Cellular Responses to Mechanical Stress

Selected Contribution: Regulatory pathways involved in mechanical induction of c-fos gene expression in bone cells

M. A. PEAKE, L. M. COOLING, J. L. MAGNAY, P. B. M. THOMAS, AND A. J. EL HAJ
Centre for Science and Technology in Medicine, School of Postgraduate Medicine, Keele University, North Staffordshire Hospital, Stoke on Trent ST4 7QB, United Kingdom

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Peake, M. A., L. M. Cooling, J. L. Magnay, P. B. M. Thomas, and A. J. El Haj. Selected Contribution: Regulatory pathways involved in mechanical induction of c-fos gene expression in bone cells. J Appl Physiol 89: 2498–2507, 2000.—The regulatory pathways involved in the rapid response of the AP-1 transcription factor, c-fos, to mechanical load in human primary osteoblast-like (HOB) cells and the human MG-63 bone cell line were investigated using a four-point bending model. HOB and MG-63 cells showed upregulation of c-fos expression on fibronectin and collagen type I substrates; however, MG-63 cells did not respond on laminin YIGSR substrates. Addition of cytochalasin D and Arg-Gly-Asp peptides during loading did not inhibit the response, whereas addition of b1-integrin antibodies inhibited the load response. The role of Ca2+ signaling has been demonstrated by blocking upregulation with addition of 2 mM EGTA, which chelates extracellular Ca2+, and gadolinium (10 μM), which inhibits stretch-activated channels. Addition of the Ca2+ ionophore A-23187 induced upregulation without loading; however, addition of nifedipine (10 μM), the L-type channel blocker, failed to prevent the load response. Inhibitors of downstream pathways indicated the involvement of protein kinase C. Our results demonstrate a key involvement of Ca2+ signaling pathways and integrin binding in the c-fos response to mechanical strain.

secondary messenger; mechanical loading; gene regulation; calcium channels; integrins

When the skeleton is subjected to mechanical forces, bone cells, in particular osteoblasts and osteocytes, have been shown to respond by releasing signaling molecules or by directly increasing bone formation and remodeling (29, 45, 50). The load-induced bone synthesis is linked to the fact that, as in many cell types, osteoblasts and osteocytes are able to induce a “load response” when subjected to even low levels of mechanical deformation. A rapid upregulation in expression of the protooncogene c-fos in response to the application of mechanical force has been documented in a number of cell types, including cardiac (54), muscle (9), and endothelial cells (1) in addition to both osteocytes (26, 27) and osteoblasts (49). In each of these cases, the upregulation of c-fos transcription has been shown to be an early member of a larger “cascade” of mechanically stimulated transcriptional responses, which may ultimately result in load-related remodeling of the matrix. Recent evidence from Moalli et al. (36) suggests that, in an actively osteogenic/remodeling in vivo environment, load induction of c-fos occurs in a biphasic manner and preempts the induction of collagen I (and alkaline phosphatase) synthesis important for de novo bone formation.

A number of intracellular signaling pathways have been implicated as playing important roles at various stages of the cellular load response (11, 13). A study of these pathways has lead to the search for a “mechano sensor” capable of receiving the mechanical physical stimulus and converting it into a biochemical signal, which regulates mRNA and protein expression and ultimately tissue growth and adaptation. Two potential components of an overall mechanosensor are membrane ion channels, such as mechanosensitive ion channels (38), and integrins, based on the tensegrity model (4). The load-induced increase in mRNA levels of the early response gene c-fos is both rapid and short lived, which presents a good model for identifying key early mechanosensors involved in early gene transcription.

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The adhesion of bone cells to the extracellular matrix (ECM) of bone is mediated through short amino acid motifs located at cell binding domains located within a number of ECM proteins, such as the Arg-Gly-Asp (RGD) tripeptide motif, which is present in fibronectin, collagen type I, and laminin (52). Immobilization of peptides containing this short motif has been shown to be sufficient to facilitate bone cell adhesion, cell spreading, and focal adhesion formation on nonadhesive synthetic polymers (35). The addition of soluble RGD peptides during cell culture has been shown to prevent adhesion to certain substrates, e.g., osteopontin and vitronectin, and to reduce adhesion to substrates such as fibronectin and collagen (20). Cell attachment, however, is not always mediated through the RGD peptide, as other peptides are also able to mediate adhesion; for example, attachment to both laminin and fibronectin can involve multiple additional sites, including the YIGSR (34) and HepII (60) motifs, respectively. Laminin YIGSR mediates cellular attachment independently of integrins through a 67-kDa receptor protein (18) shown to be involved in the shear stress-induced upregulation of endothelial nitric oxide synthase in endothelial cells (16).

The integrin family of heterodimeric cell surface receptors is known to mediate cell adhesion to specific components of the ECM (7, 24, 51). The attachments between integrins and signaling molecules, e.g., focal adhesion kinase, in focal adhesion complexes (51), in addition to their direct connection to the cytoskeleton have led to the postulation of a possible central role in the transduction of mechanical stimuli into biochemical signals (57). Combinations of the multiple α-integrin (17) and β-integrin (9) subunits so far identified allow the production of a large number of specific ligand-binding integrin receptors (7). The observation of a low degree of homology between the cytoplasmic domains of these integrin subunits led Juliano and Haskell (24) to postulate that various α/β combinations (and/or splice variants) might transduce different signals from the ECM to the cell interior. The tensegrity model (4) suggests that the cytoskeleton functions to focus mechanical forces on specific signaling molecules involved in mechanotransduction, with the application of mechanical force resulting in changes in the molecular mechanics. Studies in a variety of cell types have shown that the direct application of force to integrins (or subunits) results in increased cytoskeletal tension (63), increases in intracellular free Ca\(^{2+}\) (44), induction of mitogen-activated protein kinases, and induction of tyrosine kinase phosphorylation (57). In bone cells, modulation of integrin-mediated attachments has been shown to reduce or inhibit mechanical stimulation of DNA synthesis (64) and changes in membrane potential (56). However, these studies have not investigated the integrin-mediated link to early gene transcription.

Membrane ion channels have been proposed as one of the early critical activation steps in response to mechanical loading in a number of cell types (11, 13, 53, 61, 62). Both voltage-activated and mechano-activated Ca\(^{2+}\) channels have attracted particular interest due to the role of Ca\(^{2+}\) as a principal secondary messenger. The role of Ca\(^{2+}\) signaling in the load response has been widely noted, with increases in intracellular free Ca\(^{2+}\) found to be due to either release of intracellular Ca\(^{2+}\) stores or opening of Ca\(^{2+}\) channels in the plasma membrane (23, 62). Such increases in intracellular Ca\(^{2+}\) have been experimentally induced by short periods of cyclical mechanical loading of rat periosteal-derived osteoblasts (62). Ca\(^{2+}\) influx through a specific voltage-operated (L-type) Ca\(^{2+}\) channel has been demonstrated to be involved in the upregulation of the bone-matrix protein osteopontin (62). This study investigates the role of Ca\(^{2+}\) signaling in the upregulation of the early mechanical response gene c-fos.

In this study, we investigate regulatory pathways involved in c-fos induction in response to mechanical loading of bone cells grown to confluence on varying substrates. This model system will be used to test the hypothesis that the c-fos response is mediated via a variety of cell-matrix interactions and/or membrane ion channel-mediated signaling that converge on critical downstream regulatory pathways to initiate increased c-fos gene expression. With the rapid response time of this transcription factor, we can modulate the activity of cellular pathways using specific inhibitors and agonists to determine their requirement for initiating the load response in gene transcription within 1 h after loading.

**MATERIALS AND METHODS**

**Cell culture.** Human primary osteoblast-like (HOB) cells and MG-63 human osteosarcoma cells (3) were cultured in minimum essential medium (MEM α-modification; Sigma-Aldrich) supplemented with 10% FCS (Sigma-Aldrich) and 1% dilution of 100× antibiotic-antimycotic solution (Sigma-Aldrich) under normal culture conditions. HOB cells were prepared from small bone fragments washed several times in PBS and α-MEM supplemented with a 2% dilution of 100× antibiotic-antimycotic solution (Sigma-Aldrich). These bone fragments were then placed in 25-cm\(^2\) tissue culture flasks (Sarstedt). Outgrowing osteoblast-like cells were grown over two passages and were then trypsinized onto coated coverslips. All primary culture studies were carried out on first- or second-passage primary cultures. Cells of the HOB osteosarcoma cell line MG-63 (3) were also placed in α-MEM supplemented with 10% FCS and 1% dilution of 100× antibiotic-antimycotic solution. Confluent cultures were treated with 1× trypsin-EDTA (Sigma-Aldrich) and passaged onto coated coverslips before loading.

**Coverslip-coating protocol.** The central 24 × 30-mm region of 24 × 50-mm coverslips (thickness 2; Philip Harris) was separately coated by adsorption of ECM proteins at room temperature for 2–4 h. Collagen type I (Sigma Chemical), fibronectin (Sigma Chemical), and recombinant laminin YIGSR fragment (Sigma Chemical) were coated at final concentrations of 7.5 μg/cm\(^2\), 2.5 μg/cm\(^2\), and 2 μg/cm\(^2\), respectively. HOB and MG-63 cells were subcultured onto coated coverslips in 500-μl droplet cultures containing 2 × 10\(^5\) and 7.5 × 10\(^5\) cells, respectively. Droplet cultures were placed on the coated portion of coverslips, and cells were allowed to attach overnight. Subsequently, coverslip cultures were submerged in supplemented α-MEM and cultured until confluence was reached, and loading was carried out (7–10 days for HOB cells and 2 days for MG-63 cells).
Loading protocol. Twenty-four hours before loading, coverslip cultures were placed in a four-point bending apparatus and submerged in 5 ml of c-MEM supplemented with 2% FCS and 1× AB. Coverslips cultures were then mechanically loaded at a strain of ~1,000 microstrains (μstrains). Loading was applied homogeneously across the coverslip culture area at a 1-Hz frequency for 1,800 cycles, equivalent to 0.5 h. Control coverslip cultures were prepared in parallel using an identical procedure, with the exception of not undergoing loading. Loaded and parallel unloaded coverslip cultures were harvested and lysed in modified guanidium thiocyanate solution 1 h after the completion of loading, and total RNA was extracted using a modification of the guanidium thiocyanate method (5) as described by Ghu et al. (15). Total RNA was quantified by spectrophotometric analysis of the absorbance at 260 nm.

Four-point bending model. The four-point loading system was used to experimentally apply cyclical load to four coverslips coated with substrates and seeded with either human primary or osteosarcoma bone cells. Once the apparatus was assembled, the piston was connected to a pneumatic switching system, which in turn was attached to a pressurized gas N2 cylinder. This switching mechanism worked first by opening to allow the compressed gas through the tube connected to the pneumatic piston and then by closing to release the pressure on the load-inducing piston. This open-close switching occurred at a frequency of 1 Hz. To control the force applied to the coverslips, the pressure of gas was modulated and the thickness of the coverslip was controlled. For coverslips (thickness no. 2, 24 × 50 mm), a pressure of ~1.75 bar was used to cause the maximum amount of coverslip deformation allowed by the apparatus, resulting in the maximum possible loading of the coverslips. Repeat experiments resulted in a load of ~1,000 μstrain applied consistently over the 0.5-h period. Calibration of the load applied onto the coverslips was conducted mathematically with the use of the methods detailed by Gere and Timoshenko (14) according to the equation ε = td/a(L − 1.33a) where ε is strain, t is coverslip thickness, d is deflection, a is distance between two inner loading points, and L is distance between two outer loading parts. Strain gauge measurements were also conducted. In both cases, the levels of uniaxial strain was measured at ~1,000 μstrain.

RT-PCR analysis. Total RNA (3 μg) from each control and loaded coverslip culture was used as a template for reverse transcription with 1 μl (200 units) Superscript II reverse transcriptase (Life Technologies) in a total volume of 20 μl using random hexamer oligonucleotide primers (Life Technologies) to a final concentration of 12.5 ng/μl. Initial mRNA denaturation at 70°C for 10 min preceded preincubation at 25°C for 10 min followed by incubation and reverse transcription at 42°C for 50 min. After reverse transcriptase inactivation at 70°C for 15 min, 1 μl of the reaction products from each sample was used as a PCR template. PCR reactions were carried out using primer pairs specific for human c-fos, Cbfa1, and hypoxanthine phosphoribosyltransferase (HPRT; control) cDNA, respectively (see Table 1 for sequence information). All PCR reactions involved an initial denaturation step of one cycle at 95°C for 1 min followed by the specific conditions for each reaction (detailed in Table 1). The cycle number in each case, in conjunction with real-time RT-PCR studies, was optimized to examine the relative expression of each of the RT-PCR products within their linear range of amplification. Each reaction was completed by a final cycle of amplification with primer annealing for 2 min at the same specific temperature, followed by extension for 2 min at 72°C. In each case, amplification was carried out using 0.5 units of Taq polymerase (Promega), and all primers were used at a 0.5 μM final concentration. The forward and reverse primers used in each of these PCR experiments are detailed in Table 1.

After completion of the PCR, a 5-μl sample of the reaction products, obtained from both sets of reactions, was mixed with 2 μl of DNA loading buffer and separately loaded onto a single gel of 1% agarose in 1× Tris-borate EDTA and separated by electrophoresis at 100 V for 60 min. The resulting gel was then visualized under ultraviolet illumination, and images (see RESULTS) were captured with a digital camera-based gel documentation system (Imagestore 5000, UVP).

Treatment of coverslip cultures with agonists/antagonists. Experiments involving addition of specific agents were carried out on HOB and MG-63 coverslip cultures grown on collagen type I coverslips, coated as detailed in Coverslip-coating protocol. Chemical reagents were added to the media of control and loaded coverslip cultures 30 min before loading unless otherwise stated. Ca2+ signaling modulators EGTA (2 mM) and gadolinium III chloride (10 μM) were added 2 h before loading as was nifedipine (10 μM) in addition to a separate 30-min preload time point. The protein kinase inhibitor H7-dihydrochloride (50 μM), indomethacin (1 μM),

Table 1. Sequences of the primers and reaction conditions used for amplification of the genes, c-fos, Cbfa1, and HPRT

<table>
<thead>
<tr>
<th>PCR Primer Sequences</th>
<th>Denaturing</th>
<th>Primer Annealing</th>
<th>Extension</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-fos</td>
<td>45 s at 95°C</td>
<td>45 s at 55°C</td>
<td>45 s at 72°C</td>
<td>40</td>
</tr>
<tr>
<td>Forward primer:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-TCT-CTT-ACC-ACC-ACC-CC-3'</td>
<td></td>
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<tr>
<td>Reverse primer:</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>5'-AGT-GTA-TCA-GTG-AGC-TC-3'</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HPRT</td>
<td>45 s at 95°C</td>
<td>45 s at 55°C</td>
<td>45 s at 72°C</td>
<td>40</td>
</tr>
<tr>
<td>Forward primer:</td>
<td></td>
<td></td>
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<tr>
<td>5'-TCT-TCT-CTT-TCT-TCT-TCT-3'</td>
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<td>Reverse primer:</td>
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<tr>
<td>5'-GCC-GACC-GAC-AAA-GCA-AAG-TC-3'</td>
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<tr>
<td>Cbfa1</td>
<td>45 s at 95°C</td>
<td>45 s at 63°C</td>
<td>45 s at 72°C</td>
<td>35</td>
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<td>Forward primer:</td>
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<tr>
<td>5'-GCC-CAC-GAC-CCT-ACC-CC-AT-3'</td>
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<td>Reverse primer:</td>
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<td>5'-GCC-TCC-GCC-CAC-ATC-TC-3'</td>
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HPRT, hypoxanthine phosphoribosyltransferase.
and β1-antibody (1 μg/ml) (Chemicon International) were added 30 min before loading. Cytochalasin D (1 μM) was separately added 30 min and 2 h before loading. The Ca\(^{2+}\) ionophore A-23187 (Sigma-Aldrich) was dissolved in DMSO (10 mM) and added at a concentration of 10 μM to unloaded coverslip cultures only, with total RNA being extracted 1 h later. Parallel control coverslip cultures were treated with DMSO (1:1,000 dilution) vehicle only and were also extracted 1 h later. RGD peptide experiments were carried out with trypanized cultures of HOB and MG-63 cells preincubated for 30 min in 4 ml of α-MEM solution supplemented with 100 μg/ml GRGDS and 100 μg/ml GRGDNP peptides (Bachem) before attachment to collagen- and fibronectin-coated coverslips, respectively. These RGD peptides were also added at a 100 μg/ml concentration to the culture medium of these coverslips during attachment and proliferation and also before and during loading.

RESULTS

The early response gene, c-fos, was not detectable in control cultures of MG-63 and primary bone cells grown on collagen type I substrates using RT-PCR analysis (Fig. 1). In contrast, RT-PCR analysis of confluent primary bone cells and MG-63 cells grown on collagen type I substrates after the application of four-point bending of ~1,000 μstrain for 30 min (at a 1-Hz frequency) consistently showed amplification of a single 417-bp fragment (Fig. 1). This product was of the size expected for human c-fos cDNA, and subsequent sequence analysis confirmed this band to be amplified c-fos cDNA (data not shown). Analysis was carried out 1 h after loading. Parallel RT-PCR analysis was carried out with primers specific for the housekeeping gene HPRT; this led to the production of a 270-bp product shown by sequence analysis to be amplified HPRT cDNA (Figs. 1–5). In all samples of control, treated, and loaded samples, levels of the housekeeping gene HPRT were observed.

Although the loading experiments were carried out in reduced-serum conditions (serum starved at 2% FCS for 24 h before loading), c-fos upregulation was not found to be serum independent because an identical response was also observed in cultures of MG-63 cells incubated in serum-free medium 24 h before loading and during loading (Fig. 1B).

To define the phenotype of the primary human bone cultures and also to assess the effect of mechanical loading on osteoblast maturation, RT-PCR analysis was also carried out with primers specific for the osteoblast maturation-linked transcription factor, Cbfa1. As can be seen in Fig. 1A, a single 289-bp band was observed of the size expected for Cbfa1 cDNA, indicating that HOB cells grown to confluence on collagen type I-coated substrates with and without loading had maintained their osteoblastic phenotype. Identical results were observed in parallel MG-63 experiments (data not shown). PCR products for Cbfa1 are present in control and mechanically loaded cultures within 1 h after loading. Further confirmation of the osteoblastic nature of the human primary cultures came from the amplification of collagen I, osteocalcin, and osteopontin cDNAs using specific primers (data not shown).

Changes in the load-induced c-fos response between confluent cultures grown on different substrates were observed in MG-63 cells but not in human primary cells. Human bone cell primary and MG-63 cultures grown on fibronectin substrate showed a response to cyclical loading similar to that observed on collagen type I-coated substrates (Fig. 2). However, mechanical loading of MG-63 cells grown on the RGD-free, laminin YIGSR fragment failed to induce a c-fos response (Fig. 2B). This would appear to suggest that, although the laminin YIGSR fragment is sufficient to allow cell attachment, such attachments are not able to mediate the transfer of mechanical forces sufficient to induce a measurable c-fos response. In contrast to the situation in MG-63 cells, culture and loading of human primary bone cells grown to confluence on laminin YIGSR-coated coverslips resulted in a c-fos load response similar to that seen on both RGD-containing substrates (Fig. 2A). Such a result may in part be due to the
increased time of culture on coverslips required for the growth of confluent human primary cultures, with potentially an increased degree of breakdown of the lammin substrate and sequestration of ECM proteins present as components of serum during culture to confluence.

Integrin and cytoskeletal interactions. Subsequent experiments concentrated specifically on the role of RGD-integrin interactions, with the use of supplementary RGD peptides placed in the medium of cultures of human primary and MG-63 cells throughout the processes of cell attachment, proliferation, and mechanical loading on collagen type I and fibronectin substrates. As can be seen in Fig. 3A, pretreatment of human primary bone cells grown on collagen type I-coated coverslip with cytochalasin D failed to prevent the c-fos load response. No changes in this response were detected when cytochalasin D was added to the culture medium at varying time points before loading (data not shown).

Ca\(^{2+}\) signaling and c-fos induction. To investigate further the potential role of Ca\(^{2+}\) signaling in the c-fos response, specific modulators of Ca\(^{2+}\) membrane channels and the availability of extracellular Ca\(^{2+}\) were utilized on cultures of human primary bone cells grown on collagen type I substrates only. A similar pattern increased time of culture on coverslips required for the growth of confluent human primary cultures, with potentially an increased degree of breakdown of the lammin substrate and sequestration of ECM proteins present as components of serum during culture to confluence.

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In addition, human primary bone cells cultured on collagen substrates were incubated with anti-β1-integrin antibodies before and during mechanical loading, which inhibited the c-fos load response. c-fos cDNA was not detected in loaded or control cultures of human bone primaries after incubation with the antibody (Fig. 3B). The role of the cytoskeleton in mechanotransduction and hence the importance of maintaining cytoskeletal integrity to enable the load response were tested by the application of the cytoskeletal disrupter cytochalasin D (1 μM) to cell cultures grown on collagen type I substrates. As can be seen in Fig. 3B, pretreatment of human primary bone cells grown on collagen type I-coated coverslip with cytochalasin D failed to prevent the c-fos load response. No changes in this response were detected when cytochalasin D was added to the culture medium at varying time points before loading (data not shown).

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was observed in both cell types. Addition of the Ca\(^{2+}\)-chelating agent EGTA at 2 mM was sufficient to reduce c-fos expression in loaded human primary bone cell collagen cultures to undetectable levels (Fig. 4). To block the activity of the L-type Ca\(^{2+}\) channel, human bone primary cultures were treated with a 10 \(\mu\)M concentration of nifedipine. The levels of c-fos in response to loading were not reduced when exposed to nifedipine at this concentration and did not vary if nifedipine was added at varying time points before loading (data not shown). In contrast to the lack of inhibition observed in response to nifedipine treatment, incubation with 10 \(\mu\)M concentration of the stretch-activated Ca\(^{2+}\) channel blocker gadolinium before and during loading was sufficient to block the c-fos load response (Fig. 4). Further evidence of the importance of the role of Ca\(^{2+}\) influx in the upregulation of c-fos comes from treatment of nonloaded control cultures with the Ca\(^{2+}\) ionophore A-23187. The addition of the Ca\(^{2+}\) ionophore A-23187 to cultures of human primary bone cells grown to confluence on collagen type I-coated coverslips was sufficient to result in a pattern of c-fos upregulation equivalent to that seen in response to mechanical loading (Fig. 4). This upregulation in c-fos expression, which occurred 1 h after treatment, was absent in parallel control cultures that were treated with DMSO (vehicle) only (Fig. 4).

**DISCUSSION**

This study has examined the potential role of integrin-mediated cell-substrate interactions and Ca\(^{2+}\) signaling in the mechanostimulation of the early response gene c-fos. Although the role of c-fos in the mechanical load response of bone has not been fully characterized, expression of this AP-1 transcription factor subunit has been shown to be important for normal skeletal development (19) and may have a role in mechanotransduction through modulation of the late load-response genes through an as yet undefined transcriptional cascade mechanism.

**Fig. 5.** Effects of downstream signal pathway inhibitors on mechanical load-induced c-fos upregulation in human bone cells. Human primary bone cell cultures grown to confluence on collagen type I-coated coverslips were loaded at \(-1,000\ \mu\)strain for 1,800 cycles of 1 Hz. Parallel untreated control cultures were also treated with the Ca\(^{2+}\) ionophore A-23187 (10 \(\mu\)M) and DMSO (vehicle) 1 h before cells were harvested. Controls included untreated and DMSO vehicle only. Total RNA from all loaded and control cultures was analyzed by RT-PCR using primer pairs for c-fos and HPRT (control). Each sample lane represents a single sample taken from a set of 4 replicate samples used in each of a set of 2 or more duplicate experiments.
MacKenna et al. (33) observed that, in cardiac fibroblasts and not on laminin and elastin. In addition, the c-fos response is facilitated by collagen and fibronectin sub-laid down matrices. Varying ECM substrates have therefore been the result of interactions with these newly laid down matrices. The elongated growth time will potentially result in more matrix being laid down by the cells as well as potential turnover of the substrate. The c-fos response could therefore be the result of interactions with these newly laid down matrices. Varying ECM substrates have been shown to have potential differential effects on other load responses. Wilson et al. (64) showed that, in vascular smooth muscle cells, load-induced DNA synthesis is facilitated by collagen and fibronectin substrates and not on laminin and elastin. In addition, MacKenna et al. (33) observed that, in cardiac fibroblasts, differential load-induced activation of extracellular signal-regulated kinase and c-Jun NH$_2$-terminal kinase (JNK1) occurred on collagen, fibronectin, laminin, and vitronectin substrates.

To further explore the importance of the RGD-binding mechanism in the load response, we have used soluble RGD peptides to bind to the available sites on the membrane, before and during loading. In this way, we can identify whether alternate binding peptide motifs are still capable of initiating the cellular response to load. Our results have shown that extended blocking of the RGD cell-matrix binding sites for 2 or 7–10 days (for MG-63 and primary cells, respectively) was not sufficient to block c-fos gene expression in either cell type investigated. This may indicate that, in the absence of RGD-mediated integrin interactions, RGD-independent integrin (and/or nonintegrin)-mediated interactions are sufficient to activate the mechanical load responses of this nature. The exact nature of these interactions is not clear; however, candidates may include α3β1, α9β1, and αvβ1-integrin-mediated interactions, which variously interact with collagen I, fibronectin, and laminin in an RGD-independent manner (30).

In primary bone cells, our results indicate that β1-integrin-mediated interactions may be essential for the c-fos load response, as the presence of the anti-β1-antibody completely blocks this response in our system. In MG-63 cells, the fact that the RGD peptides do not inhibit the c-fos response and show a lack of response on the YIGSR fragment provides further support for the non-RGD-mediated β1-integrin, such as LDVP in fibronectin (39), GD-2 in laminin (42), or GFOGER in collagen type I (28), as a potential mechanism for strain transduction across the membrane. Although load transduction may occur through an RGD-mediated pathway, it may not be essential, as other non-RGD-mediated pathways are available. The β1-integrin link, however, appears to be critical.

A similar lack of RGD dependency was also observed by MacKenna et al. (33), who showed that load-induced activation of JNK1 could not be inhibited by RGD peptides, indicating that an RGD-independent integrin interaction was involved. In contrast, Salter et al. (56) showed that short-term (30 min) blocking of RGD binding at a similar peptide concentration could reduce or prevent changes in the membrane potential generated in response to load. One possible explanation is that the response may vary according to the length of incubation with the peptides. The long-term blocking of RGD-cell contacts, as detailed above, allowed sufficient time for cells to establish additional cell-substrate contacts able to support the mechanochonduction of c-fos.

In our investigations, however, we have shown the importance of the integrin-cytoskeletal signaling pathway. This study demonstrates that, by blocking the β1-integrin function through incubation with specific antibodies, we can inhibit the upregulation of c-fos in response to load in human primary bone cells. This may indicate that stretching of the membrane on the surface of the substrate via β1-integrin-mediated binding is sufficient to switch on the mechanosensitive channels and promote an influx of intracellular Ca$^{2+}$ leading to c-fos upregulation. It is possible that these pathways are working in parallel to control a cascade of genes responding to the load. However, the role of integrins in mediating such load responses may not be universal; Nebe et al. (40) demonstrated that, in a hepatocyte cell line, shear stress-induced increases in intracellular Ca$^{2+}$ concentration were not blocked after incubation with anti-integrin antibodies. Cytochalasin D treatment of bone cell cultures, in this study, failed to prevent a c-fos response, indicating that upregulation of the gene may be triggered independently of cytoskeletal integrity. Cytoskeletal integrity is also inessential for stretch activation of c-fos in cardiac myocytes, as shown by cytochalasin treatment (55). In the case of shear stress, however, the stress-induced expression of c-fos in the MC3T3-E1 osteoblast cell line was inhibited by cytochalasin D treatment and other modulators of actin-integrin interactions (43).

Our previous studies and those of other groups have indicated a key role for Ca$^{2+}$ in mechanotransduction (8, 11, 13, 22). In this study, we go further to investigate the role of these Ca$^{2+}$-mediated pathways in the c-fos upregulation in response to load. The secondary messenger pathways involved in the regulation of c-fos have been studied for many years in a wide range of cell types (21, 31, 55), and the role of ion fluxes in the regulation and stimulation of c-fos has long been recognized (37). The influx of Ca$^{2+}$ in particular has been heavily implicated in the regulation of c-fos, supported by the discovery of a consensus cAMP/Ca$^{2+}$ response element within the c-fos promoter (58). In fact, the influx of Ca$^{2+}$ has been shown to directly regulate the expression of c-fos mRNA (37, 58), first through an increased initiation of c-fos transcription and second...
through the regulation of c-fos transcript elongation (31). We have shown that activation of stretch-sensitive channels, which in bone cells have been previously demonstrated to be Ca\textsuperscript{2+} mediated (8), along with a requirement for extracellular Ca\textsuperscript{2+}, is essential for the c-fos load response. Although treatment with nifedipine, the L-channel Ca\textsuperscript{2+} blocker, alone is not sufficient to block the response, c-fos upregulation is dependant on the presence of extracellular Ca\textsuperscript{2+} in the medium and is blocked by the addition of gadolinium. The gadolinium ion is of a similar size to that of Ca\textsuperscript{2+} and is known to interact with stretch-activated channels, which show increased permeability to Ca\textsuperscript{2+} following mechanical deformation (65). This interaction has been shown to directly and selectively inhibit mechanically induced influxes of extracellular Ca\textsuperscript{2+} in a number of cell types (2, 32, 59), although the exact nature and susceptibility of such stretch-activated channels are known to vary between cell types (32, 55). Stretch-activated cation (SA-cat) channels of the type that appear to be involved in this c-fos response have been identified in both rat (10, 66) and human (8) osteoblast-like cells and osteocytes (66). Intermittent mechanical stretch has been shown to increase the activity of such SA-cat channels in both rat and human osteoblast-like cells (8, 10), and modulation of SA-cat channel activity with gadolinium is also known to abolish or reduce a number of load responses in osteoblasts and osteocytes (48). Similar gadolinium-modulated load responses were noted by Glogauer et al. (17), who showed that, in fibroblasts, Ca\textsuperscript{2+} influxes induced by manipulation of collagen-coated magnetic beads increased with increasing force application and were reduced/prevented by treatment with gadolinium chloride. In our study, further support for the role of Ca\textsuperscript{2+} signaling in the c-fos gene upregulation was provided by the upregulation of c-fos expression in the absence of loading following treatment of cultures with a Ca\textsuperscript{2+} agonist. A number of downstream signaling pathways that follow the initial Ca\textsuperscript{2+} and integrin-mediated mechano-activation mechanisms have been implicated in the cascade. Protein kinase C activation has been linked to the upregulation of c-fos in response to mechanical load (25). Our results demonstrating abolition of the c-fos response after treatment with H\textsubscript{7}-dihydrochloride would appear to confirm this and would agree with previous findings of a significant reduction in shear stress-induced c-fos induction after H\textsubscript{7} treatment of endothelial cells (46). Other blockers of protein kinase C, such as calphostin C, have been shown to significantly inhibit stretch-induced increases in c-fos mRNA expression in cardiac myocytes (25). PGE\textsubscript{2} has also been implicated in the load transduction cascade (6, 12, 27, 47). However, this may not be true for all cell types; Ogata (41) previously showed that, in osteoblast-like MC3T3-E1 cells, load-induced upregulation of the early response gene EGR-1 is not prevented by treatment with indomethacin. The failure of indomethacin treatment in our studies to block c-fos induction indicates that, in this case, PGE\textsubscript{2} also does not appear to play a role in the very rapid activation of this early response gene.

In conclusion, it is clear that rapid mechanotransducers, capable of responding within milliseconds, promote the induction of c-fos within 1 h of applied mechanical load. This activation of mechanotransducers may involve stretch or volume activation, which is followed by initiation of a series of downstream signaling pathways. The role of RGD-mediated interactions in the c-fos response in bone cells does not appear to be essential, although it may be that not all matrix binding peptides are capable of facilitating the load response. The role of Ca\textsuperscript{2+} signaling appears to be important in c-fos gene regulation in load-related responses and in particular the influx of extracellular Ca\textsuperscript{2+} through stretch-activated ion channels is critical. Although clearly the AP-1 transcription factor is one of the steps in the load cascade, further work should identify whether expression of the gene is a critical step for downstream activation of bone matrix protein genes. One of the potential functions of load-related activation of multiple cellular pathways may be to initiate independent activation of a series of genes in response to mechanical load.

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