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Mechanical Loading by Fluid Shear Stress Enhances IGF-1 Receptor Signaling in Osteoblasts in A PKCζ-Dependent Manner

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Abstract: Maintenance of optimal bone physiology requires the coordinated activity of osteoclasts that resorb old bone and osteoblasts that deposit new bone. Mechanical loading of bone and the resulting movement of interstitial fluid within the spaces surrounding bone cells is thought to play a key role is maintaining optimal bone mass. One way in which fluid movement may promote bone formation is by enhancing osteoblast survival. We have shown previously that application of fluid flow to osteoblasts in vitro confers a protective effect by inhibiting osteoblast apoptosis (Pavalko et al., 2003, J. Cell Physiol., 194: 194-205). To investigate the cellular mechanisms that regulate the response of osteoblasts to fluid shear stress, we have examined the possible interaction between fluid flow and growth factors in MC3T3-E1 osteoblast-like cells. We found that insulin-like growth factor-I (IGF-I) was significantly more effective at preventing TNF-α-induced apoptosis when cells were first subjected to mechanical loading by exposure to either unidirectional or oscillatory fluid flow compared to cells that were maintained in static culture. Additionally, downstream signaling in response to treatment with IGF-I, including ERK and Akt activation, was enhanced in cells that were subjected to fluid flow, compared to cells maintained in static culture. Furthermore, we found that PKCζ activity is essential for fluid shear stress sensitization of IGF-IR, since a specific inhibitor of PCKζ function blocked the flow-enhanced IGF-I-activated Akt and ERK phosphorylation. Together, our results suggest that fluid shear stress may regulate IGF-I signaling in osteoblasts in a PKC-ζ-dependent manner.

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1 Introduction

Physiologic levels of bone loading provide signals that promote bone formation and remodeling (1,2). Although this process is well documented, the cellular and molecular mechanisms that regulate the process are only partially defined. One way in which mechanical signals may be relayed to bone cells is through the loading-induced movement of interstitial fluid through the spaces and across the surfaces of cells in bone (3-5). Fluid movement is thought to generate shear stress at the surface of osteoblasts and osteocytes that can induce intracellular signaling events (6,7). Mechanical loading of bone cells by fluid shear stress or strain promotes an anabolic response in vitro, including upregulation of genes important for bone formation (8-10) and signals, via the mitogen activated protein kinase (MAPK) family, that stimulate cell proliferation (11,12).

In bone tissue, regulation of bone cell differentiation, proliferation and death is thought to play a key role in maintenance of healthy bone (reviewed in (13)). Apoptosis of osteoblasts, the cells responsible for deposition of new bone matrix, is a significant event in bone, as approximately 70% of osteoblasts initially present at sites of remodeling are thought to undergo apoptosis (14). The remainder of osteoblasts become bone lining cells or osteocytes (15). Signals that prolong the lifespan of osteoblasts may increase the number of osteoblasts present at sites of bone remodeling thereby increasing bone formation (16). Growing evidence indicates that mechanical forces have a

variety of effects on cell growth and metabolism (17-19). It is well established that mechanical loading of bone results in increased bone formation and remodeling (1,20). It is also clear that in the absence of loading, such as occurs in an environment of microgravity or during prolonged bed rest, bone resorption is increased, and bone mass is lost (21-24).

Insulin-like growth factor-I (IGF-I) is an abundant and potent stimulus for bone cell proliferation (25,26) and new bone formation *in vivo* (27,28). Recent studies by Sakata et al. (29) found that skeletal unloading of mice induced IGF-I "resistance" in part by downregulation of integrin signaling. And, Kapur, et al. reported that osteoblast proliferation was enhanced by a combination of fluid shear stress and IGF-I compared to either shear or IGF-I treatment alone (30). We and others have shown that mechanical stimulation of osteoblasts promotes survival signals that inhibit apoptosis (31-33). Since IGF-I is also known to elicit survival in osteoblasts, there is potential for these two potent stiimuli in bone to act together.

Cross-talk between multiple growth factor pathways and mechanical signaling has previously been demonstrated (reviewed in (34)). Often, the point of cross-talk involves a key component of both interacting signaling pathways. The protein kinase C (PKC) family can be divided into three classes of isoforms: conventional PKCs (α, β_I, β_{II}, and γ), novel PKCs (δ, ε, η, θ, and μ), and atypical PKCs (ζ, ι, and λ). Conventional PKCs require calcium, diacylglycerol, and phosphatidyl serine for activation, while novel PKCs require only diacylglycerol and phosphatidyl serine. The only requirement for activation of atypical PKCs is phospatidyl serine, making them candidates for points of cross-talk between pathways (35). The recent availability of specific inhibitors of atypical PKCs has made investigation of the role of these proteins in cellular processes a possibility. Recently, PKCζ has been implicated in regulating cell survival in both positive and negative ways (36,37). The role played by PKCζ in cell survival appears to be a cell-type- and condition-dependent phenomenon.

In this study, we report that MC3T3-E1 cells showed decreased apoptosis when subjected to fluid shear stress or treated with IGF-I, and that the combination of both stimuli was more potent at inhibiting apoptosis. Additionally, we show that this synergistic effect holds true when cells are subjected to either unidirectional or oscillatory fluid shear stress. Furthermore, we show that application of fluid shear stress sensitizes cells to treatment of IGF-I, since IGF-I receptor, ERK, and Akt phosphorylation were increased in these cells compared to those held in static culture prior to IGF-I treatment. We also show that this sensitization was not due to increases in receptor expression nor ligand binding. However, it is dependent upon PKCζ activity, since a specific inhibitor of PKCζ blocked the ability of fluid shear stress to sensitize IGF-IR. These results support the hypothesis that fluid shear stress promotes an anabolic response in bone by regulating the sensitivity of osteoblasts to signaling via the IGF-I pathway to promote osteoblast survival. And, this study demonstrates a role for PKCζ as a point of cross-talk between fluid shear stress and IGF-I signaling pathways.

2 Experimental Procedures

Cell Culture – The mouse osteoblast-like MC3T3-E1 cell line was cultured in α-minimum essential media (GIBCO, Carlsbad, CA, α-MEM) containing 10% fetal calf serum (FCS) with antibiotics. Cells were seeded at a density of approximately $10P^4$ cells/cm^2 and grown for 4-5 days with the media replaced every 48 hours, after which time they were 90-95% confluent. For all experiments, cells were in a proliferative, non-differentiated stage of growth. Cells were passaged at confluence by brief trypsinization with media changed every other day.

Fluid Flow – Unidirectional fluid flow was performed in a parallel plate flow chamber using the flow loop system designed by Frangos (38) and marketed by Cytodyne (San Diego, CA) using a total volume of 30 mL of α-MEM or MEM containing 1% FCS and antibiotics, maintained at 37^{o}C and perfused with 5% CO_2. This flow system was designed to subject the cells to fluid flow and a shear rate of 15 dynes/cm^2. Oscillatory fluid

flow was applied using a Harvard PHD2000 series programmable pump. Flow was driven by a series of sealed glass syringes with rigid walled tubing at a peak shear stress of 15 dynes/cm^2 and a frequency of 0.5 Hz. The parallel plate flow chamber was of the same dimensions as that used for unidirectional flow. Static control cells were grown on glass slides, switched to 30 mLs of α-MEM containing 1% or 0% FCS and antibiotics and placed in an incubator under static conditions at 37^{o}C with 5% CO_2 for the duration of each experiment. For analysis of apoptosis, cells were switched from growth media containing 10% FCS to media containing 1% or 0% FCS and subjected to static conditions or fluid shear for 4.5 hr. After 15 min of shear or static conditions, 50 ng/mL IGF-I or vehicle (DMSO) was added for 15 min, followed by the addition of 10 ng/mL murine TNF-α (Calbiochem, San Diego, CA) and 10 μg/mL cycloheximide for 4 hrs, while the cells remained in static culture or under shear stress conditions. For analysis of IGF-I signaling, cells were switched to media containing 0% FCS and subjected to 4 hrs of static conditions or fluid shear stress, followed by 15 min treatment of 50 ng/mL IGF-I. In some experiments, cells were treated with pseudo-substrate peptide protein kinase C ζ inhibitor (Biosource, Camarillo, CA) at a concentration of 10 μM prior to application of fluid shear stress and drug was present during the entire flow period and in static controls. DMSO (vehicle) was added to control cells not receiving drugs.

Immunoblot Analysis – Cell extracts were collected directly in SDS-PAGE sample buffer and protein concentrations were determined using the amido black assay (39). Equal protein (20 μg) was loaded onto SDS-PAGE gels for separation and transferred to nitrocellulose for immunoblotting. The following antibodies were used: mouse monoclonal anti-phosphohistone H2A.X, rabbit anti-caspase-3 (recognizing full length procaspase-3) (35 kDa) and the enzymatically active caspase-3 proteolytic fragment (17 kDa), rabbit anti-cleaved caspase-3 (recognizing only the enzymatically active caspase-3 proteolytic fragment (17 kDa), rabbit anti-phosphoAkt (serine473), rabbit anti-phosphoAkt (thr308), and rabbit anti-Akt (Cell Signaling Technology, Beverly, MA); rabbit anti-PARP (recognizing full length and cleaved PARP; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-vinculin (Sigma, St. Louis, MO). The appropriate anti-mouse Ig or anti-rabbit-Ig peroxidase-conjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA) and the antibody signal was detected by chemiluminescence using a LAS-3000plus luminescent image analyzer (Fujifilm, Sunnyvale, CA). A minimum of three separate experiments were quantified by densitometric analysis using Image Reader LAS-3000 software (Fujifilm). Statistical analysis of densitometry results for phospho-histone, Akt phosphorylation, caspase-3, and PARP cleavage was performed using Statview software. Treatment groups were compared by analysis of variance (ANOVA); $p < 0.05$ was considered significant.

3 Results

Fluid shear stress-induced inhibition of apoptosis is enhanced in the presence of serum. Previously, we found that induction of apoptosis in osteoblasts by treatment with a combination of TNF-α and cycloheximide (CHX) was significantly attenuated if the osteoblasts were subjected to mechanical loading by exposure to fluid shear stress (Pavalko et al (2003)). When exposed to a combination of 10 ng/ml TNF-α and 10μg/ml CHX, MC3T3-E1 cells showed signs of apoptosis within 4 hours, including histone phosphorylation, cleavage of caspase-3, and cleavage of poly-ADP-ribose polymerase (PARP) (Fig. 1 & data not shown). This process occurs in the presence or absence of 1 % fetal calf serum (FCS), but to no greater degree in either condition. As shown previously, serum starving MC3T3-E1 cells for 24 hours did not induce apoptosis by itself (Fig. 1 & (Pavalko et al (2003)).

To determine the potential role of growth factors present in FCS in mediating the anti-apoptotic effect of fluid shear stress, we examined whether TNF-α/CHX-induced apoptosis could be inhibited by exposure to fluid shear stress in the ab-

Table 1: Summary of changes in apoptotic markers due to IGF-I treatment, oscillatory or unidirectional flow, and the combination of flow and IGF-I.

	Change in Histone Phosphoylation vs TNF-α/CHX control			Change in Caspase-3 cleavage vs TNF-α/CHX control		
Treatment	Flow	IGF-1	Flow+IGF1	Flow	IGF-1	Flow+IGF1
OFF	-31%	-62%	-83%	-44%	-71%	-73%
UFF	-37%	-13%	-84%	-36%	-32%	-84%

Figure 1

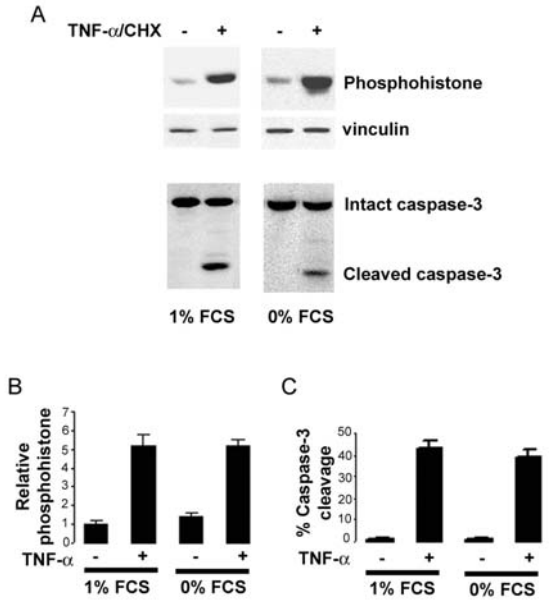

Figure 1: Analysis of TNFα/CHX-induced apoptosis in the presence and absence of serum. A) Western blot analysis of histone H2A.X phosphorylation (Ph-H2A.X), vinculin, and caspase 3 cleavage in the presence and absence of TNFα/CHX in media supplemented with 0% or 1% FCS. B) Quantification of Ph-H2A.X:vinculin densitometric ratio and caspase 3 cleavage.

sence of growth factors and other serum components using serum-free media. For these studies cells were subjected to fluid shear stress in the presence or absence of 1% FCS for 4 hours while simultaneously being exposed to TNF-α/CHX. Shear stress-induced inhibition of apoptosis, as determined by histone phosphorylation and caspase cleavage, was significantly less in the absence of FCS compared to conditions in which 1% FCS was included in the media (Fig. 2). These results suggested that growth factors present in FCS might augment the pro-survival effects of fluid shear.

Fluid shear stress-induced inhibition of apoptosis is augmented by IGF-I. Since insulin-like growth factor-I (IGF-I) plays an important role in osteoblast physiology, we investigated the role of IGF-I in fluid shear stress-induced inhibition of apoptosis in MC3T3-E1 cells treated with TNFα/CHX. When cells maintained in static culture were treated with TNFα/CHX and IGF-I, histone phosphorylation was reduced 62 or 13 % and caspase-3 cleavage was reduced 71 or 32 % compared to cells treated with TNFα/CHX alone (Figs. 3 & 4, Table 1). Additionally, TNFα/CHX-induced histone phosphorylation was reduced 31 or 37 % and caspase 3 cleavage was reduced 44 or 36 % in cells subjected to either oscillatory or unidirectional fluid shear stress, respectively, compared cells held in static culture (Figs. 3 & 4). Interestingly, the combination of either type of fluid shear stress and IGF-I was significantly more effective at inhibiting apoptosis than either fluid shear stress or IGF-I treatment alone. Histone phosphorylation was reduced 83 or 84 % and caspase 3 cleavage was reduced 73 or 84 % when cells were treated with IGF-I and oscillatory or unidirectional fluid shear stress, respectively, compared to cells held in static culture (Figs. 3 & 4, Table 1). Taken together, these results indicate that fluid shear stress augments the pro-survival effects of IGF-I, or *vice versa*.

Fluid shear stress sensitizes the IGF-I receptor to IGF-I. To evaluate potential cellular mechanisms through which fluid shear stress signaling may interact with IGF-I signaling, we considered the possibility that flow increased the ability of

Figure 2

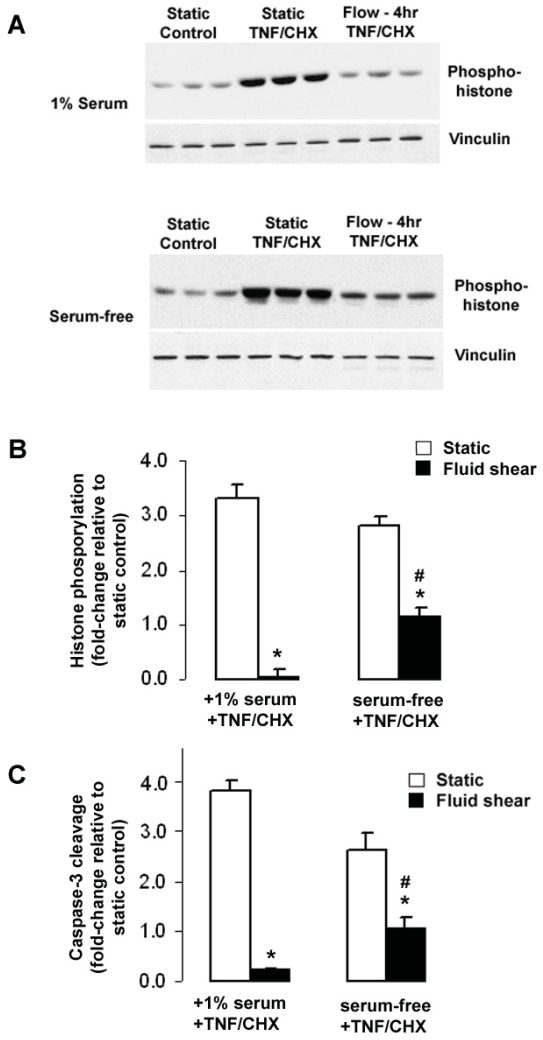

Figure 2: Analysis of fluid shear stress-induced inhibition of apoptosis in the presence and absence of serum. A) Western blot analysis of histone H2A.X phosphorylation (Ph-H2A.X) and vinculin in cells subjected to static culture or fluid shear stress in the presence or absence of TNFα/CHX and the presence (top panel) or absence (bottom panel) of 1% FCS. B) Quantification of relative fold increase in Ph-H2A.X:vinculin densitometric ratio relative to untreated static controls. $^{*}P < 0.05$ vs. Static, $^{\#}P < 0.05$ vs. 1% Serum. C) Quantification of fold increase in caspase 3 cleavage relative to untreated static controls. $^{*}P < 0.05$ vs. Static, $^{\#}P < 0.05$ vs. 1% Serum.

Figure 3

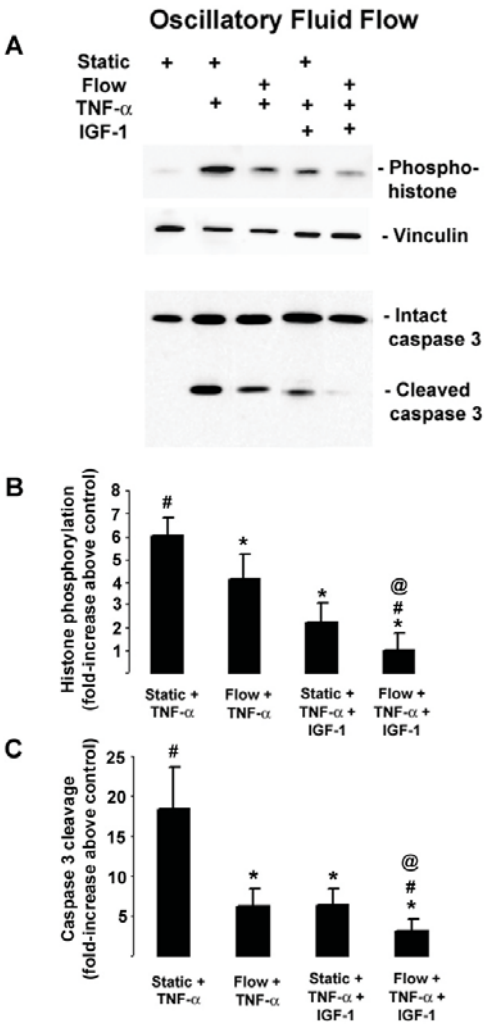

Figure 3: Analysis of the effects oscillatory fluid shear stress and IGF-I treatment on TNFα/CHX-induced apoptosis. A) Western blot analysis of Ph-H2A.X, vinculin, and caspase 3 in cells subjected to static culture or fluid shear stress in the presence of TNFα/CHX, treated with or without IGF-I. B) Quantification of fold increase in Ph-H2A.X:vinculin densitometric ratio relative to untreated static controls. $^{*}P < 0.05$ vs. Static + TNFα, $^{\#}P < 0.05$ vs. Flow + TNFα, $^{@}P < 0.05$ vs. Static + TNFα + IGF-I. C) Quantification of fold increase in caspase 3 cleavage relative to untreated static controls. $^{*}P < 0.05$ vs. Static + TNFα, $^{\#}P < 0.05$ vs. Flow + TNFα, $^{@}P < 0.05$ vs. Static + TNFα + IGF-I.

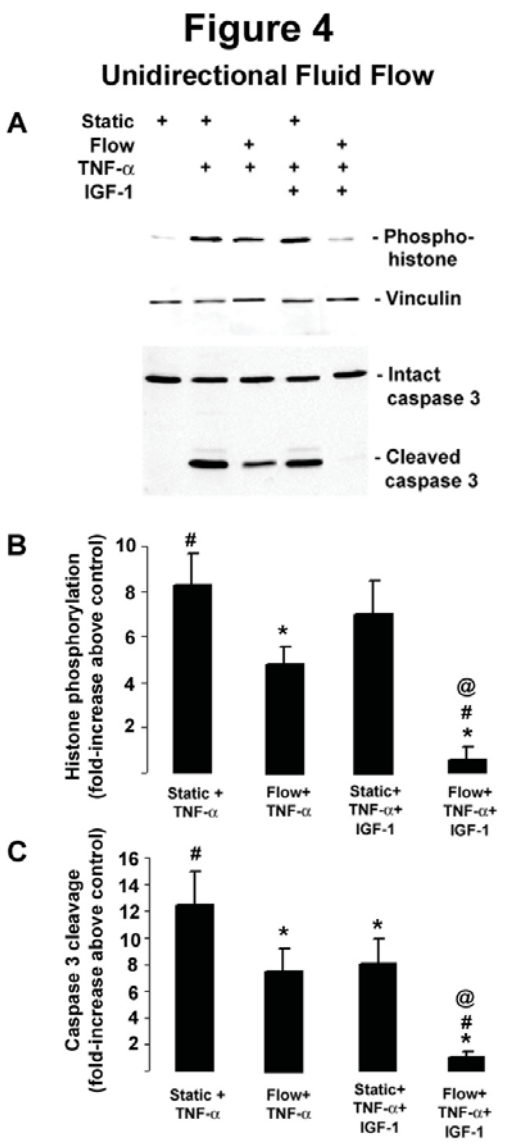

Figure 4: Analysis of the effects unidirectional fluid shear stress and IGF-I treatment on TNFα/CHX-induced apoptosis. A) Western blot analysis of Ph-H2A.X, vinculin, and caspase 3 in cells subjected to static culture or fluid shear stress in the presence of TNFα/CHX, treated with or without IGF-I. B) Quantification of fold increase in Ph-H2A.X:vinculin densitometric ratio relative to untreated static controls. $^{*}P < 0.05$ vs. Static + TNFα, $^{\#}P < 0.05$ vs. Flow + TNFα, $^{@}P < 0.05$ vs. Static + TNFα + IGF-I. C) Quantification of fold increase in caspase 3 cleavage relative to untreated static controls. $^{*}P < 0.05$ vs. Static + TNFα, $^{\#}P < 0.05$ vs. Flow + TNFα, $^{@}P < 0.05$ vs. Static + TNFα + IGF-I.

IGF-I to bind to IGF-I receptors (IGF-IR) on osteoblasts. Using radio-labeled IGF-I to assess IGF-I binding, we found no evidence of increased binding of IGF-I to the surface of osteoblasts as a consequence of 4 hrs of fluid shear stress in MC3T3-E1 cells (Fig. 5A). Furthermore, we did not observe an increase in IGF-IR expression (Fig 5B, *lower panel*), nor increased phosphorylation of IGF-IR after 4 hrs of fluid shear stress alone (not shown). We did, however, note a significant increase in phosphorylation of IGF-IR in cells treated with IGF-I for 15 min following 4 hrs of fluid shear stress compared to cells maintained in static culture prior to IGF-I treatment (Fig. 5B & C). Taken together, these data indicate that fluid shear stress does not directly regulate expression nor phosphorylation of IGF-IR, but may sensitize IGF-IR to IGF-I.

To test this possibility, we examined the effect of fluid flow on the ability of IGF-I to activate signaling pathways leading to phosphorylation and activation of Akt and extracellular signal-related MAP kinase (ERK). Treatment of MC3T3-E1 osteoblasts with IGF-I for 15 min following 4 hrs in serum free media induced a significant increase in Akt phosphorylation (Fig. 6). Cells subjected to fluid shear stress for 4 hrs in the absence of serum did not show significantly increased Akt phosphrylation (Fig. 6). However, cells treated with IGF-I following 4 hrs of fluid shear stress showed a greater than 2-fold increase in Akt phosphorylation compared to cells held in static culture prior to IGF-I treatment (Fig. 6). Furthermore, the ability of fluid shear stress to increase IGF-I induced signaling was observed when we examined the phosphorylation of ERK (Fig. 7). These data indicate that fluid shear stress sensitizes downstream elements of the IGF-I signaling pathway to IGF-I treatment.

PKCζ plays an important role in fluid shear stress-induced sensitization of IGF-I signaling. Because the atypical PKC isoform PKCζ is expressed in osteoblasts, is a putative downstream target of PI3-kinase, and has been reported to be involved in IGF-I-mediated signaling, we examined the role of PKCζ in IGF-I and fluid shear stress signaling by using a specific pseudo-

Figure 5

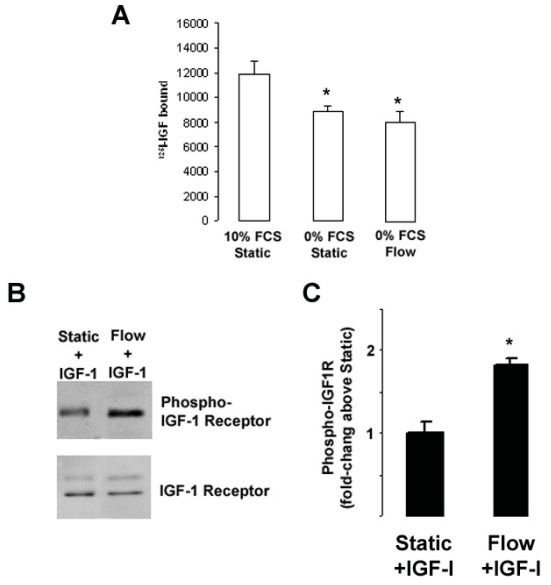

Figure 5: Analysis of fluid shear stress-induced alterations in IGF-I receptor. A) 125-I-labeled IGF-I binding assay in cells subjected to static culture in the presence or absence of 10 % FCS or subjected to fluid shear stress in the absence of serum. $^{*}P < 0.05$ vs. 10% FCS Static. B) Western blot analysis of IGF-I receptor (IGF-IR) phosphorylation in cells treated with IGF-I following 4 hours of static culture or fluid shear stress. C) Quantification of fold change in IGF-IR phosphorylation relative to cells held in static culture prior to IGF-I treatment (Static + IGF-I). $^{*}P < 0.05$ vs. Static +IGF-I.

Figure 6

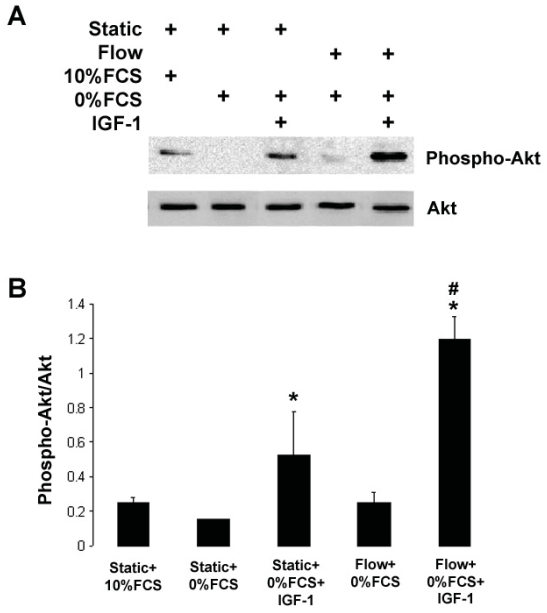

Figure 6: Analysis of the effects of fluid shear stress on IGF-I induced Akt phosphorylation. A) Western blot analysis of Phospho-Akt and total Akt in cells subjected to static culture or fluid shear stress and subsequently treated with or without IGF-I. B) Quantification of phospho-Akt:Akt densitometric ratio. $^{*}P < 0.05$ vs. Static + 0% FCS, $^{\#}P < 0.05$ vs. Static + 0% FCS + IGF-I, $^{@}P < 0.05$ vs. Flow + 0% FCS.

substrate peptide inhibitor of PKCζ (PKCζ_I). As expected, treatment of cells held in static culture with IGF-I induced increases in both Akt and ERK phosphorylation, and cells subjected to fluid shear stress prior to IGF-I treatement showed further increases in Akt and ERK phosphorylation (Fig. 8). Importantly, IGF-I was able to induce both Akt and ERK phosphorylation in cells held in static culture and treated with PKCζ_I (Fig. 8). However, we did not observe flow-enhanced IGF-I-induced increases in Akt and ERK phosphorylation were in cells treated with PKCζ_I (Fig. 8), indicating that PKCζ may play a role the fluid shear stress-induced sensitization of IGF-I signaling.

To determine if PKCζ plays a role in the synergistic inhibition of apoptosis by IGF-I and fluid shear stress, cells were treated with TNFα/CHX and subjected to static culture or fluid shear stress in the presence or absence of PKCζ_I. As expected, cells subjected to fluid shear stress and IGF-I showed reduced levels of caspase 3 cleavage compared to cells held in static culture and treated with IGF-I (Fig. 8D). Treatment of cells with PKCζ_I alone did not induce caspase 3 cleavage, in fact we observed slight decreases in total caspase 3 levels (Fig.8D). Paradoxically, treatment of cells with PKCζ_I abolished caspase 3 cleavage in cells treated with TNFα/CHX and IGF-I, regardless of whether they were held in static culture or

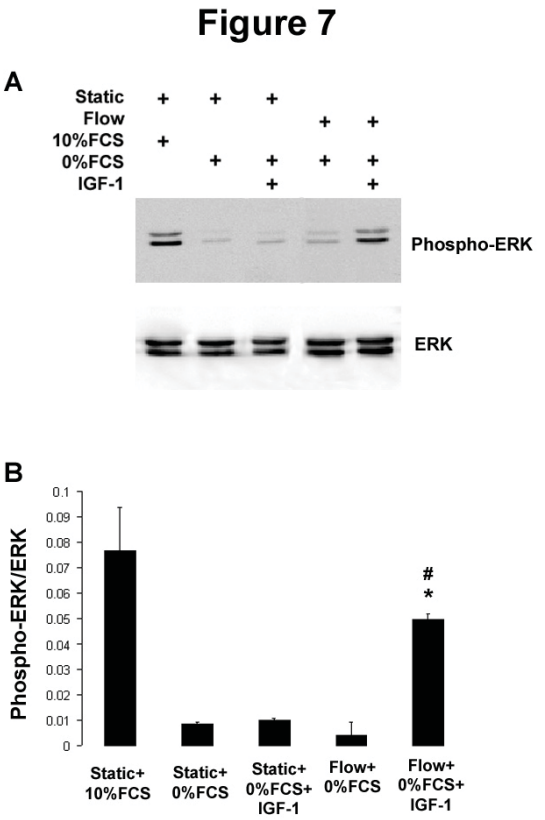

Figure 7: Analysis of the effects of fluid shear stress on IGF-I induced ERK phosphorylation. A) Western blot analysis of Phospho-ERK and total ERK in cells subjected to static culture or fluid shear stress and subsequently treated with or without IGF-I. B) Quantification of phospho-ERK:ERK densitometric ratio. $^{*}P < 0.05$ vs. Static + 0% FCS + IGF-I, $^{\#}P < 0.05$ vs. Flow + 0% FCS.

subjected to fluid shear stress (Fig. 8D). Since no caspase 3 cleavage was observed in cells held in static culture treated with TNFα/CHX and IGF-I in the presence of PKCζ_I, these data do not point conclusively to a role for PKCζ in fluid shear stress-enhanced IGF-I-induced rescue from apoptosis.

4 Discussion

In this study, we have shown that both unidirectional and a more physiological oscillatory fluid shear stress were both able to inhibit apoptosis in the osteoblast cell line, MC3T3-E1. This occurred in the presence or absence of serum, however the response in serum was significantly more robust. One of the most prominent growth factors secreted by bone cells is IGF-I (40). IGF-I has been shown to act similarly to fluid shear stress in that it can increase cell proliferation and decrease apoptosis in many cell types (reviewed in (41)). Kapur, et al recently demonstrated that steady, unidirectional fluid shear stress and IGF-I synergistically increased proliferation of TE85 osteosarcoma cells (30). Since osteoblasts undergo apoptosis at a high rate, and both fluid shear and IGF-I have protective effects against apoptotic stimuli, we tested the idea that the two stimuli might act synergistically to decrease osteoblast apoptosis. In fact, we found that the combination of either unidirectional or oscillatory fluid shear stress and IGF-I was better than either alone at inhibiting osteoblast apoptosis induced by TNFα/CHX.

Mechanical loading has been shown to increase the expression of several genes, one of which is IGF-I (42). We asked if the mechanism by which fluid shear 'sensitized' the IGF-I pathway was by a simple increase in receptor expression, as was reported by Lau, et al using DNA microarray and RT-PCR (43). Importantly, we found no increase in IGF-IR total protein due to fluid shear stress by western blotting in this cell type. Additionally, we observed no change in receptor affinity for IGF-I in cells subjected to fluid shear stress compared to static controls. These results differ from those reported by Kapur, et al in which they found a small, but significant, increase in ^{125}IGF bound to IGF-IR in cells subjected to fluid shear stress for 30 min (30). However, in that study, the authors report much higher fold increases in downstream IGF-I signaling pathways in cells subjected to fluid shear, which, by the authors' admission, are not explicable through the small increase in bound ligand alone (30). Additionally, as noted by Kapur, et al, the amount of IGF-I released by osteoblasts is high (44), and could far out-number the amount added for binding assays, making it difficult to say for certain if changes in receptor affinity occurred (30). Furthermore, cells in this

Figure 8

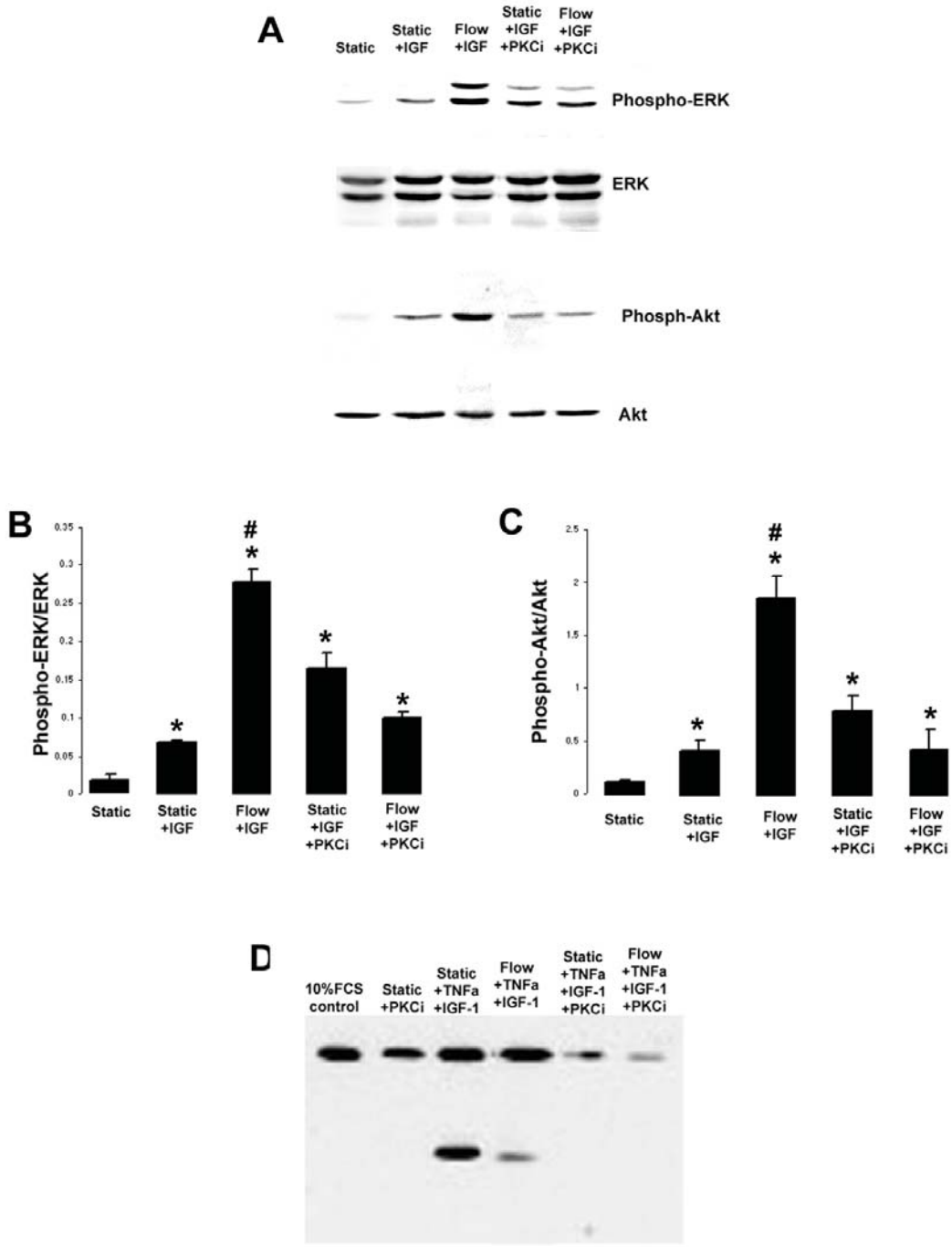

Figure 8: Analysis of the role of PKCζ in fluid shear stress-induced sensitization of IGF-I signaling. A) Western blot analysis of Phospho-ERK, ERK, Phospho-Akt, and Akt in cells maintained in static culture or subjected to fluid shear stress, treated with or without IGF-I in the presence or absence of PKCζ pseudo-substrate peptide inhibitor (PKCζ_I). B) Quantification of Phospho-ERK:ERK densitometric ratio. $^{*}P < 0.05$ vs. Static, $^{\#}P < 0.05$ vs. Static + IGF. C) Quantification of Phospho-Akt:Akt densitometric ratio. $^{*}P < 0.05$ vs. Static, $^{\#}P < 0.05$ vs. Static + IGF.

study were subjected to fluid shear stress for 4 hours prior to labeled IGF-I incubation, thus the small increase in receptor affinity may be a temporal phenomenon.

To determine where in the IGF-IR signaling pathway fluid shear stress may act to amplify IGF-I signaling, we examined the downstream activation of Akt and ERK kinases by IGF-I and fluid shear stress alone or both together. Here we report that 4 hrs of fluid shear stress itself did not increase either IGF-IR, Akt or ERK phosphorylation compared to static controls. These results differ from, but do not dispute, those reported by Kapur, et al, in which 30 min of fluid shear stress itself led to increased phosphorylation of IGF-IR and ERK, and 10 min of IGF-I treatment further enhanced this (30). In addition, these results differ from previously reported results from our lab indicating that fluid shear stress was able to increase Akt phosphorylation up to 4 hours in a PI-3K independent manner (32). Those studies were carried out in the presence of serum, while the study reported here was performed in the absence of serum, indicating a role for serum in the long-term response of Akt to fluid shear stress. Since Kapur, et al observed increased Akt phosphorylation after 30 min of flow in serum-free conditions (30), Akt activation in response to fluid shear stress may be temporally regulated. Also, several studies have reported that fluid shear stress can increase ERK phosphorylation (11,12). In this study we did not observe increased ERK phosphorylation in response to 4 hours of fluid shear stress; however, fluid shear stress-induced ERK phosphorylation is maximal at earlier time points in osteoblasts (10).

Interestingly, IGF-I-induced phosphorylation of IGF-IR, Akt, and ERK were enhanced when cells were subjected to 4 hrs of fluid shear stress prior to treatment with IGF-I compared to cells held in static culture before treatment. These results further support those reported by Kapur, et al (30), indicating that the ability of fluid shear stress to sensitize the IGF-I pathway may be a phenomenon observed in most osteoblast cell models. When we investigated further the mechanism by which this occurred, we found that PKCζ played an important role in fluid shear stress-induced sensitization of the IGF-I signaling pathway. PKCζ is an atypical PKC, activated by phosphatidyl serine but not calcium nor diacylglycerol, like conventional PKCs (45). Signaling through IGF-IR activates multiple pathways, and PKCζ has been shown to be activated in response to IGF-I treatment in vascular smooth muscle cells (46). Additionally, PKCζ has been shown to be downstream of PI-3K, but it may also be activated independently of this pathway (46). We found that PKCζ played an essential role in the ability of fluid shear stress to sensitize IGF-IR, since IGF-I induced Akt and ERK phosphorylation were not enhanced by fluid shear stress in the presence of PKCζ_I. While PKCs have been hypothesized to be involved in mechanical signaling (47), a direct role for PKCζ has not been proven. Here we show evidence that PKCζ plays an essential role in a fluid shear stress-initiated signaling pathway. However, fluid shear stress did not induce phosphorylation of PKCζ, indicating there were no changes in PKCζ activity (data not shown). These results do not preclude the possibility that PKCζ may be activated by flow at earlier time points or in a different manner (i.e. changes in subcellular localization). Further studies examining the specific role of PKCζ in this process are needed to determine the importance of PKCζ in mechanical signaling.

Recently, a role for PKCζ in regulating cell survival has been determined ((48,49) and unpublished results). Here we show that inhibition of PKCζ does not induce apoptosis by itself. In fact, we observed no caspase 3 cleavage in cells treated with PCKζ_I in the presence of IGF-I and TNFα/CHX. These results are perplexing, since the effect of PKCζ inhibition on IGF-IR sensitization was so dramatic. From these data, we cannot determine the role of PKCζ in fluid shear stress-enhanced IGF-I induced inhibition of apoptosis from these studies, but the role of PKCζ in TNFα/CHX-induced apoptosis is the focus of current work in our laboratory (Norvell, et al., manuscript in preparation).

Our results suggest that a combination of fluid shear stress and IGF-I have potent effects on os-

teoblast survival *in vitro*. The mechanism by which fluid shear sensitizes cells to IGF is not simply a means of priming components of the pathway to a more active 'basal' state nor an increase in IGF-IR protein expression or increased IGF-I binding. Our results do not rule out the possibility, though, that these components were activated at earlier timepoints. Alternatively, it is well known that components of MAPK signaling cascades are often associated with scaffold proteins that interact with multiple components of the pathway, allowing for efficient activation. Additionally, activation of Akt via PI-3K requires the localization of PI-3K at the membrane where it can phosphorylate membrane lipids to signal downstream . One hypothesis explaining the sensitization of IGF-IR is that fluid shear stress may activate the pathway at early time points, leading to localization of the proteins in signaling complexes wehre they remain until later timepoints ready to be activated quickly and efficiently. In summary, this study supports a connection between fluid shear stress and IGF-I signaling that is mediated in part by PKCζ.

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