Formation of focal adhesions on fibronectin promotes fluid shear stress induction of COX-2 and PGE\textsubscript{2} release in MC3T3-E1 osteoblasts

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Ponik, Suzanne M., and Fredrick M. Pavalko. Formation of focal adhesions on fibronectin promotes fluid shear stress induction of COX-2 and PGE\textsubscript{2} release in MC3T3-E1 osteoblasts. J Appl Physiol 97: 135–142, 2004. First published March 5, 2004; 10.1152/japplphysiol.01260.2003.—Mechanical loading of bone is important for the structural integrity of the skeleton and the maintenance of bone mass. Mechanically loading bone generates fluid shear stress (FSS) across the surface of bone cells resulting in the induction of cyclooxygenase-2 (COX-2) and release of prostaglandins, both of which are necessary for mechanically induced bone formation. However, the mechanisms by which cells transduce FSS-induced signals across the membrane and into the cell remain poorly understood. Focal adhesions, which are specialized sites of attachment between cells and the extracellular matrix, play a role in signal transduction and have been proposed to function as mechanosensors. To directly test whether focal adhesions mediate mechanotransduction in bone cells, we inhibited the formation of focal adhesions by 1) culturing MC3T3-E1 osteoblasts on bovine serum albumin (BSA), which does not contain integrin binding sites or by 2) treating cells cultured on fibronectin with soluble Arg-Gly-Asp-Ser (RGDS) peptide to specifically block integrin-fibronectin interactions. We then subjected the cells to FSS and measured COX-2 induction and PGE\textsubscript{2} release. Both COX-2 induction and PGE\textsubscript{2} release in response to FSS were significantly decreased when osteoblasts were treated with soluble RGDS peptide compared with controls. However, RGDS peptide treatment did not affect FSS-induced ERK phosphorylation. Interestingly, osteoblasts cultured on BSA to suppress focal adhesion formation secreted fibronectin and increased focal adhesion formation over time, which correlated with the induction of COX-2 in response to FSS. Together, these results suggest that fibronectin-induced formation of focal adhesions promotes FSS-induced PGE\textsubscript{2} release and upregulation of COX-2 protein.

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BONE IS A DYNAMIC TISSUE THAT models and remodels in response to mechanical loads from the external environment (16, 23, 33, 38). At the cellular level, mechanical loading of bone generates movement of interstitial fluid, creating fluid shear stress across the surface of bone cells (8, 9, 26). In response to fluid shear stress, osteoblasts increase expression of cyclooxygenase-2 (COX-2) and increase release of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) (2, 21, 30–32, 37), which are required for mechanically induced bone formation (6, 12). However, the mechanisms by which osteoblasts detect fluid shear stress and transduce the signal across the membrane to activate signaling pathways involved in bone metabolism, such as induction of COX-2 and release of PGE\textsubscript{2}, remain poorly understood.

Focal adhesions are specialized sites of cell-matrix interaction composed of integrins and many focal adhesion-associated cytoplasmic proteins including, but not limited to, vinculin, focal adhesion kinase (FAK), α-actinin, and actin filaments (3). Because of the cellular location of focal adhesions, it has been suggested that focal adhesions function as mechanoreceptors. Specifically, binding of integrins to the extracellular matrix may facilitate the transfer of mechanical signals from the external environment across the membrane to activate intracellular signaling cascades (1, 14, 18, 36). Experimental evidence supporting a role for focal adhesions as mechanoreceptors includes the observation that fluid shear stress activates intracellular focal adhesion components. For example, fluid shear stress induces integrin-Src association, depolarization of the focal adhesion, and increased phosphorylation of FAK. Each of these processes can be inhibited by integrin function-blocking antibodies or soluble Arg-Gly-Asp-Ser (RGDS) peptides that inhibit integrin binding to the RGDS sequence within many matrix proteins (19, 22, 24, 34). In addition, fluid shear stress can also cause morphological changes to focal adhesions, including increased size and number of focal adhesions and increased recruitment of \( \beta_1 \)-integrin to focal adhesions (7, 30). Although integrin activation clearly occurs in response to fluid shear stress, the induction of cyclooxygenase-2 (COX-2) and release of prostaglandins in response to fluid shear stress, which are necessary for mechanically induced bone formation, have not been directly linked to integrins or focal adhesion formation.

In this study, we investigated the role of focal adhesions as potential mechanosensors through which fluid shear stress-induced mechanical signals may be transduced across the osteoblast cell membrane to activate PGE\textsubscript{2} release and induce COX-2 protein expression. We found that inhibiting focal adhesion formation by culturing osteoblasts on bovine serum albumin (BSA), a substrate that does not contain integrin binding sites, significantly reduced PGE\textsubscript{2} release in response to fluid shear stress. When soluble RGDS peptide was used to inhibit integrin binding and limit focal adhesion formation on fibronectin, fluid shear stress-induced PGE\textsubscript{2} release as well as upregulation of COX-2 protein were significantly inhibited. However, fluid shear stress-induced ERK phosphorylation was unaltered when focal adhesion formation was inhibited with the use of soluble RGDS peptide. These results suggest that...

MATERIALS AND METHODS

Cell culture conditions. MC3T3-E1 osteoblasts were grown in α-MEM containing 10% fetal calf serum and streptomycin. To modulate focal adhesion formation, glass slides were coated with a solution of BSA (10 µg/cm²) or bovine plasma fibronectin (0.7 µg/cm²) (Sigma-Aldrich) and allowed to dry completely. After the slides were rinsed with PBS, MC3T3 osteoblasts were passaged onto the BSA- or fibronectin-coated slides and allowed to adhere for 2 h in α-MEM containing 0% fetal calf serum and 1% penicillin and streptomycin. Serum was excluded to eliminate the effect of matrix proteins contained in serum and the length of time in culture was kept short to minimize the influence of cell-secreted matrix proteins. RGDS or Arg-Gly-Glu-Ser (RGES) peptide (Sigma-Aldrich) treatment included incubating suspended MC3T3 osteoblasts for 1 h while they rotated at 4°C with 5% CO₂. Static controls were incubated in the same volume of media at 37°C with 5% CO₂. Fluid shear stress experiments were performed at 37°C with 25 ml of α-MEM containing 0% fetal calf serum and 500 µg/ml RGDS or RGES peptide or an equal volume of vehicle (PBS). After the 1-h treatment, osteoblasts were allowed to adhere to fibronectin-coated slides in the continued presence of RGDS or RGES peptide for 2 h before static or fluid shear stress conditions in the continued presence of vehicle or peptide.

Fluid flow conditions. Cells were subjected to laminar fluid shear stress (10 dyn/cm²) by using parallel-plate flow chambers and the flow-loop system designed by Frangos et al. (13) and marketed by Cytodyne (San Diego, CA). Fluid shear stress experiments were performed at 37°C with 25 ml of α-MEM subjected to a stream of 5% CO₂. Static controls were incubated in the same volume of media at 37°C with 5% CO₂. Cells cultured for 4 days in α-MEM containing 10% serum were subjected to a time course of fluid shear stress in α-MEM containing 1% serum. Serum contains 3–6 g protein/100 ml (3–6% protein). Therefore, when we subject cells to fluid shear stress in the presence of 1% serum, we are using a total protein concentration of 0.03–0.06% protein. To verify that the absence of protein did not have any adverse effects on the cells, we also cultured cells for 2 h on fibronectin-coated slides in α-MEM containing 0% serum or 0.1% BSA and then subjected the cells to a time course of fluid shear stress in α-MEM containing 0% serum or 0.1% BSA, respectively. We chose 0.1% BSA because this is close to the concentration of protein in media containing 1% serum. After verification that there was no difference in the level of COX-2 expression in the presence or absence of protein in the media (see Fig. 1), subsequent experiments in this study were performed in the absence of serum or BSA and included 2 h of adhesion followed by 5 h of fluid shear stress or static culture conditions.

Immunofluorescence microscopy. For fluorescence microscopy, cells were fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.2% Triton, and processed with the appropriate antibody. We record images with a RT Color Spots digital camera (Diagnostic Instruments) attached to an Optiphot-2 Nikon epifluorescence microscope that used a ×60 Nikon planapo objective.

Immunoblot. For immunoblot analysis, cells subjected to either static culture or fluid shear stress were harvested in SDS sample buffer. Protein concentrations were determined by using the amido black method (35). Equal cellular protein (40 µg) was loaded onto SDS-PAGE gels for separation and transferred to nitrocellulose for immunoblotting. The antibody signal was detected and analyzed with a chemiluminescence imager (Fuji, Edison, NJ) after reaction in a luminescence solution described in Norvell et al. (27). The experiments were carried out in at least triplicate.

Antibodies. For immunofluorescence analysis, we used vinculin VIN 11-5 (Sigma-Aldrich), anti-α-integrin 1928 (Chemicon), and anti-fibronectin (Transduction Laboratories). For immunoblot analysis we used, polyclonal COX-2 antibody (Cayman Chemical), monoclonal anti-actin AC-40 (Sigma-Aldrich), anti-ERK1/2 and anti-phospho-ERK1/2 (Santa Cruz). The appropriate secondary antibodies were purchased from Jackson Immunoresearch.

Prostaglandin measurement. Prostaglandin release was measured after 5 h of fluid flow or static culture by incubating the cells in 1 ml of fresh α-MEM with 0% fetal calf serum at 37°C and 5% CO₂ for 30 min. The media samples were centrifuged at 14,000 g for 1 min to pellet any particulates. The supernatant was transferred to a new tube, and the level of PGE2 present in sample was determined with enzyme immunoassay kits from Amersham Pharmacia Biotech. Statistical analysis. Statistical analysis was performed with the statistical package Statview (version 5.0.1). Differences between static and flow and between flow-fibronectin and flow-BSA were tested by using Student’s t-test for unpaired variants. Differences among group means were tested for significance by ANOVA, followed by Fisher’s protected least significant difference test for all pairwise comparisons. (P values < 0.05 were considered significant).

RESULTS

Induction of COX-2 protein in response to fluid shear stress is delayed under conditions used to modify focal adhesions. To modulate the formation of focal adhesions in MC3T3-E1 osteoblasts, cell culture conditions were carefully defined. First, serum was excluded from the media to eliminate the
confounding effect that serum matrix proteins would have if these proteins were present and allowed to adsorb to the substrate. Second, the length of time in culture was kept short to minimize the potential impact of matrix proteins secreted by osteoblasts and incorporated into the cellular microenvironment on cell-substrate interactions. We first compared COX-2 protein levels during a time course of fluid shear stress under normal-serum and serum-free conditions. We observed a dramatic difference in the length of time required for fluid shear stress to elicit a significant increase in COX-2 protein expression between these two conditions (Fig. 1). Cells cultured on glass slides under conditions that allowed the cells to secrete their own native matrix (4 days in the presence of 10% serum) upregulated COX-2 protein within 30–60 min after the onset of fluid shear stress (10 dyn/cm²) in media containing 1% serum (Fig. 1A). However, cells cultured for only 2 h in the absence of serum on slides coated with fibronectin required 3–5 h of fluid shear stress to elicit an increase in COX-2 protein (Fig. 1B).

To determine whether the absence of protein in the media may have adverse effects on the cells response to fluid shear stress (17), we cultured osteoblasts for 2 h on fibronectin-coated slides and completed a time course of fluid shear stress in the presence of media containing 0.1% BSA. Similar to our results shown in Fig. 1B in which no protein was present in the media, we found that cells cultured on fibronectin for 2 h in the presence of 0.1% BSA also required 3–5 h of fluid shear stress to elicit an increase in COX-2 protein (Fig. 1C). Therefore, to assess the role of focal adhesions as mechanosensors, experiments in this study were performed in the absence of serum and included 2 h of adhesion followed by 5 h of fluid shear stress or static culture conditions.

MC3T3-E1 osteoblasts form robust focal adhesions when cultured on fibronectin but not when cultured on BSA or when treated with soluble RGDS peptide. To determine the role of focal adhesions in PGE₂ release and induction of COX-2 protein in response to fluid shear stress, we used two methods to limit the formation of focal adhesions. MC3T3-E1 osteoblasts were cultured on glass slides coated with BSA or fibronectin in the absence of serum. BSA does not contain integrin binding sites, and therefore this substrate is used to limit focal adhesion formation. Conversely, focal adhesion formation was enhanced by culturing cells on glass slides coated with fibronectin, which contains multiple binding sites for integrins (29). Immunofluorescence analysis using an antibody to detect vinculin positive focal adhesions showed cells were well spread and formed robust focal adhesions after 2 h of adhesion on fibronectin (Fig. 2a). In contrast, cells cultured on BSA adhered and partially spread compared with cells on fibronectin but formed only a few focal adhesions (Fig. 2b).

Next, to more specifically inhibit focal adhesion formation on a fibronectin matrix, we treated osteoblasts with soluble RGDS peptide. RGDS is the primary integrin binding sequence within fibronectin and therefore treatment with soluble RGDS peptide competes with the RGDS sequence in fibronectin for integrin binding (29). A range of soluble RGDS peptide concentrations were tested, and a concentration of 500 μg/ml RGDS peptide, which did not cause cellular cytotoxicity (data not shown), was chosen for experiments. Visualization of focal adhesions by immunofluorescence microscopy showed treatment with soluble RGDS peptide inhibited the formation of focal adhesions on fibronectin after 2 h of adhesion (Fig. 2e). RGES peptide, which does not bind integrins, was used at the same concentration (500 μg/ml) as a negative control in these experiments. After 2 h in culture osteoblasts treated with vehicle (PBS) (Fig. 2c) or RGES control peptide (Fig. 2d) formed robust focal adhesions.

Fluid shear stress-induced PGE₂ release is inhibited by limiting focal adhesion formation. After 2 h of adhesion we subjected cells to 5 h of fluid shear stress or static culture conditions to determine the role of focal adhesions in fluid shear stress-induced PGE₂ release. We found that fluid shear stress-induced PGE₂ release was significantly reduced by 47% in cells cultured on BSA compared with cells cultured on fibronectin (Fig. 3A). Treatment of the MC3T3-E1 osteoblasts with soluble RGDS peptide to inhibit the formation of focal adhesions on fibronectin reduced fluid shear stress-induced PGE₂ release even more dramatically, by 77 and 78% com-

Fig. 2. Focal adhesion formation under modified culture conditions. Osteoblasts were allowed to adhere to BSA- or FN-coated slides for 2 h or treated with vehicle (PBS), Arg-Gly-Asp-Ser (RGDS), or Arg-Gly-Glu-Ser (RGES) peptide for 1 h in suspension and then allowed to adhere to FN-coated slides for 2 h. Cells were then fixed in 4% paraformaldehyde and permeabilization in 0.2% Triton, and focal adhesions were visualized by using an anti-vinculin antibody. After 2 h, cells cultured on FN formed robust vinculin positive focal adhesions (a). Osteoblasts cultured on BSA failed to form mature focal adhesions and contained only a few small vinculin-positive sites located at the cell periphery (b). Prominent vinculin-positive focal adhesions formed in vehicle- and RGES peptide (500 μg/ml)-treated controls (c and d, respectively). RGDS peptide (500 μg/ml)-treated cells failed to form mature focal adhesion and contained only a few small vinculin-positive sites after 2 h of adhesion (e).
Fig. 3. Fluid shear stress-induced prostaglandin E$_2$ (PGE$_2$) release is inhibited by limiting focal adhesions. Values are means ± SE. A: in response to 5 h of fluid shear stress, PGE$_2$ release was significantly increased compared with their static counterparts when cultured on BSA or FN. *$P < 0.05$, Flow vs. Static (by Student’s $t$-test). Fluid shear stress-induced PGE$_2$ release from cells cultured on BSA was decreased by 47% compared with cells cultured on FN. *$P < 0.05$, Flow-BSA vs. Flow-FN (by Fisher’s protected least significant difference test). B: fluid shear stress-induced a significant increase in PGE$_2$ release from FN, FN + RGDS, and FN + RGES cells compared with their static counterparts. *$P < 0.05$, Flow vs. Static (by Student’s $t$-test). Treatment with RGDS peptide significantly reduced the fluid shear stress-induced release of PGE$_2$ by 77 and 78% compared with vehicle and RGES control cells, respectively. *$P < 0.05$, Flow-FN + RGDS vs. Flow-FN and Flow-FN + RGES (by Fisher’s protected least significant difference test).

Fig. 4. Fluid shear stress induction of COX-2 is inhibited by soluble RGDS peptide treatment but not by culturing cells on BSA. A: COX-2 induction significantly increased in response to 5 h of fluid shear stress in cells cultured on both FN and BSA compared with their static counterparts ($P < 0.05$, Static (S) vs. Flow (F), by Student’s $t$-test). No difference was detected in fluid shear stress-induced COX-2 protein levels in cells cultured on BSA compared with cells cultured on FN. Equal cellular protein (40 $\mu$g) was loaded, and actin was shown as a loading control ($n = 6$ experiments for densitometry). B: fluid shear stress significantly increased COX-2 protein levels under all treatment conditions compared with their static counterparts ($P < 0.05$, Flow vs. Static, by Student’s $t$-test). Fluid shear stress-induced COX-2 protein levels in RGES-treated cells were not significantly altered compared with vehicle controls. However, treatment with RGDS peptide significantly decreased fluid shear stress-induced COX-2 protein levels by 46 and 36% compared with vehicle and RGES controls, respectively ($P < 0.05$, Flow-FN + RGDS vs. Flow-FN and Flow-FN + RGDS, by Fisher’s protected least significant difference test). Equal cellular protein (40 $\mu$g) was loaded and actin was shown as a loading control ($n = 5$ experiments for densitometry). C: induction of COX-2 protein graphed as the fold increase of COX-2 measured by densitometry in vehicle-, RGES-, or RGDS-treated cells respectively. *$P < 0.05$, FN + RGDS vs. FN, FN + RGES, and FN + RGDS, respectively. *$P < 0.05$, FN + RGDS vs. BS A, FN, and FN + RGES (by Fisher’s protected least significant difference test).
fluid shear stress, whereas cells treated with soluble RGDS peptide showed a reduced COX-2 response to fluid shear stress. Immunofluorescence analysis of vinculin-positive focal adhesions revealed that in cells cultured on fibronectin neither increasing the amount of time in static culture nor subjecting the cells to 5 h of fluid shear stress altered the formation of focal adhesions (Fig. 5, a–c). In contrast, cells cultured on BSA showed a dramatic increase in vinculin positive focal adhesions after 2 h of adhesion plus 5 h of static culture or fluid shear stress (Fig. 5, e and f) compared with cells allowed to adhere for just 2 h on BSA (Fig. 5d). Thus the formation of focal adhesions in cells cultured on BSA occurred in both static and flow conditions, indicating that the length of time in culture rather than treatment with fluid shear stress induces the formation of focal adhesions.

These results led us to investigate whether osteoblasts cultured on BSA might secrete matrix proteins, such as fibronectin, that contain binding sites for focal adhesions. Cells cultured on BSA were fixed after 2 h of adhesion or 2 h of adhesion plus 5 h of static culture or fluid shear stress, and fibronectin secretion was visualized by using an anti-fibronectin antibody. Immunofluorescence analysis showed that MC3T3-E1 osteoblasts were negative for secreted fibronectin after 2 h in culture (Fig. 5g). However, cells cultured on BSA for 2 h and then subjected to 5 h of static culture or fluid shear stress were positive for secreted fibronectin (Fig. 5, h and i), suggesting that cells cultured on BSA may form focal adhesions over time in culture by secreting fibronectin to which integrins can subsequently bind. Together, these results indicated that the secretion of fibronectin and subsequent formation of focal adhesions in cells cultured on BSA after 5 h of static culture or fluid shear stress correlates with the induction of COX-2 in response to fluid shear stress.

**RGDS peptide treatment inhibits focal adhesion formation on fibronectin even after 5 h of static culture or fluid shear stress.** To verify that treatment with soluble RGDS peptide inhibited the formation of focal adhesions on fibronectin both before and after 5 h of fluid shear stress, we visualized vinculin-positive focal adhesions by immunofluorescence microscopy. Treatment with RGDS peptide limited vinculin-positive focal adhesion formation on fibronectin after 2 h of adhesion (Fig. 6g) and continued to inhibit focal adhesions after 5 h of static culture or fluid shear stress (Fig. 6, h and i). The effect of RGDS peptide was dramatic compared with both vehicle (PBS) (Fig. 6, a–c) and RGES peptide (Fig. 6, d–f) controls, which had prominent focal adhesions after 2 h of adhesion as well as after being subjected to 5 h of static culture or fluid shear stress. These results indicate that the reduction in both fluid shear stress-induced PGE2 release and upregulation of COX-2 protein correlates with inhibition of focal adhesions on fibronectin by soluble RGDS peptide treatment.

**RGDS peptide treatment does not alter fluid shear stress-induced ERK phosphorylation.** Others studies demonstrated that COX-2 induction in response to fluid shear stress is partially dependent on ERK phosphorylation (39). Therefore, we also investigated whether focal adhesions were required for fluid shear stress-induced ERK phosphorylation in MC3T3-E1 osteoblasts. After 2 h of adhesion on glass slides coated with

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**Fig. 5.** MC3T3 osteoblasts grown on BSA secrete FN and form focal adhesions after extended time in culture. Osteoblasts allowed to adhere for 2 h or 2 h plus 5 h of static or fluid shear conditions were fixed in 4% paraformaldehyde and permeabilization in 0.2% Triton. Focal adhesions were visualized by using an anti-vinculin antibody. Osteoblasts cultured on fibronectin formed robust focal adhesion after 2 h of adhesion (a), and the focal adhesions remained prominent after 5 h of static culture or fluid shear stress (b and c). Osteoblasts cultured on BSA had very few mature focal adhesions after 2 h in culture (d) but formed more prominent vinculin-positive focal adhesions after 5 h of static culture or fluid shear stress conditions (e and f). g–i: Cells were cultured on BSA, and FN secretion was visualized by using an antibody recognizing mouse FN. After 2 h in culture, FN was undetectable in cells cultured on BSA (g). The fluorescence intensity of fibronectin increased after 5 h of static culture or fluid shear stress (h and i).
Fig. 6. RGDS peptide treatment decreases focal adhesions after 5 h of static or fluid shear stress conditions. Osteoblasts treated with vehicle (PBS), RGES, or RGDS peptide were allowed to adhere to FN-coated slides for 2 h or 2 h plus 5 h of static or flow conditions before fixation in 4% paraformaldehyde and permeabilization in 0.2% Triton. Focal adhesions were visualized by using an anti-vinculin antibody. Vehicle (PBS)-treated control cells cultured on FN formed focal adhesions after 2 h of adhesion (a), and the focal adhesions appeared unaltered by 5 h of static culture or fluid shear stress (b and c). RGES control peptide (500 μg/ml) did not have any apparent effect on vinculin containing focal adhesions under static culture or fluid shear stress (d–f) compared with vehicle-treated control cells (a–c). RGDS peptide (500 μg/ml)-treated cells formed very few mature vinculin-positive focal adhesions after 2 h of adhesion (g) as well as after 5 h of static culture or fluid shear stress (h and i). Despite limited focal adhesion formation, MC3T3-E1 osteoblasts treated with RGDS peptide remained attached when subjected to fluid shear stress.

Fig. 7. Inhibition of focal adhesions on FN does not block fluid shear stress-induced ERK phosphorylation. Western blot analysis of total ERK1/2 revealed no change in the total levels of ERK1/2 in response to fluid shear stress under any conditions (data not shown). Total ERK levels remained unchanged in response to 5 h of fluid shear stress (Fig. 7). Treatment with soluble RGDS peptide, which inhibited the induction of COX-2 in response to fluid shear stress, did not alter fluid shear stress-induced ERK phosphorylation after 5 h compared with both vehicle (PBS) and RGES peptide control cells (Fig. 7). This suggests that under our culture conditions activation of ERK is not dependent on focal adhesion formation.

DISCUSSION

To date, there has been no direct assessment of the role of focal adhesions in fluid shear-induced PGE2 release and increased COX-2 expression in osteoblasts. Here, we directly investigated the role of focal adhesions in transduction of fluid shear stress-induced mechanical signaling leading to increased COX-2 protein expression and PGE2 release in MC3T3-E1 osteoblasts. COX-2 induction and PGE2 release are necessary for mechanically induced bone formation (6, 12), but little is known about how these pathways are regulated in response to fluid shear stress. The induction of COX-2 protein, and possibly PGE2 release, in response to fluid shear stress have only been linked to signaling pathways downstream of focal adhesions. Therefore, our results are significant because we show directly that focal adhesions promote these responses. Additionally, these results focus attention on the importance of focal adhesions in the regulation of mechanically induced bone formation.

By using two methods to limit the formation of focal adhesions, we determined for the first time that fibronectin-induced formation of focal adhesions promotes fluid shear stress-induced PGE2 release and upregulation of COX-2 protein. Culturing of osteoblasts on BSA inhibited the formation of focal adhesions after 2 h of culture; however, after 5 h of static culture or fluid shear stress, osteoblasts overcame this inhibition and secreted fibronectin and formed focal adhesions. Although the secretion of fibronectin and formation of focal adhesion on BSA allowed for the induction of COX-2 after 5 h
of fluid shear stress, it was insufficient for the normal PGE₂ response. Perhaps with longer periods of fluid shear stress the normal fluid shear stress-induced PGE₂ response would be achieved. In contrast to cells cultured on BSA, focal adhesion formation was dramatically reduced both before and after 5 h of static culture or fluid shear stress when cells were treated with soluble RGDS peptide. In some studies, soluble RGDS peptide has been shown to bind to α₅β₁, α₅β₁, and αυβ₁ integrins and to induce an active conformation (4); however, we did not see any effect of RGDS treatment on static COX-2 levels or PGE₂ release compared with vehicle-treated controls or RGES peptide-treated cells. Together, these results suggest that treatment with soluble RGDS peptide did not directly activate integrins but did inhibit clustering of integrins into focal adhesions. Therefore, the significant reduction in fluid shear stress-induced COX-2 protein levels and PGE₂ release in cells treated with RGDS peptide can be more specifically attributed to the limited formation of focal adhesion on fibronectin in osteoblasts.

Wadhwa et al. (39) demonstrated that fluid shear stress-induced upregulation of COX-2 was partially dependent on the phosphorylation of ERK. In our studies, we found that fluid shear stress-induced COX-2 protein, but not ERK phosphorylation, was inhibited by blocking the formation of focal adhesions with soluble RGDS peptide. Others have also demonstrated that RGDS peptide treatments, used to block integrin-matrix interactions, did not block fluid shear stress-induced ERK phosphorylation in osteoblasts (40). Together, these results suggest that other non-focal-adhesion-mediated mechanosensers, such as receptor tyrosine kinases (5), G-protein-coupled receptors (15), the glycoalyx (11, 25), or ion channels (28), may be involved in the activation of ERK.

Studies on fluid shear stress-induced mechanotransduction have used different levels of protein in the media during shear stress, including no added protein (20), 0.05% serum (39), and 2% serum (10). We found that, under our experimental conditions on bone formation induced by mechanical stimulation. Am J Physiol Endocrinol Metab 267: E287–E292, 1994.


