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Pulsating Fluid Flow Increases Prostaglandin Production by Cultured Chicken Osteocytes—A Cytoskeleton-Dependent Process

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It has been postulated that the transduction of mechanical stress signals to bone cells occurs via loading-dependent flow of interstitial fluid through the lacuno-canalicular network of bone. We have shown earlier that chicken osteocytes release enhanced amounts of prostaglandin E_2 after 1 h treatment with pulsating fluid flow (PFF, 0.5 ± 0.02 Pa, 5 Hz). Here we study the acute response to PFF on three cell populations derived from fetal chick calvariae, namely periosteal fibroblasts (PF), an osteoblast and osteocyte containing population (OBmix), and osteocytes (OCY), and the involvement of the actin-cytoskeleton in this process. All three cell populations rapidly (OCY: within 5 min, OBmix, PF: within 10 min) increased their release of prostaglandins E_2 and I_2 in response to PFF, but the response by OCY was 2–4 times higher than that by OBmix or PF. Disruption of the actin-cytoskeleton by cytochalasin B completely abolished the response. We conclude that osteocytes are more sensitive to fluid shear stress than immature bone cells, and that the actin-cytoskeleton is involved in the response to fluid flow.

The primary function of bone is to provide mechanical support for the soft tissue. During growth, but also in the adult skeleton, individual bones are continuously modified in response to mechanical stimuli. Skeletal unloading results in bone loss, whereas increased loading leads to increased bone mass (1). The mechanisms underlying this process of mechanical adaptation are, however, poorly understood. The prevailing view is that mechanical loading causes deformation (strain) of the bone matrix, and that bone cells, i.e. osteocytes and osteoblasts, act as sensors of deformation in bone tissue and trigger metabolic effects when a deformation threshold is surpassed (2,3). Osteocytes represent by far the most abundant cell type of adult bone and are anatomically in a favorable position to detect strain within the bone matrix. Osteocytes make contact with each other and with the cells on the bone surface via gap junction-coupled cell processes that pass through bone via narrow channels, the canaliculi (4). Therefore, electrical coupling as well as intracellular and extracellular transport of signal molecules are possible throughout the bone tissue. The mechanism of mechanical activation of the sensor cells is however debated. Recent theoretical (2,3,6) as well as experimental studies suggest that, rather than the direct straining of bone cells (5), flow of interstitial fluid through osteocyte canaliculi provides the stress-derived mechanical signal for bone cells. Bone is in some ways comparable to a stiff, water soaked sponge. A compressive force on the sponge will squeeze water out of it. Similarly, mechanical loading will cause a flow of interstitial fluid through the canalicular network of bone (6,7,8). Weinbaum et al. (2) have proposed that this flow of interstitial fluid through canaliculi provides the mechanism by which osteocytes sense the very small in vivo strains of the calcified matrix. If so, osteocytes must be sensitive to very small fluid shear stresses.

Abbreviations used: AA, arachidonic acid; PFF, pulsating fluid flow; PGE_2, prostaglandin E_2; PGI_2, prostaglandin I_2; OCY, osteocytes; PF, periosteal fibroblasts; OBmix, osteoblast- and osteocyte-containing population.

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It has been shown that osteoblasts react to mechanical stimulation (9-13). Stretching of bone cells cultured on different substrates has been shown to induce PGE_2 production (9,10). Mechanical loading by continuous fluid flow was demonstrated to increase PGE_2 production and inositol triphosphate levels in cultured osteoblasts (11,13). Osteoblasts also reacted to fluid flow with increased intracellular cAMP levels, and this response was mediated by prostaglandins (12). Osteocytes were also found to be sensitive to mechanical stress (14,16,17). Mechanical loading of rat vertebrae in vivo has been shown to increase insulin-like growth factor-I mRNA expression in osteocytes, and this response was found to be mediated by prostaglandins (14). Recent development of an osteocyte isolation procedure from a heterogeneous population of bone cells (15) has enabled us to subject isolated osteocytes to mechanical stress in vitro. We found, in an earlier study, that pulsating fluid flow (PFF) stimulates a sustained PGE_2 production by isolated osteocytes in culture when these cells were subjected to 1h PFF and subsequently incubated for 1h in the absence of PFF (16). In another study, chicken calvarial osteocytes responded to PFF with a rapid increase (within 5 min) of nitric oxide production (17). In sum, ample evidence is available that bone cells, in particular osteocytes respond to mechanical stimuli in vivo and in vitro. It is, however, still a matter of speculation how an external physical stimulus such as flow is translated into an intracellular biochemical response. It has been suggested that the cytoskeleton might be involved in this mechanotransduction process (18). The cytoskeleton is physically linked through transmembrane matrix adhesion receptors such as integrins to the extracellular surface and also connected (either directly or indirectly) to intracellular structures (e.g. phospholipases, protein kinases), thus providing a route of transduction of a physical stimulus.

In this study, we tested the hypothesis that bone cells acutely react to mechanical stress with changes in prostaglandin production and that the cytoskeleton is involved in the process of mechanotransduction. We studied the acute prostaglandin response to PFF by three cell populations derived from fetal chick calvariae, namely periosteal fibroblasts (PF), an osteoblasts and osteocyte containing population (OBmix) and osteocytes (OCY), and the involvement of the actin-cytoskeleton in this process.

MATERIALS AND METHODS

Isolation and culture of chicken bone cells. Fetal chicken calvarial cells were obtained as previously described (19). Briefly, calvariae of 18-day-old chicken fetuses were aseptically dissected and the periostea removed. A mixture of osteoblasts and osteocytes (OBmix) was isolated from the bones after removal of the periostea by sequential collagenase/EDTA digestion. The cell fractions were pooled and precultured for 1 day in alpha minimum essential medium (αMEM; Gibco, Paisley, Scotland) containing 2% chicken serum (Sigma, St. Louis, MO, USA), 1.4 mM 1-glutamine (Sigma), 0.3 mM 1-ascorbic acid (Merck, Darmstadt, Germany), 5.6 mM glucose (Gibco), and 0.5 μg/ml gentamicin (Gibco) (from now on referred to as complete medium). Then they were harvested by a 5 min treatment with 0.05% trypsin (Sigma)-0.01% EDTA (Merck) in phosphate buffered saline and either seeded immediately onto chicken osteoblast conditioned medium coated glass slides at 5×10^5 cells/slide for PFF experiments, or first subjected to immunomagnetic isolation of osteocytes (OCY) using the monoclonal antibody MAb OB 7.3 as described earlier (15). Briefly, the single cell suspension was incubated with MAb OB 7.3 bound to magnetic beads. Mab OB 7.3 reacts with an antigen present on the cell surface of osteocytes, but not osteoblasts or periosteal fibroblasts. Rosetted and non-rosetted cells were separated by inserting the test tube into a magnetic field, which drew the beads with attached cells to one side of the tube, allowing the removal of the non-rosetted cells with a pipette. In this study rosetted cells (1 to 8 beads/cell) were used as isolated osteocytes unless stated otherwise. In some experiments, osteocytes were freed from beads by incubating them with excess of MAb OB 7.3 (18). More than 95% of the resulting cell population were osteocytes as shown by staining with MAb OB 7.3. Osteocytes with or without beads were seeded onto glass slides, previously coated with chicken osteoblast conditioned medium, cultured for one day in complete medium, and used for experiments the next day. Periosteal fibroblasts (PF) were isolated from the calvarial periostea by collagenase digestion (17), seeded into tissue culture flasks, and precultured for 1 day in complete medium. The next day, the cells were harvested, resuspended in complete medium, seeded onto chicken osteoblast conditioned medium coated glass slides at 5×10^5 cells/slide, and used for experiments the next day. Chicken osteoblast conditioned medium is obtained by serum starving confluent OBmix cultures during 24 hours. The resulting conditioned medium is collected and used at 1x concentration for coating (20).

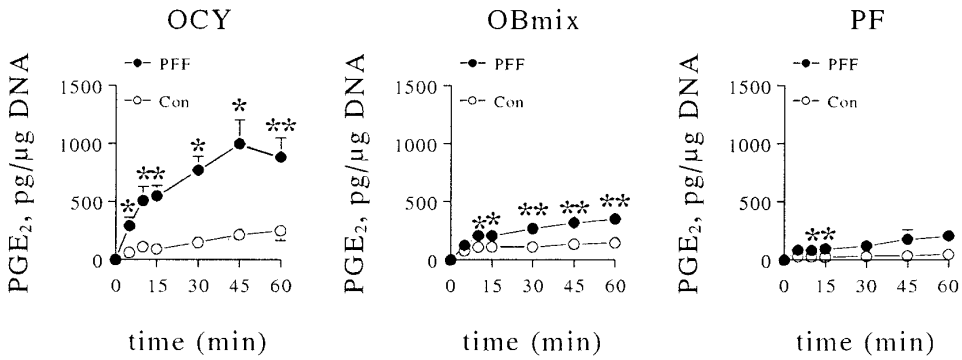

FIG. 1. Effects of pulsating fluid flow (PFF) on cumulative PGE_2 production by OCY, OBmix and PF. Values are means±SE of 4–8 separate experiments. Significant effect of PFF: $*P<0.05$, $**P<0.01$.

Pulsating fluid flow. PFF (5 Hz) was generated by pumping 15 ml of culture medium through a parallel-plate flow chamber (21) containing a glass slide with attached cells as previously described (13). Maximal fluid shear stress was 0.5 ± 0.02 Pa, and the peak stress rate 0.4 Pa/sec. All components of the apparatus were kept in a 37°C incubator. The flow medium consisting of complete medium in which serum was substituted by 0.1% bovine serum albumin (BSA), was continuously saturated with 5% CO_2 in air. Control cultures were kept in stationary culture at 37°C in a humidified atmosphere of 5% CO_2 in air. Medium samples of 2 ml each were withdrawn every 5 or 15 min from both experimental and control cultures. To maintain a constant volume the cultures were replenished with fresh medium. Samples were stored until analysis at −20°C.

Disruption of actin cytoskeleton. To investigate the role of the cytoskeleton in the prostaglandin response of cells to PFF, OBmix populations were pretreated for 60 min prior to PFF treatment with 1 μM cytochalasin B (Sigma), a potent actin-filament disrupting agent. PFF treatment was then carried out in the presence of cytochalasin B. In order to determine the optimal pretreatment procedure of OBmix cells with cytochalasin B, cells were incubated with various concentrations of cytochalasin B (1, 5, and 10 μM) for various times (10, 30 and 60 min). The actin-cytoskeleton was subsequently stained with FITC-labelled phalloidin (Sigma), according to Small and Langanger (22).

Prostaglandin extraction. Prostaglandins (PGs) were extracted from culture media using columns of octadecylsilyl-silica according to Powell (23). Medium samples were acidified to pH 3 with 1 N HCl and passed through a Sep-Pak cartridge (C_8 125 Å, Waters, Millipore, MA, USA), which was pretreated with 2 column volumes of methanol, followed by 1 column volume of water. The cartridges were placed on a vacuum container, the column was washed with 3 times 2 ml of water, 1 ml of petroleum ether, and prostaglandins eluted with 2 times 1 ml chloroform. The chloroform fractions were combined, evaporated under nitrogen, and the residu was dissolved in 200 μl of assay-buffer consisting of 0.1 M phosphate-buffer, pH 7.5, containing 0.01% thimerosal, 0.9% NaCl, and 0.1% BSA.

Medium prostaglandin E_2 (PGE_2) and I_2 and (PGI_2) determinations. Extracted samples of 50 μl were assayed for PGE_2 and 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, using a non-radioactive enzyme immunoassay system (Amersham, Buckinghamshire, UK).

DNA content. DNA content of the cell layers was determined according to Rao and Otto (24). Bisbenzimid H 33258 (Hoechst reagent; Riedel-De Haën AG, Seelze-Hannover, Germany) was used as color reagent for these determinations. DNA content was quantitated by measuring the fluorescence at 460 nm (exitation 355 nm) using purified calf thymus DNA as a standard.

Statistical analysis. The data from several independent experiments were pooled, and are expressed as mean ± SEM. Significance of differences among means was determined using Student's *t* test, for unpaired observations.

RESULTS

Figs. 1 and 2 demonstrate the effects of PFF on PGE_2 (Fig. 1) and 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2 (Fig. 2) productions in OCY, OBmix and PF cultures. The production rates and the treatment-over-control values of the two prostaglandins in all three populations were highest immediately after the onset of PFF. PFF stimulated PGE_2 production 4.5-fold in OCY, 1.3-fold in OBmix, and 2.5-fold in PF when measured after 5 min. The cumulative PGE_2 production after 1 h of PFF amounted to 882 ± 169 pg/μg DNA in PFF-treated OCY cultures and to 252 ± 87 pg/μg DNA in stationary control cultures. In OBmix and PF cultures the

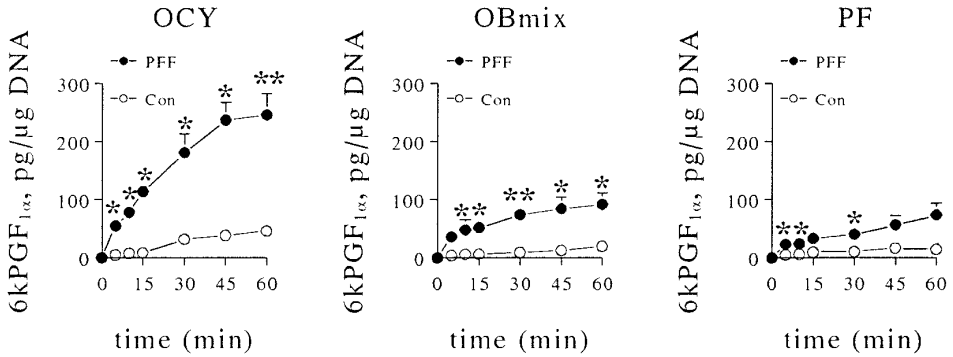

FIG. 2. Effects of pulsating fluid flow (PFF) on cumulative 6-keto-$PGF_{1\alpha}$ production by OCY, OBmix and PF. Values are means±SE of 4 separate experiments. Significant effect of PFF: $*P<0.05$, $**P<0.01$.

cumulative experimental (PFF) resp. control PGE_2 values measured over 1 h were: 376 ± 144 pg/μg DNA resp. 157 ± 58 pg/μg DNA (OBmix), and 208 ± 69 pg/μg DNA resp. 50 ± 6 pg/μg DNA (PF).

The 6-keto-$PGF_{1\alpha}$ responses of the three populations had a similar pattern as the PGE_2 responses, although at a lower level of production (Fig. 2). PFF stimulated 6-keto-$PGF_{1\alpha}$ production 9.5-fold in OCY, 8-fold in OBmix, and 3-fold in PF when measured after 5 min. After 1 h the cumulative 6-keto-$PGF_{1\alpha}$ production values in PFF resp. stationary control cultures were for OCY 246 ± 37 resp. 47 ± 8, for OBmix 92 ± 19 resp. 20 ± 6, and for PF 74 ± 20 resp. 15 ± 4 pg/μg DNA.

The PGE_2 and 6-keto-$PGF_{1\alpha}$ responses to PFF are much higher in OCY than in either OBmix or PF (Figs. 1 and 2). In order to determine whether the presence of magnetic beads on OCY was responsible for these differences, we compared the PGE_2 production of osteocytes with beads and osteocytes from which the beads had been removed by a short incubation with excess MAb OB 7.3 prior to seeding (see also Materials and Methods). Fig. 3 shows the cumulative PGE_2 production of osteocytes with beads and osteocytes without beads after 10 min of PFF. There was no significant difference in PGE_2 production between the two groups.

As the prostaglandin production rate in response to PFF rapidly decreases with time (Figs. 1 and 2) and this decrease may be related to a decrease in PG substrate, i.e arachidonic acid (AA), we tested whether addition of AA to the culture medium affected the production rate. Fig. 4 shows the PGE_2 production by OBmix cells cultured in the presence or absence of 10 μM AA. There was no significant increase in PGE_2 production in PFF cultures containing AA

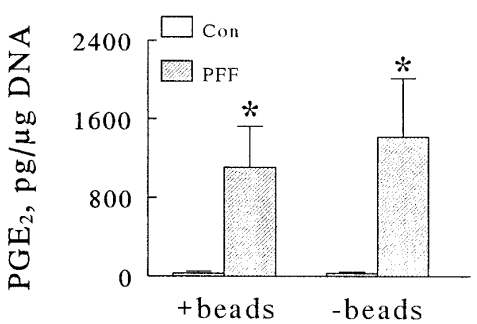

FIG. 3. Effects of 10 min PFF on cumulative PGE_2 production by osteocytes carrying beads (+beads) and osteocytes without beads (−beads). Values are means±SE of 4 separate experiments. Significant effect of PFF: $*P<0.05$.

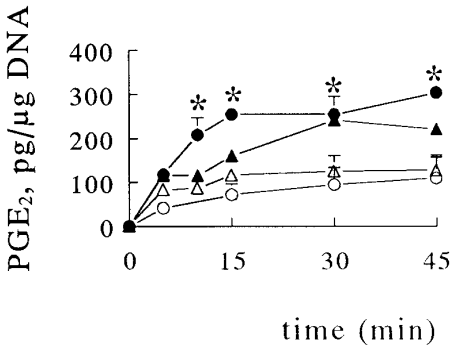

FIG. 4. Effect of PFF on cumulative PGE_2 production by OBmix cultured (flow or stationary culture) in the presence or absence of exogenously added arachidonic acid (AA, 10 μM). Open circles: stationary culture; closed circles: PFF; open triangles: stationary culture with AA; closed triangles: PFF with AA. Values are means±SE of 3 separate experiments. Significant effect of PFF: $*P<0.05$.

as compared to PFF cultures in which no AA was added to the culture medium. Both groups showed an initial increase of PGE_2 production which leveled off within 10-15 min. In stationary, control cultures 10 μM AA also did not change PGE_2 production.

Finally, we have tested whether disruption of the actin-cytoskeleton by cytochalasin B affects the prostaglandin response of chicken bone cells to PFF (Fig. 5). OBmix were treated with cytochalasin B at various concentrations and various treatment periods. The cells were stained with FITC-labelled phalloidin to visualize actin filaments. Treatment of OBmix with 1 μM cytochalasin B for 60 min, appeared to be sufficient to disrupt actin stress fibers present in these cells (results not shown). This treatment also prevented the flow-induced PGE_2 response when these cells were subsequently subjected to PFF (Fig. 5).

DISCUSSION

In the present study we examined the effects of pulsating fluid flow (PFF) on the acute prostaglandin production by isolated chicken bone cells. Osteocytes coupled to immunomagnetic beads (OCY), OBmix cells (a mixture of osteoblasts and osteocytes) and periosteal

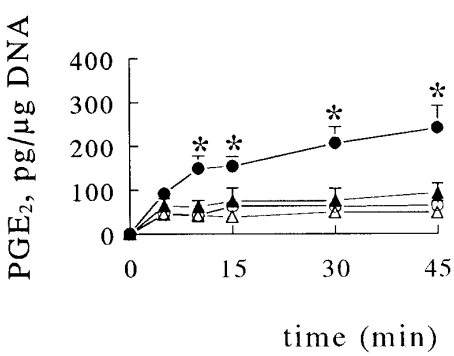

FIG. 5. Effect of cytochalasin B (1 μM) on PGE_2 response by OBmix cultured in the presence or absence of pulsatile fluid flow (PFF) for 45 min. Cells were pretreated with cytochalasin B or carrier (DMSO, 0.1 % final conc.) for 60 min and then subjected to PFF or stationary culture in the presence of cytochalasin B or carrier. Open circles: stationary culture; closed circles: PFF; open triangles: stationary culture with cytochalasin B; closed triangles: PFF with cytochalasin B. Values are means±SE of 4 separate experiments. Significant effect of PFF: $*P<0.05$.

fibroblasts (PF), all responded to PFF with increased PGE_2 and 6-keto-$PGF_{1\alpha}$ production. The onset of flow led to an initially high rate of PGE_2 and 6-keto-$PGF_{1\alpha}$ production which leveled off within 10-15 min thereafter. In studies with endothelial cells, a similar biphasic 6-keto-$PGF_{1\alpha}$ response was observed (21). The step change in flow-induced shear stress at the onset of flow provided an acute stimulation. After the initial period, the cell response attenuated to a constant but lower production rate.

The prostaglandin production was generally higher in OCY than in OBmix or PF, both in the stationary control cultures and in the flow stimulated cultures. The OCY used in the experiments described here still carried beads. Generally, the beads were not removed because of the large loss of osteocytes during the bead-removal procedure. It is reasonable to expect that osteocytes carrying beads might experience higher strains than osteocytes without beads, and that this might be reflected in higher prostaglandin responses to PFF. Therefore, we have compared the PGE_2 production of osteocytes with beads and osteocytes without beads after 10 min of PFF-treatment. The 10 min time period was chosen, because PGE_2 production rate was highest during the first 10-15 min after onset of flow. We did not find a difference in PGE_2 production, between osteocytes with beads and osteocytes without beads. This suggests that the presence of these beads does not affect the early PGE_2 response to PFF. In a recent study published by our group (16), we did find an enhanced PGE_2 production by osteocytes carrying beads as compared to osteocytes without beads. This however, was observed in a post-incubation period of 1 h, following 1 h of PFF-treatment, suggesting that the presence of beads enhanced the sustained response to PFF rather than the acute response (16). All three calvaria-derived populations, OCY, OBmix and PF, showed lower levels of 6-keto-$PGF_{1\alpha}$ than of PGE_2 production. It is still a matter of speculation whether one of these two prostaglandins (PGE_2 or 6-keto-$PGF_{1\alpha}$) should be considered more important than the other, in the cellular response to mechanical loading. We showed earlier that 1h treatment with PFF continued to enhance the production of PGE_2 but not 6-keto-$PGF_{1\alpha}$ during 1h post-incubation without flow (16). Together with the present data, this suggests that both prostaglandins are involved in the acute response of bone cells to PFF, while PGE_2 appears to be more important in long term responses.

In the present study we used perfusion media that did not contain serum. Since it has been reported that serum contains AA (13), which is converted into prostaglandins, we determined whether the decrease in PGE_2 and 6-keto-$PGF_{1\alpha}$ production rates, after the initial outburst was a result of AA limitation. Therefore, we exposed OBmix cells to PFF in the presence or absence of 10 μM AA. We did not observe any significant differences in the PGE_2 production profiles suggesting that there was no substrate limitation in the experiments reported here.

Several studies suggest that the cytoskeleton is involved in the process of mechanotransduction (18,25). Of the various components of the cytoskeleton, actin-filaments are likely linked to the prostaglandin response. It has been recently shown that actin filaments can bind to a protein kinase C isoform (protein kinase C_ϵ), thus activating this kinase (26). In another study, protein kinase C was shown to mediate flow-induced PGE_2 production in osteoblasts (13). Here, we have shown that the presence of cytochalasin B, a cytoskeleton disruptive agent, in the medium abolishes the fluid flow induced PGE_2 production in OBmix cells. This indicates that the cytoskeleton is involved in the transduction of the extracellular mechanosignal to the intracellular domain, and in the translation into cellular signals (prostaglandins). Activation of protein kinase C by the mechanostimulated cytoskeleton may lead to increased prostaglandin production.

In summary, the present data indicate that prostaglandin production plays a significant role in the transduction of the flow stimulus into a biochemical message in bone cells. Furthermore, osteocytes appeared to be the most stress sensitive cell type of bone. These cells are capable of acutely responding to fluid shear stresses as low as 0.5 Pa. This suggests that load-induced

fluid flow is the external signal involved in mechanotransduction and that chemical messengers like PGE_2 and PGI_2 act as local signalling molecules in the process of translating a mechanical strain into a biochemical response. Finally, the mechanical signal of PFF seems to be transduced intracellularly, at least in part, via the actin cytoskeleton.

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