The anabolic effect of PGE$_2$ in rat bone marrow cultures is mediated via the EP$_4$ receptor subtype

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Prostaglandins (PG) are multifaceted modulators of bone metabolism (42), and recent experimental animal studies have shown that PGE$_2$, in particular, is a powerful systemic (3, 19, 20–23, 32, 33, 52) and local (31, 59) anabolic agent. The in vivo effects have been observed both in young (22, 23, 52) and older (3, 19, 20, 21, 32, 33) rats and dogs and also in humans (24, 51). The increased bone mass (cortical as well as cancellous) of bone marrow cells by counting bone nodules and measuring alkaline phosphatase activity. PGE$_2$ increased both parameters, peaking at 100 nM, an effect that was mimicked by forskolin and was abolished by 2',3'-dideoxyadenosine (an adenylyl cyclase inhibitor) and was thus cAMP dependent, pointing to the involvement of EP$_3$ or EP$_4$. Consistently, 17-phenyltrinor PGE$_2$ (EP$_1$ agonist) and sulprostone (EP$_3$/EP$_1$ agonist) lacked any anabolic activity. Furthermore, butaprost (EP$_3$ agonist) was inactive, 11-deoxy-PGE$_1$ (EP$_3$/EP$_2$ agonist) was as effective as PGE$_2$, and the PGE$_2$ effect was abolished dose dependently by the selective EP$_4$ antagonist AH-23848B, suggesting the involvement of EP$_4$. We also found that PGE$_2$ increased nodule formation and AP activity when added for the initial attachment period of 24 h only. Thus this study shows that PGE$_2$ stimulates osteoblastic differentiation in bone marrow cultures, probably by activating the EP$_4$ receptor, and that this effect may involve recruitment of noncommitted (nonadherent) osteogenic precursors, in agreement with its suggested mode of operation in vivo.

The treatment results in increased cancellous and cortical bone mass and increased mechanical strength of the femoral neck (49). This model enabled us to explore the mechanisms of this action. Strong evidence now suggests that PGE$_2$ stimulates bone formation by recruiting new osteoblasts from their precursors. In young rats, most of the new bone produced is cancellous and therefore originates in bone marrow, and we believe, for the following reasons, that PGE$_2$ induces bone marrow osteogenic precursors in these animals to differentiate into osteoblasts. 1) We recently found, using Northern analysis, that a single anabolic dose of PGE$_2$ induces the expression of early-response genes, such as c-fos, c-jun, junB, and egr-1, in the tibia and calvaria of young rats as early as 15 min postinjection (54). Using in situ hybridization, we showed that the induced expression of these genes occurred in bone marrow cells. These data indicate that PGE$_2$ activates multiple transcription factors within the bone marrow compartment, probably to stimulate the proliferation and/or differentiation of osteogenic precursors. 2) Testing this hypothesis directly, we showed that the osteogenic capacity of bone marrow (i.e., the size of the osteoprogenitor pool) is greatly enhanced by systemic treatment with PGE$_2$ in vivo for 2 wk (55). For this purpose, we used an ex vivo bone marrow culture system that enables osteoblastic differentiation of osteogenic precursors belonging to the fibroblastic colony-forming unit population (17, 27, 30, 40). We showed that bone marrow from PGE$_2$-treated rats yielded many more osteogenic colonies (nodules) and a greater alkaline phosphatase (AP) activity compared with bone marrow from vehicle-injected rats. Because each of these colonies is believed to originate from a single precursor cell, this means that PGE$_2$ in vivo stimulates the osteogenic commitment of bone marrow precursors, as we had hypothesized.

We now seek to study the mechanisms by which PGE$_2$ recruits osteoblasts from their marrow precursors: 1) of which the PGE$_2$ receptors known today mediate the action of PGE$_2$. Prostaglandins exert their actions on various cells in the body via specific cell surface receptors that are termed EP and have been divided into four subtypes (EP$_1$–EP$_4$) according to their relative sensitivity to a range of selective agonists and antagonists (7, 12, 36, 37). In recent years, the human, rat, and mouse EP$_{1–4}$ receptors have been cloned and characterized (1, 2, 5, 16, 26, 38, 53). They all have seven transmembrane domains and are coupled to different G proteins and activate different secondary messenger systems, such as adenyl cyclase or phospholipase C. Recent data point to the possibility that EP$_2$ and EP$_4$, the two receptors using the cAMP signal

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transduction system, are the most important receptors in the effects of PGE2 on bone cells. 1) Initial localization experiments in embryonic and neonatal mice showed that EP4 is the major form found in bone tissue, especially in preosteoblasts, with some EP3 expressed in perichondrium (18). In support of the role of EP4 in bone, we recently found by Northern analysis and in situ hybridization that it is also expressed in bone marrow cells of young adult rats (M. Weinreb, M. Machwate, N. Shir, M. Abramovitz, G. A. Rodan, and S. Harada, unpublished observations). Also, expression of EP4 (originally labeled erroneously as EP2; see Refs. 39 and 53) was found in neonatal rat calvaria by in situ hybridization and in rat bone marrow cultures with PCR (25). 2) In the majority of systems examined, the stimulatory effects of PGE2 are cAMP mediated (8, 10, 12.5 U/ml nystatin (an EP3/EP1 agonist, Refs. 2 and 7, gift of Dr. F. McDonald, Schering, Germany); 11-deoxy-PGE1 (an EP2/EP4 agonist, Ref. 48, Cayman); AH-23848B (an EP4 antagonist, Ref. 13, gift of Dr. S. Lister, Glaxo, UK); forskolin (an adenylate cyclase stimulator, I CN Biomedicals, Costa Mesa, CA); and DDA (2',3'-dideoxyadenosine, an adenylate cyclase inhibitor, Sigma).

Therefore, the purpose of this study was to test which of the known EP receptors mediates the anabolic action of PGE2 in vitro by using an osteogenic bone marrow culture system in which PGE2 is anabolic and by using an array of EP agonists and antagonists.

MATERIALS AND METHODS

All animal protocols were approved by the animal experimentation committee. Sprague-Dawley rats, 6 or 7 wk old, were killed by CO2, their femora were excised and defleshed, and the epiphyses were removed. Bone marrow was flushed out, and a single cell suspension was achieved by repeated pipetting. Cells from each femur were cultured in 6-well plates (Nunc) at a density of ~2 × 10^7 cells/well in a medium containing α-MEM + 13% fetal calf serum (all reagents except where noted were from Biological Industries, Beit-Haemek, Israel) + 2 mM glutamine + 100 U/ml penicillin + 100 µg/ml streptomycin + 12.5 U/ml nystatin + 10 mM β-glycerophosphate + 50 µM ascorbic acid (Merck, Darmstadt, Germany) + 10 mM dexamethasone (Dex, Sigma). Wells were arranged in triplicates, and the different compounds were added to the culture medium. After an attachment period of 24 h, nonadherent cells were removed by a PBS (phosphate-buffered saline) rinse, and cultures were maintained in 7% CO2 at 37°C for 21 days, with medium changes twice weekly. At the end of the culture period, cultures were rinsed in PBS, fixed in a 1:1:1.5 solution of 10% Formalin-methanol-water for 2 h, and stained with the Von Kossa method for mineralized nodules (27). Mineralized nodules (completely or partially stained black) and nonmineralized nodules (stained yellow) were counted under a magnifying glass over transmitted light, and the relative proportion of mineralized nodules of the total number of nodules was determined.

The following compounds were tested: PGE2 (Cayman Chemical, Ann Arbor, MI); 17-phenyl-α-trinor PGE2 (an EP1 agonist, Refs. 4 and 48, Cayman); butaprost (an EP2 agonist, Refs. 7 and 12, gift of Dr. P. Gardiner, Bayer, UK); sulprostone (an EP3/EP4 agonist, Refs. 2 and 7, gift of Dr. F. McDonald, Schering, Germany); 11-deoxy-PGE1 (an EP2/EP4 agonist, Ref. 48, Cayman); AH-23848B (an EP4 antagonist, Ref. 13, gift of Dr. S. Lister, Glaxo, UK); forskolin (an adenylate cyclase stimulator, I CN Biomedicals, Costa Mesa, CA); and DDA (2',3'-dideoxyadenosine, an adenylate cyclase inhibitor, Sigma).

Except for the experiment in which PGE2 was added to the culture medium for varying lengths of time (see RESULTS), all compounds that were tested were added to the cultures throughout the experimental period (days 0-21 for nodules and days 0-12 for AP) and were thus replaced with the medium twice weekly. Each compound was tested on 18 wells derived from six rats × triplicate repeats.

In addition to mineralized nodule formation, osteogenic differentiation was assessed by measuring AP activity in culture (27, 55). Femoral cells were cultured as before, and on day 12, they were washed in PBS and scraped in 10 mM Tris·HCl buffer (pH = 7.6) containing 10 mM MgCl2 and 0.1% Triton X-100. AP activity was determined colorimetrically with a Sigma kit on the basis of p-nitrophenylphosphate as substrate. The protein content was measured according to Bradford with BSA as standard and a protein assay kit (Bio-Rad, Munich, Germany), and enzyme activity was expressed as units per milligram protein.

All data are presented as means ± SE. Comparison between group means was performed with one-way analysis of variance with post hoc multi-group contrasts (n = 6 animals/group).

RESULTS

First, we determined the concentration range in which PGE2 was anabolic and added it at concentrations of 10–1,000 nM for 21 days. PGE2 increased bone nodule formation with a maximal effect at 100 nM (Figs. 1 and 2). This concentration was also maximally effective in stimulating AP activity (corrected for the
protein content) in these cultures (Fig. 3). It is noteworthy that PGE2 repeatedly increased the protein content (by 10–20% at 6–12 days) but increased AP activity to a much greater degree.

In search of the signal transduction involved in the anabolic effect of PGE2, we tested whether it is mediated via increased cAMP production. Indeed, the increase in nodule formation caused by PGE2 was mimicked by forskolin, an adenylate cyclase stimulator with maximal effect at 10 µM, and was blocked by DDA, an adenylate cyclase inhibitor (Fig. 4). These data pointed to EP2 and/or EP4 as the receptor mediating the effect of PGE2.

In agreement with this finding, 17-phenyl-ω-trinor PGE2, an EP1 agonist, and sulprostone, an EP3/EP1 agonist, failed to increase nodule formation even at a concentration 10-fold higher than the most effective concentration of PGE2 (Fig. 5) and failed to stimulate AP activity (Fig. 6). When testing agonists of the cAMP stimulatory receptors (EP2 and EP4), we found that butaprost, a selective EP2 agonist, was ineffective in enhancing nodule formation, whereas 11-deoxy-PGE1, an EP3/EP2 agonist, was as effective as PGE2 in this assay, with maximal effect at 100 nM (Fig. 7). Similarly, 11-deoxy-PGE1, but not butaprost, stimulated AP activity, with maximal effect at 100 nM (Fig. 8). These data suggested that the anabolic effect of PGE2 in the bone marrow culture system was mediated via the EP4 receptor subtype. To validate this conclusion, we added to the cultures increasing concentrations of the selective EP4 antagonist AH-23848B in the presence of 100 nM PGE2. Both the increased nodule formation (Fig. 9) and enhanced AP activity (Fig. 10) caused by PGE2 were inhibited dose dependently by this compound down to the control group level, indicating the involvement of EP4 in the anabolic effect of PGE2.

All agents that increased the number of mineralized nodules also increased their proportion out of the total number of nodules (data not shown), suggesting that PGE2 recruits only osteogenic precursors to the adherent fraction of the culture. In support of this conclusion, we found that the correlation coefficient between the total number of nodules and the number of mineralized nodules in vehicle- and PGE2-treated cultures was extremely high (0.967), with a unity regression slope (Fig. 11). In all the experiments described, we counted the number of cells seeded on day 0 within each
experiment and never found any significant difference in cell number among the wells subjected to different treatments. Furthermore, we attempted a correlation analysis between the number of mineralized nodules (as a dependent variable) and the total number of cells seeded and never found any such correlation. These observations indicate that the number of mineralized nodules was affected by the compounds added to the cultures and not by the number of cells seeded.

To examine the crucial period for the anabolic effect of PGE2 in this culture system, we added it in different time schedules to the culture medium. Addition of 100 nM PGE2 for the first 24 h only was as effective as the full 21 days in stimulating nodule formation (Fig. 12). Likewise, 24 h of PGE2 were equal to the full 12 days in stimulating AP activity (Fig. 13). These data indicated that the initial attachment period of 24 h is crucial for the stimulatory action of PGE2.

Cumulatively, our data indicate that PGE2 increases the osteogenic capacity of bone marrow by recruiting osteoprogenitor cells via activation of the EP4 receptor subtype and that this effect probably occurs during the initial attachment period.

**DISCUSSION**

As reported previously by others (44, 46), PGE2 was anabolic in this bone marrow culture system, i.e., it increased the number of bone nodules and stimulated AP activity, with maximal effect at 100 nM. A similar anabolic activity was shown in a parallel system, fetal rat calvarial cells (15, 35, 50). In both systems, it is believed that each nodule originates from a single cell (fibroblastic colony-forming units), and therefore it was concluded that PGE2 recruits otherwise noncommitted osteogenic precursors. The recruitment of bone marrow osteogenic precursors in vitro is highly compatible with the proposed mode in which PGE2 enhances bone formation in vivo. Numerous animal experiments showed that both local and systemic administration of PGE2 augments bone mass by increasing the number of osteoblasts and the extent of bone forming surfaces and frequently by causing the formation of new bone tissue (3, 19, 20–23, 31–33, 52, 58). When the new bone formed was cancellous or endocortical, these new osteoblasts must have originated from bone marrow progenitors, which belong to the stromal compartment. In
support of this mechanism whereby PGE₂ stimulates bone formation, we recently showed that systemic administration of PGE₂ to young rats induced the expression of early-response genes, such as c-fos and egr-1, in bone marrow cells and increased the size of the osteoprogenitor pool in bone marrow (54, 55). Cumulatively, these data show that osteoblast recruitment from marrow precursors is the major component of the anabolic action of PGE₂ both in vivo and in vitro. Whether this recruitment involves proliferation or merely differentiation remains to be investigated.

We found that the highest concentration of PGE₂ (1,000 nM) was not as stimulatory as the maximally effective one (100 nM). A similar observation was made by others in cultures of newborn-fetal rat calvaria cells (15, 35, 50). Whether this is due to some toxic effect that sets in under this concentration is not known; however, we noted that the increase in the protein content exerted by PGE₂ (see RESULTS) was also not maximal (compared with 100 nM PGE₂).

The anabolic effect of PGE₂ in our marrow cultures was dependent on the concentration of Dex. We found that PGE₂ increased the number of bone nodules only at a lower Dex concentration (10 nM) but not at a higher concentration (100 nM), which by itself increased nodule formation (data not shown). Similar observations made by Scutt et al. (45) showed that the stimulatory effect of 100 nM PGE₂ on the number of calcified nodules, which was maximal at Dex concentrations of 1–10 nM, was greatly diminished at higher Dex concentrations. Dex is known to stimulate the differentiation of osteoblastic lineage cells in fetal rat calvaria cells (6), bone marrow cells (11, 30, 43), and other bone-related cell systems. In fact, cultures of bone marrow cells from adult rats grown without Dex do not form calcified nodules (30, 45). These observations establish that physiological concentrations of Dex are required for osteoblast differentiation in these culture systems and also permit the stimulatory (additive) effect of PGE₂ as seen in our study too. Our data suggest that when the effect of Dex in recruiting marrow osteoprogenitors is maximal, PGE₂ is no longer able to further enhance osteoblastic commitment. At higher, pharmacological doses, both effects of Dex are lost.

The exact mechanism whereby PGE₂ recruits osteogenic precursors is not known. We found that the presence of PGE₂ for the initial attachment period of 24 h is sufficient to induce the same increase in nodule formation as its presence for the full 21 days. A
somewhat similar observation was made in bone marrow cultures (44) and in cultures of fetal rat calvarial cells (50) by others. Because each nodule is believed to originate from a single cell, these findings suggest that PGE2 induces, during the attachment period, a shift from nonadherent (noncommitted) to adherent (committed) marrow osteogenic colony-forming units. In support of this conclusion, we found in this study that treatment with PGE2 adds to the cultures only mineralized colonies, suggesting that it specifically affects inducible osteogenic colony-forming units.

We present significant evidence in this study that indicates that the anabolic effect of PGE2 in our system is mediated via the EP4 receptor subtype. First, this effect was mimicked by forskolin, an adenylate cyclase stimulator, and was inhibited by DDA, an adenylate cyclase inhibitor, pointing to EP2 and/or EP4 (the 2 cAMP-related receptors) as mediators. In agreement with this conclusion, 17-phenyl-6-trinor PGE2 and sulprostone, agonists of the non-cAMP-related receptors (EP1 and EP3, respectively), were inactive. In support for a cAMP-dependent anabolic effect in bone cells, PGE2 and forskolin, but not sulprostone, were found to increase incorporation of [3H]thymidine and collagen synthesis in fetal rat calvaria organ cultures (56). Second, butaprost, a selective EP2 agonist, was ineffective in stimulating bone nodule formation and AP activity in our cultures, whereas 11-deoxy-PGE1 was as effective as PGE2. Butaprost is ~10-fold weaker than PGE2 at the EP2 receptor (7, 9, 28), but in our study even a concentration of butaprost 10-fold higher than that of PGE2 was ineffective. Although many of the agonists we tested in this study are not 100% selective for the respective EP receptors, they often have a 5- to 100-fold difference in their ability to activate the various EPs. For instance, 17-phenyl-6-trinor PGE2 is equipotent relative to PGE2 at the EP1 receptor but ~5 times less active at the EP3 receptor and 50–100 times less active at the EP4 and EP2 receptors, respectively (9, 28). Conversely, sulprostone is equipotent relative to PGE2 at the EP3 receptor but ~2–4 times less active at the EP1 receptor. To date, there is no selective EP4 agonist; however, 11-deoxy-PGE1 binds the rat EP4 receptor with an affinity identical to that of PGE2 (9) and is used in conjunction with butaprost (a selective EP2 agonist) to distinguish the involvement of EP4 from that of EP2 (14, 28, 29, 34) as was done here. Thus our data so far implicated EP4 as the mediator of the anabolic effect of PGE2 in bone marrow of young adult rats. We further validated our conclusion by showing that this effect was gradually abolished by increasing concentrations of the weak but selective EP4 antagonist AH-23848B (13, 14). The possible involvement of EP4 in the recruitment of osteoblasts from bone marrow precursors is even further strengthened by our recent finding that EP4 is expressed in bone marrow of long bones of young adult rats such as the ones used for this study, whereas EP2 is not (Weinreb et al., unpublished data). However, unequivocal demonstration of the role of EP4 vs. EP2 in this assay must await the development of (yet unavailable) 100% selective EP2 and EP4 agonists that are equipotent relative to PGE2.

Our data corroborate those of Scutt et al. (46) that the anabolic effect of PGE2 in bone marrow cells is mediated via increased cAMP production. However, these authors have concluded that such an effect was consequentely mediated via the EP2 receptor. The existence of EP4 was not known at that time and our present data, with various agonists and antagonists, point rather to EP4 as the mediator of this anabolic effect.

A recent study using RT-PCR showed that EP2 is expressed in fetal rat bones and that its expression is greatly diminished in young adult rats (38). These data, together with the report that butaprost partially stimulated proliferation and collagen synthesis in fetal rat calvariae (56), may point to a role for EP2 in fetal bone development and its possible replacement by EP4 in adult animals.

In summary, this study shows that PGE2 is anabolic in bone marrow cultures, i.e., increases the number of osteogenic colonies and enhances AP activity. This effect is apparently mediated by activation of the EP4 receptor subtype and occurs within the first 24 h, probably by recruiting noncommitted osteogenic precursors. The molecular cascade subsequent to the activation of EP4 in the noncommitted osteogenic precursors by PGE2 must be further investigated.

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