SPANDIDOS PUBLICATIONS

Synergistic effect of vasoactive intestinal peptides on TNF-α-induced IL-6 synthesis in osteoblasts: amplification of p44/p42 MAP kinase activation

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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 and phosphatidylinositol 3-kinase/Akt in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the effect of vasoactive intestinal peptide (VIP) on TNF-α-induced IL-6 synthesis in these cells. VIP, which by itself slightly stimulated IL-6 synthesis, synergistically enhanced the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. The synergistic effect of VIP on the TNF-α-induced IL-6 synthesis was concentration-dependent in the range between 1 and 70 nM. We previously reported that VIP stimulated cAMP production in MC3T3-E1 cells. Forskolin, a direct activator of adenylyl cyclase, or 8-bromoadenosine-3',5'-cyclic monophosphate (8bromo-cAMP), a plasma membrane-permeable cAMP analogue, markedly enhanced the TNF-α-induced IL-6 synthesis as well as VIP. VIP markedly up-regulated the TNF-α-induced p44/p42 MAP kinase phosphorylation. The Akt phosphorylation stimulated by TNF-α was only slightly affected by VIP. PD98059, a specific inhibitor of MEK1/2, significantly suppressed the enhancement of TNF-α-induced IL-6 synthesis by VIP. The synergistic effect of a combination of VIP and TNF-α on the phosphorylation of p44/p42 MAP kinase was diminished by H-89, an inhibitor of cAMP-dependent protein kinase. These results strongly suggest that VIP synergistically enhances TNF-α-stimulated IL-6 synthesis via up-regulating p44/p42 MAP kinase through the adenylyl cyclase-cAMP system in osteoblasts.

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Introduction

It is known that tumor necrosis factor-α (TNF-α) is a multi-functional 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 (1). Bone resorption is mediated by 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 (2,7,8). As for bone metabolism, IL-6 has been shown to stimulate bone resorption and to induce osteoclast formation (2,5,8,9). Thus, accumulating evidence indicates that IL-6 secreted from osteoblasts plays a key role as a downstream effector of bone resorptive agents. In previous studies (10-12), we have shown that TNF-α induces IL-6 synthesis through the activation of p44/p42 mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3-kinase)/Akt in osteoblast-like MC3T3-E1 cells. However, the exact mechanism of TNF-α behind the IL-6 synthesis in osteoblasts remains to be elucidated.

Vasoactive intestinal peptide (VIP) is an ubiquitous 28 amino acid peptide, structurally related to a large number of regulatory peptides belonging to the VIP/secretin/glucagon family, which is composed of pituitary adenylate cyclase-activating peptide (PACAP) 27 and 38, secretin, glucagon, etc. VIP was originally discovered in the gut with vasodilator activity. PACAPs and VIP are widely distributed in both

central and peripheral nervous systems and show a broad spectrum of biological actions in neuro-endocrine functions (13,14). VIP has also been shown to stimulate cyclic AMP (cAMP) production in various cloned osteoblastic cells including osteoblast-like MC3T3-E1 cells, which have been derived from newborn mouse calvaria (15). We previously showed that VIP stimulated cAMP production in MC3T3-E1 cells (16). As for bone metabolism, it has been reported that VIP stimulates bone resorption via a cAMP-dependent mechanism in organ cultures of mouse calvaria (17). But so far, the precise mechanism underlying the effects of VIP on bone metabolism has not been clarified.

In the present study, we investigated whether VIP plays a role in the TNF-α-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells. We show that VIP synergistically enhances TNF-α-stimulated IL-6 synthesis via up-regulating p44/p42 MAP kinase through the adenylyl cyclase-cAMP system in these cells.

Materials and methods

Materials. TNF-α was obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Mouse IL-6 ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN). 2'-Amino-3'-methoxyflavone (PD98059) was obtained from Calbiochem. Co. (La Jolla, CA). VIP was purchased from the Peptide Institute Inc. (Minoh, Japan). Forskolin and 8-bromoadenosine-3',5'-cyclic monophosphate (8-bromo-cAMP) was purchased from Sigma Chemical Co. (St. Louis, MO). *N*-[2-((p-bromo-cinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2 HCl (H-89) was obtained from Seikagaku Kogyo Inc. (Tokyo, Japan). Phospho-specific Akt antibodies, Akt antibodies, Phospho-specific p44/p42 MAP kinase antibodies and p44/p42 MAP kinase antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). ECL Western blotting detection system was purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. PD98059, H-89, forskolin and 8-bromo-cAMP were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect assay for IL-6 or Western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (18) were maintained as previously described (19). Briefly, 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. Cells were seeded into 35-mm diameter dishes ($5x10^4$/dish) or 90-mm diameter dishes ($2x10^5$/dish) in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. Cells were used for experiments after 48 h.

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. When indicated, cells were pretreated with VIP, forskolin or 8-bromo-cAMP for 1 h. Pretreatment of PD98059 was performed for 60 min prior to the stimulation by VIP. Conditioned medium was collected, and IL-6 in the medium was then measured by an IL-6 ELISA kit.

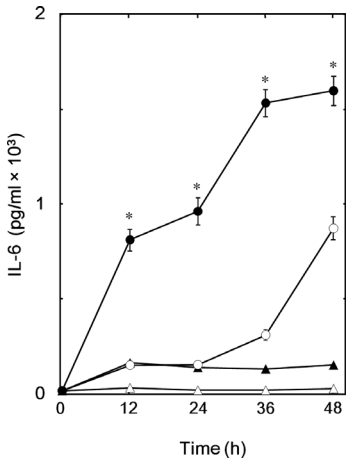

Figure 1. Effect of VIP on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. Cultured cells were pretreated with 50 nM VIP (●,▲) or vehicle (○, △) for 60 min, and then stimulated with 30 ng/ml TNF-α (circles) or vehicle (triangles) for indicated periods. Each value represents the mean ±SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p<0.05$, compared to the value of TNF-α alone.

Western blot analysis. Western blot analysis was performed as previously described (20). In brief, cultured cells were pre-treated with various doses of VIP for 60 min, and then stimulated by TNF-α in the presence of inhibitors in α-MEM containing 0.3% FCS 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/HCl; pH 6.8, 3% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli in 10% polyacrylamide gel (21). Protein (20 μg) was fractionated and transferred onto an Immun-Blot PVDF Membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween-20 (TBS-T; 20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 2 h before incubation with the primary antibodies. Western blot analysis was performed using phospho-specific Akt antibodies, Akt antibodies, phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies with peroxidase-labeled antibodies raised in goat anti-rabbit IgG which were used as second antibodies. Peroxidase activity on PVDF membranes was visualized on X-ray film by means of the ECL Western blotting detection system. All Western blot analyses were repeated at least three times in independent experiments. When indicated, cells were pretreated with H-89 for 60 min prior to VIP treatment.

Determinations. The absorbance of ELISA samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT).

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

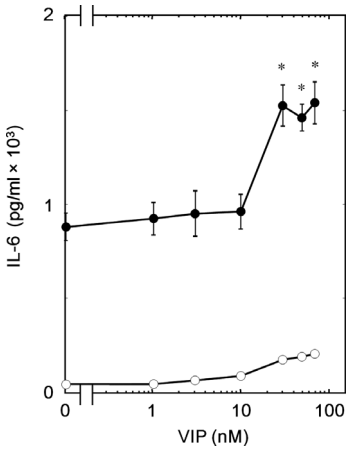

Figure 2. Effect of VIP on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. Cultured cells were pretreated with various doses of VIP for 60 min, and then stimulated with 30 ng/ml TNF-α (●) or vehicle (○) for 48 h. Each value represents the mean ±SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *p<0.05, compared to the value of TNF-α alone.

Table I. Effects of forskolin or 8-bromo-cAMP on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells.

Agent	TNF-α	IL-6 (pg/ml)
-	-	42±6
-	+	882±137*
Forskolin	-	72±4
Forskolin	+	4,786±173**
8-bromo-cAMP	-	112±11
8-bromo-cAMP	+	8,752±523**

Cultured cells were pretreated with 1 μM forskolin, 0.3 μM 8-bromo-cAMP or vehicle for 60 min, and then stimulated with 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 control. **p<0.05, compared to the value of TNF-α alone.

Results

Effect of VIP on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. We previously reported that TNF-α significantly stimulates IL-6 synthesis in osteoblast-like MC3T3-E1 cells (10). In addition, it has been shown that VIP induces IL-6 mRNA expression and protein release in calvarial osteoblasts (22). We first examined the effect of VIP on the TNF-α-stimulated IL-6 synthesis in MC3T3-E1 cells. VIP, which alone slightly stimulated IL-6 synthesis in MC3T3-E1 cells, significantly enhanced the TNF-α-induced IL-6 synthesis in a time-dependent manner up to 48 h (Fig. 1). The synergistic effect was significant 12 h later. The synergistic effect of VIP on the TNF-α-induced IL-6 synthesis was concentration-dependent in the range between 1 and 70 nM (Fig. 2). Maximum synergistic effect of VIP on IL-6 synthesis caused ~80% enhancement in the TNF-α effect (Fig. 2).

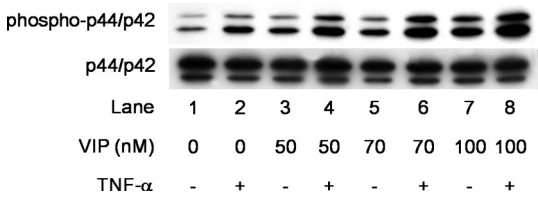

Figure 3. Effect of VIP on the TNF-α-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. Cultured cells were pretreated with various doses of VIP for 60 min, and then stimulated by 30 ng/ml TNF-α or vehicle for 10 min. Extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP or p44/p42 MAP kinases. Similar results were obtained with two additional and different cell preparations.

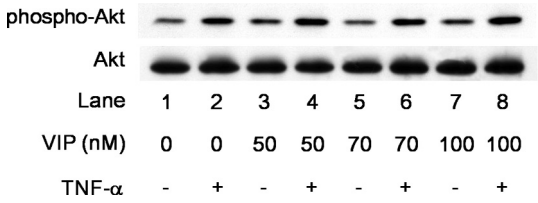

Figure 4. Effect of VIP on the TNF-α-induced phosphorylation of Akt in MC3T3-E1 cells. Cultured cells were pretreated with various doses of VIP for 60 min, and then stimulated with 30 ng/ml TNF-α or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific Akt or Akt kinase. Similar results were obtained with two additional and different cell preparations.

Effects of forskolin or 8bromo-cAMP on the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. We previously reported that VIP stimulates cAMP production in MC3T3-E1 cells (16). To clarify the role of the adenylyl cyclase-cAMP system in the TNF-α-induced IL-6 synthesis in these cells, we investigated the effects of forskolin or 8-bromo-cAMP on the TNF-α-induced IL-6 synthesis. Forskolin, a direct activator of adenylyl cyclase (23), which by itself slightly stimulated IL-6 synthesis in MC3T3-E1 cells, synergistically enhanced the TNF-α-induced IL-6 synthesis (Table I). In addition, 8-bromo-cAMP, a plasma membrane-permeable cAMP analogue, which alone slightly stimulates IL-6 synthesis, synergistically amplified the TNF-α-induced IL-6 synthesis, and the effect was greater than that of forskolin (Table I).

Effects of VIP on the phosphorylation of p44/p42 MAP kinase and Akt induced by TNF-α in MC3T3-E1 cells. We have previously shown that the p44/p42 MAP kinase and PI3-kinase/Akt pathways play a role in the TNF-α-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells (10-12). In order to elucidate whether the VIP effect on the TNF-α-induced IL-6 synthesis is mediated through p44/p42 MAP kinase activation and/or PI3-kinase/Akt activation in MC3T3-E1 cells, we next investigated the effects of VIP on the TNF-α-induced phosphorylation of p44/p42 MAP kinase or Akt in MC3T3-E1 cells. VIP markedly enhanced TNF-α-induced phosphorylation of p44/p42 MAP kinase in a dose-dependent manner (Fig. 3). On the contrary, VIP failed to affect the phosphorylation of Akt induced by TNF-α (Fig. 4).

Table II. Effect of PD98059 on the enhancement by VIP of TNF-α-induced IL-6 synthesis in MC3T3-E1 cells

PD98059	VIP	TNF-α	IL-6 (pg/ml)
-	-	-	34±7
-	-	+	768±115*
-	+	+	1,260±58**
+	+	+	537±26***

Cultured cells were preincubated with 50 μM PD98059 or vehicle for 60 min, and then pretreated with 50 nM VIP for 60 min. Cells were stimulated with 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 control. *p<0.05, compared to the value of TNF-α alone. ***p<0.05, compared to the value of TNF-α with VIP.

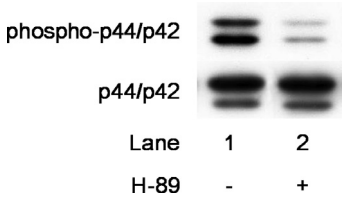

Figure 5. Effect of H-89 on the enhancement by VIP of TNF-α-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. Cultured cells were preincubated with 10 μM H-89 or vehicle for 60 min, and then pretreated by 100 nM VIP for 60 min. Cells were stimulated by 30 ng/ml TNF-α for 10 min. Extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP or p44/p42 MAP kinases. Similar results were obtained with two additional and different cell preparations.

Effect of PD98059 on the enhancement by VIP of the TNF-α-induced IL-6 synthesis in MC3T3-E1 cells. We examined the effect of PD98059, a specific inhibitor of MEK1/2, an upstream kinase that activates p44/p42 MAP kinase (24), on IL-6 synthesis induced by TNF-α in MC3T3-E1 cells in the absence or presence of VIP. We have previously 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). PD98059, which alone had little effect on the basal level of IL-6 synthesis (data not shown), markedly reduced the enhancement of IL-6 synthesis by VIP under the level of TNF-α alone (Table II). We have previously confirmed that PD98059 at the concentration used in this experiment is not toxic but specific to MEK1/2 (25).

Effect of H-89 on the enhancement by VIP of the TNF-α-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. We examined the effect of H-89, an inhibitor of cAMP-dependent protein kinase (protein kinase A) (26), on the enhancement by VIP of the TNF-α-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The enhancement by VIP of the TNF-α-induced phosphorylation of p44/p42 MAP kinase was markedly supressed by H-89 (Fig. 5).

Discussion

In the present study, we demonstrated that VIP synergistically enhanced IL-6 synthesis with TNF-α in osteoblast-like MC3T3-E1 cells. In addition, we also found that forskolin (23) or 8bromo-cAMP synergistically stimulated IL-6 synthesis with TNF-α in these cells. We previously reported that VIP stimulated cAMP production (16). Therefore, these findings suggest that VIP synergistically stimulates TNF-α-induced IL-6 synthesis through the adenylyl cyclase-cAMP system in osteoblast-like MC3T3-E1 cells.

It is well recognized that the MAP kinase superfamily plays an important role in cellular functions including proliferation, differentiation, and cell death in a variety of cells (27). Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK, are known as central elements used by mammalian cells to transduce the diverse messages. 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). Additionally, we have shown that TNF-α stimulates the IL-6 synthesis via PI3-kinase/Akt pathway independently of p44/p42 MAP kinase pathway in osteoblast-like MC3T3-E1 cells (12). In the present study, we showed that the phosphorylation of Akt stimulated by TNF-α was not affected by VIP (Fig. 3). It seems unlikely that VIP regulates the activation of Akt stimulated by TNF-α in MC3T3-E1 cells. On the other hand, we found that VIP, which by itself induced the phosphorylation of p44/p42 MAP kinase, markedly amplified the TNF-α-induced phosphorylation. It is probable that VIP up-regulates the activation of p44/p42 MAP kinase stimulated by TNF-α in osteoblast-like MC3T3-E1 cells. Furthermore, we also demonstrated that PD98059 (24) significantly suppressed the enhancement of IL-6 synthesis by VIP under the level of TNF-α alone. Therefore, taking these results into account, it is most likely that VIP synergistically enhances TNF-α-induced IL-6 synthesis through p44/p42 MAP kinase by up-regulating osteoblast-like MC3T3-E1 cells. To clarify whether protein kinase A mediates the phosphorylation of p44/p42 MAP kinase, we examined the effect of H-89 (26) on the enhancement by VIP of the TNF-α-induced phosphorylation of p44/p42 MAP kinase in these cells. The enhancement by VIP of TNF-α-induced phosphorylation of p44/p42 MAP kinase was markedly inhibited by H-89 (Fig. 5). Based on our findings as a whole, it is most probable that VIP stimulates cAMP production, and then protein kinase A activated by cAMP enhances the TNF-α-induced IL-6 synthesis through the activation of p44/p42 MAP kinase but not PI3-kinase/Akt in osteoblast-like MC3T3-E1 cells.

It is recognized that VIP is present in the peripheral nervous system including skeletal nerve fibers, which is involved in the bone remodeling processes (28). In addition, VIP is known as a regulator of inflammatory cytokine production in several cell types including osteoblasts (29). 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 suggest that VIP is implicated in the process of pathological bone resorption especially in inflammatory bone diseases in addition to bone remodeling. Osteoporosis involves multiple pathogenetic mechanisms,

clearly estrogen deficiency plays a crucial role in men and women (30). It is generally recognized that estrogen deficiency simulates the production of cytokines including TNF-α and IL-6 by hematopoietic cells such as lymphocytes (30). In addition, TNF-α may inhibit bone formation and stimulate bone resorption during bone remodeling, which causes bone loss (30). It has recently been reported that the blockade of TNF-α action prevents the acceleration of bone resorption after estrogen withdrawal in postmenopausal women (31). The signaling molecules contributing to TNF-α-induced IL-6 synthesis in osteoblasts might be therapeutic candidates for postmenopausal osteoporosis. Based on our present results, it is possible that the VIP signaling in osteoblasts is a therapeutic candidate for preventing pathological bone loss. Further investigation is necessary to clarify the exact mechanism of VIP in osteoblasts.

In conclusion, these results strongly suggest that VIP synergistically enhances TNF-α-stimulated IL-6 synthesis via up-regulating p44/p42 MAP kinase through the adenylyl cyclase-cAMP system in osteoblasts.

Acknowledgements

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