Mechanical stimulation prevents osteocyte apoptosis: requirement of integrins, Src kinases, and ERKs


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Plotkin, L. I., I. Mathov, J. I. Aguirre, A. M. Parfitt, S. C. Manolagas, and T. Bellido. Mechanical stimulation prevents osteocyte apoptosis: requirement of integrins, Src kinases, and ERKs. Am J Physiol Cell Physiol 289: C633–C643, 2005. First published May 4, 2005; doi:10.1152/ajpcell.00278.2004.—Osteocytes, former osteoblasts entombed in the bone matrix, form an extensive cell communication network that is thought to detect microdamage and mechanical strains and to transmit signals leading to repair and compensatory bone augmentation or reduction. Bone active hormones and drugs control the integrity of this network by regulating osteocyte apoptosis, which might be a determinant of bone strength. Herein we demonstrate that mechanical stimulation by stretching activates the ERKs, which in turn are responsible for the attenuation of osteocyte apoptosis. The effect of osteocyte stretching is transmitted by integrins and cytoskeletal and catalytic molecules, such as Src kinases. Stretch-induced antiapoptosis also requires nuclear translocation of ERKs and new gene transcription. The evidence linking mechanical stimulation, activation of an integrin/cytokeleton/Src/ERK signaling pathway, and osteocyte survival provides a mechanistic basis for the profound role of mechanical forces, or lack thereof, on skeletal health and disease.

bone; mechanotransduction; osteoblastic cells; caveolae; stretching

FAR FROM BEING a lifeless matrix, bone is home to several cell types. The most abundant is the osteocyte, a descendant of the bone-forming osteoblast. Osteocytes are buried in lacunae surrounded by mineralized matrix and form extensive networks with each other through long cellular processes traveling within narrow canaliculi, in a manner analogous to neuronal cells (43). Osteocytes also communicate with the osteoclasts and osteoblasts, which periodically regenerate the bone surfaces through remodeling, and might regulate the function of these cells via the production of molecules that modulate osteoclast or osteoblast formation (68, 74). Osteocytes are believed to have two separate functions: first, to detect microdamage and undergo apoptosis as part of the signals that lead to microdamage repair (62) and second, to detect changes in strain and trigger bone gain or bone loss (presumably by up- or downregulating osteoblastic bone formation or osteoclastic bone resorption) to restore local strain to the desirable range (1). Death of osteocytes is expected to compromise the mechanosensory function of the osteocyte network and to diminish the mechanical competence of the skeleton. In support of this notion, the increased bone fragility that results from glucocorticoid excess or sex steroid deficiency in animals and humans is associated with increased prevalence of osteocyte apoptosis (36, 61, 66). Conversely, bisphosphonates, intermittent parathyroid hormone (PTH) administration, and sex steroids all prevent osteocyte apoptosis, raising the possibility that preservation of osteocytes contributes to the antifracture efficacy of these agents (30, 36, 53). In support of this latter contention, blockade of glucocorticoid action on osteocytes in a transgenic mouse model preserves bone strength despite loss of bone mass, suggesting that osteocyte viability is indeed an independent determinant of bone strength (47).

The life span of osteocytes might be regulated by mechanical stimuli. Physiological levels of load imposed on bone in vivo appear to decrease the number of apoptotic osteocytes (46). On the other hand, a lack of mechanical stimulation induced by unloading of bone is associated with an increased number of hypoxic osteocytes, an effect that is reversed by loading, suggesting that mechanical forces facilitate oxygen diffusion and osteocyte survival (14).

How mechanical forces are transduced into biochemical signals in osteocytes is not understood. Osteocytes interact with the ECM in the pericellular space through discrete sites in their membranes, enriched in integrins and vinculin (2, 22), and through transverse elements that tether osteocytes to the canalicul wall (70). Therefore, fluid movement in the canaliculi resulting from mechanical loading might induce ECM deformation, shear stress, and/or tension in the tethering elements. The consequent changes in circumferential strain in the osteocyte membranes might be converted into intracellular signals by integrin clustering and their interaction with cytoskeletal and catalytic proteins at the focal adhesions (13, 20). However, the molecular machinery assembled in response to mechanical stimuli as well as the consequences of the generation of intracellular signaling for osteocyte life span have remained heretofore unknown. The findings of the present report are consistent with the transduction of mechanical forces by integrins and a signalsome comprising actin filaments, microtubules, the focal adhesion kinase FAK, and Src kinases, resulting in activation of the ERK pathway and attenuation of osteocyte apoptosis. This evidence provides the molecular basis for the profound role of mechanical forces, or lack thereof, in skeletal health and disease.

MATERIALS AND METHODS

Materials. β-Cyclodextrin, cytochalasin D, colchicine, wortmannin, gadolinium, SB-203580, actinomycin D, cycloheximide, and

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EGF from Sigma (St. Louis, MO); PD-98059 from New England Biolabs (Beverly, MA); 4-aminoo-5-(4-methylphenyl)-7-(3-butyryl)pyrazolo[3,4-d]pyrimidine (PP1) from A.G. Scientific (San Diego, CA); flexible bottom culture plates coated with collagen I or poly-l-lysine from Flexcell International (Hillsborough, NC); and ac-DEVD-AFC, recombinant caspase 3, and anti-Src antibody from Biomol (Plymouth Meeting, PA). Antibodies to ERKs, phospho-ERKs (pERKs), hemagglutinin (HA) tag, green fluorescent protein (GFP), glutathione-S-transferase (GST) and β1-integrin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to Akt, phospho-Akt, p38, and phospho-p38 were from Cell Signaling Technology (Beverly, MA). The antibody to caveolin-1 and the activating integrin antibodies were from Transduction Laboratories (Lexington, KY).

Cells. MLO-Y4 osteocytic cells derived from murine long bones and MLO-Y4 cells stably expressing GFP targeted to the nucleus (MLO-GFP) were cultured at 1–2 × 10^6 cells/cm² in phenol red-free α-MEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 2.5% FBS and 0.5–1% bovine calcitonin serum (BCS) (Hyclone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin on plates coated with calf skin collagen type I (Sigma) as previously described (31, 53).

Plasmids. The wild-type or dominant-negative (dn) constructs for HA-MEK, Src, GST-Shc, or HA-FAK were provided by N. G. Ahn (University of Colorado, Boulder, CO; Ref. 42), W. C. Horne (Yale University, New Haven, CT; Ref. 71), K. S. Ravichandran (University of Virginia, Charlottesville, VA; Ref. 63), and J.-L. Guan (Cornell University, New Haven, CT; Ref. 71), K. S. Ravichandran (University of Colorado, Boulder, CO; Ref. 42), W. C. Horne (Yale University, New Haven, CT; Ref. 71), K. S. Ravichandran (University of Virginia, Charlottesville, VA; Ref. 63), and J.-L. Guan (Cornell University, Ithaca, NY; Ref. 73), respectively. Wild-type and cytoplasmic ERK2 fused to GFP (GFP-ERK2) were provided by R. Seger (University of Virginia, Charlottesville, VA; Ref. 63), and J.-L. Guan (Cornell University, New Haven, CT; Ref. 71), K. S. Ravichandran (University of Colorado, Boulder, CO; Ref. 42), W. C. Horne (Yale University, New Haven, CT; Ref. 71), K. S. Ravichandran (University of Virginia, Charlottesville, VA; Ref. 63), and J.-L. Guan (Cornell University, Ithaca, NY; Ref. 73), respectively. The constructs encoding nuclear GFP (nGFP) or nuclear red fluorescent protein (nRFP) were described previously (36, 53).

Mechanical stimulation of cell cultures. Cells were cultured for 24 h on flexible-bottom wells coated with collagen type I, poly-l-lysine, or poly-l-lysine plates further coated with activating integrin antibodies by overnight incubation at 4°C with 10 μg/ml of each antibody in PBS. Subsequently, cultures were subjected to cycles of biaxial stretching with a FX-4000 Flexercell Strain Unit (Flexcell International) for 1–20 min. Each cycle consisted of 20 s of stretching at 2% or 5% cell elongation (corresponding to 0.02 and 0.05 strains, respectively), followed by 0.1 s of release.

Western blot analysis. Cells were serum starved for 30 min and stretched for the last 1–20 min as indicated above. For experiments using inhibitors, cells were incubated for 20 min in medium without serum containing 50 μM PD-98059, 0.4 μM cytochalasin D, 3 μM colchicine, 10 μM gadoxolinium chloride, 0.5–10 μM PP1, 1–5 mg/ml β-cyclodextrin, 0.03 μM wortmannin, or 100 μM SB-203580, followed by stretching at 5% elongation for 10 min. Cells were lysed with (in mM) 20 Tris–HCl (pH 7.5), 150 NaCl, 1 EDTA, 10 NaF, 1 sodium orthovanadate, and 1 phenylmethylsulfonyl fluoride, with 5 mg/ml leupeptin, 0.14 U/ml aprotinin, and 1% Triton X-100. Proteins were separated on 7.5% SDS-polyacrylamide gels, electrotransferred to a polyvinylidene difluoride membrane, and detected by the appropriate antibodies. Blots were developed by enhanced chemiluminescence, and the intensity of the bands in the autoradiograms was quantified by scanning and densitometry. Both ERK bands (p42 and p44) were quantified together. Results are expressed as fold increase (mean ± SD) over the respective unstretched (basal) control group, which is designated as 1. For immunoprecipitation, cell lysates (700 μg of protein per condition) were precleared with 1 μg of normal rabbit IgG together with 20 μl of protein G agarose (Santa Cruz Biotechnology) and pelleted by centrifugation at 1,500 rpm at 4°C for 5 min. Supernatants were then incubated with anti-ERK or anti-caveolin-1 antibodies for 4 h, followed by precipitation of the complexes by addition of 20 μl of protein G agarose. Immunoprecipitates were dissolved with buffer for electrophoresis, separated on SDS-PAGE gels, and electrotransferred. Blots were probed with anti-caveolin-1, β1-integrin, or ERK antibodies.

Quantification of apoptosis. MLO-GFP cells were pretreated with pharmacological inhibitors for 30 min and then stretched for 10 min at 2% or 5% elongation. Subsequently, 50 μM etoposide or 1 μM dexamethasone was added to the culture medium, and 6 h later cells were fixed and apoptosis was quantified by enumerating fluorescent cells exhibiting chromatin condensation and/or nuclear fragmentation as previously reported (53). More than 250 cells from fields selected by systematic random sampling (27) were examined for each experimental condition. The sample size required to detect differences with a 95% confidence level was calculated with a two-group continuity-corrected χ² test (15). Data are presented as percentage of etoposide- or dexamethasone-induced apoptosis in the absence of stretching. The percentage of apoptosis was calculated with the formula (%DC + st − %DCst)/(%DCp − %DCst) × 100, where DC = dead cells, p = cultures treated with proapoptotic agent(s), st = stretched cultures, and v = vehicle-treated cultures, as previously published (6, 52). To appreciate the effect of FAK overexpression on unstretched cells, data in Fig. 5D are expressed as percentage of apoptotic cells instead of percentage of apoptosis induced by each proapoptotic agent. For the experiments using dn mutants, MLO-Y4 cells were transiently transfected with nGFP or nRFP along with the indicated constructs. Forty-eight hours later, cells were subjected to stretching and apoptosis was quantified as described above.

Mechanical signals, ERKs, and osteocyte survival

Mechanical signals induced by biaxial stretching activate ERKs in an integrin-dependent manner. MLO-Y4 osteocytic cells grown for 24 h on flexible-bottom wells coated with collagen I were subjected to cycles of 2% or 5% cell elongation (corresponding to 0.02 and 0.05 strain, respectively). Whereas...
respectively, are shown. Bars depict fold increase (mean \( \pm SD \)) in the ratio of pERK/ERK over the respective unstretched (−) control group, which is designated as 1. *\( P < 0.05 \) vs. unstretched cultures, by ANOVA.

2% elongation of cells plated on collagen I had no significant effect, 5% elongation for 1–20 min gradually increased ERK phosphorylation, as assessed by Western blotting, reaching a two- to threefold increase over unstretched cells at 5 min (Fig. 1A). On the other hand, stretching did not induce ERK activation at any time or strain tested in cells plated on poly-L-lysine, a substrate that allows cell attachment without involving the interaction between cellular integrins and the ECM (Fig. 1B). Figure 2A shows a comparison between ERK stimulation induced by 5% elongation and by EGF or serum in cells plated on collagen I or poly-L-lysine. All stimuli increased ERK phosphorylation four- to sevenfold in cells plated on collagen I. Although the basal levels of pERKs were higher in cells plated on poly-L-lysine, EGF or serum induced further increases in ERK phosphorylation. However, this effect was less pronounced than that observed in cells plated on collagen, most likely reflecting the cooperative nature of ERK activation by integrins and growth factors (50). On the other hand, mechanically induced ERK activation was completely abolished in cells plated on poly-L-lysine, indicating that engagement of integrins is required for the transduction of mechanical forces into ERK activation.

To identify the integrins involved in ERK activation induced by stretching, we next examined the effect of ligation of particular integrins with activating anti-integrin antibodies. Different basal levels of phosphorylated ERKs were observed in cells plated on different integrin-activating antibodies. Nevertheless, whereas stretching had no effect on cells plated on poly-L-lysine coated with nonimmune IgG or anti-\( \alpha_1 \) or anti-\( \beta_2 \) antibody, stretching did induce ERK activation in cells plated on anti-\( \alpha_2 \), anti-\( \alpha_5 \), or anti-\( \beta_1 \) antibodies, or combinations of anti-\( \beta_1 \) with anti-\( \alpha_2 \) or anti-\( \alpha_5 \) antibodies, compared with the respective controls (Fig. 2B).

Mechanically induced ERK activation requires intact actin and tubulin cytoskeletons and Src kinase activity and is abolished by disrupting caveolae with \( \beta \)-cyclodextrin. We next investigated how integrin engagement induced by stretching activates ERKs. Outside-in signaling mediated by integrins might involve cytoskeletal and catalytic proteins (4). Consistent with this evidence, we found that disruption of actin filaments with cytochalasin D or inhibition of microtubule formation with colchicine abolished stretch-induced ERK activation (Fig. 3A). As expected, the specific inhibitor of ERK activation PD-98059 also abolished the response. On the other

Fig. 1. Stretch-induced ERK activation in osteocytic cells depends on integrins. MLO-Y4 cells plated on flexible-bottom wells coated either with collagen I (A) or with the non-integrin-dependent substrate poly-L-lysine (B) were subjected to 2% or 5% elongation for 1–20 min. Cell lysates were obtained immediately after stretching, and phospho-ERKs (pERKs) and total ERKs were analyzed by Western blotting. Representative Western blots of 8 or 3 independent experiments for cells plated on collagen or poly-L-lysine, respectively, are shown. Bars depict fold increase (mean ± SD) in the ratio pERK/ERK over the respective unstretched (−) control group, which is designated as 1. *\( P < 0.05 \) vs. unstretched cultures, by ANOVA.

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Fig. 2. Integrins are required for mechanically induced ERK activation in osteocytic cells. A: cells grown on collagen I or poly-L-lysine were left untreated (basal), subjected to 5% elongation for 10 min (stretching), or treated with 10 ng/ml EGF or 5% serum (FBS) for 10 min. B: cells grown on poly-L-lysine coated with the indicated activating anti-integrin antibodies were stretched as in A. Representative Western blots are shown. Values indicate fold increase (mean ± SD) in the ratio pERK/ERK over the respective unstretched (basal or −) control group designated as 1. *\( P < 0.05 \) vs. basal, by ANOVA or by t-test for A and B, respectively. ni IgG, non-immune IgG.
Mechanical signals prevent osteocyte apoptosis induced by etoposide or glucocorticoids. Earlier studies from our group (36, 53) established the requirement of ERK activation for the antiapoptotic effects of sex steroids as well as bisphosphonates on osteocytes and osteoblasts. On the basis of this evidence, we proceeded to determine whether mechanical stimulation influences the subsequent response of osteocytic cells to proapoptotic stimuli and whether ERK activation is involved in that effect. MLO-Y4 cells stably expressing green fluorescent protein targeted to the nucleus (MLO-GFP) were stretched at 5% elongation during 10 min, followed by induction of apoptosis by addition of either etoposide or dexamethasone. Apoptosis was evaluated 6 h later by examining the nuclear morphology. Whereas etoposide or dexamethasone increased the percentage of cells exhibiting chromatin condensation and/or nuclear fragmentation in unstretched cells, cells previously subjected to mechanical stimulation were refractory to the proapoptotic agents (Fig. 4A). Likewise, mechanical stimulation reduced etoposide-induced caspase 3 activation in a time-dependent fashion (Fig. 4B). In addition, PD-98059 reversed the antiapoptotic response induced by stretching, and, consistent with the dependence of ERK activation on Src kinases and the integrity of caveolae shown in Fig. 3, PP1 or β-cyclodextrin also abolished the antiapoptotic effect of stretching (Fig. 4C). On the other hand, wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), or SB-203580, an inhibitor of p38 MAP kinase, had no effect on antiapoptosis induced by stretching, although they effectively reduced the phosphorylation of the PI3K substrate Akt and of p38, respectively, in cells stretched or left untreated (Fig. 4D). Identical results were observed when cells were stretched at 2% elongation for 10 min (Fig. 4E). These results strongly suggest that mechanical signals interfere with proapoptotic responses via an ERK-dependent mechanism.

We next confirmed the requirement of the Src/ERK pathway in antiapoptosis induced by mechanical signals, using dn mutants of these kinases along with nGFP to allow for the quantification of apoptosis in transfected cells only (Fig. 5). The expression of the wild-type or dn mutants used in these experiments was confirmed by Western blotting and is shown in the insets of Fig. 5. Consistent with the data from the pharmacological inhibitors, cells transiently transfected with dn MEK, the kinase responsible for ERK activation, or with a kinase-defective mutant of Src (K−), did not exhibit the protective effect of stretching on etoposide- or dexamethasone-induced apoptosis (Fig. 5, A and B, respectively). We next examined the potential involvement of Shc, a Src kinase substrate that has been implicated in mechanotransduction (12). Antiapoptosis induced by mechanical signals was abolished by dn forms of Shc in which all three (FFF) or only the third (YYF) of Tyr239, Tyr240, and Tyr317, the primary site of Src-dependent phosphorylation (63), were substituted by phenylalanine (Fig. 5C). However, when Tyr239 and Tyr240, but not Tyr317, were mutated (FFY), the antiapoptotic effect of stretching was unaffected.

Moreover, cells transfected with a dn Src mutant lacking the SH2 domain (ΔSH2) also lost the responsiveness to stretching, whereas cells transfected with a dn Src mutant lacking the SH3 domain (ΔSH3) remained responsive (Fig. 5B). ERK activation initiated by integrins resulted from autophosphorylation of FAK in Tyr397 and the consequent recruitment of proteins contain-
ing SH2 domains, such as Src (18, 50). Therefore, we examined whether the survival effect induced by stretching requires FAK activation. Cells expressing wild-type FAK were protected from etoposide-induced apoptosis as effectively as cells transfected with vector. On the other hand, cells expressing the dn autophosphorylation-deficient mutant FAK Y397F were not protected (Fig. 5D). As was the case with all the constructs used in this study, transfection with the FAK constructs did not alter the effect of etoposide in untested cells. However, transfection of wild-type FAK or Y397F FAK abolished dexamethasone-induced apoptosis (Fig. 5D). For that reason, the requirement of FAK kinase activity for stretch-induced survival could be evaluated only by using etoposide as a proapoptotic stimulus. Together, the results of the experiments presented in Figs. 4 and 5 demonstrate that mechanical stimulation prevents apoptosis of osteocytic cells and that activation of FAK and the Src/Shc/ERK signaling pathway is required for this effect.

Antiapoptosis induced by mechanical signals requires nuclear accumulation of ERKs and new RNA and protein synthesis. ERKs could promote survival by phosphorylating transcription factors that regulate the expression of apoptosis-related genes.
related genes (7, 35). Because nuclear localization of ERKs is a prerequisite for this function, we sought to determine whether mechanical signals induce nuclear accumulation of ERKs, using MLO-Y4 cells transiently transfected with ERK2 fused to GFP. The percentage of cells plated on collagen I exhibiting ERK nuclear accumulation increased on stretching at 5% elongation for 10 min (Fig. 6A). As expected, blockade of ERK phosphorylation by the MEK inhibitor PD-98059 completely abolished stretching-induced ERK nuclear accumulation. Cultures stretched at 5% elongation for as little as 1 min exhibited a significant increase in the percentage of cells exhibiting ERK nuclear accumulation, and the magnitude of this effect increased with the duration of the mechanical stimulation, reaching a plateau at 5 min (Fig. 6B). The percentage of cells showing ERK nuclear accumulation induced by 20 min of mechanical stimulation was comparable to that induced by 5-min stimulation with estradiol, another inducer of ERK activation (35), in the same experiment (not shown). In contrast to the lack of an effect of 2% elongation on ERK phosphorylation shown in Fig. 1A, cultures subjected to 2% elongation also exhibited a significant increase in the percentage of cells with ERK nuclear accumulation. These results indicate that ERK nuclear accumulation is a more sensitive readout of ERK activity than ERK phosphorylation and are consistent with the demonstration that 2% elongation inhibited the proapoptotic action of etoposide or dexamethasone (Fig. 4E). In addition, and consistent with the results of ERK phosphorylation (Figs. 1 and 2), the percentage of cells exhibiting ERK nuclear...
Fig. 6. Antiapoptosis induced by mechanical signals requires nuclear accumulation of ERKs and new RNA and protein synthesis. A: MLO-Y4 cells transiently transfected with ERK2 fused to GFP (GFP-ERK2) were plated on collagen I and left untreated (basal) or stretched for 10 min at 5% elongation in the absence or presence of PD-98059. Bar indicates 20 μm. B: cells plated on collagen I or poly-L-lysine were left untreated or stretched at 2% or 5% elongation for the indicated times and immediately fixed. C: cells plated on collagen I were stretched for 10 min at 5% elongation and fixed at the indicated times after the stretching period had finished. D: cells transfected with wild-type GFP-ERK2 or a cytoplasmic GFP-ERK2 mutant, along with nuclear red fluorescent protein to allow the quantification of apoptosis by nuclear morphology, were left untreated or stretched for 10 min at 5% elongation, followed by addition of etoposide or dexamethasone. Apoptosis was assessed by nuclear morphology and quantified as in Fig. 4C. Cycloheximide treatment inhibited ~80% of [3H]leucine incorporation into protein (vehicle: 139,460 ± 4,090 cpm/mg protein; cycloheximide: 29,890 ± 1,170 cpm/mg protein). Actinomycin D treatment inhibited ~90% of [3H]uridine incorporation into RNA (vehicle: 19,170 ± 4,720 cpm/mg protein; actinomycin D: 1,840 ± 970 cpm/mg protein). Neither cycloheximide nor actinomycin D affected basal cell viability. *P < 0.05 vs. basal by ANOVA.
accumulation under basal, that is, unstretched, conditions was higher in cells plated on poly-1-lysine than in cells plated on collagen I, and mechanical stimulation (either at 2% or 5% elongation) failed to induce further ERK nuclear accumulation (Fig. 6B). We next determined the time course of the reversal of nuclear ERK accumulation. In cultures stretched for 10 min at 5% elongation, the percentage of cells exhibiting ERK nuclear accumulation remained significantly elevated for up to 1 h after the termination of the mechanical stimulus, declining to levels similar to those observed in unstimulated cultures by ~3 h (Fig. 6C). Importantly, cells expressing wild-type GFP-ERK2 were protected from etoposide- or dexamethasone-induced apoptosis by mechanical stimulation. On the other hand, cells expressing a GFP-ERK2 mutant that lacks the ability to translocate to the nucleus and is constitutively localized in the cytoplasm were not protected (Fig. 6D). These results indicate the requirement of nuclear localization of ERKs to exert their antiapoptotic effects on osteocytic cells in response to mechanical stimulation. The mechanism by which GFP-ERK2 abolishes the action of endogenous ERKs is unclear. However, earlier work demonstrated that homodimerization of ERKs on phosphorylation is required for their nuclear translocation (33). Therefore, it is likely that dimer formation of endogenous ERKs with cytoplasm-anchored GFP-ERK (largely in excess) inhibits the nuclear translocation of the endogenous kinases, thus abolishing their antiapoptotic effect. Nevertheless, consistent with the findings on cytoplasm-anchored GFP-ERK, the protein synthesis inhibitor cycloheximide and the RNA synthesis inhibitor actinomycin D abolished the antiapoptotic effect of mechanical stimulation at concentrations at which they effectively inhibited $[^3H]$leucine and $[^3H]$uridine incorporation, respectively, without affecting cell viability (Fig. 6E). Together, these results indicate that mechanical signals prevent osteocyte apoptosis via a mechanism that requires new gene transcription and strongly suggests the participation of ERK-activated transcription factors.

**DISCUSSION**

Extensive evidence reviewed elsewhere has established that changes in the prevalence of apoptosis of bone cells are a critical determinant of bone loss in various disease states and an important mechanism of action of pharmacotherapeutics (9, 41). In particular, preservation of osteocytes might be essential for the maintenance of mechanical competence of the skeleton, through mechanisms dependent on as well as independent of changes in the amount of mineral. Besides systemic factors, mechanical stimuli might also maintain osteocyte viability. However, although osteocytes are ideally positioned to detect and respond to changes in mechanical strain imposed on bone, heretofore it has remained unknown whether indeed mechanical stimuli affect directly the life span of these cells and, if so, by which mechanism.

The findings reported here demonstrate that mechanical stimuli preserve osteocyte viability via activation of ERKs and new gene transcription. The transduction of mechanical signals into ERK activation requires integrin engagement, intact actin and microtubular cytoskeletons, FAK, Src kinase activity, and the adaptor protein Shc. This evidence is consistent with earlier reports demonstrating ERK activation by mechanical stimulation in osteoblastic cells (8, 16, 29, 67).

**Convergence of systemic and mechanical survival signals on ERK activation.** Similar to the findings reported here with mechanical stimulation, ERK activation is required for the antiapoptotic actions of sex steroids and bisphosphonates on osteocytes and osteoblasts (36, 53). Nonetheless, the molecular events that lead to ERK activation as well as the mechanisms by which ERKs promote survival in each case are distinct. Indeed, whereas sex steroids activate ERKs through the estrogen and androgen receptors (35, 36), bisphosphonates activate ERKs by opening connexin43 hemichannels (52). Furthermore, whereas bisphosphonates exert their antiapoptotic effect by cytoplasm-restricted ERK signals independent of transcription (51), survival induced by sex steroids (35) or by mechanical signals (this report) requires nuclear ERK accumulation and new gene transcription. Moreover, the antiapoptotic effect of sex steroids also requires activation of PI3K (35), but as shown here this kinase is not required for antiapoptosis by mechanical stimuli. Collectively, these findings support the notion that additive or synergistic effects on osteoblast and/or osteocyte survival could explain at least partially the advantages of therapeutic regimens in which sex steroids and bisphosphonates are combined with each other or with physical exercise (10, 23, 40).

The dependence of stretch-induced survival on the nuclear localization of ERKs and on new gene transcription strongly suggests the participation of nuclear ERK substrates. Consistent with this, activation of the transcription factors Runx2/Cbfal, egr-1, and AP-1 by mechanical signals has been shown to depend on ERKs in osteoblastic cells (25, 26, 76). Furthermore, Runx2 is involved in the survival signals of PTH in osteoblasts, even though in this case PKA, but not ERKs, is required (6). The question of whether these or other transcription factors are indeed required for the antiapoptotic effect of mechanical stimulation will require further studies.

Requirement of integrins and Src for mechanically induced osteocyte survival. Engagement of specific integrins with activating antibodies revealed that $\beta_1$- (but not $\beta_2$, $\alpha_2$, and $\alpha_5$-integrins mediate stretch-induced ERK activation. This finding is consistent with the expression of high levels of $\beta_1$-integrin, but very low levels of $\beta_2$-integrin, in MLO-Y4 osteocytic cells (unpublished data) as well as with the expression of $\beta_1$-integrin in osteocytes in vivo and its involvement in osteocyte adhesion to ECM proteins (22, 28). Our results are also consistent with extensive evidence indicating that $\beta_1$- integrin triggers antiapoptotic intracellular signaling. Thus, ligatation of $\beta_1$-integrin abrogates anoikis of fibroblastic cells induced by collagen gel contraction (60). In addition, the ECM protein fibronectin, ligand of $\alpha_5\beta_1$-integrin, induces survival of different cell types, including osteoblasts (5, 21, 72), and collagen I, which binds to $\alpha_2\beta_1$-integrin, is also a survival factor (19, 60, 75).

The different basal levels of ERK activation observed in cells plated on poly-1-lysine compared with collagen I or with different integrin-activating antibodies is not unexpected, considering that engagement per se of some but not all integrins leads to modulation of the activity of ERKs themselves as well as other kinases that might influence ERK activation (24, 39, 54, 58). Nevertheless, independently of its basal levels, stretching induces an increase in ERK activation only when particular integrins are engaged. However, this effect is more modest than that observed in cells plated on collagen. This finding
MLO-Y4 cells and coimmunoprecipitates with processes (55, 59), the fact that caveolin-1 is present in cyclodextrin might also interfere with noncaveolar related responses of osteocytes to mechanical stimulation. Although antiapoptosis, suggesting that caveolae are involved in the -cyclodextrin abolishes stretch-induced ERK activation and /H9252 and the phosphorylation of the Src kinase substrate Shc (65). In the present study, we found that sequestering cholesterol with β-cyclodextrin abolishes stretch-induced ERK activation and antiapoptosis, suggesting that caveolae are involved in the response of osteocytes to mechanical stimulation. Although cyclodextrin might also interfere with noncaveolar related processes (55, 59), the fact that caveolin-1 is present in MLO-Y4 cells and coimmunoprecipitates with β1-integrin and ERKs suggests that a signalsome does indeed assemble in caveolin-rich membrane domains in these cells. This finding is consistent with previous evidence that caveolae are involved in mediating integrin-dependent ERK activation in endothelial cells (49) and that caveolin-3, the muscle-specific member of the caveolin family of proteins, is required for stretch-induced responses (32). However, further studies are required to confirm the requirement of caveolae in the survival effect of mechanical stimulation and to investigate the mechanism by which caveolin-1 controls mechanotransduction in osteocytes.

Both the kinase and the SH2 domain of Src are required for the antiapoptotic effect of stretching. This fact indicates that Src, besides playing a role in phosphorylating downstream substrates such as Shc, also plays a critical role in the assembly of the signalsome required for the transduction of mechanical signals via protein-protein interaction through its SH2 domain. Our findings show that one of these proteins, FAK, previously shown to become phosphorylated in Tyr397 on integrin engagement and to bind Src leading to ERK activation (38, 57), is required for antiapoptosis induced by stretching. Interestingly, even though phosphorylated FAK in Tyr397 also binds to the p85 subunit of the PI3K (11), only inhibition of ERKs, but not PI3K, abolishes the survival response induced by stretching.

Other proteins with affinity for the Src SH2 domain might also participate in the transduction of mechanical signals. Indeed, our recent findings (3) suggest that the estrogen receptor (ER) is also an essential component of the stretch-induced signaling cascade described here. Consistent with this, Src interacts through the SH2 domain with the ER (45). Furthermore, mice lacking ERα exhibit a poor anabolic response to bone loading (37).

**Regulation of osteocyte life span in vivo.** The relevance of the present in vitro findings to the in vivo situation is a matter of conjecture at this time. Nevertheless, the strain applied to osteocytic cells in the current studies is of a magnitude similar to that to which osteocytes might be exposed in vivo, according to the strain amplification model in which cell-level strains are at least one order of magnitude larger than bone tissue-level strains (69, 70). In addition, osteocytes have been shown to respond more readily than osteoblasts to mechanical stimulation (34), and osteoblastic cells in general appear to be more sensitive to fluid flow than to substrate stretching (44, 48), suggesting that the antiapoptotic responses reported here may be underestimated by the use of the latter approach. Furthermore, mounting evidence indicates that osteoblast and osteocyte survival is maintained not only by endocrine or autocrine/paracrine soluble factors but also by the ECM and that loss of antiapoptotic signals originated in the ECM causes osteoblastic cell apoptosis, a phenomenon referred to as “anoikis” (17). Thus neutralizing antibodies to the ECM protein fibronectin induce osteoblast apoptosis (21), and transgenic mice expressing collagenase-resistant collagen type 1 exhibit increased prevalence of osteoblast and osteocyte apoptosis compared with age-matched wild-type controls (75). Collectively, this evidence supports the notion that interactions between intact or cryptic sites of ECM proteins with cellular integrins result in “outside-in” signaling that preserves viability. Considering that osteocytes do not divide or differentiate in vivo, maintenance of osteocyte viability by promoting ECM-osteocyte interactions is likely to be one of the most relevant biological responses of these cells to mechanical forces.

In conclusion, in this report, we demonstrate that mechanical stimuli preserve osteocyte viability via activation of ERKs and new gene transcription. The evidence linking mechanical stimulation, activation of an integrin/cytoskeleton/Src/ERK signaling pathway, and osteocyte survival provides a mechanistic basis for the profound role of mechanical forces, or lack thereof, in skeletal health and disease. Furthermore, the fact that the survival responses triggered by strain activate distinct pools of the same intracellular kinases utilized by systemic stimuli provides evidence for the participation of osteocytes in the integrated response of the skeleton to both biochemical and physical stimuli.

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