Differential effects of systemic prostaglandin E_2 on bone mass in rat long bones and calvariae

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Abstract

Prostaglandin E_2 (PGE_2) has been shown to possess anabolic properties when administered systemically. All the experiments performed so far examined long bones from animals of varying age and bone status. In this study we compared the changes in bone mass of long bones (femur, tibia and humerus) to those in calvariae after a 3-week daily administration of 6 mg/kg PGE_2 into 3-week-old rats. This regimen inhibited body weight gain (by 14·1%) as well as longitudinal growth of long bones (by 2·2–3·5%) but increased their mass. Ash weight (measuring both cancellous and compact bone) increased by 10·1–14·1% but tibial cancellous bone area was elevated by 54%. Radial growth was slightly reduced due to transient inhibition of mineral apposition rate at the periosteal envelope but the expansion of the marrow cavity was inhibited to a greater extent, resulting in an 8·1% increase in the relative compact bone area. The increased bone mass was associated with greater mechanical strength of the femoral neck (24·2% increase in fracture load and 19% in stiffness). In contrast, PGE_2 administration did not affect calvarial thickness or mineral apposition rate but increased its density, i.e. reduced the area of marrow spaces due to stimulation of endocortical bone formation at this site.

The pattern of bone mass changes documented in this study closely correlates with that of the induced expression of early-response genes following a single dose of PGE_2 as we recently reported. These data, therefore, support the hypothesis that *in vivo* administration of an anabolic dose of PGE_2 increases bone formation and augments bone mass largely by stimulating the recruitment of new osteoblasts via induction of the proliferation and/or differentiation of bone marrow osteogenic precursors.

Journal of Endocrinology (1998) **156,** 51–57

Introduction

Prostaglandins (PGs) are potent modulators of bone metabolism since they can increase *in vitro* both bone resorption and bone formation (Raisz *et al.* 1993). In recent years, observations in human (Ueda *et al.* 1980, Ringel *et al.* 1982, Jorgensen *et al.* 1988, Devaric *et al.* 1989) and animal studies have shown that PGs (in particular PGE_2) are powerful anabolic agents for long bones in rats when administered systemically (Ueno *et al.* 1985, Jee *et al.* 1985, 1987, 1991, Ito *et al.* 1993). Therefore, PGE_2 treatment was used to prevent osteopenia in rats resulting from ovariectomy (Jee *et al.* 1990, Mori *et al.* 1990) and immobilization (Akamine *et al.* 1992, Jee *et al.* 1992). All the experiments conducted so far to test the anabolic effects of PGE_2 have used long bones (tibia, femur and vertebrae) as a measurement site. The question whether such an effect occurs in the calvaria, which differs from long bones in its histological structure and growth patterns, has not yet been addressed.

Osteopenia in various conditions is frequently associated with reduced mechanical strength (e.g. Peng *et al.* 1994). This relationship between bone mass and bone fragility is best illustrated in the high fracture rate in post-menopausal, osteopenic women. Therefore, it was of interest to see whether the augmentation in bone mass induced by PGE_2 results in increased mechanical strength. This has been demonstrated so far only in aged ovariectomized rats (Lauritzen *et al.* 1993).

Thus, the objectives of this study were to test: (1) whether the anabolic effect of systemic PGE_2 occurs in membranous bones (calvaria) and, using double fluorochrome labels, to determine its effect on mineral apposition rate, and (2) whether the increase in bone mass of long bones after PGE_2 treatment translates into greater mechanical strength. In addition, we have recently found that a single injection of an anabolic dose of PGE_2 induces the expression of the early-response genes c-fos, c-jun, junB and egr-1 in bone marrow cells of both the tibial metaphysis and calvariae but not the calvarial periosteum of young rats (Weinreb *et al.* 1995, 1997*a*). Therefore, in the present study we attempted to relate the changes in bone mass observed at these sites after a 3-week treatment

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with PGE_2 with the pattern of gene expression induced by a single dose.

Materials and Methods

Male Sprague-Dawley rats (3 weeks old) were used for these experiments. Six rats (first group) were killed by CO_2 inhalation to act as a baseline group. Seven rats (second group) were injected daily with a subcutaneous dose of PGE_2 (6 mg/kg, kindly donated by Dr C Hall of the Upjohn Company, Kalamazoo, MI, USA) and a similar number of rats (third group) were given daily injections of the vehicle. PGE_2 was first dissolved in 100% ethanol and further diluted in distilled water to reach the final concentration. Animals were weighed every two days to adjust the amount of PGE_2 injected. The gain in body weight throughout the experimental period was calculated for each animal. After three weeks, animals were killed by CO_2 inhalation, and both tibiae, one humerus and the calvaria were removed and fixed in 50% ethanol. One femur was cleaned of soft tissue and was frozen immediately at -20 °C. One tibia and half of the calvaria were dehydrated in ascending ethanol concentrations at 4 °C and embedded in methyl methacrylate (Baron *et al.* 1983). Tibiae were first sectioned transversally at the tibio-fibular junction and then coronally through the proximal metaphysis (both at 6 μm thickness). Calvariae were cut in a midsagittal plane just adjacent to the midline suture at 8 μm thickness. The following parameters were measured in unstained sections using a video-based image analysis system (Genias, Applied Imaging, Gateshead, UK): (1) the relative (%) area of cancellous bone at the tibial proximal secondary spongiosa between 1·5 and 2·2 mm distal to the epiphyseal growth plate; (2) tibial cross-sectional area at the tibio-fibular junction (both the periosteal and endosteal surfaces were measured) and cortical bone area (as percentage of the periosteal area); (3) calvarial thickness (in 8 equidistant points along the surface) and (4) the area within the calvaria occupied by bone as the percentage of the total area of a 5–6 mm length.

The length of one tibia and one humerus was measured with a precision caliper. These bones were then dried for 24 h at 60 °C and defatted for 24 h in ether at room temperature and the dry, fat-free weight was determined. Bones were then ashed at 700 °C for 24 h and the corrected ash weight was derived by dividing it by bone length (mg/mm). The percentage ash weight of the dry weight was calculated as a measure of mineralization.

In a separate experiment, animals were injected daily with PGE_2 or vehicle as before, but two calcein labels were injected at the following intervals: on days 0 and 7 in the first group, on days 7 and 14 in the second group and on days 14 and 21 in the third group. Each group contained six rats. Tibiae and calvariae were embedded and sectioned at 10 μm as described above. The inter-label distances were measured in unstained sections of the calvaria (at the superior and inferior surfaces) and of the tibio-fibular junction (at the medial and lateral periosteal surfaces) using a Zeiss LSM-410 confocal microscope at a final magnification of $\times 125$.

The frozen femur was thawed at a later time, cut in the middle and the proximal half was embedded vertically in a plastic plate with predrilled holes. The mechanical strength of the femoral neck was tested with a materials testing machine (Instron 4026, Instron Corporation, Canton, MA, USA) by compressing the head downward via a specially designed 2·5 mm-diameter metal cup which fitted the acetabulum and transmitted the load vertically at a progression rate of 2 mm/s until fracture. In a preliminary experiment, femora from rats of different ages (36–85 days) were tested biomechanically. The maximal load and displacement at fracture were recorded for each femur and stiffness was calculated as load/displacement. The results indicated a significant age-related increase in maximal load and stiffness and correlation coefficients of 0·89–0·95 between maximal load or stiffness and age. Since both the right (R) and left (L) femur of each animal were tested in the pilot experiment, the error associated with maximal load measurements was calculated as the square root of $\Sigma\ (d^2/2n)$ (where $d=[(L-R)/(L+R)]\star 100/0{\cdot}5$ and n=number of animals) and equaled 8·3%, in close agreement with other laboratories (Peng *et al.* 1994). Non-paired *t*-tests were used to test the differences between the two experimental groups.

Results

A three-week administration of PGE_2 decreased the gain in body weight during the experimental period by 14·1% (Table 1). This effect was repeated in the second experiment (data not shown). Each injection of PGE_2 resulted in a mild, transient diarrhea.

PGE_2 treatment reduced longitudinal growth so that bone length in PGE-injected animals was 2·2%-3·5% smaller compared with vehicle-treated animals. Bone mass of the long bones was significantly elevated – a 14·1% increase in tibial ash weight and a 10·1% increase in humeral ash weight. This parameter measures the combined mass of compact and cancellous bone. A direct measure of cortical bone mass (% compact bone at the tibio-fibular junction) increased by 8·1% while a direct measure of cancellous bone mass (% cancellous bone area) indicated a 54% increase (Table 1). There was a moderate reduction in the age-related increase in periosteal cross-sectional area while the endosteal cross-sectional area did not increase in PGE_2-treated animals (compared with a 71% increase in vehicle-treated rats). The partial inhibition in periosteal growth was probably caused by a transient diminution in mineral apposition rate during the second week of treatment (Fig. 1). Long bones of the

Table 1 Parameters of length, mass and strength in long bones and calvaria of vehicle- and PGE_2-treated rats compared with the baseline group

Parameter	Baseline	Vehicle	PGE_2	Change from vehicle group (%)
Gain in body weight (g)	—	161 ± 2	139 ± 8**	−14·1
Bone length (mm)				
Humerus	15·6 ± 0·3	23·2 ± 0·1	22·7 ± 0·2*	−2·2
Tibia	21·8 ± 0·3	34·5 ± 0·3	33·3 ± 0·2*	−3·5
Ash weight (mg/mm)				
Humerus	1·77 ± 0·07	3·75 ± 0·09	4·13 ± 0·15*	+10·1
Tibia	1·52 ± 0·04	3·62 ± 0·11	4·13 ± 0·15*	+14·1
Tibial cortical bone area (%)	64·0 ± 2·0	66·3 ± 2·0	71·7 ± 0·7*	+8·1
Tibial cancellous bone area (%)	7·2 ± 0·5	9·8 ± 1·4	15·2 ± 1·3**	+54·0
Tibial cross-sectional area (mm^2)				
Periosteal	2·76 ± 0·12	4·81 ± 0·28	3·63 ± 0·16**	−24·5
Endosteal	0·95 ± 0·06	1·63 ± 0·16	1·02 ± 0·03**	−37·4
Femoral neck fracture load (N)	N.D.	55·0 ± 3·6	68·3 ± 4·4*	+24·2
Femoral neck stiffness (N/mm)	N.D.	45·2 ± 2·5	53·8 ± 2·1*	+19·0
Calvarial thickness (microns)	185 ± 5	342 ± 19	341 ± 27	—
Calvarial density (% bone)	88·1 ± 1·7	91·5 ± 1·9	97·0 ± 0·8**	+6·0

N.D., not determined; *P<0·05, **P<0·01 compared with vehicle-treated group.

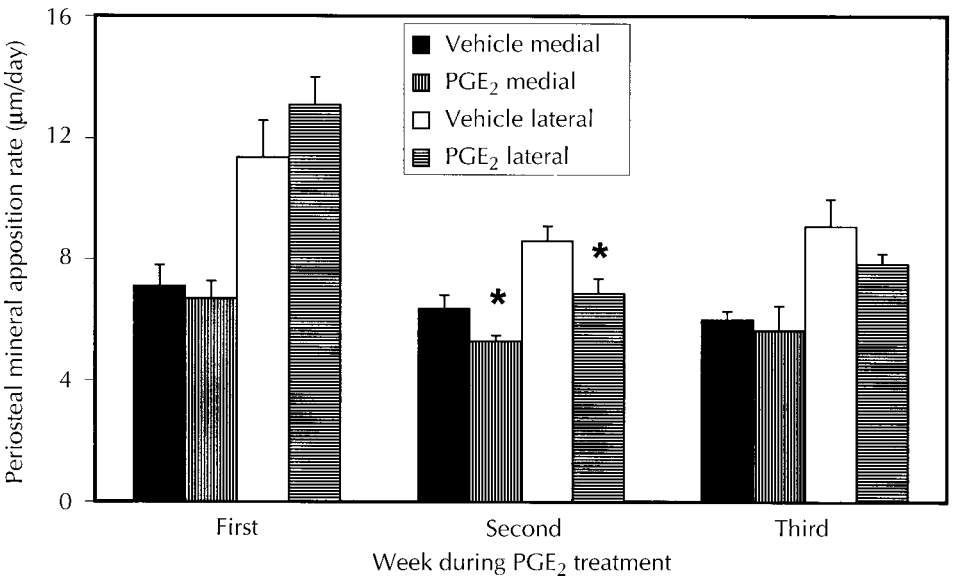

Figure 1 Reduced periosteal mineral apposition rate at the medial and lateral aspects of the tibio-fibular junction during the second week of PGE_2 treatment. *P<0·05, PGE_2-treated vs vehicle-treated group.

PGE_2-treated rats had consistently increased mineralization expressed as a higher percentage ash of the dry weight (not shown). Administration of PGE_2 increased the mechanical strength of the femoral neck – a 24·2% increase in fracture load and a 19% increase in stiffness.

In contrast with the increase in bone mass of the long bones, the thickness of the calvaria in PGE_2-treated rats did not differ from that in vehicle-treated animals (Table 1) nor did the mineral apposition rate at either the superior or the inferior calvarial surface (Fig. 2). However, the density of the calvarial plate (percentage bone area) increased significantly in PGE_2-injected animals, indicating near obliteration of the marrow spaces (Fig. 3) due to stimulation of bone formation at this site, reflected in a greater extent of double calcein labeling (Fig. 4).

Discussion

The anabolic actions of PGs (particularly PGE_2) have been known for some time (Ueda *et al.* 1980, Ringel *et al.* 1982,

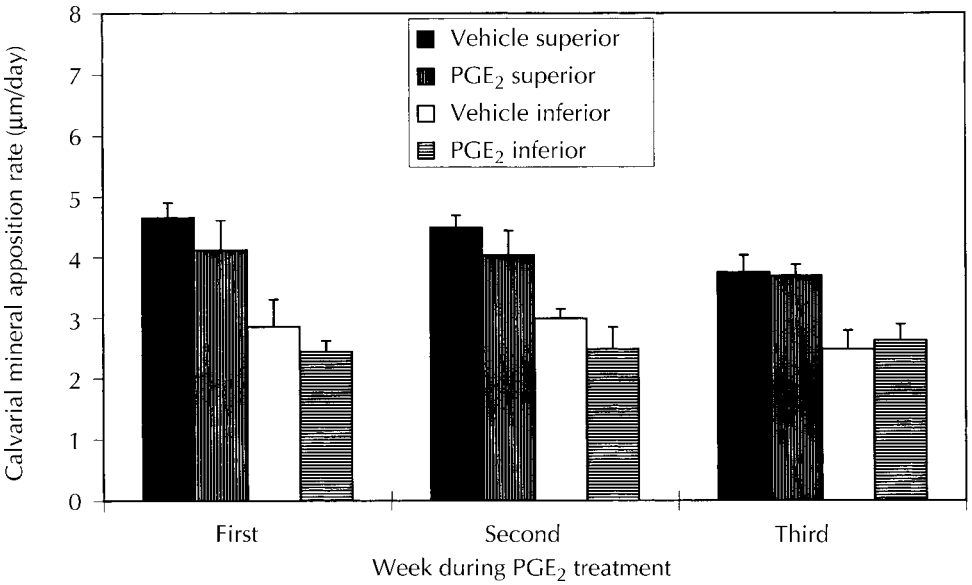

Figure 2 Lack of change in mineral apposition rate on either the superior or the inferior surface of the calvariae in PGE_2-treated rats.

Jee *et al.* 1985). Therefore PGE_2 was used to prevent or ameliorate osteopenia induced by ovariectomy or immobilization (Jee *et al.* 1990, 1992, Mori *et al.* 1990, Akamine *et al.* 1992). The only experiments using animals and treatment regimens similar to the ones in this study (Ueno *et al.* 1985, Jee *et al.* 1987) demonstrated reduced longitudinal tibial growth rate and increased cancellous bone area at the tibial proximal secondary spongiosa, similar to the data presented here. In contrast, the increased cortical bone mass presented in this study was not demonstrated previously for this animal age. Such an effect on cortical bone, however, has been shown in older animals (7–13 months old) and after longer administration periods (42–90 days) (Jee *et al.* 1990, 1992, Ke *et al.* 1993). The diminution in body weight gain and in longitudinal growth of long bones found in this study were also reported previously in male rats (Jee *et al.* 1985, Ueno *et al.* 1985, Ke *et al.* 1991, 1992).

PGE_2 treatment resulted in a 10–14% increase in bone mass determined by ash weight. However, this parameter includes changes in compact and cancellous bone and these two bone types are not expected to behave similarly. Indeed, direct measurement of cancellous bone mass indicated a much higher increase. The elevated percentage cross-sectional compact bone area in PGE_2-treated rats was caused by a combination of a smaller periosteal envelope and a much smaller endosteal envelope compared with vehicle-treated animals. At this site and age, the entire periosteal envelope is a forming surface as demonstrated by calcein labeling. We detected a transient diminution in mineral apposition rate at this site (during the second week of treatment) which could explain the smaller size of the periosteal envelope (Ueno *et al.* 1985). In contrast, only a very small fraction of the endosteal surface is forming and inhibition of its age-dependent enlargement must be related to inhibition of bone resorption. This notion is supported by the finding of a smaller number of osteoclasts in the secondary spongiosa of PGE_2-injected rats (Jee *et al.* 1987). Inhibition in the resorption of older bone may also explain the higher percentage ash weight of the dry weight in PGE-injected animals. An alternative explanation is a higher degree of mineralization of bone formed during the experimental period.

We found increased mechanical strength measured at the femoral neck in PGE_2-treated rats in parallel with the increase in bone mass in these animals. This finding corroborates data of Lauritzen *et al.* (1993) about greater mechanical strength of the femoral neck and midshaft in ovariectomized rats treated with 3 mg/kg PGE_2. The site selected for mechanical testing has been shown to reflect changes in bone mass in other conditions such as ovariectomy, bisphosphonate treatment and exercise (Toolan *et al.* 1992, Lauritzen *et al.* 1993, Peng *et al.* 1994, Sogaard *et al.* 1994*a*,*b*). The ability of PGE_2 to improve femoral neck strength is similar to that of parathyroid hormone, another anabolic agent (Li & Wronski 1995).

Despite the cumulative evidence that PGE_2 increases bone mass of long bones, the present study could not demonstrate any increase in calvarial thickness in PGE_2-injected rats. This means that bone formation was not stimulated at either the superior or inferior surfaces which are forming at this age (see Fig. 4). This is in agreement with preliminary evidence that local injections of PGE_2

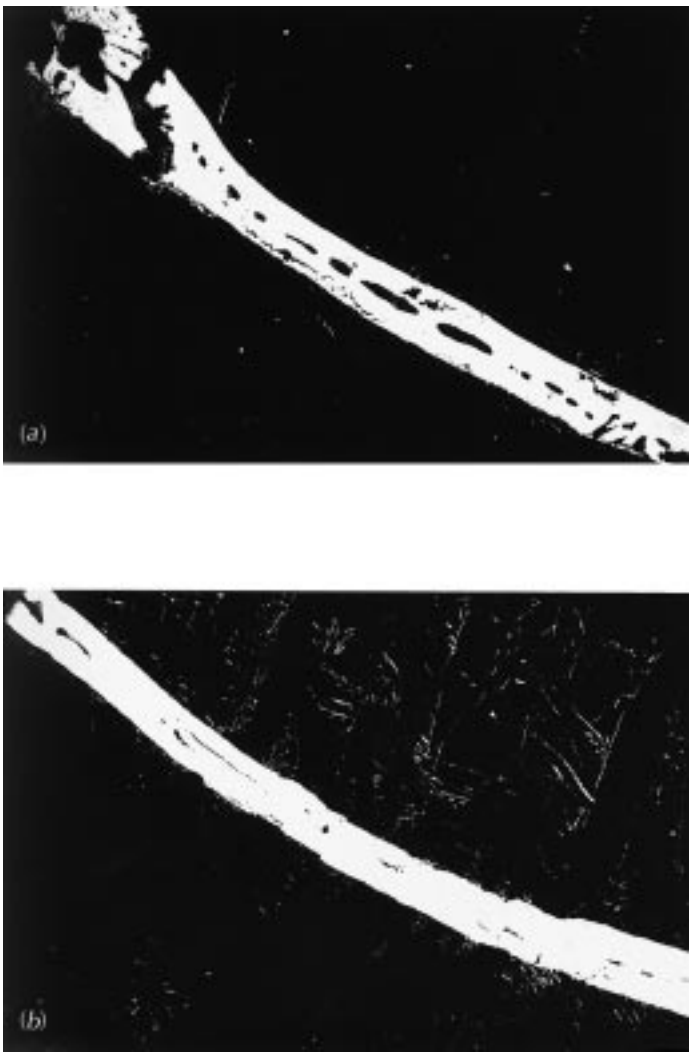

Figure 3 Photomicrographs of unstained sections of the calvarial plates using reflected light (magnification × 20). (*a*) Vehicle-treated rat; (*b*) PGE_2-treated rat. Note substantial reduction of the marrow spaces in *b*.

onto the calvarial surface of young rats does not stimulate bone formation at the superior surface (S Harada, personal communication), in contrast to local infusion of PGE_2 into the marrow cavity of long bones which causes massive new bone production (Yang *et al.* 1993, Takada *et al.* 1994). It is also compatible with the inability of PGE_2 to stimulate the already fully active periosteal envelope at the tibio-fibular junction as we report here. This means that systemic PGE_2 administration does not stimulate the activity of existing, active osteoblasts. In contrast, longer administration periods of PGE_2 to old and adult rats stimulated bone formation at the tibio-fibular junction (Jee *et al.* 1990, 1991, 1992, Ke *et al.* 1991, Ito *et al.* 1993), a site which is no longer active. This would suggest that PGE_2 recruits new osteoblasts to form new bone.

However, we found a clear stimulation of bone formation within the marrow spaces inside the calvarial plate resulting in elevated bone density and a near obliteration of these 'windows' in the central calvarial region. Others have repeatedly reported stimulated bone formation within the tibial metaphysis by systemic PGE_2. These data could be linked if one accepted the hypothesis that PGE_2 induces bone formation by recruiting new osteoblasts from their bone-marrow progenitors (osteogenic precursor cells). This is our current proposed mode of action of the anabolic effect of PGE_2 in bone, which is supported by the following additional observations: first, the pattern of bone mass changes documented in this study correlates closely with the pattern of induced expression of the early-response

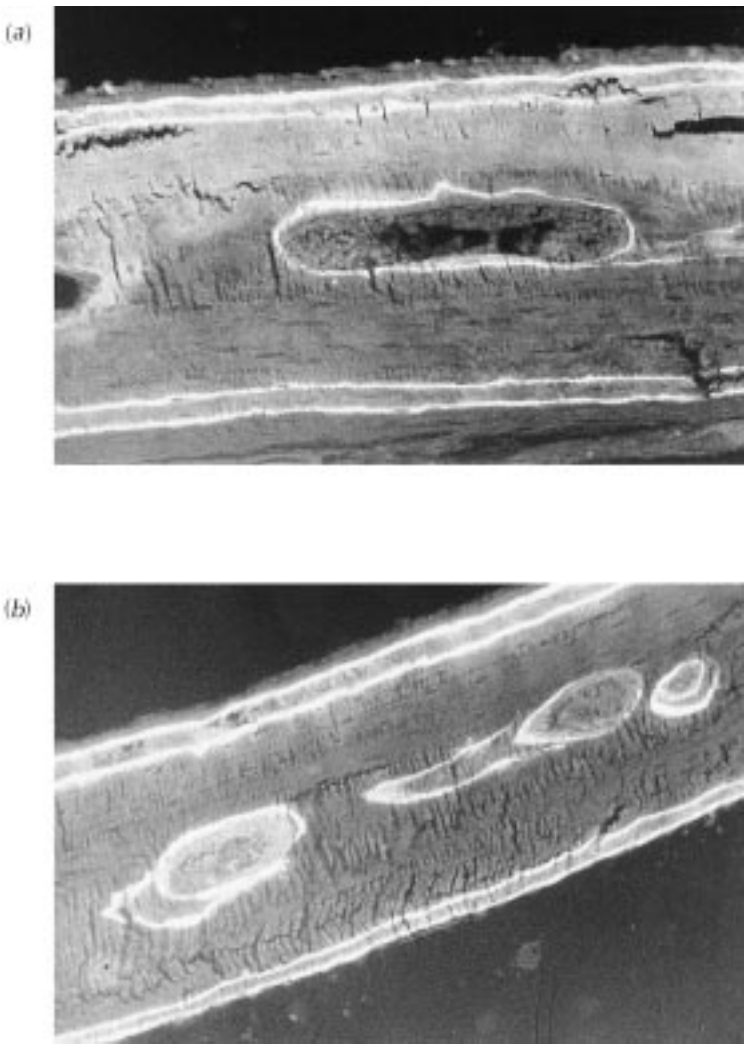

Figure 4 Photomicrographs depicting fluorescent calcein labels within the calvarial marrow spaces (magnification × 100). (a) Vehicle-treated rat; (*b*) PGE_2-treated rat. Note increased double-labeled surface in B.

genes following a single injection of the same dose of PGE_2 as we have recently reported (Weinreb *et al.* 1995, 1997*a*). This induced expression of transcription factors such as c-fos, c-jun and egr-1 occurred in bone marrow cells both in the tibial metaphysis and calvarial 'windows', two sites where PGE_2 stimulates bone formation, and did not occur within the calvarial periosteum where bone formation is not stimulated. These findings suggest that *in vivo* administration of an anabolic dose of PGE_2 into young rats stimulates the proliferation and/or differentiation of bone marrow osteogenic precursors thereby increasing bone formation and augmenting bone mass wherever bone marrow exists. This hypothesis is consistent with the observation that new bone trabeculae are forming in the tibial metaphysis of PGE_2-injected rats (Jee *et al.* 1985, 1987, Ueno *et al.* 1985). Thus, the recruitment of osteoblasts from their precursors seems to be the major mechanism of the anabolic effect of PGE_2 *in vivo*.

Secondly, such a conclusion is supported by the ability of PGE_2 to stimulate the proliferation of some bone cells *in vitro* (Quarles *et al.* 1993, Centrella *et al.* 1994). Thirdly, in support of this proposed mode of action of PGE_2 we recently found that a 2-week administration of 6 mg/kg to rats similar to the ones used in this study, increased the size of the osteoprogenitor pool within bone marrow (Weinreb *et al.* 1997*b*). We used the bone nodule formation assay in femoral bone marrow cultures and showed that PGE_2 treatment *in vivo* increased both the number of nodules formed and alkaline phosphatase activity in these cultures,

indicating increased osteogenic commitment in these animals.

In summary, a 3-week daily administration of PGE_2 at 6 mg/kg into 3-week-old rats depressed body weight gain, reduced longitudinal and radial growth rate of long bones, augmented cortical and cancellous bone mass and mechanical strength in the long bones but increased calvarial density and not thickness. These changes in bone mass are consistent with our proposed mode of action of systemic PGE_2, i.e. stimulation of bone-marrow osteoprogenitors.

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