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Formation of Mineralized Bone Nodules by Rat Calvarial Osteoblasts Decreases With Donor Age Due to a Reduction in Signaling Through EP_1 Subtype of Prostaglandin E_2 Receptor

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Abstract The effects of prostaglandin E_2 (PGE_2) on the parameters for proliferation and differentiation were studied in calvarial osteoblast-like cells isolated from rats of various ages. In cells not treated with PGE_2, it was found that mineralized bone nodule (BN) formation, alkaline phosphatase (ALP) activity, and the incorporation rate of [^{3}H]thymidine into the cells sharply decreased with the age of the cell donor at 6–50 weeks and then remained at a relatively constant level up to 120 weeks. Before studying the effects of PGE_2 on these parameters, we determined the change in the levels of PGE_2 produced by the untreated cells during the culture period and found that the endogenous PGE_2 reached a maximum on the 4th day of the culture, regardless of the cell donor age, followed by a sharp decrease. The endogenous production was blocked by pretreatment with a cyclooxygenase-2 (COX-2) inhibitor, NS-398, indicating the generation of PGE_2 through a COX-2 pathway. The area of BN was effectively suppressed by NS-398 in the cells from 10- to 35-week-old rats, whereas it was enhanced in the cells from 90- to 120-week-old rats. Treatment with PGE_2 markedly increased the BN formation and the ALP activity in the cells from 4- to 35-week-old rats (defined as young rats). By contrast, PGE_2 decreased [^{3}H]thymidine incorporation into the cells from young rats. The area of BN and the ALP activity decreased significantly, whereas [^{3}H]thymidine incorporation into the cells increased by 60–80% in the cells of 80- to 120-week-old rats (defined as aged rats). The stimulatory effects on the cell differentiation and the inhibitory effect on the proliferation in the cells from young rats was mimicked by an EP_1 agonist, 17-phenyl-ω-trinor PGE_2, while an EP_2/EP_4 agonist, 11-deoxy-PGE_1 and an adenylate cyclase activator, forskolin suppressed the differentiation and enhanced the proliferation regardless of the cell donor age. PGE_2, 11-deoxy-PGE_1 and forskolin, but not 17-phenyl-ω-trinor PGE_2 increased cyclic adenosine monophosphate (cAMP) production. Generation of inositol 1,4,5-triphosphate (IP_3) was stimulated by 17-phenyl-ω-trinor PGE_2 or PGE_2, but not by 11-deoxy-PGE_1 or forskolin increased cAMP production in the cells from young rats. By contrast, PGE_2 had little effect on IP_3 generation in aged rats. From the overall results, we concluded that PGE_2 exerts stimulatory and inhibitory effects on differentiation through the EP_1-IP_3 pathway and EP_2/EP_4-cAMP pathway, respectively, in the cells from young rats. The EP_1-IP_3 pathway seems to be inactive in the cells from aged rats. J. Cell. Biochem. 75:215–225, 1999.

Key words: osteoblasts; aging; cAMP; phosphoinositide turnover; prostaglandin E_2 (PGE_2); mineralized bone nodule

Prostaglandin E_2 (PGE_2) is a potent modulator of bone metabolism that modifies functions of osteoblasts as well as osteoclasts [Nijweide et al., 1986; Norrdin et al., 1990]. In vivo and in vitro studies showed that PGE_2 is largely involved in the regulation of bone formation [Chyun and Raisz, 1984; Yang et al., 1993], although its effect is variable, depending on the experimental conditions employed [Suponitzky and Weinreb, 1998]. In vivo administration of PGE_2 to rats enhances cortical bone mass and activates intracortical bone remodeling [Jee et al., 1985, 1990]. The addition of PGE_2 to organ cultures of fetal rat calvaria causes stimulation of DNA synthesis in the periosteum and suppression of collagen synthesis in the central bone [Raisz and Koolemans-Beynen, 1974]. PGE_2-induced cellular responses in cultured osteoblastic cells have been studied most extensively in a osteoblastic cell line, MC3T3-E1, in which the effect of PGE_2 is differentiative at low

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concentrations and proliferative at high concentrations [Hakeda et al., 1986]. The biphasic effect of PGE_2 is attributed to the difference in the signal transduction pathway leading to the cellular responses at high and low concentrations; i.e., the differentiative effect of PGE_2 is mediated by cyclic adenosine monophosphate (cAMP), while the proliferative effect is mediated through the elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). By contrast, we have recently shown, using adult rat calvarial cells, that PGE_2 stimulates bone nodule (BN) formation via the Ca^{2+} pathway at low and high concentrations [Kaneki et al., 1999]. The results indicate that the transduction mechanism utilized for PGE_2 signal may differ depending on the cell type. The mode of response to PGE_2 is known to be affected also by factors other than PGE_2 concentration. When cell fractions obtained after serial enzyme digestions of fetal parietal bone were treated with PGE_2, DNA synthesis was increased in the earliest fraction used, while it was decreased in a mixture of cells from the last three fractions [Centrella et al., 1994]. It has also been shown that the time at which PGE_2 is added during the culture period to MC3T3-E1 cells affects the responses to the agonist [Suda et al., 1996]. The diversity of the osteoblastic cell responses to PGE_2 may reflect the difference in the stage of differentiation; i.e., the signal transduction mechanism underlying the responses to PGE_2 may be modulated during the differentiation process. Furthermore, the in vivo effect of PGE_2 on bone metabolism in rats has been reported to be age dependent [Jee et al., 1985, 1990]. Within this context, it is interesting to determine the cell donor age-related change in the effect of PGE_2 on osteoblastic cells.

The purpose of this study was to examine the change in the ability of the rat calvarial osteoblast-like cells to proliferate and differentiate, to form mineralized BN with age of the cell donor. Age-related changes in the signal transduction mechanism were also studied.

MATERIALS AND METHODS

Materials

The following prostaglandin analogues were purchased from Cayman Chemical (Ann Arbor, MI): 17-phenyl-ω-trinor PGE_2 (EP_1 agonist) and 11-deoxy-PGE_1 (EP_2/EP_4 agonist). PGE_2, forskolin, and 3-(isobutyl)-1-methylxanthine (IBMX) were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), phenol red-free F-12 medium, and α-minimum essential medium (α-MEM), and trypsin were products of Gibco-BRL (Rockville, MD). Kanamycin, ascorbic acid, collagenase, and β-glycerophosphate were obtained from Wako Pure Chemical Industries (Tokyo, Japan). [Methyl-^{3}H]thymidine was from DuPont (Wilmington, DE).

Cell Culture

Cells enriched for osteoblast phenotype were enzymatically isolated from the calvaria of 4- to 120-week-old female Wistar rats (CLEA, Tokyo, Japan) according to the method of Bellows et al. [1986], modified by Kato et al. [1995]. Briefly, after five sequential digestions of calvaria with a mixture of collagenase and trypsin, the released cells from the last three digestion intervals were grown in F-12 medium containing 10% FBS. After reaching confluence, the cells were collected by a trypsin treatment, seeded in 4-well dishes at 2×10^3 cells/cm^2 in the same medium, and cultured for 4 days (designated as the proliferation period, P1-P4). At the end of day P4, the medium was changed to α-MEM supplemented with 10% FBS, 2 mM β-glycerophosphate and ascorbic acid (0.1 mg/ml), and the cells were maintained for a further 18 days (designated as the mineralization period, M1-M18).

Determination of Markers for Cell Proliferation and Differentiation

For the determination of alkaline phosphatase (ALP) activity, BN formation, and DNA synthesis, cells were incubated in serum-free F-12 medium for 24 h on day P4, with 10^{-6} M PGE_2 present in the medium as during the last 6 h; the incubation was then continued in PGE_2-free α-MEM medium, as described above. An ALP assay was performed at the beginning of day M2 according to the method of Lowry et al. [1954], using p-nitrophenylphosphate as a substrate. Quantification of BN was performed by visualization with von Kossa stain [Bhargava et al., 1988], and the total nodule area and the number of nodules were assessed on day M18, using a colony counter (BMS-400, Toyo Sokki, Tokyo, Japan). For determination of DNA synthesis, the cells were incubated for 3 h in serum-free α-MEM medium containing [^{3}H]thymidine (1.25 μCi/ml) at the beginning of day M1, and incorporation of radioactivity into DNA was measured.

Determination of cAMP and Inositol Triphosphate Formation

Intracellular cAMP was determined using a radioimmunoassay (RIA) kit (Yamasa, Chiba, Japan) as previously described [Kaneki et al. 1999]. Determination of the inositol 1,4,5-triphosphate (IP_3) generation was performed using an RIA kit (Amersham, Tokyo, Japan) as described [Kaneki et al., 1999].

Determination of PGE_2 Production

Calvarial cells were cultured for various periods under standard conditions; serum was then omitted from the medium for 18 h. The medium was replaced with fresh serum-free medium with or without NS-398 (10^{-6} M), and the incubation was continued for 3 h. The medium was changed again to serum-free standard medium, and the culture medium was collected after an additional 3-h incubation. The amount of PGE_2 was measured using the Prostaglandin E_2 [^{125}I] Assay System (Amersham Pharmacia Biotech, Tokyo, Japan).

Statistical Methods

Data were analyzed by Student's *t*-test or by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. $P < 0.01$ was considered significant. All data are presented as the mean ±SD of four cultures. The experiments with rats aged <100 weeks were repeated at least three times, while those performed with rats aged ≥100 weeks were repeated twice.

RESULTS

First, we examined the age-dependent changes in the parameters for proliferation and differentiation in cells not treated with PGE_2 (Fig. 1). BN formation in the area (Fig. 1A), number (Fig. 1B), and activity of ALP (Fig. 1C), continuously decreased with the age of the cell donor at 6–50 weeks and then remained at a relatively constant level at ≤120 weeks. The profiles of BN formed by the cells from 6- and 90-week-old rats are shown in Figure 2. A similar age-dependent decline was observed in the rate of [^{3}H]thymidine incorporation into the cells (Fig. 1D). These results suggest that the spontaneous proliferation and differentiation of the calvarial cells are suppressed as a function of donor age at 6–50 weeks.

To determine the role of endogenous PGE_2, we examined the change in the PGE_2 production during the culture of the cells from 10-, 25-, and 90-week-old rats (Fig. 3). Sharp peaks of PGE_2 production were obtained on day P4, regardless of the cell donor age. The addition of a cyclooxygenase-2 (COX-2) inhibitor, NS-398 (10^{-6} M), to the culture almost completely blocked PGE_2 production (Fig. 3), indicating that PGE_2 is generated exclusively through a COX-2 pathway. We therefore treated calvarial cells from rats of various ages with NS-398 on day P4 (Fig. 4). NS-398 decreased the area of BN by approximately 50% (Fig. 4) in the cells from 10- to 35-week-old rats, indicating that the endogenous PGE_2 increases the area of BN. By contrast, the area of BN was increased by NS-398 by approximately 20–40% (Fig. 4) in the cells from 90- to 120-week-old rats. These results suggest that the response of calvarial cells to PGE_2 differs depending on the age of the cell donor.

We next determined the effects of exogenous PGE_2 on proliferation and differentiation in cells from rats of various ages (Fig. 5). As expected from the results shown in Figure 3, the BN formation was enhanced by the PGE_2 treatment, with respect to both area (Fig. 5A) and number (Fig. 5B) by 100–180% and by 50–90%, respectively, in the cells from 4- to 35-week-old rats. Rats in this age range are hereafter referred to as young rats. The PGE_2 treatment also enhanced the ALP activity by 60–130% in the cells from young rats (Fig. 5C). The maximal percentage stimulation of BN formation and ALP activity by PGE_2 was observed in the cells from 10-week-old rats; the effect was gradually decreased with age. PGE_2 significantly decreased the area of BN (Fig. 5A) and ALP activity (Fig. 5C) in the cells from 80- to 120-week-old rats. Rats in this age range are hereafter referred to as aged rats. By contrast, PGE_2 both suppressed and enhanced [^{3}H]thymidine incorporation into the cells from young rats and aged rats, respectively (Fig. 5D). An agonist specific for the EP_1 receptor subtype of PGE_2, 17-phenyl-ω-trinor PGE_2, showed almost the same effects as PGE_2 on the proliferation and differentiation markers in the cells from young rats (Fig. 6). It increased the BN formation in both area (Fig. 6A) and number (Fig. 6B) and the ALP activity (Fig. 6C) by 130–190%, 60–100%, and 110–140%, respectively. Maximal percentage stimulation was observed in the

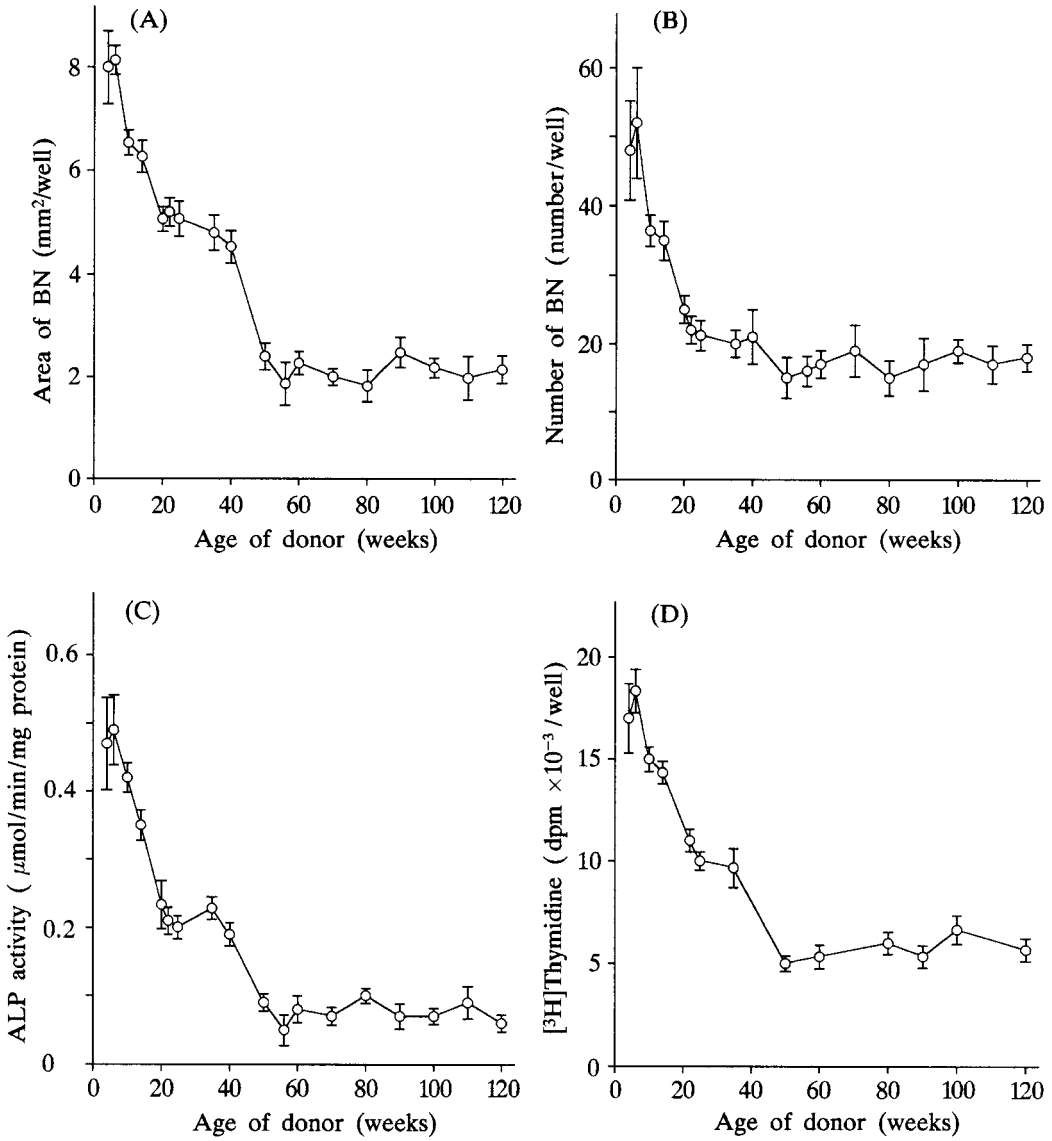

Fig. 1. Age-related changes in bone nodule (BN) formation, alkaline phosphatase (ALP) activity, and DNA synthesis in cultures of calvarial cells from rats. ALP activity **(C)** was measured at the beginning of day M2. [^{3}H]Thymidine incorporation into the cells **(D)** was measured at the beginning of day M1. The area **(A)** and number **(B)** of BN were measured on day M18. Other conditions were as described under Materials and Methods. Each value represents the mean ±SD of four cultures. The experiments with rats aged <100 weeks were repeated at least three times, while those with rats aged ≥100 weeks were repeated twice, and the results were essentially the same as those depicted.

cells from 10- to 15-week-old rats and the effects were gradually reduced with age. The EP_1 agonist decreased [^{3}H]thymidine incorporation into the cells by 20–50% in the cells from young rats (Fig. 6D). By contrast, with PGE_2, the EP_1 agonist showed no significant effect on the BN formation, ALP activity and [^{3}H]thymidine incorporation into the cells from aged rats. These results suggest that the effects of PGE_2 on both proliferation and differentiation are exerted exclusively through the EP_1 receptor subtype in the cells from young rats and that the signal transduction pathway through EP_1 is not active in the cells from aged rats.

A selective agonist for EP_2 and EP_4 receptor subtypes, 11-deoxy-PGE_1, decreased the BN formation in both area (Fig. 7A) and number (Fig. 7B) and the ALP activity (Fig. 7C) by approximately 50%, 20%, and 50%, respectively, at all cell donor ages. By contrast, 11-deoxy-PGE_1

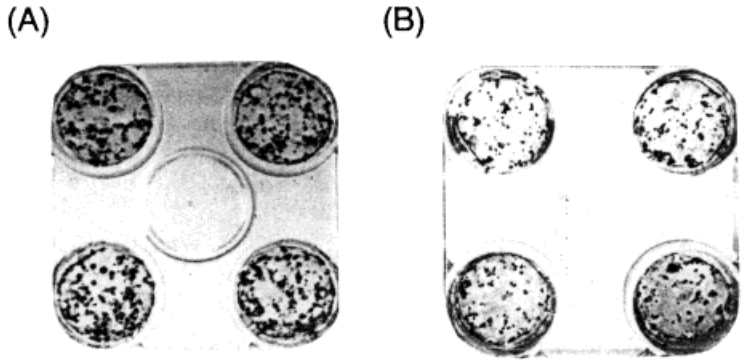

Fig. 2. Phase-contrast photomicrographs of bone nodules (BN) visualized with von Kossa stain in cultures of calvarial cells from 6-week-old rats **(A)** and 90-week-old rats **(B)**.

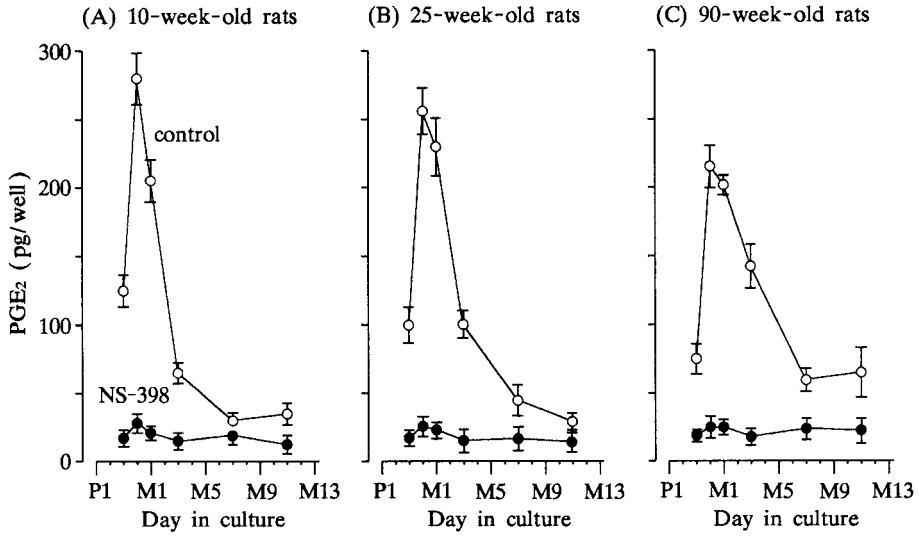

Fig. 3. Time-course of prostaglandin E_2 (PGE_2) production in the cultures of calvarial cells from 10-week-old rats **(A)**, 25-week-old rats **(B)**, and 90-week-old rats **(C)**. The culture medium was collected on the indicated days and PGE_2 in the medium was determined as described under Materials and Methods. After preincubation with (●) or without (○) NS-398 (10^{-6} M) and a further 3-h incubation in the serum-free standard medium, endogenous PGE_2 was determined on the indicated days. Each value represents the mean ±SD of four cultures. Experiments were repeated at least three times, and results were essentially the same as those depicted.

enhanced the rate of [^{3}H]thymidine incorporation into the cells by approximately 70% at all cell donor ages examined (Fig. 7D). These results indicate that activation of the EP_2/EP_4 pathway leads to the suppression of the cell differentiation and the enhancement of the cell proliferation, regardless of the cell donor age. The activation of EP_2/EP_4 pathway is known to cause an increase in the activity of adenylate cyclase to produce cAMP [Suda et al., 1996]. We therefore tested the effect of a direct activator of adenylate cyclase, forskolin, on the markers for differentiation and proliferation of osteoblastic cells (data not shown); the results with forskolin are essentially the same as those with the EP_2/EP_4 agonist shown in Figure 7. These effects of forskolin closely matched those of 11-deoxy-PGE_1, indicating that the EP_2/EP_4 agonist exerts its effects on osteoblastic cells by the activation of adenylate cyclase and the subsequent production of cAMP.

We then compared the effects of PGE_2, 17-phenyl-ω-trinor PGE_2, 11-deoxy-PGE_1 and forskolin on the production of cAMP (Fig. 8A) and IP_3 (Fig. 8B) in the cells from 25- and 90-week-old rats. PGE_2, 11-deoxy-PGE_1, and forskolin, but not 17-phenyl-ω-trinor PGE_2, induced the production of cAMP in the cells from the rats of both ages. By contrast, PGE_2 and 17-phenyl-ω-trinor PGE_2 enhanced the production of IP_3 in the cells from 25-week-old rats, but not in the cells from 90-week-old rats. These results indi-

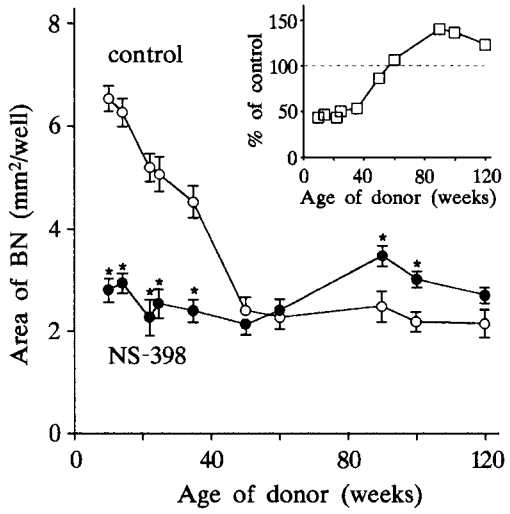

Fig. 4. Effect of the inhibition of endogenous prostaglandin E_2 (PGE_2) production by NS-398 on the area of bone nodules (BN) in cultures of calvarial cells from rats of various ages. The cells were treated with (●) or without (○) NS-398 (10^{-6} M) for 3 h on day P4, and the area of BN was measured on day M18. Inset compares the effect of NS-398 on the ratio of the area of BN in NS-398 treated cultures with that in untreated cultures. Other conditions were as described under Materials and Methods. Each value represents the mean ±SD of four cultures. The experiments with rats aged <100 weeks were repeated at least three times, while those with rats aged ≥100 weeks were repeated twice, and the results were essentially the same as those depicted.

cate that the signal transduction pathway through EP_1 receptor subtype is inactive in the cells from 90-week-old rats.

DISCUSSION

To the best of our knowledge, this is the first report to show a donor age-dependent change in the responsiveness of osteoblast-like cells to PGE_2 in primary culture. These age-dependent changes in the properties of the cells could at least partially be explained by the alteration of the signal transduction mechanisms for endogenously generated PGE_2, because the BN formation is largely affected by pretreatment of the cells with a COX-2 inhibitor; only a minor change with aging of the cell donor was observed in the rate of endogenous PGE_2 production. PGE_2 receptors are classified into four subtypes, EP_1, EP_2, EP_3, and EP_4 [Coleman et al., 1994]. Experiments using agonists specific for the PGE_2 receptor subtypes demonstrated the presence of EP_1 and EP_2/EP_4 in MC3T3-E1 osteoblast-like cells [Suda et al., 1996]. EP_1 couples to the phosphoinositide/Ca^{2+} pathway, while EP_2/EP_4 couple to the stimulative adenylate cyclase/cAMP pathway [Coleman et al., 1994]. Using adult rat (25- to 35-week-old) calvarial cells, we previously showed that PGE_2 stimulates the increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and production of cAMP through EP_1 and EP_2/EP_4 receptor subtypes, respectively [Kaneki et al., 1999], as illustrated in Figure 9. The increase in $[Ca^{2+}]_i$ leads to the activation of ALP, collagen synthesis, and BN formation, as well as suppression of cell proliferation. Although cAMP has the ability to suppress ALP activity, collagen synthesis, and BN formation and to enhance proliferation, these effects of cAMP are not observable because the newly formed cAMP is rapidly degraded by phosphodiesterase, which is activated by the increase in $[Ca^{2+}]_i$ [Kaneki et al., 1999].

In the present study, PGE_2 and an EP_1 agonist, 17-phenyl-ω-trinor PGE_2, enhanced the BN formation and ALP activity and suppressed proliferation of the cells from young rats. By contrast, an adenylate cyclase activator, forskolin, and an EP_2/EP_4 agonist, 11-deoxy-PGE_1, suppressed the BN formation and ALP activity and enhanced proliferation of these cells. These results suggest that the effect of PGE_2 on the cells from young rats is mainly exerted through the EP_1 receptor subtype (Fig. 9). In the cells from aged rats, PGE_2, forskolin, and 11-deoxy-PGE_1 significantly decreased the BN formation and ALP activity, while 17-phenyl-ω-trinor PGE_2 showed no effect on these differentiation markers. By contrast, cell proliferation was enhanced by PGE_2, forskolin and 11-deoxy-PGE_1 and not affected by 17-phenyl-ω-trinor PGE_2. These results can be explained by assuming that the signal transduction pathway through EP_1 is inactive in the cells from aged rats and PGE_2 suppresses the cell differentiation and enhances the cell proliferation through the EP_2/EP_4-cAMP pathway (Fig. 9). If the EP_1 pathway is inactive, phosphodiesterase, which degrades newly formed cAMP, is not activated; therefore, cAMP may accumulate, suppressing differentiation and enhancing proliferation of the cells.

The cellular responses through the EP_1 and EP_2/EP_4 pathway in rat calvarial cells are different from those observed with MC3T3-E1 cells in which cAMP stimulates cell differentiation and the activation of PKC results in the stimulation of proliferation and the suppression of differentiation [Hakeda et al., 1986, 1987]. Furthermore, Suda et al. [1996] have reported the presence of EP_1 and EP_2/EP_4 subtypes of PGE_2

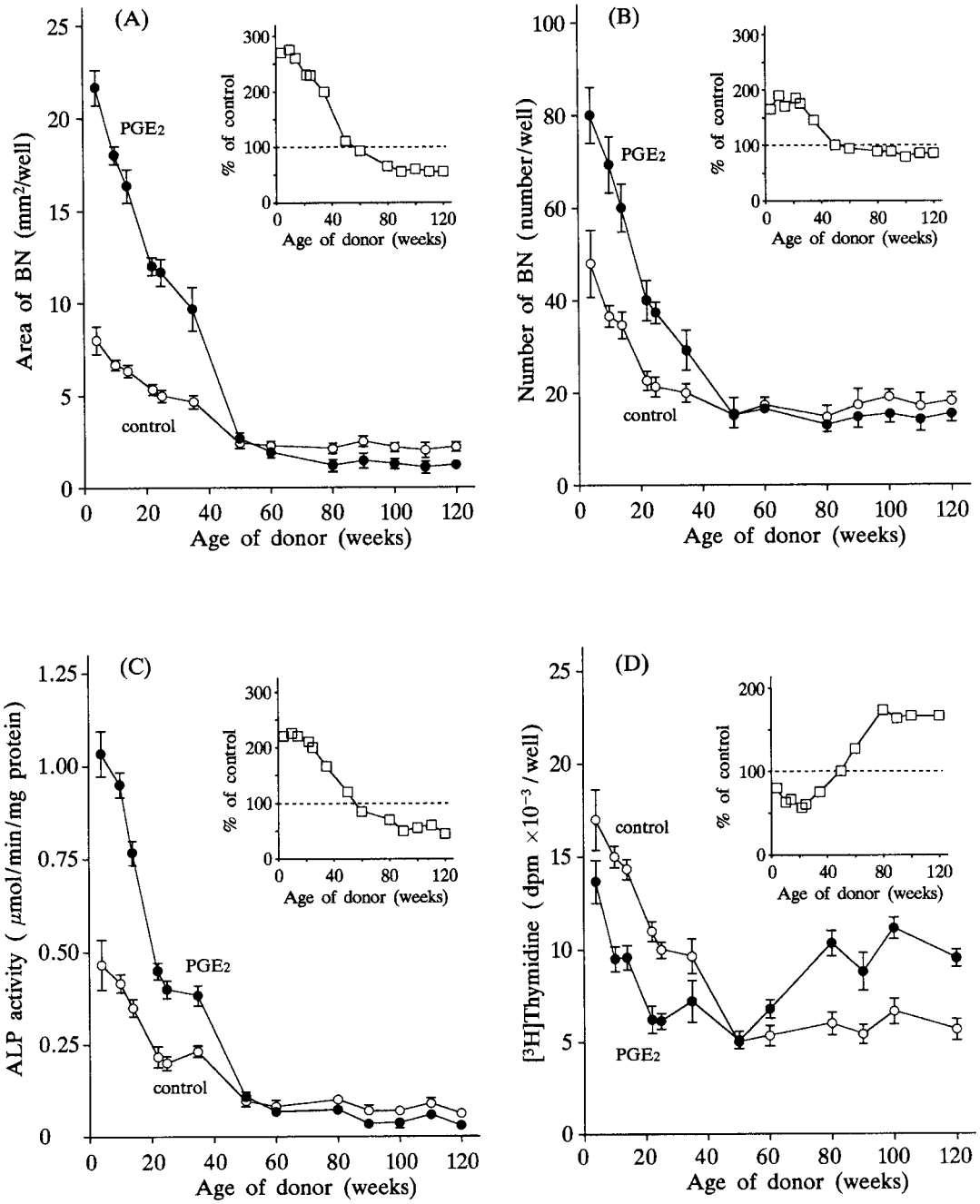

Fig. 5. Effects of prostaglandin E_2 (PGE_2) on bone nodule (BN) formation, alkaline phosphatase (ALP) activity, and DNA synthesis in cultures of calvarial cells from rats of various ages. The cells were treated with (●) or without (○) PGE_2 (10^{-6} M) during the last 6 h of day P4. ALP activity **(C)** was measured at the beginning of day M2. [^{3}H]Thymidine incorporation into the cells **(D)** was measured at the beginning of day M1. The area **(A)** and number **(B)** of BN were measured on day M18. Inset compares the effect of PGE_2 on the ratio of the BN formation, ALP activity, and [^{3}H]thymidine incorporation in PGE_2-treated cultures with that in untreated cultures. Other conditions were as described under Materials and Methods. Each value represents the mean ±SD of four cultures. The experiments with rats aged <100 weeks were repeated at least three times, while those with rats aged ≥100 weeks were repeated twice, and the results were essentially the same as those depicted.

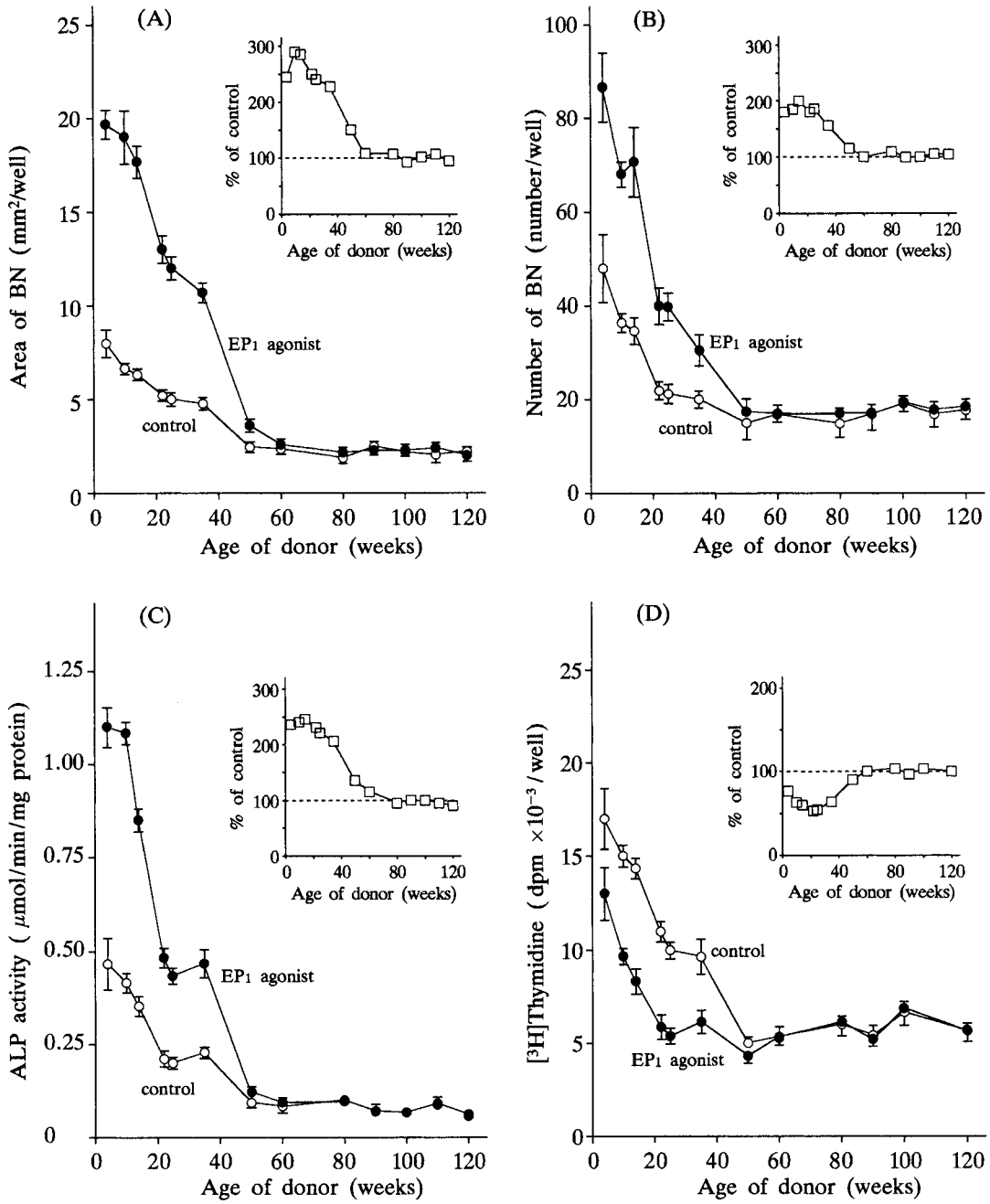

Fig. 6. Effects of EP_1 agonist, 17-phenyl-ω-trinor prostaglandin E_2 (PGE_2), on the bone nodule (BN) formation, alkaline phosphatase (ALP) activity, and DNA synthesis in cultures of calvarial cells from rats of various ages. The cells were treated with (●) or without (○) 17-phenyl-ω-trinor PGE_2 (10^{-6} M) during the last 6 h of day P4. ALP activity **(C)** was measured at the beginning of day M2. [^{3}H]Thymidine incorporation into the cells **(D)** was measured at the beginning of day M1. The area **(A)** and number **(B)** of BN were measured on day M18. Inset compares the effect of 17-phenyl-ω-trinor PGE_2 on the ratio of the BN formation, ALP activity, and [^{3}H]thymidine incorporation in 17-phenyl-ω-trinor PGE_2- treated cultures with that in untreated cultures. Other conditions were as described under Materials and Methods. Each value represents the mean ±SD of four cultures. The experiments with rats aged <100 weeks were repeated at least three times, while those with rats aged ≥100 weeks were repeated twice, and the results were essentially the same as those depicted.

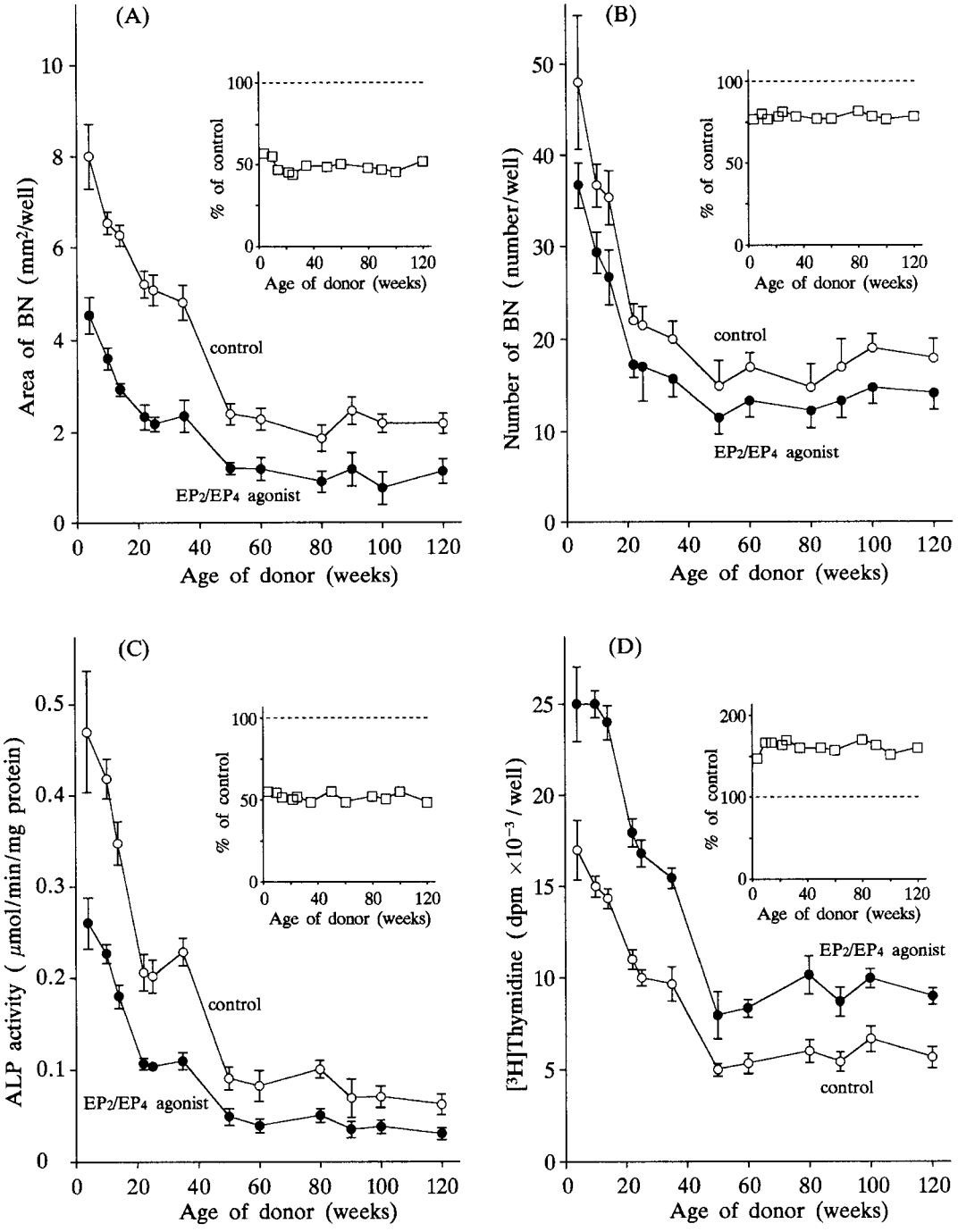

Fig. 7. Effects of EP_2/EP_4 agonist, 11-deoxy-prostaglandin E_1 (PGE_1), on bone nodule (BN) formation, alkaline phosphatase (ALP) activity, and DNA synthesis in cultures of calvarial cells from rats of various ages. The cells were treated with (●) or without (○) 11-deoxy-PGE_1 (10^{-6} M) during the last 6 h of day P4. ALP activity **(C)** was measured at the beginning of day M2. [^{3}H]Thymidine incorporation into the cells **(D)** was measured at the beginning of day M1. The area **(A)** and number **(B)** of BN were measured on day M18. Inset compares the effect of 11-deoxy-PGE_1 on the ratio of the BN formation, ALP activity, and [^{3}H]thymidine incorporation in 11-deoxy-PGE_1-treated cultures with that in untreated cultures. Other conditions were as described under Materials and Methods. Each value represents the mean ±SD of four cultures. The experiments with rats aged <100 weeks were repeated at least three times, while those with rats aged ≥100 weeks were repeated twice, and the results were essentially the same as those depicted.

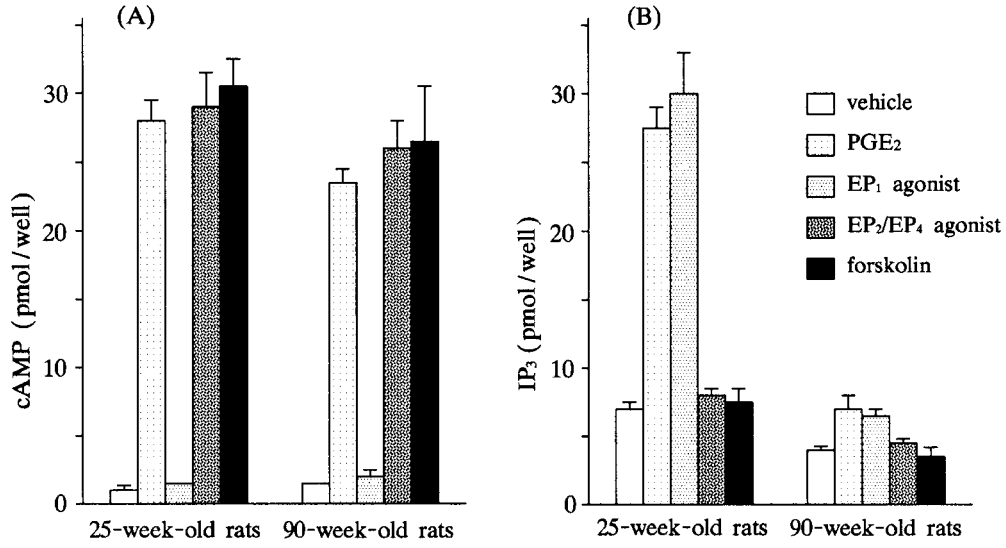

Fig. 8. Effects of prostaglandin E_2 (PGE_2), the agonists specific for PGE_2 receptor subtypes, and forskolin on the accumulation of cyclic adenosine monophosphate (cAMP) and IP_3 generation in the cultures of calvarial cells from 25-week-old rats **(A)** and 90-week-old rats **(B)**. For the determination of cAMP, the cells were incubated in serum-free F-12 medium for 18 h at the beginning of day P4 and treated with 10^{-5} M 3-(isobutyl)-1-methylxanthine (IBMX) for 20 min, followed by 10^{-6} M PGE_2, 10^{-6} M 17-phenyl-ω-trinor PGE_2 (EP_1 agonist), 10^{-6} M 11-deoxy-PGE_1 (EP_2/EP_4 agonist) or 10^{-6} M forskolin for 5 min. For the determination of IP_3, the cells were incubated in inositol- and serum-free medium containing 0.01% bovine serum albumin for 18 h at the beginning of day P4. The cells were then treated with the four agonists (10^{-6} M) for 0.5 min. Other conditions were as described under Materials and Methods. Each value represents the mean ±SD of four cultures. The experiments were repeated at least three times, and the results were essentially the same as those depicted.

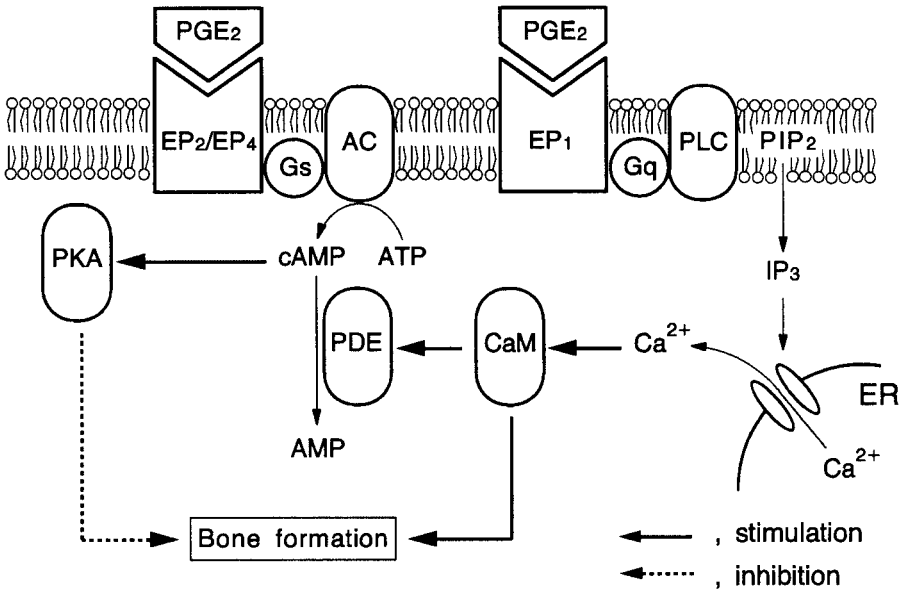

Fig. 9. Scheme of the signal transduction pathway for prostaglandin E_2 (PGE_2)-induced cellular responses in rat calvarial cells. Binding of PGE_2 to the EP_2/EP_4 receptor subtype leads to the activation of adenylate cyclase (AC) to produce cyclic adenosine monophosphate (cAMP), which inhibits bone formation through the activation of cAMP-dependent protein kinase (PKA). Binding of PGE_2 to the EP_1 receptor subtype leads to the activation of phospholipase C (PLC) to produce IP_3, which binds to its receptor on endoplasmic reticulum (ER), resulting in the release of Ca^{2+}. The increase in $[Ca^{2+}]_i$ causes the activation of bone formation. Ca^{2+} also activates a phosphodiesterase (PDE) that blocks the signaling through EP_2/EP_4 receptor subtype by degrading cAMP.

receptor and proposed that the former promotes cell growth, whereas the latter mediates differentiation of osteoblasts. In the present study, the EP_1 agonist promoted differentiation and suppressed growth, while the EP_2/EP_4 agonist promoted growth and suppressed differentiation. As described in a previous paper [Kaneki et al., 1999], the increase in $[Ca^{2+}]_i$, but not the activation of PKC transduces EP_1 signal in rat calvarial cells. The reason for the discrepancy remains unknown, but one possible explanation would be the difference in the stage of differentiation. It has been reported that PGE_2 treatment increases DNA synthesis in the first fraction of five serial collagenase digestion, while it inhibits DNA synthesis in the mixture of third to fifth fractions [Centrella et al., 1994]. The results were explained by the difference in the differentiation stage between the two cell populations. The mode of the response to PGE_2 is also affected by the time of addition during the culture period [Suda et al., 1996; Kaneki et al., 1999]. These differences in the cellular responses to PGE_2 may be a reflection of the difference in the differentiation stage, because osteoblastic cells are likely to differentiate spontaneously during the culture. The present study suggested that the EP_1 pathway is not functioning in the aged rats. The lack of signaling through EP_1 receptor subtype may be attributable to the donor age-dependent loss of the receptor subtype. The changes in the expression of the receptor subtypes with age remain to be examined.

REFERENCES

Bellows CG, Aubin JE, Heersche JNM, Antosz ME. 1986. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif Tissue Int 38:143–154.

Bhargava U, Bar-Lev M, Bellows CG, Aubin JE. 1988. Ultrastructural analysis of bone nodules formed in vitro by isolated fetal rat calvaria cells. Bone 9:155–163.

Centrella M, Casinghino S, McCarthy T. 1994. Differential actions of prostaglandins in separate cell populations from fetal rat bone. Endocrinology 135:1611–1620.

Chyun YS, Raisz LG. 1984. Stimulation of bone formation by prostaglandin E_2. Prostaglandins 27:96–103.

Coleman RA, Smith WL, Narumiya S. 1994. International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 46:205–229.

Hakeda Y, Yoshino T, Nakatani Y, Kurihara N, Maeda N, Kumegawa M. 1986. Prostaglandin E_2 stimulates DNA synthesis by a cyclic AMP-independent pathway in osteoblastic clone MC3T3-E1 cells. J Cell Physiol 128:155–161.

Hakeda Y, Hotta T, Kurihara N, Ikeda E, Maeda N, Yagyu Y, Kumegawa M. 1987. Prostaglandin E_1 and $F_{2\alpha}$ stimulate differentiation and proliferation, respectively, of clonal osteoblastic MC3T3-E1 cells by different second messengers in vitro. Endocrinology 121:1966–1974.

Jee WSS, Ueno K, Deng YP, Woodbury DM. 1985. The effects of prostaglandin E_2 in growing rats: increased metaphyseal hard tissue and cortico-endosteal bone formation. Calcif Tissue Int 37:148–157.

Jee WSS, Mori S, Li XJ, Chan S. 1990. Prostaglandin E_2 enhances cortical bone mass and activates intracortical bone remodeling in intact and ovariectomized female rats. Bone 11:253–266.

Kaneki H, Takasugi I, Fujieda M, Kiriu M, Mizuochi S, Ide H. 1999. Prostaglandin E_2 stimulates the formation of mineralized bone nodules by a cAMP-independent mechanism in the culture of adult rat calvarial osteoblasts. J Cell Biochem 73:36–48.

Kato H, Matsuo R, Komiyama O, Tanaka T, Inazu M, Kitagawa H, Yoneda T. 1995. Decreased mitogenic and osteogenic responsiveness of calvarial osteoblasts isolated from aged rats to basic fibroblast growth factor. Gerontology 41:20–27.

Lowry OH, Roberts NR, Wu ML, Hixton WS, Crawford EJ. 1954. The quantitative histochemistry of brain. II. Enzyme measurements. J Biol Chem 207:19–37.

Nijweide PJ, Burger EH, Feyer JHM. 1986. Cells of bone: proliferation, differentiation and hormonal regulation. Physiol Rev 66:855–886.

Norrdin RW, Jee WSS, High WB. 1990. The role of prostaglandins in bone in vivo. Prostaglandins Leukot Essent Fatty Acids 41:139–149.

Raisz LG, Koolemans-Beynen AR. 1974. Inhibition of bone collagen synthesis by prostaglandin E_2 in organ culture. Prostaglandins 8:377–385.

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

Suponitzky I, Weinreb M. 1998. Differential effects of systemic prostaglandin E_2 on bone mass in rat long bones and calvariae. J Endocrinol 156:51–57.

Yang RS, Liu TK, Lin-Shiau SY. 1993. Increased bone growth by local prostaglandin E_2 in rats. Calcif Tissue Int 52:57–61.