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The effect of cytoskeletal disruption on pulsatile fluid flow-induced nitric oxide and prostaglandin E_2 release in osteocytes and osteoblasts

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Abstract

Fluid flowing through the bone porosity might be a primary stimulus for functional adaptation of bone. Osteoblasts, and osteocytes in particular, respond to fluid flow in vitro with enhanced nitric oxide (NO) and prostaglandin E_2 (PGE_2) release; both of these signaling molecules mediate mechanically-induced bone formation. Because the cell cytoskeleton is involved in signal transduction, we hypothesized that the pulsatile fluid flow-induced release of NO and PGE_2 in both osteoblastic and osteocytic cells involves the actin and microtubule cytoskeleton. In testing this hypothesis we found that fluid flow-induced NO response in osteoblasts was accompanied by parallel alignment of stress fibers, whereas PGE_2 response was related to fluid flow stimulation of focal adhesions formed after cytoskeletal disruption. Fluid flow-induced PGE_2 response in osteocytes was inhibited by cytoskeletal disruption, whereas in osteoblasts it was enhanced. These opposite PGE_2 responses are likely related to differences in cytoskeletal composition (osteocyte structure was more dependent on actin), but may occur via cytoskeletal modulation of shear/stretch-sensitive ion channels that are known to be dominant in osteocyte (and not osteoblast) response to mechanical loading.

Keywords: Osteocytic; Osteoblastic; Pulsatile fluid flow; Nitric oxide; Prostaglandin E_2; Cytoskeleton; Actin; Microtubules

Optimal bone mass and architecture is maintained throughout life by adapting to the prevailing mechanical environment in a continuous sequence of bone remodeling. Bone remodeling is presumably initiated and sustained by a disparity between the actual loads experienced by bone cells and the normal homeostatic loads. Several studies suggest that the interstitial fluid through the bone porosity provides the stimulus for bone adaptation [1]. Fluid flow through the bone porosity has been experimentally observed by visualizing molecular tracer transport in rat tibia under four-point bending [2] and it has been estimated that strain-derived fluid shear stress in the lacunar–canalicular porosity ranges from 0.8 to 3 Pa [3]. Indeed, many in vitro studies have reported that bone cells respond to fluid shear stress in this range with, for example, increased intracellular calcium concentration [4], the release of nitric oxide (NO) and prostaglandin E_2 (PGE_2) signaling molecules [5–9], and increased cyclooxygenase-2 (COX-2) expression [10,11]. Studies which have compared the effects of fluid flow and strain on bone cells report increased NO and PGE_2 responsiveness to fluid flow stimulation [12,13], which appears to be as a result of the larger cellular deformation caused by fluid flow at commonly applied 'physiological' magnitudes [14]. It has also been shown that osteocytes are more responsive to fluid flow stimulation than other osteogenic cells [5,15]. While the hypothesized osteocyte responsiveness in vivo may be attributed to the mechanical environment associated with their anatomical location [7,14], the higher responsiveness of osteocytes in vitro is not understood, but may be related to differences in cell

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morphology or cytoskeletal composition. It is evident that fluid flow stimulates certain biological responses in osteogenic cells, although the mechanisms by which these cells detect mechanical forces and transduce such signals across the membrane to activate signaling pathways involved in bone cell metabolism are not clear.

In vivo, NO mediates mechanically induced bone formation [16,17] and inhibition of NO production suppresses bone formation [18]. Prostaglandins are also involved in adaptive response to loading [19]. Mechanical loading of canine cancellous bone explants has been found to increase prostaglandin release [20]. Indomethacin, which blocks PGE_2 activity, administered just prior to mechanical loading modulates this osteogenic response [21]. As already mentioned, several in vitro studies confirm that mechanical stimulation induces both NO and PGE_2 release in osteogenic cells over a range of time points [1,6–8,13,22]. It has been reported that NO and PGE_2 responses to fluid flow are caused by the magnitude and rate of the fluid shear stress [9,22,23], and not due to fluid flow-induced changes in streaming potential or nutrient diffusion. Activation of NO has been shown to involve conversion of L-arginine to NO via endothelial nitric oxide synthase (ecNOS) [8,24], and PGE_2 release involves COX-2 mediated conversion of arachidonic acid to PGE_2 [25]. However, how mechanical forces can influence and alter these signaling pathways in osteogenic bone cells remains unknown.

It is possible that both the integrin-cytoskeletal structure [26,27] and mechanosensitive cation channels [28,29] mediate the signaling pathways that lead to enhanced mechanically induced NO and PGE_2 response. The cytoskeleton has a large negatively charged surface area for linkage of mechanosensitive molecules [30] and for localization of enzymatic activity [27]. For example, protein kinase C [31], G protein dependent pathways [32], and phospholipases [33] have all been found to mediate mechanotransduction. The interconnected and viscoelastic nature of the cytoskeleton together with its coupling to the extracellular environment via receptor–ligand complexes is suited to the detection and transmission of mechanical stress to deep within cells, to organelles, and to the nucleus [27,34]. The cytoskeleton also affects membrane distortion, which can influence mechanically induced signaling in adherent cells [26,27]. Indeed, a role for the cytoskeleton in the transduction of mechanical signals has been confirmed in previous studies that establish cytoskeletal involvement in various mechanically induced responses: e.g., COX-2 production and c-fos expression in bone cells [11]; flow-induced osteocyte PGE_2 response [25]; and NO response in endothelial cells [35].

In the present study, we hypothesized that the actin and microtubule cytoskeleton in osteocytes and osteoblasts are involved in the transduction of mechanical forces to early NO and PGE_2 responses. If this would be true, differences in cytoskeletal composition may explain why osteocytes are more responsive than osteoblasts to fluid flow. To test this hypothesis, we subjected normal, actin-disrupted, and microtubule-disrupted osteoblastic and osteocytic cells to pulsatile fluid shear stress for 10 min in a parallel plate chamber, and subsequently: (i) measured the cumulative NO and PGE_2 released by each cell type, and (ii) obtained fluorescent images of the effects of both pulsatile fluid flow and cytoskeleton disruption on the actin cytoskeleton.

Methods

Cell culture. Osteoblastic MC3T3-E1 cells (OB) were cultured in alpha minimum essential medium (α-MEM; Gibco, Paisley, UK), supplemented with 10% fetal bovine serum (FBS; Gibco), 1% β-glycerophosphate, 1% L-glutamine (Sigma–Aldrich, St. Louis, MO, USA), 1% ascorbic acid (Merck, Darmstadt, FRG), and 0.5% fungizone and gentamicin antibiotics (Gibco). Osteocytic MLO-Y4 cells (OCY) were cultured in α-MEM, supplemented with 5% FBS, 5% calf serum (Gibco), 0.1% penicillin and streptomycin (Sigma–Aldrich), and 0.5% fungizone and gentamicin. The day preceding an experiment, 5×10^5 of either OB or OCY cells were plated on polylysine-coated (50 μg/ml; poly-L-lysine hydrobromide, mol wt $15–30 \times 10^4$; Sigma–Aldrich) glass slides such that the cells could be subjected to pulsatile fluid flow (PFF) stimulation in a parallel plate chamber (Fig. 1). Cells were cultured in petri dishes for 24 h in the appropriate medium (as described above) and were maintained at 37 °C in a 95% air and 5% CO_2 environment throughout the experiment.

Disruption of the actin and microtubule cytoskeleton. Prior to fluid flow stimulation, plated cells were incubated for 1 h in 13 ml of the appropriate medium with the total serum content reduced to 2% (referred to as experimental medium). Cells were then divided at random into three groups:

(i) untreated control medium;
(ii) actin-disrupted: medium supplemented with 1 μM cytochalasin B (Sigma–Aldrich), a toxin that prevents actin polymerization. The concentration of cytochalasin B used was based on a previous study in which it was shown that PFF-induced PGE_2 release in embryonic chicken calvarial osteocytes was dependent on the actin cytoskeleton [25];
(iii) microtubule-disrupted: medium supplemented with 1 μM colchicine (Sigma–Aldrich), a toxin that prevents microtubule polymerization. 1 μM of colchicine was used as that concentration was considered a medium concentration in a previous study [36].

We observed that these concentrations of both cytoskeleton-disrupting agents had a significant non-toxic effect on cell morphology within 1 h.

Pulsatile fluid flow stimulation. Cells were then selected at random from the three groups, i.e., untreated, actin-disrupted, or microtubule-disrupted. After the 1 h pre-incubation the 13 ml experimental medium was refreshed, i.e., replaced with the appropriate medium for that group. Cells were either: (i) cultured under static conditions in petri dishes containing 13 ml experimental medium for 10 min or (ii) subjected to PFF-derived shear stress of magnitude 0.39 Pa (3 Hz) or 0.64 Pa (5 Hz) for 10 min, as described in Bakker et al. [9]. The glass slides to which cells were attached served as the base of the parallel plate chamber. The pulsatile flow was generated by pumping 13 ml of experimental medium for each group through a parallel plate chamber using a roller pump in an incubator, also at 37 °C with the cycling

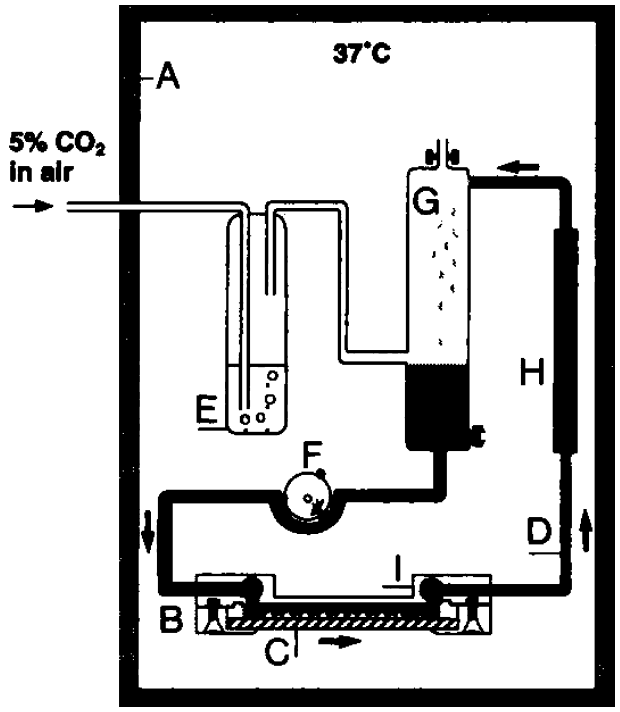

Fig. 1. Schematic illustration of the apparatus used to apply pulsatile fluid shear stress to osteocytes or osteoblasts by pumping experimental medium through a parallel plate chamber. 37° incubator (A); parallel plate chamber (B); polylysine-coated glass slide containing cells (C); experimental medium (D); H_2O (E); revolving pump (F); 5% CO_2–air mixture (G); flow probe (H); and plate with two openings through which experimental medium enters and exits chamber (I). See Methods for further explanation.

medium in the presence of a steady flow of CO_2 (Fig. 1). In both static and pulsatile fluid flow cultures, a 1 ml sample of medium was taken from the flow loop after 10 min for biochemical analysis. The rate of flow was confirmed using a small animal blood flow meter (T206, Transonic systems Inc.) included in the flow loop to confirm the accuracy of the flow characteristics above.

NO and PGE_2 measurement. Nitric oxide was measured as nitrite (NO_2^-) accumulated in the experimental medium using Griess reagent, consisting of 0.1% naphthylethylene–diamine–dihydrochloride, 1% sulfanilamide, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in medium were used to obtain a standard curve for each measurement. The absorbance was measured at 550 nm. The detection threshold of Griess reagent assay is 0.1 μM [37], which we have confirmed with $NaNO_2$ standards.

PGE_2 release in the conditioned medium was measured by an enzyme immunoassay (EIA) system (Amersham, Buckinghamshire, UK) using an anti-body raised against mouse PGE_2. The detection limit was 16 pg/ml. Absorbance was measured at 450 nm.

Statistical analysis. Data obtained from separate experiments were pooled and expressed as mean values ± SEM. Comparisons between groups were made using the Mann–Whitney *U* test. Differences were considered significant if $p < 0.05$.

Cytoskeletal staining. Immediately after 10 min pulsatile fluid flow (or as static controls) the glass slides containing either OBs or OCYs were removed from the parallel plate chamber and, along with static controls, were washed twice in phosphate-buffered saline (PBS) in petri dishes. The cells were then fixed using 4% formaldehyde in PBS for 15 min, washed in PBS, and permeabilized using 0.1% Triton in PBS for 10 min followed by two washes in PBS. To reduce non-specific binding, cells were then incubated in PBS containing 1% bovine serum albumin for 15 min. For actin staining, cells were incubated in ~80 μl FITC-phalloidin (Molecular Probes, Leiden, The Netherlands) for 45 min in a covered container and were then washed twice in PBS. FITC-phalloidin is a fluorescent label that binds to an antibody directed against f-actin. Hence, changes in the actin cytoskeleton due to pulsatile fluid flow and due to both actin and microtubule disruption could be visualized. The percentage of cells in which alignment of stress fibers occurred in each of the images obtained from four separate experiments was calculated as mean values ± SEM. Fluorescent images were obtained at 488 nm using a fluorescent microscope.

Results

The effects of pulsatile fluid flow and cytoskeletal disruption on the actin cytoskeleton

Application of pulsatile fluid flow increased the number of OBs in which formation and parallel alignment of stress fibers (actin filaments bundled into long fibers) occurred, from 6.6 ± 2.6% to 73 ± 7% of OBs (mean ± SEM) (e.g., Figs. 2A and B). Treatment of OB cultures with actin or microtubule disrupting agents (Figs. 2C–F) significantly reduced the number of OBs in which parallel alignment of stress fibers occurred when subjected to pulsatile fluid flow (to 12 ± 4% and 11 ± 5%, respectively). In OBs, both actin and microtubule disrupting treatments caused spots of actin intensity, presumably clustering at focal adhesion sites, in both static and pulsatile fluid flow-treated OB cultures (Figs. 2C–F).

Applying pulsatile fluid flow to untreated OCYs slightly increased formation and parallel alignment of stress fibers (e.g., Figs. 3A and B); the percentage of OCYs with this 'combed' appearance increased from 22.6 ± 3.6% to 36 ± 11% of OCYs (mean ± SEM, $n = 4$). Several OCYs treated with actin-disruptors had reduced cell body size, with rounded stellate morphologies extending thinner processes (e.g., Figs. 3C and D). Microtubule disruption had a less severe effect on morphology than actin disruption but did cause ruffling of actin boundaries/outer edges (Figs. 3E and F). The number of OCYs with parallel alignment of stress fibers due to pulsatile fluid flow treatment was significantly reduced (to 10 ± 0.3% of OCYs) when treated with actin disrupting agents (e.g., Fig. 3D).

The effects of pulsatile fluid flow and cytoskeleton disruption on NO and PGE_2 response

Neither the NO or PGE_2 response in OBs or OCYs when subjected to 3 Hz (0.39 Pa) was statistically different to those responses induced by 5 Hz (0.64 Pa) pulsatile fluid flow; results were therefore combined and expressed as PFF-treated relative to static controls. In OBs, a PFF-induced increase in NO release was inhibited by disrupting actin alone (Fig. 4A). A PFF-induced increase in PGE_2 release could be observed by

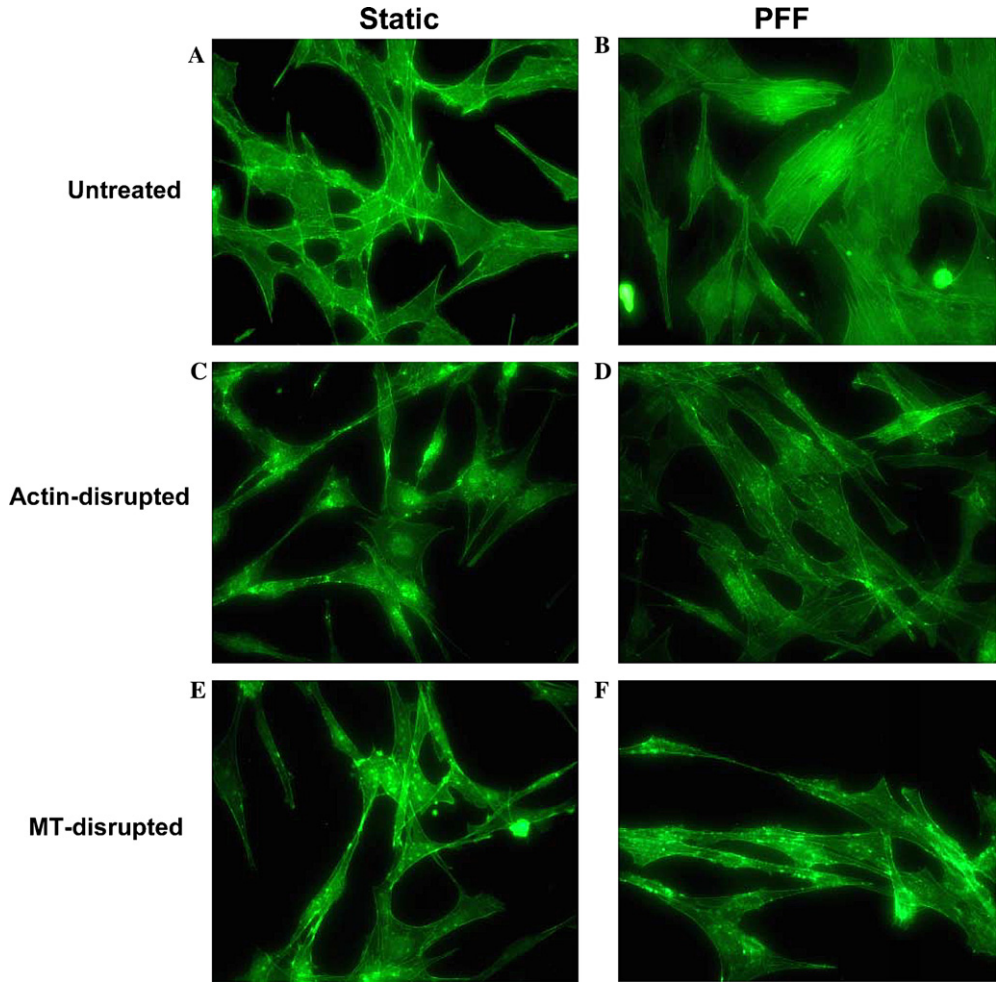

Fig. 2. Fluorescent staining of the actin cytoskeleton of osteoblastic (MC3T3-E1) cells illustrating the effect of pulsatile fluid flow and disruptors of both actin and microtubule (MT) polymerization (400× magnification).

disrupting either the actin or microtubule cytoskeleton (Fig. 4B).

In OCYs, there was a significant PFF-induced increase in NO release, which did not require an intact actin cytoskeleton but was enhanced with microtubule disruption (Fig. 5A). A large PFF-induced increase in PGE_2 release in OCYs required both an intact actin and microtubule cytoskeleton (Fig. 5B), i.e., it was inhibited if either cytoskeletal element was disrupted.

Discussion

Several of the early NO and PGE_2 responses to pulsatile fluid flow stimulation reported here (in the absence of cytoskeletal disruption) confirm previous results [5,6,9,14,22,25]. Furthermore, by visualizing the actin cytoskeleton we could explore the changes in the actin cytoskeleton that accompany the PFF-induced NO and PGE_2 responses measured—this approach yielded the following insights:

(i) there are considerable differences in actin and microtubule cytoskeleton involvement in NO and PGE_2 release between both OB and OCY cells; and
(ii) there are opposite effects of cytoskeletal disruption on OB and OCY PGE_2 responses.

In summary (as discussed in detail below), we can report that the mechanism by which the cytoskeleton modulates PFF-induced NO response in OBs appears to involve stress fiber formation and alignment, while the enhanced PGE_2 response seen in OBs is likely related to pulsatile fluid flow stimulation of focal adhesions formed when the cytoskeleton is disrupted in OBs. In OCYs (and also in OBs), we observe modulation of NO response by cytoskeleton disruption, which has also been seen in endothelial cells [38]. This suggests that NO response in osteogenic cells proceeds via a similar mechanism to that in endothelial cells. Finally, inhibition of PFF-induced PGE_2 response in OCYs (but not OBs) with cytoskeleton disruption may be due to the greater

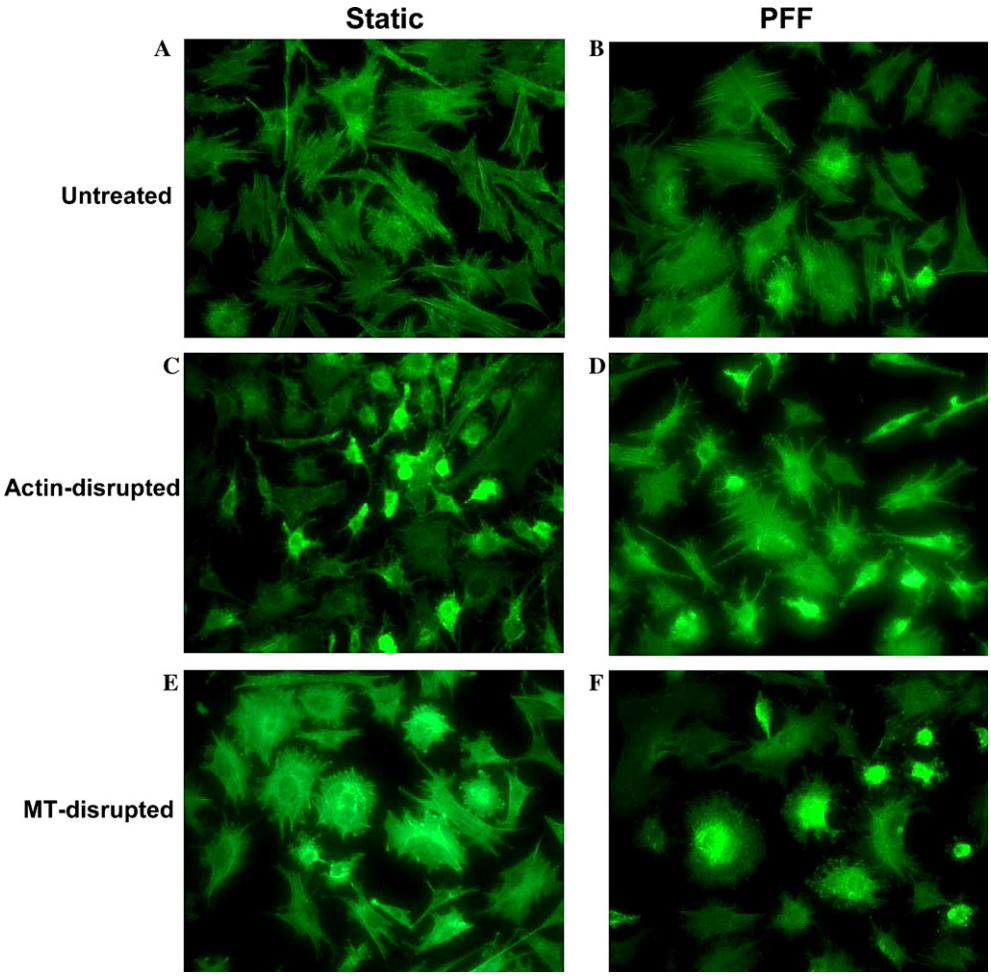

Fig. 3. Fluorescent staining of the actin cytoskeleton of osteocytic (MLO-Y4) cells illustrating the effect of pulsatile fluid flow and disruptors of both actin and microtubule (MT) polymerization (400× magnification).

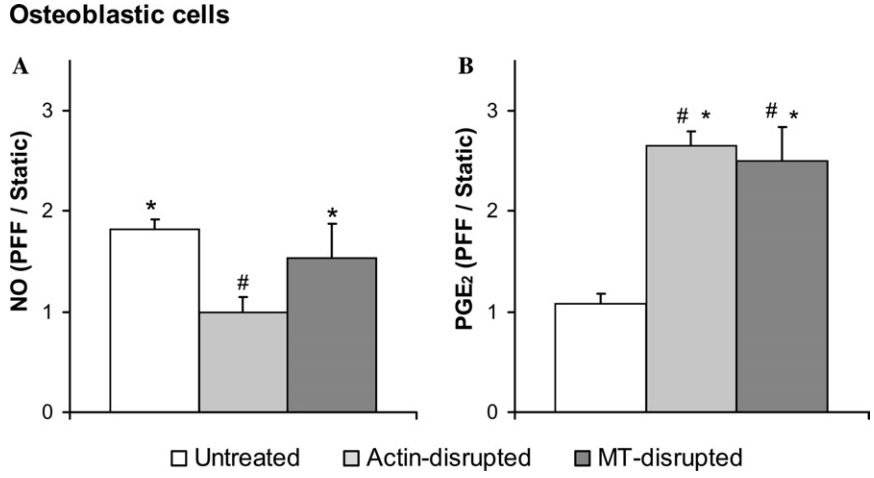

Fig. 4. The effect of actin and microtubule (MT) cytoskeleton disruption on pulsatile fluid flow-induced NO (A) and PGE_2 (B) release in osteoblastic cells. *Significantly different from 1; #significantly different from untreated. Values are means ± SEM, $p < 0.05$.

dependence of OCYs on their actin-rich cytoskeleton. However, it is also possible that cytoskeletal disruption may be modulating OCY PGE_2 responses to pulsatile fluid flow via shear/stretch-sensitive ion channels that are known to be more dominant in OCY response to mechanical loading (than in OBs) [29].

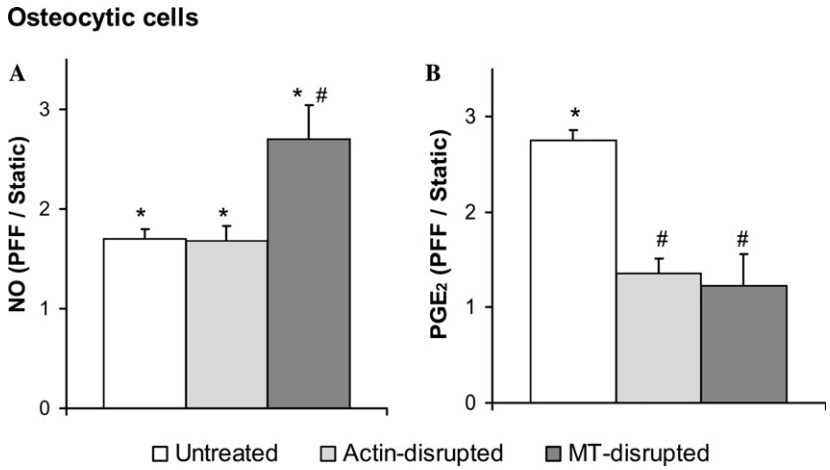

Fig. 5. The effect of actin and microtubule (MT) cytoskeleton disruption on pulsatile fluid flow-induced NO (A) and PGE_2 (B) release in osteocytic cells. *Significantly different from 1; #significantly different from untreated. Values are means ± SEM, $p < 0.05$.

In OBs, complete inhibition of PFF-induced NO response by actin cytoskeleton disruption (Fig. 4A) was accompanied by significantly reduced parallel alignment of actin bundles/stress fibers. This suggests that the mechanism of PFF-induced NO response is mediated by the actin cytoskeleton—this has not been previously observed in OBs, but has been reported for fluid flow-induced NO response in endothelial cells [39,40], which is of interest here because it has been shown that shear stress-induced NO production in primary OB cells proceeds via activation of endothelial cell nitric oxide synthase (ecNOS) [8] as it does in endothelial cells [38]. Accordingly, our result that the actin cytoskeleton (but not the microtubule cytoskeleton) is required for PFF-induced NO response in OBs suggests that NO response in bone cells may proceed via the same actin cytoskeleton-regulated pathway as in endothelial cells.

In OBs, disruption of the actin and microtubule cytoskeleton resulted in decreased stress fiber formation and alignment (Fig. 2), but simultaneously activated PFF-induced PGE_2 response (Fig. 4B). This indicates that (unlike NO response as discussed above) PFF-induced PGE_2 response is independent of actin cytoskeleton reorganization. In both static and PFF-stimulated OBs, we observe that cytoskeletal disruption causes actin clustering at focal adhesions. These results allow us to suggest that increased stimulation of these focal adhesions when subjected to pulsatile fluid flow elevates PGE_2 response in OBs. Interestingly, Ponik and Pavalko [41] similarly report that focal adhesion formation promotes fluid shear stress-induced PGE_2 release and COX-2 production (a key enzyme for PGE_2), also suggesting that focal adhesions mediate transduction of mechanical forces in OBs. Similarly, in the present study, increased PGE_2 response occurred only when increased formation of focal adhesions (via cytoskeletal disruption) was accompanied by pulsatile fluid flow. This does not, however, agree with Norvell et al. [42] in which higher concentrations of cytoskeleton disrupting agents than we have used here do not alter OB PGE_2 response to 90 min steady fluid shear stress. A key difference is that in their study the OBs were subjected to steady fluid flow—we have used the more physiologically relevant *pulsatile* fluid flow, which may explain why we measure PGE_2 response, also over a much shorter time-scale.

Few studies have investigated the effects of mechanical stimulation on osteocytes or OCY cell lines, as in the present study. We had anticipated higher responsiveness from OCYs relative to OBs, as osteocytes have been previously shown to be more mechanosensitive [5,15]. Although this was not the case with the cell lines we used, our results do uncover interesting differences between cytoskeletal involvement in OCY and OB responses to pulsatile fluid flow. As pulsatile fluid flow caused less stress fiber alignment in OCYs than in OBs, we cannot conclusively say that actin cytoskeleton reorganization is related to PFF-induced OCY NO or PGE_2 responses, as was the case for PFF-induced NO response in OBs. Zaman et al. [43] have shown that mechanical strain-induced NO production in OCYs proceeds via activation of ecNOS. As ecNOS activity in endothelial cells is mediated by the actin cytoskeleton (see above), we had anticipated that the actin cytoskeleton would also be involved in NO response in OCYs (as in OBs), but this was not the case. A possible explanation is that the cytoskeleton disrupting agents do not equally affect OBs and OCYs because of differences in actin cytoskeleton composition (see paragraph below). We additionally report that the PFF-induced increase in OCY NO response was further enhanced by microtubule disruption, which has not been reported previously for OCYs. However, an equivalent result for endothelial cells has been reported in which microtubule disruption

enhances fluid shear stress-induced ecNOS response [35]. Hence, this supports the possibility that mechanically induced OCY bone cell responses to mechanical loading also involve the same ecNOS mediated mechanisms as in endothelial cells (as mentioned above for OBs). Our results indicate that both an intact actin and microtubule cytoskeleton are required for PFF-induced PGE_2 response in OCYs. Although Ajubi et al. [25] also report that an intact actin cytoskeleton is required for PGE_2 response (in embryonic chicken calvarial osteocytes), to our knowledge no previous studies report microtubule involvement in OCY responses to mechanical stimulation. Since it has been suggested that cytoskeletal disruption modulates PGE_2 response via passage of calcium ions [28], it is therefore possible that the PFF-induced PGE_2 responses we have observed arise because cytoskeletal disruption alters mechanosensitive ion channel activity in OCYs (as discussed below).

The opposite effects of cytoskeletal disruption on OCY and OB PFF-induced responses, in particular PGE_2 release, are probably related to differences in cytoskeleton composition between both cells. The more significant effect of actin disruption (than microtubule disruption) on OCY morphology indicates that OCY structure is largely dependent on actin. This has also been observed by Tanaka-Kamioka et al. [44]. The membrane in actin-disrupted OCYs may therefore become incapable of maintaining tension [44] which would alter OCY [26,27] via altered mechanosensitive ion channel activity [45]. Indeed, this greater dependence of OCYs on actin in maintaining cell structure may impact on the shear/stretch sensitive ion channels that are known to be involved in mechanically induced PGE_2 release [25,29] when OCYs are treated with cytoskeleton disrupting agents, as in the present study. This may explain why we observe inhibition of PGE_2 in OCYs, but not in OBs. Indeed, it is interesting to compare our results to those of Rawlinson et al. [29], in which it was found that blocking shear/stretch sensitive cation channels abolishes glucose-6-phosphate dehydrogenase (G6PD) activity (for which PGE_2 response is required: [46]) in OCYs. However, in OBs, voltage-dependent calcium channels have the dominant influence in mechanically induced responses, and blocking shear/stretch sensitive cation channels only slightly reduces PGE_2 release. In the present study, that cytoskeletal disruption abolishes PGE_2 response in OCYs (but not in OBs) may be because of this greater dominance of stretch/shear-sensitive ion channels in OCY response to mechanical loading, i.e., the cytoskeleton may modulate PFF-induced PGE_2 response in OCYs via these mechanosensitive ion channels. A mechanism by which this is possible is provided by numerous studies in which treating cells with cytoskeleton-disrupting agents alters cell mechanical integrity [36,47], which can in turn alter mechanosensitivity [26,34].

Acknowledgments

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References

[1] E.H. Burger, J. Klein-Nulend, Mechanotransduction in bone—role of the lacuno-canalicular network, FASEB J. 13 (1999) S101–S112.
[2] M.L. Knothe-Tate, R. Steck, M.R. Forwood, P. Niederer, In vivo demonstration of load-induced fluid flow in the rat tibia and its potential implications for processes associated with functional adaptation, J. Exp. Biol. 203 (2000) 2737–2745.
[3] S. Weinbaum, S.C. Cowin, Y. Zeng, A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses, J. Biomech. 27 (1994) 339–360.
[4] C.T. Hung, F.D. Allen, S.R. Pollack, C.T. Brighton, What is the role of the convective current density in the real-time calcium response of cultured bone cells to fluid flow?, J. Biomech. 29 (1996) 1403–1409.
[5] J. Klein-Nulend, A. van der Plas, C.M. Semeins, N.E. Ajubi, J.A. Frangos, P.J. Nijweide, E.H. Burger, Sensitivity of osteocytes to biomechanical stress in vitro, FASEB J. 9 (1995) 441–445.
[6] D.L Johnson, T.N. McAllister, J.A. Frangos, Fluid flow stimulates rapid and continuous release of nitric oxide in osteoblasts, Am. J. Physiol. 271 (1996) E205–E208.
[7] R. Smalt, F.T. Mitchell, R.L. Howard, T.J. Chambers, Induction of NO and prostaglandin E_2 in osteoblasts by wall-shear stress but not mechanical strain, Am. J. Physiol. 273 (1997) E751–E758.
[8] J. Klein-Nulend, M.H. Helfrich, J.G. Sterck, H. MacPherson, M. Joldersma, M.H. Ralston, C.M. Semeins, E.H. Burger, Nitric oxide in response to shear stress by human bone cell cultures is endothelial nitric oxide synthase dependent, Biochem. Biophys. Res. Commun. 250 (1998) 108–114.
[9] A.D. Bakker, K. Soejima, J. Klein-Nulend, E.H. Burger, The production of nitric oxide and prostaglandin E_2 by primary bone cells is shear stress dependent, J. Biomech. 34 (2001) 671–677.
[10] A.D. Bakker, J. Klein-Nulend, E.H. Burger, Mechanotransduction in bone cells proceeds via activation of COX-2, but not COX-1, Biochem. Biophys. Res. Commun. 305 (2003) 677–683.
[11] F.M. Pavalko, N.X. Chen, C.H. Turner, D.B. Burr, S. Atkinson, Y.-F. Hsieh, J. Qui, R.L. Duncan, Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton–integrin interactions, Am. J. Physiol. 275 (1998) C1591–C1601.
[12] I. Owan, D.B. Burr, C.H. Turner, J. Qui, Y. Tu, J.E. Onyia, R.L. Duncan, Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain, Am. J. Physiol. 273 (1997) C810–C815.
[13] M.G. Mullender, A.J. El Haj, Y. Yang, M.A. van Duin, E.H. Burger, J. Klein-Nulend, Mechanotransduction of bone cells in vitro: mechanobiology of bone tissue, Med. Biol. Eng. Comput. 42 (2004) 14–21.
[14] J.G. McGarry, J. Klein-Nulend, M.G. Mullender, P.J. Prendergast, A comparison of strain and fluid shear stress in stimulating bone cell responses—a computational and experimental study, FASEB J. (2004) published December 29, doi:10.1096/fj.04-2210fje.

[15] I. Westbroek, N.E. Ajubi, M.J. Alblas, C.M. Semeins, J. Klein-Nulend, E.H. Burger, P.J. Nijweide, Differential stimulation of prostaglandin G/H synthase-2 in osteocytes and other osteogenic cells by pulsating fluid flow, Biochem. Biophys. Res. Commun. 268 (2000) 414–419.
[16] T.J. Chambers, J.W. Chow, S.W. Fox, C.J. Jagger, J.M. Lean, The role of prostaglandins and nitric oxide in the response of bone to mechanical stimulation, Adv. Exp. Med. Biol. 433 (1997) 295–298.
[17] S.W. Fox, T.J. Chambers, J.W. Chow, Nitric oxide is an early mediator of the increase in bone formation by mechanical stimulation, Am. J. Physiol. 270 (1996) E955–E960.
[18] C.H. Turner, Y. Takano, I. Owan, G.A. Murrell, Nitric oxide inhibitor L-NAME suppresses mechanically induced bone formation in rats, Am. J. Physiol. 270 (1996) E634–E639.
[19] T. Akamine, W.S.S. Jee, H.Z. Ke, J. Li, B.Y. Lin, Prostaglandin E_2 prevents bone loss and adds extra bone to immobilized distal femoral metaphysic in female rats, Bone 13 (1992) 11–22.
[20] S.C.F. Rawlinson, A.J. El Haj, S.L. Minter, I.A. Tavares, A. Bennett, L.E. Lanyon, Loading related increases in prostaglandin production in cores of adult canine cancellous bone in vitro: a role for prostacyclin in adaptive bone remodeling?, J. Bone Miner Res. 6 (1991) 1345–1351.
[21] M.J. Pead, L.E. Lanyon, Indomethacin modulation of new load-related stimulation of new bone formation in vivo, Calcif. Tissue Int. 45 (1989) 34–40.
[22] T.N. McAllister, J.A. Frangos, Steady and transient fluid shear stress stimulate NO release in osteoblasts through distinct biochemical pathways, J. Bone Miner. Res. 14 (1999) 930.
[23] R.G. Bacabac, T.H. Smit, M.G. Mullender, S.J. Dijcks, J.J.W.A. van Loon, J. Klein-Nulend, Nitric oxide production by bone cells is fluid shear stress rate dependent, Biochem. Biophys. Res. Commun. 315 (2004) 823–829.
[24] M.H. Helfrich, D.E. Evans, P.S. Grabowski, J.S. Pollock, H. Ohshima, M.H. Ralston, Expression of nitric oxide synthase isoforms in bone and bone cell cultures, J. Bone Miner. Res. 12 (1997) 1108–1115.
[25] N.E. Ajubi, J. Klein-Nulend, P.J. Nijweide, T. Vrijheid-Lammers, M.J. Alblas, E.H. Burger, Pulsating fluid flow increases prostaglandin by cultured chicken osteocytes: a cytoskeleton dependent process, Biochem. Biophys. Res. Commun. 225 (1996) 62–68.
[26] N. Wang, J.P. Butler, D.E. Ingber, Mechanotransduction across the cell surface and through the cytoskeleton, Science 21 (1993) 1124–1127.
[27] P.A. Janmey, The cytoskeleton and cell signaling: component localisation and mechanical coupling, Physiol. Rev. 78 (1998) 763–781.
[28] N.E. Ajubi, J. Klein-Nulend, M.J. Alblas, E.H. Burger, P.J. Nijweide, Signal transduction pathways involved in fluid flow-induced PGE_2 production by cultured osteocytes, Am. J. Physiol. 276 (1999) E171–E178.
[29] S.C. Rawlinson, A.A. Pitsillides, L.E. Lanyon, Involvement of different ion channels in osteoblasts' and osteocytes' early responses to mechanical strain, Bone 19 (1996) 609–614.
[30] F.J. Alenghat, D.E. Ingber, Mechanotransduction: all signals point to cytoskeleton, matrix, and integrins, Sci. STKE 12 (2002) 119:PE6.
[31] K.M. Reich, J.A. Frangos, Protein kinase C mediates flow-induced prostaglandin E_2 production in osteoblasts, Calcif. Tissue Int. 52 (1993) 62–66.
[32] K.M. Reich, T.N. McAllister, S. Gudi, J.A. Frangos, Activation of G proteins mediates flow-induced prostaglandin E_2 production in osteoblasts, Endocrinology 138 (1997) 1014–1018.
[33] C.M. Brophy, I. Mills, O. Rosales, C. Isales, B.E. Sumpio, Phospholipase C: a putative mechanotransducer for endothelial cell response to acute hemodynamic changes, Biochem. Biophys. Res. Commun. 190 (1993) 576.
[34] A.J. Maniotis, C.S. Chen, D.E. Ingber, Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure, Proc. Natl. Acad. Sci. USA 94 (1997) 849–854.
[35] H.L. Knudsen, J.A. Frangos, Role of cytoskeleton in shear stress-induced endothelial nitric oxide production, Am. J. Physiol. 273 (1997) H347–H355.
[36] W.R. Trickey, T.P. Vail, F. Guilak, The role of the cytoskeleton in the viscoelastic properties of human articular chondrocytes, J. Orthop. Res. 22 (2004) 131–139.
[37] D.J. Kleinhenz, X. Fan, J. Rubin, C.M. Hart, Detection of endothelial nitric oxide release with the 2,3-diaminonaphthalene assay, Free Radic. Biol. Med. 34 (2003) 856–861.
[38] R. Busse, I. Fleming, Regulation and functional consequences of endothelial nitric oxide formation, Ann. Med. 27 (1995) 331–340.
[39] Y. Su, S. Edwards-Bennett, M.R. Bubb, E.R. Block, Regulation of endothelial nitric oxide synthase by the actin cytoskeleton, Am. J. Physiol. 284 (2003) C1542–C1549.
[40] S.I. Zharikov, A.A. Sigova, S. Chen, M.R. Bubb, E.R. Block, Cytoskeletal regulation of the L-arginine/NO pathway in pulmonary artery endothelial cells, Am. J. Physiol. Lung Cell. Mol. Physiol. 280 (2001) L465–L473.
[41] S.M. Ponik, F.M. Pavalko, Formation of focal adhesions on fibronectin promotes fluid shear stress induction of COX-2 and PGE_2 release in MC3T3-E1 osteoblasts, J. Appl. Physiol. 97 (2004) 135–142.
[42] S.M. Norvell, S.M. Ponik, D.K. Bowen, R. Gerard, F.M. Pavalko, Fluid shear stress induction of COX-2 protein and prostaglandin release in cultured MC3T3-E1 osteoblasts does not require intact microfilaments or microtubules, J. Appl. Physiol. 96 (2004) 957–966.
[43] G. Zaman, A.A. Pitsillides, S.C. Rawlinson, R.F. Suswillo, J.R. Mosley, M.Z. Cheng, L.A. Platts, M. Hukkanen, J.M. Polak, L.E. Lanyon, Mechanical strain stimulates nitric oxide production by rapid activation of endothelial nitric oxide synthase in osteocytes, J. Bone Miner. Res. 14 (1999) 1123–1131.
[44] K. Tanaka-Kamioka, H. Kamioka, H. Ris, S.-S. Lim, Osteocyte shape is dependent on actin filaments and osteocyte processes are unique actin-rich projections, J. Bone Miner. Res. 13 (1998) 1555.
[45] R.L. Duncan, K.A. Hruska, Chronic, intermittent loading alters mechanosensitive channel characteristics in osteoblast-like cells, AJP—Renal Physiol. 267 (1994) F909–F916.
[46] A.J. El Haj, S.L. Minter, S.C. Rawlinson, R. Suswillo, L.E. Lanyon, Cellular responses to mechanical loading in vitro, J. Bone Miner. Res. 5 (1990) 923–932.
[47] G.T. Charras, M.A. Horton, Single cell mechanotransduction and its modulation analyzed by atomic force microscopy indentation, Biophys. J. 82 (2002) 2970–2981.