

Systemic prostaglandin E₂ increases cancellous bone formation and mass in aging rats and stimulates their bone marrow osteogenic capacity *in vivo* and *in vitro*

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Abstract

Prostaglandin E₂ (PGE₂) has been shown to exert a bone anabolic effect in young and adult rats. In this study we tested whether it possesses a similar effect on bone formation and bone mass in aging rats. Fifteen-month-old rats were injected daily with either PGE₂ at 5 mg/kg or vehicle for 14 days. PGE₂ treatment stimulated the rate of cancellous bone formation (a ~5.5-fold increase in bone formation rate), measured by the incorporation of calcein into bone-forming surfaces at the tibial proximal metaphysis. This effect resulted in increased cancellous bone area (+54%) at the same site. Since PGE₂ treatment resulted in a much higher proportion of bone surface undergoing bone formation and thus lined with osteoblasts, we tested the hypothesis that PGE₂ stimulates osteoblast differentiation from bone marrow precursor cells both *in vivo* and *in vitro*. We found that *ex vivo* cultures of bone marrow stromal cells from rats injected for 2 weeks with PGE₂ at 5 mg/kg per day yielded more (~4-fold) mineralized nodules and exhibited a greater (by 30–40%) alkaline phosphatase activity compared with cultures from vehicle-injected rats, attesting to a stimulation of osteoblastic differentiation by PGE₂.

We also compared the osteogenic capacity of bone marrow from aging (15-month-old) versus young (5-week-old) rats and its regulation by PGE₂ *in vitro*. Bone marrow stromal cell cultures from aging rats exhibited a greatly diminished osteogenic capacity, reflected in reduced nodule formation (~6% of young animals) and lower alkaline phosphatase activity (~60% of young animals). However, these parameters could be stimulated in both groups of animals by incubation with 10–100 nM PGE₂. The magnitude of this stimulation was greater in cultures from aging rats (+550% vs +70% in nodule formation of aging compared with young rats).

In conclusion, we demonstrate here that PGE₂ exerts a bone anabolic effect in aging rats, similar to the effect we and others have reported in young, growing rats. The PGE₂-stimulated bone formation, which augments bone mass, most likely results from recruitment of osteoblasts from their bone marrow stromal precursors.

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Introduction

In the last two decades, prostaglandins of the E series (PGEs), especially PGE₂, have been shown to be potent stimulators of bone formation *in vivo* (reviewed by Norrdin *et al.* 1990 and Jee & Ma 1997). The anabolic effect of systemically administered PGEs was reported in many species including young (Ueno *et al.* 1985, Jee *et al.* 1987, Suponitzki & Weinreb 1998) and adult (Ke *et al.* 1991, 1992) rats as well as humans (Ueda *et al.* 1980, Jorgensen *et al.* 1988). Detailed analysis of the effects of PGE₂ in these growing rats showed that it increases bone formation and bone mass mainly by increasing the number of osteoblasts present on bone surfaces and by inducing the production of new bone. However, the anabolic properties of PGE₂ in aging or old animals have been poorly studied so far.

Aging in humans is associated with diminished bone mass and consequently higher fracture incidence (Davis *et al.* 1994, Parfitt *et al.* 1995, Heersche *et al.* 1998, Rizzoli & Bonjour 1999). Bone loss in aging humans is the result of both increased bone resorption and reduced bone formation. The latter is thought to be a consequence of paucity in mature osteoblasts. It is well known that osteoblasts are derived from osteoprogenitor cells (OPC), which are present within the bone marrow stromal compartment (Friedenstein 1990, Simmons 1996). These stromal cells, called CFUs (colony-forming unit-fibroblastic) can be cultured *in vitro* where they differentiate into osteoblast-like cells and produce mineralizing matrix (Friedenstein *et al.* 1968, Maniopoulos *et al.* 1988, Owen 1988). Therefore, it is not surprising that many studies revealed that the number of CFUs is diminished in

aging animals (Tsuji *et al.* 1990, Egrise *et al.* 1992, Kahn *et al.* 1995, Bergman *et al.* 1996) and humans (D'Ippolito *et al.* 1999).

Therefore, the purpose of this study was twofold: (A) to test whether systemically administered PGE₂ exerts an anabolic effect (i.e. stimulates bone formation and increases bone mass) on cancellous bone of aging rats, and (B) to examine whether the potential of bone marrow stromal cells to differentiate into osteoblasts, which is expected to be diminished in these aging animals, can be stimulated by *in vivo* or *in vitro* treatment with PGE₂.

Materials and Methods

In vivo testing

Animal treatment For each experiment, 15-month-old male Sprague–Dawley rats were divided into two groups (seven or eight rats each). One group received daily subcutaneous injections of PGE₂ (a generous gift of Dr C Hall, the Upjohn Company, Kalamazoo, MI, USA) at 5 mg/kg per day in 10% ethanol for 14 days, while the second group received the vehicle alone. In some experiments, both groups were injected with calcein (Sigma, St Louis, MO, USA) at 15 mg/kg, 9 and 2 days prior to sacrifice to measure dynamic parameters of bone formation. At the end of the injections, animals were killed with CO₂, and both hind limbs were removed. Tibiae were fixed in cold 50% ethanol for histomorphometry and femora were used for bone marrow cultures and then for endocortical label measurements (see below).

Histomorphometry Tibiae and femoral shafts (after the initiation of marrow cultures) were dehydrated in increasing concentrations of ethanol and were embedded in methyl methacrylate (Weinreb *et al.* 1991, Suponitzki & Weinreb 1998). Ten micrometer thick frontal (tibia) or transverse (femur) sections were cut with a Polycut microtome (Leica, Nussloch, Germany) and were mounted on gelatin-coated slides. Unstained sections were photographed at a × 83 magnification and scanned in a Polaroid Sprintscan Scanner. Bone area in the tibial proximal metaphysis was measured with the Adobe Photoshop software in an area extending 0.5 to 1.75 mm distal to the growth plate. For measurements of dynamic parameters of bone formation, unstained sections were viewed in a fluorescence microscope and an area extending 1–1.9 mm distal to the tibial growth plate was photographed at a final magnification of × 110. The percent bone surface with single (sL) or double (dL) calcein labels was determined as percent of the total bone surface (BS) using a computerized digitizer (Summagraphics, Fairfield, CT, USA) and the SigmaScan software (Jandel Corporation, San-Rafael, CA, USA). The mineralizing surface (MS) was calculated according

to the formula $MS = (dL + 1/2 sL) \times 100/BS$ (Parfitt *et al.* 1987). A similar analysis was performed on the endosteal surface of the distal femoral shaft.

Bone marrow cultures Femora were defleshed and the epiphyses were removed. Bone marrow was flushed out and a single-cell suspension was achieved by repeated pipetting. Cells were counted with a hemocytometer and were cultured in six-well plates (Nunc, Roskilde, Denmark) at a density of $5-6 \times 10^7$ cells/well in a medium containing α minimal essential medium + 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 µg/ml ascorbic acid (Merck, Darmstadt, Germany) + 10 nM dexamethasone (Sigma). After an attachment period of 24 h, non-adherent cells were removed by rinsing with PBS and cultures were maintained in 7% CO₂ and 37 °C for 21 days with twice-a-week medium changes. 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 (Keila *et al.* 1994, Weinreb *et al.* 1997a). Mineralized nodules (stained black) and non-mineralized nodules (stained yellow) were counted macroscopically with a magnifying glass over transmitted light and the number of each class of nodules per well and the relative proportion of mineralized nodules of the total number of nodules was determined.

In addition to mineralized nodule formation, osteoblastic differentiation was assessed by measuring alkaline phosphatase (AP) activity in culture (Weinreb *et al.* 1997a, 1999). Femoral cells were cultured as before, and on days 8 and 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 using a Sigma kit based on *p*-nitrophenyl phosphate as substrate. The protein content was measured according to Bradford using BSA as standard and a protein assay kit (Bio-Rad Laboratories, Munich, Germany) and enzyme activity was expressed as units/mg protein.

In vitro testing

Bone marrow cultures We have previously reported that PGE₂ stimulates *in vitro* the osteogenic capacity of bone marrow in very young (4-week-old) rats (Weinreb *et al.* 1997a). In this study we initiated parallel cultures from both young (4 week) and aging (15 months) rats to compare the effect of aging and PGE₂. Femoral bone marrow cells were seeded in six-well plates as described above and PGE₂ (0–1000 nM) in 0.1% ethanol was added upon plating and in each subsequent medium change.

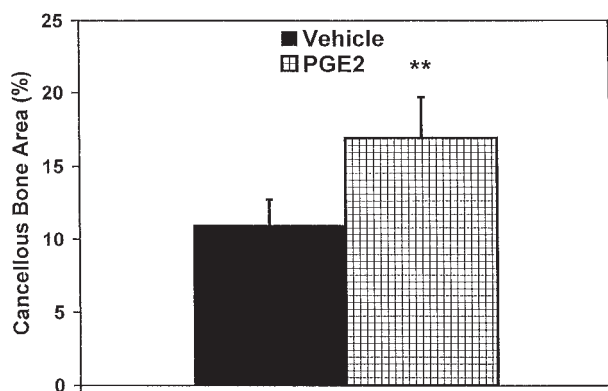


Figure 1 Systemic administration of PGE₂ (5 mg/kg per day) for 14 days increases cancellous bone area of the tibial secondary spongiosa by ~54%. ** $P < 0.01$ (PGE₂ vs vehicle). Data are presented as means \pm S.E.M.S.

Cells were cultured for either 12 days for determination of AP activity or 21 days for measurement of nodule formation (both performed as indicated above).

All data are presented as means \pm S.E.M.S. Comparison between group means was performed using one-way analysis of variance with *post hoc* multi-group contrasts.

Results

In vivo testing

The aging rats used in this study gained very little weight throughout the experiment and their epiphyseal growth plates were almost closed.

Systemic administration of PGE₂ at 5 mg/kg for 14 days to aging rats increased cancellous bone mass in the tibial secondary spongiosa by ~54% (Fig. 1). The increase in bone mass was associated with increased bone formation, reflected in a much greater fraction of bone surface involved in formation (sL, dL, MS) (Fig. 2). In addition, mineral apposition rate increased by ~36% (Fig. 3). The resulting calculated rate of bone formation increased ~5.5-fold in cancellous bone of PGE₂-injected animals (Fig. 3). In addition to stimulation of bone formation on the cancellous envelope, PGE₂ treatment stimulated bone formation on the endosteal surface, facing the marrow cavity. As in the case of cancellous bone, PGE₂ stimulated both osteoblast recruitment (reflected in the fraction surface involved in formation; Fig. 4) and osteoblastic activity, reflected in mineral apposition rate (MAR) (Fig. 5). Consequently, the calculated endosteal bone formation rate was 2.5-fold greater in PGE₂-injected animals (Fig. 5).

Since these measurements indicated that the extent of bone surface involved in bone formation increased greatly in PGE₂-treated animals, a stimulation of osteoblast

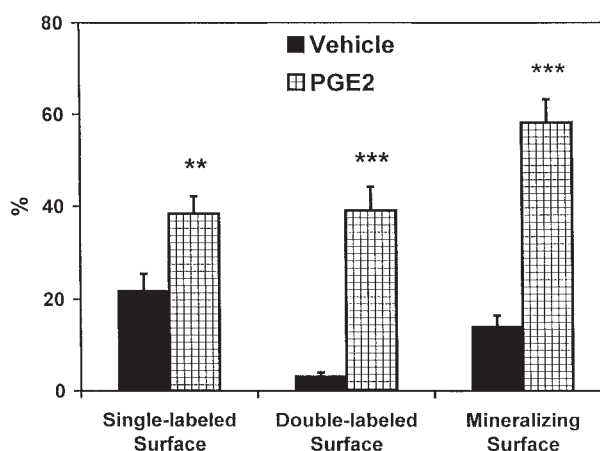


Figure 2 Systemic PGE₂ increases the fraction of cancellous bone surface bearing single- or double-calcein labels or as MS (mineralizing surface). ** $P < 0.01$, *** $P < 0.005$ (PGE₂ vs vehicle). Data are presented as means \pm S.E.M.S.

recruitment from precursor cells present in bone marrow was expected. Indeed, bone marrow from PGE₂-treated rats showed increased overall cellularity (Fig. 6) and specifically, an expanded osteoprogenitor pool as evidenced by increased mineralized nodule formation (Fig. 7). Both the number of nodules per well and their relative proportion out of the total number of nodules increased significantly following systemic PGE₂ administration. Furthermore, this treatment increased AP activity in *ex vivo* cultures of bone marrow cells (Fig. 8). As we reported previously (Weinreb *et al.* 1997a), AP activity increases between 8 and 12 days of culture, parallel with the progressing osteoblastic differentiation of these cells. However, at each of these time points, AP activity in cultures taken from PGE₂-treated rats was greater than

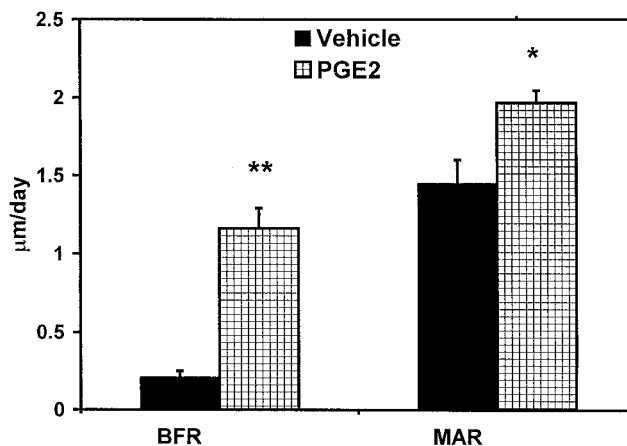


Figure 3 Systemic PGE₂ increases cancellous mineral apposition rate (MAR) and consequently the calculated bone formation rate (BFR). * $P < 0.05$, ** $P < 0.01$ (PGE₂ vs vehicle). Data are presented as means \pm S.E.M.S.

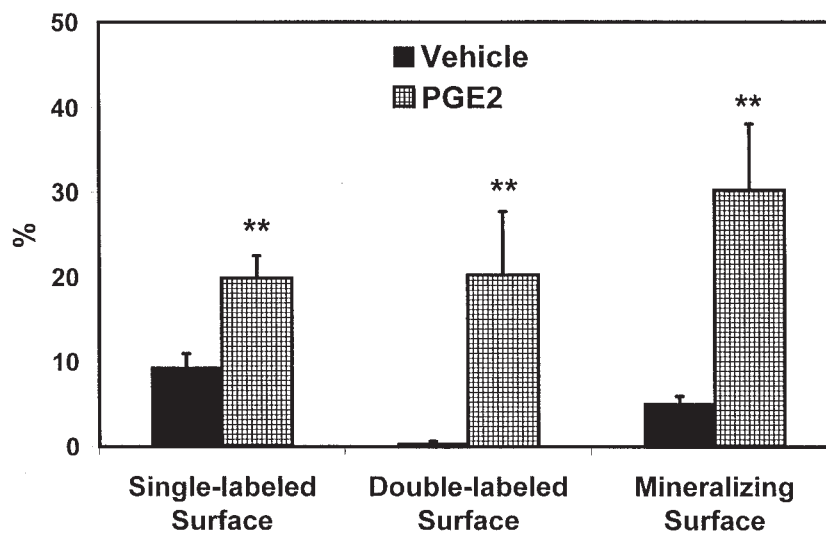


Figure 4 Systemic PGE₂ increases the fraction of endosteal bone surface bearing single- or double-calcein labels or as MS (mineralizing surface). ** $P < 0.01$ (PGE₂ vs vehicle). Data are presented as means \pm S.E.M.s.

that in cultures taken from vehicle-treated rats. AP activity, expressed as units/mg protein, increased in cultures from PGE₂-treated animals in addition to an increase in their protein content (Fig. 9).

These data demonstrated that an anabolic dose of PGE₂ increased bone formation and bone mass in aging rats and that this effect can be attributed to stimulation of the osteoprogenitor pool present in bone marrow. Thus bone tissue of aging rats is responsive to the anabolic effect of PGE₂ with a mechanism similar to that reported by us in very young (3–4-week-old) rats.

In vitro testing

In addition to the ability of systemically administered PGE₂ to exert an anabolic effect, we tested its *in vitro*

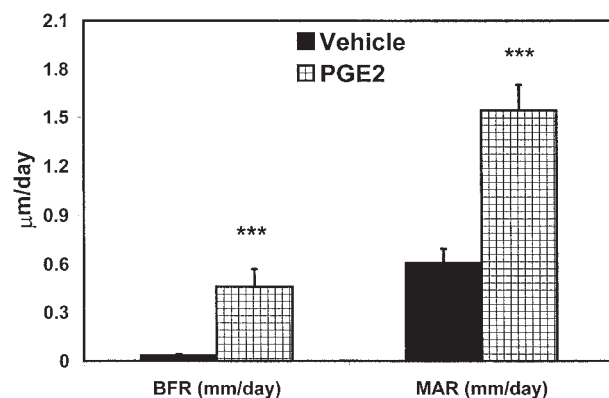


Figure 5 Systemic PGE₂ increases endosteal mineral apposition rate (MAR) and the calculated bone formation rate (BFR). *** $P < 0.005$ (PGE₂ vs vehicle). Data are presented as means \pm S.E.M.s.

stimulatory activity on osteoblastic differentiation in parallel bone marrow cultures from young and aging rats. Despite seeding of a similar number of bone marrow cells, the number of mineralized nodules per well with no PGE₂ added was much greater (~ 16 -fold) in cultures from young compared with aging rats (Fig. 10). These data reflect the great diminution in the proportion of osteoprogenitor cells in aging animals. Addition of PGE₂ at 10–100 nM to bone marrow cultures of the two animal groups increased significantly the number of nodules formed while a concentration of 1000 nM was no longer effective (as we previously reported (Weinreb *et al.* 1999)). However, comparing the magnitude of the stimulation exerted by PGE₂ on nodule formation, the effect on cultures from aging rats was more pronounced than that on cultures from young rats (+550% vs +70% respectively).

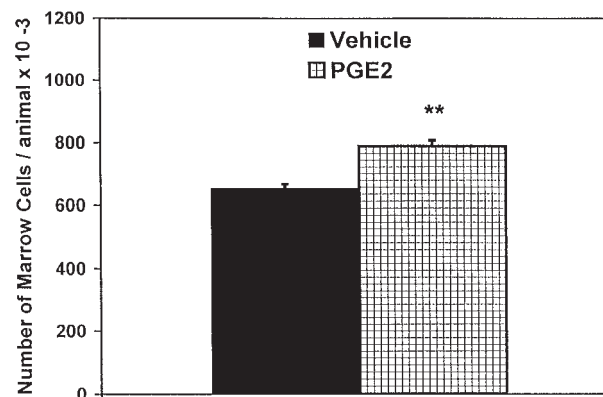


Figure 6 Systemic administration of PGE₂ increases total bone marrow cellularity. ** $P < 0.01$ (PGE₂ vs vehicle). Data are presented as means \pm S.E.M.s.

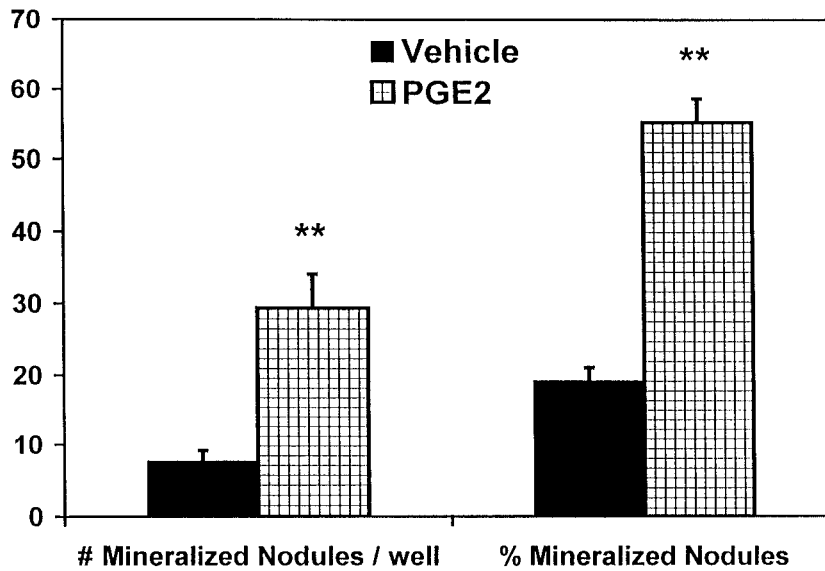


Figure 7 Systemic PGE₂ increases the number and proportion of mineralized nodules in *ex vivo* bone marrow stromal cultures. ***P*<0.01 (PGE₂ vs vehicle). Data are presented as means ± S.E.M.S.

Similarly, AP activity on day 12 in cultures from aging rats was ~40% lower than that in cultures from young rats (Fig. 11). In both cultures, addition of 100 nM PGE₂ increased AP activity significantly. Again, AP activity was stimulated by PGE₂ on top of its increase of the protein content of the cultures (Fig. 12).

Cumulatively, the data of this study demonstrate the ability of systemic PGE₂ to exert an anabolic affect on bones of aging (15-month-old) rats and strongly suggest

that stimulation of osteoblastic differentiation from bone marrow osteogenic precursors is a major mechanism for this effect.

Discussion

We and others have previously shown that PGE₂ increases bone formation and mass in intact young, rapidly growing as well as adult rats (Ueno *et al.* 1985, Jee *et al.* 1987, Ke

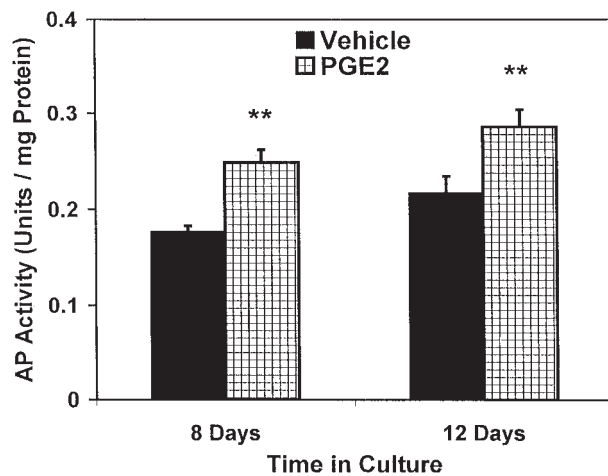


Figure 8 Systemic PGE₂ increases alkaline phosphatase (AP) activity in *ex vivo* bone marrow stromal cultures. ***P*<0.01 (PGE₂ vs vehicle for each time point). Data are presented as means ± S.E.M.S.

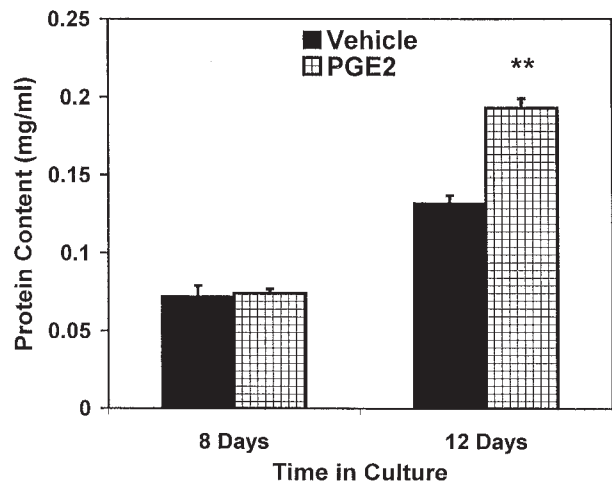


Figure 9 Systemic PGE₂ increases the 12-day protein content of *ex vivo* bone marrow stromal cultures. ***P*<0.01 (PGE₂ vs vehicle). Data are presented as means ± S.E.M.S.

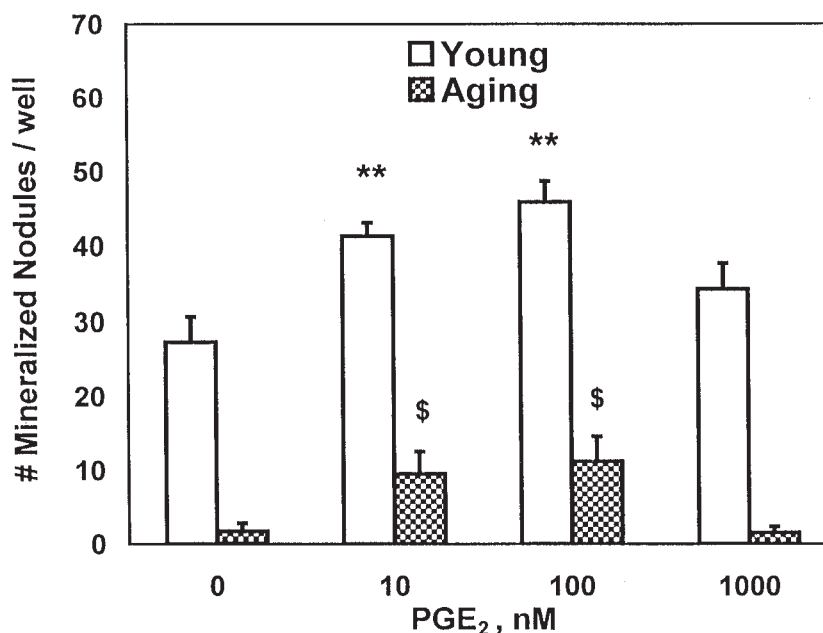


Figure 10 Incubation of bone marrow cells *in vitro* with PGE₂ at 10–100 nM increases nodule formation in both young (5-week-old) and aging (15-month-old) rats. ** or \$, $P < 0.01$ (vs PGE₂=0). Data are presented as means \pm S.E.M.s.

et al. 1991, 1992, Jee & Ma 1997, Saponitzki & Weinreb 1998). Recently, PGE₂ was also shown to increase cortical bone formation rate and red/yellow bone marrow ratio in the tibial shaft of intact old male rats (Yao *et al.* 1999). Our study shows that PGE₂ also increases bone formation rate and consequently bone area of the cancellous bone of the

tibial proximal secondary spongiosa of aging rats. Thus, systemic administration of PGE₂ into aging or old rats can stimulate bone formation rate in both types of bone tissue. Given the ability of PGE₂ to increase cancellous and cortical bone mass, it has previously been used to treat animals with various models of osteopenia such as

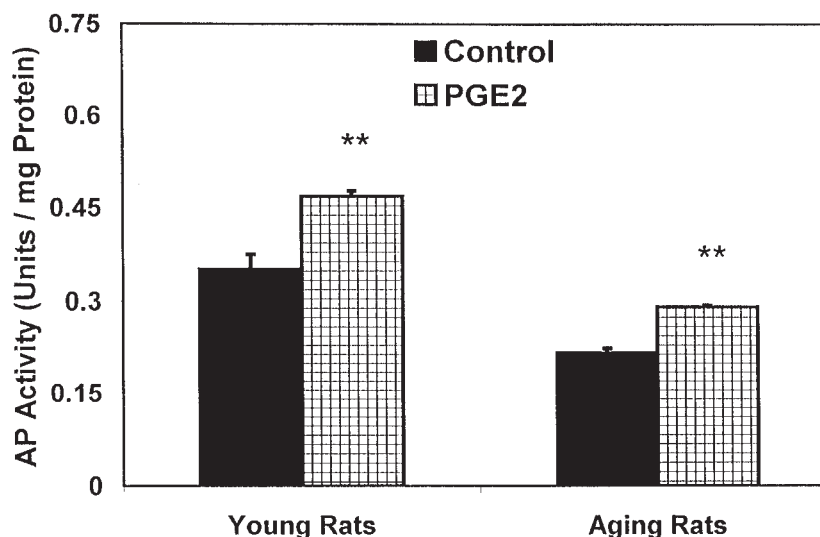


Figure 11 Incubation of bone marrow cells *in vitro* with PGE₂ at 100 nM increases AP activity in both young (5-week-old) and aging (15-month-old) rats. ** $P < 0.01$ (PGE₂ vs control for both time points). Data are presented as means \pm S.E.M.s.

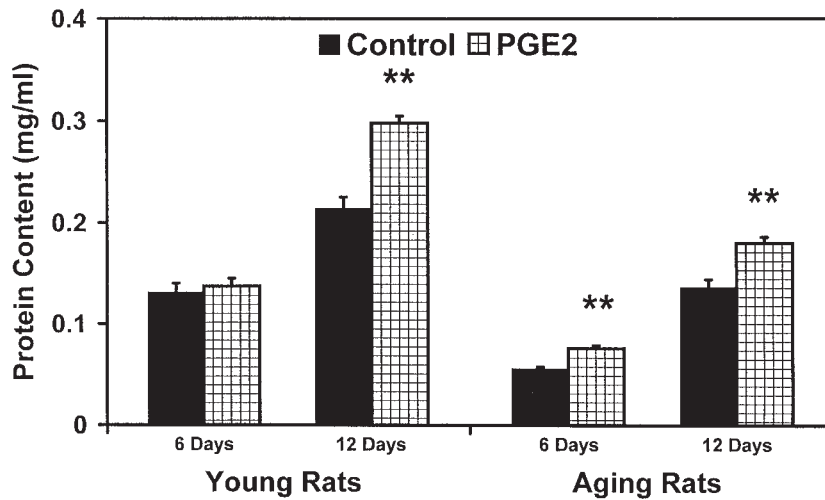


Figure 12 Incubation of bone marrow cells *in vitro* with PGE₂ at 100 nM increases the protein content of cultures from both young (5-week-old) and aging (15-month-old) rats. ** $P < 0.01$ (PGE₂ vs control for each time point). Data are presented as means \pm S.E.M.S

ovariectomy (Mori *et al.* 1990) or mechanical unloading (Akamine *et al.* 1992, Jee *et al.* 1992). Taken together, these studies clearly show that bone tissue of aging rats is responsive to the anabolic action of PGE₂.

Our data of increased bone marrow cellularity caused by PGE₂ treatment may be relevant to the PGE₂-induced changes in bone marrow composition reported in old rats (Yao *et al.* 1999). In general, osteoblastogenesis and adipogenesis behave in opposite manners in bone tissue (e.g. increase in bone marrow fat content versus the decline in bone formation and osteoblast number with aging (Meunier *et al.* 1971)). Thus the improvement in red/yellow marrow ratio reported by others or the increased number of cells that can be harvested from the femoral marrow cavity as shown here may reflect a shift away from adipocytic toward osteoblastic commitment, caused by PGE₂.

This study documented a significant stimulation of cancellous and endosteal bone formation rate induced by systemic treatment with PGE₂. As previously mentioned, osteoblasts differentiating on cancellous and endocortical surfaces originate from marrow osteoprogenitors. Our data clearly show that systemic PGE₂ increases the potential of the marrow stromal compartment to produce osteoblasts. The nodule-formation assay used here is based on the observations that these CFUfs are clonogenic (Owen 1988) and that the number of mineralized nodules reflects the number of recruitable osteoprogenitors. These data from the nodule formation assay are corroborated by the PGE₂ stimulation of AP activity as shown here, and this effect is compatible with the increase in the number of osteoblasts on bone-forming surfaces measured by the calcein labeling. These stimulatory effects of PGE₂ in

aging rats was previously reported by us in young, growing rats (Weinreb *et al.* 1997a). Whether this stimulation of osteoblastogenesis involves effects on proliferation, differentiation or apoptosis is not known. We have shown that a single injection of an anabolic dose of PGE₂ induces the expression of various early-response genes in bone marrow cells (Weinreb *et al.* 1997b), pointing again to the presence of PGE₂ target cells within bone marrow.

In this study we found, as others have reported, that aging is associated with a marked reduction in the osteogenic capacity of bone marrow, when compared with that of young rats (Tsuiji *et al.* 1990, Egrise *et al.* 1992, Kahn *et al.* 1995, Quarto *et al.* 1995, Bergman *et al.* 1996, Inoue *et al.* 1997). However, this capacity can be stimulated by PGE₂ in both young and aging animals, peaking at a concentration of 100 nM (Weinreb *et al.* 1999). Nevertheless, the fold increase in nodule formation in the aging rats was more pronounced than that in the young rats and the *in vitro* data strongly point to a direct effect of PGE₂ on the stromal cells.

Both the effects of aging (reduction) and PGE treatment (stimulation) on the marrow osteogenic capacity are thus examples of the positive correlation between the rate of bone formation and the number of osteoprogenitors. Our previous report that the osteogenic capacity of bone marrow is greatly diminished in mechanical unloaded rat limbs, in which bone formation is markedly depressed (Keila *et al.* 1994), is another example of this correlation. The mechanism underlying the aging-associated diminution in osteoprogenitor cells is not known. One possibility is that the abundance of or response to a variety of growth factors is deficient in old animals. A correlation between age-related decline in bone formation and in the

concentration of insulin-like growth factor-I (IGF-I) in bone tissue was suggested (Boonen *et al.* 1997) while others associated aging-related osteopenia with diminished activity and/or availability of transforming growth factor- β (Gazit *et al.* 1998). Whether the stimulation of bone formation and osteoblastic commitment by PGE₂ is mediated via changes in these or other growth factors remains to be determined.

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