Prostaglandin E₂ Increases the Expression of B-Type Natriuretic Peptide Receptor through EP1 Receptor, Ca²⁺ Mobilization and Protein Kinase C Signaling Pathway in Rat Calvarial Osteoblasts

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(Received December 22, 2008; Accepted March 21, 2009; Published online March 25, 2009)

The C-type natriuretic peptide stimulates osteoblastic functions through the B-type natriuretic receptor (NPR-B). In this study, we examined the signaling pathway behind the regulation of NPR-B expression through the prostaglandin E₂ (PGE₂) receptor, EP1 subtype using rat calvarial osteoblasts. A23187 as a Ca²⁺ ionophore increased NPR-B expression dose-dependently. PGE₂ or 17-phenyl- ω -trilor PGE₂ (EP1A), an EP1 agonist, increased NPR-B expression, and the potentiating effects were blocked by treating with BAPTA-AM as an intracellular Ca²⁺ chelator. Activators of protein kinase C (PKC), 1-oleoyl-2-acetyl-*sn*-glycerol, a membrane-permeable diacylglycerol, and 12-*o*-tetradecanoyl-phorbol-13-acetate, also increased NPR-B expression, and the potentiating effects were blocked by treating with BAPTA-AM. The treatment of cells with GF109203X, a PKC inhibitor, blocked the PGE₂- and EP1A-induced increase in NPR-B expression. From these results, we concluded that EP1-mediated increase in the expression of NPR-B requires not only Ca²⁺ mobilization but also PKC activation through the activation of phosphatidylinositol-specific phospholipase C.

Key words — B-type natriuretic peptide receptor, prostaglandin E₂, EP1, osteoblast, protein kinase C

INTRODUCTION

Osteoporosis in the elderly is characterized by a loss of bone mass most likely resulting from the suppression of bone formation by osteoblasts with aging.^{1,2)} It is recognized that osteoblastic bone formation is an event regulated by local factors.³⁾ Prostaglandin E₂ (PGE₂), a local factor, has multiple effects on bone metabolism through four well-characterized receptors, termed EP1, EP2, EP3, and EP4.^{4,5)} Previously, we have shown that EP1 contributes to the stimulation of bone formation by PGE₂ in rat calvarial osteoblasts.^{6,7)} The binding of PGE₂ to the EP1 receptor leads to the activation of phosphatidylinositol-specific phospholipase C (PI-PLC) to produce inositol-1,4,5-triphosphate (IP₃), which binds to its specific receptor on the endoplasmic reticulum (ER), resulting in the rapid release of

Ca²⁺. The EP1-mediated Ca²⁺ mobilization stimulates alkaline phosphatase activity, type I collagen synthesis, and mineralized bone nodule formation, which are markers for osteoblastic bone formation, through the activation of calmodulin (CaM). Interestingly, the signaling pathway does not function in cells from aged rats due to a decrease in the expression of EP1, resulting in the impaired osteoblastic functions.

C-type natriuretic peptide (CNP) stimulates the growth of endochondral bone *in vivo*^{8–10)} and the differentiation of osteoblastic lineage cells including rat calvarial osteoblasts *in vitro*^{11–13)} through the B-type natriuretic peptide receptor (NPR-B). Recently, we have shown that PGE₂ increases the expression of NPR-B through EP1 in rat calvarial osteoblasts.¹⁴⁾ However, the EP1-mediated signaling pathway, which increases the expression of NPR-B, remains unknown. In this study, we examined whether the EP1-induced Ca²⁺ mobilization increases the expression of NPR-B using calvarial osteoblasts from 25-week-old rats.

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MATERIALS AND METHODS

Materials——PGE₂, 17-phenyl- ω -trinor PGE₂ (EP1A, an EP1 agonist), and 1-oleoyl-2-acetyl-sn-glycerol [OAG, a protein kinase C (PKC) activator] were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). A23187 (a Ca²⁺ ionophore), BAPTA-AM (an intracellular Ca²⁺ chelator), GF109203X (a PKC inhibitor), and W-7 (a CaM inhibitor) were purchased from BIOMOL International (Plymouth Meeting, PA, U.S.A.). The antibody for β -actin and 12-*o*-tetradecanoylphorbol-13-acetate (TPA, a PKC activator) were purchased from Sigma (St. Louis, MO, U.S.A.). The antibody for NPR-B was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The agonists, activators, and inhibitors were dissolved in dimethylsulfoxide, and these solutions were kept at -20°C before use.

Animals——Female Wistar rats (4 weeks old) were purchased from CLEA (Tokyo, Japan), and maintained in the animal facility of Toho University under specific pathogen-free conditions. All animal experiments were performed in accordance with the Care and Use of Laboratory Animals Committee of the Faculty of Pharmaceutical Sciences, Toho University.

Cell Culture——Cells enriched for osteoblast phenotype were enzymatically isolated from calvariae of 25-week-old female Wistar rats (CLEA) as described previously.¹⁴⁾ The cells were plated in 35-mm dishes at a density of 4×10^3 cells/cm², and grown in F-12 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Rockville, MD, U.S.A.) at 37°C for 6 days.

Reverse Transcription (RT)-PCR Analysis——RNA extraction, RT and quantitative real-time PCR amplification were performed according to the method described previously.¹⁴⁾ In brief, after the treatment of cells with vehicle or a reagent for 24 hr on day 5, the cells were homogenized using 1 ml of TRIzol reagent (Invitrogen, Rockville, MD,

U.S.A.), and total RNA was extracted according to the manufacturer's protocol. cDNA was synthesized using 20 μ l of reverse transcription reaction solution (Roche Applied Science, Indianapolis, IN, U.S.A.) containing 1 μ g of total RNA, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM deoxynucleoside triphosphates, 2.5 μ M random hexamers, 20 U of ribonuclease inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase. Quantitative real-time PCR amplification was performed in an iCycler real-time PCR machine using an iQ SYBR Green supermix (both from Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's instruction. The sequences of the primer sets for NPR-B and β -actin mRNAs, the target sites on mRNAs and the product sizes determined by PCR are shown in Table 1. To minimize the background of the products amplified from genomic DNAs, the primers were designed to exist on two different exons. The quantity of NPR-B mRNA in each sample was normalized using the C_T (threshold cycle) value obtained for β -actin mRNA amplification.

Western Blot Analysis——On day 6, cellular lysates were prepared and subjected to Western blot analysis as described previously.¹⁴⁾ In brief, after the treatment of cells with vehicle or a reagent for 24 hr on day 5, the cells were washed with cold phosphate buffered saline and whole cell lysates were prepared by adding a mammalian protein extraction reagent (PIERCE, Rockford, IL, U.S.A.) containing a protease inhibitor cocktail (Roche Applied Science) to the cells. Twenty micrograms of protein was loaded per lane and fractionated on a 10% polyacrylamide gel; the fractionated proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) by electroblotting. The membrane was blocked for nonspecific binding in 3% nonfat dry milk, followed by incubation with the antibody for NPR-B or β -actin at 4°C. After the membrane was washed, the blots were probed with a horseradish peroxidase-conjugated secondary an-

Table 1. Sequences of Primers Used in Real-Time PCR Analysis

Gene	GenBank TM accession number	Sequence of primers ^{a)}	Product size (bp)
NPR-B	NM_053838	F: 5'-TGAGCAAGCCACCCACTTC-3' R: 5'-CAGCGGGCCGCGAGATA-3'	111
β -actin	NM_031144	F: 5'-CATGAAGATCAAGATCATTGCTCCT-3' R: 5'-CTGCTTGCTGATCCACATCTG-3'	109

a) F, forward primer; R, reverse primer.

tibody (Bio-Rad Laboratories) and visualized using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, U.S.A.) according to manufacturer's instructions.

Statistical Methods—Data were analyzed by Student's *t*-test or one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. *p* < 0.05 was considered significant. All data are represented as the mean ± S.D. of four cultures.

RESULTS AND DISCUSSION

To investigate a Ca^{2+} -dependent signaling pathway for NPR-B expression, we first examined the effect of A23187 as a calcium ionophore on the expression level of NPR-B (Fig. 1). A23187 increased the mRNA level of NPR-B dose-dependently and the maximal mRNA level of NPR-B obtained at 1 μM A23187 was 3.6-fold higher than the control (Fig. 1A). A23187 (10 μM) also increased the protein level of NPR-B (Fig. 1B). These results suggest that the increase in the concentration of intracellular Ca^{2+} leads to the increase in NPR-B expression of rat calvarial osteoblasts. To explore this further, we next examined the effect of BAPTA-AM as an intracellular Ca^{2+} chelator on the EP1-mediated increase in NPR-B expression (Fig. 2). PGE_2 (10 μM) and EP1A (10 μM) increased the mRNA levels of NPR-B 5.7-fold and 6.1-fold over the control, respectively, and the treatment of cells with BAPTA-AM decreased the de-

Fig. 1. Effect of Ca Ionophore on NPR-B Expression

On day 5, cells were treated with vehicle or the indicated concentrations of A23187 as a calcium ionophore for 24 hr. After the treatment, total RNA and protein were extracted from the cells and subjected to real-time RT-PCR and Western blot analyses. (A) The relative mRNA level of NPR-B was normalized to that of β -actin in the same sample. Each point represents the mean ± S.D. of four cultures. **p* < 0.05, compared with the control. (B) Protein levels of NPR-B and β -actin. The experiment was repeated twice and the results were essentially the same as those depicted.

gree of EP1 agonist-induced increases in the mRNA level of NPR-B dose-dependently (Fig. 2A and 2B). The BAPTA-AM treatment also blocked the potentiating effect of PGE_2 or EP1A on the protein level of NPR-B (Fig. 2C). These results suggest that PGE_2 increases NPR-B expression through the Ca^{2+} -dependent signaling pathway in rat calvarial

Fig. 2. Effect of Intracellular Ca^{2+} Chelator on EP1-Mediate Increase in NPR-B Expression

On day 5, cells were pretreated with vehicle or the indicated concentrations of BAPTA-AM as an intracellular Ca^{2+} chelator in the Ca^{2+} -free medium containing 0.01% BSA for 1 hr, and then treated with PGE_2 (10 μM) or 17-phenyl- ω -trilor PGE_2 (EP1A, 10 μM) in the same medium containing the chelator for 24 hr. After the treatment, total RNA and protein were extracted from the cells and subjected to real-time RT-PCR and Western blot analyses. (A, B) The relative mRNA level of NPR-B was normalized to that of β -actin in the same sample. Each point represents the mean ± S.D. of four cultures. **p* < 0.05, compared with the control. ***p* < 0.05, compared with level in cells treated only with EP1 agonist. (C) Protein levels of NPR-B and β -actin. The experiment was repeated twice and the results were essentially the same as those depicted.

osteoblasts.

We have shown that the PGE₂- or EP1A-induced Ca²⁺ mobilization stimulates the bone formation through the activation of CaM in rat calvarial osteoblasts, and that W-7 (1 μ M) as a CaM inhibitor blocks the potentiating effect of PGE₂ or EP1A completely.^{6,7} Then, we examined the effect of W-7 (1 μ M) on the EP1-mediated increase in NPR-B expression (Fig. 3). Contrary to our expectation, the treatment of cells with W-7 had no effect on the EP1 agonist-induced increases in the mRNA level of NPR-B, suggesting that the activation of CaM is not involved in the EP1-mediated increase in NPR-B expression in rat calvarial osteoblasts.

PI-PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) to produce two second messengers, IP₃ and diacylglycerol (DAG).^{15,16} It has been known that DAG activates PKC isoforms, α , β and γ , in a Ca²⁺-dependent manner.¹⁷ Yang *et al.* reported that several PKC isoforms, α , β , ϵ , and δ , exist in rat calvarial osteoblasts¹⁸ In addition, Tang *et al.* showed that PGE₂ increases the expression of fibronectin, a bone protein, through the EP1/PI-PLC/PKC α pathway in the cells.¹⁹ From these findings, there is a possibility that the accumulation of DAG and the increase in the concentration of Ca²⁺, which are induced by the action of PI-PLC, cause an increase in NPR-B expression through the activa-

Fig. 3. Effect of Calmodulin Inhibitor on EP1-Mediate Increase in NPR-B Expression

On day 5, cells were pretreated with vehicle or W-7 (1 μ M) as a CaM inhibitor for 1 hr, and then treated with PGE₂ (10 μ M) or 17-phenyl- ω -trinor PGE₂ (EP1A, 10 μ M) in the medium containing W-7 for 24 hr. After the treatment, total RNA and protein were extracted from the cells and subjected to real-time RT-PCR analysis. The relative mRNA level of NPR-B was normalized to that of β -actin in the same sample. Each point represents the mean $\pm$ S.D. of four cultures. n.s., not significant. The experiment was repeated twice and the results were essentially the same as those depicted.

tion of PKC. To clarify the hypothesis, we examined the effects of OAG as a membrane-permeable DAG and TPA as a PKC activator on the expression level of NPR-B (Fig. 4). OAG (1 μ M) or TPA (1 μ M) increased the mRNA and protein levels of NPR-B, and the potentiating effects of OAG and TPA were blocked by the treatment of cells with BAPTA-AM, suggesting that the activation of PKC increases NPR-B expression in a Ca²⁺-dependent manner. To explore this further, we examined the effect of GF109203X as a PKC inhibitor on the EP1-mediated increase in NPR-B expression (Fig. 5). The treatment of cells with GF109203X inhibited the PGE₂- or EP1A-induced increases in the mRNA level of NPR-B dose-dependently (Fig. 5A and 5B). The GF109203X treatment also blocked the potentiating effect of PGE₂ or EP1A on the protein level of NPR-B (Fig. 5C), suggesting that the activation of PKC is involved in the EP1-mediated increase in

Fig. 4. Effects of PKC Activators on NPR-B Expressions

On day 5, cells were pretreated with vehicle or BAPTA-AM (10 μ M) for 1 hr, and then treated with OAG (1 μ M) as a membrane-permeable DAG (1 μ M) or TPA (1 μ M) as a PKC activator in the same medium for 24 hr. After the treatment, total RNA was extracted from the cells and subjected to real-time RT-PCR and Western blot analyses. (A) The relative expression levels of NPR-B mRNA were normalized to that of β -actin in the same sample. Each point represents the mean $\pm$ S.D. of four cultures. * p < 0.05; compared with the control. ** p < 0.05; compared with level in cells treated only with PKC activator (1 μ M) in the same stimulation group. (B) NPR-B and β -actin protein levels. The experiment was repeated twice and the results were essentially the same as those depicted.

NPR-B expression in rat calvarial osteoblasts. From overall result, we concluded that EP1-induced increase in the expression of NPR-B requires not only Ca^{2+} mobilization but also PKC activation through the activation of PI-PLC in rat calvarial osteoblasts (Fig. 6). The precise mechanism of PKC-induced signaling pathway is now under investigation in our laboratory.

Fig. 5. Effect of PKC Inhibitor on EP1-Mediate Increase in NPR-B Expression

On day 5, cells were pretreated with vehicle or the indicated concentrations of GF-109203X as a PKC inhibitor for 1 hr, and then treated with PGE₂ (10 μM) or 17-phenyl- ω -trilor PGE₂ (EP1A, 10 μM) in the medium containing the PKC inhibitor for 24 hr. After the treatment, total RNA and protein were extracted from the cells and subjected to real-time RT-PCR and Western blot analyses. (A, B) The relative mRNA level of NPR-B was normalized to that of β -actin in the same sample. Each point represents the mean $\pm$ S.D. of four cultures. * p < 0.05, compared with the control. ** p < 0.05, compared with level in cells treated only with EP1 agonist. (C) Protein levels of NPR-B and β -actin. The experiment was repeated twice and the results were essentially the same as those depicted.

It has been established that the balance of two peptides produced by osteoblastic lineage cells, osteoprotegerin (OPG) and receptor activator of nuclear factor- κ B ligand (RANKL), is critical for the bone resorption process. The binding of RANKL, expressed on the surface of osteoblastic lineage cells, to its receptor RANK, expressed on the surface of osteoclastic lineage cells, induces differentiation, activation and survival of osteoclasts, leading to increased bone resorption.^{20–22)} The effect of RANKL is neutralized by its decoy receptor OPG, which plays an important inhibitory role in regulation of osteoclastic bone resorption.^{23, 24)} Many local and systemic factors important in bone remodeling are associated with changes in OPG/RANKL ratio in osteoblastic lineage cells. PGE₂, parathyroid hormone, 1,25-dihydroxyvitamin D₃, and glucocorticoids decrease OPG/RANKL ratio, thereby favor osteoclast recruitment.^{25–29)} By contrast, estrogen, androgen and leptin have the reverse effect, subsequently decreasing bone resorption and slowing bone turnover.^{30, 31)} Furthermore, extensive evidence suggests that the main mechanism by which PGE₂ stimulates osteoclastogenesis is an increase in RANKL expression and a decrease in OPG expression through EP2 and EP4 receptors, both of which lead to the activation of adenylate cyclase to produce cyclic adenosine monophosphate (cAMP).^{25, 32)} Previously, we have shown that the EP1-induced Ca^{2+} mobilization activates a phosphodiesterase that blocks the signaling through EP2 and EP4 receptors by degrading cAMP in calvar-

Fig. 6. Scheme of the Signaling Pathway for PGE₂-Induced Enhancement of NPR-B Expression in Calvarial Osteoblasts

ial osteoblasts from young rats, and that the EP1-induced signaling is not functioning due to a decrease in EP1 expression.^{6,7,13)} These findings and our results in the present study suggest that the age-dependent decrease in EP1 expression can cause decreases in OPG/RANKL ratio and NPR-B expression, which may be associated with a sustained bone loss due to impaired osteoblastic bone formation and improved osteoclastic bone resorption in senile osteoporosis.

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