Mechanical Strain Differentially Regulates Endothelial Nitric-oxide Synthase and Receptor Activator of Nuclear κB Ligand Expression via ERK1/2 MAPK*

Received for publication, March 19, 2003, and in revised form, May 30, 2003
Published, JBC Papers in Press, June 24, 2003, DOI 10.1074/Jbc.M302822200

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Exercise promotes positive bone remodeling through controlling cellular processes in bone. Nitric oxide (NO), generated from endothelial nitric-oxide synthase (eNOS), prevents resorption, whereas receptor activator of nuclear κB ligand (RANKL) promotes resorption through regulating osteoclast activity. Here we show that mechanical strain differentially regulates eNOS and RANKL expression from osteoprogenitor stromal cells in a magnitude-dependent fashion. Strain (0.25–2%) induction of eNOS expression was magnitude-dependent, reaching a plateau at 218 ± 36% of control eNOS. This was accompanied by increases in eNOS protein and a doubling of NO production. Concurrently, 0.25% strain inhibited RANKL expression with increasing response up to 1% strain (44 ± 3% of control RANKL). These differential responses to mechanical input were blocked when an ERK1/2 inhibitor was present during strain application. Inhibition of NO generation did not prevent strain-activated ERK1/2. To confirm the role of ERK1/2, cells were treated with an adenovirus encoding a constitutively activated MEK; Ad.caMEK significantly increased eNOS expression and NO production by more than 4-fold and decreased RANKL expression by half. In contrast, inhibition of strain-activated c-Jun kinase failed to prevent strain effects on either eNOS or RANKL. Our data suggest that physiologic levels of mechanical strain utilize ERK1/2 kinase to coordinately regulate eNOS and RANKL in a manner leading to positive bone remodeling.

The capacity of bone to remodel to meet functional structural demands was recognized by Wolff in 1892 as the “law of bone transformation” (1). Studies in humans (2, 3) and animals (4, 5) have shown that applied loads are associated with changes in bone density as well as skeletal macro- and microstructure (6, 7). Removing load results in bone loss (8, 9), whereas application of load causes bone apposition (10–12), confirming that normal bone recognizes its loading environment and adapts to maintain an optimal functional structure. Only recently has understanding of the cellular processes underlying the ability of the skeleton to remodel (i.e. to serve Wolff’s Law) made possible a search for the specific mechanisms by which mechanical force is translated into alterations in bone structure. Studying cellular response to mechanical strain in vitro, we have previously shown that murine osteoprogenitor cells respond to the application of strain by decreasing the expression of receptor activator of NF-κB ligand (RANKL)1 mRNA (13). RANKL is the dominant molecule controlling osteoclastogenesis (14) and is up-regulated in response to hormones and factors that are known to promote bone resorption (15, 16). We then showed that strain-induced reduction in RANKL expression required activation of ERK1/2 kinase, which was rapidly and sensitively activated by mechanical strain (17). This process in bone mirrors a signal cascade known to be pertinent to many of the responses of vascular tissue to shear forces (18–21) as well as to strain (22, 23). Since the diminished expression of RANKL by bone cells is inextricably linked to a repression of osteoclast formation, we wondered whether other “proformative” events might be associated with signals initiated by mechanical factors.

In this work, we show that endothelial nitric oxide synthase (eNOS) is regulated by strain in a divergent fashion; strain induces the expression of eNOS at the same time that this mechanical input decreases expression of RANKL. eNOS, which generates nitric oxide (NO), appears to promote an anabolic picture in bone (24, 25). NO has been shown to have an inhibitory effect on both osteoclast formation and activation (26, 27). There is also growing evidence that NO (and eNOS specifically) has roles in bone formation during growth (28, 29) and in response to loading (30). We will show in this work that induction of eNOS expression, similarly to mechanical regulation of RANKL, also requires activation of ERK1/2 kinase. To confirm the role of ERK1/2 in these mechanically controlled events, we utilize an adenovirus causing constitutive ERK1/2 activity and show that both eNOS is up-regulated and that RANKL is down-regulated in the presence of an activated MAPK signaling system.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Antibodies to total ERK1/2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and those to 48-phosphorylated ERK1/2 were from New England Biolabs (Beverly, MA). ERK1/2 inhibitor PD98059 was obtained from Calbiochem, as was the JNK inhibitor (JNKi; SP600125). Fetal bovine serum was from Hyclone.

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1 The abbreviations used are: RANKL, receptor activator of nuclear κB ligand; NOS, nitric oxide synthase; eNOS, endothelial NOS; iNOS, inflammatory NOS; nNOS, neuronal NOS; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; JNKi, JNK inhibitor; ERK1/2, ERK inhibitor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MOI, multiplicity of infection; L-NAME, Nω-nitro-L-arginine methyl ester.
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Logan, UT). Other chemicals and supplies were purchased from Sigma.

**Cell Culture**—To generate primary stromal cell cultures, murine marrow cells collected from the tibiae and femurs of 3–5-week-old male C57BL/6 mice were plated in 6-well plates at 1.6 × 10^6 cells/cm² as previously published (17). After 60 min, nonadherent cells containing the stromal elements were transferred to Bioflex collagen I-coated plates (Flexcell Corp., McKeesport, PA) in α-minimal essential medium plus 10% fetal bovine serum. The next day nonadherent cells were discarded; adherent stromal cells were cultured with 10 μM 1,25-dihydroxyvitamin D3 added to stimulate RANKL expression on day 4. Strain regimens were applied on day 6.

For experiments where lysates were made for Western analyses, inhibitors were added 30 min prior to strain, and the experiment was stopped on the following day. When the end point was to measure nitrite, total mRNA was isolated 24 h after beginning strain induction.

**Application of Mechanical Strain**—Uniform equibiaxial mechanical stress was generated using a Flexcell Bioflex instrument (Flexcell Corp., McKeesport, PA) as previously described (13). Strain magnitudes were as noted from 0.25 to 2%, with strain frequency fixed at 10 cycles/min (0.17 Hz). Similar plates containing control cultures were kept in the same incubator but were not subjected to strain regimens.

**Measurement of NO**—A fluorometric assay was used to measure nitrite in samples using the reagent 2,3-diaminonaphthalene with comparison with a NaNO2 standard curve (0–10 μM) as described previously (31, 32). Briefly, 100 μl of samples and standards were added to microtiter 96-well plates (DYNEX Technologies, Inc.) and mixed with 10 μl of fresh 2,3-diaminonaphthalene (prepared in 0.62 M HCl) for 10 min at room temperature. The reactions were terminated with 5 μl of 2.8 N NaOH. Formation of the 2,3-diaminonaphthotriazole end product was measured using an LB 50 plate reader (PerkinElmer Life Sciences) with excitation at 380 nm and emission at 440 nm. All standards and samples were measured in triplicate.

**Western Blot Analysis**—For NOS expression, proteins were extracted from stromal cells in boiling lysis buffer (10 mM Tris, pH 7.4, 1% SDS, and 1 mM sodium orthovanadate). Cell lysates were boiled for an additional 5 min and passed three times through a 26-gauge needle. After centrifugation at 16,000 × g for 5 min to remove insoluble material, protein concentrations in the supernatant were determined using the Bradford assay (Bio-Rad). The proteins were electrophoresed through a 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane. For NOS expression, 10 μg of protein was chromatographed on a 4.5-μm polyvinylidene difluoride membrane. Membranes were immersed in blocking buffer containing TBS with 0.1% Tween 20 (TBST) and 5% nonfat milk overnight at 4 °C. The eNOS and inflammatory NOS (iNOS) isoforms were identified using respective polyclonal antibodies (1:1000; Transduction Laboratories, Lexington, KY); the 42- and 44-kDa phosphorylated bands as in Ref. 17. To assess c-Jun N-terminal protein kinase (JNK) activity, a c-Jun phosphorylation assay was used as previously described; briefly, soluble cell lysates in cold Triton lysis buffer with proteinase inhibitors were incubated with 2.5 μg of agonist-induced glutathione GGT-1a (Caltbiochem) in a total volume of 400 μl of Triton lysis buffer overnight at 4 °C on a rotating platform (17, 37). Washed agarose beads were mixed with 100 μM ATP and 5 μCi of [γ-32P]ATP, and reactions were carried out at 30 °C for 30 min. Phosphoproteins were separated on a 15% SDS-PAGE gel, and phosphorylated glutathione S-transferase-c-Jun (1–79) was visualized at ~37 kDa.

**Statistical Analysis**—Results are expressed as the mean ± S.E. Statistical significance was evaluated by Dunnett or Bonferroni one-way analysis of variance (GraphPad Prism).

**RESULTS**

**Mechanical Strain Induces eNOS Expression in Murine Stromal Cells**—Bone cells express multiple forms of NOS, with eNOS being the most prominent isoform. iNOS and neuronal NOS (nNOS) have both been described in bone during growth and fracture healing but are clearly present in lower amounts than eNOS in bone cells themselves (38, 39). Using Western blotting to identify species in C57BL/6 murine stromal cells, we were able to identify eNOS protein in samples where 200 μg of lysate protein was loaded. To assess whether the NOS might be residing in the membrane component, we also separated membrane protein and found that similar loading of 200 μg of membrane protein did not alter the amount of eNOS reacting with the antibody. In cells exposed to 2% magnitude mechanical strain overnight, eNOS increased, as shown in a representative blot (Fig. 1a). iNOS was not visible in Western blots from these lysates, despite successful blotting of an assay positive control for the iNOS species.

Because of sensitivity in assessing amounts of eNOS protein, we confirmed the strain inducive response using a sensitive real time PCR assay for eNOS mRNA. This assay was designed to recognize only eNOS, and not iNOS or nNOS, on the basis of primer specificity. In Fig. 1b, compiled from three separate experiments where cells were exposed to 24 h of 2% strain, eNOS mRNA significantly increased by more than 2-fold. Using primers specific for murine iNOS, we demonstrated a lack of response to strain of this NOS isoform; we performed real time PCR on control and cells strained for 24 h. Grouped real time PCR data from three experiments showed no significant difference in levels of iNOS mRNA between control (100 ± 10%) and strained cells (90 ± 25%). These data ruled out a...
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Fig. 1. Strain increases eNOS expression in stromal cells. a, mouse stromal cells were strained on the last 24 h of cultures. Protein was extracted and run at 200 μg/lane on 7.5% SDS-PAGE. Western blot showed that eNOS synthesis significantly increased after 24 h of mechanical strain. The positive control was purchased from Transduction Laboratories (eNOS). The experiment was repeated twice, confirming elevated eNOS protein after strain. b, real-time PCR for eNOS expression in cells strained for 24 h shows that eNOS mRNA from strained cells significantly increased 2-fold (218 ± 36% of control; *p < 0.05 by t test). Three experiments grouped (mean ± S.E.) for statistical analysis are shown in the graph.

Gene Expression Is Differentially Sensitive to Strain Magnitude—The response of eNOS was in the opposite direction to the strain-induced decrease in RANKL gene expression, which we had explored previously (17). To understand better the differences in mechanical regulation of these two genes, eNOS and RANKL, we performed a series of experiments where strain magnitude was varied. Our current strain instrumentation allowed application of uniform strain as low as 0.25%, and strain magnitude was varied. Our current strain instrument allowed application of uniform strain as low as 0.25%, and strain magnitude was varied. At least three separate experiments were performed at each strain magnitude and compiled in Fig. 2. For each sample, eNOS, RANKL, and 18 S were amplified from samples subjected to reverse transcription with random decamers as described under “Experimental Procedures.” The data was expressed as percentage of target control mRNA compared with 18 S in the sample. eNOS mRNA responded to 0.25% strain, rising to 120%, and continued to increase, reaching a plateau at 181 ± 28% compared with eNOS mRNA measured in unstrained cells (p < 0.001), a rise comparable with the previous series shown in Fig. 1b. RANKL also responded to application of 0.25% strain, reaching a nadir by 1% strain, as shown in Fig. 2. This result can be compared with previous results where strains less than or equal to 1% were not effective when dosed for only 6 h (17). In the experiments presented here, application of strain for 24 h allowed us to generate reproducible mechanical effects on both eNOS and RANKL gene expression with strains significantly lower than 1%. Results showing that

Fig. 2. Strain magnitude dose-dependently regulates eNOS and RANKL gene expression. Real time PCR data shown in the graph ± S.E. is compiled from 16 experiments. Each experiment included strained and unstrained wells, with strain in each experiment limited to a single magnitude. Three separate experiments were run for each strain magnitude, with magnitude chosen in no special order. Total RNA was collected from three wells/sample, two samples/condition for real time assessment of eNOS, RANKL, and 18 S. eNOS, shown in gray bars, increased in response to strain in a magnitude-dependent fashion, becoming significant by 1% strain magnitude (*, p < 0.01; ###, p < 0.001). For strain-induced inhibition of RANKL mRNA expression, all strain magnitudes examined were significant (*, p < 0.001), and the 1% magnitude was significantly more inhibited than the 0.25% (**, p < 0.05).

Strain caused a divergent response of these two genes at all magnitudes studied suggested that mechanical regulation of eNOS and RANKL share a proximal signaling pathway.

NO Release Is Stimulated by Strain and Reflects Increases in eNOS—We next measured nitric oxide production from murine cells during strain. NO did not rise immediately, in contrast to the immediate activation in response to fluid shear in cellular NOS of both osteocytes and osteoblasts (40, 41). Even at 60 min, as measured by a more sensitive assay (31, 32) than used in the latter studies, NO was not significantly different from that secreted into media by unstrained cell cultures (Fig. 3a). However, NO was significantly increased by 2-fold after 24 h of strain, as shown in Fig. 3b. Furthermore, the strain induction of NO required the activity of endogenous NOS, as the competitive inhibitor, L-NAME, blocked the effect of strain on NO production, shown in Fig. 3c. Because iNOS was not affected by the strain protocol (see Fig. 1c), we inferred that the increased NO resulted from increased gene and protein expression of the endothelial form of NOS.

Inhibition of ERK Activation Indicates That ERK Has Primary Effects on Both eNOS and RANKL—We had previously shown that straining cells at magnitudes less than 2% caused activation of both ERK1/2 and JNK (17). Here we set out to prove that strain induction of eNOS expression is blocked by inhibition of ERK1/2 activation. The effect of overnight strain in this series of five experiments was to increase eNOS expression to 195 ± 26% of the unstrained control, as expected (Fig. 4a). Treatment with ERKi during the strain protocol prevented any strain-induced increase in eNOS as shown in the second gray bar in Fig. 4a, which shows an insignificant difference between the unstrained and strained cultures in the presence of ERKi. Exposure of unstrained controls overnight to an efficacious concentration of ERK inhibitor (ERKi) unexpectedly increased eNOS expression to 136 ± 8% of unstrained control levels, suggesting that basal ERK1/2 activity operates some
regulatory control over eNOS expression in bone cells. However, when ERK1/2 was inhibited, application of strain failed to cause further increases in eNOS mRNA levels.

The effect of strain on control cells in this series of experiments was to diminish RANKL expression to 58 ± 4% of control (Fig. 4a). The strain inhibition was ablated in cultures where ERK1/2 activation was blocked by treatment with ERKi; RANKL expression was not significantly different from that in unstrained cells treated with ERKi. However, as shown in Fig.

ERK1/2 activation was blocked by treatment with ERKi; RANKL expression was not significantly different from that in unstrained cells treated with ERKi. However, as shown in Fig.
Constitutive Activation of MEK Reproduces the Strain Effect on Both Genes—To further understand the relationship between ERK activation and its divergent regulation of eNOS and RANKL, we generated an adenovirus to deliver a constitutively activated MEK to cells. The use of adenovirus to transfer the gene was necessitated because these primary stromal cells are not very susceptible to infection by retro- and adenovirus-associated viruses as well as to liposome enhanced transient transfection (data not shown). We designed an infection protocol using a type 5 replication-deficient adenovirus encoding a green fluorescent protein driven by the strong cytomegalovirus promoter (Ad.GFP) (gift of Dr. Peter Thule, Emory University). Ad.GFP virus combined with LipofectAMINE infected more than 60% of the stromal cells in culture as assessed by percentage of green fluorescence-positive cells measured by fluorescence-activated cell sorting analysis (data not shown). To study the effect of activated ERK in culture, we thus selected adenovirus as the means of gene delivery.

The Ad.caMEK virus was used after amplification and titering in H293 cells by viral cytopathology. Plaque-purified empty adenovirus (Ad.empty) was used as a control. 24 h after infection with viruses, cell lysates were made to examine activation of ERK1/2. As shown in Fig. 5a, an MOI of 2 was sufficient to cause ERK phosphorylation. Increases in MOI above 4 did not further increase ERK activation. This may be due to other distal and unexplained effects of highly activated ERK systems, such as a balancing check on the MAPK system.

In compiled experiments where stromal cells were infected with Ad.caMEK virus and eNOS and RANKL mRNA was assessed 24 h after infection, the effect of mechanical strain was reproduced. As shown in Fig. 5b, Ad.caMEK stimulated eNOS expression by more than 4-fold at an MOI of 4 (423 ± 117% of that expressed in cultures infected with Ad.empty).

Also, Ad.caMEK inhibited RANKL mRNA expression to a nadir of 56 ± 12% as shown in Fig. 5c. Interestingly, the effect of this sustained ERK1/2 phosphorylation had a reproducibly equivalent effect to the plateau effect of strain on RANKL mRNA inhibition.

To further explore the effect of the Ad.caMEK on eNOS and to prove that NO production increased as a distal result, we also were able to show that NO was altered by ERK1/2 activation. As shown in Fig. 5d, Ad.caMEK caused a dose-dependent increase in NO release into the medium (measured as nitrite). Compiling three experiments where Ad.empty was used as control (100%) for comparison with Ad.caMEK infection, we found that an MOI of 5 increased cell-generated nitrite to 397 ± 87% of that of control cells, and an MOI of 10 caused increases to 374 ± 66% of Ad.empty control (p < 0.01 for both conditions). Thus, caMEK delivery reproduced the effects of strain to cause divergent responses in eNOS and RANKL expression.

Inhibition of JNK Does Not Prevent the Strain Response—Strain also causes a sustained activation of JNK, specifically JNK2 kinase (17). With the current availability of a JNKi, we were able to examine the role that strain-induced JNK might play in the differential effects of mechanical factors on eNOS and RANKL. We first replicated our data showing that c-jun phosphorylation was increased after 15 min of strain, as shown in Fig. 6a. Also shown in this figure is a demonstration that 50 μM concentration of the JNKi, SP600125, was able to inhibit the strain-induced increase in c-jun phosphorylation. Thus, we were able to inhibit JNK during the application of mechanical strain.

As shown in Fig. 6b, eNOS responded to overnight strain with a doubling of expression and continued to respond significantly to strain even in the presence of JNKi, although the effect was blunted. The question of whether this blunting was due to an already nearly maximal stimulation of eNOS expression or JNK represents a separate pathway by which strain up-regulates eNOS expression remains for future investigation. Similarly to effects on basal cultures caused by inhibition of ERK1/2, JNKi generated an increase in basal eNOS mRNA expression to 168 ± 28% of basal levels.

JNKi also caused a significant increase in basal expression of RANKL as shown in Fig. 6c. In contrast to the effect of inhibition of the ERK signaling pathway, the strain effect prevailed in the presence of JNKi, as shown by the significant difference between control and strain cultures treated with the JNKi (right set of bars).

DISCUSSION

Complex interactions between signals controlling both osteoblast and osteoclast functions are ultimately responsible for the adaptive response of the skeleton to its loading environment. In data presented here, we have shown that application of mechanical strain to bone stromal cells induces a coordinate regulation through MAPK of two genes, eNOS and RANKL, which are predicted to work in synergy to promote positive bone remodeling. The effective levels of strain (~0.25%) in bone stromal cells are far below those affecting vascular tissue, which continue to increase response until at least 20% strain (22), a level well above the fracture threshold of the hard skeleton (43).

Strain, through activation of ERK1/2, caused a doubling in the amount of eNOS expressed and NO generated by primary bone stromal cells. NO has long been implicated as one of the signals transducing mechanical stress in cells (25, 44). In the vasculature, both shear (18) and strain (23, 45, 46) induce nitric oxide, and blocking NO synthesis prevents many of the downstream effects of biophysical inputs. Skeletal response to biophysical input also appears to involve NO metabolism. One of the earliest reports linking NO to bone remodeling showed that NOs inhibitors potentiated ovariectomized bone resorption in rats (27). Since then, studies have shown that NO can slow bone loss in animals (47) and even humans (48, 49). Mechanical stimulation has been shown to initiate NO production: loading rat vertebrae and ulnae causes activation of NO
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Fig. 5. Constitutive activation of MEK reproduces the strain effect. 

a. Ad.caMEK increases levels of stromal cell ERK-P as shown in the Western blot. Phosphorylated ERK1/2 appears in the upper row (42- and 44-kDa bands); the first lane shows stromal cells infected 24 h previously with an empty adenovirus. Phosphorylation increases and NO release (50). Inhibition of NO production further appears to impair response to loading; in rats treated with an inhibitor of NOS, a regimen of four-point bending of tibiae induced less new bone formation than in controls (51). Further studies in rats showed that mechanically induced osteogenesis required and was enhanced by endogenous NO production, whereas inhibition of NO production decreased bone apposition (52). In vitro, studies of the effects of mechanical stimulation on osteoblast NO production have shown that both shear and deformation (strain) increase NO production (40, 50, 53–55) and that nitric oxide causes proliferation of bone-forming cells (56). NO is likely to affect bone remodeling, therefore, through targeting the formation and function of both osteoclasts and osteoblasts.

NO in bone is largely generated through eNOS, which is the predominant isoform expressed in skeletal tissue (38, 39, 57). Transgenic mice lacking eNOS show decreased bone volume and formation until at least 12 weeks, when other adaptive processes must overcome the deficiencies caused by the absence of eNOS (29). eNOS null mice are furthermore unable to respond to estrogen with an anabolic response (58). In terms of the mechanical response, studies have implicated iNOS as also being involved in the reloading bone apposition after loss through hind limb raising; in the iNOS null transgenic, bone loss was similar to control during the unweighting protocol, but reloading failed to induce bone formation in the transgenic animal (5). In our studies, we did not see a response of iNOS to strain. The roles of eNOS and iNOS and their production by the many cells involved in bone remodeling will be important to explore in future studies.

Whereas eNOS is induced by strain, RANKL is coordinately repressed, as demonstrated by the divergence of response during the strain magnitude-dosing protocol (Fig. 3). RANKL inhibition by strain in vitro is discrete, rarely dropping below 50% of control levels, an effect faithfully replicated by activating ERK1/2 through delivery of an adenovirus encoding constitutively active MEK. In contrast, where strain induction of eNOS caused less than a doubling response, constitutive activation of MEK induced anywhere between 2- and 10-fold increases in eNOS expression, reflected in much greater increases in NO generation. This suggests that whereas eNOS response to strain appears to require similar activated MAPK pathways, a constitutively activated ERK1/2 might lead to additional pathways affecting eNOS expression.

Since strain also induces JNK activation, indeed prolonged beyond the transient activation of ERK1/2 (17), we tested the role of JNK in strain-induced increases in eNOS and decreases in RANKL. The JNKi used in these studies was shown to inhibit strain-induced JNK activation. Overnight application of JNKi had significant effects on basal levels of both eNOS and RANKL, suggesting that JNK is involved in regulating the expression of important genes in bone cells. However, JNKi failed to prevent either strain induction of eNOS or strain after infection with Ad.caMEK. Total ERK, show in the bottom row, does not change. b, stromal cells were infected with empty Ad (4 MOI, represented as “0” Ad.caMEK on the x-axis) or the indicated doses of Ad.caMEK, and total RNA was assayed for eNOS levels 24 h later, eNOS rose significantly in the presence of activated MEK (the asterisks show significance at p < 0.05). c, RANKL was examined in the same samples as for b showing a dose-dependent increase in RANKL expression in the presence of the Ad.caMEK viral infection. An asterisk shows significance at p < 0.01. d, stromal cells infected with increasing MOI of Ad.caMEK showed a dose-dependent increase in NO production as assayed in the media using the sensitive nitrite assay. Empty adenovirus was used as control (there was no difference between nitrite in media of uninfected and Ad.empty-infected cells; data not shown). The asterisks show significant difference from Ad.empty at p < 0.05.
Our results do not rule out modulation of the ERK1/2 pathway by other signaling pathways that are known to be activated by mechanical factors. Shear stress, another relevant mechanical factor in the skeleton, is also known to activate ERK1/2 (42, 59) as well as nitric-oxide synthase (40, 60). Differences between these signaling pathways activated by shear and stress are subtle; for instance, both shear and strain activate ERK in osteoblast-like cells but through putatively divergent upstream pathways (18, 42). Recent papers underline the complexity of shear-induced signaling in osteoblasts in terms of proliferative and differentiative responses, including cyclooxygenase, G-proteins, and nitric-oxide synthases (61, 62). This level of complexity is almost certain to be present in the cellular response to mechanical strain. It is possible that many signaling pathways will impact on both the upstream and downstream pathways involving ERK1/2 that regulate eNOS and RANKL expression.

The mechanisms by which ERK1/2 regulates eNOS and RANKL gene expression in bone cells are currently unknown. In the case of endothelial cell eNOS, mechanical shear is known to transiently increase gene transcription, a process dependent on the Src-Ras-MEK-ERK cascade, as well as to invoke a non-ERK-dependent increase in eNOS mRNA stability (18). We were not able to measure strain induction of eNOS in the presence of ERK inhibition, suggesting that strain, at least at magnitudes relevant to skeletal physiology, may be effected largely through increases in eNOS transcription. In terms of RANKL, mechanistic control of this important transcript has so far eluded analysis. Whereas endogenous RANKL expression is acutely sensitive to osteotropic factors (15, 16), transient transfection of up to 7000 nucleotides of the promoter does not respond to agents known to effectively increase expression (64, 65). Understanding RANKL regulation will require novel techniques to probe endogenous gene activity.

Thus, we have shown that strain has a magnitude-specific ability to control two genes in entirely divergent fashions, one inhibitory and one stimulatory. For regulation of eNOS and RANKL, this ability relies on activation of the ERK1/2 signaling cascade, suggesting that ERK represents a common distal pathway where multiple mechanical signals converge. The difference in effect suggests that the final regulatory result is determined by recruitment of specific co-regulators. In total, in vitro strain represents a paradigm for understanding how loading the skeleton results in positive bone remodeling.

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