Calcif Tissue Int (1990) 47:35–39

Calcified Tissue International
© 1990 Springer-Verlag New York Inc.

The Effect of Strain on Bone Cell Prostaglandin E_2 Release: A New Experimental Method

D. W. Murray and N. Rushton

University of Cambridge Orthopaedic Research Unit, Addenbrooke's Hospital, Cambridge, UK

Summary. A new method of investigating the mechanisms of strain-induced bone remodeling has been developed. Bone cells were subjected to cyclical strains *in vitro* by computer-controlled stretching of the plastic substrate on which they were cultured, enabling both physiological and pathological strains to be investigated. Physiological strains have not previously been investigated *in vitro*. The prostaglandin E_2 (PGE_2) released by the cells was found to depend on the strain magnitude. It was independent of cycle time, and 5 hours after straining had ceased, it had returned to control levels. These results are similar to the *in vivo* findings that bone remodeling is dependent on strain magnitude and not strain frequency, indicating that PGE_2 may play an important role in strain-induced bone remodeling. The relationship between PGE_2 release and strain magnitude was biphasic, with particularly high levels being released at strains that would be associated with either abnormally strenuous activity or microstructural bone damage. It is therefore possible that PGE_2 stimulates the osteogenesis caused by increased functional demands, and initiates the remodeling caused by bone damage. This new method of investigating strain-induced remodeling is useful, as any cell type, any mediator, and any strain pattern or parameter can be individually studied.

Key words: Bone strain — PGE_2 — Osteoblasts — Bone remodeling.

Send reprint requests to D. W. Murray, M.D., FRCS, University of Cambridge Orthopaedic Research Unit, Level E6, Addenbrook's Hospital, Cambridge, CB2 2QQ England.

Bone remodels in order to withstand the mechanical stresses exerted upon it (Wolff's law). Any stress, be it tensile compressive or torsional, will cause a small change in length of the bone. This strain, which is defined as the relative change in length, will be detected by the cells within the bone. In response to altered strain, these cells may release mediators that stimulate remodeling. In order to investigate strain-induced bone remodeling *in vitro,* it is necessary to know the mechanical properties of bone, the strains that occur physiologically, and the types of strains that cause bone remodeling. The mechanical properties of bone are complex. Cortical bone behaves elastically up to about 3 millistrain (a thousandth of change in length ÷ initial length), and then plastically until it fails at about 30 millistrain [1]. The initial part of this plastic deformation, up to about 10 millistrain, is viscous, whereas larger strains are associated with microstructual damage which is soon repaired [1]. The strains that occur in human bones are thought to be similar to those that occur in animals [2]. An experiment on a human tibia demonstrated that during walking, the peak strain and the peak strain rate were 0.4 millistrain and 4 millistrain/second respectively, and during running they were 0.85 millistrain and 13 millistrain/second [2]. In weight-bearing bones of a range of animals, the peak strains during strenuous activity were between 2 and 3 millistrain [3]. The magnitude and direction of the strain varied with the actual location within the bone and was altered by the insertion of a prosthesis [4]. The physiological range of strain, which includes strains encountered with normal activity and those that are not associated with microstructural bone damage, therefore extends up to about 5 millistrain. Bone remodeling depends partly on strain magnitude. In general, increased strains cause hypertrophy and decreased

strains are associated with disuse atrophy. A few months after the loading on a bone increases, the bone has remodeled to such an extent that the strains, which initially rise, return to normal [5]. Bone remodeling also depends on the distribution of the strain and on the strain rate, with higher strain rates inducing more hypertrophy than lower ones [6, 7]. Bone remodeling is independent of the frequency of load cycles provided there are more than about 10 cycles per day [6].

Recent studies investigating the effect of strain on bone cells, by stretching the substrate on which they were cultured, have suggested that PGE_2 may play a role in mediating strain-induced bone remodeling; the release of PGE_2 by bone cells increases when they are strained [8, 9]. Bone cell cyclic AMP and DNA synthesis is increased by both PGE_2 and strain, the latter being inhibited by indomethacin [10, 11]. PGE_2 stimulates bone formation and resorption, both *in vivo* and *in vitro* [12, 13]. The bone cells that respond to strain are thought to be osteoblasts [11]. However, in all these studies the strains used were different from those that occur physiologically; the magnitudes of the strains were between 10 and 100 millistrain, which are out of the physiological range and would be associated with bone damage. The strain rates were neither controlled nor physiological, and in most studies the cells were strained once and not repeatedly. The aim of this investigation was to subject bone cells to physiological as well as pathological strains, and to determine how much PGE_2 is released.

Methods

Bone cells were cultured in special wells, the bases of which were subjected to cyclical strains in a stretching machine. The PGE_2 released both during a control period prior to straining and during the straining period was determined. From these the percentage increase in PGE_2 release was calculated.

Bone Cell Cultures

A method based on that of Wong and Cohn [14] was used to obtain a population of bone cells rich in osteoblasts. Calveria from 4-day-old mice, having been carefully prepared by microdissection so as to remove all fibrous tissue, were sequentially digested in 1 mg/ml collagenase (Sigma type 1, Sigma Ltd, Poole, UK) at 37°C. The cells released between 30 and 120 minutes were washed and then cultured in the stretching wells. After 3 days the cells were confluent and ready to be used in experiments. They were cultured in Hepes buffered Medium 199 with 5% fetal bovine serum (FBS) and 1% antibiotics (penicillin 1 U/ml and streptomycin 5 mg/ml) in a humidified atmosphere at 37°C.

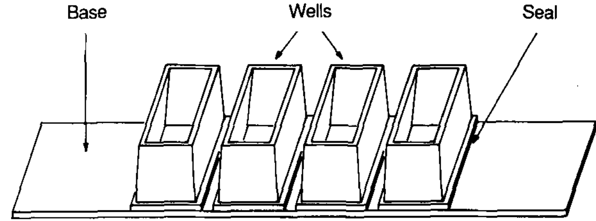

Fig. 1. A set of stretching wells.

The Stretching Wells

Special wells were constructed with transparent bases that could be stretched repeatedly and elastically (Fig. 1). The well sides were formed from the sides of Lab-tek tissue culture chamber-slides (Miles Labs Ltd, Slough, UK.). The bases were either made from tissue culture-treated Permanox (the base of Lab-tek slides) or polycarbonate. So that cells would adhere strongly to the polycarbonate, it was coated with a 50 nm layer of gold using a gold sputtering technique. The well sides were sealed to the polycarbonate base with silicone grease. Although Permanox-based wells were simple to construct, being Lab-tek slides with the end chamber removed, they could only be used for experiments of short duration involving small strains because the plastic seals joining the wells' sides to the Permanox base occasionally leaked and Permanox broke at about 15 millistrain. Polycarbonate, however, behaved elastically up to approximately 30 millistrain, and then deformed plastically.

The Stretching Machine

The stretching machine consisted of a lead screw and a series of levers that converted the rotation of a computer-operated stepper motor into movement of clamps that held the well bases (Fig. 2). The strain was applied repeatedly in the form of a ramped square wave for the duration of the test period. The strain rate was 10 millistrain/second. The duration of both test and control periods was 5 hours for Polycarbonate-based wells and 1.5 hours for Permanox-based wells. In different experiments, different strains and different cycle times were used.

Prostaglandin Assay

The amount of PGE_2 released by the cells was determined by a radioimmunoassay (New England Nuclear, Stevenage, UK) which was very sensitive (0.13 pg/sample), and specific (cross-reactivity with PGE_1 3.7%, and with other biological compounds similar to PGE_2 $<0.5\%$).Medium samples (100 μl) were centrifuged (1,000 rpm, 10 minutes) buffered (20 μl of 0.5 M phosphate buffer), and then diluted to bring their PGE_2 concentration to within the range of the assay (0.25–25 pg/sample). Tracer (100 μl^{125} I-labeled PGE_2), antibody (100 μl lyophilized rabbit anti-PGE_2), and following overnight incubation, cold precipitating reagent (1 ml) were added. After 25 minutes in ice, the tubes were centrifuged (1,500 rpm, 30 minutes, 4°C), and the radioactivity in the resultant pellet was determined. For each assay a standard curve was generated from 20 standard samples, and from the standard curve the sample PGE_2 concentrations were calculated.

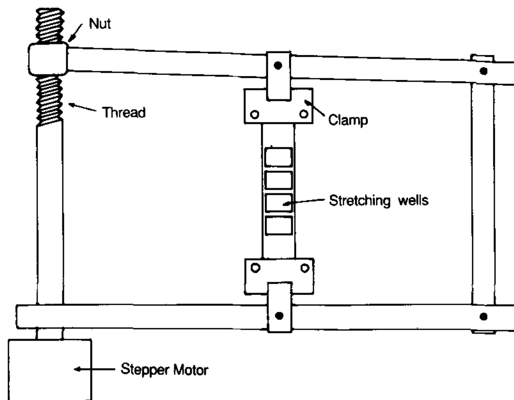

Fig. 2. Schematic representation of the stretching machine.

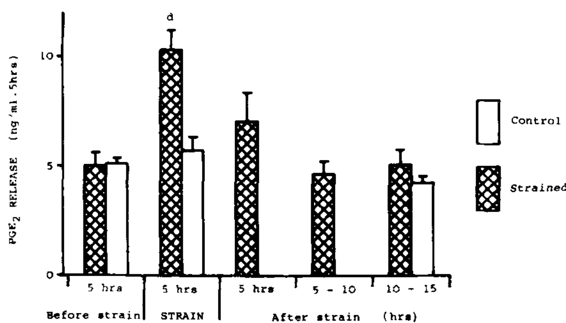

Fig. 3. Time course of PGE_2 release. Significant increase (d) $P <$ 0.001.

The PGE_2 released during a control period prior to straining, during the straining period, and during poststrain periods were determined. For statistical analysis, *t* tests were used when two conditions were compared, and analysis of variance (ANOVA) was used when a number of different conditions (e.g., strain magnitudes or time intervals) were compared. For each test condition n = 4. Means and standard errors are plotted. The increased PGE_2 release was calculated from the release during the prestrain and the strain periods, and was expressed as a percentage, thus, the cells acted as their own controls. A logarithmic transformation was used when percentage increases were compared statistically.

Results

PGE$_2$ Released by Strained Bone Cells

Two sets of polycarbonate-based wells containing bone cells cultured from the same population were treated in an identical manner, except that the test set was subjected to strain, and the control set was not. Every 5 hours for a period of 25 hours the medium was renewed and the PGE_2 production was assayed (Fig. 3). During the second 5-hour period, the test cells were strained repeatedly, with a cycle

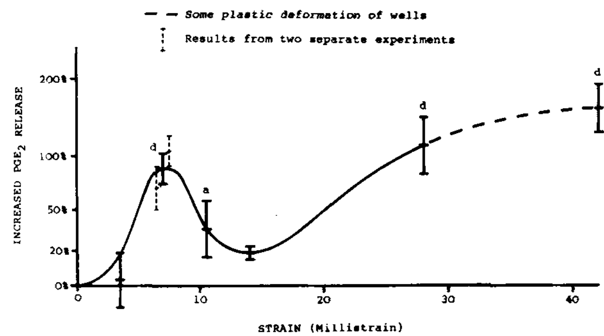

Fig. 4. The increased PGE_2 release caused by different strains. Significant increase (a) $P < 0.01$, (d) $P < 0.001$.

time of 10 minutes, by 28 millistrain (approximately the strain at which bone fractures). It was found that the PGE_2 release doubled when the cells were strained (a significant increase $P < 0.001$). When the straining ceased these cells continued to release an increased amount of PGE_2, but this gradually decreased until after about 5 hours when it returned to the unstretched level and remained at that level. The PGE_2 released by the nonstrained control cells did not alter significantly druing the 25-hour period.

The Influence of Strain Magnitude on PGE$_2$ Release

Bone cells in polycarbonate-based wells were subjected to various strain magnitudes, with a constant cycle time of 10 minutes. The percentage increase in PGE_2 release was determined for each well (Fig. 4). At different strains, the PGE_2 release was significantly different ($P < 0.005$, ANOVA). The experiment was repeated twice at 7 millistrain and no significant difference was found between the two sets of results, indicating that the results were repeatable. The graph comparing strain magnitude to PGE_2 release was biphasic. At 7 millistrain, and at 28 millistrain and above, the PGE_2 release was approximately twice the unstrained level, and these increases were highly significant ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$). At 3 millistrain and between 7 millistrain and 28 millistrain, the increases were small and not always significant. The increased release decreased from about 100% at 7 millistrain and 28 millistrain to 20% at 14 millistrain, decreases that were significant ($P < 0.01$, $P < 0.01$).

At all strains tested, it was found that the PGE_2 release had returned to the control levels by about 12 hours after straining had ceased. The percentage difference in PGE_2 releases between the poststrain levels (approximately 12 hours after straining had ceased) and the prestrain levels were as follows: at

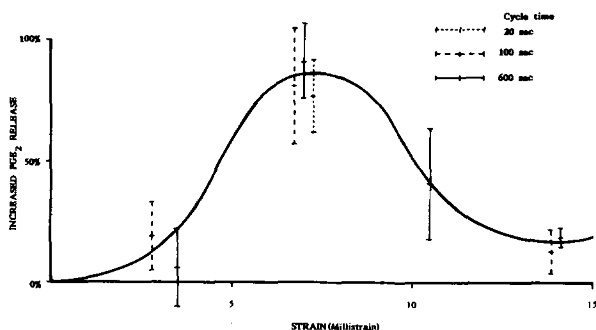

Fig. 5. The PGE_2 release at different cycle times.

3 millistrain 4% (SE 10), at 7 millistrain 4% (SE 16), at 10 millistrain 19% (SE 37), and at 28 millistrain 3% (SE 16).

The Influence of Cycle Time on PGE_2 Release

Bone cells in polycarbonate-based wells were subjected to cyclical strains of 7 millistrain, with cycle times of either 20 seconds or 600 seconds. In both cases the PGE_2 release approximately doubled (Fig. 5). There was no significant difference in the increased PGE_2 release at the different cycle times. In another series of experiments, Permanox-based wells containing bone cells were subjected to various strains with a cycle time of 100 seconds. (These experiments using Permanox were repeated three times.) The percentage increase in PGE_2 release was determined and is plotted with the results of other experiments in which the same range of strains and different cycle times were used (Fig. 5). It was found that at all strains tested, the PGE_2 release depended only on the magnitude of the strain and was the same whether the cycle time was 20s, 100s, or 600s. PGE_2 release therefore appears to be independent of cycle time.

Histology

Both strained and nonstraineed bone cells were stained and examined microscopically. It was found that strain did not cause a morphological change in the cells. A large proportion of cells stained positively for alkaline phosphatase (azo-dye coupling method) confirming that they were probably osteoblasts (Fig. 6).

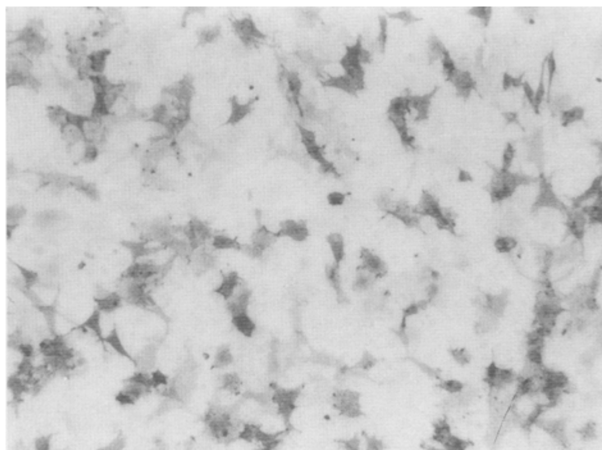

Fig. 6. Bone cells stained with alkaline phosphatase.

Discussion

These results confirm the experimental findings of other workers: When bone cells are subject to strain they release more PGE_2. This study has also shown that the amount of PGE_2 released depends on the magnitude of the applied strain and that for strain cycles of 10 minutes or less, the PGE_2 release was independent of cycle time (Figs. 4 and 5). As it takes approximately 5 hours for the PGE_2 release to return to normal after straining has ceased, the PGE_2 release is likely to be independent of cycle time provided this is less than a few hours (Fig. 3). Other workers have shown *in vivo* that bone remodeling depends on strain magnitude, and is independent of cycle time provided it is less than a few hours. This validates the experimental method, and also suggests that PGE_2 released by bone cells may play a role in strain-induced bone remodeling.

It is interesting that the graph comparing applied strain to PGE_2 release is biphasic and not a straightforward relationship (Fig. 4). The graph first rises to a maximum at 7 millistrain, it then falls to a minimum at 14 millistrain, and finally rises up again. Within the physiological range of strains, which extends up to the first maximum on the graph, an increase in strain causes an increase in PGE_2 release. At the lower end of this range, at strains that occur with normal gentle activity, there is no significant increase in PGE_2 release. This suggests that PGE_2 is unlikely to play an important role in mediating disuse atrophy. However, with strains at the upper end of the physiological range, the PGE_2 release is about twice the control level, suggesting that PGE_2 may stimulate the osteogenesis that occurs with increased functional demands. At strains above the physiological range, the PGE_2 release decreases until very high strains are encountered when it increases again, being approximately twice control levels at 28 millistrain. These very high

strains would be associated at least with microstructural bone damage *in vivo* and might cause fracture. It is therefore possible that PGE_2 may have a role in initiating the remodeling that is necessary after microstructural damage.

It is difficult to explain why the graph comparing applied strain with PGE_2 release is biphasic. It is unlikely that it is a statistical quirk as the release at 14 millistrain is significantly ($P < 0.01$ and $P < 0.01$) less than the release at either 7 millistrain or 28 millistrain. A possible explanation is that there are two mechanisms that control the release of PGE_2. There may be a specialized receptor that only responds to low physiological strains and that controls the release of PGE_2. At very high strains the increased release of PGE_2 may result from cell membrane disturbance. If there is a specialized receptor that responds to physiological strains then it is likely to play a central role in strain-induced bone remodeling. The PGE_2 released by this receptor would be related to the strain that the cells are subjected to for the majority of the time, rather than strains that are only applied momentarily. This is demonstrated by the finding that cells strained to 14 millistrain show little increase in PGE_2 release even though they have been strained momentarily by 7 millistrain (as the strain was applied), whereas cells strained to 7 millistrain for awhile release a large amount of PGE_2 (Fig. 4).

The amount of PGE_2 released by bone cells depends on the magnitude of the strain and not on how frequently it is applied, providing it is applied at least once every few hours. Therefore, if strains of different magnitudes occur, the PGE_2 released will depend on the strains that cause the largest release of PGE_2. Within the physiological range, the larger the strain the larger the PGE_2 release, so that in physiological situations the PGE_2 released by bone cells will depend on the peak strains. As bone remodeling is a slow process it is likely that it will depend on the average amount of PGE_2 released by bone cells. Therefore, PGE_2-mediated strain-induced bone remodeling will depend on the peak strains that occur in bone.

The results of control experiments are important. The release of PGE_2 by nonstrained control cells did not alter during the experimental period. This indicates that the increased PGE_2 release from test cells was caused by strain rather than by an experimental artifact like the silicone grease or the gold coating. When an experiment was repeated under identical conditions the same results were obtained, indicating that the results were repeatable. Medium containing 5% FBS was assayed and found to contain 15 (SE 1) pg/ml of PGE_2. The amount of PGE_2 released by the bone cells was more than an order of magnitude greater than this so the FBS did not influence the results.

A new *in vitro* method of investigating strain-induced bone remodeling has been developed and has been shown to produce valid results. Unlike other published methods, it is particularly useful as physiological strains can be investigated. It is adaptable as any strain pattern or parameter (strain magnitude, rate, frequency, etc.) and any cell type or mediator can be studied. The cells can also be microscopically examined before, during, and after straining.

Acknowledgment. This work was supported by a grant from the Hospital Trust Fund, Addenbrooke's Hospital, Cambridge, UK.

References

1. Dumbleton JH, Black J (1975) Mechanical properties of tissues. In: An introduction to orthopaedic materials. CC Thomas, Springfield, Illinois, pp 106–131
2. Lanyon LE, Hampson WGH, Goodship AE, Shah JS (1975) Bone deformation recorded in vivo from strain gauges attached to the human tibial shaft. Acta Orthop Scand 46:256–268
3. Rubin CT (1984) Skeletal strain and the functional significance of bone architecture. Calcif Tissue Int 36:S11–18
4. Indong OH, Harris WH (1978) Proximal strain distribution in the loaded femur. J Bone Joint Surg 60A:75–85
5. Goodship AE, Lanyon LE, McFie H (1979) Functional adaption of bone to increased stress. J Bone Joint Surg 61A:539–546
6. Rubin CT, Lanyon LE (1984) Regulation of bone formation by applied dynamic loads. J Bone Joint Surg 66:397–402
7. O'Conner JA, Lanyon LA, McFie H (1982) The influence of strain rate on adaptive bone remodeling. J Biomech 15:767–781
8. Somjen D, Binderman I, Berger E, Harell A (1980) Bone remodeling induced by physical stress is prostaglandin E_2 mediated. Biochim Biophys Acta 627:91–100
9. Yeh C, Rodan A (1984) Tensile forces enhance PGE synthesis in osteoblasts grown on collagen ribbon. Calcif Tissue Int 36:S67–71
10. Hasegawa S, Sato S, Saito S, Suzuki Y, Brunette DM (1985) Mechanical stretching increases the number of cultured bone cells synthesizing DNA and alters their pattern of protein synthesis. Calcif Tissue Int 37:431–436
11. Binderman I, Shimshoni Z, Somjen D (1984) Biochemical pathways involved in the translation of physical stimulus into biological message. Calcif Tissue Int 36:S82–85
12. Raisz LG, Martin TJ (1983) Prostaglandins in bone and mineral metabolism. In: Peck WA (ed) Bone and mineral research, vol 2. Excerpta Medica, Amsterdam, pp. 286–310
13. Shin MS, Norridin RW (1986) Effects of prostaglandins on regional remodeling changes during tibial healing in beagles: a histomorphometric study. Calcif Tissue Int 39:191–197
14. Wong G, Cohn DV (1974) Separation of PTH and calcitonin-sensitive cells from nonresponsive bone cells. Nature 252:713–715

Received March 7, 1989, and in revised form June 2, 1989.