Recently fluid flow has been shown to be a potent physical stimulus in the regulation of bone cell metabolism. However, most investigators have applied steady or pulsing flow profiles rather than oscillatory fluid flow, which occurs in vivo because of mechanical loading. Here oscillatory fluid flow was demonstrated to be a potentially important physical signal for loading-induced changes in bone cell metabolism. We selected three well known biological response variables including intracellular calcium (Ca^{2+}), mitogen-activated protein kinase (MAPK) activity, and osteopontin (OPN) mRNA levels to examine the response of MC3T3–E1 osteoblastic cells to oscillatory fluid flow with shear stresses ranging from 2 to \(-2\) Newtons/m\(^2\) at 1 Hz, which is in the range expected to occur during routine physical activities. Our results showed that within 1 min, oscillatory flow induced cell Ca^{2+} mobilization, whereas two MAPKs (ERK and p38) were activated over a 2-h time frame. However, there was no activation of JNK. Furthermore 2 h of oscillatory fluid flow increased steady-state OPN mRNA expression levels by approximately 4-fold, 24 h after exposure to fluid flow. The presence of both ERK and p38 inhibitors and thapsigargin completely abolished the effect of oscillatory flow on steady-state OPN mRNA levels. In addition, experiments using a variety of pharmacological agents suggest that oscillatory flow induces Ca^{2+} mobilization via the L-type voltage-operated calcium channel and the inositol 1,4,5-trisphosphate pathway.

Mechanical loading plays an important role in regulating bone metabolism. Increased mechanical loading increases bone formation and decreases bone resorption (1). The absence of mechanical stimulation causes reduced bone matrix protein production, mineral content, and bone formation, as well as an increase in bone resorption (2). However, the mechanism by which bone cells sense and respond to their physical environment is still poorly understood. In this study we examine a novel physical stimulus and loading-induced oscillatory fluid flow and demonstrate that when applied to cultured osteoblastic cells at levels expected to occur in vivo it regulates mRNA levels for an important bone matrix protein, osteopontin (OPN). Furthermore, this regulation occurs via an increase in intracellular calcium (Ca^{2+}) and mitogen-activated protein kinases (MAPKs). The sensitivity of bone tissue to mechanical loading has been proposed to involve a variety of cellular biophysical signals including loading-induced electric fields, matrix strain, and fluid flow. The latter effect of loading, originally described by Piekar ski et al. (3), has recently been proposed to directly regulate bone cell metabolism in vivo (4, 5). Furthermore, relative to other loading-induced biophysical signals applied to cells in vitro, fluid flow appears to be significantly more potent at physiological levels (6–10). The origin of loading-induced fluid flow is a consequence of the fact that a significant component of bone tissue is unbound fluid. Bone tissue contains an extracellular fluid compartment that has been demonstrated to communicate with the vascular compartment, and mechanical loading has been shown to enhance fluid exchange between the two spaces (11).

When bone is exposed to mechanical loading fluid in the matrix is pressurized and tends to flow into haversian canals. As loading is removed (e.g. during the gait cycle) the pressure gradients, and consequently the direction of fluid flow, are reversed resulting in a flow-time history experienced by the cells that is oscillatory in nature. In vitro experiments have shown fluid flow to have a number of effects on bone cells including Ca^{2+} mobilization (12), production of nitric oxide and prostaglandin E\(_2\) (8, 13), and regulation of the expression of genes for OPN, Cyclooxygenase-2, and c-Fos (9, 14, 15). However, it is important to note that only one study to date utilized a reversing flow profile and found significantly different results when contrasted with nonreversing flow (16). Thus, the aim of this study is to detail important aspects of the biochemical response pathway including immediate, interme-
Oscillatory Flow Activated Ca$^{2+}$, MAP Kinases, and Osteopontin

diate, and long term effects of oscillatory fluid flow on bone cells, as well as on their inter-relationships.

To achieve this goal, we first investigated three well known biological osteogenic response variables. Ca$^{2+}$, a known second messenger transducing extracellular signals to the cell interior, was our immediate response variable. Activity of MAPKs is important for regulating cell differentiation and apoptosis by transmitting extracellular signals to the nucleus (17, 18) and was our intermediate response variable. OPN is characterized as one of the predominant noncollagenous proteins that accumulate in the extracellular matrix of bone (19, 20) and is also believed to be an important factor associated with bone remodeling caused by mechanical stress in vivo (21). Recently, strong evidence suggests that OPN is an important factor in loading induced bone cell metabolism (22–24). Furthermore, the role of osteopontin in extracellular matrix is more than structural. It has been shown to be involved in regulating bone cell attachment, osteoclast function, and mineralization, suggesting a central role in both the initiation and regulation of bone remodeling (25, 26). Therefore, we quantified steady-state OPN mRNA levels as a long term response to oscillatory flow.

Recently MAPK family members including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase have been shown to be important signaling components linking mechanical stimuli to cellular responses, including cell growth, differentiation, and metabolic regulation, in endothelial cells, smooth muscle cells, and myocytes (27–30). However, the role of MAPKs in bone cell mechanotransduction has not been determined. Moreover the role of Ca$^{2+}$, in osteogenic gene transcription is unclear, especially in the case of oscillatory fluid flow. Therefore, the second goal of this study is to elucidate the roles of Ca$^{2+}$ and the three major MAPKs in bone cell osteopontin gene expression induced by oscillatory flow.

Finally, the mechanism responsible for fluid-flow-induced Ca$^{2+}$ mobilization has not been fully established, particularly for the oscillatory flow profiles expected to occur in vivo. Yelowley et al. (31) demonstrated that the steady flow-induced Ca$^{2+}$ responses in bovine articular chondrocytes involved both influx of external Ca$^{2+}$ and release of internal Ca$^{2+}$ from IP$_7$-sensitive stores and that the mechanism is G-protein-activated. Similar results were observed in bone cells stimulated by steady fluid flow (14, 32). However there is evidence to suggest that steady and oscillatory fluid flow may have different biochemical effects on bone cells (16). Therefore, the third goal of this study is to elucidate the mechanism contributing to oscillatory flow-induced Ca$^{2+}$ mobilization in bone cells. Steady flow (32), substrate stretch (33), and whole bone loading experiments (34) suggest that either stretch-activated mechanosensitive channels and/or L-type voltage-operated calcium channels (L-type VOCCs) may be involved. Additionally, it is not known whether the involvement of the IP$_7$-sensitive stores is as important in the response to oscillatory fluid flow or whether other internal pathways (the ryanodine-sensitive pathway) may be involved in Ca$^{2+}$ mobilization.

MATERIALS AND METHODS

Cell Culture—The mouse osteoblastic cell line MC3T3–E1 was cultured in minimal essential medium (MEM–a, Life Technologies, Inc.) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT), 1% penicillin and streptomycin (Life Technologies, Inc.) and maintained in a humidified incubator at 37 °C with 5% CO$_2$. All cells were subcultured on glass slides for 2 days prior to experiments, with the exception of cells cultured for Ca$^{2+}$ studies, for which quartz slides were used, for UV transparency. $3 \times 10^6$ cells seeded were seeded on the glass slides (75 × 38 × 1.0 mm), and $8.5 \times 10^5$ cells were seeded on the quartz slides (76 × 26 × 1.6 mm). There are no significant differences observed in the behavior of MC3T3–E1 cells grown on normal glass slides versus quartz slides. It is important to note that under these conditions the cells had not reached confluency nor did the medium (which did not include ascorbic acid or β-glycerophosphate) or time in culture (2 days) support differentiation or mineralization (35). Cells were exposed to oscillatory fluid flow in MEM–a and 2% FBS for calcium imaging experiments, and in MEM–a and 10% FBS for 2-h MAPK activity assays.

Oscillatory Fluid Flow Device—Two different parallel plate flow chamber sizes were utilized. Larger chambers with a rectangular fluid volume of $56 \times 24 \times 0.28$ mm were employed for long term flow to accommodate the larger glass slides. This size of slide was necessary to obtain adequate amounts of cell protein and mRNA. The smaller chamber with a fluid volume of $420 \times 26.8 \times 0.28$ mm, was employed in the calcium imaging studies where total cell number is not an issue. The oscillatory flow device was described in our previous study (16). Briefly, a Hamilton glass syringe was mounted in a small servopneumatic loading frame (EnduraTec, Eden Prairie, MN). The flow rate was monitored with an ultrasonic flowmeter with a 100-Hz frequency response (Transonic Systems Inc., Ithaca, NY).

Calcium Imaging—Intracellular calcium ion concentration ([Ca$^{2+}$]) was quantified with the fluorescent dye fura-2. fura-2 exhibits a shift in absorption when bound to Ca$^{2+}$ such that the emission intensity when illuminated with ultraviolet light increases with calcium concentration at a wavelength of 340 nm and decreases with calcium concentration at 380 nm. The ratio of light intensity between the two wavelengths corresponds to the calcium concentration. A calibration curve of intensity ratio and calcium concentration was obtained using fura-2 in buffered calcium standards supplied by the manufacturer (Molecular Probes, Inc., Eugene, OR).

Preconfluent (80%) cells were washed with MEM–a and 2% FBS at 37 °C, incubated with 10 μM fura-2-acetoxyethyl ester (Molecular Probes, Inc., Eugene, OR) solution for 30 min at 37 °C, then washed again with fresh MEM–a and 2% FBS prior to experiments.

Cell ensembles were illuminated at wavelengths of 340 and 380 nm in turn. Emitted light was passed through a 510-nm interference filter and detected with an intensifier charge coupled device camera (International Ltd., Sterling, VA). Images were recorded, one every 2 s, and analyzed using image analysis software (Metamorph; Universal Imaging, West Chester, PA). Basal [Ca$^{2+}$], was sampled for 3 min and followed by 3 min of oscillatory fluid flow (peak shear stress 2 nN/m$^2$, 1 Hz).

MAPK Activity Assay—There are three major MAPKs, p38 MAPK, ERK, and JNK. 100 μg of lysate protein from either control or flow treated cells was immunoprecipitated with anti-p38 MAPK, anti-ERK1/2, or anti-JNK antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight. Following addition of 15 μl of protein A/G for 2 h, the immunoprecipitate was collected by centrifugation, and the kinase reaction was then conducted in a kinase reaction buffer containing substrates myelin basic protein (for p38 MAPK or ERK) or c-Jun glutathione S-transferase (for JNK) in the presence of [γ-32P]ATP as described before (36). The reaction mix was subjected to SDS polyacrylamide gel electrophoresis, and phosphorylation of substrates was determined by autoradiography.

Osteopontin mRNA Analysis—The steady-state osteopontin mRNA level was quantified by quantitative real time reverse transcription polymerase chain reaction (QRT-RT-PCR) (9). Briefly, this technique is based on the detection of a fluorescent signal produced by an SNP-specific oligonucleotide probe during PCR primer extension (Prism 7700 sequence detection system; Applied Biosystems, West Chester, PA). Basal [Ca$^{2+}$], was sampled for 3 min and followed by 3 min of oscillatory fluid flow (peak shear stress 2 nN/m$^2$, 1 Hz).

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Unpublished data.
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yanodine (1 and 20 μM), 2-aminoethoxydiphenyl borate (2APB; 100 mM), U73122 and U73343 (4 or 5 μM). Thapsigargin is an inhibitor of the ATP-dependent Ca\(^{2+}\) pump of intracellular Ca\(^{2+}\) stores that causes Ca\(^{2+}\) discharge (38) and was used to empty the intracellular calcium stores. Gadolinium chloride (10 μM) (Aldrich) is a putative stretch-activated channel blocker (39). Nifedipine is a blocker of the L-type VOCC (40). Ryanodine, which affects ryano...
inhibitor PD98059 (PD; 10 μM) were obtained with a reduction of steady-state OPN mRNA to 1.76 ± 0.21 × no flow levels. Moreover the presence of both inhibitors (SB + PD) completely abolished the effect (0.84 ± 0.05 × no flow levels; not statistically different). Those results suggest that activation of p38 MAPK and ERKs is synergistically involved in flow-mediated OPN expression.

Sources of Ca\(^{2+}\) Mobilization in Response to Oscillatory Fluid Flow—The number of cells that responded to oscillatory fluid flow with a change in intracellular calcium in the presence of GdCl\(_3\), nifedipine, or c-Jun glutathione S-transferase (for JNK). The reaction mix was subjected to SDS polyacrylamide gel electrophoresis, and phosphorylation of substrates was determined by autoradiography. A representative autoradiograph is shown. The experiments were repeated with similar results. The appearance of double bands is an accepted occurrence with this assay (64, 65) and is because of impurity, phosphorylation, or degradation of the myelin basic protein (MBP) substrate but cannot be ascribed to differential ERK1/2 activity.

**Fig. 2.** Time courses for p38, ERK1/2, and JNK activation during oscillatory flow (2 N/m\(^2\), 1 Hz). At each time point the cells from two slides were combined to yield sufficient protein for the MAPK activity assay. Kinase activity was assayed by incubating lysates with \(\left[\gamma^{32}\right]ATP\) and myelin basic protein (for p38 MAPK and ERK1/2) or c-Jun glutathione S-transferase (for JNK). The reaction mix was subjected to SDS polyacrylamide gel electrophoresis, and phosphorylation of substrates was determined by autoradiography. A representative autoradiograph is shown. The experiments were repeated with similar results. The appearance of double bands is an accepted occurrence with this assay (64, 65) and is because of impurity, phosphorylation, or degradation of the myelin basic protein (MBP) substrate but cannot be ascribed to differential ERK1/2 activity.

Although a large number of *in vitro* studies have been aimed at discovering the regulatory effect of mechanical loading in bone adaptation, little consensus can be found in the literature regarding the appropriate biophysical signals. For example, bone cells have been shown to respond with metabolic changes to deformation induced by stretching of the substrate to which they are attached (22, 47–49). However, these studies employed either hyperphysiologic levels of strain or systems known to induce mechanical effects other than pure strain (50). More recent studies have suggested that bone cells are more responsive to the fluid flow induced by mechanical strain than directly to the strain in the tissue (7–9). However, loading-induced fluid flow *in vivo* involves a reversal of flow direction associated with the cyclical unloading that occurs in the vast majority of physical activities. To date, the ability of the resulting oscillatory flow profiles to regulate bone cell behavior *in vitro* has not been investigated beyond its ability to mobilize cytosolic calcium (16). In this study a novel oscillatory fluid flow system was designed to demonstrate that oscillatory fluid flow is capable of regulating bone cell gene expression via ERK and p38 MAPK activity and intracellular calcium signaling involving IP\(_3\)-mediated calcium release. Additionally, we were able to demonstrate some potentially important differences in the characteristics of the response of bone cells to oscillatory flow when contrasted with published experiments on steady/pulsatile flow. The study of the effects of oscillatory fluid flow on bone cells will allow us to more accurately understand the mechanism of mechanotransduction in bone cells *in vivo*, for which the other *in vitro* systems may not be as suitable.

Our experimental data have shown that oscillatory fluid flow induced three biological responses that are believed to be important in the response of bone tissue to mechanical load. In the short term, within 2 min of the start of oscillatory flow, 59.1 ± 4.6% of cells increased [Ca\(^{2+}\)]\(_i\), which was significantly different from the no flow period. This is consistent with prior observations of the Ca\(^{2+}\) response of bovine aortic endothelial cells (51), articular chondrocytes (52), and bone cells (12, 16) to steady/pulsatile fluid flow. However, oscillatory flow appears to be significantly less stimulatory than steady/pulsatile flow for bone cells in terms of the Ca\(^{2+}\) response (16). This suggests that the mechanotransduction pathways induced by oscillatory flow could be different in part or in whole from those activated by steady/pulsatile flow.

Recently MAPK activity has been shown to be modulated by various external stimuli such as growth factors, cytokines, and physical stresses (ultraviolet radiation, hyperosmolarity, hypoxia, and fluid flow shear stress) (17, 18, 53) and is known to play a pivotal role in a variety of cell functions. Our results are the first to examine the regulation of MAPK activity in response to biophysical stimulation in bone cells. We show that fluid flow induces an increase in the activity of two of three major MAPKs (ERKs and p38) over a period of 2 h for bone cells. p38 activity started to increase at 15 min and reached a maximum at 30 min and then returned to initial levels 90 min after the onset of oscillatory flow. A similar pattern was observed for ERK1/2 activity with some delay; at 60 min it reached a peak and returned to its pre-flow value at 90 min. JNK activity was unchanged during 90 min of oscillatory fluid flow stimulus. Our biphasic time course ERK1/2 and p38 activity results are consistent with previous studies in endothelial cells and smooth muscle cells (27, 28). However, our time to peak ERK1/2 activity (60 min) is slower than observed for
steady fluid flow (5 min), possibly because of the different mechanical stimuli. Another difference is that oscillatory fluid flow did not induce JNK activity in bone cells; however steady fluid flow is capable of activating JNK in endothelial cells within 60 min (27). Although possibly because of differences between the cell types, it may be a result of differences in the effects of the physical signals applied. This is consistent with the possibility that oscillatory fluid shear stress may stimulate different mechanotransduction pathways from steady/pulsatile fluid shear stress.

It was suggested previously that the ERK pathway is involved in the regulation of cell proliferation and differentiation whereas p38 and JNK are important signaling pathways in the regulation of cell apoptosis (54). However, recent information demonstrated that p38 MAPK may also play a critical role in the regulation of differentiation (55, 56). In this study, both the p38 inhibitor SB and the ERK1/2 inhibitor PD were applied to determine whether the increased MAPK activity we observed was required for the effect of oscillating flow on steady-state OPN mRNA levels. Either MAPK inhibitor alone was found to greatly attenuate (80%) the flow effect on steady-state OPN mRNA, whereas the presence of both inhibitors (SB + PD) completely abolished the effect of flow on steady-state OPN mRNA levels. This indicates that oscillatory flow-induced OPN expression involves both ERK and p38 MAPK activity with mild redundancy but does not require JNK activity. It is interesting to note that JNK activity has been observed in endothelial cells in response to the steady flow associated with apoptosis (27). In contrast, bone cells experiencing more moderate oscillatory shear stress exhibit increased ERK1/2 activity associated with proliferation and differentiation but no change in JNK activity. These findings support the view that oscillatory fluid flow may be a potent cellular physical signal in bone remodeling in vivo.

Our results also suggest that the biochemical mechanism of Ca\(^{2+}\) mobilization is different between nonreversing steady/pulsatile fluid flow and oscillatory flow. The results of the calcium experiment using nifedipine show that the L-type VOCC membrane channel is involved in the calcium response to oscillatory flow in contrast to steady flow experiments in primary bone cells (32) and in the same cell line (14). However our data are in agreement with substrate stretch experiments on primary osteoblasts in which the calcium response was inhibited by nifedipine (33). In those experiments fluid flow may have been induced in the system, as well as substrate stretch (50). Thus, it is possible that the Ca\(^{2+}\) response that the investigators observed was because of the pathway we describe here in response to oscillatory flow. Furthermore, the nitric oxide and prostaglandin E\(_2\) response of loaded whole rat bones in an in vivo model has been shown to be eliminated by nifedipine (34). This is again consistent with the view that oscillatory flow, rather than steady flow, is the cellular physical signal that regulates the adaption of bone to mechanical load in vivo.

Our data also suggest that the stretch-activated membrane channel, blocked using gadolinium chloride, is not important for response to oscillatory fluid flow. This is in contrast to data for steady flow where calcium responses were inhibited by blocking this channel (14, 32). However, our finding that GdCl\(_3\) did not influence steady-state OPN gene mRNA is consistent with the results of Chen et al. (14) that showed that the effect of steady flow on cytoskeletal reorganization and Cyclooxygenase-2 mRNA involved IP\(_3\)-mediated intracellular calcium release but not extracellular calcium. One interpretation is that both oscillatory and steady flow activate an IP\(_3\) cascade that is important in bone adaptation; however steady flow also stimulates an GdCl\(_3\)-sensitive calcium influx whereas oscillatory flow does not.

Our finding that thapsigargin completely blocked the calcium response to oscillatory flow demonstrated that the source of Ca\(^{2+}\) is release from intracellular stores. The next series of experiments were designed to further elucidate the mechanism of this release. The combination of the U73122 and the 2APB data strongly suggest that the IP\(_3\) pathway is involved. We achieved a partial block of the calcium response using U73122. This may be because there are other pathways to the formation of IP\(_3\) besides the phospholipase C and phospholipase A\(_2\) pathways blocked by U73122. However the effect of U73122 is
calcium to leave the stores before the flow was applied, in a similar way to thapsigargin, did have an inhibitory effect on the response, though less than that of thapsigargin. However the blocking of the ryanodine-sensitive channel with high concentration ryanodine had no significant effect on the calcium response. This suggests that ryanodine-sensitive Ca\textsuperscript{2+} stores, which can also be mobilized by the IP\textsubscript{3} pathway (60), were partially depleted by the low concentration ryanodine but that ryanodine-sensitive channels were not affected by oscillatory fluid flow.

Interestingly, our finding that the source of the calcium response is IP\textsubscript{3}-mediated release from intracellular stores seems to be contradicted by our finding that the L-type VOCC is also involved. If the VOCC is important to a calcium response mechanism one might expect to observe some residual calcium increase of extracellular origin, even in the presence of blockers of intracellular stored calcium. However, in our study both 2APB and thapsigargin totally abolished the calcium response to oscillatory fluid flow. Similar results were found in the Walker et al. substrate stretch study (33) in which thapsigargin inhibited the calcium response more than would be expected if only the residual calcium released after nifedipine treatment was from intracellular stores sensitive to thapsigargin. An explanation for these results may be that the IP\textsubscript{3} receptor on the endoplasmic reticulum has been shown to be coregulated by cytosolic calcium concentration. Thus, the VOCC could potentiate the fluid flow calcium response by regulating the local calcium concentration surrounding the endoplasmic reticulum IP\textsubscript{3} receptor but at levels that are not detectable with our imaging system. This mechanism would require that the VOCC and endoplasmic reticulum IP\textsubscript{3} receptor are in close association. Such an arrangement has previously been described in muscle cells between the VOCC and ryanodine-sensitive channels (61).

In the final phase of our investigation we related these intracellular signaling pathways to the regulation of gene expression. OPN has been implicated as an important factor in triggering bone remodeling caused by mechanical stress in vivo (21). Our OPN data are consistent with the in vitro results of Owan et al. (7). Steady-state OPN mRNA levels increased almost 4-fold within 24 h after 2-h oscillatory fluid flow. To elucidate the role of Ca\textsuperscript{2+} in bone cell mechanotransduction and OPN gene regulation, thapsigargin was employed to empty Ca\textsuperscript{2+}\textsubscript{i} stores, which prevents Ca\textsuperscript{2+} from being available to the cells during the oscillatory flow period. Thapsigargin completely abolished the increase in steady-state OPN mRNA levels that occurred on application of fluid flow. This finding combined with the role of OPN in mechanically mediated remodeling suggests a prominent role of cytosolic calcium mobilization in the adaptation of bone to mechanical loading.

Although our results suggest that both Ca\textsuperscript{2+}\textsubscript{i} and MAPK are involved in the mechanical stress-induced OPN expression in bone cells via oscillatory flow, the relationships between Ca\textsuperscript{2+}\textsubscript{i} and MAPK are still unclear. Our results show that Ca\textsuperscript{2+} is required for OPN expression induced by oscillatory flow. However some investigators demonstrated that steady flow in chondrocytes activated ERK1/2 in a way that did not require Ca\textsuperscript{2+}, and Ca\textsuperscript{2+} alone was not sufficient for MAPK activation by steady flow (62). Therefore the role of Ca\textsuperscript{2+} in MAPK activation under oscillatory flow remains to be determined. However, both IP\textsubscript{3} and Ca\textsuperscript{2+} have been shown to be a necessary step in G-protein-mediated MAPK activation in smooth muscle cells (63). Little is known about the signaling pathways between MAPK and target genes, although some investigations have shown that MAPK phosphatase-1 may act as a mediator to regulate target gene expression in vascular smooth muscle cells.
(29). Further investigation of the whole cascade of mechanotransduction in bone cells is necessary.

In summary, our study demonstrates that oscillatory fluid flow is a potent physiological stimulator that induces Ca\(^{2+}\) release and OPN gene expression via ERK1/2 and p38 activation but not JNK. OPN gene expression required Ca\(^{2+}\) mobilization. Ca\(^{2+}\) is mobilized using primarily the IP\(_3\) pathway, with the L-type VOCC membrane channel also playing a role. Although we did not compare oscillatory fluid flow directly to steady/pulsatile flow in this study, when compared with previously published studies on steady/pulsatile flow, our findings suggest that there are some potentially important differences in the response of bone cells to these two stimuli. This contrast indicates that there may exist multiple mechanotransduction pathways in bone cells that are activated depending on stimulus type and that determining an appropriate cellular mechanical stimulus is critical in understanding the role of mechanical loading in the regulation of bone.

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REFERENCES

1. Morey, E. R., and Baylink, D. J. (1978) Science 201, 1138–1141
2. Sessions, N. D., Halloran, B. P., Bilk, D. D., Wronski, T. J., Cone, C. M., and Donahue, H. J. (1995) J. Bone Miner. Res. 10, 935–941
3. Morooka, T., and Nishida, E. (1998) Matrix Biol. 17, 23–30
4. Seger, R., and Krebs, E. G. (1995) J. Biol. Chem. 270, 1475–1482
5. Geiger, R. V., Berk, B. C., Alexander, R. W., and Nerem, R. M. (1992) J. Biomech. 25, 231–252
6. Chao, P. H., Mow, V. C., Ratcliffe, A., and Valhmu, W. B. (2000) J. Biomech. 33, 278–285
7. Seger, R., and Krebs, E. G. (1995) J. Biol. Chem. 270, 1475–1482
8.Ștefănescu, D., and Băjenaru, C. (2001) J. Biomech. 34, 791–796
9. Schaffler, M. D., and Hollinger, J. O. (1994) Arch. Biochem. Biophys. 310, 222–229
10. Chin, T. Y., and Chueh, S. H. (1998) Arch. Biochem. Biophys. 355, 263–269
11. Chin, T. Y., and Chueh, S. H. (1998) Arch. Biochem. Biophys. 355, 263–269
12. Hung, C. T., Pollack, S. R., and Brighton, C. T. (1996) J. Bone Miner. Res. 11, 1243–1248
13. Toma, C. D., Ashkar, S. A., Gray, M. L., Schaffer, J. L., and Gerstenfeld, L. C. (1997) J. Bone Miner. Res. 12, 1626–1636
14. Hartner, L. V., Hruska, K. A., and Duncan, R. L. (1995) Endocrinology 136, 528–535
15. Zhen, X., Uryu, K., Hanley, M. B., and Friedman, E. (1998) Mol. Pharmacol. 54, 453–458
16. Miyazaki, Y., Setoguchi, M., Yoshida, S., Higuchi, Y., Akizuki, S., and Yamamoto, S. (1990) J. Biol. Chem. 265, 14432–14438
17. Takahashi, M., Okuda, M., Lee, J. D., and Berk, B. C. (1999) J. Biol. Chem. 274, 615–622
18. Yan, C., Takahashi, M., Okuda, M., Lee, J. D., and Berk, B. C. (1999) J. Biol. Chem. 274, 615–622
19. Li, C., Hu, Y., Mayr, M., and Xu, Q. (1999) J. Biol. Chem. 274, 25273–25280
20. Toma, C. D., Ashkar, S. A., Gray, M. L., Schaffer, J. L., and Gerstenfeld, L. C. (1997) J. Bone Miner. Res. 12, 1626–1636
21. Terai, K., Takano-Yamamoto, T., Obha, Y., Hiraoka, K., Sugimoto, T., Sato, M., Kawahata, H., Imaeguchi, N., Kitamura, Y., and Nomura, S. (1999) J. Bone Miner. Res. 14, 809–814
22. Toma, C. D., Ashkar, S. A., Gray, M. L., Schaffer, J. L., and Gerstenfeld, L. C. (1997) J. Bone Miner. Res. 12, 1626–1636
23. Hartner, L. V., Hruska, K. A., and Duncan, R. L. (1995) Endocrinology 136, 528–535
24. Kubota, T., Yamauchi, M., Onozaki, J., Sato, S., Suzuki, Y., and Sodek, J. (1993) Arch. Oral Biol. 38, 23–30
25. Reinholt, F. P., Hultenby, K., Oldberg, A., and Heinegard, D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4473–4475
26. Giachelli, C. M., and Steitz, S. (2000) Matrix Biol. 19, 615–622
27. Jo, H., Sipos, K., Go, Y. M., Law, R., Rong, J., and McDonald, J. M. (1997) J. Biol. Chem. 272, 1395–1401
28. Yan, C., Takahashi, M., Okuda, M., Lee, J. D., and Berk, B. C. (1999) J. Biol. Chem. 274, 143–150
29. Li, C., Hu, Y., Mayr, M., and Xu, Q. (1999) J. Biol. Chem. 274, 25273–25280
30. Liang, F., and Gardner, D. G. (1999) J. Clin. Invest. 104, 1603–1612
31. Yellowley, C. E., Jacobs, R. C., and Pollack, H. J. (1999) J. Cell. Physiol. 180, 402–408
32. Hung, C. T., Allen, F. D., Pollack, S. R., and Brighton, C. T. (1996) J. Biomech. 29, 1411–1417
33. Walker, L. M., Publicover, S. J., Preston, M. R., Said Ahmed, M. A., and El Haj, A. J. (2000) J. Cell. Biochem. 76, 648–651
34. Robinson, S. C., Pitsillides, A. A., and Lanyon, L. E. (1996) Bone 19, 609–614
35. McCauley, L. K., Koh, A. J., Beecher, C. A., Cui, Y., Decker, J. D., and Franceschi, R. T. (1995) J. Bone Miner. Res. 10, 1234–1255
36. Zhen, X., Uryu, K., Hanley, M. B., and Friedman, E. (1998) Mol. Pharmacol. 54, 453–458
37. Miyazaki, Y., Setoguchi, M., Yoshida, S., Higuchi, Y., Akizuki, S., and Yamamoto, S. (1990) J. Biol. Chem. 265, 14432–14438
38. Toma, C. D., Ashkar, S. A., Gray, M. L., Schaffer, J. L., and Gerstenfeld, L. C. (1997) J. Bone Miner. Res. 12, 1626–1636
39. Hartner, L. V., Hruska, K. A., and Duncan, R. L. (1995) Endocrinology 136, 528–535
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