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CHAPTER 4

Mechanical Loading and the Synthesis of 1,25(OH)$_2$D in Primary Human Osteoblasts

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ABSTRACT

The metabolite 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) is synthesized from its precursor 25-hydroxyvitamin D (25(OH)D) by human osteoblasts leading to stimulation of osteoblast differentiation in an autocrine or paracrine way. Osteoblast differentiation is also stimulated by mechanical loading through activation of various responses in bone cells such as nitric oxide signaling. Whether mechanical loading affects osteoblast differentiation through an enhanced synthesis of 1,25(OH)$_2$D by human osteoblasts is still unknown. We hypothesized that mechanical loading stimulates the synthesis of 1,25(OH)$_2$D from 25(OH)D in primary human osteoblasts. Since the responsiveness of bone to mechanical stimuli can be altered by various endocrine factors, we also investigated whether 1,25(OH)$_2$D or 25(OH)D affect the response of primary human osteoblasts to mechanical loading.

Primary human osteoblasts were pre-incubated in medium with/without 25(OH)D$_3$ (400 nM) or 1,25(OH)$_2$D$_3$ (100 nM) for 24 h and subjected to mechanical loading by pulsatile fluid flow (PFF). The response of osteoblasts to PFF was quantified by measuring nitric oxide, and by PCR analysis. The effect of PFF on the synthesis of 1,25(OH)$_2$D$_3$ was determined by subjecting osteoblasts to PFF followed by 24 h post-incubation in medium with/without 25(OH)D$_3$ (400 nM).

We showed that 1,25(OH)$_2$D$_3$ reduced the PFF-induced NO response in primary human osteoblasts. 25(OH)D$_3$ did not significantly alter the NO response of primary human osteoblasts to PFF, but 25(OH)D$_3$ increased osteocalcin and RANKL mRNA levels, similar to 1,25(OH)$_2$D$_3$. PFF did not increase 1,25(OH)$_2$D$_3$ amounts in our model, even though PFF did increase CYP27B1 mRNA levels and reduced VDR mRNA levels. CYP24 mRNA levels were not affected by PFF, but were strongly increased by both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$.

In conclusion, 1,25(OH)$_2$D$_3$ may affect the response of primary human osteoblasts to mechanical stimuli, at least with respect to NO production. Mechanical stimuli may affect local vitamin D metabolism in primary human osteoblasts. Our results suggest that 1,25(OH)$_2$D$_3$ and mechanical loading, both stimuli of the differentiation of osteoblasts, interact at the cellular level.

INTRODUCTION

The vitamin D metabolite 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) is considered the most active vitamin D metabolite, and plays a key role in mineral and bone homeostasis [25]. It is mainly produced in the kidney through hydroxylation of its precursor 25-hydroxyvitamin D (25(OH)D) by the enzyme 1α-hydroxylase (CYP27B1 gene) [25]. Maintenance of mineral and bone homeostasis is primarily accomplished by the stimulating actions of 1,25(OH)$_2$D on intestinal calcium absorption through the vitamin D receptor (VDR) [9;45]. In vitro studies also show direct 1,25(OH)$_2$D actions on bone homeostasis which is possible by the presence of the VDR in bone cells [22;42;44]. The metabolite 1,25(OH)$_2$D inhibits cell growth and enhances osteoblast differentiation by regulating the expression of collagenous and non-collagenous proteins, including osteocalcin and osteopontin [38;42]. In addition, 1,25(OH)$_2$D enhances the mineralization of osteoid tissue produced by osteoblasts [44;48]. Osteoblasts also respond to 1,25(OH)$_2$D by secreting RANKL which binds to RANK and subsequently induces osteoclast differentiation from hematopoetic progenitors [30].

In addition to the kidney, osteoblasts in vitro are also able to synthesize 1,25(OH)$_2$D from 25(OH)D by expressing the 1α-hydroxylase gene CYP27B1 [3;42;43]. The locally synthesized 1,25(OH)$_2$D has been proposed to stimulate osteoblast differentiation and mineralization of osteoid made by osteoblasts [3;43]. Moreover, transgenic mice that overexpress CYP27B1 in osteoblasts and osteocytes show increased trabecular bone volume, suggesting that CYP27B1 activity within these cells has an anabolic effect on bone tissue [37;41]. The metabolite 1,25(OH)$_2$D regulates its own metabolism by inducing CYP24 activity in osteoblasts, which results in the synthesis of 1,24R,25-trihydroxyvitamin D (1,24R,25(OH)$_3$D) [1]. Although osteoblasts can convert 25(OH)D to 1,25(OH)$_2$D, the regulation of this conversion is completely different compared to that in the kidney [2]. Bone CYP27B1 is not influenced by the renal regulators such as PTH [43], which raises the question whether local regulatory mechanisms are present.

An important local factor that affects the activity of bone cells is mechanical loading [16]. Mechanical loading causes extracellular fluid flow in the bone canalicular network resulting in mechanosensing by the osteocyte [10;16]. Osteocytes are the most abundant bone cells and are essential for the adaptive response of bone tissue to mechanical loading [17]. Although the osteocyte is the most mechanosensitive bone cell, osteoblasts are also very responsive to mechanical stimuli in vitro [19]. In response to mechanical stimuli, osteocytes and osteoblasts produce signaling molecules, including the short-lived chemical second messenger nitric oxygen (NO) [6;20]. NO can modulate the activity of osteoblasts and osteoclasts in an autocrine or paracrine way leading to adaptation of bone mass and structure to mechanical stimuli [20]. The responsiveness of bone to mechanical stimuli can be altered by endocrine factors, including PTH, estrogen, insulin-like growth factors [31;33] and

1,25(OH)$_2$D [47]. In mouse osteoblasts, 1,25(OH)$_2$D stimulates the NO production under static conditions, while under pulsatile fluid flow conditions 1,25(OH)$_2$D inhibits the production of NO [47]. In human osteoblasts, it is still unknown whether 1,25(OH)$_2$D affects the response to mechanical loading.

Mechanical loading stimulates a number of responses in bone cells which eventually result in the recruitment of mesenchymal stem cells and osteoblast differentiation [31]. Osteoblast differentiation is also stimulated by locally synthesized 1,25(OH)$_2$D by human osteoblasts [3;42;43]. However, whether mechanical loading stimulates osteoblast differentiation through an enhanced synthesis of 1,25(OH)$_2$D by human osteoblasts is still unknown. After all, an enhanced conversion of 25(OH)D to 1,25(OH)$_2$D may increase local concentrations of 1,25(OH)$_2$D, which may reduce osteoblast proliferation and enhance osteoblast differentiation.

We hypothesized that mechanical loading increases the expression of CYP27B1 and the synthesis of 1,25(OH)$_2$D in primary human osteoblasts. The other hypothesis is that both 1,25(OH)$_2$D and 25(OH)D affect the response of primary human osteoblasts to mechanical loading.

MATERIALS AND METHODS

Primary human osteoblast culture

Primary human osteoblasts were isolated from redundant trabecular bone fragments obtained from healthy donors undergoing pre-implant bony reconstruction of the mandible or maxilla with autologous bone from the anterior iliac crest. The donor group consisted of 2 males and 9 females with a mean age of 35.1 ± 5.7 years. The protocol was approved by the Medical Ethical Review Board of the VU University Medical Center, Amsterdam, the Netherlands, and all donors gave their written informed consent.

A modification of the methods of Beresford et al. and Marie et al. [7;27] was used to obtain a primary human osteoblast culture. Shortly, the trabecular bone fragments were minced into small pieces and washed extensively with phosphate buffered saline (PBS). The bone pieces were treated with 2 mg/ml collagenase type II (300 U/mg; Worthington Biochemical Corporation, Lakewood, NJ, USA) for 2 h in a shaking waterbath at 37°C. The pieces were placed in culture flasks with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Gibco, Life technologies, Grand Island, NY, USA) supplemented with 10% Fetal Clone I (HyClone; Thermo Fisher Scientific, Rockford, IL, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco; Life technologies), 1.25 μg/ml fungizone (Gibco; Life technologies) and incubated at 37°C in a humidified air with 5% CO$_2$. Medium was changed twice a week until cells reached confluence.

Pulsatile fluid flow (PFF) and static treatments

To study the effects of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ on the response of primary human osteoblasts to PFF, pre-incubations of 24 h were performed in medium with 25(OH)D$_3$ (0 or 400 nmol/l) or 1,25(OH)$_2$D$_3$ (0 or 100 nmol/l) (Sigma–Aldrich, St. Louis, MO, USA) under static conditions. The concentrations of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ were chosen based on a previous study in which 400 nmol/l 25(OH)D$_3$ and 100 nmol/l 1,25(OH)$_2$D$_3$ affected osteoblast proliferation and differentiation to a similar extend [42]. Thereafter, cells were subjected to 1 h PFF or static treatment as described previously [6]. Shortly, primary human osteoblasts were plated out on polylysine-coated glass slides (15 cm^2) at a cell density of 2×10^5 cells per slide. PFF was generated by pumping 13 ml medium through a parallel-plate flow chamber using a roller pump at 37°C in a humidified air with 5% CO$_2$. The cells were exposed to 5 Hz pulse with an amplitude of 0.7 Pa. Static cultures (control) were kept in petri dishes under similar conditions as the PFF treated cultures. After the onset of PFF or static treatment, medium samples were collected at 5, 10 and 60 min for NO measurement. At 0 or 3 h after PFF or static treatment, cells were lysed for total RNA isolation as described below.

To study the effects of PFF on the conversion of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$, post-incubations of 24 h were performed after 1 h PFF or static treatment in medium with 0 or 400 nmol/l 25(OH)D$_3$. High cell density of 5×10^5 cells per slide was used to reach detectable levels of 1,25(OH)$_2$D$_3$. After 24 h of post-incubation, cells were lysed for total RNA isolation and medium was collected for 1,25(OH)$_2$D$_3$ measurements.

All pre- and post-incubations as well as PFF and static treatments were carried out in medium containing 0.2% bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO, USA) and 0.1% ethanol (vehicle).

Nitric oxide (NO)

NO was measured as nitrate (NO$_2^-$) accumulation in the medium using Griess reagent, containing 1% sulfanilamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 mol/l H$_3$PO$_4$. NO concentrations were determined using a standard curve with known concentrations of NaNO$_2$ in non-conditioned medium. Absorbance was measured by a spectrophotometer (Bio-Rad Laboratories, Veenendaal, The Netherlands) at 540 nm.

RNA isolation and RT-qPCR

Total RNA isolation of primary human osteoblasts was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For removing residual DNA amounts an additional on-column DNase treatment was accomplished during the RNA isolation procedure. Total RNA concentration was measured by the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

RNA was reverse transcribed from 100 ng total RNA in a 20 μl reaction mixture containing 5 mmol/l MgCl$_2$ (Eurogentec, Maastricht, The Netherlands), 1x RT buffer (Pro-

mega, Madison, WI, USA), 1 mmol/l dATP, 1 mmol/l dCTP, 1 mmol/l dGTP, 1 mmol/l dTTP (Roche Diagnostics, Mannheim, Germany), 1 mmol/l betaïne, 10 ng/ul random primer, 0.4 U/µl RNAsin (Promega) and 5 U/µl M-MLV RT-enzyme (Promega). The PCR reaction of total 25 µl contained 3 µl cDNA, 300 nmol/l reverse and forward primer (Table 1) and SYBR Green Supermix (Bio-Rad). The PCR was performed on an iCycler iQ™Real-Time PCR Detection System (Bio-Rad): 3 min at 95°C, 40 cycles consisting of 15 s at 95°C and 1 min at 60°C. The relative gene expression was calculated by the $2^{-\Delta Ct}$ method and TATA binding protein (TBP) was used as housekeeping gene.

Table 1. Primer sequence

Gene	Primer sequence (5'- 3')
CYP27B1	Forward: TGGCCCAGATCCTAACACATTT
	Reverse: GTCCGGGTCTTGGGTCTAACT
CYP24	Forward: CAAACCGTGGAAGGCCTATC
	Reverse: AGTCTTCCCCTTCCAGGATCA
Vitamin D receptor (VDR)	Forward: GGACGCCCACCATAAGACCTA
	Reverse: CTCCCTCCACCATCATTCACA
RANKL	Forward: CGGGGGTGACCTTATGAGAAA
	Reverse: GCGCTAGATGACACCCTCTC
OPG	Forward: GGCAACACAGCTCACAAGAA
	Reverse: CGCTGTTTTCACAGAGGTCA
Osteocalcin	Forward: GGCGCTACCTGTATCAATGG
	Reverse: TCAGCCAACTCGTCACAGTC
Osteopontin	Forward: TTCCAAGTAAGTCCAACGAAAG
	Reverse: GTGACCAGTTCATCAGATTCAT
TATA binding protein (TBP)	Forward: GGTCTGGGAAAATGGTGTGC
	Reverse: GCTGGAAAACCCAACTTCTG

1,25(OH)$_2$D$_3$ measurement

The metabolite 1,25(OH)$_2$D$_3$ was measured in medium samples by a radioimmunoassay (Immunodiagnostic Systems, Boldon, UK). Cross-reactivity with 25(OH)D$_3$ was <0.01%. The intra-assay variation was 8% at a level of 25 pmol/l and 9% at a level of 70 pmol/l. The inter-assay variation was 11% at a concentration of 25 and 70 pmol/l.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Differences between groups were assessed using Wilcoxon signed rank test or Friedman test followed by Dunn's post hoc test. A p-value <0.05 was considered to be significant. Data were analyzed using Graphpad Prism 4 (Graphpad Software, San Diego, CA, USA).

RESULTS

Effects of 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ on primary human osteoblasts

Both 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ did not affect CYP27B1 and VDR mRNA levels, but both vitamin D$_3$ metabolites markedly increased CYP24 mRNA levels by respectively 1.9×10^5 and 1.5×10^5 fold (p<0.01 and p<0.05, respectively; Fig. 1). Both 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ stimulated osteocalcin mRNA levels by respectively 56.2 and 52.6 fold (p<0.05 and p<0.01, respectively), but neither 1,25(OH)$_2$D$_3$ nor 25(OH)D$_3$ affected osteopontin mRNA levels. The metabolite 1,25(OH)$_2$D$_3$ increased RANKL mRNA levels by 5.8 fold (p<0.01), while 25(OH)D$_3$ did not significantly affect RANKL mRNA levels. OPG mRNA levels were not affected by either 1,25(OH)$_2$D$_3$ or 25(OH)D$_3$.
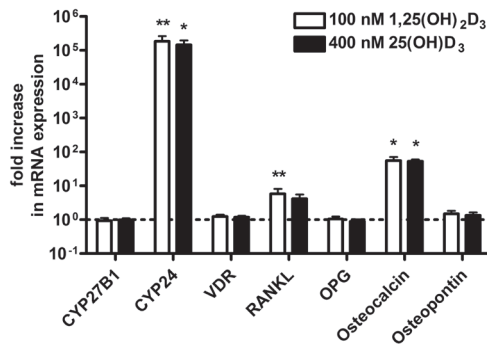

Figure 1. Effects of 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ on primary human osteoblasts. Primary human osteoblasts were pre-incubated for 24 h in the presence or absence of 100 nM 1,25(OH)$_2$D$_3$ or 400 nM 25(OH)D$_3$ and mRNA levels of CYP27B1, CYP24, VDR, RANKL, OPG, osteocalcin and osteopontin were determined. Results (mean ± SEM) are expressed as treatment versus control ratios (control was set at 1.0; dashed line) using cells from 7 different donors. Effects of 1,25(OH)$_2$D$_3$ or 25(OH)D$_3$ on mRNA levels of each gene were analysed using Friedman test followed by Dunn's post hoc test (*p<0.05, **p<0.01).

Effects of 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ on the PFF response of primary human osteoblasts

To study the response of primary human osteoblasts to fluid shear stress, cells were subjected to 1 h PFF. PFF did not affect mRNA levels of osteocalcin, osteopontin, RANKL and OPG directly after cessation of PFF (data not shown). Three hours after PFF treatment, RANKL mRNA levels were increased by 4.2 fold compared to static cultures (p<0.05; Fig. 2A). At this time-point, PFF did not significantly affect osteocalcin, osteopontin and OPG mRNA levels or the RANKL/OPG ratio.

At 5 and 10 min after the onset of PFF treatment, primary human osteoblasts responded to PFF with a higher NO production compared to static cultures (5.9 and 8.5 fold, respectively; p<0.05; Fig. 2B). Pre-incubation with 1,25(OH)$_2$D$_3$ decreased the production of NO compared to cultures without 1,25(OH)$_2$D$_3$ pre-incubation at
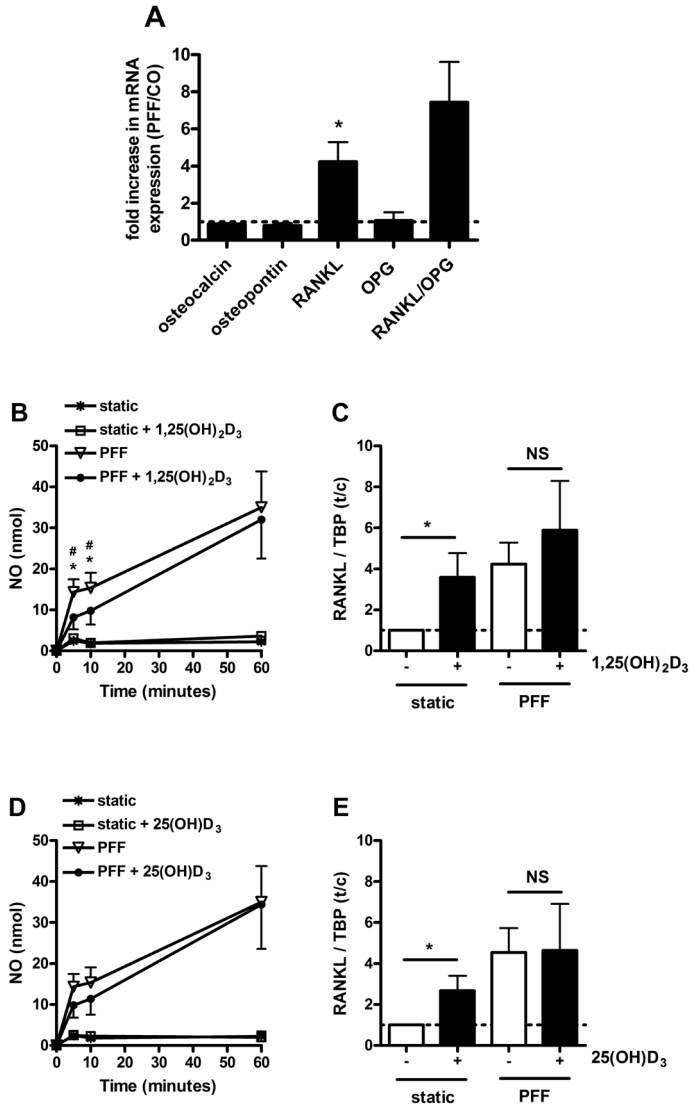
Figure 2. Effects of 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ on the PFF response of primary human osteoblasts. (**A**) At 3 h after PFF or static treatment, mRNA levels of osteocalcin, osteopontin, RANKL, OPG and RANKL/OPG ratio were determined. (**B**) Osteoblasts were pre-incubated for 24 h in medium supplemented with 0 or 100 nM 1,25(OH)$_2$D$_3$ and subjected to 1 h PFF or static treatment. NO was measured at 5, 10 and 60 min after the onset, and (**C**) 3 h after PFF or static treatment RANKL mRNA levels were determined. (**D**) Osteoblasts were also pre-incubated for 24 h in medium supplemented with 0 or 400 nM 25(OH)D$_3$ and subjected to 1 h PFF or static treatment. NO was measured at 5, 10 and 60 min after the onset, and (**E**) 3 h after PFF or static treatment RANKL mRNA levels were determined. Results (mean ± SEM) are expressed as treatment versus control ratios (control was set at 1.0; dashed line) using cells from 5-7 different donors. PFF treatment was compared with static treatment using Wilcoxon signed rank test (B). The effects of vitamin D supplementation were analysed using Wilcoxon signed rank test for each time-point (B,D) or gene (A,C) (*p<0.05, #p<0.05) (*PFF vs. static, #PFF vs. PFF+1,25(OH)$_2$D$_3$).

5 and 10 min after the onset of PFF (0.6 fold; p<0.05; Fig. 2B). Pre-incubation with 1,25(OH)$_2$D$_3$ increased RANKL mRNA levels by 3.6 fold in static cultures (p<0.05; Fig. 2C), but an additional effect in PFF-treated cultures was not observed.

Pre-incubation of cell cultures with 25(OH)D$_3$ did not significantly affect the production of NO during PFF treatment (Fig. 2D). Pre-incubation with 25(OH)D$_3$ increased RANKL mRNA levels by 2.7 fold in static cultures (p<0.05; Fig. 2E), but an additional effect in PFF-treated cultures was not observed.

Effects of PFF on mRNA levels of genes involved in vitamin D metabolism

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PFF did not affect mRNA levels of CYP27B1, CYP24 and VDR directly after cessation of PFF treatment (Fig. 3A-C). However, at 3 h after cessation, PFF increased CYP27B1 mRNA levels by 2.0 fold and decreased VDR mRNA levels by 0.6 fold compared to those in static cultures (p<0.05; Fig. 3A and B). PFF did not significantly affect mRNA levels of CYP24 at 3 h after treatment (Fig. 3C).
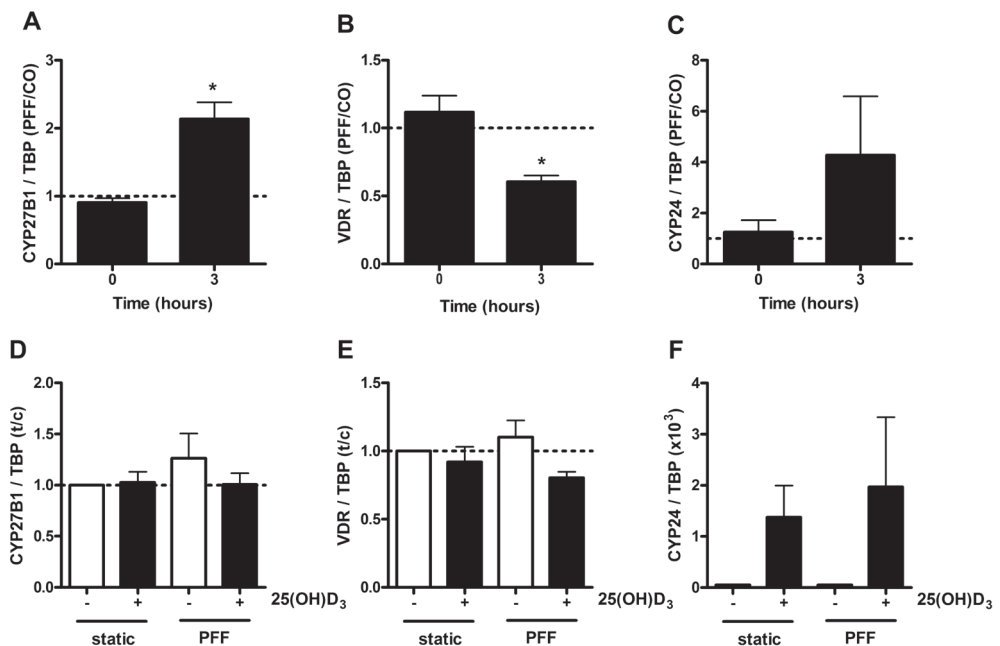

Figure 3. Effects of PFF on mRNA levels of genes involved in vitamin D metabolism. Primary human osteoblasts were subjected to 1 h PFF or static treatment and 0 or 3 h after PFF or static treatment mRNA levels of CYP27B1 (**A**), VDR (**B**) and CYP24 (**C**) were determined. Primary human osteoblasts were also subjected to 1 h PFF or static treatment followed by 24 h static post-incubation in medium supplemented with 0 or 400 nM 25(OH)D$_3$ and mRNA levels of CYP27B1 (**D**), VDR (**E**) and CYP24 (**F**) were determined. Results (mean ± SEM) are expressed as treatment versus control ratios (A-E; control was set at 1.0; dashed line) using cells from 7 (A-C) or 4 (D-F) different donors. PFF treatment was compared with static treatment using Wilcoxon signed rank test for each time-point (A-C). The effects of vitamin D supplementation and PFF on mRNA levels were analysed using Friedman test followed by Dunn's post hoc test (D-F) (*p<0.05).

At 24 h post-PFF incubation in medium with or without 25(OH)D$_3$, primary human osteoblasts did not respond to PFF by altered mRNA levels of CYP27B1, VDR and CYP24 (Fig. 3D-F).

Effects of PFF on the synthesis of 1,25(OH)$_2$D$_3$ from 25(OH)D$_3$

Because PFF stimulated CYP27B1 mRNA levels at 3 h after PFF treatment, we investigated whether PFF treatment followed by 24 h post-incubation in medium supplemented with 400 nmol/l 25(OH)D$_3$ resulted in an increase in 1,25(OH)$_2$D$_3$ synthesis by primary human osteoblasts compared to static treatment of cells. Primary human osteoblasts synthesized 1,25(OH)$_2$D$_3$ after 25(OH)D$_3$ exposure for 24 h under static and PFF treated conditions (mean levels of 146 and 121 pmol/l, respectively), but 1,25(OH)$_2$D$_3$ concentrations in medium from PFF treated cultures were not different from static cultures (Fig. 4).

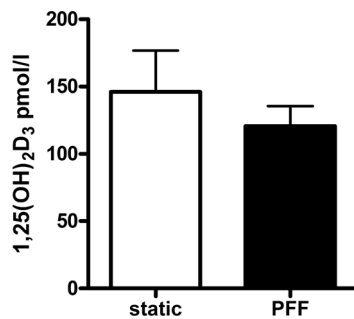**Figure 4. Effects of PFF on the synthesis of 1,25(OH)$_2$D$_3$ from 25(OH)D$_3$.** Primary human osteoblasts were subjected to 1 h PFF or static treatment followed by 24 h static post-incubation in medium supplemented with 400 nM 25(OH)D$_3$ and medium concentrations of 1,25(OH)$_2$D$_3$ were determined. Results are expressed as mean ± SEM using cells from 4 different donors. Results were analysed using Wilcoxon signed rank test.

DISCUSSION

The purpose of the present study was twofold. First, we investigated whether mechanical loading can affect vitamin D metabolism within primary human osteoblasts. We found that PFF temporarily increased CYP27B1 and decreased VDR expression, and that PFF treatment did not result in increased 1,25(OH)$_2$D$_3$ concentrations after 25(OH)D$_3$ exposure. Secondly, we investigated whether 1,25(OH)$_2$D$_3$ or 25(OH)D$_3$ affect the response of primary human osteoblasts to mechanical loading. We found that 1,25(OH)$_2$D$_3$ inhibited PFF-induced NO production, but PFF-induced expression of RANKL was not altered by either 1,25(OH)$_2$D$_3$ or 25(OH)D$_3$.

Bone adaptation to mechanical loading is governed by osteocytes which are the major mechanosensitive cells in bone tissue [17]. In addition to osteocytes, several studies have shown that osteoblasts are also very mechanosensitive [4;5;28;40;47]. In osteocyte-ablated mice subjected to reloading following unloading, the recovery of bone mass is not affected which suggests that osteoblasts are able to respond to the reloading stimulus [40]. In vitro studies have also shown that not only osteocytes respond to mechanical stimuli [18], but osteoblasts as well [4;5;28;47]. In our model, we used bone cells derived as outgrowth from trabecular bone fragments treated with collagenase, which results in a heterogeneous cell population. These cells show mainly characteristics that are typical for osteoblasts such as the activity of alkaline phosphatase and the expression and secretion of osteocalcin as well as P1NP [42], but characteristics that are typical for osteocytes have also been shown in our cell culture model such as the expression of matrix extracellular phosphoglycoprotein (MEPE) and fibroblast growth factor 23 (FGF23) (see sFig. 1) [8]. Thus the cells in our model are useful for studying bone cell mechanosensitivity.

Consistent with previous studies [4-6;47], we found that osteoblasts subjected to PFF treatment showed a marked increase of NO production. Moreover, we showed that the PFF-induced NO response was inhibited by pre-incubation with 1,25(OH)$_2$D$_3$, which is in line with a previous study in which wild type mouse long bone cells were used [47]. An inhibited PFF-induced NO response by 1,25(OH)$_2$D$_3$ pre-incubation could be due to a lower cell number since it has been shown that 1,25(OH)$_2$D$_3$ inhibits osteoblast proliferation [42]. However, after 24 h of treatment an effect of 1,25(OH)$_2$D$_3$ on cell number was not observed (unpublished observations) showing that cell number is not the reason for the lower PFF-induced NO response by 1,25(OH)$_2$D$_3$ pre-incubation. In addition to a reduction of osteoblast proliferation, 1,25(OH)$_2$D$_3$ is also able to enhance osteoblast differentiation. Therefore, 24 h pre-incubation with 1,25(OH)$_2$D$_3$ may result in more differentiated cells than non-treated cells in our model. Because osteocytes are more mechanosensitive than osteoblasts, it can be speculated that a more differentiated cell may produce more NO after mechanical stimuli, and not less. Thus if cell differentiation state is affected by 1,25(OH)$_2$D$_3$, it does not explain the inhibited NO response to PFF treatment.

It has been proposed that mechanical loading and 1,25(OH)$_2$D$_3$ interact as both factors can affect intracellular calcium concentrations [47]. Mechanically stimulated osteoblasts show an increase of intracellular calcium concentrations essential for the activation of the calcium-calmodulin dependent nitric oxide synthase (NOS) enzymes, whereas the non-genomic effect of 1,25(OH)$_2$D$_3$ is also to increase intracellular calcium concentrations [29;46;47]. Indeed, it has been shown that 1,25(OH)$_2$D$_3$ not only diminished the mechanical loading-induced response in wild type mouse long bone cells, but also in VDR knockout mouse long bone cells, which suggests that 1,25(OH)$_2$D$_3$ can act independently of genomic VDR [47]. Therefore, in our model the inhibited PFF-induced NO-response by 1,25(OH)$_2$D$_3$ may be due to effects on

intracellular calcium concentrations. Supportive is that calcium-modulating agents such as gadolinium trichloride (GdCl$_3$), EGTA, and TMB-8 also reduce the NO production by primary human osteoblasts in response to PFF (data not shown).

Under static conditions we did not find any effect of 1,25(OH)$_2$D$_3$ on NO production. This result is supported by a study in mouse osteoblastic MC3T3 cells in which no effect of 1,25(OH)$_2$D$_3$ (10^{-7} mol/l) on basal NO synthesis was observed [32]. However, this effect is probably concentration dependent, because at low 1,25(OH)$_2$D$_3$ (10^{-11} mol/l) levels MC3T3-E1 osteoblasts show an increased NO production and iNOS gene expression [47].

In addition to the PFF-induced NO response, primary human osteoblasts responded to PFF by increased RANKL mRNA levels which is in line with previous studies performed in an in vitro murine and human osteoblast model [21;28]. In vivo, increased RANKL mRNA levels have also been shown in a murine tibia exposed to vibration compared to a non-exposed tibia [15] and during mandibular distraction osteogenesis in rats [49]. Since RANKL is essential for osteoclast differentiation as well as activation [24], our result indicates that mechanical loading is not only involved in bone formation, but also in bone resorption. In contrast to our study, several studies show that mechanical stimuli lead to reduced RANKL mRNA levels, but those results were obtained from bone marrow stromal cell cultures [12;34-36]. In MC3T3-E1 osteoblasts [39] and in primary human osteoblasts [23] also reduced RANKL mRNA levels were observed after mechanical stimuli, but these cells were exposed to mechanical stimuli in the form of cyclic tensile strain and a longer period of mechanical stimulation was applied. Thus effects of mechanical stimuli on RANKL expression levels depend partly on maturation state as well as method and duration of mechanical stimulation. Furthermore, we showed that pre-incubation with 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ did not affect the RANKL-response to PFF. Possibly RANKL mRNA is already maximally stimulated by 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$, whereby no synergistic effects of PFF were observed. Thus, 1,25(OH)$_2$D$_3$ may affect the response of osteoblasts to mechanical loading with respect to NO, but not to RANKL.

The precursor 25(OH)D$_3$ did not significantly affect the mechanosensitivity of primary human osteoblasts. However, 25(OH)D$_3$ is able to influence osteoblast function as shown by increased mRNA levels of CYP24, RANKL and osteocalcin, similar to 1,25(OH)$_2$D$_3$. Effects of 25(OH)D$_3$ are most likely to occur after conversion to 1,25(OH)$_2$D$_3$ by 1α-hydroxylase [3;14;42;43]. However, it can not be excluded that 25(OH)D has also direct effects on osteoblasts, as discussed previously [42].

A higher conversion of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ and subsequently a higher local availability of 1,25(OH)$_2$D$_3$ may be beneficial for bone tissue. We showed that mechanical stimuli increased mRNA levels of CYP27B1 in primary human osteoblasts. This result is supported by a study in human macrophages in which increased CYP27B1 mRNA levels were demonstrated in response to mechanical loading in the form of cyclic hydrostatic pressure. After 25(OH)D$_3$ exposure, that study also ob-

served an increased synthesis of 1,25(OH)$_2$D$_3$ in macrophages [11]. We showed an increased expression of CYP27B1 after PFF which suggests that PFF may enhance the conversion rate of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$. However, this could not be demonstrated in our experimental setup. It is possible that the conversion to 1,25(OH)$_2$D$_3$ by 1α-hydroxylase was actually enhanced by PFF, but was immediately followed by the further conversion of 1,25(OH)$_2$D$_3$ to other metabolites via 24-hydroxylation such as 1,24R,25(OH)$_3$D$_3$, thereby neutralizing the effect. Supportive is that CYP24 mRNA levels were strongly increased after both 25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ treatment, indicating that 1,25(OH)$_2$D$_3$ is further metabolized to 1,24R,25(OH)$_3$D$_3$ by the enzyme 24-hydroxylase. Primary human osteoblasts have a fast vitamin D$_3$ metabolism which has been demonstrated in a previous study in which after 24 h 25(OH)D$_3$ levels in conditioned medium were reduced to 29% of values in non-conditioned medium [42]. This study also showed that 25(OH)D$_3$ was soon converted to 24R,25(OH)$_2$D$_3$ by 24-hydroxylase [42]. Thus, our results suggest that CYP27B1 may be regulated by mechanical stimuli, but additional research is needed to find out whether an increased CYP27B1 expression leads to an enhanced conversion to 1,25(OH)$_2$D$_3$ on earlier time-points and whether the quantity of 1,24R,25(OH)$_3$D$_3$ is affected.

Primary human osteoblasts increased their CYP27B1 mRNA levels, but reduced their VDR mRNA levels in response to PFF treatment. VDR mRNA level regulation is important for the responsiveness to 1,25(OH)$_2$D$_3$, as the biological activity of 1,25(OH)$_2$D$_3$ has been shown to be proportional to the amount of the VDR [26;30]. Thus mechanical stimuli may attenuate the responsiveness of osteoblasts to 1,25(OH)$_2$D$_3$ in our model. However, 1,25(OH)$_2$D$_3$ not only exerts genomic effects through the nuclear VDR, but also non-genomic effects which probably occur through a plasma membrane receptor and second messengers [13;25]. These rapid non-genomic effects, such as the opening of calcium and chloride channels in osteoblasts [13], may not be diminished after mechanical loading in our model.

A limitation of this study is that only one time-point (24 h post-incubation) was tested to investigate whether PFF-treated osteoblasts produce higher 1,25(OH)$_2$D$_3$ levels in medium than static-treated osteoblasts. However, it has been shown that after an incubation time of 24 h osteoblasts metabolize sufficient 1,25(OH)$_2$D from 25(OH)D$_3$ to detect in medium [42]. Moreover, an increased quantity of 1,25(OH)$_2$D$_3$ in medium at later time-points is less likely because CYP27B1 expression levels were only temporarily increased. Another limitation is the use of relatively high concentrations of 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$ compared to normal serum concentrations which makes it difficult to translate the results to in vivo. However, the high concentrations of 25(OH)D$_3$ used in our model were necessary for the synthesis of detectable levels of 1,25(OH)$_2$D$_3$ by osteoblasts. Another point that can be made is that this study focused on the acute response of osteoblasts to pulsatile fluid flow, but long-term effects of pulsatile fluid flow on osteoblast function and local vitamin D metabolism may be different.

In conclusion, $1,25(OH)_2D_3$ may affect the response of primary human osteoblasts to mechanical stimuli, at least with respect to NO production. The conversion rate of $25(OH)D_3$ to $1,25(OH)_2D_3$ was not affected by mechanical stimuli in our model, but mechanical stimuli did increase CYP27B1 mRNA levels and reduced VDR mRNA levels suggesting a role for mechanical loading in local vitamin D metabolism in bone tissue. Thus, $1,25(OH)_2D_3$ and mechanical loading, both stimuli of the differentiation of osteoblasts, may interact at the cellular level.

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SUPPLEMENTARY MATERIAL
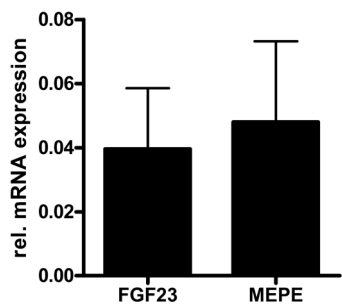
sFigure 1. FGF23 and MEPE mRNA levels in primary human osteoblasts. FGF23 and MEPE mRNA levels were determined in primary human osteoblasts cultured in medium without $25(OH)D_3$ or $1,25(OH)_2D_3$. Results are expressed as mean ± SEM using cells from 5 or 6 different donors.