PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 156, 373–377 (1977)

Bone Resorption *in Vitro* and *in Vivo* in PGE-Treated Mice[1] (39939)

M. G. SANTORO, B. M. JAFFE, AND D. J. SIMMONS[2]

Departments of Surgery and the Division of Orthopedic Surgery, Washington University School of Medicine, St. Louis, Missouri 63110

In 1970, Klein and Raisz (1) demonstrated that prostaglandins promote resorption of ^{45}Ca-labeled fetal rat bones *in vitro*. Several experimental tumors which produce large amounts of prostaglandins have been shown to cause bone resorption *in vitro* (2, 3) and hypercalcemia and histologic changes in bone *in vivo* (4, 5). Hyperprostaglandinemia has also been implicated in the pathogenesis of hypercalcemia in a number of patients bearing adenocarcinomas (6–8). These observations suggest that, in specific instances, circulating prostaglandins play a major role in bone resorption and resultant hypercalcemia *in vivo*. Although repeated subcutaneous injections of PGE_1 caused local bone loss (9) and continuous intravenous infusions of PGE_2 produced hypercalcemia (10), both in rats, it has not yet been possible to demonstrate a bone-resorbing effect of systemically administered (circulating) prostaglandins *in vivo* (1, 11), presumably due to the short biological half-lives of those compounds (12).

The present study was undertaken to determine if chronic systemic administration of a long-acting synthetic analog of PGE_2 (16,16-dimethyl-PGE_2-methyl ester) produced bone resorption and hypercalcemia in mice *in vivo*. Moreover, since there has been controversy about the relative activity of the different prostaglandins in resorbing bone, the effects of PGE_2, $PGF_{2\alpha}$, and PGA_1 on ^{45}Ca-labeled infant mouse bones *in vitro* have also been evaluated.

Materials and methods. In vitro studies. Calcium resorption from ^{45}Ca-prelabeled bones in culture was evaluated using a modification of the *in vitro* radiocalcium release bioassay described by Tashjian *et al.* (3). Infant (1- to 2-day-old) N2W Webster-Swiss origin mice (National Laboratories) were injected subcutaneously with 1.0 μCi of ^{45}Ca (New England Nuclear) in 0.05 ml of saline on 2 consecutive days, sacrificed 48 hr later, and their calvaria were cultured for 96 hr. PGE_2, $PGF_{2\alpha}$, and PGA_1 (Upjohn Company) were dissolved in absolute ethanol, maintained as stock solutions at 4°, and diluted to the desired concentration in modified Bigger's medium (Gibco). Both test and control media contained 0.1% ethanol, a concentration which did not affect resorption in our assay system. The study was further controlled by testing bones which had been killed by repeated (3 × 10 min) freezing-thawing cycles at −70°. The ^{45}Ca contents of bone and media were determined and the percentage of calcium released was calculated.

In vivo studies. Seventy-nine C57/B1/6J female mice were injected intraperitoneally with 0.1 ml of sterile saline containing either 25% absolute ethanol or ethanolic solutions of 16,16-dimethyl-PGE_2-methyl ester (di-M-PGE_2). The detailed experimental protocols are summarized in Table I. None of the di-M-PGE_2-treated animals developed diarrhea or suffered unusual changes in weight gain patterns. At the end of each of the four studies, the animals were weighed, and sacrificed by decapitation. Blood samples were collected in heparinized centrifuge tubes, and total blood calcium concentrations (mg%) were determined by atomic absorption spectrophotometry. With respect to the blood calcium determinations, it is important to note that most of the animals were sacrificed in the afternoon, but the work reported in Study I was performed during the morning hours. At autopsy, the left femurs were resected, completely cleaned of soft tissues, and the epi-

[1] Supported in part by grants from the St. Louis Institute for Medical Education and Research and from the U.S. Public Health Service (AM-16931).

[2] Send reprint requests to Dr. David J. Simmons, Division of Orthopedic Surgery, Washington University School of Medicine, 4960 Audubon Avenue, St. Louis, Missouri 63110.

ISSN 0037-9727

TABLE 1. CHANGES IN BONE HISTOMORPHOMETRY AND BONE CALCIUM FOLLOWING Di-M-PGE_2 TREATMENT.

	Group			
	I	II	III	IV
Age	4 Months	4 Months	30 Days	5 Months
No. of mice				
Control	9	9	20	8
Treated	9	7	10	7
Dose	10 μg/day	10 μg/day	10 μg/day	25 μg
Days of treatment	4	20	4	Single dose
Weight (g)	23.1 ± 0.4	23.4 ± 0.5	17.4 ± 0.5	25.9 ± 0.5
Study time	AM	PM	PM	PM
	Percentage trabecular bone (mean ± SEM)			
Diluent control	20.8 ± 1.3	15.6 ± 0.6	30.3 ± 2.3	–
Di-M-PGE_2	13.9 ± 1.6*	13.5 ± 0.9	22.8 ± 2.3**	–
	Percentage surface with osteoclasts (mean ± SEM)			
Diluent control	5.26 ± 0.37	1.11 ± 0.26	1.43 ± 0.15	1.5 ± 0.2
Di-M-PGE_2	11.45 ± 1.67*	6.30 ± 1.20*	3.47 ± 0.44*	1.4 ± 0.2
	Percentage bone calcium (mean ± SEM)			
Diluent control	25.5 ± 0.74	20.4 ± 1.27	21.6 ± 0.7	18.1 ± 0.34
Di-M-PGE_2	21.7 ± 1.20***	21.5 ± 0.76	21.5 ± 0.7	18.2 ± 0.23
	Plasma calcium (mg%, mean ± SEM)			
Diluent control	9.32 ± 0.31	7.96 ± 0.18	–	8.17 ± 0.28
Di-M-PGE_2	8.39 ± 0.21**	8.30 ± 0.18	–	8.06 ± 0.22

* $P < 0.01$.
** $P < 0.05$.
*** $P < 0.02$.

physes were dissected off. The shafts were weighed, dissolved in concentrated HNO_3, and aliquots of the solutions were analyzed for bone calcium (mg/g tissue wet wt). The tibias were fixed in 10% neutral formalin, decalcified in a formic acid–citric acid solution, and then embedded in paraffin for routine histologic procedures. The bones were sectioned parallel to their long axes on a rotary microtome at 6 μm, and the sections were stained with hematoxylin and eosin and Azure II. The slides were coded and the volume (mean ± SEM) of bone trabeculae in the proximal metaphyses and the percentage of the trabecular surfaces covered by osteoclasts were estimated microscopically in six to eight sections by point counting techniques using a Zeiss II integrating eyepiece at 200×.

Statistical analyses. Statistical analyses of intergroup differences were performed by the Student's *t* test for unpaired data, and *P* values of <0.05 were considered significant.

Results. In vitro studies. In control cultures, the viable bones lost significantly more ^{45}Ca (20.3 ± 1.9%) than did bones that had been killed by freezing–thawing (13.2 ± 0.6%, $P < 0.01$). In test bone cultures, the bone resorbing activities of PGE_2, $PGF_{2\alpha}$, and PGA_1 were tested at different concentrations (from 1.0 ng to 10 μg/ml) on ^{45}Ca-prelabeled infant murine calvaria cultured for 96 hr. At 1.0 ng/ml, none of the prostaglandins caused significant stimulation of bone resorption (Fig. 1). At higher concentrations, PGE_2 was the most active compound. The bone resorptive effect of PGE_2 was maximal at a concentration of 1.0 μg/ml (3×10^{-6} *M*), causing an 89.8% increase in the mobilization of bone calcium. Higher concentrations of PGE_2 were less effective; at 10 μg/ml, calcium

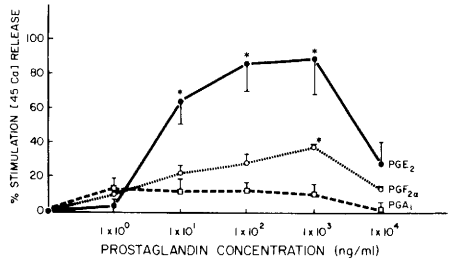

FIG. 1. The effect of prostaglandins on bone resorption *in vitro*. Each point represents the mean ± SEM of at least five values. * $0.01 < P < 0.05$.

losses were stimulated by only 22.2%. The same dose-related effect was evident with $PGF_{2\alpha}$, where a concentration of 1.0 μg/ml stimulated resorption by 36.7% ($P < 0.001$). In contrast, PGA_1 did not cause significant bone calcium mobilization over the dose range tested.

In vivo studies. The effect of chronic systemic administration of di-M-PGE_2 was studied *in vivo* in a series of four different experiments. Histomorphometric and bone calcium changes are summarized in Table I.

Study I. Four-month-old mice received four consecutive daily intraperitoneal injections of di-M-PGE_2 (10 μg/day/mouse) or the equivalent volume of PGE-free diluent. This di-M-PGE_2 treatment regimen resulted in a slight (↓ 15%) but significant loss of bone calcium and in the percentage trabecular bone (↓ 33.2%). The percentage of the trabecular surface lined by osteoclasts increased by 117%. Serum calcium levels were not increased by di-M-PGE_2; in contrast, they were significantly lower in the di-M-PGE_2-treated mice than in the controls.

Study II. Four-month-old mice received either 10 μg of di-M-PGE_2 or the equivalent amount of diluent intraperitoneally every day for 20 days. Di-M-PGE_2 appeared less effective after long periods of treatment than after shorter exposures (Study I). There were no significant differences between the control and di-M-PGE_2-treated mice with respect to bone and blood calcium concentrations or the percentage trabecular bone volume. Nevertheless, the percentage of bone surfaces lined with osteoclasts was markedly greater (↑ 450%) in the di-M-PGE_2-treated mice than in the control group.

Study III. Since Study I involved older mice which had passed their peak growth phase, this study was performed to determine if the effects of di-M-PGE_2 on bone resorption would be more severe in young, actively growing animals. Six-week-old mice were divided into two control groups of 10 mice each, one injected with 0.1 ml of saline (saline control) and one given 0.1 ml of saline + 25% ethanol (ethanol control). Control and di-M-PGE_2-treated animals were injected for 4 consecutive days and sacrificed 3 hr after the last injection. In all parameters evaluated, saline- and ethanol-treated control groups behaved identically. Ethanol administration did not produce a significant hypocalcemic effect as previously described (13). Although bone calcium levels were not significantly different in the two control and the di-M-PGE_2 groups, bones from di-M-PGE_2-treated mice showed a mean 24.8% decrease in the volume of trabecular bone as well as a mean 144% increase in the area lined by osteoclasts.

Study IV. Fifteen 5-month-old mice were divided into a di-M-PGE_2 group which received a single dose of 25 μg of di-M-PGE_2 and a control group which received an identical amount of PGE-free diluent. The animals were sacrificed 2 hr after injection. After this period, no differences were noted in any chemical or histomorphometric parameters.

Discussion. Using fetal rat long bones prelabeled with ^{45}Ca, Klein and Raisz (1) first demonstrated that prostaglandins induced bone resorption *in vitro*. Subsequent reports dealing with the prostaglandin specificity of these effects have been contradictory. Dietrich and his colleagues (14) reported that, although prostaglandins of the E, F, A, and B groups all possessed bone resorptive activity, PGE_1 and PGE_2 were 10 times as active as any other prostaglandin tested. At concentrations of 10^{-5}–10^{-7} *M*, PGE stimulated resorption, but, at higher concentrations, the effect on bone resorption declined. In contrast, using calvaria from 5-day-old mice in culture, Tashjian and associates (3) reported that neither PGF or PGB had any apparent resorptive activity. In the present study, PGA_1 was found to be inactive and $PGF_{2\alpha}$ appeared to have a resorptive effect which was one third

as potent as that of PGE_2. The resorptive effects of PGE_2 and $PGF_{2\alpha}$ decreased considerably at concentrations above 1.0 μg/ml.

In a number of tumor and nontumor *in vitro* systems, endogenous PGE has been shown to have effects similar to those of exogenously administered prostaglandins. Bone resorption has been demonstrated by benign dental cysts (15), monkey gingiva (16), rheumatoid synovia (17), and fetal thyroid (18), tissues which synthesize significant amounts of PGE_2. PGE_2-mediated enhancement of bone resorption has also been ascribed to the $HSDM_1$ mouse fibrosarcoma (19) and human breast cancer (2) *in vitro*.

These *in vitro* findings suggest that prostaglandins synthesized by tumors may be responsible for the bone resorption associated with many neoplastic diseases. In fact, by virtue of the resorptive effects, prostaglandins may prepare suitable sites for circulating tumor cells to seed the bones, and thus facilitate the development of osseous metastases. This has been substantiated for a number of experimental tumors, including $HSDM_1$ mouse fibrosarcoma (3, 20), rabbit VX_2 carcinoma (4), and Walker carcinosarcomas in rats (21), as well as several human neoplasms, including renal cell adenocarcinomas (6, 7), breast cancers (22, 23), and others (8, 24, 25), all of which synthesize large amounts of prostaglandins. In these neoplastic syndromes, hypercalcemia appears to be a major consequence of the bone resorption.

In the present study, intraperitoneal administration of a long-acting analog of PGE_2 to mice from 4 to 20 days resulted in a significant increase in the osteoclast census, and, after short-term treatment, a significant increase in bone resorption. Mice treated with di-M-PGE_2 for 4 days (Studies I and III) sustained a mean 33% loss of trabecular volume, a 15% loss of bone calcium, and a 140% increase in the osteoclast census. In animals treated for 20 days (Study II), the volume of bone-lined osteoclasts increased by 450%, but there was only a very small decrease in trabecular volume. These data indicated that both short- and long-term treatment with di-M-PGE_2 causes osteoclasts to concentrate in bone, but did not provide an explanation for the discrepancy between osteoclast number and trabecular bone volume in Study II; one possibility is that long-term di-M-PGE_2 administration interferes with the functional ability of the mobilized osteoclasts. However, di-M-PGE_2-treated animals did *not* develop hypercalcemia, and, in fact, in one experiment, their mean serum calcium levels were lower than those of the controls. This effect on serum calcium has been reported by Klein and Raisz in rats (1). Although the blood calcium levels for the controls and di-M-PGE_2-treated mice in Studies II and IV were relatively low, this was not unexpected, since these experiments were performed in the late afternoon and under *ad lib.* feeding conditions. Several groups of investigators have reported that plasma calcium and phosphorus levels have a circadian rhythm which is controlled by the feeding schedule (26–29). If feeding is restricted to either the day or night, then plasma calcium and phosphorus values begin to decline by 10–20% several hours before "food presentation," due to secretion of gastrin, resulting in hypercalcitoninemia (30). Under the *ad lib.* feeding conditions of the present study, mice and other rodents are nocturnal consumers and lower calcium values occur in the afternoon. The absence of hypercalcemia in mice with osteolysis warrants comment. A number of possible explanations have been entertained, including an increased rate of urinary calcium excretion, binding of calcium to soft tissue, and hypercalcitoninemia. The studies of Silva and colleagues (31, 32), which reported that normocalcemic patients with various types of malignancies associated with rarified bones had elevated serum levels of thyrocalcitonin, support the final possible explanation.

Although this series of studies has confirmed *in vitro* bone loss as a result of PGE_2 treatment, we do not intend to suggest that the mechanism *in vivo* is necessarily a direct one. The experiments performed were not designed to assess the possible mechanisms of the calcium-mobilizing action of the prostaglandins. Having demonstrated that long-acting analogs of PGE_2 cause bone resorption *in vivo*, possible mechanisms of action

will be evaluated in subsequent studies.

Summary. The bone-resorbing activity of prostaglandin was assessed in neonatal mouse calvaria maintained in culture as well as in the bones of adult mice *in vivo*. In an *in vitro* ^{45}Ca-release bioassay, PGE_2, the most active compound, produced a maximal stimulation of radiocalcium bone depletion of 89% versus 38% for $PGF_{2\alpha}$ and 12% for PGA_1. Daily intraperitoneal injection of 10 μg of 16,16-dimethyl-PGE_2-methyl ester, a long-acting synthetic analog of PGE_2, in several groups of C57/B1/6J mice produced a mean 33% loss of trabecular bone and a 140% increase in the area of bone lined by osteoclasts. Moreover, treatment of 4-month-old mice with di-M-PGE_2 for 4 days caused a mean 15% depletion of bone calcium. There were no significant changes in blood calcium with the protocol employed.

1. Klein, D. C., and Raisz, L. G., Endocrinology **86,** 1436 (1970).
2. Powles, T. J., Clark, S. A., Easty, D. M., Easty, G., and Neville, A. M., Brit. J. Cancer **28,** 316 (1973).
3. Tashjian, A. H., Jr., Voelkel, E. F., Levine, L., and Goldhaber, P., J. Exp. Med. **136,** 1329 (1972).
4. Voelkel, E. F., Tashjian, A. H., Jr., Franklin, R., Wasserman, E., and Levine, L., Metabolism **24,** 973 (1975).
5. Galasko, C. S. B., and Bennett, A., Nature (London) **263,** 508 (1976).
6. Brereton, H. D., Halushka, P. V., Alexander, R. W., Mason, D. M., Keiser, H. R., and DeVita, V. T., Jr., N. Engl. J. Med. **291,** 83 (1974).
7. Robertson, R. P., Baylink, D. J., Marini, J. J., and Adkison, H. W., J. Clin. Endocrinol. Metab. **41,** 164 (1975).
8. Seyberth, H. W., Segre, G. V., Morgan, J. L., Sweetman, B. J., Potts, J. T., and Oates, J. A., N. Engl. J. Med. **293,** 1278 (1975).
9. Goodson, J. M., McClatchy, K., and Revell, C., J. Dent. Res. **53,** 670 (1974).
10. Franklin, R. B., and Tashjian, A. H., Jr., Endocrinology **97,** 240 (1975).
11. Beliel, O. M., Singer, F. R., and Coburn, J. W., Prostaglandins **3,** 237 (1973).
12. Dietrich, J. W., and Raisz, L. G., Clin. Orthop. Related Res. **111,** 228 (1975).
13. Peng, T.-C., and Gitelman, H. J., Endocrinology **94,** 608 (1974).
14. Dietrich, J. W., Goodson, J. M., and Raisz, L. G., Prostaglandins **10,** 231 (1975).
15. Harris, M., Jenkins, M. V., Bennett, A., and Wills, M. R., Nature (London) **245,** 213 (1973).
16. Gomes, B. C., Hansmann, E., Weinfeld, N., and DeLuca, C., Calc. Tiss. Res. **19,** 285 (1976).
17. Robinson, D. R., Tashjian, A. H., Jr., and Levine, L., J. Clin. Invest. **56,** 1181 (1975).
18. Feinblatt, J. D., Tai, L., and Leone, R. G., Prog. Endocrine Soc. **58,** 187 (1976).
19. Goldhaber, P., Proc. Amer. Assoc. Cancer Res. **3,** 113 (1960).
20. Tashjian, A. H., Jr., Voelkel, E. F., Goldhaber, P., and Levine, L., Prostaglandins **3,** 515 (1973).
21. Minne, H., Raue, F., Bellwinkel, S., and Ziegler, R., Acta Endocrinol. **78,** 613 (1975).
22. Bennett, A., Charlier, E. M., McDonald, A. M., Simpson, J. S., and Stamford, J. F., Prostaglandins **11,** 461 (1976).
23. Powles, T. J., Dowsett, M., Easty, D. M., Easty, G. C., and Neville, A. M., Lancet **1,** 608 (1976).
24. Demers, L., Allegra, J., Harvey, H., Lipton, A., Luderer, J., White, D., Gillin, M., and Brenner, D., Clin. Res. **24,** 359A (1976).
25. Powell, D., Singer, F. R., Murray, T. M., Minkin, C., and Potts, J. T., N. Engl. J. Med. **289,** 176 (1973).
26. Talmage, R. V., Roycroft, J., and Anderson, J. J. B., Calc. Tissue Res. **17,** 91 (1975).
27. Milhaud, G., Perrault-Staub, A. M., and Staub, J. F., J. Physiol. **222,** 559 (1972).
28. Perrault-Staub, A. M., Staub, J. F., and Milhaud, G., Endocrinology **94,** 480 (1974).
29. Talmage, R. V., Doppelt, S. H., and Cooper, C. W., Proc. Soc. Exp. Biol. Med. **149,** 855 (1975).
30. Cooper, C. W., Doppelt, S. H., and Talmage, R. V., Proc. Soc. Exp. Biol. Med. **153,** 16 (1976).
31. Silva, O. L., Becker, K. L., Primack, A., Doppman, J. L., and Snider, R. H., Chest **69,** 495 (1976).
32. Silva, O. L., Becker, K. L., Primack, A., and Snider, R. H., Pharmacologist **15,** 252 (1973).

Received March 7, 1977. P.S.E.B.M. 1977, Vol. 156.