

## Additive effects of estrogen and mechanical stress on nitric oxide and prostaglandin E<sub>2</sub> production by bone cells from osteoporotic donors

A.D. Bakker · J. Klein-Nulend · E. Tanck · G.H. Albers  
P. Lips · E.H. Burger

Received: 11 February 2004 / Accepted: 29 September 2004 / Published online: 16 November 2004  
© International Osteoporosis Foundation and National Osteoporosis Foundation 2004

**Abstract** Mechanical loading is thought to provoke a cellular response via loading-induced flow of interstitial fluid through the lacuno-canalicular network of osteocytes. This response supposedly leads to an adaptation of local bone mass and architecture. It has been suggested that loss of estrogen during menopause alters the sensitivity of bone tissue to mechanical load, thereby contributing to the rapid loss of bone. The present study aimed to determine whether estrogen modulates the mechanoresponsiveness of bone cells from osteoporotic women. Bone cell cultures from nine osteoporotic women (aged 62–90 years) were pre-cultured for 24 h with  $10^{-11}$  mol/l  $17\beta$ -estradiol (E2) or vehicle, and subjected to 1 h of pulsating fluid flow (PFF) or static culture. E2 alone enhanced prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and nitric oxide (NO) production by 2.8-fold and 2.0-fold, respectively, and stimulated endothelial nitric oxide synthase protein expression by 2.5-fold. PFF, in the absence of E2, stimulated PGE<sub>2</sub> production by 3.1-fold and NO production by 3.9-fold. Combined treatment with E2 and PFF increased PGE<sub>2</sub> and NO production in an additive manner. When expressed as PFF-treatment-over-control ratio, the response to fluid shear stress was

similar in the absence or presence of E2. These results suggest that E2 does not affect the early response to stress in bone cells. Rather, E2 and shear stress both promote the production of paracrine factors such as NO and PGE<sub>2</sub> in an additive manner.

**Keywords** Bone cell culture · Estrogen · Mechanosensitivity · Nitric oxide · Osteoporosis · Prostaglandin E<sub>2</sub>

### Introduction

Osteoporosis is a condition characterized by a compromised resistance of the skeleton to mechanical loads as a result of reduced bone mass and an impaired mechanical competence of the trabecular architecture. The cause of bone loss in patients with osteoporosis is likely to be multifactorial, as bone mass and the skeleton's mechanical performance are affected by many factors. Systemic factors that affect bone mass are calcium-regulating hormones, such as parathyroid hormone, calcitonin and vitamin D, growth hormone, and the sex hormones [1, 2, 3]. Of the latter, estrogen is probably the best studied, due to its role in bone loss after menopause.

While all of these hormones are capable of affecting overall bone mass, local bone mass and architecture are primarily determined by mechanical signals. Several studies have shown that mechanical loading determines bone mass, shape, and trabecular architecture [4, 5, 6]. The purpose of this process of mechanical adaptation is to obtain bone that combines a proper resistance against mechanical failure with a minimum use of material. It is currently believed that mechanotransduction, the process whereby mechanical signals are converted into chemical messages, proceeds as follows: when bones are loaded, the hard matrix will undergo a very small deformation (strain). This deformation drives the thin

A.D. Bakker · J. Klein-Nulend (✉) · E.H. Burger  
Department of Oral Cell Biology,  
ACTA-UvA and Vrije Universiteit,  
Van der Boechorststraat 7, 1081 BT  
Amsterdam, The Netherlands  
E-mail: J.KleinNulend@vumc.nl  
Tel.: +31-20-4448660  
Fax: +31-20-4448683

E. Tanck  
Orthopedic Research Laboratory, UMC Nijmegen,  
Nijmegen, The Netherlands

G.H. Albers  
Department of Orthopedics, Hilversum Hospital,  
Hilversum, The Netherlands

P. Lips  
Department of Endocrinology, VU-University Medical Center,  
Amsterdam, The Netherlands

layer of interstitial fluid surrounding the osteocyte network to flow from regions under high pressure to regions under low pressure [7, 8, 9]. Although osteoblasts are sensitive to strain, and might thus sense mechanical loading directly through the deformation of their supporting substratum, it has been found by several investigators that the amount of strain does not correlate with bone formation *in vivo* [10, 11, 12]. Rather, the strain rate of the applied stimulus, which is directly related to the flow of interstitial fluid within the bone, is related to bone formation [10, 11, 12]. Loading-induced flow of fluid exerts a shear stress on the osteocyte cell membrane, which provokes a cellular response [13, 14]. It is thought that the osteocytes subsequently signal the osteoblasts and osteoclasts to change their bone remodeling activities [15]. In this concept the osteocytes act as the mechanosensors of bone, while the osteoblasts and osteoclasts are the effector cells of bone adaptation.

Bone mass diminishes with increasing age, in part due to declining levels of physical activity and changed mobility patterns. Decreased estrogen levels following menopause also have a strong negative effect on bone mass [16]. Administration of exogenous estrogens has been shown to increase bone mineral density in humans [17, 18], and large doses of estrogens have been shown to induce new bone formation in mice [19, 20]. These effects of estrogen on bone can be explained by its opposite effects on osteoclasts and osteoblasts. Both osteoclasts and osteoblasts express estrogen receptors, and estrogen is able to suppress bone resorption and stimulate bone formation [16, 21]. In addition, it has been suggested that estrogen alters the response of the bone mechanosensing cells to mechanical loading, thereby modulating the anabolic response of bone tissue to mechanical loading [22].

This hypothesis is supported by studies which show that estrogen and mechanical loading can synergistically affect bone formation [18, 23]. On the other hand, there are also several *in vivo* studies that were unable to detect a synergistic effect of estrogen and mechanical loading on bone formation [17, 24, 25, 26]. Estrogen application was shown to affect mechanically induced bone formation whether applied before or after loading [24], and estrogen did not affect the number of osteocytes expressing *c-fos* and IGF-I mRNA in response to mechanical loading of rat vertebrae [24]. These results do not support the idea of an interaction between mechanical loading and estrogen at the level of mechanosensation in osteocytes.

To study direct effects of E2 on mechanoresponsiveness in an early phase of mechanotransduction we investigated whether E2 modifies the response to shear stress in cultured bone cells from postmenopausal osteoporotic women. Women with bone cells that are highly sensitive to the effects of estrogen are likely to experience severe bone loss during the menopause, which results in an osteoporotic phenotype. Therefore, we specifically chose to investigate bone cells obtained from postmenopausal osteoporotic women. Nitric oxide

(NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) release were determined as parameters of bone cell responsiveness, since both signaling molecules have been shown to be essential for the anabolic response of bone to mechanical loading [27, 28]. We applied mechanical stress by submitting the cells to a pulsating laminar fluid flow (PFF) [13, 14].

We hypothesized that estrogen would affect the magnitude of the fluid flow-induced NO and PGE<sub>2</sub> production of the bone cells in an additive manner.

---

## Materials and methods

### Donors

Trabecular bone samples (surgical waste) were taken from the femoral neck during hip surgery after osteoporotic, subcapital hip fracture. Nine female osteoporotic donors (mean age 80; range 62–90 years), without other metabolic bone disease, were included in this study. Osteoporotic fracture was defined as a fracture without preceding trauma or in response to minimal trauma. People suffering from hip fracture after severe trauma were excluded from the study. The protocol was approved by the Ethical Review Board of the Vrije University Medical Center, and all subjects gave informed consent.

### Culture of primary human bone cells

Trabecular bone biopsies were placed in sterile phosphate-buffered saline (PBS), chopped into small fragments and washed extensively with PBS. The bone fragments were incubated with 2 mg/ml collagenase II (Sigma, St. Louis, Mo., USA) for 2 h at 37°C in a shaking water bath to remove all soft tissue from the bone chip's surface, after which the denuded bone fragments were transferred to 25 ml flasks (Nunc, Roskilde, Denmark). The fragments were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Paisley, UK) supplemented with 100 U/ml penicillin (Sigma), 50 µg/ml streptomycin sulfate (Sigma), 50 µg/ml gentamicin (Gibco), 1.25 µg/ml fungizone (Gibco), 100 µg/ml ascorbate (Merck, Darmstadt, Germany), and 10% fetal bovine serum (FBS; Hyclone, Logan, Utah, USA). Culture medium was replaced three times per week.

When the cell monolayer growing from the bone fragments reached confluency, the cells were harvested with 0.25% trypsin (Difco Laboratories, Detroit, Mich., USA) and 0.1% EDTA (Sigma) in PBS, plated at 25×10<sup>3</sup> cells per well in six-well culture dishes (Costar, Cambridge, Mass., USA) and cultured in 3 ml medium as described above, until the cell layer reached confluency again. Then the cells were characterized as described below, or used for pulsating fluid flow and E2 experiments.

### Cell characterization: cbfa1 mRNA expression and 1,25-dihydroxyvitamin D<sub>3</sub> challenge

To test the osteoblastic phenotype, we isolated RNA from the bone-derived cell cultures, using TRIzol reagent (Gibco), in accordance with the manufacturer's instructions. cDNA synthesis was performed with 1 µg of total RNA in 50 µl reaction mix consisting of 1x first-strand buffer, 500 µmol dNTPs, 10 units RNase inhibitor, 8 mmol dithiothreitol, 50 units Superscript RT (Gibco) and 2 pmol primer p(dT)15 (Boehringer, Mannheim, Germany). For the amplification of the cbfa1 product, 4 µl of cDNA was added to the PCR reaction mixture consisting of 1x Thermal Ace buffer (Invitrogen, Carlsbad, Calif., USA), 0.2 mmol dNTP, 6 mmol sense primer, 6 mmol antisense primer and 1 U Thermal Ace polymerase (Invitrogen), in a final volume of 50 µl. The cbfa1 upstream and downstream primer sequences were 5' atg ctt cat teg cct cac aac 3' for the forward primer, and 5' ttt gat gcc ata gtc cct cct t 3' for the reverse primer, respectively. The samples were pre-heated 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.

Alternatively, bone cell cultures were incubated for 3 days in the presence or absence of 10<sup>-8</sup> mol/l 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] in medium supplemented with 0.2% bovine serum albumin (BSA). After 3 days' incubation, the 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 use of *p*-nitrophenyl phosphate (Merck) as a substrate at pH 10.3, in accordance with the method described by Lowry [29]. The absorbance was read at 410 nm with a Dynatech MR7000 microplate reader (Dynatech, Billingham, UK). The amount of protein in the cell layer was measured with a BSA protein assay reagent kit (Pierce, Rockford, Ill., USA); the absorbance was read at 570 nm. ALP values were expressed per amount of protein in the cell layer.

### Pulsating fluid flow in the presence or absence of estrogen

Two days before PFF treatment, the cells were harvested from the six-well plates and seeded onto polylysine-coated (50 µg/ml; poly-L-lysine hydrobromide, molecular weight 15–30×10<sup>4</sup>; Sigma) glass slides (size 2.5 cm×6.5 cm). The cells were plated at 5×10<sup>5</sup> cells/glass slide and cultured overnight in Petri dishes with 13 ml culture medium as described above in "Culture of primary human bone cells". The next morning the culture medium was replaced by DMEM without phenol red (Gibco) containing 10<sup>-11</sup> mol/l 17β-estradiol (E2; Sigma) or vehicle, and supplemented with 0.2% BSA.

The medium also contained antibiotics and ascorbate as described above. Similar medium, with or without E2, was used the next day during the PFF experiments.

The cells were subjected to 1 h of PFF as described previously [30]. Briefly, we generated PFF by pumping 13 ml of culture medium through a parallel-plate flow chamber containing the bone cells. The cells were subjected to a 5 Hz pulse with a mean shear stress of 0.6 Pa, a pulse amplitude of 0.3 Pa and a peak shear stress rate of 8.4 Pa/s. Stationary control cultures were kept in a Petri dish under similar conditions as the experimental cultures, i.e., at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After 5 min of PFF or static culture, the medium was collected and assayed for NO production. After 1 h the medium was collected for determination of PGE<sub>2</sub> production. After 1 h PFF treatment was terminated, and the cells were either directly harvested in TRIzol reagent for determination of total DNA content or harvested in ice-cold cell lysis buffer (R&D systems, Minneapolis, Minn., USA) for determination of human endothelial nitric oxide synthase (eNOS) protein expression.

### Nitric oxide and prostaglandin E<sub>2</sub>

We measured NO as nitrite (NO<sub>2</sub><sup>-</sup>) accumulation in the conditioned medium, using Griess reagent consisting of 1% sulfanilamide, 0.1% naphthylethylene-diamine-dihydrochloride and 2.5 mol H<sub>3</sub>PO<sub>4</sub>. Serial dilutions of NaNO<sub>2</sub> in non-conditioned medium were used as the standard curve. The absorbance was measured at 540 nm.

PGE<sub>2</sub> release in the medium was measured by an enzyme immunoassay (EIA) system (Amersham, Buckinghamshire, UK) that used an antibody raised against mouse PGE<sub>2</sub>. The detection limit was 16 pg/ml, and the absorbance was measured at 450 nm.

### Isolation of total DNA

DNA was isolated from the bone cell cultures with TRIzol reagent in accordance with the manufacturer's instructions. DNA content was determined by measurement of the absorbance in water at 260 nm with an Ultrospec III spectrophotometer (Amersham).

### Determination of endothelial nitric oxide synthase protein

Human eNOS protein expression was quantified with an eNOS Quantikine immunoassay kit for determination of eNOS in endothelial cells (R&D systems), in accordance with the manufacturer's protocol. This kit employs the quantitative sandwich enzyme immunoassay [enzyme-linked immunosorbent assay (ELISA)] technique and uses antibodies raised against *E. coli*-expressed recombinant human eNOS.

## Statistical analysis

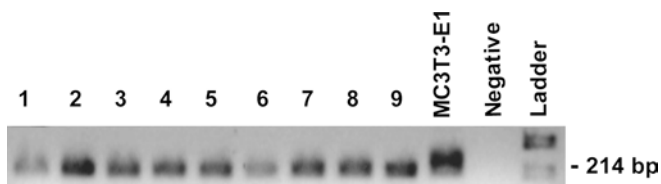
Data of absolute NO and PGE<sub>2</sub> production were not normally distributed and were, therefore, analyzed with the Wilcoxon signed-rank test. When we were comparing treated-over-control (T/C) values, the data were analyzed by a paired *t*-test. Differences were considered significant when  $P < 0.05$ .

## Results

Cells started to grow out of the collagenase-stripped bone chips along the bottom of the culture flask after approximately 6 days in culture and reached confluency within 19 days, when they were passaged. Expression levels of *cbfa1* mRNA by the osteoporotic (OP) bone cell cultures were comparable to that of a MC3T3-E1 mouse osteoblastic cell line (Fig. 1), while treatment of the passaged cells with  $10^{-8}$  mol/l 1,25(OH)<sub>2</sub>D<sub>3</sub> resulted in a significant 1.6-fold (range 1.2-fold to 2.2-fold) mean increase in alkaline phosphatase activity.

Treatment of the cells with PFF and/or E2 did not affect the total amount of DNA per culture (static,  $0.60 \pm 0.13$   $\mu$ g; static + E2,  $0.59 \pm 0.12$   $\mu$ g; PFF,  $0.57 \pm 0.14$   $\mu$ g; PFF + E2,  $0.64 \pm 0.12$   $\mu$ g. Values are mean  $\pm$  SEM of nine cultures), which demonstrated that the cells were not removed by the application of fluid flow and that 24 h of E2 treatment did not affect cell numbers.

Treatment of the cells with  $10^{-11}$  mol/l E2 for 24 h stimulated NO and PGE<sub>2</sub> production by 2.0-fold and 2.8-fold, respectively (Fig. 2a, b) and increased eNOS protein expression by 2.5-fold (Fig. 2c). One hour's



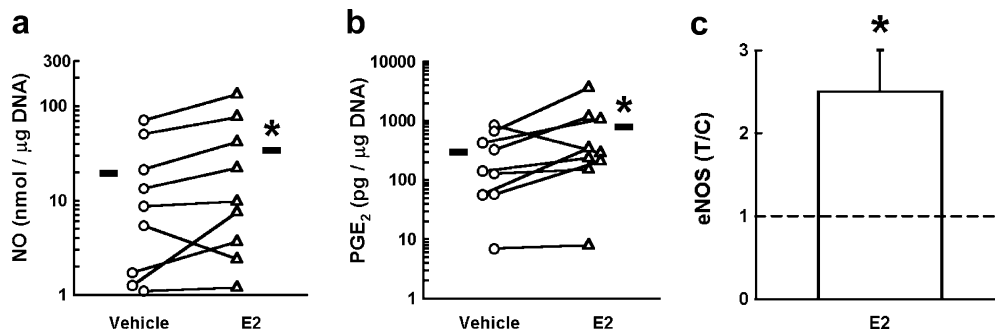
**Fig. 1** *Cbfa1* mRNA expression by untreated primary human bone cell cultures from osteoporotic donors (numbers 1–9). Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. MC3T3-E1, a mouse osteoblast cell line, served as a positive control for *cbfa1* expression. *Negative* negative control, *Ladder* 100 bp ladder

treatment with PFF slightly, but not significantly, increased eNOS expression (data not shown). Application of PFF rapidly stimulated NO production, in both the vehicle and the E2-treated cells (Fig. 3a). PFF also stimulated the production of PGE<sub>2</sub>, both in the absence and presence of E2 (Fig. 3b). For both NO and PGE<sub>2</sub> the combined treatment with PFF and E2 seemed to have an additive, but not synergistic, effect when mean group values (Fig. 3a, b) were considered.

When the same data were expressed as PFF-treated over static culture (PFF/static) ratios this observation became even clearer (Fig. 4a, b). Five minutes of PFF treatment stimulated the NO production by 3.9-fold in the vehicle-treated cells and by 3.5-fold in the E2-treated cultures (Fig. 4a). After 1 h, PFF increased PGE<sub>2</sub> production by 3.1-fold in the vehicle-treated cells and by 2.7-fold in E2-treated cultures (Fig. 4b). Thus, PFF had a similar stimulatory effect on the production of NO and PGE<sub>2</sub> in the presence and absence of E2.

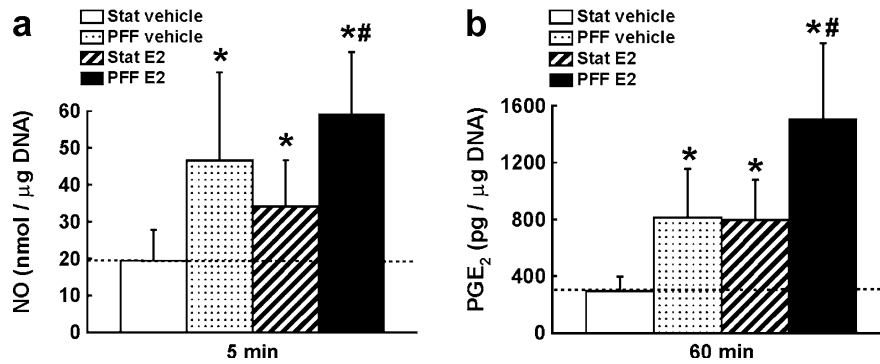
## Discussion

In the present study we tested whether estrogen modulates the early response of bone cells from post menopausal osteoporotic donors to a mechanical stimulus, applied in the form of a pulsating fluid flow. Bone cells were grown from surgical waste trabecular bone, obtained during hip surgery for osteoporotic, subcapital femur fracture. Our population did not include any premenopausal donors. It is, however, unlikely that factors such as hormonal status of the donor would have affected the mechanosensitivity of the bone cells *in vitro*, since the cells were cultured for an average of 30 days, in the presence of 10% fetal calf serum containing low concentrations of estrogen, before they were used for the



**Fig. 2a–c** Effect of 24 h treatment with E2 on NO and PGE<sub>2</sub> production, and eNOS protein expression by OP bone cells. **a, b** Each symbol represents the mean NO and PGE<sub>2</sub> production, normalized for the amount of DNA, by duplicate cultures from one donor. *Lines* connect data of vehicle-treated cultures (*circles*) with data of E2-treated cultures (*triangles*) from the same donor. *Horizontal bars* express mean NO or PGE<sub>2</sub> production. Note that the y-axis is logarithmic. **c** Treatment over control (T/C) ratio of eNOS expression. E2 stimulated NO (**a**) and PGE<sub>2</sub> (**b**) production and eNOS protein expression (**c**). \*Significant effect of E2,  $P < 0.05$





**Fig. 3a,b** Effect of E2 and/or PFF on NO and PGE<sub>2</sub> production by OP bone cells. Bars represent mean  $\pm$  SEM. Both PFF and E2 treatment significantly enhanced NO (a) and PGE<sub>2</sub> (b) production by the bone cell cultures. Combined treatment with PFF and E2 further increased NO and PGE<sub>2</sub> production in an additive, but not synergistic, manner. \*Significantly different from static (stat) culture + vehicle; # significantly different from stat + E2,  $P < 0.05$

PFF experiments. The bone fragments were treated with collagenase to remove all soft tissue from the bone surface before being put into culture, and it took 6 days before cells started to grow out of the denuded bone chips. This suggests that they were derived from a cell source within the bone matrix rather than from its surface.

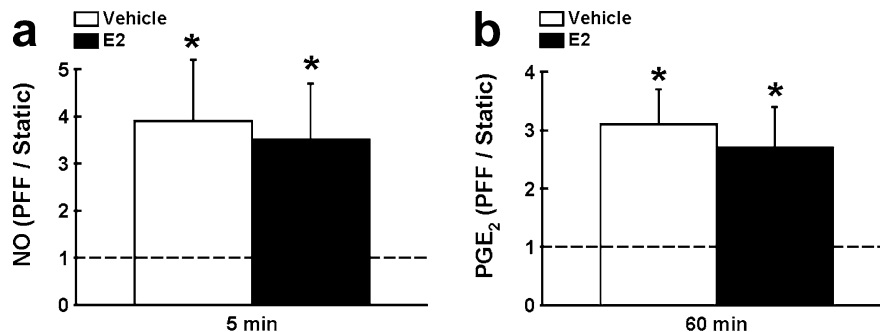
The “osteocytic osteoblast”, a cell stage between osteoblast and osteocyte [31] seems a good candidate. Treatment of the cell cultures with 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulated ALP activity, and the cells expressed *cbfa1* mRNA, indicating that they were of the osteoblast lineage. We have previously shown that human cells isolated as outgrowth from denuded bone chips respond to treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> with increased osteocalcin expression [32] and do not express the endothelial cell-specific factor VIII, von Willebrand factor [33]. Osteocytes, the terminally differentiated cells from the osteo-

blastic lineage, are highly responsive to mechanical loading in the form of a fluid flow [13]. The strong response to PFF of the cells isolated as outgrowth from denuded bone chips supports our contention that, to a certain extent, they mimic osteocytes.

We measured NO and PGE<sub>2</sub> production as parameters of the bone cell response to mechanical stimulation because animal studies have shown that NO as well as PGE<sub>2</sub> are essential for the induction of new bone formation in reaction to mechanical loading [27, 28]. In addition, both NO and PGE<sub>2</sub> production are influenced by estrogen [34, 35, 36, 37], and the anabolic effect of estrogen on bone seems to be mediated by prostaglandins and NO [20, 34, 38]. We used the physiological concentration of 10<sup>-11</sup> mol/l E2, because higher concentrations did not affect NO production in human and mouse osteoblastic cells or in bovine endothelial cells [36, 39]. Moreover, this E2 concentration has previously been shown to increase PGE<sub>2</sub> production by primary human bone cells [40]. The limited amount of donor bone precluded an extensive dose/effect study. Mechanical loading was applied by a pulsating fluid flow of 0.6  $\pm$  0.3 Pa at 5 Hz because this regime has been shown to cause a submaximal stimulation of NO and PGE<sub>2</sub> production [14].

The question whether estrogen and mechanical loading act synergistically to increase bone mass and bone strength is still unresolved. An in vitro study found a synergistic effect of estrogen and mechanical loading on <sup>3</sup>H-thymidine and <sup>3</sup>H-proline incorporation in cultured ulnae of female rats [23], and a clinical study reported a synergistic effect of weight-bearing exercise and hormone replacement therapy (HRT) on whole-body bone mineral density (BMD) in elderly women [18].

**Fig. 4a,b** Effect of E2 on the magnitude of the NO and PGE<sub>2</sub> response to PFF by OP bone cells. Data are expressed as PFF-treated over static culture (PFF/static) ratios. Bars are mean  $\pm$  SEM of nine donors. Application of PFF for 5 min enhanced NO production both in vehicle and E2-treated cultures in a similar manner (a). There was no difference in magnitude of the PGE<sub>2</sub> response to PFF between E2 and vehicle-treated cells (b). \*Significant effect of PFF,  $P < 0.05$



However, that study found no synergistic effect of exercise and HRT on BMD of the spine and hip, two sites prone to osteoporotic fracture [18].

In another clinical study HRT and exercise both increased BMD of the proximal tibia of postmenopausal women, but no synergistic effect was observed at this site either [17]. In two separate studies with rats, the anabolic response of bone to treadmill exercise was not negatively affected by ovariectomy, which suggests that estrogen status does not affect the mechanoresponsiveness of bone tissue [25, 26]. In rat bone, estrogen did not significantly alter the proportion of osteocytes that expressed *c-fos* and IGF-1 mRNA in response to mechanical loading, and affected mechanically induced osteogenesis similarly when administered before or after application of loading [24]. This latter observation suggests that even *if* estrogen alters the mechano-adaptive response of bones as an organ, estrogen does not act on the strain-sensing mechanism itself.

Our present *in vitro* data support that conclusion. We found that E2 and fluid flow-induced shear stress both promote the rapid production of NO and PGE<sub>2</sub> and that the combined application of E2 and PFF had an additive effect on NO and PGE<sub>2</sub> production, rather than a synergistic one. Our results are in agreement with an earlier study that showed that estrogen enhanced fluid shear stress-induced prostaglandin production by bone cells from elderly women [40]. When the results of that study are expressed as PFF-treated over static culture (T/C) ratios, it becomes clear that the response to shear stress was similar in the absence or presence of E2 (PGE<sub>2</sub> production, mean  $\pm$  SEM, vehicle treated  $3.6 \pm 1.3$ ; E2 treated  $3.5 \pm 1.4$ ). In the latter study, cells were used from osteoarthritic rather than osteoporotic donors. The lack of a synergistic effect does not, therefore, appear to be related to the osteoporotic status of the donor.

We found that treatment for 24 h with E2 more than doubled eNOS expression, in agreement with other studies [35, 36, 37]. The enhanced eNOS protein expression after 24 h E2 might explain the enhanced NO production under static culture conditions. The finding that E2 stimulated eNOS expression in the cells, but that PFF and E2 nonetheless acted in an additive manner, suggests that eNOS protein expression in the cells was not rate limiting for PFF-induced NO production. We had found earlier that mRNA levels for eNOS had doubled after 1 h of static post-incubation following 1 h of PFF treatment [33]. In the present study eNOS protein expression was not significantly enhanced immediately after 1 h exposure to PFF, likely because the time period was too short to allow for a significant increase in eNOS protein expression.

Our results are also consistent with the finding by Damien et al. that estrogen and mechanical strain had an additive, rather than synergistic, effect on osteoblast proliferation *in vitro* [41]. That study by Damien et al., as well as subsequent papers, found evidence for an important role of the estrogen receptor  $\alpha$  (ER $\alpha$ ) in the proliferative response of bone cells to mechanical stress

[31, 42, 43, 44, 45]. In addition, in ER $\alpha$  knockout mice the adaptive bone formation in response to mechanical loading was blunted [46]. Thus, mechanical loading and estrogen do seem to share a common signaling pathway in the regulation of osteoblast proliferation and bone formation. Of course, the responses of osteoblasts, which are the effector cells of bone adaptation, are downstream of the response of the mechanosensors to a mechanical stimulus [15]. It is the latter response that we have studied in the present paper.

Although we found no synergistic effect of E2 and PFF on NO and PGE<sub>2</sub> production by OP bone cells, we did find additive effects, both on NO and PGE<sub>2</sub> production. The production of both factors was highest when shear stress was applied in the presence of estrogen and lowest when neither estrogen nor shear stress was applied. If we assume that both factors are related to anabolic events further downstream in the adaptive response to mechanical loading [27, 28], this suggests that estrogen and mechanical usage both promote bone mass and strength. As such, these *in vitro* results are in agreement with clinical studies [17, 18]. Our results do not rule out the possibility that estrogen modulates the adaptive response of whole bones to mechanical load but do contradict the concept that estrogen, by itself, promotes mechanotransduction in osteocytes, the principal bone mechanosensing cells in the adaptive cascade.

**Acknowledgments** The authors thank C.M. Semeins and H.F. Teshale for their expert technical assistance. We thank Drs P. Patka, A. van Kampen, E. Hoebink and T. Patt for their assistance in obtaining osteoporotic bone material, M.N. Helder for kindly providing PCR-primers for *cbfa1*, and M. Joldersma for providing the raw data of her study on osteoarthritic women. The Netherlands Organization for Scientific Research supported the work of A.D. Bakker (NWO Grant 903-41-193) who also received a financial contribution from the Stichting Anna Fonds. The European Community supported the work of J. Klein-Nulend (fifth framework grant QLK3-1999-00559).

---

## References

1. Parfitt AM (1976) The actions of parathyroid hormone on bone: relation to bone remodeling and turnover, calcium homeostasis, and metabolic bone diseases II. PTH and bone cells: bone turnover and plasma calcium regulation. *Metabolism* 25:909–955
2. Pondel M (2000) Calcitonin and calcitonin receptors: bone and beyond. *Int J Exp Pathol* 81:405–422
3. Lips P (2001) Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. *Endocr Rev* 22:477–501
4. Rubin CT, Lanyon LE (1984) Regulation of bone formation by applied dynamic loads. *J Bone Joint Surg Am* 66:397–402
5. Mosley JR, Lanyon LE (1998) Strain rate as a controlling influence on adaptive modeling in response to dynamic loading of the ulna in growing male rats. *Bone* 23:313–318
6. Turner CH, Forwood MR, Otter MW (1994) Mechanotransduction in bone: do bone cells act as sensors of fluid flow? *FASEB J* 8:875–878
7. Piekarski K, Munro M (1977) Transport mechanism operating between blood supply and osteocytes in long bones. *Nature* 269:80–82

8. Cowin SC, Weinbaum S (1998) Strain amplification in the bone mechanosensory system. *Am J Med Sci* 316:184–188
9. Knothe Tate ML, Knothe U (2000) An ex vivo model to study transport processes and fluid flow in loaded bone. *J Biomech* 33:247–254
10. Luo G, Cowin SC, Sadegh AM, Arramon YP (1995) Implementation of strain rate as a bone remodeling stimulus. *J Biomech Eng* 117:329–338
11. Lanyon LE, Rubin CT (1984) Static versus dynamic loads as an influence on bone remodelling. *J Biomech* 17:897–905
12. Turner CH, Owan I, Takano Y (1995) Mechanotransduction in bone: role of strain rate. *Am J Physiol* 269:E438–E442
13. Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH (1995) Sensitivity of osteocytes to biomechanical stress in vitro. *FASEB J* 9:441–445
14. Bakker AD, Soejima K, Klein-Nulend J, Burger EH (2001) The production of nitric oxide and prostaglandin E<sub>2</sub> by primary bone cells is shear stress dependent. *J Biomech* 34:671–677
15. Burger EH, Klein-Nulend J (1999) Mechanotransduction in bone—role of the lacunocanalicular network. *FASEB J* 13:S101–S112
16. Turner RT, Riggs BL, Spelsberg TC (1994) Skeletal effects of estrogen. *Endocr Rev* 15:275–300
17. Cheng S, Sipila S, Taaffe DR, Puolakka J, Suominen H (2002) Change in bone mass distribution induced by hormone replacement therapy and high-impact physical exercise in postmenopausal women. *Bone* 31:126–135
18. Kohrt WM, Snead DB, Slatopolsky E, Birge SJ Jr (1995) Additive effects of weight-bearing exercise and estrogen on bone mineral density in older women. *J Bone Miner Res* 10:1303–1311
19. Samuels A, Perry MJ, Tobias JH (1999) High-dose estrogen induces de novo medullary bone formation in female mice. *J Bone Miner Res* 14:178–186
20. Samuels A, Perry MJ, Gibson RL, Colley S, Tobias JH (2001) Role of endothelial nitric oxide synthase in estrogen-induced osteogenesis. *Bone* 29:24–29
21. Chow J, Tobias JH, Colston KW, Chambers TJ (1992) Estrogen maintains trabecular bone volume in rats not only by suppression of bone resorption but also by stimulation of bone formation. *J Clin Invest* 89:74–78
22. Frost HM (1987) The mechanostat: A proposed pathogenic mechanism of osteoporoses and the bone mass effects of mechanical and nonmechanical agents. *Bone Miner* 2:73–85
23. Cheng MZ, Zaman G, Rawlinson SC, Suswillo RF, Lanyon LE (1996) Mechanical loading and sex hormone interactions in organ cultures of rat ulna. *J Bone Miner Res* 11:502–511
24. Jagger CJ, Chow JW, Chambers TJ (1996) Estrogen suppresses activation but enhances formation phase of osteogenic response to mechanical stimulation in rat bone. *J Clin Invest* 98:2351–2357
25. Tromp AM, Bravenboer N, Tanck E, Kostense PJ, Lips P (2002) The effects of additional weight bearing during exercise and estrogen on bone mass and structure in female rats. *Acta Bioeng Biomech* 4 [Suppl 1]:399
26. Järvinen TLN, Kannus P, Pajamäki I, Vuohelainen T, Tukkaneen J, Järvinen M, Sievänen H (2003) Estrogen deposits extra mineral into bones of female rats in puberty, but simultaneously seems to suppress the responsiveness of female skeleton to mechanical loading. *Bone* 32:642–651
27. Forwood MR (1996) Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. *J Bone Miner Res* 11:1688–1693
28. Turner CH, Takano Y, Owan I, Murrell GA (1996) Nitric oxide inhibitor L-NAME suppresses mechanically induced bone formation in rats. *Am J Physiol* 270:E634–E639
29. Lowry OH (1955) Micromethods for the assay of enzyme. II Specific procedure. Alkaline phosphatase. *Methods Enzymol* 4:371
30. Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH (1995) Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts: correlation with prostaglandin upregulation. *Biochem Biophys Res Commun* 217:640–648
31. Nijweide PJ, Van der Plas A, Scherft JP (1981) Biochemical and histological studies on various bone cell preparations. *Calcif Tissue Int* 33:529–540
32. Sterck JG, Klein-Nulend J, Lips P, Burger EH (1998) Response of normal and osteoporotic human bone cells to mechanical stress in vitro. *Am J Physiol* 274:E1113–E1120
33. Klein-Nulend J, Helfrich MH, Sterck JG, MacPherson H, Joldersma M, Ralston SH, Semeins CM, Burger EH (1998) Nitric oxide response to shear stress by human bone cell cultures is endothelial nitric oxide synthase dependent. *Biochem Biophys Res Commun* 250:108–114
34. Samuels A, Perry MJ, Tobias JH (1999) High-dose estrogen-induced osteogenesis in the mouse is partially suppressed by indomethacin. *Bone* 25:675–680
35. Armour KE, Ralston SH (1998) Estrogen upregulates endothelial constitutive nitric oxide synthase expression in human osteoblast-like cells. *Endocrinology* 139:799–802
36. Riancho JA, Zarrabeitia MT, Fernandez-Luna J, Gonzalez-Macias J (1995) Mechanisms controlling nitric oxide synthesis in osteoblasts. *Mol Cell Endocrinol* 107:87–92
37. Chambliss KL, Shaul PW (2002) Estrogen modulation of endothelial nitric oxide synthase. *Endocr Rev* 23:665–686
38. Armour KE, Armour KJ, Gallagher ME, Godecke A, Helfrich MH, Ralston SH (2001) Defective bone formation and anabolic response to exogenous estrogen in mice with targeted disruption of endothelial nitric oxide synthase. *Endocrinology* 142:760–766
39. Stewart KG, Zhang Y, Davidge ST (1999) Estrogen decreases prostaglandin H synthase products from endothelial cells. *J Soc Gynecol Investig* 6:322–327
40. Joldersma M, Klein-Nulend J, Oleksik AM, Heyligers IC, Burger EH (2001) Estrogen enhances mechanical stress-induced prostaglandin production by bone cells from elderly women. *Am J Physiol* 280:E436–E442
41. Damien E, Price JS, Lanyon LE (1998) The estrogen receptor's involvement in osteoblasts' adaptive response to mechanical strain. *J Bone Miner Res* 13:1275–1282
42. Jessop HL, Sjöberg M, Cheng MZ, Zaman G, Wheeler-Jones CP, Lanyon LE (2001) Mechanical strain and estrogen activate estrogen receptor alpha in bone cells. *J Bone Miner Res* 16:1045–1055
43. Zaman G, Cheng MZ, Jessop HL, White R, Lanyon LE (2000) Mechanical strain activates estrogen response elements in bone cells. *Bone* 27:233–239
44. Cheng MZ, Rawlinson SCF, Pitsillides AA, Zaman G, Mohan S, Baylink DJ, Lanyon LE (2002) Human osteoblasts' proliferative responses to strain and 17 $\beta$ -estradiol are mediated by the estrogen receptor and the receptor for insulin-like growth factor I. *J Bone Miner Res* 17:593–602
45. Jessop HL, Suswillo RFL, Rawlinson SCF, Zaman G, Lee K, Das-Gupta V, Pitsillides AA, Lanyon LE (2004) Osteoblast-like cells from estrogen receptor  $\alpha$  knockout mice have deficient responses to mechanical strain. *J Bone Miner Res* 19:938–946
46. Lee K, Jessop HL, Suswillo RFL, Zaman G, Lanyon LE (2003) Bone adaptation requires estrogen receptor- $\alpha$ . *Nature* 424:389