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Nitric Oxide Response to Shear Stress by Human Bone Cell Cultures Is Endothelial Nitric Oxide Synthase Dependent

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Bone cells, in particular osteocytes, are extremely sensitive to shear stress, a phenomenon that may be related to mechanical adaptation of bone. In this study we examined whether human primary bone cells produce NO in response to fluid shear stress and established by RT/PCR which NOS isoforms were expressed before and after application of shear stress. One hour pulsating fluid flow (PFF; 0.7 ± 0.02 Pa, 5 Hz) caused a rapid (within 5 min) 2 to 4-fold increase in NO production. NO release was only transiently increased during the first 15 min of exposure to PFF, and remained at control levels during a 1-24 hr postincubation period. In both control and PFF-treated cells, mRNA was easily detected for ecNOS, but not nNOS, and only minimal amounts iNOS were found. mRNA levels for ecNOS increased 2-fold at 1 hr after 1 hr PFF treatment. These results suggest that the rapid production of NO by human bone cells in response to fluid flow results from activation of ecNOS. PFF also leads to an increase in ecNOS mRNA which is likely related to the shear stress responsive element in the promoter of ecNOS.

Key Words: **fluid shear stress; mechanical stress; nitric oxide; ecNOS; nitric oxide synthase; bone cells.**

Living bone is able to adapt its mass and internal architecture to the prevailing loads acting upon it (1-3). However the cell biological explanation of Wolff's Law is still far from complete. Several studies agree that canalicular flow of interstitial fluid, resulting from mechanical strain, could act as the physical stimulus that informs the osteocytes about the magnitude of local strains (4-6). In this concept osteocytes are the bone mechanosensors which transform the physical stimuli derived from mechanical loading into cellular signals. Theoretical (7) and experimental studies (8,9) have shown that fluid flow does occur in the osteocyte canaliculi as a result of physiological strain, and experimental evidence shows that fluid shear stress of the order of 0.5 Pa and more stimulates cAMP and prostaglandin production as well as prostaglandin G/H synthase-2 mRNA expression by isolated calvarial bone cells (4,6,10). These studies suggest that, rather than direct straining of bone cells, flow of interstitial fluid through osteocyte canaliculi provides the stress-derived mechanical signal for adaptive bone modeling. Osteoblastic bone cell cultures, although not expressing the typical morphology of osteocytes, are quite sensitive to fluid shear stress, probably because of their close relationship with osteocytes (6,10).

We and others have shown that avian osteocytes and avian and rodent osteoblasts produce nitric oxide (NO) when exposed to fluid shear stress in vitro, and this is the earliest cellular response to stress so far reported (5,11). In vivo studies have indicated that NO mediates the adaptive response of bone tissue to mechanical loading (12,13). NO is a short-lived free radical, which is generated from L-arginine by a family of NO synthase (NOS) enzymes (14,15). Two isoforms, endothelial (ecNOS) and neuronal NOS (nNOS), are constitutively expressed and produce

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Abbreviations; PFF, pulsating fluid flow; NO, nitric oxide; NOS, nitric oxide synthase; ecNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ALP, alkaline phosphatase; PNP, p-nitrophenyl phosphate; vWF, Von Willebrand factor VIII; VIP, very intense purple; RT/PCR, reverse transcription polymerase chain reaction.

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small quantities of NO in response to changes in intracellular calcium concentrations. The inducible form of NOS (iNOS) in contrast, can be expressed by a wide variety of mammalian cells when they are exposed to bacterial endotoxin or inflammatory cytokines (16). iNOS is calcium-independent and, once induced, produces large amounts of NO, which have been implicated in the control of host defense mechanisms and immunoregulation (17,18). Helfrich et al. (19) have shown that all three isoforms are expressed in rat bone and also demonstrated evidence of differential regulation and cell-specific expression in bone and bone cell cultures. Fox and Chow (20) have demonstrated that the predominant isoform in human bone is ecNOS, and is highly expressed in osteocytes and lining cells.

The recently reported increase in NO release in avian and rodent bone cells as a result of mechanical stress (5,11) shows kinetics that are consistent with activation of a constitutive form of NOS, given the promptness of the response, but it has not been established which NOS isoform is responsible. In this study we examined whether human primary bone cells also produce NO in response to fluid shear stress, and established by RT/PCR which NOS isoforms were expressed before and after application of shear stress.

METHODS

Donors

Transiliac bone biopsies were obtained from 18 donors between 7 and 90 years of age, with a mean age of 29 ± 6 years. They were all without metabolic bone disease and entered the hospital for maxillofacial surgery (cleft palate or mandibular reconstruction using iliac crest bone) or orthopaedic surgery (elective total hip replacement). The protocols were approved by the ethical review board of the Academic Hospital Vrije Universiteit and all donors gave informed consent.

Isolation and Culture of Cells

Human primary bone cell cultures were established according to earlier described methods (21). Briefly, the trabecular bone specimens were placed in cold sterile phosphate buffered saline (PBS) and were dissected within one hour after removal. Bone specimens were minced into small fragments, washed with PBS, and incubated with 2 mg/ml collagenase (type II, Worthington, NJ) for 2 hr at 37°C in a shaking water bath. The collagenase-treated bone fragments were washed once with medium containing 10% fetal bovine serum (FBS; Gibco, Paisley, UK) to inhibit collagenase activity, and transferred to 25 cm^2 or 75 cm^2 flasks (Nunc, Roskilde, Denmark), depending on the amount of bone tissue obtained. Roughly, 5-10 mg of bone fragments were added per cm^2 flask surface. The bone fragments were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 100 U/ml of penicillin (Sigma, St. Louis, MO), 50 μg/ml of streptomycin sulphate (Sigma), 50 μg/ml of gentamycin (Gibco), 1.25 μg/ml of fungizone (Gibco), 100 μg/ml of ascorbate (Merck, Darmstadt, Germany), and 10% FBS. Culture medium was replaced 3 times per week. Bone cells started to migrate from the bone chips after 4 to 14 days. When the cell monolayer growing from the bone fragments reached confluence, after 2-5 weeks, cells were trypsinized using 0.05% trypsin (Difco Laboratories, Detroit, MI) and 0.02% EDTA (Sigma) in PBS for 5 to 10 min, and plated at 25 x 10^3 cells per well in 6-well culture dishes (Costar, Cambridge, MA) containing 3 ml DMEM with 10% FBS. Cells were grown until subconfluency, when they were challenged with PFF or 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$).

TABLE 1

PCR Primer Sequences (5′-3′) for Human NOS

iNOS forward:	CTC TAY RTT TGY GGI GAT GTG CG
iNOS reverse:	GGA TAT CAC TTT CCT CCA TCT CCC CA
product size 334 bp	
ecNOS forward:	AAG CCG CAT ACG CAC CCA GAG
ecNOS reverse:	TGG GGT ACC GCT GCT GGG AGG
product size 346 bp	
nNOS forward:	AAA GCG ACC ATC CTC TAT GCC
nNOS reverse:	CAT TTC CAT CAA AGC ACA GCC
product size 225 bp	
β-actin forward:	ATC GTG GGG CGC CCC AGG CAC C
β-actin reverse:	CTC CTT AAT GTC ACG CAC GAT TTC
product size 541 bp	

Note. Ambiguity codes: R = A/G, Y = C/T, I = inosine.

Characterization of Bone Cell Cultures

To confirm their osteoblastic phenotype, cell cultures of 17 donors were incubated for 3 days in the presence or absence of 10^{-8} M $1,25(OH)_2D_3$ as described (21), followed by measurement of osteocalcin production in the conditioned medium by radioimmunoassay (Incstar, Stillwater, MN) using an antibody raised against bovine osteocalcin. The detection limit of this assay is 0.2 ng/ml. All cultures responded to $1,25(OH)_2D_3$ treatment with an 8-fold increase in osteocalcin production (unstimulated 44.9 ± 14.8 ng/mg protein, mean ± SEM; $1,25(OH)_2D_3$-stimulated 367.3 ± 118.3 ng/mg protein, $P < 0.01$). In addition, alkaline phosphatase (ALP) activity was determined in the cell lysate using p-nitrophenyl phosphate (PNP, Merck) as a substrate at pH 10.3, according to the method as described by Lowry (22). Treatment with $1,25(OH)_2D_3$ increased the ALP activity 4-fold from 2.9 ± 0.9 to 11.7 ± 3.1 nmole PNP/h/μg protein (mean ± SEM, $P < 0.01$), clearly demonstrating a differentiating effect of $1,25(OH)_2D_3$.

Finally, to exclude the presence of endothelial cells in the bone cell cultures, monolayers were stained for expression of the endothelial cell-specific factor VIII, Von Willebrand factor (vWF), by immunofluorescence using a monoclonal vWF antibody (DAKO Corporation, Carpinteria, CA, USA). Cultures of primary human umbilical cord-derived endothelial cells served as positive control for the human bone cell cultures.

Immunocytochemistry

Bone cell cultures were stained before and after application of PFF with antibodies to the NOS isoforms. The iNOS antibody was raised to rat iNOS (gift of Dr. Ohshima, Lyon, France; 23) and known to cross react with human iNOS (17); the ecNOS antibody raised to bovine ecNOS, but cross reactive with human ecNOS, was obtained from Calbiochem (Beeston, UK), and nNOS antibody was obtained from Santa Cruz Antibodies (Insight Biotechnologies, Wiltshire, UK). Cells were fixed in acetone for 10 minutes and incubated with the antibodies for 30 minutes, followed by detection using an biotin/avidin/peroxidase system and very intense purple (VIP) as the substrate (Vector Laboratories, Peterborough, UK).

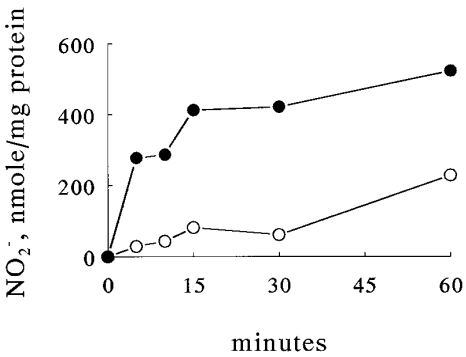


FIG 1. Time-course of the effect of pulsating fluid flow (PFF) on NO release by human bone cells. Cells were incubated for 1 hr with or without PFF, and the conditioned medium assayed for NO, measured as NO_2^-. Values are means of duplicate cell cultures from one donor, which are representative results from 3 separate studies on 3 donors. Note that after 15 minutes no further increase occurred between stressed and control cell cultures.

Pulsating Fluid Flow (PFF)

PFF (5 Hz, 0.7 ± 0.03 Pa, 12.2 Pa/sec) was generated using a flow apparatus containing a parallel-plate flow chamber as described earlier (4-6). For PFF experiments cells were trypsinized and plated onto polylysine-coated (50 μg/ml; poly-l-lysine hydrobromide, mw 15-30x10^4; Sigma) glass slides, which served as the bottom of the flow chamber. Cells were plated at 5 x 10^5 cells per glass slide (size 2.5 x 6.5 cm) and preincubated overnight in DMEM with 10% FBS, resulting in a subconfluent monolayer. Then the medium was changed to DMEM supplemented with 2% FBS, antibiotics, and 100 μg/ml of ascorbate, and the cells were incubated for 1 hr in the absence (static control) or presence of PFF. Subsequently, the glass slide with the cells was removed from the flow apparatus, a culture well was created around the cells by securing a rectangular silicone rubber ring (Dow Corning Corporation, Midland, MI) on the glass slide, 1 ml fresh culture medium was added, and the cells postincubated for 24 hr in the absence of PFF (post-PFF). Static control cultures were cultured in 13 ml static medium during the first "PFF" hour and in 1 ml medium thereafter. Medium samples were taken at 5, 10, 15, 30, and 60 minutes, and after 1 and 24 hr post-incubation. The conditioned medium was assayed for NO_2^- concentrations as described below.

Nitric Oxide

NO release was measured as nitrite (NO_2^-) accumulation in the conditioned medium using Griess reagent (24) as described earlier (5).

Reverse Transcription Polymerase Chain Reaction

In 7 cases total RNA was extracted from non-stressed control cultures, cells subjected to 1 hr PFF, and cells cultured for 1 hr after PFF, by using TRIzol Reagent (Gibco). Briefly, cells were harvested with a solution containing 4 M guanidinium isothiocyanate, and cellular RNA was precipitated with isopropanol (Sigma). After centrifugation, the pellet was washed in 75% ethanol. The RNA pellet was air dried, redissolved in sterile, double-distilled water (ddH_2O), heated at 60°C for 10 minutes, and stored at −80°C until use.

cDNA from each sample was prepared from total RNA using Moloney Murine Leukaemia Virus reverse transcriptase (Superscript, Gibco) and oligo-dT_{13} as primer, heat inactivated at 95°C for 5 minutes, and the cDNA resuspended in 100 μl of ddH_2O, and stored at −20°C prior to use in the polymerase chain reaction (PCR). The PCR reactions were carried out using Taq polymerase (Promega, Madison, WI) according to the manufacturer's instructions with each primer at a final concentration of 1 μM. PCR amplification was performed using specific primers for ecNOS, nNOS and inducible NOS (iNOS) (Table 1). Beta-actin was used as an internal control for efficiency of the extraction, reverse transcription, and PCR steps. The thermal cycling protocol for β-actin, iNOS, and ecNOS was 94°C for 50 s, 65°C for 60 s, and 72°C for 90 s, for 35 cycles of amplification. During the first cycle, the 94°C step was extended to 4 minutes, and on the final cycle the 72°C step was extended to 5 minutes. For nNOS amplification the annealing temperature was 50°C. PCR products were separated on 1.5% agarose gels. Specificity of the primers was confirmed by amplifying products from tissues known to express the specific isoforms of NOS (nNOS from brain, iNOS from cytokine-stimulated osteoblasts, and ecNOS from aorta).

Quantitation of PCR Products

The linear range of PCR amplification products was established by gradually increasing the cycle number. At a suitable point within the linear range the optical density of the products obtained for ecNOS and β-actin was measured and the ratio ecNOS/β-actin compared for the 3 groups (non-stressed controls, cells subjected to 1 hr PFF, and cells cultured for 1 hr after PFF). Optical densities were calculated using Herolab Easywin software (Scotlab, Coatbridge, UK).

Protein

After 24 hr post-PFF the protein content of the cell layer was measured using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) (25).

Statistics

Data are expressed as mean ± SEM. Data at 24 hr post-PFF represent the cumulative production during the whole post-PFF period. The effects of treatment with $1,25(OH)_2D_3$, and the effect of treatment with pulsatile fluid flow (PFF) on ecNOS mRNA expression were analysed using paired two-tailed student's t-test. The effects of treatment with PFF on NO release were analyzed using Wilcoxon's signed-rank test, because the data did not meet the requirements for normal distribution. Differences were considered significant at a p value < 0.05.

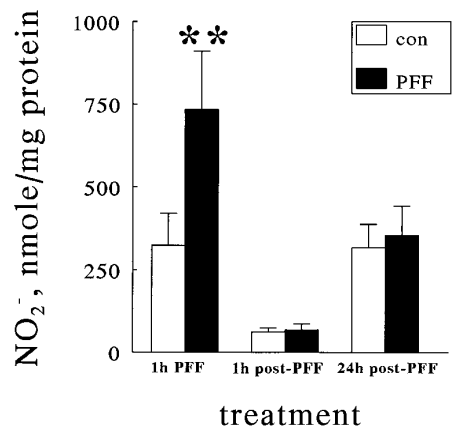


FIG. 2. Effect of pulsating fluid flow (PFF) on NO release by human bone cells. Cells were incubated for 1 hr with or without PFF, and post-incubated for 1 to 24 hr after PFF treatment. The conditioned medium was assayed for NO, measured as NO_2^-. Values are means ± SEM of duplicate cell cultures from 10 donors at 1 h PFF, and from 18 donors for post-PFF incubation. **Significant effect of PFF, $P < 0.01$.

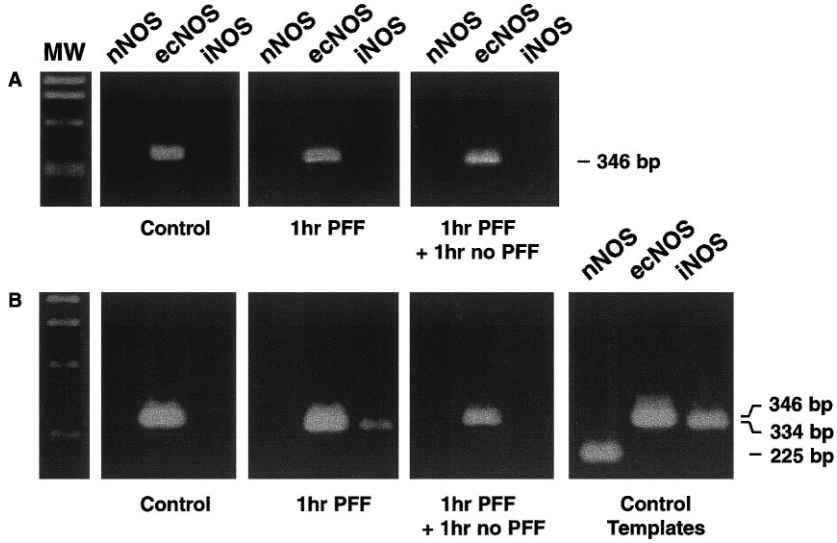


FIG. 3. Detection of NOS isoform mRNA expression in human bone cells cultured with or without pulsating fluid flow (PFF). Total RNA was extracted from non-stressed controls, cells subjected to 1 hr PFF, and cells cultured for 1 hr after PFF. RNA was reverse transcribed and cDNA subjected to PCR amplification using specific primers for ecNOS, nNOS and iNOS. The top row (A) shows that only ecNOS product is visible after 40 rounds of PCR amplification. After another 40 rounds of amplification (bottom row, B), using 1 μl of a 1/100 dilution of the first PCR as template, a faint band for iNOS was seen in approximately 30% of the samples, irrespective of treatment. nNOS mRNA was undetectable in any sample.

RESULTS

Cells became visible as outgrowth of the collagenase-stripped bone chips after 1 or 2 weeks in culture. They formed a subconfluent layer within 2 to 3 weeks, when they were passaged. The speed of cell outgrowth (measured as time of first visible cell outgrowth, time of monolayer subconfluency) was somewhat faster in the younger donors, below 44 years of age, than in the older donors (data not shown), but in all cases the cells responded to 10^{-8} M $1,25(OH)_2D_3$ with increased ALP activity and osteocalcin release, demonstrating characteristics of mature osteoblasts.

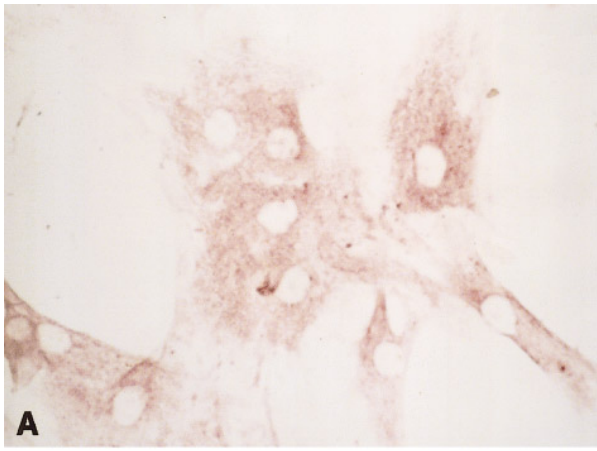
Application of mechanical stress by PFF for 1 hr did not result in permanent changes in cell shape or alignment of the cells in the direction of the flow, and did not affect cell viability as determined by trypan blue exclusion (data not shown). The amount of protein per cell culture was also not affected when measured 24 hr after PFF treatment (static controls, 85 $\pm$ 11 μg; PFF-treated, 82 $\pm$ 8 μg, mean $\pm$ SEM).

Mechanical stimulation by PFF resulted rapidly in increased NO production (Figure 1). Maximal stimulation values were reached within 5 minutes after onset of flow, indicating the presence of constitutive NOS activity. The stimulatory effect was transient and disappeared after 10 minutes because there was a parallel increase in both control and fluid flow-treated cultures (Figure 1).

All human primary bone cell cultures released detectable amounts of NO into the medium under control conditions, 1 hour after changing the culture medium (mean $\pm$ SEM, 324 $\pm$ 95 nmole/mg protein) (Figure 2). PFF caused a rapid increase in NO production with levels after 1 hour that were 2-4 fold higher (mean $\pm$ SEM, 734 $\pm$ 177 nmole/mg protein) than in controls. NO release was only increased during exposure to PFF, but not after removal of PFF, as shown by the return to control levels during 1 to 24 hours of postculture (Figure 2).

We then investigated the effect of 1 hour PFF on the mRNA levels of NOS isoforms in human bone cells (Figure 3). Messenger-RNA for ecNOS was easily detected in control and PFF-treated cells, whereas only minimal amounts of iNOS mRNA were present in both conditions. mRNA for nNOS could not be detected in control nor PFF-treated cells (Figure 3).

Confirming these results, we were unable to detect nNOS and iNOS protein in primary bone cell cultures by immunocytochemistry, whereas ecNOS protein was easily detected (Figure 4a). Application of PFF did not produce detectable changes in NOS isoform protein expression. To confirm that ecNOS was indeed expressed by human bone cells, and not by endothelial cells contaminating the bone cell cultures, we tested the cultures for expression of von Willebrand factor VIII (vWF), an established endothelial cell-specific characteristic. Cultures of human umbilical cord-derived endothelial cells served as positive control (Figure 4c). Immunostaining for vWF revealed that vWF was abundantly expressed in the umbilical cord cell cultures, but was not seen in the human



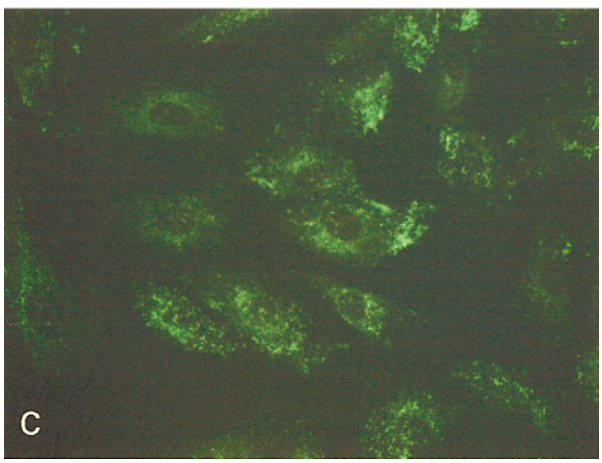


FIG. 4. Expression of high levels of ecNOS protein in human primary bone cells (A), but no expression of von Willebrand factor VIII (B), and expression of high levels of von Willebrand factor VIII in cultures of primary human endothelial cells (C). In primary human bone cells high levels of ecNOS protein were expressed (A), whereas iNOS and nNOS protein were undetectable (not shown). No difference was seen in expression levels for NOS isoforms after application of PFF (not shown). Expression of von Willebrand factor VIII was only visible in cultures of primary human endothelial cells (C), but not in cultures of primary bone cells (B) as shown by immunofluorescence using a monoclonal von Willebrand factor VIII antibody. Magnification: x 400.

bone cell cultures (Figure 4b). These observations confirm that ecNOS mRNA expression and upregulation by PFF indeed occurred in bone cells, and that the PCR amplification ecNOS product was not caused by endothelial cells contaminating the bone cell cultures (Figure 4).

Semi-quantitative PCR showed that mRNA levels for ecNOS increased approximately 2-fold after PFF (Figure 5a,b). No difference in ecNOS mRNA levels was observed between control and 1 hour PFF groups (Figure 5a,b).

DISCUSSION

Mounting evidence suggests that in loaded bone the flow of interstitial fluid through osteocyte canaliculi, and more specifically the resulting shear stress acting on the bone cell membrane, provides the strain-derived signal in bone cell mechanotransduction (4-6,9,26,27). The present study shows in primary human bone cell cultures, that fluid flow rapidly enhances the release of NO, and that ecNOS is the likely isoenzyme involved in this response.

ecNOS, the constitutive NOS isoform that co-purifies with the cell membrane fraction, was originally isolated from endothelial cells (28). In the vasculature ecNOS is involved in the production of NO in response to changes in blood flow (29). It has been shown that the structure and internal diameter of arteries are intimately linked to the hemodynamic forces to which they are subjected (30). The flow of blood, a viscous fluid, results in shear stress, the tractive force per unit area, which acts on the surface of the inner wall of the blood vessel. Fluid shear stress promotes the release of NO from the endothelium (31,32). NO derived from endothelial cells regulates the tone of the underlying vascular smooth muscle, and hence contributes to the control of regional blood flow (15). Shear stress-stimulated NO formation by endothelial cells is associated with an enhanced expres-sion of ecNOS (33). ecNOS activity in endothelial cells is predominantly associated with the plasma membrane (33). The localization of the enzyme in the plasma membrane may be related to the transduction mechanism by which physical forces such as shear stress elicit the formation of NO, for instance by rendering the enzyme susceptible to activation by a shear stress-induced change in the fluidity of the membrane (34).

In bone, recent in vivo studies indicate that NO is involved in mechanically induced bone formation (12,13). The present study suggests that the mechanically-induced formation of NO results from activation of ecNOS in bone cells. It seems remarkable that two so widely different biological systems as the vascular system and bone would use a similar mechanism for the detection of mechanical signals, as part

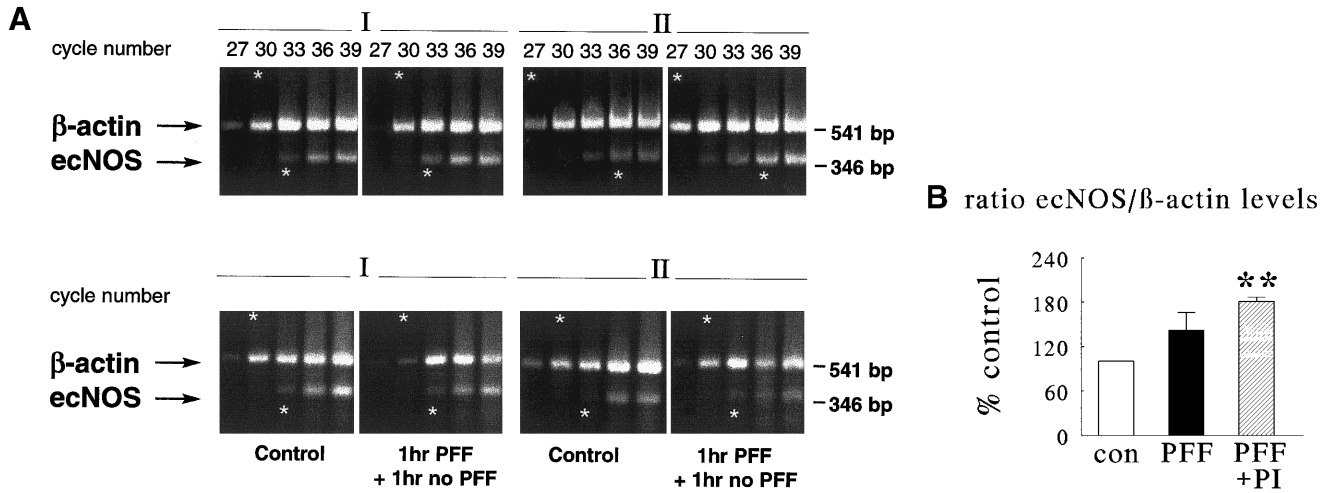


FIG. 5. Semi-quantitative PCR of ecNOS mRNA expression in human bone cells cultured with or without pulsating fluid flow (PFF). Total RNA was extracted from non-stressed controls, cells subjected to 1 hr PFF, and cells cultured for 1 hr after PFF. RNA was reverse transcribed and cDNA subjected to PCR amplification using specific primers for ecNOS. A) Example of semi-quantitative PCR reactions. Flow experiments were performed in duplicate (panel I and panel II are duplicates) with cells obtained from the same donor (the experiments were repeated three times with cells from different donors, see Figure 5b). The appropriate point within the linear range of product amplification was established for each pair of experimental/control conditions, and is indicated by asterisks. Optical densities of the products obtained for ecNOS and β-actin was measured and calculated using Herolab Easywin software. B) ecNOS mRNA levels after PFF. The graph illustrates the ratio of ecNOS to β-actin levels expressed as percent of control increase (mean $\pm$ SEM) in 6 experiments with cells from 3 different donors. PFF + PI, 1 hr PFF + 1 hr postincubation without PFF. **Significant effect of PFF, $P < 0.006$.

of a process of functional adaptation. The kinetics of this adaptive response are widely different in the two systems, that in bone taking at least some 10 days (35), while a change of arterial diameter may only take minutes (15). Still the very first steps of stress detection and signal transduction seem to be comparable. We speculate that the mechanism of NO upregulation in bone cells, like endothelial cells, involves increases in intracellular calcium and opening of cation channels (36), as gadolinium and nifedipine-sensitive cation channels were shown to mediate the early response of osteoblasts to applied strain, including the NO response (37).

We observed only a modest degree of stimulation of ecNOS expression, and only at one hour postincubation after 1 hour treatment with flow, or 2 hours after flow treatment was started. As NO release did not continue after flow treatment was withdrawn, the enhanced expression of ecNOS does not seem involved in further amplification of the stress signal as was suggested for PGHS-2 (6). Rather, enhanced ecNOS expression after loading might sensitize the cell to repeated bouts of mechanical loading. In vivo, such a cellular response might be part of the effect of training, where repeated bouts of loading have more effect than an isolated loading event. In vascular tissue it has been shown that even a modest degree of regulation of NO synthase may have important physiological or pathophysiological implications (38). Within a narrow range of NO concentrations (2 to 3 fold), large changes of vascular tone may occur as a result of vascular relaxation (15). Therefore, it is likely that also in bone small changes in ecNOS expression may have important effects on the responsiveness of the tissue to mechanical loads. The data presented in this paper support the notion that the ecNOS gene is constitutively expressed in human bone cells, and that there are elements in the promoter which may confer responsiveness to mechanical influences. The degree of regulation of the ecNOS by this factor may be modest, but nevertheless physiologically important.

ACKNOWLEDGMENTS

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