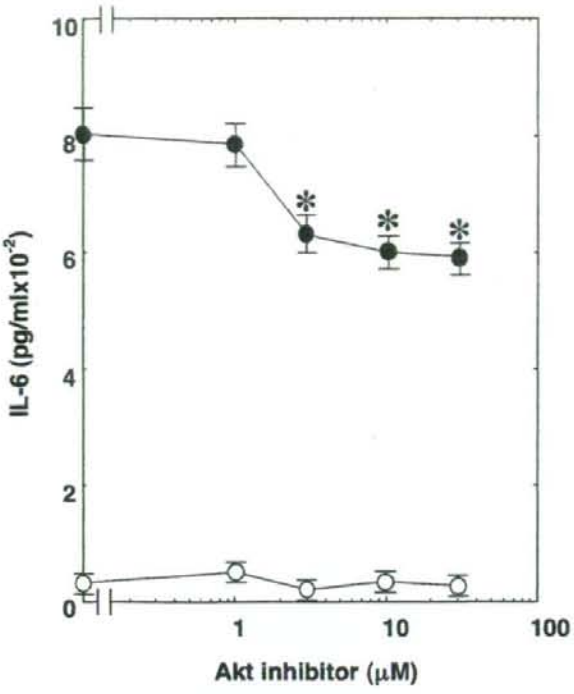

Fig. 3 Effect of Akt-inhibitor on TNF-α-stimulated IL-6 synthesis in primary osteoblasts. The cultured cells were pretreated with various doses Akt inhibitor for 60 min, and then stimulated by 30 ng/ml TNF-α (●) or vehicle (○) for 48 h. Each value represents the mean ± SEM of triplicate determinations.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

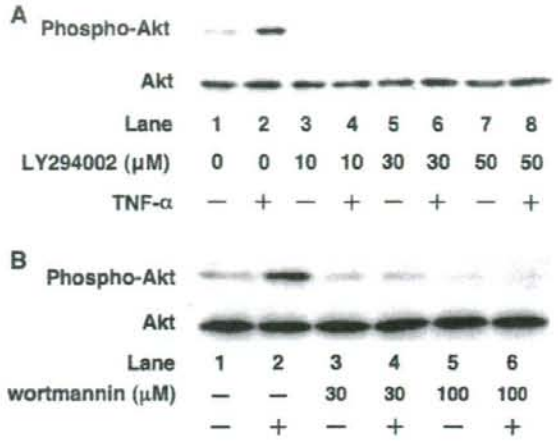

Fig. 4 Effect of LY294002 or wortmannin on the TNF-α-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 (**A**) or wortmannin (**B**) for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 min. The extracts of cells were subjected 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

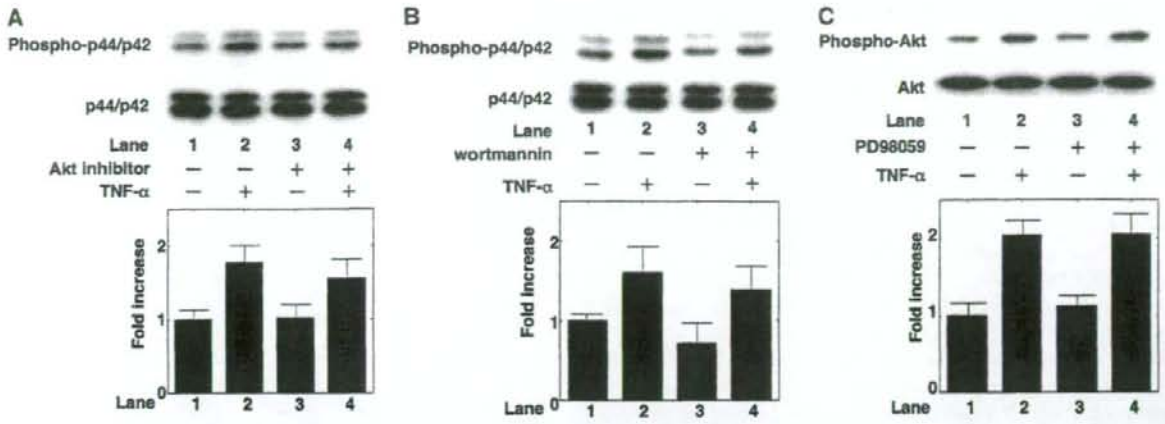

Fig. 5 Effect of Akt inhibitor or wortmannin on the TNF-α-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. Effect of PD98059 on the TNF-α-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 50 nM Akt inhibitor (**A**) or wortmannin (**B**) for 60 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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Up-regulation by zinc of FGF-2-induced VEGF release through enhancing p44/p42 MAP kinase activation in osteoblasts

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Abstract

We previously reported that basic fibroblast growth factor (FGF-2) activates stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p44/p42 mitogen-activated protein (MAP) kinase resulting in the stimulation of vascular endothelial growth factor (VEGF) release in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether zinc affects the VEGF release by FGF-2 in MC3T3-E1 cells. The FGF-2-induced VEGF release was significantly enhanced by $ZnSO_4$ but not Na_2SO_4. The enhancing effect of $ZnSO_4$ was dose-dependent between 1 and 100 μM. $ZnSO_4$ markedly enhanced the FGF-2-induced phosphorylation of p44/p42 MAP kinase while having little effect on the SAPK/JNK phosphorylation. PD98059 significantly reduced the amplification by $ZnSO_4$ of the FGF-2-stimulated VEGF release. Taken together, our findings strongly suggest that zinc enhances FGF-2-stimulated VEGF release resulting from up-regulating activation of p44/p42 MAP kinase in osteoblasts.
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Keywords: Zinc; FGF-2; VEGF; MAP kinase; Osteoblast

Introduction

Zinc is an important regulator of biological functions in humans and many animals (Vallee and Falchuk, 1993). It is generally recognized that growth failure and impaired wound healing occur due to the absence of adequate dietary zinc (Vallee and Falchuk, 1993). In bone metabolism, zinc deficiency results in bone loss. It has been shown that zinc has a stimulatory effect on bone formation and mineralization in vivo and in vitro (Yamaguchi and Yamaguchi, 1986; Hall et al., 1999). Two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption, regulate bone metabolism (Nijweide et al., 1986). As for osteoblasts, it has been reported that zinc increases alkaline phosphatase activity and protein concentration (Hashizume and Yamaguchi, 1994). In addition, the activity of 1,25-dihydroxyvitamin D_3-dependent promoters and the anabolic effect of insulin-like growth factor-I are reportedly enhanced by zinc (Matsui and Yamaguchi, 1995; Lutz et al., 2000). We have recently shown that zinc reduces prostaglandin $F_2\alpha$-stimulated interleukin-6 (IL-6) synthesis via suppression of phosphoinositide-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D in osteoblast-like MC3T3-E1 cells (Hatakeyama et al., 2002). However, the exact role of zinc in osteoblasts has not yet been clarified.

Vascular endothelial growth factor (VEGF) is a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells (Ferrara and Davis-Smyth, 1997). VEGF that is produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells (Ferrara and Davis-Smyth, 1997). As for bone metabolism, it has been shown that inactivation of VEGF causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate (Gerber et al., 1999). Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in

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response to various humoral factors (Ferrara and Davis-Smyth, 1997; Goad et al., 1996; Wang et al., 1996; Schlaeppi et al., 1997). During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, migrate into the resorption lacuna. Currently, it is well recognized that the activities of osteoblasts, osteoclasts and capillary endothelial cells are closely coordinated via humoral factors as well as by direct cell-to-cell contact, and these cells cooperatively regulate bone metabolism (Erlebacher et al., 1995). Therefore, it is thought that VEGF secreted from osteoblasts may play a crucial role in the regulation of bone metabolism. However, the mechanism behind VEGF synthesis in osteoblasts is not fully known.

Basic fibroblast growth factor (FGF-2) is embedded in bone matrix, and osteoblasts synthesize FGF-2 (Baylink et al., 1993; Hurley et al., 1993). FGF-2 expression in osteoblasts is detected during fracture repair (Bolander, 1992). Therefore, it is thought that FGF-2 may play a pivotal role in fracture healing, bone remodeling and osteogenesis (Marie, 2003). We have previously reported that FGF-2 stimulates VEGF release in MC3T3-E1 cells, and that among the mitogen-activated protein (MAP) kinase superfamily (Tokuda et al., 2003), p44/p42 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) play as positive regulators in the VEGF release (Tokuda et al., 2000, 2003). In the present study, we investigated the effect of zinc on the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. We here show that $ZnSO_4$ up-regulates FGF-2-stimulated VEGF release via enhancing activation of p44/p42 MAP kinase in these cells.

Materials and methods

Materials

FGF-2 and mouse VEGF enzyme immunoassay kit were purchased from R&D Systems, Inc. (Minneapolis, MN). Zinc sulfate ($ZnSO_4$) and Sodium sulfate (Na_2SO_4) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). PD98059 was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies 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. PD98059 was dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for VEGF or the analysis of MAP kinases.

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 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 h.

VEGF assay

The cultured cells were stimulated by FGF-2 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with $ZnSO_4$ or Na_2SO_4 for 20 min. The pretreatment of PD98059 was performed for 60 min before the addition of $ZnSO_4$. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

Analysis of p44/p42 MAP kinase and SAPK/JNK

The cultured cells were stimulated by FGF-2 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 p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies or SAPK/JNK antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the PVDF sheet was

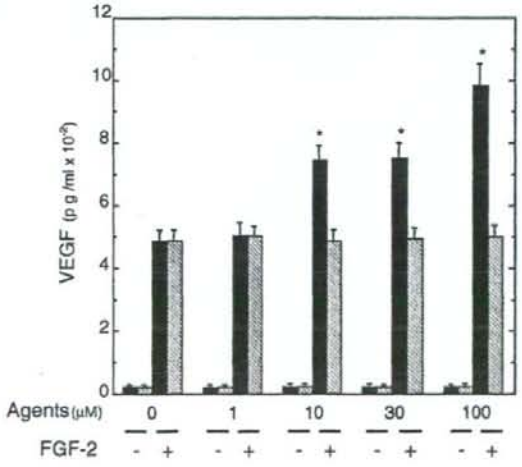

Fig. 1. Effects of $ZnSO_4$ or Na_2SO_4 on FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of $ZnSO_4$ (closed bar) or Na_2SO_4 (hatched bar) for 20 min, and then stimulated by 70 ng/ml FGF-2 or vehicle for 24 h. Each value represents the mean+SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p<0.05$, compared to the value of FGF-2 alone.

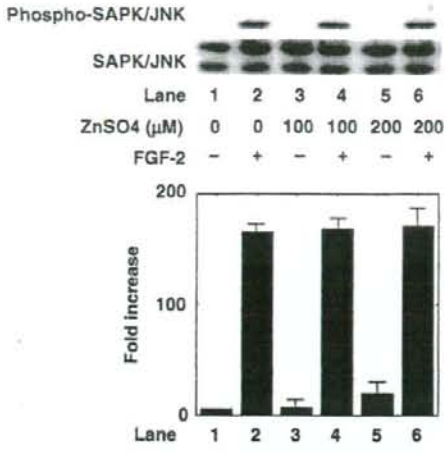

Fig. 2. Effect of $ZnSO_4$ on the phosphorylation of SAPK/JNK induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of $ZnSO_4$ or vehicle for 20 min, and then stimulated by 70 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-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.

visualized on X-ray film by means of the ECL Western blotting detection system. When indicated, the cells were pretreated with $ZnSO_4$ 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 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+SEM of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effects of $ZnSO_4$ on the VEGF release by FGF-2 in MC3T3-E1 cells

In our previous study (Tokuda et al., 2000), we have demonstrated that FGF-2 stimulates VEGF release in osteoblast-like MC3T3-E1 cells. To clarify whether zinc affects FGF-2-stimulated VEGF release in MC3T3-E1 cells, we first examined the effect of $ZnSO_4$ on the VEGF release. $ZnSO_4$, which by itself had little effect on the VEGF levels, significantly amplified the FGF-2-stimulated release of VEGF (Fig. 1). The amplifying effect of $ZnSO_4$ was dose-dependent between 1 and 100 μM (Fig. 1). $ZnSO_4$ at 100 μM caused about 110% enhancement in the FGF-2 alone. Na_2SO_4 failed to affect the FGF2-stimulated VEGF release (Fig. 1).

Effects of $ZnSO_4$ on the phosphorylation of p44/p42 MAP kinase and SAPK/JNK induced by FGF-2 in MC3T3-E1 cells

We have previously reported that FGF-2 stimulates VEGF release at least in part via p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells (Tokuda et al., 2000, 2003). In order to elucidate if $ZnSO_4$-effect on the FGF-2-stimulated VEGF release is mediated via p44/p42 MAP kinase activation or SAPK/JNK activation in these cells, we next examined the effect of $ZnSO_4$ on the FGF-2-induced phosphorylation of SAPK/JNK kinase. However, $ZnSO_4$ failed to affect the phosphorylation of SAPK/JNK induced by FGF-2 (Fig. 2). On the other hand, the FGF-2-induced phosphorylation of p44/p42 MAP kinase was significantly enhanced by $ZnSO_4$ (Fig. 3). According to the densitometric analysis, $ZnSO_4$ (200 μM) caused about 50% amplification of the FGF-2-effect on the p44/p42 MAP kinase phosphorylation. In addition, $ZnSO_4$ enhanced the p44/p42 MAP kinase phosphorylation in a dose-dependent manner (Fig. 3).

Effect of PD98059 on the enhancement by $ZnSO_4$ of FGF-2-stimulated VEGF release in MC3T3-E1 cells

To furthermore investigate whether the up-regulating effect of $ZnSO_4$ on FGF-2-induced VEGF release is due to

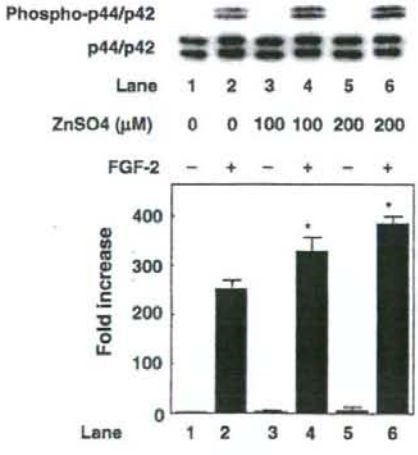

Fig. 3. Effect of $ZnSO_4$ on the phosphorylation of p44/p42 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of $ZnSO_4$ or vehicle for 20 min, and then stimulated by 70 ng/ml FGF-2 or vehicle for 75 min. The extracts of cells were subjected 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 FGF-2-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. *$p<0.05$, compared to the value of FGF-2 alone.

Table 1
Effect of PD98059 on the enhancement by $ZnSO_4$ of the FGF-2-stimulated VEGF synthesis in MCT3T3-E1 cells

PD98059	$ZnSO_4$	FGF-2	VEGF (pg/ml)
−	−	−	23±10
−	−	+	502±45*
−	+	−	21±10
−	+	+	955±65**
+	−	−	25±10
+	−	+	263±25**
+	+	−	20±10
+	+	+	272±25***

The cultured cells were pretreated with 10 μM PD98059 or vehicle for 60 min, and then incubated by 100 μM $ZnSO_4$ for 20 min. The cells were stimulated by 70 ng/ml FGF-2 or vehicle for 24 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 FGF-2 alone. ***$p<0.05$, compared to the value of FGF-2 with $ZnSO_4$ pretreatment.

enhancement of p44/p42 MAP kinase activation in MC3T3-E1 cells, we examined the effect of PD98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase a highly specific inhibitor of MEK (Alessi et al., 1995), on the enhancement by $ZnSO_4$. PD98059, which by itself had no effect on the basal levels of VEGF, significantly reduced the enhancement by $ZnSO_4$ of FGF-2-induced VEGF release to the levels of the FGF-2 with PD98059 (Table 1).

Discussion

In the present study, we showed that $ZnSO_4$, which alone did not affect the levels of VEGF, significantly amplified the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. On the contrary, Na_2SO_4 had little effect on the VEGF release in these cells. Therefore, it is probable that the FGF-2-induced VEGF release is enhanced by zinc in MC3T3-E1 cells.

We next investigated the mechanism of zinc behind the amplification. It is well recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays an important role in cellular functions including proliferation, differentiation, and apoptosis 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 transduce the diverse messages (Widmann et al., 1999). In our previous studies (Tokuda et al., 2000; Tokuda et al., 2003), we have shown that FGF-2 activates p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells, and these MAP kinases act as positive regulators in FGF-2-induced VEGF release. In the present study, we demonstrated that $ZnSO_4$ did not affect the FGF-2-induced phosphorylation of SAPK/LNK. Therefore, it seems unlikely that $ZnSO_4$ amplified the FGF-2-induced VEGF release through up-regulating the activation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. On the contrary, we showed that the FGF-2-induced phosphorylation of p44/p42 MAP kinase was markedly amplified by $ZnSO_4$ in a dose-dependent manner. It seems that zinc enhances the FGF-2-stimulated VEGF release via up-regulating activation of p44/p42 MAP kinase. We next demonstrated that PD98059 (Alessi et al., 1995) markedly suppressed the enhancement of VEGF release by $ZnSO_4$ almost to the levels of FGF-2 alone. Therefore, it is probable that the enhancement in the FGF-2-induced VEGF release is mediated through the activation of p44/p42 MAP kinase. Based on our findings as a whole, it is most likely that zinc up-regulates FGF-2-stimulated VEGF release through enhancing the activation of p44/p42 MAP kinase but not SAPK/JNK in osteoblast-like MC3T3-E1 cells. Further investigation is necessary to clarify the exact mechanism of zinc in the amplification of VEGF release in osteoblasts.

It is well known that the expansion of capillary network providing microvasculature is an essential process of bone remodeling (Goad et al., 1996). Since VEGF is a specific mitogen of vascular endothelial cells (Ferrara and Davis-Smyth, 1997), it is generally recognized that VEGF secreted by osteoblasts functions as an important intercellular mediator between osteoblasts and vascular endothelial cells in bone metabolism. Moreover, it has been reported that VEGF is involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in epiphyseal growth plate of mouse (Gerber et al., 1999), supporting the significance of VEGF in bone metabolism. On the other hand, in bone metabolism, zinc plays as an important regulator in stimulating bone formation and mineralization (Yamaguchi and Yamaguchi, 1986; Hall et al., 1999). It has been reported that zinc enhances the activity of 1,25-dihydroxyvitamin D_3-dependent promoters and the anabolic effect of insulin-like growth factor-I (Matsui and Yamaguchi, 1995; Lutz et al., 2000). In addition, zinc reportedly induces expression of macrophage colony stimulating factor gene in osteoblasts (Kanekiyo et al., 2002). Based on these findings, it is probable that zinc-enhanced VEGF release from osteoblasts plays a pivotal role in the process of bone remodeling via up-regulating the proliferation of capillary endothelial cells. We have previously reported that zinc reduces prostaglandin $F_2\alpha$-stimulated synthesis of IL-6 in osteoblast-like MC3T3-E1 cells (Hatakeyama et al., 2002). It is well recognized that IL-6 is a potent bone resorptive agent which induces osteoclast formation and stimulates osteoclast activity to resorb bone (Rifas, 1999). Taking our results into account as a whole, therefore, it is most likely that zinc stimulates osteogenesis through increasing VEGF activity in addition to the suppression of interleukin-6 activity in bone metabolism. Further investigations are required to elucidate the precise role of zinc in osteoblasts.

Conclusion

Our present results strongly suggest that zinc enhances FGF-2-stimulated VEGF release resulting from up-regulating activation of p44/p42 MAP kinase in osteoblasts.

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認知症に対する作業療法

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はじめに[1~3]

高齢化社会の進展に伴い、認知症高齢者の数は増加し、その治療やケアが重要な問題となっている。

認知症の中核症状は、「物忘れ」や「時間や今いる場所がわからない」などの記憶・見当識障害であり、副次症状は、うつ状態、夜間せん妄、徘徊、尿・便失禁、易怒などの行動がある。中核症状に対して、薬物療法が症状の進行を遅らせる効果が期待されている。また、周辺症状の問題行動には、介護者をなぐる、ける、かみつくなどの身体的行動、不適切な行動、同じ言葉を繰り返したり、同じような要求をする、大きな声を出したり、悪口雑言を言ったりするなどが含まれる。問題行動は、認知症を有する者のうち80%で起きるとされている。大声は、ナーシングホームで60%の発生率である。問題行動は、認知症ではよく見られ、介護者にとっては非常に大きな負担となっている。過去では、問題行動に対しては、向精神薬、身体拘束、または無視することで対応してきたが、人道上からもQOLの観点からも好ましい対応ではなかった。

認知症を介護していくうえでの目標は、認知症の生活の快適さを向上させること、そして、人間として尊厳ある生活を維持していくことが大切である。つまり、問題行動を向精神薬で穏やかにコントロールされた状態ではなく、むしろ、少々落ち着きがなく騒がしくとも家族や他人と会話したり、ときには逸脱行動があっても表情豊かで元気な状態を求めることにある。このような中で現在、リハビリテーションなどの非薬物療法は、認知症の問題行動の対処法として重要であると考えられている。

認知症に伴うさまざまな問題行動を改善させたり、認知症の精神機能を活性化させ、自発性、集中力や意欲面を向上させたり、認知症の症状を遅らせる目的でさまざまな活動が行われている。

認知症高齢者に対する非薬物療法としては、具体的には、回想法、リアリティ・オリエンテーション、音楽療法、理学療法（筋力強化、バランス訓練、関節可動域訓練）、作業療法（レクリエーション療法、認知トレーニング、家事・家庭内役割作業、手工芸・工作）、園芸療法、演芸療法、社会心理療法、ダンス、散歩、各種体操（ラジオ体操、リズム体操、民謡体操、ストレッチ体操）などがあり、また、環境の整備、介護者への教育・指導なども含まれる。

作業療法とは、身体的・精神的・社会的・職業的な機能の回復を最大限に図り、自立した生活ができる能力を取り戻すことであり、この目的のために作業活動を用いて治療、訓練、指導および援助を行うことである。作業療法で行う作業活動は、日常生活動作の諸動作、仕事・遊び、教育活動など人間の生活全般に関わる諸活動が対象となる。

認知症に対して、現在、当院で行っている具体的な作業療法的アプローチ方法として、レクリエーション療法と認知トレーニングを紹介する。

レクリエーション療法

レクリエーション療法は、音楽、体操、いろいろな種類のゲームなどから構成されている。なかには、回想法、リアリティ・オリエンテーションのプログラムが含まれることもある。当院では、認知症高齢者に対してレクリエーション療法を行ってきた。レクリエーション療法は、リハビリ体操、風船バレーボール、ボウリング、ちぎり絵、ダンスなどからなり、月曜日から金曜日までの週5日、90分間からなり、作業療法士1名と看護師1名で行ってきた。

レクリエーション療法の効果を、脳血管性認知症患者