Interactive effects of PTH and mechanical stress on nitric oxide and PGE$_2$ production by primary mouse osteoblastic cells

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Bakker, Astrid D., Manon Joldersma, Jenneke Klein-Nulend, and Elisabeth H. Burger. Interactive effects of PTH and mechanical stress on nitric oxide and PGE$_2$ production by primary mouse osteoblastic cells. Am J Physiol Endocrinol Metab 285: E608–E613, 2003. First published May 13, 2003; 10.1152/ajpendo.00501.2002.—Parathyroid hormone (PTH) and mechanical stress both stimulate bone formation but have opposite effects on bone resorption. PTH increased loading-induced bone formation in a rat model, suggesting that there is an interaction of these stimuli, possibly at the cellular level. To investigate whether PTH can modulate mechanotransduction by bone cells, we examined the effect of $10^{-8}$ M human PTH-(1–34) on fluid flow-induced prostaglandin E$_2$ (PGE$_2$) and nitric oxide (NO) production by primary mouse osteoblastic cells in vitro. Mechanical stress applied by means of a pulsating fluid flow (PFF; 0.6 ± 0.3 Pa at 5 Hz) stimulated both NO and PGE$_2$ production twofold. In the absence of stress, PTH also caused a twofold increase in PGE$_2$ production, but NO release was not affected and remained low. Simultaneous application of PFF and PTH nullified the stimulating effect of PFF on NO production, whereas PGE$_2$ production was again stimulated only twofold. Treatment with PTH alone reduced NO synthase (NOS) enzyme activity to undetectable levels. We speculate that PTH prevents stress-induced NO production via the inhibition of NOS, which will also inhibit the NO-mediated upregulation of PGE$_2$ by stress, leaving only the NO-independent PGE$_2$ upregulation by PTH. These results suggest that mechanical loading and PTH interact at the level of mechanotransduction.

parathyroid hormone; prostaglandin E$_2$; nitric oxide synthase

MECHANICAL LOADING, resulting from daily activity, and parathyroid hormone (PTH) are both able to modulate bone remodeling. PTH, being a systemic hormone, stimulates overall bone turnover and increases bone resorption and formation throughout the whole skeleton (28). This is most clearly shown in chemical and histomorphological studies in patients with hyperparathyroidism, in whom both osteoclastic and osteoblastic activity are increased as a result of the elevated PTH levels (6, 34, 44). Mechanical loading, on the other hand, serves to locally enhance osteoblastic bone formation but inhibit local osteoclastic bone resorption (11, 35, 36). PTH and mechanical loading interact at the tissue level, as suggested by several studies in rats, in which administration of PTH increased mechanical stress-induced bone formation (5, 21) and reduced the effect of disuse (21, 40). It is, however, still unclear how mechanical stress and PTH interact at the cellular level.

Both PTH and mechanical loading can modulate the production of local signaling molecules by cells of the osteoblastic lineage. In vitro studies (12, 16, 32, 47) have shown that mechanical stress stimulates the production of signaling molecules such as nitric oxide (NO) and prostaglandin E$_2$ (PGE$_2$) (2a), which are powerful local modulators of osteoclast and osteoblast activity. PGE$_2$ regulates bone formation in several ways, such as by recruitment and stimulation of cells of the osteoblastic lineage (8, 43), and PGE$_2$ as well as NO have been shown to directly inhibit the activity and motility of mature osteoclasts (23, 25). In animal studies, both NO and prostaglandins were crucial for the anabolic effect of mechanical stress (9, 39). PTH is also known to stimulate PGE$_2$ production by bone cells and bone organ cultures (15, 17, 22). PTH does not seem to modulate NO production (30) in bone cells (33), although a stimulatory effect of human (h)PTH-(1–34) on NO production by endothelial cells has been reported (13). In the present study, bone cell cultures from adult mouse long bones were subjected to mechanical stress, hPTH-(1–34), or both. Mechanical stress was applied by subjecting the cells in monolayer to pulsating fluid flow (PFF). This approach is based on the assumption that interstitial fluid flow in the canaliculi of strained bone provides the stimulus for mechanoperception (42). Mechanoperception is considered to be primarily the task of osteocytes, which then send signals to alter the activity of (pre)osteoblasts and/or osteoclasts (3, 4, 37). In vitro, osteoblasts and osteocytes show similar responses to strain, but osteocytes seem to respond stronger (19). Both osteocytes and osteoblasts express the PTH receptor and respond to PTH in a similar manner in vitro (7, 29). In this study, we investigated whether mechanical stress and PTH interact at the

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cellular level in cells of the osteoblastic lineage. Because stress-induced PGE₂ production is dependent on NO synthesis (14, 18), both PGE₂ and NO production were studied as parameters of bone cell responsiveness.

We tested the hypothesis that application of PTH will modify the response of osteoblastic cells to mechanical loading, measured as NO and PGE₂ production.

**MATERIALS AND METHODS**

Isolation and culture of primary mouse bone cells. Mouse long bone cells were obtained from the limbs of adult Swiss albino mice. The long bones were aseptically harvested, the epiphyses were cut off, and bone marrow was flushed out using a syringe and needle. The diaphyses were chopped into small fragments, washed with PBS, and incubated with collagenase II (Sigma, St. Louis, MO) at 2 mg/ml in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO, Paisley, UK) at 37°C in a shaking water bath to remove all adhering cells from the bone chip surfaces. The bone fragments were washed with medium containing 10% fetal calf serum (FCS) and transferred to 25-cm² flasks (Nunc, Roskilde, Denmark).

Bone fragments were cultured in DMEM supplemented with 100 U/ml penicillin (Sigma), 50 µg/ml streptomycin sulfate (GIBCO), 50 µg/ml gentamicin (GIBCO), 1.25 µg/ml fungizone (GIBCO), 100 µg/ml of ascorbate (Merck, Darmstadt, Germany), and 10% FCS. Upon reaching confluence, cells were harvested using 0.25% trypsin and 0.1% EDTA in PBS, plated at 25 × 10⁶ cells/well in six-well culture dishes (Costar, Cambridge, MA), and cultured in 3 ml of medium as described above, until the cell layer reached confluence again. Then the cells were characterized as described below or used for PTH and/or PFF experiments as follows. Cells were trypsinized from the six-well plates (day 0) and seeded onto polystyrene-coated (50 µg/ml; poly-L-lysine hydrobromide, mol wt 15–30 × 10⁴; Sigma) glass slides (2.5 × 6.5 cm) that fitted the parallel-plate flow chamber used for PFF. Cells were plated at 5 × 10⁶ cells/glass slide and were cultured overnight in petri dishes with 12 ml of culture medium as described above. The following day (day 1), culture medium was replaced by DMEM containing 0.2% bovine serum albumin (BSA) and supplemented with antibiotics and ascorbate as before. Similar medium, but supplemented with PTH or vehicle, was used during PTH and/or PFF treatment on day 2.

**Cell characterization.** To test their osteoblastic phenotype, expression of the bone-specific gene cbfa-1 was studied in the cell cultures as well as their responsiveness to vitamin D. cDNA was isolated from the bone-derived cell cultures with TRIzol reagent according to the manufacturer’s instructions. cDNA synthesis was performed using 1 µg of total RNA in 50 µl of reaction mix consisting of the following final concentrations: 1× first-strand buffer (GIBCO), 500 µM dNTPs (GIBCO), 10 U of RNase inhibitor (GIBCO), 8 mM dithiothreitol (GIBCO), 50 U of Superscript RT (GIBCO), and 2 pmol primer p(dT)₁₅ (Boehringer, Mannheim, Mannheim, Germany). For the amplification of the cbfa1 product, 4 µl of cDNA were added to the PCR reaction mixture, consisting of 1× Thermal Ace buffer (Invitrogen, Carlsbad, CA), 0.2 mM dNTP, 6 mM sense primer, 6 mM antisense primer, and 1 U of Thermal Ace polymerase (Invitrogen) in a final volume of 50 µl. The cbfa1 upstream and downstream primer sequences were 5’-AGT CTT CAT TCG CCT CAC AAA C-3’ for the forward primer, and 5’-TCT GAT GCC ATA GTC CCT T-3’ for the reverse primer, respectively. The samples were preheated for 10 min at 95°C, followed by a three-step PCR procedure, consisting of 45 s at 95°C, 15 s at 53°C, and 15 s at 74°C, for 45 cycles. The PCR products were subjected to electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. As a positive control, the cloned osteoblastic mouse cell line MC3T3 was used.

Alternatively, bone cell cultures were incubated for 3 days in the presence or absence of 10⁻⁸ M 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in medium supplemented with 0.2% BSA. After a 24-h incubation, cells were harvested for determination of alkaline phosphatase (ALP) activity and total protein content of the cell layer. ALP activity was determined in the cell lysate by using p-nitrophenyl phosphate (Merck) as a substrate at pH 10.3, according to the method as described by Lowry (20). The absorbance was read at 410 nm with a Dynatech MR7000 microplate reader (Dynatech, Billinghurst, UK). The amount of protein in the cell layer was measured using a BSA protein assay reagent kit (Pierce, Rockford, IL), and the absorbance was read at 570 nm. ALP values were expressed per amount of protein in the cell layer.

To test the presence of endothelial cells in the bone cell cultures, cells were stained for expression of the endothelial cell-specific factor VIII, von Willebrand factor (vWF), and stained by immunofluorescence using a monoclonal vWF antibody, and a red endothelial cell line served as a positive control.

**PTH.** hPTH (1–34) (Sigma) was dissolved in 0.005% HAc buffer containing 0.1% BSA to a final concentration of 10⁻⁸ M (stock solution) and stored at −80°C until further use. For determination of the dose-response relationship as well as the time course of the effect of PTH treatment, 10⁻¹⁰ to 10⁻⁷ M PTH or vehicle alone was added to bone cell cultures for 24 h. Medium samples were collected at 0.5, 1, 2, 3, 6, 12, and 24 h after addition of PTH and assayed for PGE₂ production as described below.

**PFF.** PFF, at a 5-Hz pulse frequency, was generated by pumping 12 ml of culture medium in a pulsatile manner through a parallel-plate flow chamber containing the bone cells, as described previously (2). Mean fluid shear stress was 0.6 Pa, with a pulse amplitude of 0.3 Pa, and the estimated peak stress rate was 8.4 Pa/s. Control cultures were kept under stationary conditions in a petri dish, under similar conditions as the experimental cultures, i.e., at 37°C in a humidified atmosphere of 5% CO₂ in air. To study the interaction of mechanical loading and PTH treatment, 10⁻⁹ M PTH or vehicle was added to the medium before PFF treatment or stationary incubation. Medium was collected after 5 and 30 min of treatment and assayed for PGE₂ content. NO release was only measured after 5 min of treatment.

**PGES and NO.** PGES release in the conditioned medium was measured by an enzyme immunoassay system (Amer sham, Buckinghamshire, UK), using an antibody raised against mouse PGES. Absorbance was read at 450 nm. NO was measured as nitrite (NO₂⁻) accumulation in the conditioned medium with the use of Griess reagent, consisting of 1% sulfanylamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5 M H₃PO₄. Serial dilutions of NaN₁₀₂ in nonconditioned medium were used as standard curve. Absorbance was measured at 540 nm.

**Total protein and DNA.** Total protein and DNA were isolated from the bone cells using TRIzol reagent (GIBCO) according to the manufacturer’s instructions. The amount of protein was determined using a bicinchoninic acid protein assay reagent kit as described above. DNA content was determined by measuring absorbance at 260 nm using an Ultrospec III spectrophotometer (Amersham).

**NOS activity assay.** Cells were incubated for 45 min with culture medium containing 10⁻⁹ M PTH or vehicle, washed
with PBS, and collected in 200 μl of homogenization buffer. NOS activity was measured using an NOS-Detect Assay Kit (Stratagene), based on the conversion of L-arginine to L-citrulline by NOS enzyme, following the manufacturer’s instructions. Briefly, radioactive L-arginine was added to the cells as a substrate for NOS enzyme. After incubation, reactions were stopped with a buffer containing EDTA, which chelates the calcium required by NOS. Sample reactions were applied to spin cups containing equilibrated resin, which binds L-arginine but not the radioactive L-citrulline, being ionically neutral at pH 5.5. Radioactivity was measured in the eluate with a β-scintillation counter.

Statistical analysis. Mean values of data obtained from duplicate cultures from at least three separate experiments were calculated and analyzed using the Wilcoxon signed rank test or univariate analysis of variance (post hoc: Bonferroni). Differences were considered significant if P was <0.05.

RESULTS

Cells started to grow out of the collagenase-stripped bone chips along the bottom of the culture flask after 3–5 days of culture and reached confluence within 13–17 days, when they were passaged. Expression levels of cbfa1 mRNA by these bone cell cultures were comparable to those of the MC3T3-E1 mouse osteoblastic cell line (Fig. 1). Treatment of the passaged cells with 10⁻⁸ M 1,25(OH)₂D₃ resulted in a 1.9-fold (range 1.4–2.7) mean increase in ALP activity. Together, these findings demonstrate the osteoblastic characteristic of the cell population. Immunostaining for the endothelial cell-specific vWF showed that all rodent endothelial cells stained positive, whereas none of the primary mouse bone cell cultures expressed vWF (data not shown). PFF treatment, in the presence or absence of PTH, did not affect the total amount of protein or DNA, demonstrating that no cells were lost as a result of the treatment with fluid flow (data not shown).

To study the dose dependence of PTH-induced PGE₂ production, cells were incubated for 24 h with a range of PTH concentrations. We found that 10⁻⁸ and 10⁻⁹ M PTH significantly stimulated PGE₂ production, but this effect was lost at lower or higher PTH concentrations (Fig. 2A). Because a maximal 1.6-fold stimulation was found with 10⁻⁹ M PTH (Fig. 2A), this concentration was used for all further experiments. The time curve of PTH-stimulated PGE₂ production showed that stimulation of PGE₂ production occurred primarily during the first 6 h of the incubation period, with the highest production rate during the first 30 min (Fig. 2B).

The effects of PTH and PFF were first studied separately by culturing cells either under static conditions in the presence of 10⁻⁹ M PTH or vehicle or by subjecting them to PFF. In the latter case, PTH vehicle was added to the medium to allow comparison with subsequent combination treatment. As expected from earlier studies, PFF treatment rapidly increased NO production from 61.7 to 110.3 nmol/mg protein (Fig. 3A). PFF stimulated PGE₂ production from 29.9 to 53.3 ng/mg protein (Fig. 3B). PTH treatment alone did not affect NO release (Fig. 3A) but significantly stimulated PGE₂ production (Fig. 3B) to 80.7 ng/mg protein.

![Fig. 1. Cbfa1 mRNA expression by three separate primary mouse bone cell cultures and the osteoblastic cell line MC3T3. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. PMBC, primary mouse bone cell culture.](image)

![Fig. 2. Effect of parathyroid hormone (PTH) on prostaglandin E₂ (PGE₂) production by mouse bone cells. A: dose-response curve of PGE₂ production in reaction to 24-h incubation with PTH. Values, obtained from 3 separate experiments, are expressed as means ± SE of PTH treated over control (T/C) ratios. Dashed line, T/C = 1 (no effect). B: cumulative PGE₂ production during 24-h treatment with 10⁻⁹ M PTH or vehicle. Values, obtained from 3 separate experiments, are expressed as means ± SE. *Significant effect of PTH; #significantly different from 10⁻⁸ M PTH, P < 0.05. Treatment with PTH stimulated PGE₂ production in a dose-dependent and biphasic manner, with maximal stimulation after 6 h of incubation with 10⁻⁹ M PTH.](image)

![Fig. 3. Effect of PTH or pulsating fluid flow (PFF) on nitric oxide (NO) and PGE₂ production by mouse bone cells. A: NO production by bone cells treated for 5 min with PFF or 10⁻⁹ M PTH. B: PGE₂ production by bone cells treated for 30 min with PFF or 10⁻⁹ M PTH. Values were derived from 5 (A) or 7 (B) separate experiments and are expressed as means ± SE. Stat, stationary incubation without flow; vehicle, PTH vehicle. *Significant effect of PTH or PFF, P < 0.05. PFF significantly stimulated both NO and PGE₂ production by the cells, whereas 10⁻⁹ M PTH significantly stimulated PGE₂ production but did not affect on NO production.](image)
Cell cultures were then subjected to a combined treatment of PTH and PFF to study the interaction between PTH and mechanical stimulation. Surprisingly, all treatments, i.e., PTH alone, PFF alone, and PTH combined with PFF, resulted in an approximately twofold increase of PGE$_2$ production, indicating that the simultaneous treatment with PFF and PTH did not have an additive or synergistic effect (Fig. 4B). The effect of PTH on PFF-induced NO production was even more striking, as the 1.9-fold stimulation of NO production by PFF was completely abolished in the presence of PTH (Fig. 4A).

In cells of the osteoblastic lineage, NO production induced by shear stress results from the activation of endothelial cell NOS (ecNOS) (16, 18, 47). Therefore, we tested whether PTH treatment modulated NOS activity. Treatment of the bone cell cultures with 10$^{-9}$ M PTH dramatically reduced NOS activity to values below the detection limit of the assay (Fig. 5).

**DISCUSSION**

The purpose of the present study was to investigate whether PTH can interfere in the signaling pathways used by osteoblastic cells during transduction of mechanical signals. To this aim, we studied the effect of hPTH-(1–34) treatment on fluid flow-induced NO and PGE$_2$ production by mouse primary bone cell cultures. We hypothesized that application of PTH would modify the response of osteoblastic cells to mechanical loading, measured as NO and PGE$_2$ production. In line with earlier studies (2, 12, 18), we found that fluid shear stress rapidly stimulated NO and PGE$_2$ production. Treatment with PTH in the absence of shear stress stimulated PGE$_2$ production but not NO production, as was also found by others (15, 17, 22, 30, 33). When PTH was added shortly (<1 min) before fluid shear stress was applied, it completely blocked the shear stress-induced NO production. We therefore tested whether PTH modulates NOS enzyme activity and found that this activity is severely inhibited by 10$^{-9}$ M PTH. ecNOS, the isoform of NOS enzyme that is sensitive to shear stress, is abundantly present in bone cells (10, 16, 47). The present results therefore suggest that the stimulating effect of shear stress on NO production was prevented by an inhibiting effect of PTH on ecNOS, which blocked the PFF-induced NO production.

To our knowledge, there is no literature describing any actions of PTH on NOS in osteoblastic cells, but there are indications that PTH does affect NOS in cells of the vascular wall. PTH is known to relax vascular smooth muscle and to acutely lower blood pressure in rats when administered in high concentrations (26), but whether this action of PTH involves endothelium-derived NO is unresolved. Evidence for a possible inhibiting effect of PTH on NO was provided by a more recent study, in which rats with chronic renal failure were parathyroidectomized, resulting in lowered blood pressure, increased urinary excretion of stable NO metabolites, enhanced vascular NOS activity, and ecNOS and inducible NOS expression in the thoracic aorta and remnant kidney (41). Of course, one must be cautious when interpreting these results, because many variables are affected by chronic renal failure as well as by parathyroidectomy, and the observed effect on NOS does not necessarily imply a direct action of PTH on NOS enzyme activity. Using a highly sensitive porphyrinic microsensor, Kalinowski et al. (13) found that administration of hPTH-(1–34) stimulated NO release from a single endothelial cell in culture. The stimulation of NO production by PTH was through the PTH/PTH-related protein (PTHrP) receptors and mediated via the calcium/calmodulin pathway (13). We found no such stimulation of NO production by PTH in our primary bone cell cultures when PTH was administered in the absence of shear stress. This is in accord with results of others, who observed no effect of PTH on
NO release by primary human osteoblastic cells, bone cells derived from neonatal mouse calvariae, or by cells from the mouse osteoblast cell line MC3T3 (30, 33). This difference in NO response to PTH between cultured bone cells and endothelial cells is remarkable and suggests that NO-related signaling pathways may differ among endothelial cells and bone cells.

NO is known to activate cyclooxygenase (COX) enzyme activity in osteoblastic MC3T3-E1 cells (14), and we showed earlier (18) that inhibition of NOS enzyme in mouse primary osteoblastic cells leads to a severe decrease of stress-induced PGE2 production. This suggests that the effect of mechanical loading on PGE2 production is dependent on NOS activity, and inhibition of NOS enzyme by PTH could thus prevent activation of prostaglandin synthesis by shear stress. The stimulation of PGE2 production by shear stress was therefore probably decreased during combined treatment with shear stress and PTH. However, PGE2 production could still have been stimulated by PTH through a NOS-independent pathway. We have recently found that PFF-induced prostaglandin production is totally dependent on activity of the inducible form of COX, COX-2 (2a). NO-dependent PGE2 production by COX-2 might thus be inhibited during combined treatment with shear stress and PTH, whereas prostaglandin production by COX-1 persists unaffected. Binding of PTH to its receptor activates the cAMP-protein kinase A and -protein kinase C pathways, as well as calcium-signaling pathways (1), and all of these pathways have been shown to be involved in COX activation and/or COX mRNA induction (27, 31, 32, 38). Thus we can explain how combined treatment with PFF and PTH lead to PGE2 levels that were similar to those found after treatment with PTH alone.

Our in vitro results do not explain how PTH and mechanical stress have synergistic effects on bone formation, as was shown in animal studies (5, 21); rather, they seem to relate to the opposite effects of stress and PTH on bone resorption. Several studies in animals have shown unequivocally that mechanical stress inhibits bone resorption (11, 35, 36). PTH, on the other hand, stimulates bone resorption as well as the subsequent formation of bone, leading to a generally enhanced remodeling of bone, as demonstrated in patients with hyperparathyroidism (6, 34, 44). Thus mechanical stress and PTH act in opposite directions in the regulation of bone resorption. In this respect, it is important to note that NO is an inhibitor of bone resorption. Administered to osteoclasts in vitro, NO causes the retraction of the osteoclasts from their resorbing substrate and inhibition of resorbing activity (23, 24). In vivo, administration of NO has been reported to inhibit bone loss in animal studies as well as in patients (45, 46). We have therefore proposed that stress-induced NO production by osteoblastic cells serves to inhibit osteoclastic bone resorption (4, 37). Inhibition by PTH of stress-induced NO production could then lead to enhanced resorption, in line with the clinical observation of enhanced resorption in patients with hyperparathyroidism. The opposite effects of PTH and mechanical loading on NO production, as found in the present study, might thus relate to their opposite effects on bone resorption. This subject needs further study, preferably using an in vivo approach.

In sum, the present study suggests that PTH can directly interfere in the transduction of mechanical signals by osteoblastic cells by inhibiting stress-induced NO production. As the stress-induced rise in NO production subsequently mediates an increase in PGE2 production, the inhibitory effect of PTH on NO also leads to lack of an additive effect of PTH and stress on PGE2 production. We speculate that the opposite effects of stress and PTH on NO production, as found in this in vitro study, may relate to their inhibiting vs. stimulating effect on bone resorption in vivo.

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## DISCLOSURES

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