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Fluid shear stress induction of COX-2 protein and prostaglandin release in cultured MC3T3-E1 osteoblasts does not require intact microfilaments or microtubules

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Norvell, Suzanne M., Suzanne M. Ponik, Deidre K. Bowen, Rita Gerard, and Fredrick 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: 957–966, 2004. First published November 14, 2003; 10.1152/japplphysiol.00869.2003.—Cultured osteoblasts express three major types of cytoskeleton: actin microfilaments, microtubules, and intermediate filaments. The cytoskeletal network is thought to play an important role in the transmission and conversion of a mechanical stimulus into a biochemical response. To examine a role for the three different cytoskeletal networks in fluid shear stress-induced signaling in osteoblasts, we individually disrupted actin microfilaments, microtubules, and intermediate filaments in MC3T3-E1 osteoblasts with multiple pharmacological agents. We subjected these cells to 90 min of laminar fluid shear stress (10 dyn/cm^2) and compared the PGE_2 and PGI_2 release and induction of cyclooxygenase-2 protein to control cells with intact cytoskeletons. Disruption of actin microfilaments, microtubules, or intermediate filaments in MC3T3-E1 cells did not prevent a significant fluid shear stress-induced release of PGE_2 or PGI_2. Furthermore, disruption of actin microfilaments or microtubules did not prevent a significant fluid shear stress-induced increase in cyclooxygenase-2 protein levels. Disruption of intermediate filaments with acrylamide did prevent the fluid shear stress-induced increase in cyclooxygenase-2 but also prevented a PGE_2-induced increase in cyclooxygenase-2. Thus none of the three major cytoskeletal networks are required for fluid shear stress-induced prostaglandin release. Furthermore, although neither actin microfilaments nor microtubules are required for fluid shear stress-induced increase in cyclooxygenase-2 levels, the role of intermediate filaments in regulation of cyclooxygenase-2 expression is less clear.

osteoblasts; bone; stress-strain; mechanical loading

THE THREE MAJOR CYTOSKELETAL elements, microfilaments, microtubules, and intermediate filaments, are thought to play an important role in the response of cells to mechanical stimulation. Together with the extracellular matrix, the cytoskeleton is thought to respond to changes in mechanical load in part according to the theory of tensegrity, which suggests that the microtubules act as load-bearing compression elements and the actin microfilaments and intermediate filaments as tension elements (20). In addition to cell-matrix interactions, a role for cell-cell junctions, including gap junctions and adherens junctions, in mechanotransduction is beginning to be elucidated and may or may not require cytoskeleton association (10, 27). However, the role of the cytoskeletal network in mechanosensitive signaling is controversial. Although there is accumulating evidence suggesting that the function of many potential mechanotransducing molecules may require their linkage to the cytoskeleton (3, 21), it is also possible that membrane distortion alone may be sufficient for mechanical stimulation-induced signaling in the cell (5, 18, 26). For example, the activity of many cellular proteins, including stretch-activated calcium channels and G proteins, is known to be induced by mechanical stimulation, and they by themselves may act as mechanosensors as well as mechanotransducers (16).

Cell surface integrin receptors, all three major cytoskeletal filaments, and nuclear scaffolds are mechanically coupled in living cells (29). This is consistent with the idea that the three filament systems together make up a tensionally integrated complex that is not simply an additive system (19, 45). The integrins, which link the extracellular matrix to the cell surface and attach to the cytoskeleton inside the cell, are thought to play a central role in mechanotransduction in response to fluid shear stress (FSS). For example, in endothelial cells, FSS-induced vasodilation of coronary arteries was blocked by integrin-blocking peptides or integrin-blocking antibodies (30). In addition, our laboratory showed that in osteoblasts a mutant focal adhesion protein blocked FSS-induced expression of cyclooxygenase (COX)-2 and c-Fos (35).

Application of a mechanical load to bone tissue induces new bone formation (22, 44). Osteoblasts are the cells that actively deposit new bone matrix. Osteoblasts that have stopped producing bone matrix differentiate into bone lining cells and osteocytes. The dendritic processes of osteocytes that traverse the canals in bone known as lacunae and canaliculi are exposed to interstitial fluid flow when bone is strained (7, 25, 28, 43). In fact, FSS alone has been shown to be a more potent stimulus for osteoblastic cells than mechanical strain alone (34, 38). Our laboratory and others have demonstrated that subjecting osteoblasts to FSS increases the expression of genes, including c-Fos and COX-2 (23, 33, 35). FSS also causes increased release of prostaglandins, the synthesis of which depends on COX-1 and COX-2 (23, 36). Cyclooxygenases and prostaglandins are important for the mechanoadaptation of bone tissue because blocking prostaglandin synthesis in vivo by using inhibitors of cyclooxygenase activity before loading inhibits load-induced bone formation (9, 13).

For this study, we sought to determine whether in osteoblasts the individual cytoskeletal elements were required for the response of osteoblasts to mechanical stimulation. Given the important role for COX-2 and prostaglandin metabolism in

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bone biology (12), we focused on these molecules as our end points for testing FSS-induced responses in osteoblasts. We found that in MC3T3-E1 osteoblasts neither actin stress fiber formation, insoluble F-actin, intact microtubules, nor intact intermediate filaments are necessary for FSS-induced prostaglandin release, suggesting that individually none of these cytoskeletal elements is required for FSS-induced prostaglandin release. Induction of COX-2 protein expression by FSS or PGE_2 also does not appear to require actin stress fiber formation, insoluble F-actin, or intact microtubules; however, the role of intermediate filaments in regulation of COX-2 expression remains unclear.

MATERIALS AND METHODS

Cell culture and fluid flow. Mouse MC3T3-E1 osteoblasts were cultured in α-MEM containing 10% fetal calf serum (FCS) and 1% penicillin-streptomycin. Cells were grown to 90% confluency on glass slides and were therefore still in a proliferative state at the time of each experiment. The cells were in contact with other cells, and cell-cell junctions were abundant at the time of the experiment as verified by immunofluorescence analysis (data not shown). For each static and FSS experiment, cells on glass slides were transferred to 30 ml of α-MEM containing 1% FCS for the duration of each experiment. Cells were subjected to 90 min of static incubation or 90 min of laminar FSS (10 dyn/cm^2). Fluid flow was performed in parallel plate flow chambers at 37°C with the media subjected to a stream of 5% CO_2 by use of the flow loop system designed by Frangos et al. (14) and marketed by Cytodyne (San Diego, CA). After the 90-min static or FSS treatment, slides of cells were overlaid with 1 ml of α-MEM (containing 1% FCS) and incubated for 30 min at 37°C, 5% CO_2 under static conditions. After the 30-min incubation, the media were collected for determination of PGE_2 and PGI_2 levels and the cells were harvested in SDS sample buffer for whole cell lysis and subsequent immunoblot analysis.

Drug treatment. Cells were pretreated for 1 h with the appropriate cytoskeleton-disrupting drug or vehicle (DMSO for actin and microtubule drugs, H_2O for acrylamide), and the drug or vehicle was included during the 90-min treatment (static or FSS) time and 30-min posttreatment incubation periods. Cytoskeleton drug concentrations were as follows: 0.5 μM latrunculin A (LAT A), 5 μM cytochalasin B (CYT B), 5 μM cytochalasin D (CYT D), 10 μM nocodazole (NOC), and 10 μM colchicine (COLC) (all purchased from Calbiochem, San Diego, CA), 1 μM jasplakinolide (JAS P; purchased from Molecular Probes, Eugene, OR), 25 mM acrylamide (purchased from Research Organics, Cleveland, OH). For inhibition of COX-2 enzyme activity, cells were pretreated overnight with 10 μM NS-398 (purchased from Calbiochem) or vehicle (ethanol) and also treated during the 90-min treatment (static or FSS) time and 30-min posttreatment incubation periods.

Antibodies. For immunofluorescence, we used rhodamine-phalloidin (Molecular Probes), anti-tubulin antibody B-512 (Sigma Chemical, St. Louis, MO), anti-vimentin antibody VIM 13.2 (Sigma Chemical). For immunoblot analysis, we used anti-COX-2 polyclonal antibody (Cayman Chemical, Ann Arbor, MI), anti-actin antibody AC-40 (Sigma Chemical), anti-c-Jun monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA), and anti-c-Fos polyclonal antibody sc-7202 (Santa Cruz Biotechnology, Santa Cruz, CA). The appropriate conjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA).

Fluorescence microscopy and immunoblot analysis. Cells for immunofluorescence microscopy of actin microfilaments or microtubules were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton-PBS. Cells for immunofluorescence microscopy of vimentin intermediate filaments were fixed in ice-cold methanol for 2 min. Images were recorded by use of a RT Color Spotdigital camera (Diagnostic Instruments, Sterling Heights, MI) attached to an Optiphot-2 Nikon epifluorescent microscope using a ×60 Nikon Planapo objective (1.4 numerical aperture).

For immunoblot analysis, cells grown on glass slides and subjected to either static or fluid flow conditions were harvested in SDS sample buffer, and protein concentrations were determined by using the amido black method (37). Equal protein (20 μg) was loaded onto 7.5% SDS-PAGE gels for separation and transferred to nitrocellulose for immunoblotting. Antibody signal was detected and analyzed by using a Fuji chemiluminescence imager (Fuji, Edison, NJ) after reaction in a luminescence solution described in Norvell and Green (32). The experiments were carried out in triplicate.

Measurement of insoluble F-actin. A modified version of the assay used by Frigeri and Apgar (15) was used to measure the relative levels of insoluble F-actin in MC3T3-E1 cells. Cells grown on glass slides were pretreated with LAT A, CYT B, CYT D, JAS P, or vehicle (DMSO) alone and incubated under static or FSS conditions for 180 min at 37°C. The cells were then rinsed twice in PBS and permeabilized by the addition of 1 ml ice-cold solubilizing buffer [10 mM imidazole (pH 7.2), 40 mM KCl, 10 mM EGTA, 1% Triton X-100] and protease inhibitors (PMSF, aprotinin, and leupeptin) for 15 min. After being rinsed twice in PBS for 5 min, cells were fixed in 4% paraformaldehyde in PBS for 15 min followed by two more PBS rinses. Next, cells were incubated with 1 ml of nitrobenzoxadiazole (NBD)-phallacidin (3.3×10^{-8} M) for 2 h at room temperature. Cells were then washed three times with PBS, and the bound NBD-phallacidin was extracted by scraping the cells with 1.5 ml of methanol and incubating overnight in the dark. Cell debris was removed by centrifugation at 12,000 *g*, and the supernatant was assayed by using a spectrofluorometer with an excitation wavelength of 465 nm and an emission wavelength of 535 nm.

Prostaglandin measurement. Media samples from the 30-min posttreatment incubation were centrifuged at 14,000 *g* for 1 min to pellet any particulates. The supernatants were transferred to a new tube, and the levels of PGE_2 and the PGI_2 stable metabolite, 6-keto-$F1_\alpha$, present in samples were determined by using the appropriate enzymeimmunoassay kit from Amersham Pharmacia Biotech (Piscataway, NJ).

Statistical analysis. Statistical analysis was performed by use of the statistical package Statview, version 5.0.1.

RESULTS

FSS induces actin stress fiber formation without increasing insoluble F-actin levels. Actin microfilaments were analyzed in MC3T3-E1 osteoblasts subjected to 90 min of laminar FSS (10 dyn/cm^2) or incubated under static conditions. Ninety minutes of 10 dyn/cm^2 FSS was chosen because it induces significant and consistent stress fiber formation as well as induction of prostaglandin release and COX-2 expression, which are analyzed in this study. Immunofluorescence analysis revealed that FSS induced robust actin stress fiber formation (Fig. 1, *A* and *B*), as has been reported previously (35). To quantitate the changes in the actin cytoskeleton reorganization, we analyzed insoluble F-actin levels in osteoblasts subjected to FSS or static conditions. Analysis of insoluble F-actin levels using NBD-phallacidin to specifically label insoluble F-actin filaments revealed that insoluble F-actin levels were not significantly altered by FSS (Fig. 2). Immunoblot analysis of total actin levels also revealed no significant change in total levels of actin in cells subjected to FSS (data not shown). This suggests that the actin stress fiber formation induced by FSS is due primarily to a rearrangement of the existing F-actin.

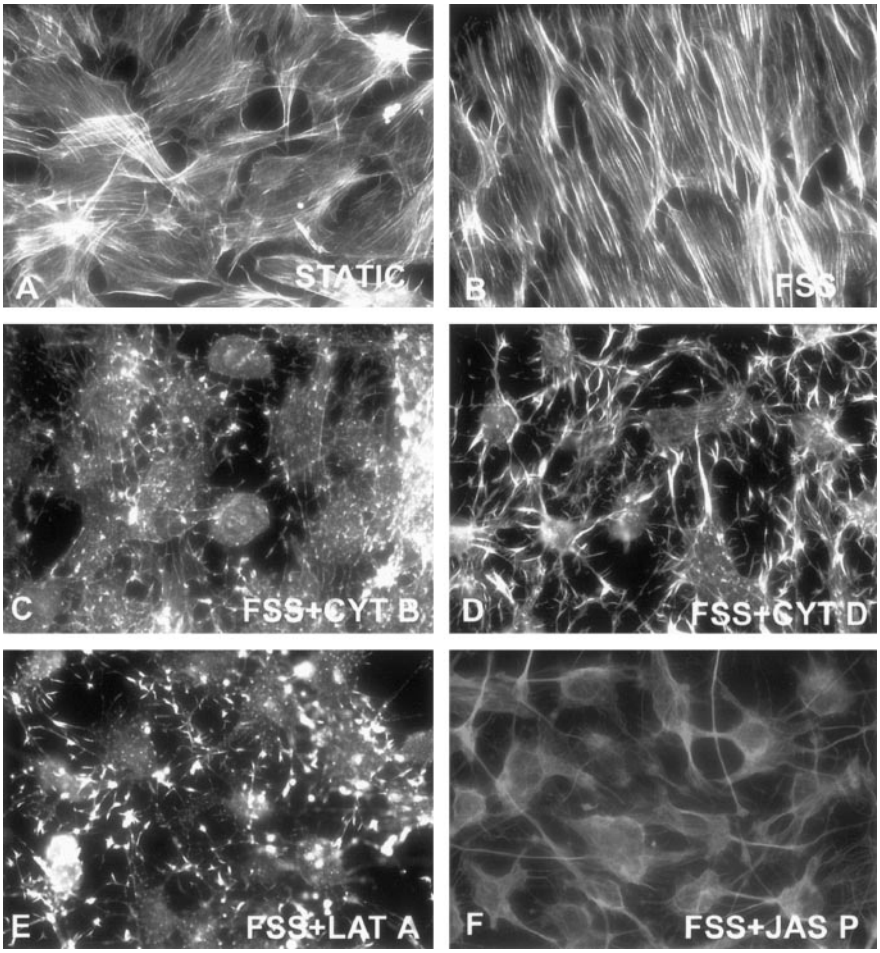

Fig. 1. Disruption of fluid shear stress (FSS)-induced actin stress fiber formation with cytochalasin B (CYT B), cytochalasin D (CYT D), latrunculin A (LAT A), and jasplakinolide (JAS P). Immunofluorescence analysis actin microfilaments in MC3T3-E1 cells. Actin microfilaments were visualized with rhodamine phalloidin. FSS induces the formation of stress fibers. *A*: static culture. *B*: 90 min of FSS. Cells treated with 5 μM CYT B (*C*), 5 μM CYT D (*D*), 0.5 μM LAT A (*E*), or 1 μM JAS P (*F*) and subjected to FSS exhibited disrupted actin microfilament organization and no stress fiber formation.

Pharmacological agents that disrupt the actin microfilament network reduce insoluble F-actin to varying degrees. To examine the role of actin microfilaments in mechanotransduction in osteoblasts, we disrupted the actin cytoskeleton with several known pharmacological disruptors of actin filaments. Cells were pretreated for 1 h and treated during the experiment with the following concentrations of drug or vehicle control (DMSO): 5 μM CYT D, 5 μM CYT B, 0.5 μM LAT A, and 1 μM JAS P. Multiple drug concentrations were tested, and the above concentrations were chosen on the basis of a lack of cytotoxicity and complete disruption of the actin filament network without causing cell detachment. In both cells held under static conditions (data not shown) and cells subjected to FSS, immunofluorescence analysis of actin microfilaments revealed that each of the actin disruptors prevented stress fiber formation and altered cellular morphology. CYT B-, CYT D-, and LAT A-treated cells appeared to have spotty, bright patches of remaining filaments (Fig. 1, *C–E*), whereas actin filaments in JAS P treated cells appeared to be more completely disrupted (Fig. 1*F*).

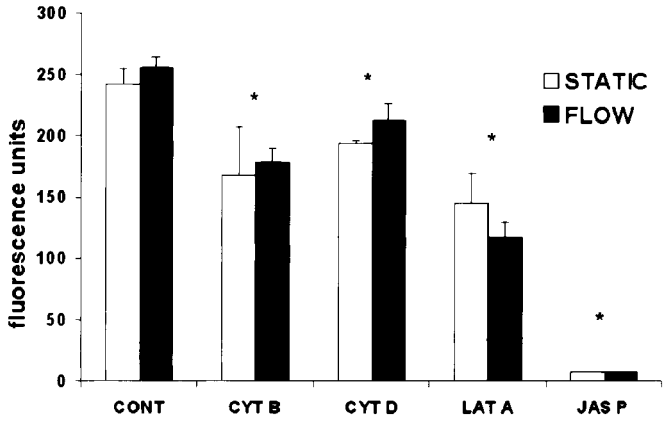

Fig. 2. FSS does not change insoluble F-actin levels, but CYT B, CYT D, LAT A, and JAS P significantly reduce insoluble F-actin levels to varying degrees. Levels of insoluble F-actin were determined in static cells and cells subjected to FSS using a fluorescence-based insoluble F-actin assay. FSS (solid bars) did not significantly affect insoluble F-actin levels compared with static controls (open bars) in control (CONT) or drug-treated cells. However, 5 μM CYT B, 5 μM CYT D, 0.5 μM LAT A, and 1 μM JAS P significantly reduced insoluble F-actin levels by 30, 19, 55, and 98%, respectively, compared with control cells (set to 100%). *$P < 0.05$, individual drug treatment vs. control, ANOVA.

Next, we examined the actin drugs' efficacy by measuring the levels of insoluble F-actin in cells held under static conditions or subjected to 90 min of laminar FSS. Interestingly, the drugs affected insoluble F-actin levels to varying degrees. On average, CYT B, CYT D, LAT A, and JAS P reduced insoluble F-actin levels by 30, 19, 55, and 98%, respectively, making JAS P the most effective disruptor of insoluble F-actin (Fig. 2). As in control cells, FSS did not induce a significant change in the levels of insoluble F-actin in cells treated with actin drugs (Fig. 2), and there was no FSS-induced change in total actin levels (data not shown).

Disruption of actin microfilaments does not inhibit FSS-induced prostaglandin release. To determine whether disruption of stress fiber formation or decreasing insoluble F-actin levels affected the response of osteoblasts to FSS, we first measured the FSS-induced release of the prostaglandins PGE_2 and PGI_2. As expected, control cells released 3.4-fold more PGE_2 and 4.8-fold more PGI_2 in response to FSS compared with cells in static conditions (Fig. 3). Cells treated with any of the actin-disrupting drugs released similar levels of PGE_2 and PGI_2 in response to FSS. Cells treated with CYT D, CYT B, LAT A, or JAS P released 3.3, 2.8, 2.8, and 3.6-fold, respectively, more PGE_2 than drug-treated static counterparts (Fig. 3*A*). Cells treated with CYT D, CYT B, LAT A, or JAS P released 5.1, 4.6, 7.1, and 9.9-fold, respectively, more PGI_2 than drug-treated static controls (Fig. 3*B*). These results indicate that an intact actin cytoskeleton is not required for FSS-induced prostaglandin release. Interestingly, in both the control and drug-treated cells, the FSS-induced release of both PGE_2 and PGI_2 was completely inhibited by blocking COX-2 activity by use of 10 μM NS-398 (Fig. 3), indicating that all of the shear-induced prostaglandin release was due to COX-2 activity.

Disruption of actin microfilaments does not inhibit the FSS or the PGE_2 induction of COX-2 protein. Because the FSS-induced prostaglandin release was entirely due to the activity of COX-2, we next measured the FSS induction of COX-2 protein in cells with disrupted actin cytoskeletons. In control cells, FSS induced a 3.8-fold increase in COX-2 protein (Fig. 4*A*). None of the actin disruptors significantly prevented a shear-induced increase in COX-2 levels by FSS. Cells treated with CYT D, CYT B, LAT A, or JAS P significantly increased COX-2 levels in response to FSS 4.7-, 3.0-, 3.5-, and 3.1-fold, respectively, more than their drug-treated static counterparts (S vs. F, $P < 0.05$) (Fig. 4*A*). This indicates that neither the FSS-induced reorganization of insoluble F-actin into stress fibers nor basal levels of insoluble F-actin are required for the induction of COX-2 by FSS in osteoblasts.

PGE_2 is also known to increase COX-2 levels in osteoblasts (41). Therefore, we examined whether preventing the FSS-induced prostaglandin release with NS-398 (see Fig. 3) af-

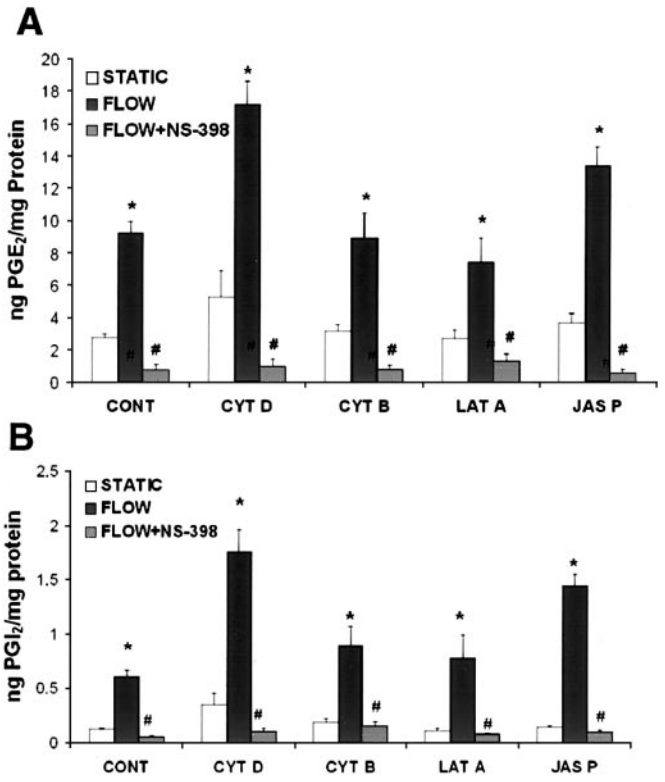

Fig. 3. Treatment with CYT D, CYT B, LAT A, or JAS P to disrupt actin microfilaments does not inhibit FSS-induced, cyclooxygenase-2 (COX-2)-dependent PGE_2 and PGI_2 release. PGE_2 (*A*) and PGI_2 (*B*) release was measured in control and drug-treated cells (5 μM CYT D, 5 μM CYT B, 0.5 μM LAT A, and 1 μM JAS P) in static conditions (open bars) and after 90 min of FSS (solid bars). Inhibition of COX-2 activity with 10 μM NS-398 (hatched bars) completely blocked the FSS-induced PGE_2 and PGI_2 release. $^*P < 0.05$, static vs. flow, Student's *t*-test. $\#P < 0.05$, flow vs. flow + NS-398, Student's *t*-test.

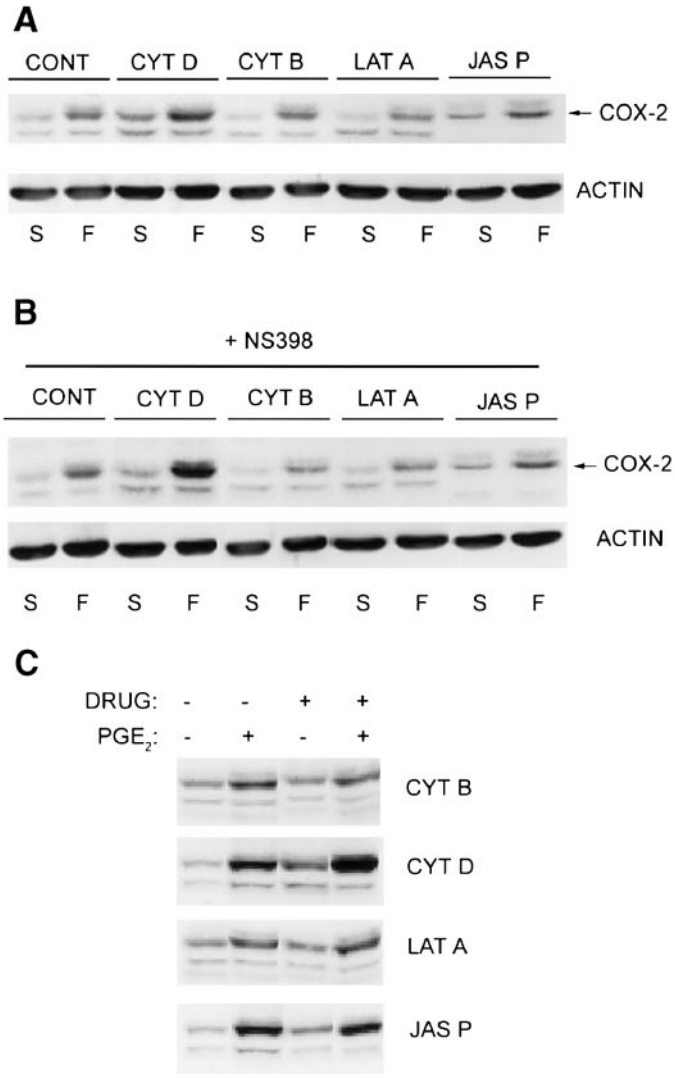

Fig. 4. Treatment with CYT D, CYT B, LAT A, or JAS P to disrupt actin microfilaments does not inhibit FSS or PGE_2-induced COX-2 levels. Immunoblot analysis of COX-2 and actin in control and drug-treated cells (5 μM CYT D, 5 μM CYT B, 0.5 μM LAT A, and 1 μM JAS P). Protein was loaded in each lane in 20-μg amounts. *A*: cells in static culture (S) or cells subjected to FSS (F). *B*: cells treated with NS-398 in static culture or subjected to FSS. *C*: cells in static culture treated with 10 μM PGE_2 in the presence or absence of actin poisons. Densitometric analysis of a minimum of 3 experiments was performed. $P < 0.05$, static vs. flow, $+PGE_2$ vs. $-PGE_2$, Student's *t*-test.

fected the FSS induction of COX-2 protein. Although treatment with 10 μM NS-398 completely inhibited the FSS induction of prostaglandin release, it did not prevent a significant induction of COX-2 protein in both control and drug-treated cells (S vs. F, $P < 0.05$) (Fig. 4*B*), suggesting that FSS-induced prostaglandin release is not necessary for the FSS induction of COX-2 protein.

To assess whether osteoblasts with disrupted actin cytoskeletons could still respond to PGE_2 by increasing COX-2 protein levels, we treated cells with 10 μM PGE_2 and measured COX-2 levels in the presence of all of the actin disruptors. Similar to all flow experiments, cells were pretreated for 1 h with the actin disruptors and then treated with PGE_2 in the presence of the actin disruptors for 2 h. A 2-h treatment with 10 μM PGE_2 resulted in a significant threefold increase in COX-2 levels compared with vehicle controls [with (+) PGE_2 vs. without (−) PGE_2, $P < 0.05$] (Fig. 4*C*). Similar to flow results, disruption of actin filaments with any of the actin disruptors did not prevent a significant PGE_2-induced increase in COX-2 levels. PGE_2 induced a significant 3.3-, 2.2-, 2.4-, and 2.2-fold increase in COX-2 levels even in the presence of CYT D, CYT B, LAT A, or JAS P, respectively ($+PGE_2$ vs. $-PGE_2$, $P < 0.05$) (Fig. 4*C*).

Disruption of microtubules does not inhibit the FSS-induced prostaglandin release. Next, we investigated the contribution of microtubules to FSS-induced responses in osteoblasts. Immunofluorescence analysis revealed that FSS does not induce major changes in the organization of microtubules in MC3T3-E1 osteoblasts (Fig. 5, *A* and *B*). To disrupt microtubules, cells were treated with 10 μM NOC or 10 μM COLC. Drug concentrations were chosen that were not cytotoxic and did not cause cell detachment during static or flow conditions. Like the experiments with actin disruption, control cells (DMSO) and cells treated with microtubule disruptors were subjected to laminar FSS (10 dyn/cm^2) or static conditions, and microtubules were then visualized by immunofluorescence. Both NOC and COLC completely disrupted the microtubule network (Fig. 5, *C* and *D*), as tubulin appeared diffuse and dispersed throughout the cytoplasm.

To determine whether disruption of microtubules affects the response of osteoblasts to FSS, we first measured release of the prostaglandins PGE_2 and PGI_2. These experiments were done in parallel with the experiments in which actin filaments were disrupted so the controls were pooled. Control cells released 3.4-fold more PGE_2 and 4.8-fold more PGI_2 in response to FSS compared with cells in static conditions (Fig. 6). Cells treated with either NOC or COLC released significantly increased levels of PGE_2 and PGI_2 in response to FSS. Cells treated with NOC released 3.1- and 4.1-fold more PGE_2 and PGI_2, respectively, than their static counterpart (Fig. 6). Cells treated with COLC released 1.8- and 4.7-fold more PGE_2 and PGI_2, respectively, than their static counterpart (Fig. 6). The increase in prostaglandin release in static cells somewhat decreased the total fold increase; however, microtubule inhibitors have been reported to induce release of prostaglandins in epithelial cells (40). Just as discovered for actin microfilaments, intact microtubules were not necessary for FSS-induced prostaglandin release. Also, in both the control and microtubule drug-treated cells, the FSS-induced release of both PGE_2 or PGI_2 was completely inhibited by blocking COX-2 activity by use of 10 μM NS-398 (Fig. 6), indicating that all of the shear-induced prostaglandin release was due to COX-2 activity.

Disruption of microtubules does not inhibit the FSS or the PGE_2 induction of COX-2 protein. We next measured the FSS induction of COX-2 protein in cells with disrupted microtubule cytoskeletons. In control cells, FSS induced a significant 3.8-fold increase in COX-2 protein (S vs. F, $P < 0.05$) (Fig. 7*A*). Neither NOC nor COLC treatment prevented a significant increase in COX-2 levels by FSS. Cells treated with NOC significantly increased COX-2 2.9-fold, and cells treated with COLC significantly increased COX-2 3.3-fold in response to FSS (S vs. F, $P < 0.05$) (Fig. 7*A*). Also, just as we found in experiments with actin disruptors, treatment with NS-398, which completely blocks flow-induced prostaglandin release,

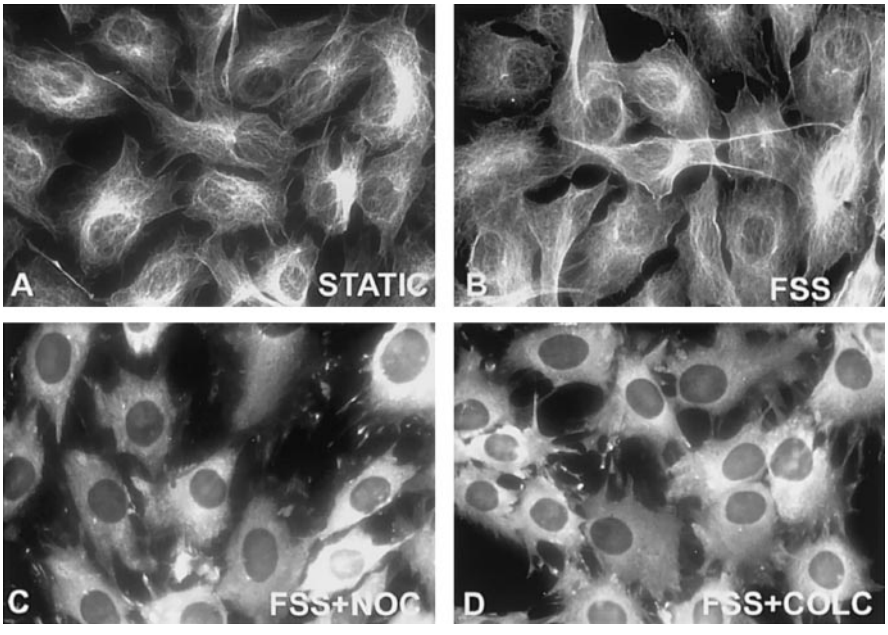

Fig. 5. Disruption of microtubules with nocodazole (NOC) and colchicine (COLC). Immunofluorescence analysis of microtubules using an antibody that recognizes tubulin. Microtubule organization appears the same in cells in static culture (*A*) or in cells subjected to FSS (*B*); 10 μM NOC and 10 μM COLC disrupt intact microtubules in cells subjected to FSS (*C* and *D*) or held in static culture (data not shown).

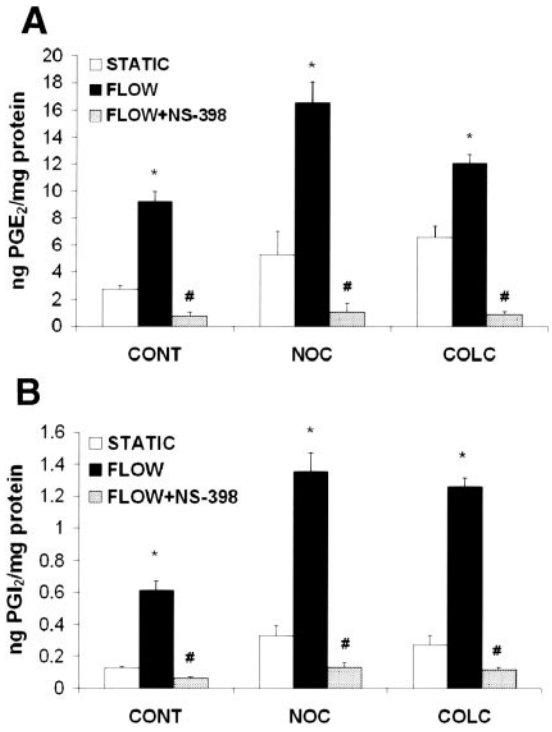

Fig. 6. Treatment with NOC or COLC to disrupt microtubules does not inhibit FSS-induced, COX-2-dependent PGE_2 and PGI_2 release. PGE_2 (*A*) and PGI_2 (*B*) release in control and drug-treated cells (10 μM NOC and 10 μM COLC) in static conditions (open bars) and after FSS (solid bars). Inhibition of COX-2 activity with 10 μM NS-398 (hatched bars) completely blocked the FSS-induced PGE_2 and PGI_2 release. $*P < 0.05$, static vs. flow, Student's *t*-test. $\#P < 0.05$, flow vs. flow + NS-398, Student's *t*-test.

had no effect on the induction of COX-2 protein in both control and drug-treated cells (Fig. 7*B*).

Again, to examine whether this phenomenon was specific for FSS induction of COX-2, we treated cells with 10 μM PGE_2 and measured COX-2 levels in the presence of NOC and COLC. PGE_2 treatment resulted in a significant threefold increase in COX-2 levels compared with vehicle controls (Fig. 7*C*), and, in the presence of NOC or COLC, PGE_2 induced a 4.8-fold and a 5.1-fold, respectively, increase in COX-2. Thus, similar to the flow results, disruption of microtubules did not prevent a PGE_2-induced increase in COX-2 levels.

Disruption of vimentin intermediate filaments with acrylamide does not inhibit FSS-induced prostaglandin release. Lastly, we investigated the contribution of intermediate filaments to FSS-induced responses in osteoblasts. Although FSS did not induce major changes in vimentin filament organization, vimentin did mimic the more elongated shape of the cell induced by FSS (Fig. 8, *A* and *B*). To disrupt vimentin, a dose of acrylamide was tested in the range of 5–25 mM (data not shown), and 25 mM was chosen because it significantly disrupted the organization of vimentin filaments without being cytotoxic or causing cell detachment during static or flow conditions. Treatment with 25 mM acrylamide caused collapse of the vimentin filaments around the nucleus in both static conditions (data not shown) and after FSS (Fig. 8*C*) compared with vehicle-treated controls. Interestingly, at all concentrations at which acrylamide disrupted vimentin organization actin microfilaments were also disrupted (Fig. 8*D*).

We first examined FSS-induced prostaglandin release in the presence of acrylamide. Control cells released 3.2-fold more PGE_2 and 4.3-fold more PGI_2 in response to FSS compared with cells in static conditions (Fig. 9). Cells treated with acrylamide also released significantly increased levels of PGE_2 and PGI_2 in response to FSS: 2.3-fold and 3.8-fold PGE_2 and PGI_2, respectively (Fig. 9). Although these fold increases are slightly lower than control fold increases, they are still significantly elevated compared with their static, acrylamide-treated counterpart. The decrease in the fold increase is at least in part due to the fact that acrylamide appears to increase prostaglandin release in static culture. Again, these data suggest that, like microtubules and actin microfilaments, intact intermediate filaments are not required for FSS-induced prostaglandin release. Also, in both the control and acrylamide-treated cells, the FSS-induced release of both PGE_2 and PGI_2 was completely

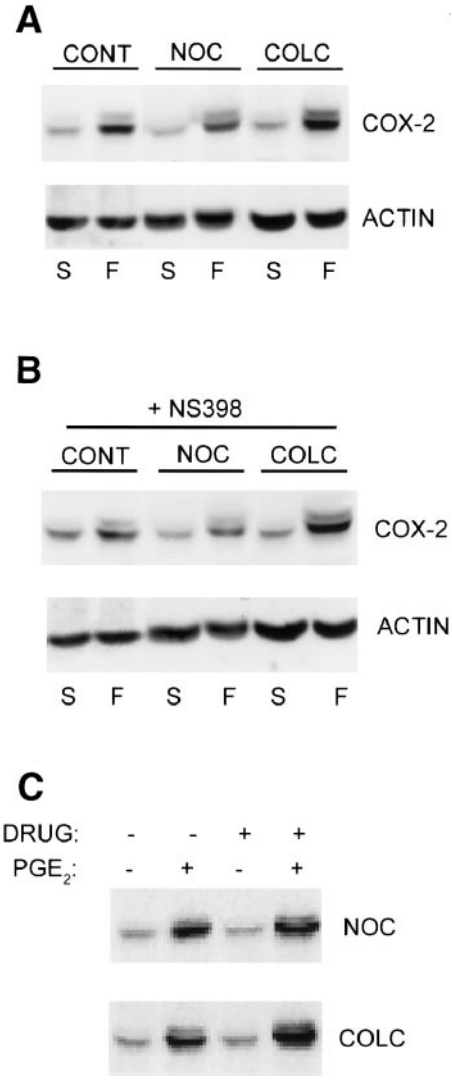

Fig. 7. Treatment with NOC or COLC to disrupt microtubules does not inhibit FSS or PGE_2-induced increased COX-2 levels. Immunoblot analysis of COX-2 and actin in control and drug-treated cells (10 μM NOC and 10 μM COLC); 20 μg of protein was loaded in each lane. *A*: cells in static culture or cells subjected to FSS. *B*: cells treated with NS-398 in static culture or subjected to FSS. *C*: cells in static culture treated with 10 μM PGE_2 in the presence or absence of actin drugs. Densitometric analysis of at least 3 experiments was performed. $P < 0.05$, static vs. flow, $+PGE_2$ vs. $-PGE_2$, Student's *t*-test.

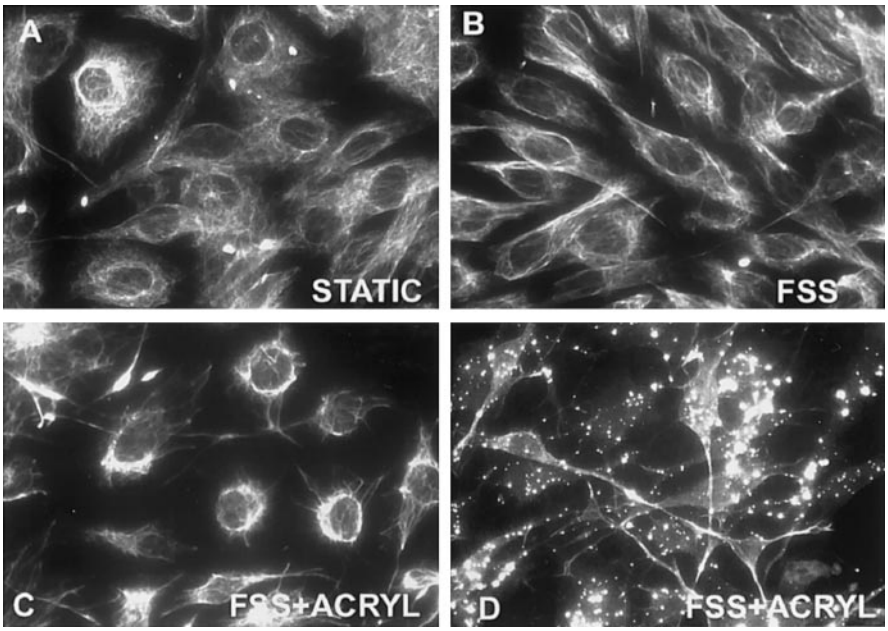

Fig. 8. FSS induces modest changes in vimentin organization, and acrylamide disrupts both vimentin organization and actin microfilaments. Immunofluorescence analysis of vimentin intermediate filaments with an antibody that recognizes murine vimentin. Control cells in static culture (*A*) and after FSS (*B*). Note the elongated appearance of vimentin in cells subjected to FSS. Acrylamide treatment (25 mM) significantly disrupted vimentin filaments (*C*) but also disrupted actin microfilaments as visualized by use of rhodamine phalloidin (*D*).

inhibited by blocking COX-2 activity by use of 10 μM NS-398 (Fig. 9), indicating that all of the shear-induced prostaglandin release was due to COX-2 activity.

Disruption of vimentin intermediate filaments with acrylamide inhibits the FSS induction of COX-2, c-Jun, and c-Fos protein and also inhibits the PGE_2 induction of COX-2 protein. We next measured the FSS induction of COX-2, c-Fos, and c-Jun protein in acrylamide cells with disrupted vimentin. In control cells, FSS induced a significant 4.1-fold increase in COX-2 protein (S vs. F, $P < 0.05$) (Fig. 10*A*). Interestingly, cells treated with acrylamide failed to significantly increase COX-2 in response to FSS (Fig. 10*A*). Just as we found in experiments with actin disruptors and microtubule disruptors, treatment with NS-398, which completely inhibits flow-induced prostaglandin release, had no significant effect on COX-2 levels in drug-treated cells in static conditions and subjected to FSS (Fig. 10*A*). We previously demonstrated that FSS increased c-Fos levels in osteoblasts (35). To test the specificity of the effect of acrylamide on COX-2, we also examined the levels of c-Fos and c-Jun protein. c-Fos and c-Jun protein levels were increased significantly 5.7-fold and 3.6-fold (S vs. F, $P < 0.05$), respectively, in control cells subjected to FSS, and acrylamide treatment inhibited this response (Fig. 10*B*).

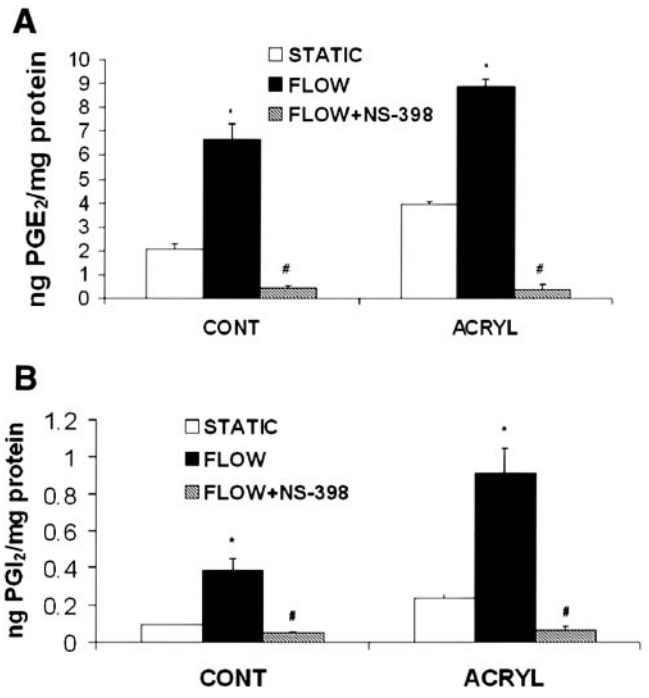

Fig. 9. Treatment with acrylamide to disrupt intermediate filaments does not inhibit FSS-induced, COX-2-dependent PGE_2 and PGI_2 release. PGE_2 (*A*) and PGI_2 (*B*) release in control and acrylamide-treated cells in static conditions (open bars) and after FSS (solid bars). Inhibition of COX-2 activity with 10 μM NS-398 (hatched bars) completely blocked the FSS-induced PGE_2 and PGI_2 release. $*P < 0.05$, static vs. flow, Student's *t*-test. $\#P < 0.05$, flow vs. flow + NS-398, Student's *t*-test.

To examine whether acrylamide would also block a nonmechanotransduction pathway, we treated cells with 10 μM PGE_2 and measured COX-2 levels in the presence of acrylamide. PGE_2 treatment resulted in a significant threefold increase in COX-2 levels compared with vehicle controls ($+PGE_2$ vs. $-PGE_2$, $P < 0.05$) (Fig. 10*C*). Treatment with acrylamide completely inhibited the PGE_2 induction of COX-2 protein (Fig. 10*C*).

DISCUSSION

In this study, we examined whether any of the three major cytoskeletal networks were required for FSS-induced signaling by individually disrupting these networks and measuring the effects on FSS-induced prostaglandin release and COX-2 induction. We found that in MC3T3-E1 osteoblasts neither actin stress fibers, insoluble F-actin, microtubules, nor intermediate filaments are by themselves required for FSS-induced prostaglandin release. We also found that regulation of FSS-induced, as well as PGE_2-induced, COX-2 protein does not require actin stress fiber formation, insoluble F-actin, or intact microtubules.

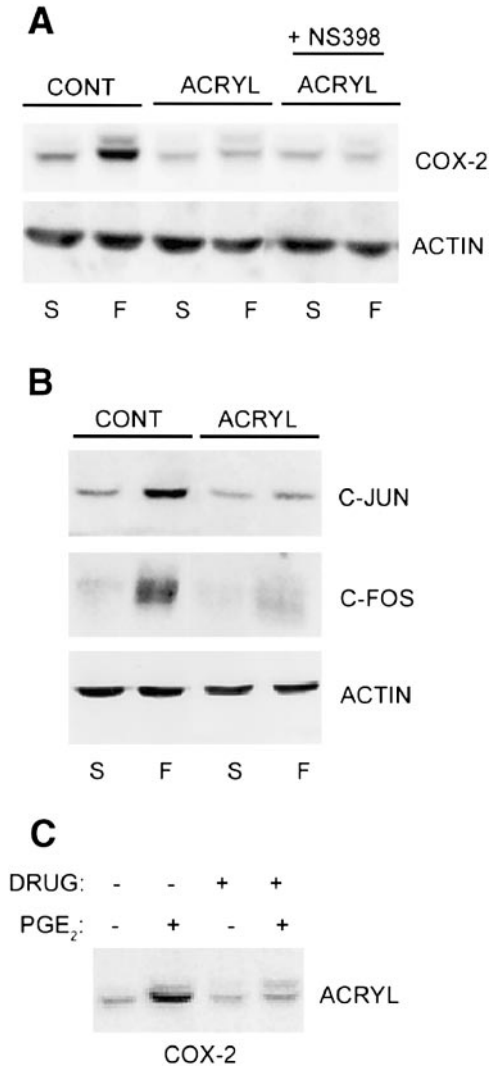

Fig. 10. Treatment with acrylamide to disrupt intermediate filaments inhibits the FSS induction of COX-2, c-Jun, and c-Fos protein and the PGE_2-induced increase in COX-2 levels. Immunoblot analysis of COX-2, c-Fos, and c-Jun proteins in control and acrylamide-treated cells. Protein was loaded in each lane in 20-μg amounts. *A*: immunoblot analysis of COX-2 and actin in control cells and cells treated with 25 mM acrylamide ± 10 μM NS-398. *B*: immunoblot analysis of c-Jun, c-Fos, and actin in control cells and cells treated with 25 mM acrylamide. *C*: immunoblot analysis of COX-2 protein in cells in static conditions treated with 10 μM PGE_2 ± 20 mM acrylamide. Densitometric analysis of at least 3 experiments was performed. FSS and PGE_2 induced significant increases in controls cells, but not in acrylamide-treated cells. For control cells, $P < 0.05$, static vs. flow, $+PGE_2$ vs. $-PGE_2$, Student's *t*-test.

We were somewhat surprised by these results given the accumulating evidence that suggests that cytoskeleton may play a role in cellular mechanotransduction (3). However, in this study, we tested only the individual requirement of each type of cytoskeleton. We cannot rule out the possibility that all three cytoskeletal networks work together to promote mechanotransduction and that one filament system may substitute for another in this capacity. In fact, it is likely that disruption of all three major cytoskeletal systems would be cytotoxic to the cell. Furthermore, we cannot rule out the possibility that in vivo, where bone cells are surrounded by a three-dimensional matrix, the cytoskeleton might play a pivotal role in the response of bone cells to mechanical stimulation.

Others have also investigated whether the different cytoskeletal systems are required for specific cellular signaling mechanisms. Knudsen and Frangos (26) and Stamatas and McIntire (39) found that FSS-induced nitric oxide release and calcium response, respectively, did not depend on intact, individual cytoskeletal networks. In contrast to the above studies, however, Charras and Horton (8) found, using atomic force microscopy, that both intermediate filaments and microtubules were required for activation of stretch-activated calcium channels. In a study similar to ours, Ajubi et al. (2) showed that disruption of actin microfilaments in osteocytes with cytochalasin B did block the pulsating fluid flow induction of PGE_2 release. Although Ajubi et al. used a pulsating fluid flow model, they reported similar levels of PGE_2 release as we did in this study using our laminar FSS system. The most important difference seems to be that they used osteocytes in their study and we used osteoblasts. Lastly, in 1998, our laboratory reported that cytochalasin D blocked the FSS induction of COX-2 (35). There are several differences between our present study and previous studies from our laboratory and others. Most importantly, in this study we investigated the role of the three major cytoskeletal networks and utilized multiple cytoskeleton-disrupting drugs. Furthermore, our present study was performed by using a different flow time course and a concentration of cytochalasin that more completely prevents stress fiber formation than our previous study. Also, we were able to utilize the cytochalasins and LAT A to disrupt only actin filament organization and use JAS P to rid the cell of essentially all insoluble F-actin. We were initially surprised that JAS P disrupted insoluble F-actin so severely; however, it has previously been shown to disrupt actin polymerization in cultured cells (6). Results of these studies clearly show that none of the actin poisons or microtubule poisons prevented FSS-induced signaling.

We previously reported that FSS induced the formation of robust actin stress fibers (35) in osteoblasts and proposed a potential role for stress fiber formation in mechanotransduction. Here, we attempted to quantitate the stress fiber formation and found that FSS-induced stress fiber formation is simply due to a reorganization of existing actin filaments and not due to measurable increase in total actin (data not shown) or insoluble F-actin levels. In addition, although the actin cytoskeleton does not seem to be required for the FSS-induced responses we measured, our present data do not rule out the possibility that integrins and focal adhesions function as mechanosensors. However, the role of an intact cytoskeleton in transmission of those signals is now questionable. It will be interesting to test in the future whether integrins can act as mechanosensors and transducers without being linked to an intact cytoskeleton. Also, we cannot rule out that the cortical actin cytoskeleton may be essential for signaling through focal adhesions, and we do not know the extent to which we disrupted the cortical actin cytoskeleton in these studies. These questions require further investigation.

Lastly, our results show that disruption of vimentin intermediate filaments with acrylamide blocks the FSS induction of COX-2, c-Fos, and c-Jun protein as well as the PGE_2 induction of COX-2 protein. The most straightforward explanation for these results is that intermediate filaments may play a pivotal

role in mechanotransduction in osteoblasts. Although this may indeed be true, acrylamide has also been reported to block protein synthesis (1, 24), and unfortunately we cannot rule this out as a possible explanation for our results. As discussed above, FSS-induced prostaglandin release still occurred in the presence of acrylamide and was dependent on the activity of the existing COX-2. One explanation for the ability of the cells to still release prostaglandins in a COX-2 dependent manner in the presence of acrylamide is that acrylamide affects arachidonic acid production. In fact, although acrylamide treatment did not increase PLA_2 activity in our experiments (data not shown), Muller-Decker et al. (31) previously demonstrated that acrylamide does indeed increase arachidonic acid production. Future studies using osteoblasts from vimentin knockout mice, for example, will provide a more direct analysis of the contribution of vimentin to mechanotransduction in osteoblasts. In fact, analysis of fibroblasts from vimentin knockout mice revealed that the fibroblasts display impaired mechanical stability and migration (11, 17), suggesting a role for vimentin in the mechanical stability of cells.

Interestingly, we also discovered that all of the FSS-induced prostaglandin release in control and drug-treated cells was due to the activity of COX-2. This indicates that COX-2, but not COX-1, plays a critical role in FSS-induced prostaglandin release, as has also been recently demonstrated by Bakker et al. (4). Also, PGE_2 treatment alone has been shown to increase COX-2 levels in MC3T3-E1 cells (41). To determine whether the FSS-induced release of PGE_2 contributes to the FSS-induced increase in COX-2 levels, we blocked all FSS-induced prostaglandin release with NS-398 and measured COX-2 protein levels. We found that blocking the FSS-induced prostaglandin release did not prevent a significant increase in COX-2 levels. This result indicates for the first time that an autofeedback mechanism involving FSS-induced prostaglandin release is not required for FSS-induced increases in COX-2 protein.

In summary, our results demonstrate that FSS-induced prostaglandin production in osteoblasts is dependent primarily on COX-2 activity and that prostaglandin release is not prevented by disruption of any of the three major cytoskeletal networks.

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GRANTS

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