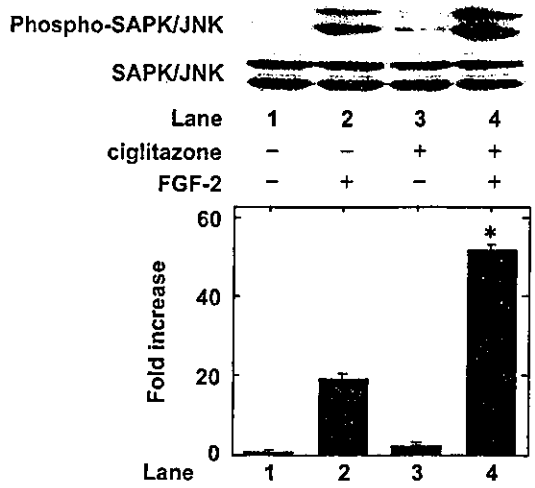

Fig. 3. Effect of ciglitazone on the phosphorylation of SAPK/JNK induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with 3 μM ciglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent 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. $*p < 0.05$, compared to the value of FGF-2 alone.

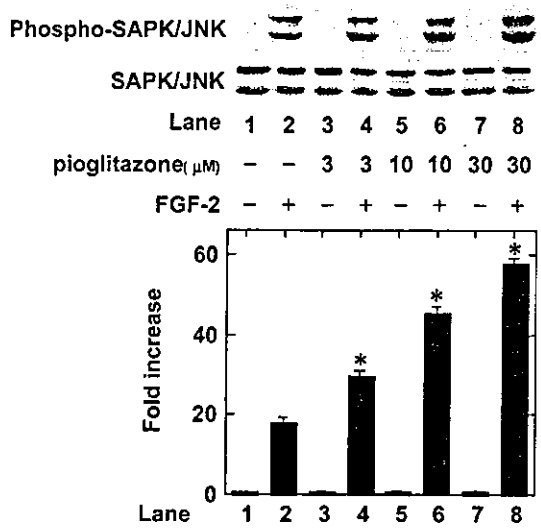

Fig. 4. Effect of pioglitazone on the phosphorylation of SAPK/JNK induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of pioglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE 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. $*p < 0.05$, compared to the value of FGF-2 alone.

through p44/p42 MAP kinase activation or SAPK/JNK in MC3T3-E1 cells, we next examined the effect of ciglitazone on the FGF-2-induced phosphorylation of p44/p42 MAP kinase. However, ciglitazone failed to affect the phosphorylation of p44/p42 MAP kinase induced by FGF-2 (Fig. 2). On the other hand, the FGF-2-induced SAPK/JNK phosphorylation was markedly amplified by ciglitazone (Fig. 3). According to the densitometric analysis, ciglitazone (3 μM) caused about 70% enhancement of the FGF-2-effect on the SAPK/JNK phosphorylation. In addition, pioglitazone enhanced the SAPK/JNK phosphorylation in a dose-dependent manner (Fig. 4). According to the densitometric analysis, pioglitazone (30 μM) caused about 80% enhancement of the FGF-2-effect on the SAPK/JNK phosphorylation.

Effect of ciglitazone on the phosphorylation of p38 MAP kinase induced by FGF-2 in MC3T3-E1 cells

We have previously reported that the FGF-2-stimulated VEGF release is negatively regulated by FGF-2-activated p38 MAP kinase in MC3T3-E1 cells [15]. In order to investigate whether PPAR-γ-effect on the FGF-2-stimulated VEGF release is mediated through p38 MAP kinase activation in MC3T3-E1 cells, we next

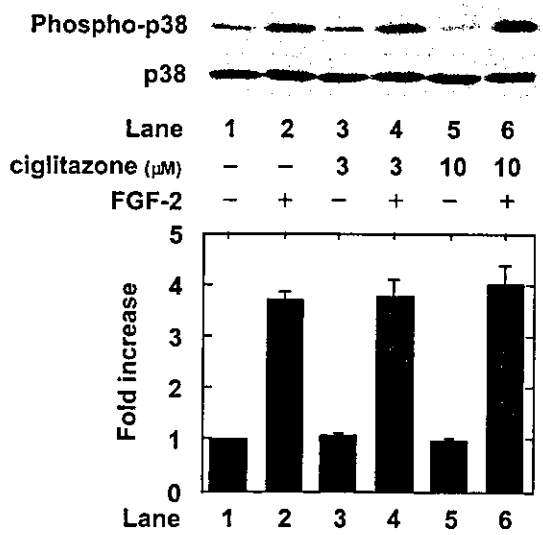

Fig. 5. Effect of ciglitazone on the phosphorylation of p38 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of ciglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 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.

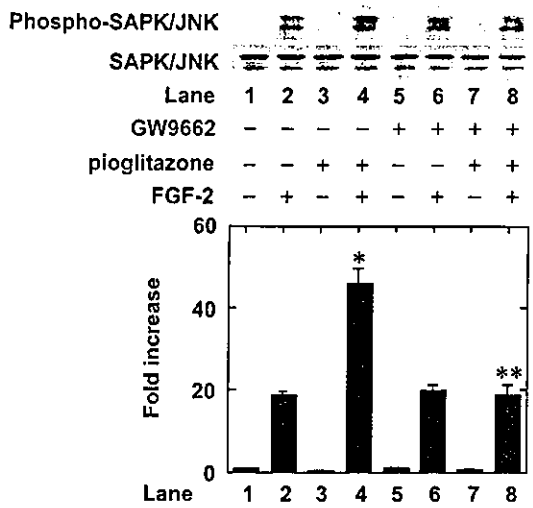

Fig. 6. Effect of GW9662 on the enhancement by pioglitazone of the FGF-2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM GW9662 or vehicle for 60 min, and then incubated by 10 μM pioglitazone for 8 h. The cells were stimulated by 30 ng/ml FGF-2 or vehicle for 24 h. 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. $^{*}p < 0.05$, compared to the value of FGF-2 alone. $^{**}p < 0.05$, compared to the value of FGF-2 with pioglitazone pretreatment.

examined the effect of ciglitazone on the FGF-2-induced phosphorylation of p38 MAP kinase. However, ciglitazone hardly affected the FGF-2-induced phosphorylation of p38 MAP kinase (Fig. 5).

Effect of GW9662 on the enhancement by pioglitazone of FGF-2-induced SAPK/JNK phosphorylation in MC3T3-E1 cells

To clarify whether the amplifying effect of ciglitazone or pioglitazone on FGF-2-induced SAPK/JNK phosphorylation is mediated through PPAR-γ in MC3T3-E1 cells, we examined the effect of GW9662 on the enhancement by pioglitazone. GW9662, which alone hardly affected the basal level of VEGF or the FGF-2-induced SAPK/JNK phosphorylation, suppressed the enhancement by pioglitazone almost completely to the levels of FGF-2 alone (Fig. 6).

Discussion

In the present study, we demonstrated that ciglitazone, a PPAR-γ-ligand, which by itself did not affect the levels of VEGF, significantly enhanced the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. In addition, pioglitazone, another ligand of PPAR-γ, amplified the VEGF release as well as ciglitazone. Thus, our findings suggest that the FGF-2-stimulated VEGF release is enhanced via the activation of PPAR-γ. To clarify whether the effects of ciglitazone and pioglitazone are mediated through PPAR-γ activation in MC3T3-E1 cells, we next examined the effect of GW9662, a PPAR-γ antagonist [22], on the amplification by ciglitazone. GW9662 significantly suppressed the ciglitazone-induced enhancement of VEGF release while it failed to affect the FGF-2-stimulated VEGF release. Taking these findings into account, it is most likely that activation of PPAR-γ amplifies the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells.

It is well recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions including proliferation, differentiation, and cell death in a variety of cells [17]. 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 [17]. In our previous studies [15,16], we have shown that FGF-2 activates three MAP kinases in osteoblast-like MC3T3-E1 cells, and p44/p42 MAP kinase and SAPK/JNK act as positive regulators in FGF-2-induced VEGF release while p38 MAP kinase negatively regulates the VEGF release. In the present study, we showed that ciglitazone did not affect the FGF-2-induced phosphorylation of p44/p42 MAP kinase. Thus, it seems unlikely that ciglitazone amplified the FGF-2-induced VEGF release through up-regulating the activation of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells. In addition, ciglitazone had little effect on the FGF-2-induced p38 MAP kinase phosphorylation. Therefore, it seems unlikely that the ciglitazone-induced enhancement of FGF-2-stimulated VEGF release is due to the inhibition of p38 MAP kinase activation.

On the contrary, we showed that the FGF-2-induced SAPK/JNK phosphorylation was markedly amplified by ciglitazone. Furthermore, pioglitazone dose-dependently strengthened the SAPK/JNK phosphorylation as well as ciglitazone. These results suggest that the PPAR-γ activation up-regulates the FGF-2-stimulated activation of SAPK/JNK. We next demonstrated that GW9662 [22] did not affect the SAPK/JNK phosphorylation induced by FGF-2 alone, but markedly suppressed the enhancement by pioglitazone almost to the levels of FGF-2 alone. Therefore, it is probable that the amplification in the SAPK/JNK phosphorylation is mediated through the activation of PPAR-γ. Based on our findings as a whole, it is most likely that PPAR-γ activation up-regulates FGF-2-stimulated VEGF release through enhancing the activation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. Further

investigations are required to elucidate the precise mechanism of PPAR-γ activation in the amplification of VEGF release.

It is generally recognized that the expansion of capillary network providing microvasculature is an essential process of bone remodeling [11]. Since VEGF is a specific mitogen of vascular endothelial cells [6], it is speculated that VEGF synthesized by osteoblasts acts as a crucial intercellular mediator between osteoblasts and vascular endothelial cells. 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 [7], supporting the importance of VEGF in bone metabolism. On the other hand, it is well known that PPAR-γ transcription factor determines the differentiation of progenitors into adipocytes [1]. In addition, a mesenchymal stem cell gives rise to adipocytes, osteoblasts, endothelial cells, and chondrocytes [23]. It has recently been reported that PPAR-γ is expressed also in osteoblasts including MC3T3-E1 cells [3]. Taking our results into account, it is probable that PPAR-γ ligand-enhanced VEGF release in osteoblasts plays an important role in the process of bone remodeling through regulating the capillary endothelial cells proliferation.

As for effects of PPAR-γ ligands on osteoblasts, it has recently been shown that PPAR-γ activators modulate osteoblast maturation such as alkaline phosphatase activity, Cbfa1 activity, and the expression of osteocalcin [3–5]. Therefore, it is probable that activation of PPAR-γ in osteoblasts functions as a pivotal role in bone metabolism. Further investigations are necessary to clarify the exact role of PPAR-γ activation in osteoblasts.

In conclusion, our present results strongly suggest that PPAR-γ activation up-regulates FGF-2-stimulated VEGF release via enhancing activation of SAPK/JNK among the MAP kinase superfamily in osteoblasts.

Acknowledgment

This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] M.J. Kersten, B. Desvergne, B.W. Wahli, Roles of PPARs in health and disease, Nature 405 (2000) 421–424.
[2] P.J. Nijweide, E.H. Burger, J.H.M. Feyen, Cells of bone: proliferation, differentiation, and humoral regulation, Physiol. Rev. 66 (1986) 855–886.
[3] S.M. Jackson, L.L. Demer, Peroxisome proliferator-activated receptor activators modulate the osteoblastic maturation of MC3T3-E1 preosteoblasts, FEBS Lett. 471 (2000) 119–124.
[4] M.J. Jeon, J.A. Kim, S.H. Kwon, S.W. Kim, K.S. Park, S.W. Park, S.Y. Kim, C.S. Shin, Activation of peroxisome proliferator-activated receptor-gamma inhibits the Runx2-mediated transcription of osteocalcin in osteoblasts, J. Biol. Chem. 278 (2003) 23270–23277.
[5] E. Khan, Y. Abu-Amer, Activation of peroxisome proliferator-activated receptor-gamma inhibits differentiation of preosteoblasts, J. Lab. Clin. Med. 142 (2003) 29–34.
[6] N. Ferrara, T. Davis-Smyth, The biology of vascular endothelial growth factor, Endocr. Rev. 18 (1997) 4–25.
[7] H.P. Gerber, T.H. Vu, A.M. Ryan, J. Kowalski, Z. Werb, N. Ferrara, VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation, Nat. Med. 5 (1999) 623–628.
[8] D.L. Goad, J. Rubin, H. Wang, A.H. Tashijian Jr., C. Patterson, Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I, Endocrinology 137 (1996) 2262–2268.
[9] D.S. Wang, K. Yamazaki, K. Nohtomi, K. Shizume, K. Ohsumi, M. Shibuya, H. Demura, K. Sato, Increase of vascular endothelial growth factor mRNA expression by 1,25-dihydroxyvitamin D_3 in human osteoblast-like cells, J. Bone Miner. Res. 11 (1996) 472–479.
[10] J.M. Schalaeppi, S. Gutzwiller, G. Finlenzeller, B. Fournier, 1,25-dihydroxyvitamin D_3 induces the expression of vascular endothelial growth factor in osteoblastic cells, Endocr. Res. 23 (1997) 213–229.
[11] A. Erlebacher, E.H. Filvaroff, S.E. Girelman, R. Derynck R, Toward a molecular understanding of skeletal development, Cell 80 (1995) 371–378.
[12] D.J. Baylink, R.D. Finkleman, S. Mohan, Growth factor to stimulate bone formation, J. Bone Miner. Res. 8 (1993) S565–S572.
[13] M.M. Hurley, C. Abreu, J.R. Harrison, A.C. Lichtler, L.G. Raisz, B.E. Kream, Basic fibroblast growth factor inhibits type I collagen gene expression in osteoblastic MC3T3-E1 cells, J. Biol. Chem. 268 (1993) 5588–5593.
[14] M.E. Bolander, Regulation of fracture repair by growth factors stimulate tyrosine kinase activity in vivo, Proc. Soc. Exp. Biol. Med. 200 (1992) 165–170.
[15] H. Tokuda, O. Kozawa, T. Uematsu, Basic fibroblast growth factor stimulates vascular endothelial growth factor release in osteoblasts: divergent regulation by p44/p42 mitogen-activated protein kinase and p38 mitogen-activated protein kinase, J. Bone Miner. Res. 15 (2000) 2371–2379.
[16] H. Tokuda, K. Hirade, X. Wang, Y. Oiso, O. Kozawa, Involvement of SAPK/JNK in basic fibroblast growth factor-induced VEGF release in osteoblasts, J. Endocrinol. 177 (2003) 101–107.
[17] C. Widmann, S. Gibson, M.B. Jarpe, G.L. Johnson, Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human, Physiol. Rev. 79 (1999) 143–180.
[18] H. Sudo, H. Kodama, Y. Amagai, S. Yamamoto, S. Kasai, In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria, J. Cell Biol. 96 (1983) 191–198.
[19] O. Kozawa, H. Tokuda, M. Miwa, J. Kotoyori, Y. Oiso, Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells, Exp. Cell Res. 198 (1992) 130–134.
[20] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[21] K. Kato, H. Ito, K. Hasegawa, Y. Inaguma, O. Kozawa, T. Asano, Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 glioma cells, J. Neurochem. 66 (1996) 946–950.

[22] L.M. Leesnitzer, D.J. Parks, R.K. Bledsoe, J.E. Cobb, J.L. Collins, T.G. Consler, R.G. Davis, E.A. Hull-Ryde, J.M. Lenhard, L. Patel, K.D. Plunket, J.L. Shenk, J.B. Stimmel, C. Therapontos, T.M. Willson, S.G. Blanchard, Functional consequences of cysteine modification in the ligand binding sites of peroxisome proliferator activated receptors by GW9662, Biochemistry 41 (2002) 6640–6650.
[23] J.E. Dennis, P. Charbord, Origin and differentiation of human and murine stroma, Stem Cells 20 (2002) 205–214.

ELSEVIER

Molecular and Cellular Endocrinology 229 (2005) 75–82

MCE
Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

Adenylyl cyclase-cAMP system inhibits thyroid hormone-stimulated osteocalcin synthesis in osteoblasts

Yosuke Kanno[a,b], Akira Ishisaki[a], Minoru Yoshida[a], Keiichi Nakajima[a], Haruhiko Tokuda[a,c], Osamu Numata[b], Osamu Kozawa[a,*]

[a] *Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan*
[b] *Institute of Biological Science, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan*
[c] *Department of Internal Medicine, Chubu National Hospital; National Institute for Longevity Sciences, Obu, Aichi 474-8511, Japan*

Received 7 January 2004; accepted 23 September 2004

Abstract

It is generally recognized that thyroid hormone modulates osteoblast cell function. We have previously shown that triiodothyronine (T_3) activates p38 mitogen-activated protein (MAP) kinase, resulting in the synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells. In the present study, we investigated the effect of the adenylyl cyclase-cAMP system on thyroid hormone-stimulated osteocalcin synthesis in these cells. Dibutyryl-cAMP (DBcAMP) reduced the osteocalcin synthesis stimulated by T_3. Forskolin and cholera toxin suppressed the osteocalcin synthesis while dideoxyforskolin, a forskolin derivative that does not activate adenylyl cyclase, had little effect on the synthesis. KT5720, a selective inhibitor of protein kinase A, reversed the inhibitory effect of forskolin or DBcAMP. DBcAMP and forskolin markedly reduced the phosphorylation of p38 MAP stimulated by T_3. Pituitary adenylate cyclase-activating polypeptide (PACAP) significantly inhibited the T_3-stimulated osteocalcin synthesis. These results strongly suggest that the adenylyl cyclase-cAMP system has an inhibitory role in thyroid hormone-stimulated osteocalcin synthesis via suppression of p38 MAP kinase activation in osteoblasts.

Keywords: Triiodothyronine; Osteocalcin; cAMP; Osteoblast

1. Introduction

Thyroid hormone is an important regulator of skeletal function, resulting in modulating bone metabolism and hyperthyroidism causes secondary osteoporosis (Khosla and Melton, 1995). In hyperthyroidism, the serum levels of alkaline phosphatase and osteocalcin, markers of osteoblast phenotype, and the excretion of pyridinoline and hydroxypyridinoline cross-link, which reflects bone resorption, are elevated (Stern, 1996). It is well known that both increased bone resorption and decreased bone formation contribute to the loss of bone mass by hyperthyroidism (Stern, 1996). Two functional cells, osteoblasts and osteoclasts mainly regulate bone metabolism (Nijweide et al., 1986). The former cells are responsible for bone formation and the latter for bone resorption. The receptor for triiodothyronine (T_3) has been shown to exist on osteoblasts (Stern, 1996). It has been reported that thyroid hormone stimulates alkaline phosphatase activity and secretion of osteocalcin and insulin-like growth factors in osteoblasts and that it modulates proliferation of osteoblasts (Stern, 1996; Rizzoli et al., 1986; Kasono et al., 1988). In a previous study (Tokuda et al., 1998), we have demonstrated that T_3 modulates interleukin-6 synthesis at two points in osteoblast-like MC3T3-E1 cells as follows; one is exerted at the point between adenylyl cyclase and protein kinase A, and the other is at a point downstream from protein kinase C activation. However, the exact mechanism of thyroid hormone in osteoblasts has not been fully clarified.

It is generally recognized that the receptor of thyroid hormone belongs to the nuclear hormone receptor superfamily (Evans, 1988). The effects of thyroid hormone, as well as

* Corresponding author. Tel.: +81 58 230 6214; fax: +81 58 230 6215.
E-mail address: okozawa@cc.gifu-u.ac.jp (O. Kozawa).

0303-7207/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2004.09.004

other steroid hormones, are exerted through binding to its specific intracellular receptors and subsequently activating the expression of the gene network (Evans, 1988). As for the intracellular signal transduction of thyroid hormone, it has recently been reported that the activation of p44/p42 mitogen-activated protein (MAP) kinase, which belongs to the MAP kinase superfamily (Widmann et al., 1999), is involved in the regulation of thyroid hormone-inhibited p53 transcriptional activity in human kidney cells (Shih et al., 2001). The MAP kinase superfamily plays pivotal roles in intracellular signaling of a variety of agonists to transduce the diverse messages (Widmann et al., 1999). In our recent studies (Kozawa et al., 2001; Ishisaki et al., 2004), we have demonstrated that the T_3 activates p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells and that p38 MAP kinase plays a part in T_3-stimulated synthesis of osteocalcin, an osteoblastic-specific phenotype marker (Ducy et al., 1996).

cAMP is produced from ATP by adenylyl cyclase and then activates cAMP-dependent protein kinase (protein kinase A) (Defer et al., 2000). It is generally recognized that the adenylyl cyclase-cAMP system plays a crucial role in osteoblast differentiation and proliferation (Siddhanti and Quarerles, 1994). We previously showed that pituitary adenylate cyclase-activating polypeptide (PACAP), a physiological agent for osteoblasts, stimulates cAMP production while having no effect on protein kinase C activation or Ca^{2+} mobilization in osteoblast-like MC3T3-E1 cells (Suzuki et al., 1994). In the present study, we investigated whether adenylyl cyclase-cAMP system affects the thyroid hormone-stimulated synthesis of osteocalcin in MC3T3-E1 cells. We herein show that adenylyl cyclase-cAMP system has an inhibitory role in T_3-stimulated osteocalcin synthesis in these cells.

2. Materials and methods

2.1. Materials

T_3, forskolin, dideoxyforskolin, dibutyryl-cAMP (DB-cAMP), cholera toxin were obtained from Sigma Chemical Co. (St. Louis, MO). Osteocalcin radioimmuassay (RIA) kit was obtained from Biomedical Technologies Inc. (Stoughton, MA). PACAP was obtained from Peptide Institute, Inc. (Minoh, Japan). KT5720 was purchased from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific p38 MAP kinase antibodies and p38 MAP kinase antibodies were obtained from New England BioLabs (Beverly, MA). An enhanced chemiluminescence (ECL) Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. T_3 was dissolved in 0.1 M NaOH. Forskolin, dideoxyforskolin, KT5720 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect assay for osteocalcin and Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells, which have been 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.

2.3. Assay for osteocalcin

The cultured cells were pretreated with forskolin, DB-cAMP, KT5720 or PACAP for 20 min, and then stimulated by T_3 in 1 ml of α-MEM containing 0.3% FCS, and then incubated for the indicated periods. The pretreatment with cholera toxin was performed for 2 h. The conditioned medium was collected, and osteocalcin in the conditioned medium was then measured by an osteocalcin RIA kit.

2.4. Western blot analysis

The cultured cells were pretreated with forskolin or DB-cAMP for 20 min, and then stimulated by T_3 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–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. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (Laemmli, 1970) in 10% polyacrylamide gels. Western blotting was performed as described previously (Kato et al., 1996) by using phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by use of an ECL Western blotting detection system. When indicated, the cells were pretreated with DBcAMP or forskolin for 20 min.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized as follows: 1 μg of DNase-treated total RNA together with 0.5 μg of oligo dT12-18 (Pharmacia, Uppsala, Sweden) in a total volume of 11 μl were heated to 70 °C for 10 min and then chilled on ice. A mix consisting of 4 ml of five times first strand cDNA buffer (Gibco-BRL, Gaithersburg, MD), 2 μl of 100 mM dithiothreitol, 1 μl of 10 mM dNTPs and 1 μl of RNase block (40 U/μl, Stratagene, La Jolla, CA) was added to the tube and heated at

42 °C for 2 min. SuperScriptk II RNase H-Reverse Transcriptase (Gibco-BRL) (200 U) was then added and the reaction was continued at 42 °C for 50 min. After a 15 min inactivation step at 70 °C, the cDNA was stored at −20 °C until use. RT-PCR was performed by co-amplification of the gene in question using cDNA template generated as described. The primer sequences for osteocalcin were as follows: 5′-TgC gCT CTg TCT CTC TgA CC-3′(sense) and 5′-CTg TgA CAT CCA TAC TTg Cag g-3′(antisense). PCR was carried out in a total volume of 10 μl containing 0.5 μl of cDNA solution, 0.5 U of Taq DNA polymerase (Sigma), 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of sense and antisense primers, 10 mM Tris–HCl, pH 8.3, and 50 mM KCl in a Robot Thermal Cycler (Stratagene) as follows: initial denaturation for 5 min at 94 °C, 29–35 cycles with denaturation at 94 °C for 30 s, annealing at 56–61 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension step for 5 min at 72 °C. After PCR, 7 μl of products was run on an agarose gel, stained with ethidium bromide and the intensity of bands was quantified using 1-D Image Analysis Software (Kodak Digital Sciencek, USA).

2.6. Determination

The radioactivity of radioimmunoassay samples was determined using a Wallac 1480 WIZARD3" automatic gamma counter (Turk, Finland). The densitometric analysis was performed using Molecular Analysis/Macintosh (Bio-Rad Laboratories, Hercules, CA).

2.7. Statistical analysis

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

3. Results

3.1. Effect of DBcAMP on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells

We have previously reported that T_3 activates p38 MAP kinase in osteoblast-like MC3T3-E1 cells, resulting in the synthesis of osteocalcin (Ishisaki et al., 2004). To clarify the role of the adenylyl cyclase-cAMP system in the thyroid hormone-stimulated osteocalcin in MC3T3-E1 cells, we examined the effects of each direct activator of the adenylyl cyclase-cAMP system on the osteocalcin synthesis. DBcAMP, a permeable analogue of cAMP, which alone did not affect the levels of osteocalcin in MC3T3-E1 cells, significantly suppressed the osteocalcin synthesis stimulated by T_3 (Fig. 1A). The inhibitory effect of DBcAMP on the T_3-stimulated osteocalcin synthesis was dose dependent in the range between 0.1 and 3 mM (Fig. 1B). The effect of DBcAMP was observed at 3 mM, a dose that caused about 80% reduction in the T_3-effect (Fig. 1B). We also examined the effect of DBcAMP on the T_3-induced mRNA for osteocalcin in MC3T3-E1 cells. DBcAMP markedly suppressed T_3-induced mRNA for osteocalcin (Fig. 1C).

Table 1
Effect of dideoxyforskolin on the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

	T_3	Osteocalcin (ng/ml)
−	−	5.0 ± 0.7
−	+	305.0 ± 28.2*
Dideoxyforskolin	−	4.3 ± 1.6
Dideoxyforskolin	+	301.2 ± 43.2
Forskolin	−	4.6 ± 3.5
Forskolin	+	122.0 ± 15.4**

The cultured cells were pretreated with 10 μM dideoxyforskolin, 10 μM forskolin or vehicle for 20 min, and then stimulated by 10 nM T_3 or vehicle for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared with the value of T_3 alone.

** $P < 0.05$, compared with the value of T_3 with forskolin-pretreatment.

3.2. Effects of forskolin or dideoxyforskolin on the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

Forskolin, a direct activator of adenylyl cyclase (Seamon and Daly, 1981), which by itself had little effect on the levels of osteocalcin, significantly reduced the T_3-stimulated osteocalcin synthesis (Fig. 2A). The inhibitory effect of forskolin on the T_3-stimulated osteocalcin synthesis was dose dependent in the range between 0.1 and 50 μM (Fig. 2B). The effect of forskolin was observed at 50 μM, a dose that caused about 80% reduction in the T_3-effect. We also examined the effect of forskolin on the T_3-induced mRNA for osteocalcin in MC3T3-E1 cells. Forskolin markedly suppressed T_3-induced mRNA for osteocalcin (Fig. 2C).

On the contrary, dideoxyforskolin (10 μM), a forskolin derivative that does not activate cAMP (Seamon et al., 1984), failed to suppress the osteocalcin synthesis while forskolin (10 μM) significantly reduced the synthesis (Table 1).

3.3. Effect of cholera toxin on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells

Cholera toxin, which alone did not affect the levels of osteocalcin, significantly inhibited the T_3-stimulated synthesis of osteocalcin (Table 2).

3.4. Effect of KT5720 on the inhibition by forskolin or DBcAMP in T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells

To clarify whether cAMP-dependent protein kinase (protein kinase A) mediates the inhibitory effect of forskolin

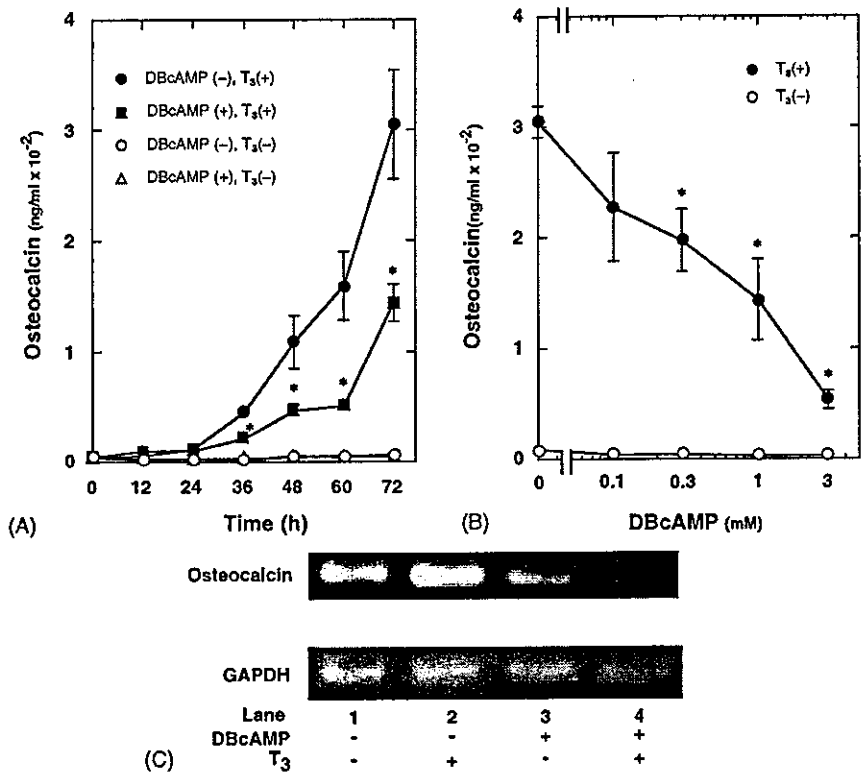

Fig. 1. Effect of DBcAMP on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells. (A) The cultured cells were pretreated with 1 mM DBcAMP (triangles) or vehicle (circles) for 20 min, and then stimulated by 10 nM T_3 (closed symbols) or vehicle (open symbols) for the indicated periods. (B) The cultured cells were pretreated with various dose of DBcAMP for 20 min, and then stimulated by 10 nM T_3 (closed circles) or vehicle (open circles) for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of T_3 alone. (C) Effect of DBcAMP on the T_3-induced levels of the mRNA for osteocalcin in MC3T3-E1 cells. The cultured cells were pretreated with 1 mM DBcAMP or vehicle for 20 min, and then stimulated by 10 nM T_3 or vehicle for 12 h.

or DBcAMP in osteoblast-like MC3T3-E1 cells, we next examined the effect of KT5720, a specific inhibitor of protein kinase A (Kase et al., 1987), on the inhibition by forskolin in the T_3-stimulated synthesis of osteocalcin. KT5720 significantly reversed the suppressive effect of forskolin on the T_3-stimulated synthesis of osteocalcin (Table 3). Additionally, the inhibition by DBcAMP of the osteocalcin synthesis was reversed by KT5720 (data not shown).

Table 2
Effect of cholera toxin on the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

Cholera toxin	T_3	Osteocalcin (ng/ml)
−	−	6.5 ± 0.4
−	+	304.6 ± 28.6*
+	−	4.1 ± 1.3
+	+	63.6 ± 11.5**

The cultured cells were pretreated with 1 μg/ml cholera toxin or vehicle for 6 h, and then stimulated by 10 nM T_3 or vehicle for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared with the value of T_3 alone.

** $P < 0.05$, compared with the value of T_3 with cholera toxin-pretreatment.

3.5. Effect of PACAP on the T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells

We previously showed that PACAP induces cAMP production in osteoblast-like MC3T3-E1 cells (Suzuki et al., 1994). Therefore, we investigated whether a physiological

Table 3
Effect of KT5720 on the inhibition by forskolin in the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

KT5720	Forskolin	T_3	Osteocalcin (ng/ml)
−	−	−	4.8 ± 2.4
−	−	+	306.2 ± 36.9
−	+	−	7.0 ± 3.2
−	+	+	34.6 ± 5.2*
+	−	−	6.3 ± 2.9
+	−	+	266.4 ± 28.1
+	+	−	3.8 ± 3.1
+	+	+	90.0 ± 6.6**

The cultured cells were pretreated with 10 μM KT5720 or vehicle for 20 min, and then incubated with 10 μM forskolin for 20 min. The cells were stimulated by 10 nM T_3 or vehicle for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared with the value of T_3 alone.

** $P < 0.05$, compared with the value of T_3 with forskolin-pretreatment.

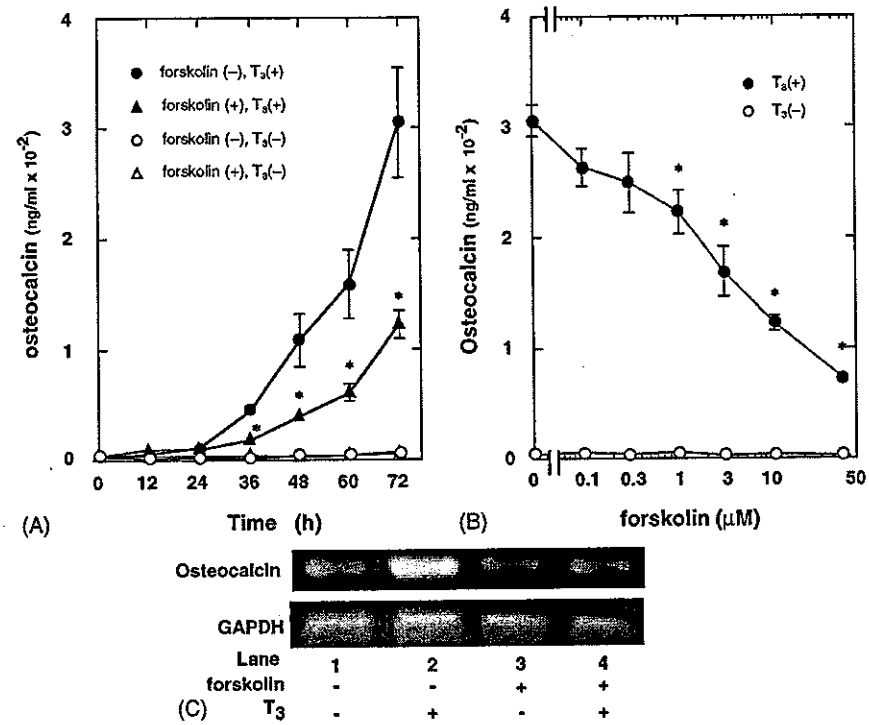

Fig. 2. Effect of forskolin on T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells. (A) The cultured cells were pretreated with 10 μM forskolin (triangles) or vehicle (circles) for 20 min, and then stimulated by 10 nM T_3 (closed symbols) or vehicle (open symbols) for the indicated periods. (B) The cultured cells were pretreated with various dose of forskolin for 20 min, and then stimulated by 10 nM T_3 (closed circles) or vehicle (open circles) for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P<0.05$, compared with the value of T_3 alone. (C) Effect of forskolin on the T_3-induced levels of the mRNA for osteocalcin in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM forskolin or vehicle for 20 min, and then stimulated by 10 nM T_3 or vehicle for 12 h.

agonist such as PACAP that stimulates cAMP production affects the T_3-stimulated synthesis of osteocalcin in these cells. PACAP actually significantly reduced the osteocalcin synthesis stimulated by T_3 (Table 4). PACAP (0.1 μM) caused a 35% reduction in the effect of T_3.

3.6. Effects of DBcAMP and forskolin on the T_3-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells

We previously showed that p38 MAP kinase acts as positive regulator in the T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells (Ishisaki et al., 2004). We already demonstrated that T_3 time dependently induces p38 MAP kinase phosphorylation in these cells (Ishisaki et al., 2004). Thus, we next investigated whether adenylyl cyclase-cAMP system affects the T_3-induced activation of p38 MAP kinase. DBcAMP markedly reduced the T_3-induced phosphorylation of p38 MAP kinase (Fig. 3A). In addition, forskolin suppressed the p38 MAP kinase phosphorylation by T_3 (Fig. 3B).

Table 4
Effect of PACAP on the T_3-stimulated osteocalcin synthesis in MC3T3-E1 cells

PACAP	T_3	Osteocalcin (ng/ml)
–	–	9.2 ± 4.4
–	+	305.2 ± 24.6*
+	–	8.5 ± 4.1
+	+	198.6 ± 39.8**

The cultured cells were pretreated with 0.1 μM PACAP or vehicle for 20 min, and then stimulated by 10 nM T_3 or vehicle for 72 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P<0.05$, compared with the value of T_3 alone.

** $P<0.05$, compared with the value of T_3 with PACAP-pretreatment.

4. Discussion

We have previously demonstrated that T_3 activates p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells and that p38 MAP kinase is involved in T_3-stimulated synthesis of osteocalcin (Kozawa et al., 2001; Ishisaki et al., 2004). In the present study, we showed that forskolin suppressed T_3-stimulated synthesis of osteocalcin in these cells. It is well known that forskolin is a direct activator of adenylyl cyclase (Seamon and Daly, 1981) and that we have shown that forskolin truly stimulates cAMP accumulation in MC3T3-E1 cells (Kozawa et al., 1992). In addition, dideoxyforskolin, which is a biologically inactive forskolin analogue (Seamon et al., 1984), failed to suppress the T_3-stimulated synthesis of osteocalcin. Thus, it seems that cAMP inhibits T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells. We next demonstrated that cholera

toxin, a direct and continuous activator of Gs (Gilman, 1987), reduced the T_3-stimulated synthesis of osteocalcin in these cells. It is well known that Gs, a heterotrimeric GTP-binding protein that mediates stimulative signals from the receptor to adenylyl cyclase, and the activation of adenylyl cyclase results in the production of cAMP (Gilman, 1987). Therefore, these results suggest that cAMP produced by adenylyl cyclase activation suppresses the osteocalcin synthesis stimulated by T_3 in MC3T3-E1 cells. Moreover, DBcAMP, a permeable analogue of cAMP, reduced the T_3-stimulated synthesis of osteocalcin. In a previous study (Suzuki et al., 1994), we have reported that PACAP stimulated cAMP accumulation by various doses in osteoblast-like MC3T3-E1 cells, and the effect of PACAP reached a maximum value at 10 nM. PACAP stimulated in the cAMP production without affecting the activity of protein kinase C in these cells. In addition, we found that PACAP had little effect on Ca^{2+} influx in these cells. Thus, we investigated whether PACAP, a physiological agonist for osteoblasts and aselective stimulator of cAMP production, extracellulary affects the synthesis of osteocalcin stimulated by T_3 in these cells. We demonstrated that PACAP truly reduced the T_3-stimulated synthesis of osteocalcin. Taking our findings into account, it is most likely that the adenylyl cyclase-cAMP system has an inhibitory role in T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells.

Parathyroid hormone (PTH) functions as a major mediator of bone remodeling as well as an essential regulator of calcium homeostasis (Strewler et al., 1987). PTH affects both cortical and cancellous bone mass and architecture, and PTH leads to increased bone resorption. PTH also activates rat osteocalcin transcription via cAMP-dependent protein kinase A pathway (Yu and Chandrasekhar, 1997). Thus, PTH is probably the most important agent that acts on osteoblasts through the cAMP signaling system. Therefore, we investigated that the effect of PTH on T_3-induced osteocalcin expression. However, PTH did not have an effect on T_3-stimulated synthesis of osteocalcin (data not shown). It is possible that PTH-dependent cAMP may have little effect on T_3-stimulated synthesis of osteocalcin in MC3T3-E1 cells.

In the present study, we showed that KT5720, a specific inhibitor of protein kinase A (Kase et al., 1987), salvaged the suppressive effect of forskolin on the T_3-stimulated osteocalcin synthesis. Thus, it seems that the inhibitory effect of forskolin is mediated through protein kinase A in MC3T3-E1 cells. In addition, we found that the suppression by DBcAMP of the T_3-stimulated synthesis of osteocalcin was reversed by KT5720. Therefore, our results strongly suggest that protein kinase A activated by cAMP negatively

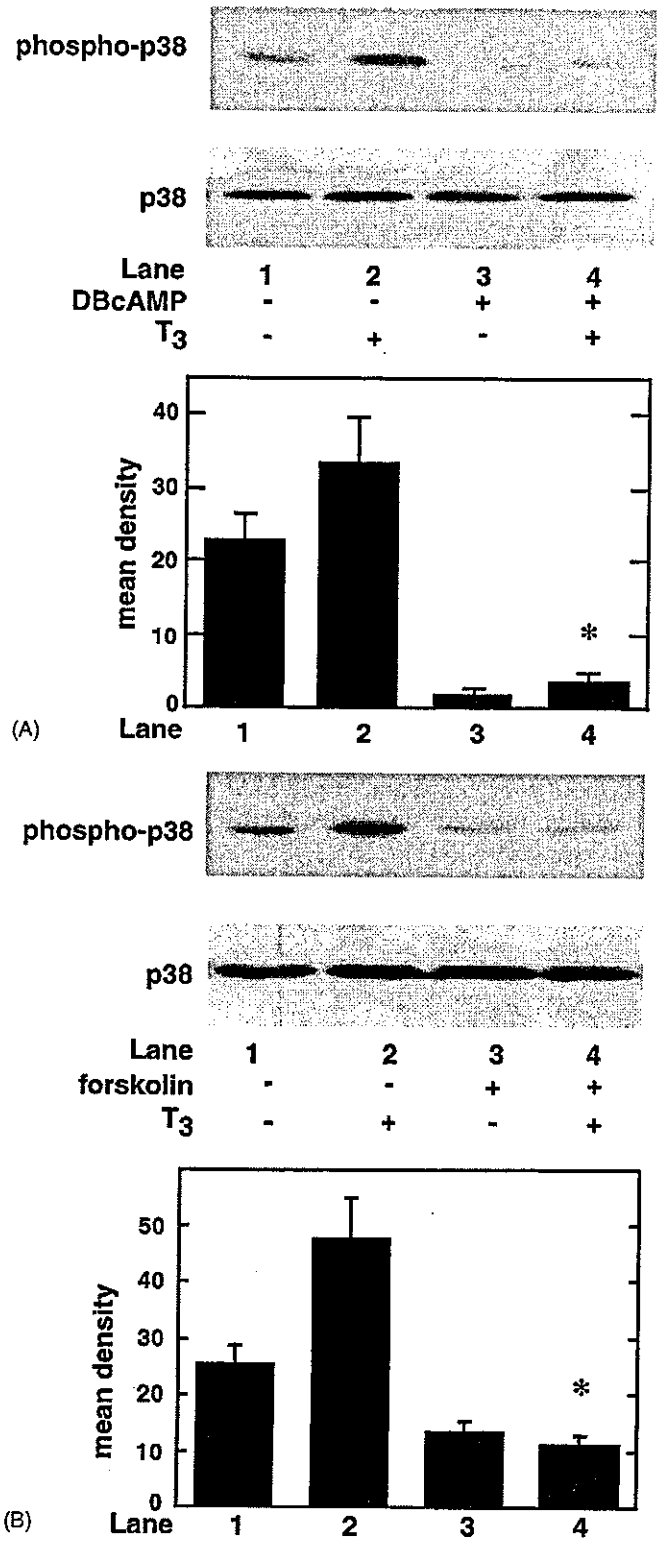

Fig. 3. Effects of DBcAMP or forskolin on T_3-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with (A) 1 mM DBcAMP or (B) 10 μM forskolin for 20 min, and then stimulated by 10 nM T_3 for 3 h. The extracts of cells were subjected to SDS–PAGE against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies. The histogram shows quantitative representations of the levels of T_3-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of T_3 alone.

regulates T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells.

We have shown that not p44/p42 MAP kinase but p38 MAP kinase among the MAP kinase superfamily plays a part in the T_3-stimulated synthesis of osteocalcin in osteoblast-like MC3T3-E1 cells (Ishisaki et al., 2004). To investigate whether the adenylyl cyclase-cAMP system affects T_3-induced p38 MAP kinase activation in these cells, we examined the effect of forskolin on the p38 MAP kinase phosphorylation stimulated by T_3. We showed that forskolin markedly reduced the phosphorylation of p38 MAP kinase induced by T_3. It is well known that MAP kinase is activated by phosphorylation on tyrosine and threonine by dual specificity MAP kinase (Raingeaud et al., 1995). These findings suggest that the adenylyl cyclase-cAMP system inhibits the T_3-induced activation of p38 MAP kinase at a point upstream from p38 MAP kinase in MC3T3-E1 cells. In addition, we found that the T_3-induced phosphorylation of p38 MAP kinase was truly inhibited by DBcAMP. Based on our results as a whole, it is most likely that T_3-stimulated osteocalcin synthesis is negatively regulated by protein kinase A at a point upstream from p38 MAP kinase in osteoblast-like MC3T3-E1 cells.

Osteocalcin is well known as an osteoblastic-specific marker, which is a γ-carboxylated, calcium-binding protein produced and secreted only by mature osteoblasts (Ducy et al., 1996; Stein and Lian, 1993). The levels of initially expressed osteocalcin are low and then up-regulated with time in cultured osteoblasts (Franchesi and Iyer, 1992). Osteocalcin is one of the most abundant noncollagenous proteins in the extracellular matrix of bone, and it contains glutamic acid residues (Shearer, 1997). Vitamin K is essential for carboxylation of glutamic acid residues, and Vitamin K promotes osteocalcin accumulation and mineralization in human osteoblasts (Koshihara et al., 1996; Koshihara and Hoshi, 1997). Osteocalcin-deficient mice develop hyperostosis (Ducy et al., 1996), suggesting that γ-carboxyglutamic acid (Gla)-containing osteocalcin promotes normal bone mineralization. In vitro and in vivo data provide conflicting insights into the role of osteocalcin in the regulation of biomineralization. When γ-carboxylated by a Vitamin K-dependent carboxylase, osteocalcin binds to bone mineral (apatite) with high affinity (Poser and Price, 1979). This affinity enables it to regulate the rate of hydroxyapatite crystal growth in solution (Boskey et al., 1985; Hauschka et al., 1983; Hunter et al., 1996; Romberg et al., 1986), suggesting that osteocalcin can affect mineral formation and mineral crystal growth. In culture, osteocalcin is chemotactic for osteoclasts (Malone et al., 1982), implying that it is involved in bone remodeling. Taking our findings into account, osteocalcin synthesis is negatively regulated by adenylyl cyclase-cAMP system. It has probable a crucial role as a regulator of bone metabolism.

In conclusion, these results strongly suggest that the adenylyl cyclase-cAMP system has an inhibitory role in thyroid hormone-stimulated osteocalcin synthesis via suppression of p38 MAP kinase activation in osteoblasts.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (09671041, 12470015) from the Ministry of Education, Science, Sports and Culture of Japan, the Research Grants for Longevity Sciences (15A-1 and 15C-2), Health and Labour Sciences Research Grants for Research on Dimentia and Fracture, and Research on Proteomics from the Ministry of Health, Labour and Welfare of Japan.

References

Boskey, A.L., Wians Jr., F.H., Hauschka, P.V., 1985. The effect of osteocalcin on in vitro lipid-induced hydroxyapatite formation and seeded hydroxyapatite growth. Calcif. Tissue Int. 37, 57–62.

Defer, N., Best-Belpomme, M., Hanoune, J., 2000. Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. Am. J. Physiol. Renal. Physiol. 279, 400–416.

Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., Karsenty, G., 1996. Increased bone formation in osteocalcin-deficient mice. Nature 382, 448–452.

Evans, R.M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240, 889–895.

Franchesi, RT., Iyer, B.S., 1992. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J. Bone Miner. Res. 7, 235–246.

Gilman, A.G., 1987. G protein: transducers of receptor-generated signals. Annu. Rev. Biochem. 56, 615–649.

Hauschka, P.V., Frenkel, J., DeMuth, R., Gundberg, C.M., 1983. Presence of osteocalcin and related higher molecular weight 4-carboxyglutamic acid-containing proteins in developing bone. J. Biol. Chem. 258, 176–182.

Hunter, G.K., Hauschka, P.V., Poole, A.R., Rosenberg, L.C., Goldberg, H.A., 1996. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. Biochem. J. 317, 59–64.

Ishisaki, A., Tokuda, H., Yoshida, M., Hirade, K., Kunieda, K., Hatakeyama, D., Shibata, T., Kozawa, O., 2004. Activation of p38 mitogen-activated protein kinase mediates thyroid hormone-stimulated osteocalcin synthesis in osteoblasts. Mol. Cell. Endocrinol. 214, 189–195.

Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A., Kaneko, M., 1987. K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem. Biophys. Res. Commun. 142, 436–440.

Kasono, K., Sato, K., Han, D.C., Fujii, Y., Tsushima, T., Shizume, K., 1988. Stimulation of alkaline phosphatase activity by thyroid hormone in mouse osteoblast-like (MC3T3-E1): a possible mechanism of hyperalkaline phosphatasis in hypewrthyroidism. Bone Miner. 4, 355–363.

Kato, K., Ito, H., Hasegawa, K., Inaguma, Y., Kozawa, O., Asano, T., 1996. Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 rat glioma cells. J. Neurochem. 66, 946–950.

Khosla, S., Melton III, L.J., 1995. Secondary osteoporosis. In: Riggs, L.G., Melton III, L.J. (Eds.), Osteoporosis; Etiology, Diagnosis and Management, second ed. Lippincott-Raven Publishiers, Philadelphia, pp 183–204.

Koshihara, Y., Hoshi, K., Ishibashi, H., Shiraki, M., 1996. Vitamin K2 promotes 1alpha 25(OH)2 vitamin D3-induced mineralization in human periosteal osteoblasts. Calcif. Tissue Int. 59, 466–473.

Koshihara, Y., Hoshi, K., 1997. Vitamin K2 enhances osteocalcin accumulation in the extracellular matrix of human osteoblasts in vitro. J. Bone Miner. Res. 12, 431–438.

Kozawa, O., Tokuda, H., Miwa, M., Kotoyori, J., Oiso, Y., 1992. Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E2 in osteoblast-like cells. Exp. Cell Res. 198, 130–134.

Kozawa, O., Hatakeyama, D., Yoshida, M., Kamiya, Y., Kondo, C., Matsuno, H., Uematsu, T., 2001. Activation of p44/p42 mitogen-activated protein kinase limits triiodothyronine-stimulated alkaline phosphatase activity in osteoblasts. Biochem. Biophys. Res. Commun. 286, 1140–1143.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Malone, J.D., Teitelbaum, S.L., Griffin, G.L., Senior, R.M., Kahn, A.J., 1982. Recruitment of osteoclast precursors by purified bone matrix constituents. J. Cell Biol. 92, 227–230.

Nijweide, P.J., Burger, E.H., Feyen, JH.M., 1986. Cells of bone: proliferation, differentiation, and hormonal regulation. Physiol. Rev. 66, 855–886.

Poser, J.W., Price, P.A., 1979. A method for decarboxylation of gamma-carboxyglutamic acid in proteins. Properties of the decarboxylated gamma-carboxyglutamic acid protein from calf bone. J. Biol. Chem. 254, 431–436.

Raingeaud, J., Guspa, S., Rogers, J.S., Dickens, M., Hans, J., Ulevitch, R.J., Davis, R.J., 1995. J. Biol. Chem. 270, 7420–7426.

Rizzoli, R., Poser, J., Burgi, U., 1986. Nuclear thyroid hormone receptor in cultured bone cells. Metabolism 35, 71–74.

Romberg, R.W., Werness, P.G., Riggs, B.L., Mann, K.G., 1986. Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. Biochemistry 25, 1176–1180.

Seamon, K., Daly, J.W., 1981. Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. J. Biol. Chem. 256, 9799–9801.

Seamon, K., Vaillancourt, R., Edwards, M., Daly, J.W., 1984. Binding of [^{3}H]forskolin to rat brain membranes. Proc. Natl. Acad. Sci. U.S.A. 81, 5081–5085.

Shearer, M.J., 1997. The roles of vitamins D and K in bone health and osteoporosis prevention. Proc. Nutr. Soc. 56, 915–937.

Shih, A., Lin, H.Y., Davis, F.B., Davis, P.J., 2001. Thyroid hormone promotes serine phosphorylation of p53 by mitogen-activated protein kinase. Biochemistry 40, 2870–2878.

Siddhanti, S.R., Quarerles, L.D., 1994. Molecular to pharmacologic control of osteoblast proliferation and differentiation. J. Cell Biochem. 55, 310–320.

Stein, G.S., Lian, J.B., 1993. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr. Rev. 14, 424–442.

Stern, P.H., 1996. Thyroid hormone and bone. In: Bilezikian, J.P., Raisz, L.G., Rodan, GA. (Eds.), Principles of Bone Biology. Academic Press, San Diego, pp. 521–531.

Strewler, G.J., Stern, P.H., Jacobs, J.W., Eveloff, J., Klein, R.F., Leung, S.C., Rosenblatt, M., Nissenson, R.A., 1987. Parathyroid hormone-like protein from human renal carcinoma cells Structural and functional homology with parathyroid hormone. J. Clin. Invest. 80, 1803–1807.

Sudo, H., Kodama, H., Amagai, Y., Yamamoto, S., Kasai, S., 1983. In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J. Cell Biol. 96, 191–198.

Suzuki, A., Kotoyori, J., Oiso, Y., Kozawa, O., 1994. Pituitary adenylate cyclase-activating polypeptide induces cAMP production independently from vasoactive intestinal polypeptide in osteoblast-like cells. Cell Signal 6, 11–16.

Tokuda, H., Kozawa, O., Harada, A., Isobe, K., Uematsu, T., 1998. Triiodothyronine modulates interleukin-6 synthesis in osteoblasts: inhibition in protein kinase A and C pathways. Endocrinology 139, 1300–1305.

Widmann, C., Gibson, S., Jarpe, M.B., Johnson, G.L., 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. 79, 143–180.

Yu, X.P., Chandrasekhar, S., 1997. Parathyroid hormone (PTH 1-34) regulation of rat osteocalcin gene transcription. Endocrinology 138, 3085–3092.

ELSEVIER

Molecular and Cellular Endocrinology 220 (2004) 89–95

MCE
Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

Involvement of SAPK/JNK in prostaglandin E_1-induced VEGF synthesis in osteoblast-like cells

Y. Kanno [a,b], H. Tokuda [a,c], K. Nakajima [a], A. Ishisaki [a], T. Shibata [d], O. Numata [b], O. Kozawa [a,*]

[a] *Department of Pharmacology, Gifu University School of Medicine, Gifu 500-8705, Japan*
[b] *Institute of Biological Science, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan*
[c] *Department of Internal Medicine, Chubu National Hospital, National Institute for Longevity Sciences, Obu, Aichi 474-8511, Japan*
[d] *Department of Oral and Maxillo-Facial Surgery, Gifu University School of Medicine, Gifu 500-8705, Japan*

Received 21 October 2003; accepted 25 March 2004

Abstract

We previously reported that prostaglandin E_1 (PGE_1) activates both p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase via cAMP-dependent protein kinase in osteoblast-like MC3T3-E1 cells, and that p38 MAP kinase but not p42/p44 MAP kinase is involved in PGE_1-induced synthesis of vascular endothelial growth factor (VEGF). In the present study, we investigated the involvement of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in the PGE_1-induced VEGF synthesis in MC3T3-E1 cells. PGE_1 induced the phosphorylation of SAPK/JNK. SP600125, a specific inhibitor of SAPK/JNK, markedly reduced the PGE_1-induced VEGF synthesis. Forskolin, a direct activator of adenylyl cyclase, elicited the phosphorylation of SAPK/JNK, and 8bromo-cAMP, a plasma membrane-permeable cAMP analogue-stimulated VEGF synthesis was significantly reduced by SP600125. SP600125 suppressed the PGE_1-induced phosphorylation of SAPK/JNK without affecting the phosphorylation of p38 MAP kinase induced by PGE_1. The phosphorylation of c-Jun induced by PGE_1 was also inhibited by SP600125. SB203580, a p38 MAP kinase inhibitor, failed to reduce the PGE_1 induced phosphorylation of SAPK/JNK. A combination of SP600125 and SB203580 suppressed the PGE_1-stimulated VEGF synthesis in an additive manner. These results strongly suggest that PGE_1 activates SAPK/JNK in osteoblasts, and that SAPK/JNK plays a part in PGE_1-induced VEGF synthesis.

Keywords: PGE_1; VEGF; SAPK/JNK; Osteoblast

1. Introduction

Vascular endothelial growth factor (VEGF) has been characterized as a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells (Ferrara and Davis-Smyth, 1997). It is well recognized that VEGF, which 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). Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption (Nijweide et al., 1986). Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in response to various humoral factors (Goad et al., 1996; Wang et al., 1996; Ferrara and Davis-Smyth, 1997; Schalaeppi 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. It is currently 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). Based on these findings, there

* Corresponding author. Tel.: +81-58-267-2233; fax: +81-58-267-2959.
E-mail address: okozawa@cc.gifu-u.ac.jp (O. Kozawa).

0303-7207/$ – see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2004.03.010

is no doubt that VEGF secreted from osteoblasts plays a pivotal role in the regulation of bone metabolism. However, the exact mechanism behind VEGF synthesis in osteoblasts has not yet been fully clarified.

Prostaglandins (PGs) are well known as autocrine/paracrine modulators of osteoblasts, and play important roles in their cell functions (Nijweide et al., 1986; Smith, 1986). Among them, PGE_1 reportedly stimulates cyclic AMP (cAMP) production and induces alkaline phosphatase activity, a marker of osteoblast phenotype (Robinson et al., 1973), in osteoblasts (Pilbeam et al., 1996). It has been shown that PGE_1 increases the levels of mRNA for VEGF and produces VEGF in primary cultured rat calvaria cells and RCT-3 osteoblast-like cells, and that cAMP mediates the synthesis of VEGF (Harada et al., 1994). The mitogen-activated protein (MAP) kinase superfamily is well-recognized to play crucial roles in the intracellular signaling of variety of agonists (Widmann et al., 1999). Three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) are known as central elements used by mammalian cells to transduce the various messages (Widmann et al., 1999). We previously reported that PGE_1 activates both p44/p42 MAP kinase and p38 MAP kinase via cAMP-dependent protein kinase (protein kinase A) in osteoblast-like MC3T3-E1 cells, and that p38 MAP kinase but not p44/p42 MAP kinase takes part in the VEGF synthesis induced by PGE_1 (Tokuda et al., 2001). However, the exact roles of SAPK/JNK in osteoblasts have not yet been clarified.

In the present study, we investigated whether SAPK/JNK is involved in the PGE_1-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. We here show that PGE_1 activates SAPK/JNK as well as p44/p42 MAP kinase and p38 MAP kinase in these cells, and that SAPK/JNK plays a part in the PGE_1 induced VEGF synthesis in addition to p38 MAP kinase.

2. Materials and methods

2.1. Materials

PGE_1, forskolin and 8bromo-cAMP were obtained from Sigma (St. Louis, MO). Mouse VEGF enzyme immunoassay kit was purchased from R & D Systems Inc. (Minneapolis, MN). SP600125 and SB203580 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific c-Jun antibodies and c-Jun 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. PGE_1 and forskolin were dissolved in ethanol. SP600125 or SB203580 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of ethanol or DMSO was 0.1%, which did not affect the assay for VEGF or the analysis of MAP kinases.

2.2. 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.

2.3. VEGF assay

The cultured cells were stimulated by PGE_1 or 8bromo-cAMP in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with SP600125 or SB203580 for 60 min. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

2.4. Analyses of MAP kinases and c-Jun

The cultured cells were stimulated by PGE_1 or forskolin in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lyzed, 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 SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific c-Jun antibodies, c-Jun antibodies, phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system. When indicated, the cells were pretreated with SP600125 or SB203580 for 60 min.

2.5. 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).

2.6. 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 ± S.E.M. of triplicate determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effect of PGE_1 on the phosphorylation of SAPK/JNK in MC3T3-E1 cells

We previously reported that PGE_1 activates p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells, and that p38 MAP kinase but not p44/p42 MAP kinase is involved in the VEGF synthesis induced by PGE_1 (Tokuda et al., 2001). To clarify whether PGE_1 activates SAPK/JNK in MC3T3-E1 cells, we examined the effect of PGE_1 on the SAPK/JNK phosphorylation. PGE_1 markedly induced the phosphorylation of SAPK/JNK in a time-dependent manner up to 60 min (Fig. 1).

3.2. Effect of SP600125 on the PGE_1-induced VEGF synthesis in MC3T3-E1 cells

To investigate whether SAPK/JNK is involved in the PGE_1-induced VEGF synthesis in MC3T3-E1 cells, we examined the effect of SP600125, a highly specific inhibitor of JNK on the synthesis (Bennett et al., 2001). SP600125, which alone hardly affected the basal level of VEGF, significantly reduced the PGE_1-induced VEGF synthesis (Fig. 2). The inhibitory effect of SP600125 on the PGE_1-induced VEGF synthesis was dose-dependent in the range between 1 and 50 μM (Fig. 3). The maximum inhibitory effect of SP600125 was observed at 50 μM, which caused about 85% reduction in the PGE_1-effect.

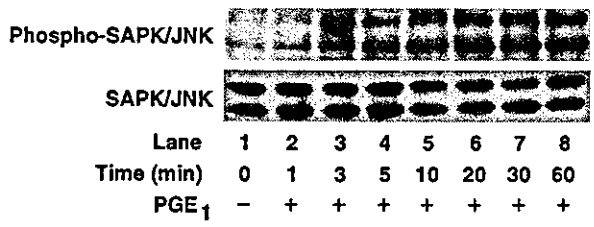

Fig. 1. Effect of PGE_1 on the phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were stimulated by 10 μM PGE_1 for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The upper bands and the lower ones indicated p54 and p46 SAPK/JNK, respectively. Lane 1, control cells.

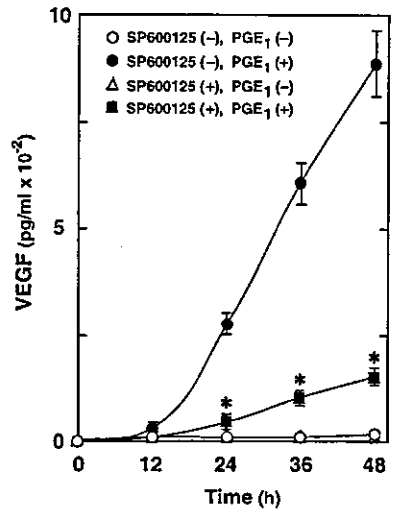

Fig. 2. Effect of SP600125 on the PGE_1-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM SP600125 (triangles) or vehicle (circles) for 60 min, and then stimulated by 10 μM PGE_1 (closed symbols) or vehicle (open symbols) for the indicated periods. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared to the value of PGE_1 alone.

3.3. Effects of SP600125 on the phosphorylation of SAPK/JNK or c-Jun induced by PGE_1 in MC3T3-E1 cells

We found that SP600125 truly inhibited the phosphorylation of SAPK/JNK induced by PGE_1 (Fig. 4). According to the densitometric analysis, SP600125 caused about 50% reduction of the PGE_1-effect on the SAPK/JNK phosphorylation.

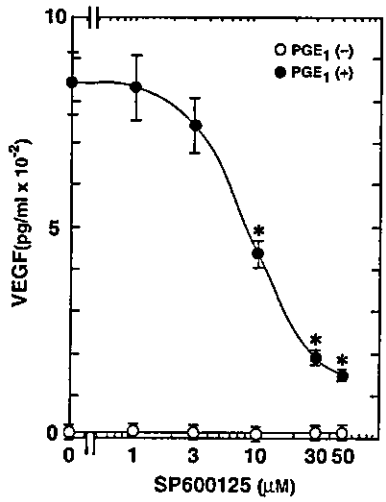

Fig. 3. Dose-dependent effect of SP600125 on the PGE_1-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SP600125 for 60 min, and then stimulated by 10 μM PGE_1 (closed circles) or vehicle (open circles) for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared to the value of PGE_1 alone.

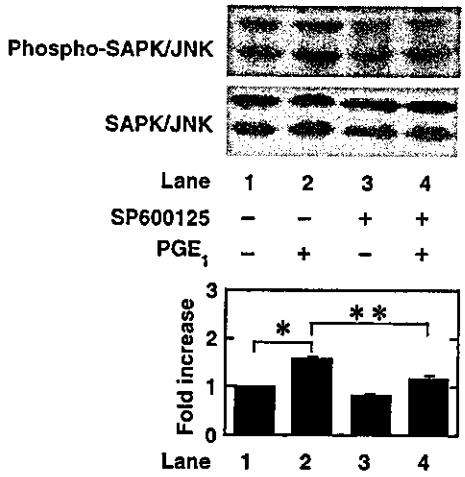

Fig. 4. Effect of SP600125 on the PGE_1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM PGE_1 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of PGE_1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. 'Fold increase' indicates the relative expression levels of phosphorylated SAPK/JNK after normalization with that of each total SAPK/JNK. $^*P < 0.05$, compared with the value of vehicle. $^{**}P < 0.05$, compared with the value of PGE_1 alone. Similar results were obtained with two additional and different cell preparations.

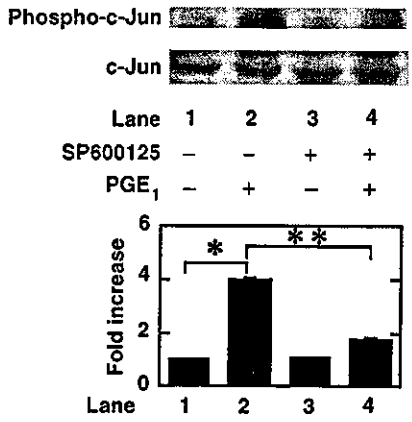

Fig. 5. Effect of SP600125 on the PGE_1-induced phosphorylation of c-Jun in MC3T3-E1 cells. The cultured cells were pretreated with 3 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM PGE_1 or vehicle for 60 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific c-Jun or c-Jun. The histogram shows quantitative representations of the levels of PGE_1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. 'Fold increase' indicates the relative expression levels of phosphorylated c-Jun after normalization with that of each total c-Jun. $^*P < 0.05$, compared with the value of vehicle. $^{**}P < 0.05$, compared with the value of PGE_1 alone. Similar results were obtained with two additional and different cell preparations.

In order to further clarify that SP600125 is actually effective at inhibiting SAPK/JNK, we firstly examine the effect of PGE_1 on c-Jun phosphorylation. PGE_1 time-dependently induced the phosphorylation of c-Jun and the maximum effect of PGE_1 was observed at 60 min after the stimulation (data not shown). We next examined the effect of SP600125 on the PGE_1-induced c-Jun phosphorylation, and found that the phosphorylation of c-Jun stimulated by PGE_1 was suppressed by SP600125 (Fig. 5). SP600125 elicited about 45% reduction of the PGE_1-effect on the c-Jun phosphorylation.

3.4. Effect of forskolin on the phosphorylation of SAPK/JNK and effect of SP600125 on 8bromo-cAMP-induced VEGF synthesis in MC3T3-E1 cells

We have previously shown that PGE_1 activates adenylyl cyclase, resulting in the formation of cAMP in osteoblast-like MC3T3-E1 cells and that PGE_1 activates both p44/p42 MAP kinase and p38 MAP kinase via cAMP-dependent protein kinase (protein kinase A) in these cells (Ito et al., 1996; Tokuda et al., 2001). We next examined the effect of forskolin, a direct activator of adenylyl cyclase (Seamon and Daly, 1981), on the phosphorylation of SAPK/JNK in these cells. Forskolin-induced time-dependent phosphorylation of SAPK/JNK was seen up to 20 min, after which it decreased (Fig. 6).

In addition, we examined the effect of SP600125 on the VEGF synthesis induced by 8bromo-cAMP, a plasma membrane-permeable cAMP analogue. We have demonstrated that 8bromo-cAMP alone stimulates VEGF synthesis in MC3T3-E1 cells (Tokuda et al., 2001). SP600125 significantly reduced the 8bromo-cAMP-induced VEGF synthesis (Table 1).

3.5. Effect of SP600125 on the phosphorylation of p38 MAP kinase induced by PGE_1 in MC3T3-E1 cells

SP600125 did not suppress the PGE_1-induced phosphorylation of p38 MAP kinase (Fig. 7). According to the densitometric analysis, SP600125 slightly but significantly enhanced the phosphorylation of p38 MAP kinase.

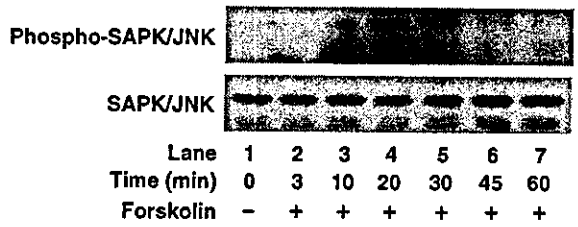

Fig. 6. Effect of forskolin on the phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were stimulated by 50 μM forskolin for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The upper bands and the lower ones indicated p54 and p46 SAPK/JNK, respectively. Lane 1, control cells.

Table 1
Effect of SP600125 on the 8bromo-cAMP-stimulated VEGF synthesis in MC3T3-E1 cells

SP600125	8bromo-cAMP	VEGF (pg/ml)
−	−	14 ± 2
−	+	258 ± 23
+	−	13 ± 2
+	+	173 ± 14*

The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then stimulated by 0.3 mM 8bromo-cAMP or vehicle for 24 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared to the value of 8bromo-cAMP alone.

3.6. Effect of SB203580 on the PGE_1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells

We previously reported that SB203580, a specific inhibitor of p38 MAP kinase (Cuenda et al., 1995), significantly reduced both VEGF synthesis and the phosphorylation of p38 MAP kinase induced by PGE_1 in MC3T3-E1 cells (Tokuda et al., 2001). Therefore, we examined the effect of SB203580 on the PGE_1 induced SAPK/JNK phosphorylation in these cells. However, SB203580 did not reduce the PGE_1 induced phosphorylation of SAPK/JNK (Fig. 8).

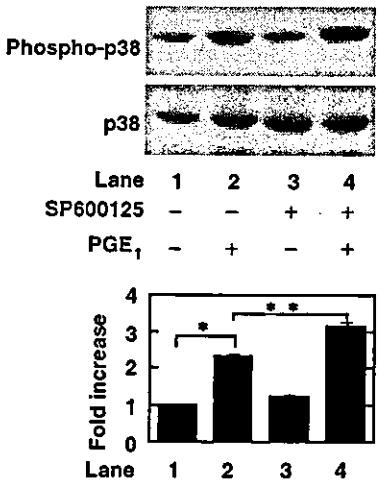

Fig. 7. Effect of SP600125 on the PGE_1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM PGE_1 or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of PGE_1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. 'Fold increase' indicates the relative expression levels of phosphorylated p38 MAP kinase after normalization with that of each total p38 MAP kinase. * $P < 0.05$, compared with the value of vehicle. ** $P < 0.05$, compared with the value of PGE_1 alone. Similar results were obtained with two additional and different cell preparations.

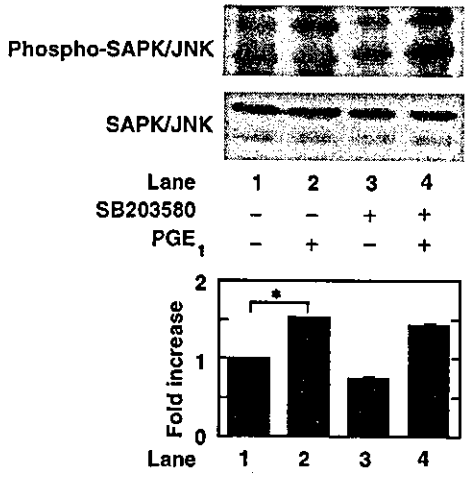

Fig. 8. Effect of SB203580 on the PGE_1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min, and then stimulated by 10 μM PGE_1 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of PGE_1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. 'Fold increase' indicates the relative expression levels of phosphorylated SAPK/JNK after normalization with that of each total SAPK/JNK. * $P < 0.05$, compared with the value of vehicle. Similar results were obtained with two additional and different cell preparations.

3.7. Effect of a combination of SP600125 and SB203580 on the PGE_1-stimulated VEGF synthesis in MC3T3-E1 cells

We further examined the effect of a combination of SP600125 and SB203580 on the PGE_1-stimulated VEGF synthesis in MC3T3-E1 cells. A combination of SP600125 and SB203580 significantly reduced the PGE_1-stimulated VEGF synthesis in an additive manner (Table 2).

Table 2
Effect of a combination of SP600125 and SB203580 on the PGE_1-stimulated VEGF synthesis in MC3T3-E1 cells

SP600125	SB203580	PGE_1	VEGF (pg/ml)
−	−	−	16 ± 2
−	−	+	842 ± 75
−	+	−	13 ± 3
−	+	+	308 ± 27*
+	−	−	14 ± 2
+	−	+	435 ± 38*
+	+	−	13 ± 2
+	+	+	55 ± 5**

The cultured cells were pretreated with 10 μM SP600125, 30 μM SB203580 or vehicle for 60 min, and then stimulated by 10 mM PGE_1 or vehicle for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared to the value of PGE_1 alone.

** $P < 0.05$, compared to the value of PGE_1 with SB203580 or SP600125.

4. Discussion

In the present study, we demonstrated that PGE_1 induces the phosphorylation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. It is currently understood that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions including proliferation, differentiation and cell death in a variety of cells (Widmann et al., 1999). Three major MAP kinase, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK are known as central elements used by mammalian cells to transducer the diverse messages (Widmann et al., 1999). We have previously shown that PGE_1 activates both p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells (Tokuda et al., 2001). It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase (Raingeaud et al., 1995; Widmann et al., 1999). Therefore, these results strongly suggest that PGE_1 activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. This is probably the first report showing the PGE_1-induced SAPK/JNK activation in osteoblasts as far as we know.

Herein, we showed that SP600125, a specific inhibitor of SAPK/JNK (Bennett et al., 2001), significantly suppressed the PGE_1-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. In addition, we found that SP600125 truly attenuated the phosphorylation of SAPK/JNK induced by PGE_1 in these cells. We previously reported that activation of p38 MAP kinase plays as a positive regulator in the PGE_1-induced VEGF synthesis in MC3T3-E1 cells (Tokuda et al., 2001). However, SP600125 did not suppress but rather enhanced the PGE_1-induced phosphorylation of p38 MAP kinase. On the other hand, we here showed that SP600125 reduced the c-Jun phosphorylation elicited by PGE_1 in these cells. Thus, it is probable that the suppression by SP600125 of PGE_1-stimulated VEGF synthesis is truly due to the inhibition of SAPK/JNK activation in MC3T3-E1 cells. These results strongly suggest that the activation of SAPK/JNK is involved in PGE_1-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. In addition, we showed that SB203580, a specific inhibitor of p38 MAP kinase (Cuenda et al., 1995), did not reduce the levels of the phosphorylation of PGE_1-induced SAPK/JNK. Furthermore, the PGE_1-stimulated VEGF synthesis was suppressed additively by a combination of SP600125 and SB203580. Therefore, our findings suggest that SAPK/JNK participates at least in part in the PGE_1-stimulated VEGF synthesis independently of p38 MAP kinase. Taking these findings into account, it is most likely that the VEGF synthesis stimulated by PGE_1 is mediated through the activation of both p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells.

It has been reported that PGE_1 increases mRNA for VEGF and the synthesis through the cAMP production in primary cultured rat calvaria cells and RCT-3 osteoblast-like cells (Harada et al., 1994). We previously reported that PGE_1 induces VEGF synthesis via activation of protein kinase A in osteoblast-like MC3T3-E1 cells (Tokuda et al., 2001). Herein, we demonstrated that forskolin, a direct activator of adenylyl cyclase (Seamon and Daly, 1981), induced the phosphorylation of SAPK/JNK in these cells, and that SP600125 truly reduced the 8bromo-cAMP-stimulated VEGF synthesis. These results suggest that SAPK/JNK acts at a point downstream from protein kinase A, and plays a pivotal role in PGE_1-induced VEGF synthesis. Based on our findings as a whole, it is most likely that PGE_1 induces VEGF synthesis through the protein kinase A-dependent activation of both p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells.

The expansion of capillary network providing microvasculature is an essential process of bone remodeling (Erlebacher et al., 1995). Since VEGF is a specific mitogen of vascular endothelial cells (Ferrara and Davis-Smyth, 1997), it is probable that VEGF synthesized by osteoblasts acts as an important intercellular mediator between osteoblasts and vascular endothelial cells. 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 importance of VEGF in bone metabolism. PGs are known to be autocrine/paracrine modulators of osteoblasts (Nijweide et al., 1986, Smith, 1986). It is well established that PGs of the E series are the most potent activators of bone resorption in organ cultures (Pilbeam et al., 2002). The clinical use of aspirin or non-steroidal anti-inflammatory drugs, which inhibits the PGs synthesis, has been found to be associated with significant increase in bone mineral density (Bauer et al., 1996). In addition, it is recognized that both resorption and formation of bone are impaired in cyclooxygenase-2 null mice (Pilbeam et al., 2002). These findings indicate the crucial roles of PGs in the bone remodeling process. Although PGE_1 is not a physiological but a synthetic prostaglandin, the effect is known to be mediated through EP receptors (Narumiya et al., 1999). Among them, EP_2 and EP_4 are known to be coupled to increase cAMP levels (Narumiya et al., 1999), and both of them reportedly exist in MC3T3-E1 cells (Suda et al., 1996). Using the pharmacological tools, we have previously shown that not EP_2 but EP_4 possibly mediates the PGE_1-induced VEGF synthesis in these cells (Tokuda et al., 2001). From the knock-out mice studies (Li et al., 2000; Miyaura et al., 2000), it is recognized that EP_2 and EP_4 in osteoblasts are involved in the osteoclastogenesis, resulting in bone resorption. In addition to the well-established role to migrate vascular endothelial cells, it has recently been reported that VEGF induces osteoclast chemotaxix (Henriksen et al., 2003). Taking these findings into account as a whole, it is probable that VEGF synthesis by osteoblasts through the stimulation of EP_4 and/or EP_2 plays an important role in the bone remodeling process even under the physiological conditions. Further investigations would be required to clarify the details.

In conclusion, our present results strongly suggest that PGE_1 activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblasts, and that SAPK/JNK plays as a positive regulator at least in part in PGE_1-induced VEGF synthesis.

Acknowledgements

This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

References

Bauer, D.C., Orwoll, E.S., Fox, K.M., Vogt, T.M., Lane, N.E., Hochberg, M.C., Stone, K., Nevitt, M.C., 1996. Aspirin and NSAID use in older women: effect on bone mineral density and fracture risk. Study of Osteoporotic Fractures Research Group. J. Bone Miner. Res. 11, 29–35.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., Anderson, D.W., 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal Kinase. Proc. Natl. Acad. Sci. U.S.A. 98, 13681–13686.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., Lee, J.C., 1995. SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. 364, 229–233.

Erlebacher, A., Filvaroff, E.H., Girelman, S.E., Derynck, R., 1995. Toward a molecular understanding of skeletal development. Cell 80, 371–378.

Ferrara, N., Davis-Smyth, T., 1997. The biology of vascular endothelial growth factor. Endocr. Rev. 18, 4–25.

Gerber, H.-P., Vu, T.H., Ryan, A.M., Kowalski, J., Werb, Z., Ferrara, N., 1999. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med. 5, 623–628.

Goad, D.L., Rubin, J., Wang, H., Tashijian Jr., A.H., Patterson, C., 1996. Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinology 137, 2262–2268.

Harada, S., Nagy, J.A., Sullivan, K.A., Thomas, K.A., Endo, N., Rodan, G.A., 1994. Induction of vascular endothelial growth factor expression by prostaglandin E_2 and E_1 in osteoblasts. J. Clin. Invest. 93, 2490–2496.

Henriksen, K., Karsdal, M., Delaiss, J.-M., Engsig, M.T., 2003. RANKL and vascular endothelial growth factor (VEGF) induce osteoclast chemotaxis through an ERK1/2-dependent mechanism. J. Biol. Chem. 278, 48745–48753.

Ito, Y., Suzuki, A., Watanabe-Tomita, Y., Oiso, Y., Kozawa, O., 1996. Okadaic acid enhances prostaglandin E_1 induced alkaline phosphatase activity in osteoblast-like cells: regulation at a point downstream from protein kinase A. Prostaglandins Leukot. Essent. Fatty Acids 55, 357–361.

Kato, K., Ito, H., Hasegawa, K., Inaguma, Y., Kozawa, O., Asano, T., 1996. Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 glioma cells. J. Neurochem. 66, 946–950.

Kozawa, O., Tokuda, H., Miwa, M., Kotoyori, J., Oiso, Y., 1992. Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells. Exp. Cell Res. 198, 130–134.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Li, X., Okada, Y., Pilbeam, C.C., Lorenzo, J.A., Kennedy, C.R.J., Breyer, R.M., Raisz, L.G., 2000. Knockout of the murine prostaglandin EP_2 receptor impairs osteoclastogenesis in vitro. Endocrinology 141, 2054–2061.

Miyaura, C., Inada, M., Suzawa, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., Narumiya, S., Suda, T., 2000. Impaired bone resorption to prostaglandin E receptor EP4-knockout mice. J. Biol. Chem. 275, 19819–19823.

Narumiya, S., Sugimoto, Y., Ushikubi, F., 1999. Prostanoid receptors: structures properties and functions. Physiol. Rev. 79, 1193–1226.

Nijweide, P.J., Burger, E.H., Feyen, J.H.M., 1986. Cells of bone: proliferation, differentiation, and humoral regulation. Physiol. Rev. 66, 855–886.

Pilbeam, C.C., Harrison, J.R., Raisz, L.G., 1996. Prostaglandins and bone metabolism. In: Bilezikian, J.P., Raisz, L.G., Rodan, G.A. (Eds.), Principles of Bone Biology. Academic Press Inc., San Diego, pp. 715–728.

Pilbeam, C.C., Harrison, J.R., Raisz, L.G., 2002. Prostaglandins and bone metabolism. In: Bilezikian, J.P., Raisz, L.G., Rodan, G.A. (Eds.), Principles of Bone Biology, second ed. Academic Press Inc., San Diego, pp. 979–994.

Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J., David, R.J., 1995. Pro-inflammatory cytokines and enviromental stress cause p38 MAP kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. 270, 7420–7426.

Robinson, R.J., Doty, S.B., Cooper, R.R., 1973. Electron microscopy of mammalian bone. In: Zipkin, I. (Ed.), Biological Mineralization. Academic Press Inc., New York, pp. 257–296.

Schalaeppi, J.M., Gutzwiller, S., Finlenzeller, G., Fournier, B., 1997. 1,25-dihydroxyvitamin D_3 induces the expression of vascular endothelial growth factor in osteoblastic cells. Endocr. Res. 23, 213–229.

Seamon, K.B., Daly, J.W., 1981. Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res. 7, 201–224.

Smith, W.L., 1986. The eicosanoids and their biochemical mechanism of action. Biochem. J. 259, 315–324.

Suda, M., Tanaka, K., Natsui, K., Uui, T., Tanaka, I., Fukushima, M., Shigeno, C., Konishi, J., Narumiya, S., Ichikawa, A., Nakao, N., 1996. Prostaglandin E receptor subtypes in mouse osteoblastic cell line. Endocrinology 137, 1698–1705.

Sudo, H., Kodama, H., Amagai, Y., Yamamoto, S., Kasai, S., 1983. In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J. Cell Biol. 96, 191–198.

Tokuda, H., Kozawa, O., Miwa, M., Uematsu, T., 2001. p38 Mitogen-activated protein (MAP) kinase but not p44/p42 MAP kinase is involved in prostaglandin E1-induced vascular endothelial growth factor (VEGF) synthesis in osteoblasts. J. Endocrinol. 170, 629–638.

Wang, D.S., Yamazaki, K., Nohtomi, K., Shizume, K., Ohsumi, K., Shibuya, M., Demura, H., Sato, K., 1996. Increase of vascular endothelial growth factor mRNA expression by 1,25-dihydroxyvitamin D_3 in human osteoblast-like cells. J. Bone Miner. Res. 11, 472–479.

Widmann, C., Gibson, S., Jarpe, M.B., Johnson, G.L., 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. 79, 143–180.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Prostaglandins, Leukotrienes and Essential Fatty Acids 70 (2004) 441–447
Prostaglandins Leukotrienes Essential Fatty Acids
www.elsevier.com/locate/plefa

Involvement of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) in prostaglandin $F_{2\alpha}$-induced heat shock protein 27 in osteoblasts

H. Tokuda[a,b], M. Niwa[b], A. Ishisaki[b], K. Nakajima[b], H. Ito[c], K. Kato[c], O. Kozawa[b,*]

[a] *Department of Internal Medicine, Chubu National Hospital, National Institute for Longevity Sciences, Obu, Japan*
[b] *Department of Pharmacology, Gifu University School of Medicine, Gifu 500-8705, Japan*
[c] *Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Japan*

Received 23 June 2003; accepted 18 September 2003

Abstract

We have reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) activates p44/p42 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells, and that p44/p42 MAP kinase plays a role in the $PGF_{2\alpha}$-induced heat shock protein 27 (HSP27). In the present study, we investigated the involvement of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), a member of the MAP kinase superfamily, in $PGF_{2\alpha}$-induced HSP27 in MC3T3-E1 cells. $PGF_{2\alpha}$ time dependently induced the phosphorylation of SAPK/JNK. SP600125, a specific inhibitor of SAPK/JNK, markedly reduced the $PGF_{2\alpha}$-stimulated HSP27 accumulation. The inhibitory effect of SP600125 was dose dependent in the range between 0.1 and 30 μM. SP600125 reduced the $PGF_{2\alpha}$-increased level of HSP27 mRNA. SP600125 suppressed the phosphorylation of SAPK/JNK induced by $PGF_{2\alpha}$, but did not affect the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase. On the other hand, PD98059, a specific inhibitor of the upstream kinase of p44/p42 MAP kinase, which reduced the phosphorylation of p44/p42 MAP kinase stimulated by $PGF_{2\alpha}$, had little effect on the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK. These results strongly suggest that SAPK/JNK plays a part in $PGF_{2\alpha}$-induced HSP27 in addition to p44/p42 MAP kinase in osteoblasts.

Keywords: $PGF_{2\alpha}$; HSP27; SAPK/JNK; Osteoblast

1. Introduction

Heat shock proteins (HSPs) are produced by cells when the cells are exposed to the biological stress such as heat stress and chemical stress [1]. HSPs are classified into high- and low-molecular-weight HSPs based on apparent molecular sizes. It is well known that the high-molecular-weight HSPs such as HSP90 and HSP70 act as molecular chaperones in protein folding, oligomerization and translocation [1]. 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 [1]. It is currently recognized that the low-molecular-weight HSPs may have chaperoning functions like the high-molecular-weight HSPs [1]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [2]. The formation of bone structures and bone remodeling results from the coupling process, bone resorption by activated osteoblasts with subsequent deposition of new matrix by osteoblasts. Accumulating evidence indicates that osteoblasts are responsible for bone resorptive factors such as parathyroid hormone and 1.25-$(OH)_2$ vitamin D_3 [2] through the upregulation of RANKL expression [3], suggesting that osteoblasts play crucial role in the regulation of bone remodeling. In osteoblasts, the expression of HSP27 is induced by heat, and the heat-induced HSP27 expression is reportedly facilitated by estrogen [4,5]. In addition, it has been shown that the downregulation of proliferation is accompanied by a transient increase of the expression of HSP27 mRNA [4,5]. We have reported that physiological agents for bone metabolism such as endothelin-1 and transforming

*Corresponding author. Tel.: +81-58-267-2233; fax: +81-58-267-2959.
E-mail address: okozawa@cc.gifu-u.ac.jp (O. Kozawa).

0952-3278/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.plefa.2003.09.006