Induction of NO and prostaglandin E$_2$ in osteoblasts by wall-shear stress but not mechanical strain

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Smalt, R., F. T. Mitchell, R. L. Howard, and T. J. Chambers. Induction of NO and prostaglandin E$_2$ in osteoblasts by wall-shear stress but not mechanical strain. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E751–E758, 1997.—The nature of the stimulus sensed by bone cells during mechanical usage has not yet been determined. Because nitric oxide (NO) and prostaglandin (PG) production appear to be essential early responses to mechanical stimulation in vivo, we used their production to compare the responsiveness of bone cells to strain and fluid flow in vitro. Cells were incubated on polystyrene film and subjected to unidirectional linear strains in the range 500–5,000 microstrain (µε). We found no increase in NO or PGE$_2$ production after loading of rat calvarial or long bone cells, MC3T3-E1, UMR-106–01, or ROS 17/2.8 cells. In contrast, exposure of osteoblastic cells to increased fluid flow induced both PGE$_2$ and NO production. Production was rapidly induced by wall-shear stresses of 148 dyn/cm$^2$ and was observed in all the osteoblastic populations used but not in rat skin fibroblasts. Fluid flow appeared to act through an increase in wall-shear stress. These data suggest that mechanical loading of bone is sensed by osteoblastic cells through fluid flow-mediated wall-shear stress rather than by mechanical strain.

nitric oxide; bone

ONE OF THE PRIMARY functions for which bones have evolved is to act as a structural support. To achieve this, bones remodel throughout life so that their structure remains optimal for the prevailing mechanical environment. Failure of bones to maintain structural adaptation leads to the increased incidence of fractures in diseases such as osteoporosis.

Recently, the development of avian and rodent experimental models has provided a substantial body of information concerning the mechanisms by which mechanical forces act on bone in vivo. It has been found that even relatively brief exposure of bones to mechanical stimulation, by strains within the range experienced under physiological circumstances, is followed by bone formation. This response is associated with very early expression of mRNA in osteocytes and bone surface cells, including c-fos (20) and insulin-like growth factor I (19, 28), which precedes bone formation. Prostaglandin (PG) and nitric oxide (NO) synthesis is also required (4, 11, 26, 38) and appears to play a role very early in the signaling process, since inhibition of either pathway around the time of mechanical stimulation abrogates the osteogenic response.

Little is known, however, of the cellular mechanisms underlying the osteogenic response. Even the nature of the mechanical signal that activates a sensor cell in bone is a matter of debate. Suggestions include cell deformation as a direct result of strain in the load-bearing matrix, or strain-induced fluid flow through the lacunocanalicular network of bone, which might be detected as changes in solute transport, or through streaming potentials or wall-shear stress (8, 9, 14, 16, 39). It would clearly be advantageous, for a clarification of the molecular and cellular processes underlying mechanical adaptation, to identify the nature of the mechanical stimulus acting on bone cells. The recent observations that the response of bone to osteogenic mechanical stimuli is suppressed by inhibition of cyclooxygenase and NO synthase (NOS) (4, 11, 26, 38) predict that the transduction of mechanical signals should be associated with PG and NO synthesis.

There is extensive literature documenting PG production by bone cells in vitro in response to mechanical strain (2, 3, 22, 23, 27, 40). However, previous studies have used very large or unquantified strains for their experiments and have not uncoupled strain from fluid flow effects, used prolonged periods of stimulation (minutes) compared with the duration of external loading stimulus needed in vivo, or imposed strains by four-point bending, which causes medium perturbation with potential fluid flow effects, one of which is PG synthesis in bone cells.

Even less is known concerning the nature of the stimulus causing NO production. A recent report found that a fluid shear stress of 6 dyn/cm$^2$ caused NO production continuously for 12 h (15). Dexamethasone resistance suggested that NO production was due to constitutive rather than inducible NOS. This is consistent with expression of mRNA for neuronal NOS in bone cells (27, 33). There is also a report showing that the imposition of physiological levels of strain in vitro by four-point bending causes NO production by bone cells (27).

In an attempt to clarify the nature of the mechanical stimulus to which bone cells respond, we compared the ability of mechanical strain and fluid flow to induce NO and PG production in bone cells. To do this, we developed a novel method whereby cells could be exposed to measurable and physiological strain magnitude with less perturbation of the culture medium than occurs during the four-point bending system for imposition of strain. For fluid flow experiments we adapted a parallel plate flow model commonly used in the assessment of endothelial cell responses to fluid flow. We could detect no induction in either PG or NO production by strains up to 5,000 microstrain (µε). In contrast, low levels of fluid flow induced rapid production of both agents.

MATERIALS AND METHODS

Osteoblastic cells. Primary rat calvarial cells were isolated by collagenase digestion. Calvaria from 3-day-old Wistar rats
were prepared free from adherent tissue and digested in 1 mg/ml collagenase II (Sigma, Poole, Dorset, UK) in N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-buffered medium 199 (Imperial, Andover, Hants, UK) for 15 min at 37°C. The medium was then discarded and replaced with fresh medium containing 3 mg/ml of collagenase. After a further 90-min incubation, the calvaria were incubated with trypsin-EDTA (Imperial) for 10 min. The second collagenase and the trypsin-EDTA digests were pooled, and cells were pelleted and resuspended in α-modified Eagle’s medium (α-MEM; Imperial) for subsequent incubations. Primary rat long bone cells were isolated using the same digestion protocol with mid-bone shafts of femora, tibiae, and humeri, which had been scraped clean of all adherent tissue, split longitudinally, and cleared of all bone marrow. Primary rat skin fibroblasts were prepared from neonatal skin digested in 3 mg/ml collagenase for 90 min at 37°C. MC3T3-E1 cells were a kind gift from Dr. M. Kumegawa (Mekai University School of Dentistry, Sakada, Japan) and were used up to passage 15. UMR-106-01 and ROS 17/2.8 were obtained from Dr. T. Martin (St. Vincent’s Institute for Medical Research, Melbourne, Australia) and Dr. G. Rodan (Merck, West Point, NY), respectively. Primary cells were incubated in α-MEM, and cell lines were maintained in MEM (Imperial). Ten percent newborn calf serum (NCS; Imperial), glutamine, penicillin, and streptomycin (all from Imperial) were added to culture media. Primary cells were incubated overnight before use in experiments. For use, primary cells or osteoblastic cell lines were released into suspension by incubation in trypsin-EDTA, washed, and resuspended for use in the strain or fluid flow apparatus. Samples of the suspension of calvarial and long bone cells were further incubated for assessment of alkaline phosphatase and the ability to form mineralized nodules.

Strain experiments. Cells resuspended as above were added (1 ml at 10^5 cells/ml in α-MEM or MEM and NCS) to wells formed by mounting the upper 2.2 cm of a 7-ml screw-top bijou bottle (15 mm diam; Bibby-Sterilin, Staffs, UK) on a tissue culture-treated strip of polystyrene film (Trycite 3001, bijou bottle (15 mm diam; Bibby-Sterilin, Staffs, UK)) with mid-bone shafts of femora, tibiae, and humeri, which had been scraped clean of all adherent tissue, split longitudinally, and cleared of all bone marrow. Primary rat skin fibroblasts were prepared from neonatal skin digested in 3 mg/ml collagenase for 90 min at 37°C. MC3T3-E1 cells were a kind gift from Dr. M. Kumegawa (Mekai University School of Dentistry, Sakada, Japan) and were used up to passage 15. UMR-106-01 and ROS 17/2.8 were obtained from Dr. T. Martin (St. Vincent’s Institute for Medical Research, Melbourne, Australia) and Dr. G. Rodan (Merck, West Point, NY), respectively. Primary cells were incubated in α-MEM, and cell lines were maintained in MEM (Imperial). Ten percent newborn calf serum (NCS; Imperial), glutamine, penicillin, and streptomycin (all from Imperial) were added to culture media. Primary cells were incubated overnight before use in experiments. For use, primary cells or osteoblastic cell lines were released into suspension by incubation in trypsin-EDTA, washed, and resuspended for use in the strain or fluid flow apparatus. Samples of the suspension of calvarial and long bone cells were further incubated for assessment of alkaline phosphatase and the ability to form mineralized nodules.

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Flow experiments. A single-pass flow-through system was used. Glass slides (2 × 3 in.; Horwell, London, UK) were placed in tissue culture dishes (100 mm diam; Bibby-Sterilin). Cell suspensions obtained as described above were added (2 × 10^5 cells/ml, 10 ml in MEM/NCS) and incubated for 1–4 days before exposure to fluid flow. For exposure to fluid flow, the glass slide was carefully removed and mounted on a parallel plate flow chamber, as previously described by McIntyre and Eskin (21) (Fig. 1). Nesco film (Nippon Shoji Kaisha, Osaka, Japan) was used as a gasket, creating a flow channel 80 μm deep × 31.5 mm wide × 51.5 mm long. The flow rate of HEPES-buffered medium 199 with 0.1% bovine serum albumin was controlled with a syringe pump (duo infusion/withdrawal pump model 944; Harvard Apparatus, Edenbridge, Kent, UK). Flow rate and shear stress have a linear relationship in a channel of fixed dimensions. Shear stresses between 0 and 80 dyn/cm^2 were calculated from velocity of flow, medium viscosity, and the dimensions of the chamber, which were validated by measurement of pressure gradient across the chamber, and related to flow rates. Outflow fluid was collected for analysis. For a typical experiment, the chamber was perfused at a low flow rate (unless otherwise stated, 0.1 dyn/cm^2; 31 μl/min) immediately after construction, until a steady baseline became established. Flow was then increased briefly before fluid flow rate was returned to the previous low level. After sufficient time for a response to be characterized, the cells were again exposed to an episode of increased flow. In some experiments N-nitro-L-arginine methyl ester (L-NAME, 100 μM; Sigma) was added to the medium in the syringe. Methylcellulose (1.2%; 1,500 centipoise at 2%; Sigma) was added to the medium for the increased viscosity experiments. Strain experiments were performed in a warm room (37°C).

Measurement of PGE2. PGE2 was measured by an adaptation of the dextran-coated charcoal radioimmunoassay. Briefly, 500 μl of a 1:4,000 dilution of a polyclonal rabbit anti-PGE2 antiserum (Sigma) were added to duplicate 100-μl samples of supernatants and incubated at room temperature for 30 min. [3H]PGE2 solution (100 μl; 823 Bq/ml; Amersham, Little...
Bone cells were used due to the absence of response in calvarial cells, since it seemed possible that responsiveness to strain might be a feature of cells derived from bones that are most clearly adapted for mechanical usage. Although 30–70% of such cells stained positive for alkaline phosphatase and the cultures were able to form mineralized bone nodules (data not shown), we could detect no increase in release of PGF₂α or NO. In two experiments using long bone cells, we measured 6-keto PGF₁α (the product of spontaneous decay of PGI₂) and found no detectable increase after exposure to 5,000 µe (1 Hz) for 20 min.

In contrast, when similar cell populations were exposed to fluid flow, we noted rapid induction of NO production (Fig. 3). An increase in flow rate was frequently associated with a decrease in NO concentration during the phase of increased flow, presumably due to dilution of NO. [Each 100-µl aliquot contained NO generated during ~3 min of osteoblast activity at basal (31 µl/min) but only 20, 4, or 1 s at the higher flow rates.] However, when flow rate returned to normal, chamber effluent contained substantially greater concentrations of NO than those measured before exposure to increased fluid flow. The volume of fluid between the most upstream cells and the collecting chamber (150 µl) was contained within the first two samples, collected during 6 min after each episode of stimulation. Therefore, the increased NO levels we detected in samples removed up to 15 min after an episode of increased flow suggest that NO is generated during flow and also for some minutes subsequently. This response was observed with calvarial cells, long bone cells, and the osteoblastic cell lines (MC3T3 E-1, ROS 17/2.8, and UMR-106-01) but not skin fibroblasts. We noted a flow rate-dependent relationship for osteoblastic cells between flow rate (expressed as wall-shear stress) and NO production at the three levels of wall-shear stress used (Figs. 3 and 4). Wall-shear stress of 1 dyn/cm²...
was sufficient to induce NO production in osteoblastic cells. Because NO has an extremely short half-life time in aqueous solution, it was not practical to measure NO directly. Instead, we assessed the amount of nitrite, which is the stable oxidation product of NO, formed in the medium. To confirm that the increases we found in the nitrite concentration were actually the result of an

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**Fig. 3.** Effect of wall-shear stress on NO production by osteoblastic and non-osteoblastic cells in vitro. Cells were sequentially exposed to defined wall-shear stresses of 1.0, 4.6, and 21.5 dyn/cm², respectively, for 1 min each. Baseline shear stress was 0.1 dyn/cm². Osteoblastic cells show a clear dose-dependent increase in NO production immediately after the stimulus, returning to baseline levels after 20–30 min: primary rat calvarial (A) and long bone (B) cells, and MC3T3-E1 (C). Primary rat skin fibroblasts (D) do not show this response to wall-shear stresses. Each figure shows a typical response of individual cultures. Each experiment was repeated for at least 4 cultures.

**Fig. 4.** Effect of wall-shear stress on NO production by osteoblastic and nonosteoblastic cells in vitro. Cells were exposed to wall-shear stress as in Fig. 3. Data show aggregate results of 4 experiments for each cell type: calvarial cells (A), long bone cells (B), MC3T3-E1 (C), and fibroblasts (D). Values represent peak level observed after the episode of increased flow, less concentration in sample taken immediately before flow rate was increased. Each peak was observed in samples removed within 15 min of stimulation. Results show mean (± SE). *P < 0.05 vs. baseline value (Student's t-test).
increase in NO production, we stimulated cells with shear stress in the absence and presence of the NOS inhibitor L-NAME. Addition of the inhibitor completely blocked the NO response that was seen without the inhibitor (Fig. 5), demonstrating that the changes in the nitrite concentration we found are indeed the direct result of changes in the NO production rate.

Fluid flow can affect cells in two distinct ways. The first is through wall-shear stress, expressed in dyn/cm² (or Pa; 1 Pa = 10 dyn/cm²). A second flow-mediated effect on cells is electrical in nature and is caused by the movement of charged molecules in the fluid over the cell surface. This can generate potential differences across the cell membrane, which could either affect the cellular ion balance directly or act indirectly through, for example, activation of voltage-gated ion channels.

To differentiate between these fluid-flow-generated stimuli, we used methylcellulose to increase the viscosity of the medium. Methylcellulose does not contain charged groups and should, therefore, not affect the electrical properties of the fluid. When the flow rate was kept constant but normal medium was replaced with medium of an 80-fold greater viscosity (1.2% methylcellulose), we found a substantial increase in NO production by osteoblastic cells, indicating that the increased NO production induced by increased fluid flow is due to changes in mechanical rather than electrical stimuli (Fig. 6). This effect was observed at flow rates of 0.1 and 0.2 ml/min.

Reich and Frangos (30) have found that fluid flow causes PG production by osteoblastic cells. This was confirmed in experiments in which we found a flow-induced increase in the PGE₂ secretion, first observed 10 min after the start of stimulation (Fig. 7). The increase leveled off at 30 min and showed a second, more pronounced increase, starting after 60–90 min.
DISCUSSION

It is still a matter of debate whether mechanical adaptation of bone occurs as a direct response to strain or indirectly, as a response to strain-induced fluid flow. Experiments in which bone is subjected to mechanical stimulation suggest that the osteogenic response is dependent on the production of PG and NO at or soon after the time of loading (4, 11, 26, 38). Therefore, we used these responses to compare the sensitivity of bone cells in vitro with the levels of strain and fluid flow-induced wall-shear stress likely to be experienced by bone cells in vivo. We found that, although bone cells were unresponsive to mechanical strain, both primary cultures containing osteoblastic cells and osteoblastic cell lines responded to even low levels of wall-shear stress. Although there are differences in experimental conditions between the two systems that might influence the magnitude or sensitivity of the mechanical response, it seems unlikely that these could account for the complete absence of detectable response to mechanical strain and high sensitivity to fluid flow, and our data suggest that mechanical stimulation is primarily transduced by wall-shear stress.

There is a substantial body of evidence for a role for PG production in the mechanical responsiveness of bone. PGs, which increase bone formation when administered systemically (see Ref. 24), are released by bone in organ culture when bone is subjected to applied loads (29). When PG production is inhibited by indomethacin in vivo, the osteogenic response of bone to mechanical stimulation is markedly reduced (4, 10, 26). PGs have also been shown to be produced after stimulation of bone cells by fluid flow (17, 30, and present data).

It has previously been reported, with the use of a recirculating flow system (15), that dexamethasone-resistant NO production can be detected within 1 h of initiation of fluid flow at 6 dyn/cm². Using a sensitive detection system, we have found that the induction of fluid flow occurs over the range 1–48 dyn and that NO production is induced very soon after stimulation. This strongly suggests that NO production is attributable to a Ca²⁺-dependent form of NOS rather than the inducible form.

We failed to detect a response to mechanical strain, despite exposure of the cells to a similar regimen to those found to cause a substantial osteogenic response in vivo (5). This failure contrasts with responses observed by others in cells exposed to mechanical strain in vitro (2, 3, 22, 23, 27, 40). However, previous studies used very large or unquantified strains for their experiments and have not uncoupled strain from fluid flow effects, used prolonged periods (minutes) of stimulation compared with the duration of external loading stimulus needed in vivo, or imposed strains by four-point bending, which causes medium perturbation with potential fluid flow effects.

There are two caveats to our conclusion that strain may not be the stimulus to which bone cells normally respond. First, the detection of strain by cells depends on adequate substrate adhesion. It is thus possible that greater strains are required to provide cells cultured on an artificial substrate with a physiological degree of strain sensation. However, we detected no response even to strains almost 10-fold greater than those that generate a substantial response in vivo (5), and the cultures were sensitive to wall-shear stress, which is similarly dependent on substrate adhesion and an intact cytoskeleton (1, 8).

The second caveat concerns the nature of the cells we tested in vitro, since it is generally considered that osteocytes represent the primary transducers of mechanical information. The response of shear stress we have observed suggests that, if osteocytic differentiation is required for mechanical responsiveness, then those aspects of the osteocytic phenotype required for mechanical responsiveness are expressed in some or all of the cells in our cultures. This might be expected in populations of cells of the lineage that forms osteocytes in vivo. However, the osteocyte is not the only cell in bone that shows a rapid response to mechanical stimulation in vivo. Immediate gene responses are also observed on bone surfaces (20), and osteocytes in situ might owe their mechanical responsiveness to their anatomic location rather than to some special characteristic not shared with other cells of the lineage.
Mechanical loading of bone results in flow of interstitial fluid through the canalicular network (8, 14, 18). It has been suggested that fluid flow through canaluli provides the mechanism by which bone cells transduce the very small strains measured in bone during mechanical loading (30, 31). Such a mechanism implies that bone cells must be sensitive to fluid-induced wall-shear stresses of ~8–30 dyn/cm² that have been predicted to result from normal mechanical usage of bone (39, 41). The sensitivity of bone cells to wall-shear stress described in the present experiments is similar to the responsiveness previously observed in vitro (13, 17) and is consistent with the notion that fluid-induced wall-shear stresses could account for the mechanical sensitivity of bone.

The stimulatory effects of fluid flow may be due to 1) electric potentials induced by the flow of charged molecules (streaming potentials), 2) effects on agonist or metabolite availability, or 3) direct perturbation of the cells by wall-shear stress (6, 7, 9, 41). For a given flow rate, the former are independent of viscosity, whereas wall-shear stress is directly proportional to viscosity. The increased response to perfusion by medium of increased viscosity at the same flow rate we noted suggests that osteoblastic NO production is at least largely attributable to wall-shear stress. This is consistent with the previously observed enhancement of PG and adenosine 3',5'-cyclic monophosphate production by osteoblasts perfused in viscous medium (17, 31) and with the mechanism by which endothelial cells are thought to respond to fluid flow (12, 32).

We used a dynamic strain regimen because it has been shown that the stimulus for mechanical adaptation is not the absolute strain but the rate of change of strain. If strain is transduced by fluid flow, we would anticipate that the rate of flow would be proportional to the rate of change of strain (25, 37). Therefore, constant flow rates were used in our experiments to enable us to compare the direct response of cells to strain with the response to the consequence of those strains. It is likely that in vivo normal mechanical usage, which frequently consists of repetitive movements, generates a pulsatile waveform for fluid flow in bone, although there is no information either on the characteristics of the waveform or whether it reverses during cycles to enable this to be reproduced. This makes it difficult to design more appropriate experiments than those we have used. In blood vessels, where flow is pulsatile, the response to continuous vs. pulsatile fluid flow is quantitative rather than qualitative (12).

Because NO and PG production appears to be essential for mechanical responsiveness in vivo, our observation that even relatively large mechanical strains do not cause detectable production in vitro, whereas fluid flow induces both in the same cell populations, suggests that fluid flow is more likely to be the stimulus acting on bone cells in vivo. The mechanisms by which detection of fluid flow by bone cells could be translated into information that allows bone to adapt its structure in a way appropriate for the mechanical environment is unknown. The remodeling of capillary beds by fluid flow during development suggests that information derived from fluid flow can be translated into morphogenetic decisions. Although PG and NO production might both represent signals that directly regulate bone formation and resorption, it is equally possible that they and other signals generated by fluid flow are involved in the processing of mechanically generated information by bone cells. Whatever the role of PG and NO, our experiments suggest that fluid flow is the primary stimulus that causes bone cells to initiate the process whereby the skeleton adapts its structure to meet the challenges of the mechanical environment.

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