Hylonome

Bone anabolic therapy with selective prostaglandin analogs

J.R. Hartke, M.W. Lundy

Procter and Gamble Pharmaceuticals, Mason, OH, USA

Abstract

Prostaglandin E_2 has been shown to increase bone mass in animals and humans but it also has considerable dose limiting systemic side effects. The molecular description of multiple seven transmembrane domain G protein coupled prostanoid receptors offered the opportunity to probe the skeletal effects of specific receptors using selective agonists. Bone effects have been reported with many of the prostanoid receptors, with most interest focused on the anabolic effects of EP2, EP4, and FP receptors. Current data suggests activity at the EP2 receptor stimulates formation, activity at the EP4 receptor stimulates resorption (and possibly formation), and activity at the FP receptor produces new trabeculae. However, caution must be exercised in extending the effects of prostanoids in isolated systems to systemic skeletal effects, since tissue level effects are the cumulative result of bone formation and bone resorption. Furthermore, species differences in receptor sequence and density confound extrapolation of effects from one model to another model. While these molecular targets increase our insight into how the skeleton can be affected pharmacologically, they still do not answer questions about the role of naturally occurring prostaglandins in skeletal health. This manuscript will review some of the recent advances in knowledge of the bone anabolic effects of selective prostanoid ligands.

Keywords: Prostanoid, Bone Anabolic, Osteoblast

The skeleton has been optimized through evolution to support two basic processes - 1) to provide a rigid framework (for locomotion and protection of internal organs), and 2) as a reservoir for mineral homeostasis. While the debate continues on whether bone strength or mineral homeostasis is most important for the health of the organism, there is little doubt that both are essential to host survival. The controls for mineral homeostasis (parathyroid hormone, calcitonin, and Vitamin D) have been identified through removal of specific organs such as the parathyroid and thyroid glands. However, little is known about the controls regulating mechanical homeostasis. Bone is known to respond locally to mechanical stress, such as the unilateral increase in forearm bone density in tennis players[1,2]. Conversely, bone that is no longer subject to mechanical stresses, as seen with denervation injury, is resorbed, although not completely[3]. Likewise, bone that is added during anabolic therapy is removed once therapy is discontinued[4]. Bones such as the skull and the femur respond differently to mechanical stress for maintenance of "normal" mass. This

should not be surprising since evolutionary pressures for the skull integrity and femur integrity are likely to be quite different.

Osteocytes are well situated to sense the strain on bone, but they must communicate the need for increased bone mass through mediators to periosteal or endosteal osteoblasts. These mediators most likely diffuse rapidly and are inactivated in the systemic circulation. Both prostaglandins and nitric oxide are good candidates for mechanical signal transduction[5] and may be useful targets for systemic bone anabolic therapy. In an osteocyte model system, cultured mouse calvarial cells exposed to pulsatile fluid flow increased their release of PGE_2, $PGF_2\alpha$, and PGI_2, but with a different time course and to a different extent[6]. $PGF_2\alpha$ was increased in 5-10 minutes, PGE_2 release was increased by 10 minutes and continued over 60 minutes of treatment, and PGI_2 increased the slowest. The pulsed fluid flow also increased expression of prostaglandin G/H synthase-2 (PGHS-2) but not PGHS-1, which is responsible for production of prostaglandins, and may be important for long-term potentiation of the response[6]. These same mediators can also dilate local blood vessels causing an increase in blood flow[7,8] or prevent osteoblast apoptosis[9]. The prevention of apoptosis in osteoblasts or osteocytes by prostaglandins may also play a role in the tissue level

preservation or addition of bone mass. Peptides are also likely involved in mediation of prostanoid effects, as shown by the up regulation of IGF-1 in response to PGE$_2$ in cultured fetal rat calvaria[10]. While these examples demonstrate local control of bone mass by prostaglandins, most studies have investigated the effects of systemically administered prostaglandins, since these studies are easier to perform and provide data on the potential use of prostanoids for osteoporosis treatment.

Systemic effects of prostaglandins

Systemic administration of prostaglandins has been known for many years to produce an increase in bone mass, bone volume, and bone strength[11-14]. Prostaglandins have also been shown to increase bone mass in humans, both in neonates administered PGE$_1$ to maintain patency of the ductus arteriosus[15-18], and in cases of infantile cortical hyperostosis[19,20]. PGE$_2$ adds bone to the endosteal envelope by a modeling and remodeling dependent bone gain, often with formation of new trabeculae composed of woven bone at high doses[21,22]. Periosteal formation occurs through a modeling dependent bone gain[22-24]. *In vitro* cultures of periosteal osteoblasts from neonatal mouse calvaria demonstrate increased osteoblastic proliferation and bone formation in response to PGE$_2$[25]. The timing and location of bone formation in response to PGE$_2$ has been summarized by Jee and Ma[22]. It must be emphasized that the increase in bone mass is a cumulative result of a net positive bone balance, or when the rate of bone formation is greater than the rate of bone resorption. This point is especially important when predicting bone anabolic effects while studying prostaglandins *in vitro*, where typically only formation or resorption is measured at one time, but not both at the same time. Further discordance of experimental results is evident when using different animal models since species variation in receptor sequence and expression can result in a predominance of resorption and a net bone loss or a predominance of formation and a net bone gain. These species differences are discussed later in this manuscript in the sections on EP2 and EP4 receptors. Likewise, the absence of a receptor during fetal development and during growth in receptor knockout animals can result in compensation by other receptor systems, and may not predict the result of receptor stimulation or inhibition in adult animals.

When PGE$_2$ treatment is discontinued, remodeling processes remove most of the trabecular and endocortical bone, while sparing the periosteal bone[26]. While the bone anabolic effects of PGE$_2$ have been well described, the specific receptors responsible for the increase in bone mass are only beginning to be understood. This is caused by the poor selectivity of most naturally occurring prostaglandins for a single receptor. For example, PGE$_2$ has the greatest affinity for the EP3 receptor (dissociation constant or K_i of 0.33 nM), followed closely by the EP4 (K_i 0.79 nM), EP2 (K_i 4.9 nM), EP1 (K_i 9.1 nM) and FP (K_i 119 nM) receptors[27]. From the administration of PGE$_2$, it is not clear which receptor is responsible for the bone anabolic effect and whether it is a different receptor that is responsible for the side effects. To probe the specific skeletal effects of a single prostanoid receptor, selective ligands need to be identified and their effects compared.

Receptor types and signaling pathways

During the past decade, the identification of specific G protein coupled membrane receptors for prostaglandins has greatly facilitated the study of the roles for specific prostanoid receptors[7,8]. The prostanoid receptors are named after the naturally occurring prostanoid with the highest affinity. For example, the FP receptor binds prostaglandin F$_2\alpha$ with the highest affinity; the DP receptor binds prostaglandin D$_2$ with the highest affinity, and so forth[7]. Prostaglandin E$_2$ binds with the highest affinity to four receptors, which are designated EP1, EP2, EP3 and EP4 (Fig. 1)[7]. Specific analogs have been identified for some of these receptors and will be discussed later.

In general, prostanoid receptors can be subdivided into two basic classes based upon their coupling to G proteins[7,28]. The relaxant prostanoid receptors (EP2, EP4, DP, and IP) are grouped by their general ability to relax vascular smooth muscle (Fig. 1). They are coupled to guanosine triphosphate regulatory proteins which elevate cAMP (G$_s$ proteins)[8]. The excitatory prostanoids (FP, EP1, and TP) are grouped by their ability to cause contraction of vascular smooth muscle. They are coupled to G$_q$ proteins and act through increases in calcium and inositol triphosphate[8] (Fig. 1). The EP3 receptor can couple to G$_s$, G$_i$, or G$_q$ proteins depending on splice modifications to the intracellular carboxyl terminus. Knockout mice now exist for all of these receptors. The reported phenotypes of these knockout mice have not included any gross skeletal abnormalities[28]. This suggests either redundancy of pathways that locally control skeletal mass, or that the absence of one prostanoid receptor results in a compensatory increase in sensitivity of another prostanoid receptor.

Alternatively, mice may use different prostanoid receptors to control bone mass and may not predict the effects in other species. Based upon the receptor affinity profile, PGE$_2$ should stimulate all four EP receptors, resulting in an increase in both cAMP and calcium/inositol triphosphate in cells with all four receptors. This intracellular messenger profile is similar to the intracellular messenger profile of parathyroid hormone[29]. The short half-life of PGE$_2$ also suggests the presence of a mechanism to potentiate the effect of PGE$_2$. The up-regulation of prostaglandin G/H synthase-2 and the production of additional prostaglandins have been suggested as a possible mechanism to prolong (potentiate) the effect of PGE$_2$[30]. Potentiation also likely occurs through the production of peptides such as IGF-1[10].

Selectivity of ligands

Selective ligands are required to study specific receptor effects *in vivo*. The selectivity of numerous compounds have
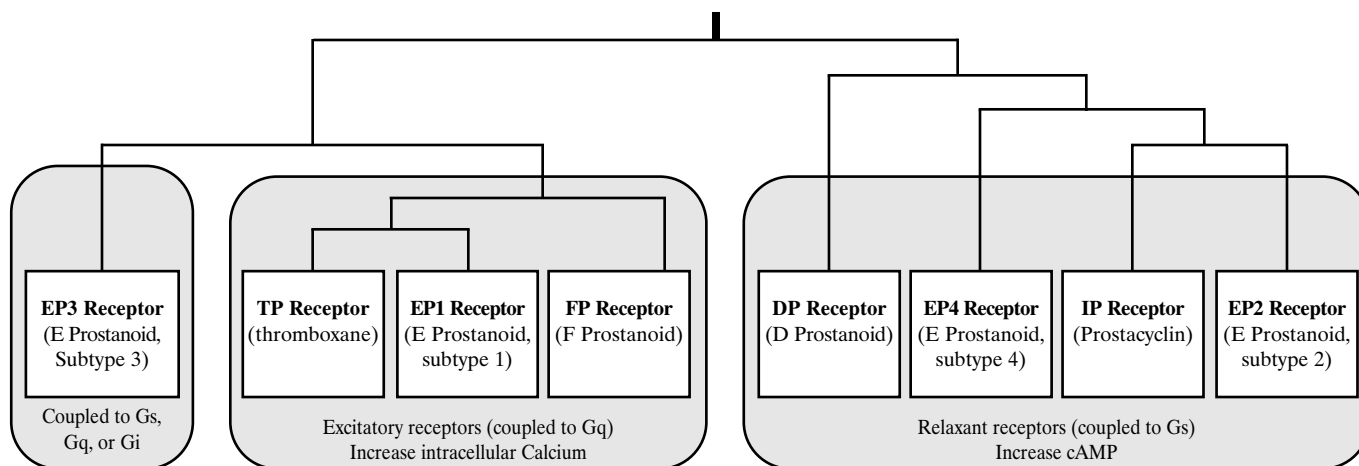
Figure 1. Prostanoid receptor relationships.

been published for the mouse receptors and the human receptors[27,31]. Doses at five to ten fold above the half maximal inhibitory concentration (IC_{50}) of most naturally occurring prostanoids stimulates multiple receptors, confusing the measurement of specific effects of individual receptors[27]. The lack of specificity of naturally occurring prostanoids may also be responsible for the biphasic or variable effects seen *in vitro* since as the dose level of a non-specific prostanoid is increased, additional receptors are stimulated, often with opposing activities[32]. For example, PGE_2 and $PGF_2\alpha$ when combined with cortisol, both increased tritiated (^3H) thymidine incorporation. However, the selective FP agonist, fluprostenol was ineffective[33], suggesting the $PGF_2\alpha$ effect was due to activity at an EP receptor rather the FP receptor. Conversely, since different prostanoid receptors use the same intracellular pathways, stimulation of two different receptors on a cell can result in similar elevations of an intracellular messenger (such as the EP2 and EP4 receptors, both of which elevate cAMP). This redundancy in the prostanoid pathways may also explain the lack of skeletal effects in prostanoid receptor knockout mice. Furthermore, induction of prostaglandin G/H synthase-2 to increase production of prostaglandins (auto amplification) further convolutes the study of specific agonists *in vitro* or *in vivo*[30].

While the most studied prostanoid is PGE_2, the specific receptors mediating the effects on bone formation and resorption are not known. Specific agonists are known for the FP, TP, EP2 and IP receptors[7,27]. New receptor specific compounds offer the potential to study the bone effects of additional individual receptors[27]. Recent evidence points to the two relaxant receptors, EP2 and EP4, which both elevate cAMP, as playing a major role in mediating the effect of PGE_2.

EP2 effects

The EP2 receptor is expressed in both long bones and calvaria of rats as shown by reverse transcriptase polymerase chain reaction (RT-PCR) and in situ hybridization[34]. The mRNA levels were higher in growing rats compared to mature adults. Cultures of fetal rat calvaria respond to EP2 agonism with an elevation in cAMP resulting in replication and differentiation of osteoblasts. EP2 agonism in this system was proposed to be the major receptor responsible for PGE_2 anabolic activity[33]. Recent work with new receptor selective EP2 analogs demonstrates an increase in bone volume and strength after local administration to the tibia of rats[35,36].

Resorption in mouse calvaria appears to be controlled by both EP2 and EP4 receptors[37]. In highly purified osteoclasts from rabbits, PGE_2 inhibited bone resorption[38], which was attributed to EP4 activity, since it was the most abundantly expressed receptor and was inhibited by an EP4 agonist. However, an EP2 agonist (butaprost) also inhibited resorption in this rabbit *in vitro* osteoclast model as well, suggesting a contribution of the EP2 receptor[38]. In EP4 knockout mice, the EP2 agonist butaprost also increased resorption slightly[39]. These effects on resorption by butaprost in these two models may be a result of either a role for the EP2 receptor in resorption or inadequate receptor selectivity of butaprost. In summary, activity at the EP2 receptor appears to stimulate osteoblast differentiation with less stimulation of osteoclast resorption, resulting in a net effect of a bone mass increase. *In vivo* studies using local delivery[35,36] have helped confirm these *in vitro* findings. However, as stated earlier, species specific effects due to receptor distribution and sequence are likely responsible for subtle differences in net bone balance, and care should be taken in extrapolating effects between species.

EP4 effects

The EP4 receptor is expressed in osteoblastic cell lines (MC3T3, RCT-1, RCT-3, TRAB-11 and RP-1), in the tibia of young rats[40] and in primary human osteoblasts[41,42]. Additional *in vitro* studies suggested that EP4 was the major prostanoid receptor subtype present in bone, was responsible for increasing cAMP, and up-regulates in response to ligand binding[42].

In mouse calvarial cultures, resorption levels were increased with an EP4 agonist to a greater extent when compared to other EP receptor selective compounds[39]. However, PGE_2 still produced the greatest increase in resorption (most likely since it stimulates all four EP receptors). There was no resorption increase in these calvarial cultures in response to EP1 or EP3 agonists[39]. Additional evidence of the importance of the EP4 receptor in bone resorption comes from an impaired bone resorption response to PGE_2 in EP4 knockout mice[43]. Cultured calvaria from EP4 knockout mice had no increase in resorptive activity when exposed to PGE_2. Calvaria from EP4 knockout mice still responded to dibutyryl cAMP with an increase in resorption, demonstrating the potential for normal osteoclastic activity. In contrast, calvaria from normal (wild type) mice-increased cAMP and resorption in response to PGE_2 or dibutyryl cAMP[43]. Further evidence for the role of the EP4 receptor in resorption comes from the prevention of osteoclast formation by an EP4 antagonist[44]. In an *in vivo* study, PGE_2 administered to young rats along with an EP4 antagonist greatly suppressed the bone formation response to PGE_2[45]. This result was interpreted as evidence demonstrating a significant role for the EP4 receptor in stimulation of formation[45]. However, this depressed formation could also represent an indirect result of depressed resorption which was not discussed in the publication. In summary, most of the literature suggests a role for the EP4 receptor in stimulation of resorption. However, there is indirect evidence that the EP4 receptor is also involved in bone formation. The relative balance of these effects in different species may lead to confusion on the bone anabolic potential of an EP4 agonist.

The EP4 receptor also appears to play a role in osteoclast activity associated with inflammation. In EP4 knockout mice, lipopolysaccharide administration caused no increase in osteoclastic activity as measured by urinary deoxypyridinoline. This increase in resorptive activity through the EP4 receptor appears to require both osteoblasts and osteoclasts, since in situ hybridization detected the EP4 receptor on osteoblasts, but not in multinucleated cells of mice[46]. Furthermore, when a mixed culture from EP4 knockout mice was examined, IL-1α, TNFα, and bFGF were not elevated in response to PGE_2[46]. This data was taken to suggest that the EP4 receptor plays a major role in inflammation-induced bone resorption (periodontal disease and osteomyelitis)[47].

EP1 effects

The EP1 receptor is present on osteoblasts (Northern analysis in MC3T3 cells)[48]. The administration of an EP1 agonist, 17-phenyl trinor PGE_2, forced replication of cells rather than differentiation as measured by an increase in DNA synthesis and a decrease in alkaline phosphatase[48]. The administration of 17-phenyl trinor PGE_2 also resulted in cell shape changes resulting in a more fibroblastic appearance. The EP1 receptor appears to contribute to the up regulation of PGE_2 production rates during PGE_2 administration (auto-

amplification) based upon the increase in prostaglandin G/H synthase activity after *in vitro* addition of 17-phenyl trinor PGE_2[49]. This auto-amplification may be crucial for prolonging bone growth in response to fractures or mechanical stress. No increase in resorption was seen in mouse calvarial cultures after treatment with an EP1 agonist[37,39].

FP effects

The FP receptor is present in the Py1a (rat), MC3T3-E1 (mouse), and stem cell cultures (MN-7 and mouse marrow) using RT-PCR[50]. The FP receptor is also present in cultures of primary human osteoblasts after three weeks of culture[41]. Inhibition of collagen synthesis in bone cells appears to be mediated by the FP receptor[50]. The effects of $PGF_2\alpha$ may be mediated in part by up regulation of prostaglandin production[37], since indomethacin, an inhibitor of prostaglandin G/H synthase, blocks the $PGF_2\alpha$ response in neonatal parietal bone cell cultures, but had no effect on PGE_2. Furthermore, prostaglandin G/H synthase message was increased in bone cells stimulated with FP receptor agonists[50]. Prostaglandin $F_2\alpha$ increased inositol phosphates and elevated intracellular calcium but did not increase cAMP in quiescent NIH-3T3 cells. In this same study, DNA synthesis was increased in a dose-dependent manner by an FP agonist[51]. In another study, $PGF_2\alpha$ and the prostaglandin F receptor (FP) selective agonist, fluprostenol, significantly inhibited $[^3H]$ proline incorporation (>90%) into collagen and noncollagen protein[52]. $PGF_2\alpha$ inhibited 3T3-L1 adipocyte differentiation through an FP receptor-mediated increase in intracellular calcium and associated increase in DNA synthesis[53]. $PGF_2\alpha$ and fluprostenol also have been shown to increase basic FGF-2[54].

In summary, the FP receptor *in vitro* appears to increase osteoblast replication rather than promoting differentiation, and is capable of up-regulating the production of PGE_2. The *in vitro* effects reported with FP agonists suggest against an anabolic effect *in vivo*. However, as we have stated earlier in this review, bone anabolic activity is a summation of bone formation and resorption activity. When FP selective agonists were examined *in vivo*, a significant increase in bone anabolic activity was seen[55-57].

In our own work, we examined prostaglandin FP receptor selective agonists (cloprostenol and fluprostenol) in an osteopenic rat model ovariectomized (OVX) at 6 months of age and treated starting at 8 months of age for 60 days. FP receptor agonists significantly increased bone volume compared to the pre-treatment and final OVX groups (Table 1), returning BV/TV to levels similar to final intact controls, with increases in both trabecular thickness and number. In the mid- and distal femur, both FP receptor agonists significantly increased BMD compared to the final OVX group. In this aged rat model, selective FP agonists not only increased bone volume and density over pre-treatment values, but they also built sufficient bone to completely reverse the loss due to estrogen deficiency. This data demonstrates an unexpected bone anabolic effect of selective FP agonists,

which *in vitro* had only been shown to increase osteoblast proliferation and not promote osteoblast differentiation.

Discussion

In this review, we have summarized some of the current knowledge on the effect of prostaglandins on bone. The biphasic or variable effects seen with many studies of naturally occurring prostaglandins *in vitro* are most likely caused by stimulation of multiple receptors with compounds that lack sufficient selectivity. These occur less commonly *in vivo*, since there are limits to dose levels that are tested *in vivo* and stimulation of multiple receptors probably occurs to a lesser extent. This data also suggests that *in vitro* experiments with prostanoids should be interpreted with caution since most studies only examine formation or resorption and not both at the same time.

In the future as more selective compounds are described, we will achieve greater certainty of the role of each receptor and identify which may be the best target for a bone anabolic agent. The more selective compounds offer hope to identify a prostanoid that builds bone but does not suffer from numerous side effects such as increases in gastrointestinal motility and changes in blood pressure. Furthermore, since naturally occurring prostaglandins are rapidly inactivated or cleared from the circulation by a variety of mechanisms, such as oxidation of the 15-hydroxyl group in the lungs or removal of two carbons from the carboxyl terminus in the liver, new analogs may be designed with increased metabolic stability and increased half-lives.

While we have learned about many effects of specific receptors *in vitro*, we have limited *in vivo* knowledge of the activity of selective compounds. From our work with the FP selective prostanoids, there is a reminder that *in vitro* study of specific portions of the bone balance equation may help to understand, but will not always predict *in vivo* bone anabolic activity. A positive bone balance at the tissue level or the organism level is the result of a sustained greater rate of bone formation compared to bone resorption and must be measured *in vivo* with the full complement of mechanical, hormonal, and vascular components.

References

1. Haapasalo H, Kontulainen S, Sievanen H, Kannus P, Jarvinen M, Vuori I. Exercise-induced bone gain is due to enlargement in bone size without a change in volumetric bone density: A peripheral quantitative computed tomography study of the upper arms of male tennis players. Bone 2000; 27(3):351-7.
2. Kannus P, Haapasalo H, Sievanen H, Oja P, Vuori I. The site specific effects of long-term unilateral activity on bone mineral density and content. Bone 1994; 15(3):279-84.
3. Chow JW, Jagger CJ, Chambers TJ. Reduction in dynamic indices of cancellous bone formation in rat tail vertebrae after caudal neurectomy. Calcif Tissue Int 1996; 59(2):117-20.
4. Ke HZ, Li XJ, Jee WSS. Partial loss of anabolic effect of prostaglandin E2 on bone after its withdrawal in rats. Bone 1991; 12(3):173-83.
5. Tang LY, Cullen DM, Yee JA, Jee WSS, Kimmel DB. Prostaglandin E-2 increases the skeletal response to mechanical loading. J Bone Miner Res 1997; 12:276-82.
6. Klein-Nulend J, Burger EH, Semeins CM, Raisz LG, Pilbeam CC. Pulsating fluid flow stimulates prostaglandin release and inducible prostaglandin G/H synthase MRNA expression in primary mouse bone cells. J Bone Miner Res 1997; 12(1):45-51.
7. Coleman RA, Smith WL, Narumiya S. International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 1994; 46(2):205-29.
8. Baxter GS, Clayton JK, Coleman RA, Marshall K, Sangha R, Senior J. Characterization of the prostanoid receptors mediating constriction and relaxation of human isolated uterine artery. Br J Pharmacol 1995; 116(1):1692-6.
9. Mancini L, Moradi-Bidhendi N, Becherini L, Martineti V, MacIntyre I. The biphasic effects of nitric oxide in primary rat osteoblasts are CGMP dependent. Biochem Biophys Res Commun 2000; 274(2):477-81.
10. Raisz LG, Fall PM, Gabbitas BY, McCarthy TL, Kream BE, Canalis E. Effects of prostaglandin E2 on bone formation in cultured fetal rat calvariae: role of insulin-like growth factor-I. Endocrinology 1993; 133(4):1504-10.
11. Ueno K, Haba T, Woodbury D, Price P, Anderson R, Jee WSS. The effects of prostaglandin E2 in rapidly growing rats: depressed longitudinal and radial growth and increased metaphyseal hard tissue mass. Bone

	Vertebral BV/TV (%)	PTM BV/TV (%)	Distal Femur-BMD (mg/cm^2)
Pre-Trt Intact	28.52±4.04	16.98±6.99	.266±.015
Pre-Trt OVX	25.45±6.11	7.69±2.72	.236±.015
Final Intact	*29.10±4.22	**12.95±1.83	*.254±.007
Final OVX	21.92±2.74	2.75±1.51	.233±.021
Clo-100 ug/kg	*29.81±2.59	*9.61±1.89	**.269±.009
Flu-1000 ug/kg	**32.31±3.04	**12.01±1.96	**.265±.012
Mean ± SD. * p<0.001 vs. final OVX; + p<0.01 vs. pre-treatment OVX			

Table 1. Cloprostenol (0.3 to 100 μg/kg) and fluprostenol (3 to 1000 μg/kg) were administered at ½ log intervals for a total of six doses each (n=6/group). Cancellous bone volume was measured in the fourth lumbar vertebra and in the proximal tibial metaphysis (PTM). Bone mineral density (BMD) was measured in the mid- and distal femur by DXA.

1985; 6(2):79-86.

12. Jee WSS, Ueno K, Deng YP, Woodbury DM. The effects of prostaglandin E2 in growing rats: increased metaphyseal hard tissue and cortico-endosteal bone formation. Calcif Tissue Int 1985; 37(2):148-57.

13. Norrdin RW, Jee WSS, High WB. The role of prostaglandins in bone *in vivo*. Prostaglandins Leukot. Essent Fatty Acids 1990; 41(3):139-49.

14. Jee WSS, Akamine T, Ke HZ, Li XJ, Tang LY, Zeng QQ. Prostaglandin E2 prevents disuse-induced cortical bone loss. Bone 1992; 13(2):153-9.

15. Ueda K, Saito A, Nakano H, Aoshima M, Yokota M, Muraoka R, Iwaya T. Cortical hyperostosis following long-term administration of prostaglandin E1 in infants with cyanotic congenital heart disease. J Pediatr 1980; 97(5):834-6.

16. Alpert BS, Jureidini SB, Vanderzalm T. Resolution of prostaglandin-induced cortical hyperostosis in an infant. South Med J 1984; 77(8):1039-40.

17. Faye-Petersen OM, Johnson WH Jr, Carlo WA, Hedlund GL, Pacifico AD, Blair HC. Prostaglandin E1-induced hyperostosis: clinicopathologic correlations and possible pathogenetic mechanisms. Pediatr Pathol Lab Med 1996; 16:489-507.

18. Nadroo AM, Shringari S, Garg M, Sowailem AM. Prostaglandin induced cortical hyperostosis in neonates with cyanotic heart disease. J Perinat Med 2000; 28(6):447-52.

19. Heyman E, Laver J, Beer S. Prostaglandin synthetase inhibitor in caffey disease. J Pediatr 1982; 101(2):314.

20. Thometz JG, DiRaimondo CA. A case of recurrent Caffey's disease treated with naproxen. Clin Orthop 1996; (323):304-9.

21. Mori S, Jee WSS, Li XJ. Production of new trabecular bone in osteopenic ovariectomized rats by prostaglandin E2. Calcif Tissue Int 1992; 50(1):80-7.

22. Jee WSS, Ma YF. The *in vivo* anabolic actions of prostaglandins in bone. Bone 1997; 21(4):297-304.

23. Jee WSS, Mori S, Li XJ, Chan S. Prostaglandin E2 enhances cortical bone mass and activates intracortical bone remodeling in intact and ovariectomized female rats. Bone 1990; 11(4):253-66.

24. Jee WSS, Ke HZ, Li XJ. Long-term anabolic effects of prostaglandin-E2 on tibial diaphyseal bone in male rats. Bone Miner 1991; 15(1):33-55.

25. Gronowicz GA, Fall PM, Raisz LG. Prostaglandin E2 stimulates preosteoblast replication: an autoradiographic study in cultured fetal rat calvariae. Exp Cell Res 1994; 212(2):314-20.

26. Jee WSS, Ke HZ, Li XJ. Loss of Prostaglandin E2-induced extra cortical bone after its withdrawal in rats. Bone Miner 1992; 17(1):31-47.

27. Abramovitz M, Adam M, Boie Y, Carriere M, Denis D, Godbout C, Lamontagne S, Rochette C, Sawyer N, Tremblay NM, Belley M, Gallant M, Dufresne C, Gareau Y, Ruel R, Juteau H, Labelle M, Ouimet N,

Metters KM. The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. Biochim Biophys Acta 2000; 1483(2):285-93.

28. Sugimoto Y, Narumiya S, Ichikawa A. Distribution and function of prostanoid receptors: studies from knockout mice. Prog Lipid Res 2000; 39(4):289-314.

29. Juppner H, Abou-Samra AB, Freeman M, Kong XF, Schipani E, Richards J, Kolakowski LF, Jr, Hock J, Potts JT, Jr, Kronenberg HM. A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. Science 1991; 254(5034): 1024-6.

30. Kawaguchi H, Raisz LG, Voznesensky OS, Alander CB, Hakeda Y, Pilbeam CC. Regulation of the two prostaglandin G/H synthases by parathyroid hormone, interleukin-1, cortisol, and prostaglandin E2 in cultured neonatal mouse calvariae. Endocrinology 1994; 135(3): 1157-64.

31. Kiriyama M, Ushikubi F, Kobayashi T, Hirata M, Sugimoto Y, Narumiya S. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. Br J Pharmacol 1997; 122(2):217-24.

32. Raisz LG, Fall PM. Biphasic effects of prostaglandin E2 on bone formation in cultured fetal rat calvariae: interaction with cortisol. Endocrinology 1990; 126(3): 1654-9.

33. Woodiel FN, Fall PM, Raisz LG. Anabolic effects of prostaglandins in cultured fetal rat calvariae: structure-activity relations and signal transduction pathway. J Bone Miner Res 1996; 11(9):1249-55.

34. Nemoto K, Pilbeam CC, Bilak SR, Raisz LG. Molecular cloning and expression of a rat prostaglandin E2 receptor of the EP2 subtype. Prostaglandins 1997; 54(4):713-25.

35. Qi H, Crawford DT, Chidsey-Frink KL, Thompson DD, Ke HZ. Local delivery of CP-463755, a novel, non-prostanoid EP2 receptor subtype selective prostaglandin E2 agonist, restores bone mass to the proximal tibial metaphysis of osteopenic, OVX rats. J Bone Miner Res 2000; 15:SA377.

36. Ke HZ, Qi H, Crawford DT, Chidsey-Frink KL, Cameron KO, Lefker BA, Paralkar VM, Owen TA, Li M, Lu B, Grasser WA, Yu L, DaSilva-Jardine P, Thompson DD. Discovery of a non-prostanoid EP2 receptor selective prostaglandin E2 (PGE2) agonist that mimics the local anabolic effects of PGE2. J Bone Miner Res 2000;15: SU189.

37. Gardner CR, Blanque R, Cottereaux C. Mechanisms involved in prostaglandin-induced increase in bone resorption in neonatal mouse calvaria. Prostaglandins Leukot Essent Fatty Acids 2001; 64(2):117-25.

38. Mano M, Arakawa T, Mano H, Nakagawa M, Kaneda T, Kaneko H, Yamada T, Miyata K, Kiyomura H, Kumegawa M, Hakeda Y. Prostaglandin E2 directly inhibits bone-resorbing activity of isolated mature osteoclasts mainly through the EP4 receptor. Calcif Tissue Int 2000; 67(1):85-92.

39. Suzawa T, Miyaura C, Inada M, Maruyama T, Sugimoto Y, Ushikubi F, Ichikawa A, Narumiya S,

Suda T. The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. Endocrinology 2000; 141(4):1554-9.

40. Weinreb M, Grosskopf A, Shir N. The anabolic effect of PGE2 in rat bone marrow cultures is mediated via the EP4 receptor subtype. Am J Physiol 1999; 276 (2 Pt 1): E376-E383.

41. Sarrazin P, Bkaily G, Hache R, Patry C, Dumais R, Rocha FAC, Brum-Fernandes AJ. Characterization of the prostaglandin receptors in human osteoblasts in culture. Prostaglandins Leukot Essent Fatty Acids 2001; 64(3):203-10.

42. Weinreb M, Machwate M, Shir N, Abramovitz M, Rodan GA, Harada S. Expression of the prostaglandin E-2 (PGE(2)) receptor subtype EP4 and its regulation by PGE(2) in osteoblastic cell lines and adult rat bone tissue. Bone 2001; 28(3):275-81.

43. Miyaura C, Inada M, Suzawa T, Sugimoto Y, Ushikubi F, Ichikawa A, Narumiya S, Suda T. Impaired bone resorption to prostaglandin E2 in prostaglandin E receptor EP4-knockout mice. J Biol Chem 2000; 275(26):19819-23.

44. Ono K, Akatsu T, Murakami T, Nishikawa M, Yamamoto M, Kugai N, Motoyoshi K, Nagata N. Important role of EP4, a subtype of prostaglandin (PG) E receptor, in osteoclast-like cell formation from mouse bone marrow cells induced by PGE2. J Endocrinol 1998; 158(3):R1-R5.

45. Machwate M, Harada S, Leu CT, Seedor G, Labelle M, Gallant M, Hutchins SM, Lachance N, Rodan SB, Young R, Rodan GA. Prostaglandin receptor EP4 mediates the bone anabolic effects of PGE2. J Bone Miner Res 2000; 15:SU184.

46. Sakuma Y, Tanaka K, Suda M, Yasoda A, Natsui K, Tanaka I, Ushikubi F, Narumiya S, Segi E, Sugimoto Y, Ichikawa A, Nakao K. Crucial involvement of the EP4 subtype of prostaglandin E receptor in osteoclast formation by proinflammatory cytokines and lipopolysaccharide. J Bone Miner Res 2000; 15(2):218-27.

47. Sakuma Y, Tanaka K, Suda M, Komatsu Y, Yasoda A, Miura M, Ozasa A, Narumiya S, Sugimoto Y, Ichikawa A, Ushikubi F, Nakao K. Impaired bone resorption by lipopolysaccharide *in vivo* in mice deficient in the prostaglandin E receptor EP4 subtype. Infect Immun 2000; 68(12):6819-25.

48. Suda M, Tanaka K, Natsui K, Usui T, Tanaka I, Fukushima M, Shigeno C, Konishi J, Narumiya S, Ichikawa A, Nakao N. Prostaglandin E receptor subtypes in mouse osteoblastic cell line. Endocrinology 1996; 137(5):1698-705.

49. Suda M, Tanaka K, Yasoda A, Natsui K, Sakuma Y, Tanaka I, Ushikubi F, Narumiya S, Nakao K. Prostaglandin E2 (PGE2) autoamplifies its production through EP1 subtype of PGE receptor in mouse osteoblastic MC3T3-E1 cells. Calcif Tissue Int 1998; 62(4):327-31.

50. Nemoto K, Bernecker PM, Pilbeam CC, Raisz LG. Expression and regulation of prostaglandin F receptor MRNA in rodent osteoblastic cells. Prostaglandins 1995; 50(5-6):349-58.

51. Watanabe T, Satoh H, Togoh M, Taniguchi S, Hashimoto Y, Kurokawa K. Positive and negative regulation of cell proliferation through prostaglandin receptors in NIH-3T3 cells. J Cell Physiol 1996; 169(2):401-9.

52. Fall PM, Breault DT, Raisz LG. Inhibition of collagen synthesis by prostaglandins in the immortalized rat osteoblastic cell line Py1a: Structure-activity relations and signal transduction mechanisms. J Bone Miner Res 1994; 9(12):1935-43.

53. Miller CW, Casimir DA, Ntambi JM. The mechanism of inhibition of 3T3-L1 preadipocyte differentiation by prostaglandin F2alpha. Endocrinology 1996; 137(12): 5641-50.

54. Sabbieti MG, Marchetti L, Abreu C, Montero A, Hand AR, Raisz LG, Hurley MM. Prostaglandins regulate the expression of fibroblast growth factor-2 in bone. Endocrinology 1999; 140(1):434-44.

55. Froman CA, Jee WSS, Setterberg R, Cui L, Yao W, Chmielewski PA, Lundy MW, Hartke JR. Nitric oxide modulates fluprostenol-induced bone formation. J Bone Miner Res 2000; 15:SU389.

56. Hartke JR, Jankowsky ML, deLong MA, Soehner ME, Jee WSS, Lundy MW. Prostanoid FP agonists build bone in the ovariectomized rat. J Bone Miner Res 1999; 14:T326.

57. Borah B, Dufresne TE, Chmielewski PA, Pierce SA, Hartke JR, Lundy MW. Fluprostenol, a prostaglandin FP agonist, improves 3- dimensional trabecular architecture in rat vertebrae as measured by micro-computed tomography. J Bone Miner Res 1999; 14:SA368.