Signal Transduction and Mechanical Stress

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Bone undergoes a constant process of remodeling in which mass is retained or lost in response to the relative activity of osteoblasts and osteoclasts. Weight-bearing exercise—which is critical for retaining skeletal integrity—promotes osteoblast function, whereas a lack of mechanical stimulation, as seen during spaceflight or prolonged bed rest, can lead to osteoporosis. Thus, understanding mechanotransduction at the cellular level is key to understanding basic bone biology and devising new treatments for osteoporosis. Various mechanical stimuli have been studied as in vitro model systems and have been shown to act through numerous signaling pathways to promote osteoblast activity. Here, we examine the various types of stress and the sequential response of transduction pathways that result in changes in gene expression and the ensuing proliferation of osteoblasts.

Introduction

Healthy bone is in a constant state of remodeling, with the osteoclast breaking down bone matrix and the osteoblast forming new bone. The mechanisms underlying osteoporosis are still unknown (*1*). However, in postmenopausal women, bone loss occurs as a result of an increase in the rate of remodeling—that is, an imbalance between the activities of the osteoclast and osteoblast (*1*). Exercise is essential for maintaining skeletal integrity and bone mass. Suppression of this stimulus under conditions such as long-term bed rest or spaceflight can result in osteoporosis (*2*, *3*). Experiments in growing rats indicate that spaceflight osteoporosis occurs primarily through loss of osteoblast function (*4*), whereas exercise causes the skeleton to initiate adaptive responses (*5*, *6*) through intracellular signaling and gene induction, which leads to increased osteoblast activity. Because mechanical stimuli regulate various osteoblastic physiological functions, including gene induction, protein synthesis, proliferation, and differentiation, understanding mechanotransduction at the cellular level is key to understanding basic bone biology and devising new treatments for osteoporosis.

Mechanotransduction is the process of translating mechanical force on a cell into a biological response. Over the past decade, in vitro studies have indicated that mechanotransduction involves the extracellular matrix, integrins, signals from the cytoskeleton, calcium channels, guanosine triphosphatases (GTPases), adenylate cyclase, phospholipase C (PLC), and mitogen-activated protein kinases (MAPKs), all of which play important roles in early mechanical signaling (Fig. 1). The primary techniques used to study mechanotransduction in vitro have included fluid flow, four-point bending, and substrate stretch; however, some studies have used gravity force, vibration, magnetic bead twisting, atomic force, or shockwaves.

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Mechanical stress is the physical condition that exists within any cell as a result of strain or deformation by external forces and is expressed quantitatively as units of force per unit area. When stress is applied to a cell and it is stretched, linear strain is defined as the ratio of increase in length to original length (other forms of cell deformation resulting in more complex strains may also occur). Elastic modulus is the ratio of stress to strain in bone. The duration of experimental stress has ranged from seconds to days, with levels of strain ranging from 60 µstrain (µε) to 10,000 µε [strain from shear forces is calculated here according to the formulas described in (*7*) by assuming that the cells have a modulus of elasticity of 500 N/m^2 (*8*)]. Physiological levels of strain above the 5000-µε range can cause fractures or breaks in human bone, depending on the condition of the bone.

Osteoblast response depends on the type, duration, and level of stress. The various methods of introducing strain result in the osteoblast being mechanically stimulated; all methods were designed to replicate stress in the bone. The relevance of in vitro studies of mechanical stress in the osteoblast is that they are translatable to the whole animal. Rats given mechanical stress of 3000 µε to the tibia for 3 min had increased ratios of mineralizing surface to bone surface, mineral apposition rate, and bone formation rate on the endocortical surface in loaded tibias (*9*). In vitro, we see that with higher ranges of strain, calcium channels are probable signal transduction mediators. Li *et al.* (*9*) found this to be true in vivo: Animals treated with calcium channel blockers had up to a 61% decrease in bone formation factors after mechanical stress. In addition, we know that the Wnt pathway is important in mechanical stress, as shown in LPR5 mutant mice with abnormally high bone density in weight-bearing areas (*10*).

Here, we examine the various types of stress and the sequential response of transduction pathways that result in early changes in gene expression and the ensuing proliferation of osteoblasts.

Fluid Shear Stress

Fluid shear stress (FSS) is a technique that uses directed flow of fluid over a cell layer. Most investigators use FSS ranging from 0.7 Pa (460 µε) to 2.4 Pa (1600 µε) (*11–13*). Studies applying FSS of 800 µε demonstrated actin stress fiber formation and induction of mRNA for *cox-2* (cyclooxygenase-2) and the immediate early gene c-*fos* within an hour of application (*14*). c-*fos* (*15*, *16*), *cox-2*, and prostaglandin E_2 (PGE_2) (*17–19*) are needed for transition from G_1 to S phase of the cell cycle, and all are induced by growth factors or mechanical stress. This is also true in vivo; mechanically induced bone formation is preceded by expression of the transcription factor c-Fos and prostaglandin production in the whole animal (*20*). Induction of Cox-2 is key in bone formation in vivo; Forwood (*21*) showed that animals with stressed tibias (65 N) had increased bone formation,

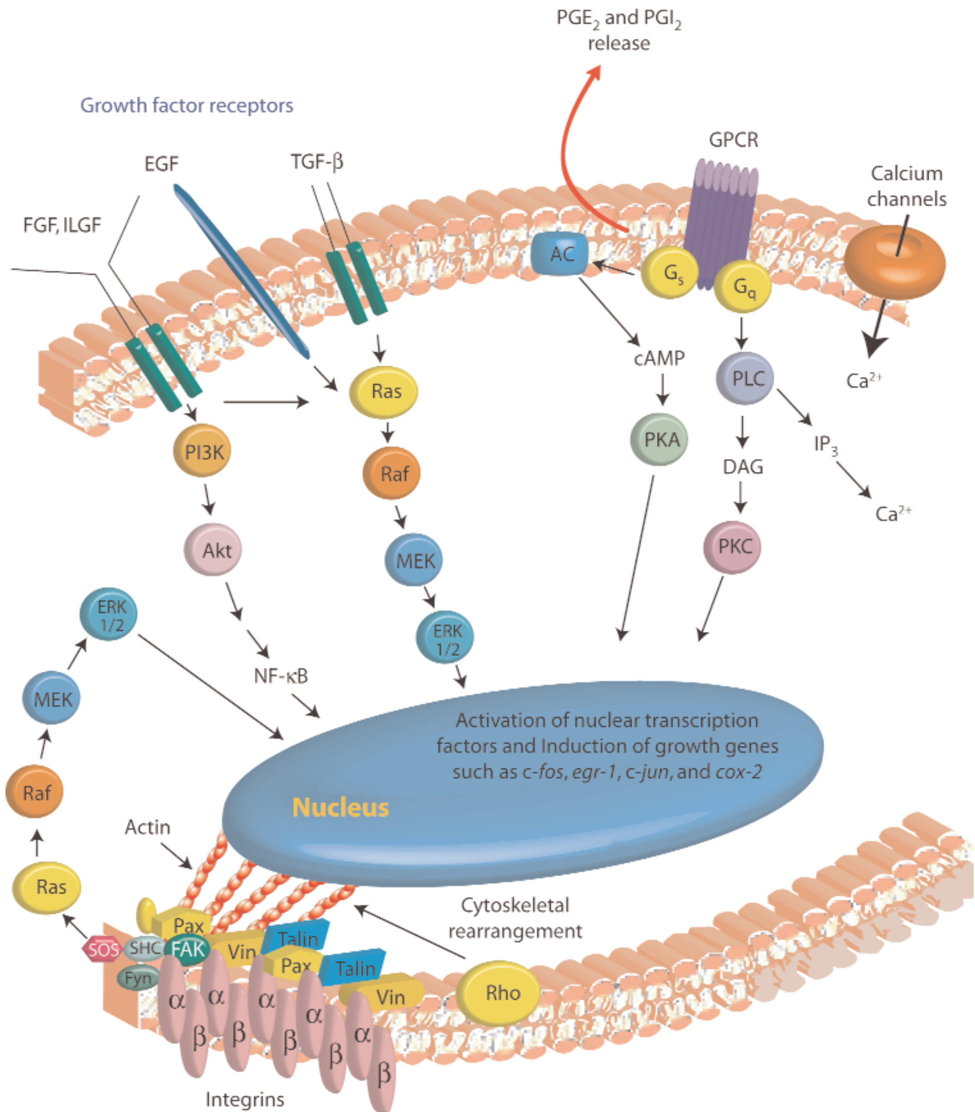

Fig. 1. Schematic diagram of the pathways that work together in the osteoblast mechanotransduction response. The upper membrane shows relevant growth factor receptors and subsequent pathways that are activated. FGF and ILGFs act through receptor tyrosine kinases to activate PI3K (phosphoinositide 3-kinase) and Akt (protein kinase B), causing release of NF-κB and its translocation to the nucleus. Ras is activated by growth factor receptors, seven-transmembrane-domain G protein–coupled receptors, and interaction with the integrins through the adaptor protein SHC and the guanine nucleotide exchange factor SOS. The pathway ends with phosphorylation of ERK-1 or ERK-2. Released PGE_2 and PGI_2 act through their seven-transmembrane–coupled receptors to activate the adenylate cyclase system. G proteins such as Gα have been shown to be directly activated by mechanical disturbance of the cell membrane, leading to PKA activation and subsequent phosphorylation of CREB transcription factor. Mechanical stress has been shown to activate PLC, causing activation of IP_3 and increases in intracellular calcium as well as activation of PKC. The growth factors FGF, TGF-β, and ILGF are all induced by mechanical stress, as is the receptor for EGF. On the lower membrane of the cell, the network including integrin and the cytoskeleton is activated by mechanical stress and causes binding of cytoskeletal elements such as talin, paxcillin (PAX), vinculin (VIN), or focal adhesion kinase (FAK), eventually leading to transcription factor activation. The GTPase Rho causes realignment of actin stress fibers during FSS and is associated with induction of gene expression. Together, these pathways work as a unit, resulting in full osteoblast activation.

whereas all mechanically induced bone formation was lost when animals were given a specific Cox-2 inhibitor ($P < 0.05$). Cox-2 transcript can be induced by various pathways implicated in mechanical stress response, including pathways involving protein kinase A (PKA), Akt, and Wnt (*10*, *22*, *23*). Application of cytochalasin D, which disrupts the actin cytoskeleton, or expression of a dominant negative form of the small GTPase Rho blocked formation of fluid shear–induced actin stress fibers and immediate early gene expression; this finding suggests that Rho-mediated formation of stress fibers bound to integrin in focal adhesions may promote the transcriptional activation of *cox-2* and c-*fos* (*14*). Integrins, as the main receptors that connect the cytoskeleton to the extracellular matrix (ECM), play an important role in transmitting mechanical stresses across the plasma membrane as stresses are applied to cells from the ECM. Because integrins also regulate signaling pathways (*24*), they are positioned to transduce physical forces into chemical signals. As seen in Fig. 1, mechanical stress applied to osteoblast ECM (and hence integrins) leads to signaling through a MAPK pathway that can cause an increase in the expression of immediate early genes such as *cox-2* and c-*fos*. Chen *et al*. applied a steady fluid flow of 800 με on MC3T3-E1 osteoblasts, causing substantial changes in actin stress fiber rearrangement and increases in c-*fos* and *cox-2* transcript (*25*). Inhibitors of calcium stretch or L-channels had no effect on gene activation. However, thapsigargin (which inhibits calcium adenosine triphosphatase,

an endoplasmic reticulum enzyme) completely blocked the increase in c-*fos* and *cox-2* expression, which suggests that calcium stores were involved in fluid shear mechanotransduction. Neomycin or U73122 were used to inhibit PLC; both drugs blocked actin cytoskeleton rearrangement as well as activation of inositol 1,4,5-trisphosphate (IP_3) and the consequent release of intracellular calcium. Taken together, these studies suggested that fluid flow initiates filamentous actin (F-actin) formation and immediate early gene activation through a PLC-IP_3–mediated pathway involving IP_3-dependent intracellular Ca^{2+} release (*25*) (Fig. 1). Intracellular Ca^{2+} release caused nuclear factor κB (NF-κB) translocation to the nucleus and stimulated *cox-2* gene expression during fluid shear (*13*). Inhibition of PLC or of intracellular calcium release, or blocking NF-κB translocation with nuclear localization signal (NLS) peptides, inhibited shear-induced up-regulation of *cox-2* (*13*). Neither cytochalasin D nor nocodazole altered NF-κB translocation, which suggests that neither an intact actin cytoskeleton nor functioning microtubules are required for this signaling event (*13*).

FSS was also shown to mediate increases in the intracellular concentration of IP_3. Shear stress of 24 dynes/cm^2 (1600 με) substantially increased IP_3 concentrations for up to 2 hours (*26*). Stopping flow resulted in a gradual return of IP_3 to basal concentrations. This increase in IP_3 concentration was partially inhibited by 20 μM ibuprofen and 14 μM indomethacin, indicating that the IP_3 response was partly dependent on flow-induced prostaglandin synthesis (*26*). Using a parallel-plate flow chamber and fura-2 fluorescence microscopy, Hung *et al*. (*12*) measured intracellular calcium cell by cell in preconfluent primary culture rat calvarial bone cells at 1200, 2333, and 4600 με of FFS; a dose-dependent relationship was observed between the number of responsive cells (responding >50% over basal levels) and shear-stress magnitude.

In other studies, fluid flow increased intracellular adenosine 3′,5′-monophosphate (cAMP) concentrations in cultured osteoblasts, an effect mediated through prostaglandin synthesis. Fluid flow caused an increase by factors of 9 (at 400 με) or 20 (at 1600 με) in the rate of PGE_2 production in osteoblasts, which suggests a role for prostaglandin and its receptors in mechanotransduction (*26*). Adenylate cyclase has also been implicated in mechanotransduction (*27*). In reductionist experiments, Frangos and colleagues (*28*) purified heterotrimeric guanine nucleotide binding proteins (G proteins) and reconstituted them into phospholipid vesicle liposomes (*28*). The vesicles were then loaded with [γ-^{32}P]GTP and subjected to physiological levels of fluid shear stress. Shear stress activated the G proteins in a dose-dependent manner over a range of 0 to 2000 με. When membrane fluidity was increased with the use of lysophosphatidylcholine, mechanical stress caused an increase in GTPase activity by a factor of 3 to 5. Conversely, incorporation of cholesterol into the membrane reduced the activation of G protein by shear. These results demonstrate the ability of the phospholipid bilayer to mediate shear stress–induced activation of GTPase and adenylate cyclase; they also show that the physical properties of the bilayer modulate mechanotransduction (*28*).

Wadhwa *et al*. (*22*), using MC3T3-E1 cells stably transfected with the *cox-2* promoter fused to a luciferase reporter, examined involvement of the PKA and protein kinase C (PKC) signaling responses to FSS at 666 με (*22*). Neither inhibition nor down-regulation of the PKC pathway affected the FSS stimulation of *cox-2* mRNA or luciferase activity (*22*). In contrast, inhibitors of the PKA pathway, used at doses that inhibited forskolin-stimulated luciferase activity by 70 to 80%, reduced FSS-stimulated *cox-2* mRNA expression and luciferase activity by 50 to 80%. Hence, this group found that peak FSS stimulation of *cox-2* expression in MC3T3-E1 osteoblastic cells is largely dependent on the PKA signaling pathway (*22*).

The extracellular signal-regulated kinase 1 and 2 (ERK 1/2) signaling pathway has been implicated in fluid shear induction of *cox-2*. Using 10 dynes/cm^2 (660 με) of shear flow, Wadhwa *et al*. found that within 30 min of initiating FSS, ERK 1/2 became activated, with phosphorylation peaking at 8 hours (*29*). Inhibiting ERK phosphorylation with the specific inhibitor PD98059 inhibited FSS induction of *cox-2* mRNA by 55 to 70% and FSS stimulation of luciferase activity by >80% in both MC3T3-E1 and primary osteoblast (POB) cells. The authors concluded that FSS transcriptionally activated *cox-2* gene expression in osteoblasts and that induction occurs largely through an ERK signaling pathway. In addition to the role of ERK 1/2, nitric oxide (NO) also plays an important role in osteoblast mechanotransduction. Kapur *et al*. (*30*) showed that PD98059 (MEK1 inhibitor), U0126 (MEK 1/2 inhibitor), or *N*(Ω)-nitro-L-arginine methyl ester (p38 MAPK inhibitor) completely blocked the shear stress–induced increases in ERK phosphorylation, [^{3}H]thymidine incorporation, and alkaline phosphatase without any effect on integrin β_1 expression. This indicates that the ERK and NO synthase pathways are essential for shear stress–induced proliferation and differentiation of normal human osteoblasts and that each involves ERK activation but not integrin β_1 up-regulation. Indomethacin blocked shear stress–induced osteoblast proliferation and differentiation as well as integrin β_1 up-regulation, but not ERK activation; this result suggests that the cyclooxygenase pathway [i.e., prostacyclin (PGI_2) or PGE_2, or both] mediates the shear stress–induced osteoblast proliferation in an ERK-independent manner.

You *et al*. (*31*) studied the effect of oscillating fluid flow (OFF) (2 N/m^2 = 1330 με) on osteoblasts and found that L-type calcium channels and IP_3 induction of intracellular calcium mobilization activated ERK and p38 MAPK, which was followed 24 hours later by an increase in osteopontin (OPN) induction. Osteopontin is a multifunctional ECM molecule involved in cell adhesion and migration and is an early marker of differentiation. Blocking ERK, p38 phosphorylation, or intracellular calcium release with specific inhibitors blocked the OPN response (*31*). Pulsating fluid flow (PFF) of 0.7 N/m^2 at 5 Hz (466 με) also stimulated both the influx of calcium through calcium channels and release of internal Ca^{2+} stores. Blocking either pathway inhibited release of PGE_2, as did disruption of the cytoskeleton by cytochalasin B (*32*).

Remarkably, FSS regulates portions of many major signaling pathways, including translocation of NF-κB; activation of ERK 1/2, PLC, and IP_3; release of intracellular Ca^{2+}; activation of adenylate cyclase; release of PGE_2 to its EP receptor; and Rho signaling. Duncan and colleagues (*13*) showed that an intact cytoskeleton was not an exclusive requirement for FSS-mediated translocation of NF-κB to the nucleus; however, the work of You *et al*. (*31*) showed that disruption of the cytoskeleton by cytochalasin B caused an inhibition of OPN induction by OFF, suggesting different mechanisms for these two responses to fluid flow.

Gravity Stress

Miwa *et al*. (*33*) first demonstrated that 5*g* hypergravity (50 με) stimulated DNA synthesis in a time-dependent manner, increasing DNA levels to about 150% of the control. It has been calculated that such forces are routinely encountered in vivo with walking (*7*). Their data suggested that PGE_2 mediates the sig-

naling of hypergravity on the proliferation of osteoblast-like cells. These data were confirmed by Nose and Shibanuma with a force of 50*g* (500 με) (*34*). Fitzgerald and Hughes-Fulford (*35*) showed that c-*fos* was significantly induced with as little as a 10-min pulse at 3*g* (30 με) of force. Later, these authors (*36*) showed that a factor of 10 induction of c-*fos* by gravity force was mediated through a cAMP pathway. Down-regulation of PKC activity by chronic TPA (12-*O*-tetradecanoyl phorbol-β-acetate) treatment failed to reduce c-*fos* induction, which suggests that TPA-sensitive isoforms of PKC are not responsible for c-*fos* up-regulation. Pretreatment with the cyclooxygenase inhibitors indomethacin or flurbiprofen did not hinder the early induction of c-*fos* by mechanical stimulation. These data suggest that c-*fos* expression induced by mild mechanical loading is dependent primarily on cAMP, not PKC, and that the initial induction of c-*fos* is not necessarily dependent on the action of newly synthesized PGE_2 (*37*, *38*).

Hatton *et al*. (*7*) showed that a 15-min pulse of gravity stress on osteoblasts increased osteoblast proliferation after 24 hours. Calculations showed that 4 to 30*g* corresponds to in vivo physiological levels such as those seen in walking or running ranging from 40 to 300 με. Short-term gravity loading (15 min) induced increases in expression of the growth-related immediate early gene c-*fos*, the early growth response factor *egr-1*, and the autocrine factor *fgf-2* (by factors of 15, 5, and 3, respectively). Genes that do not promote early osteoblast proliferation, such as those encoding EP-1 (PGE_2 receptor 1), TGF-β (transforming growth factor–β), and 18S, were unaffected by gravity loading. Short-term physiological loading induced ERK 1/2 phosphorylation in a dose-dependent manner, with maximum phosphorylation saturating at mechanical loading levels of 12*g* ($P < 0.001$) but no effect on total ERK. The phosphorylation of focal adhesion kinase (FAK) remained unaltered by mechanical force, and *g*-loading did not activate p38 MAPK or c-Jun N-terminal kinase (JNK). Additionally, a gravity pulse resulted in the localization of phosphorylated ERK 1/2 to the nucleus; this did not occur in unloaded cells. The induction of c-*fos* was inhibited 74% by the MEK 1/2 inhibitor U0126 ($P < 0.001$) but was not affected by MEK1 or p38 MAPK-specific inhibitors, which suggests that induction of c-*fos* is partially due to the ERK 1/2 signaling cascade. The 24-hour consequence of a single 15-min gravity pulse was a 64% increase in cell growth ($P < 0.001$). U0126 significantly inhibited gravity-induced growth by 50% ($P < 0.001$). These studies suggest that short periods of physiological mechanical stress stimulate immediate early gene expression and proliferation in MC3T3-E1 osteoblasts primarily through an ERK 1/2–mediated pathway. It is currently unknown whether these long-term mechanical stress responses are due to signaling through new synthesis or release of PGE_2 or new synthesis or release of autocrine proteins.

Four-Point Bending

ERK activation in osteoblasts has also been observed using the four-point bending technique. Using a range of inhibitors, Jessop *et al*. (*39*) demonstrated specific differences in the pathways by which ERK-1 and ERK-2 are activated in ROS17/2.8 rat cells in response to fluid movement alone or in response to strain (10,000 με for 10 min) in addition to its associated fluid movement. ERK-1 activation induced by fluid movement alone was markedly reduced by nifedipine and therefore appears to involve L-type calcium channels. However, ERK-1 activation was unaffected by either nitro-1-arginine methyl ester (L-NAME), a blocker of NOS, or indomethacin, a blocker of Cox-1 and Cox-2. This suggests independence from NO and PGI_2 production in the response to fluid flow alone. In contrast, ERK-1 activation, induced by application of strain (and its associated fluid disturbance), was abrogated by octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8, a blocker of calcium release from intracellular stores), L-NAME, or indomethacin. This is consistent with the dependence of strain-induced ERK-1 activation on calcium mobilization from intracellular stores by PLC and IP_3, resulting in production of NO and PGI_2, respectively. ERK-2 activation appears to be mediated by a separate mechanism; fluid movement alone involves both PGI_2 and NO production, but its activation by strain is not affected by TMB-8, L-NAME, or indomethacin. Pertussis toxin, an inhibitor of G_i- and G_o-type G proteins, did not cause a reduction in the activation of ERK-1 or -2 in response to either stimulus. These results demonstrate that these flow and mechanical strains stimulate osteoblasts through distinct signaling pathways (*39*).

Other studies using four-point bending at 3400 με revealed a relationship between estrogen and mechanical response in osteoblasts (*40*–*43*). In these studies, estrogen increased mechanical induction of proliferation by 20 to 40%, which may partially explain the loss of bone after menopause. Gallagher *et al*. showed that treatment of postmenopausal women with a daily dose of 0.625 mg of estrogen increased spine BMD (bone mass density) by 6.6% and hip BMD by 3.1%; later studies showed that even lower doses of estrogen could be effective in restoring BMD (*44*, *45*). That said, some types of exercise (mechanical stress) can overcome the lack of estrogen and increase BMD. Young female gymnasts have a higher rate of amenorrhea than their athletic running counterparts (*46*), yet BMC of the spine and femoral neck were highest in the gymnasts and lowest in the runners. The authors concluded that the higher BMD was due to high-impact loading in the gymnasts, which had a greater osteogenic effect than did the increased resorption induced by amenorrhea (*46*).

A study of regulation of c-*fos* induction using four-point bending also demonstrated the role of extracellular calcium responding to forces from 1000 με (*47*, *48*). This response was mediated in part by the calcium stretch channel and thought by Peake and co-workers to be activated by means of the shear stress response element (SSRE) (*49*). However, Peake *et al*. established that multiple stress pathways were at work, because experiments that used SSRE or cAMP response element (CRE) deletion constructs indicated that neither element was fully responsible for biological mechanotransduction in this system (*47*, *48*). In contrast, Chen *et al*. (*25*) found no effect on the L-channel or stretch channel with 800 με of steady flow alone when examining the induction of *cox-2* and c-*fos*; this finding suggests that larger loads of strain (>800 με) may be required to activate these calcium channels.

Substrate Stretch

Substrate stretch is accomplished by applying force to cells grown on flexible membrane. For instance, the Flexcell (Flexcell International, Hillsborough, NC) consists of a multichambered dish in which osteoblasts are cultured on the bottom silastic membrane surface. The dish bottom is then fitted to a variable vacuum that delivers mechanical stretch to the membranes. Although early Flexcell studies involved strains as high as

180,000 με (*50*), here we review experiments using stress with more physiological relevance ranging from 1000 to 10,000 με.

Granet *et al*. (*51*) investigated the expression and nuclear translocation of Egr-1 and NF-κB after substrate stretch was applied to the osteoblastic ROS17/2.8 cells. The Flexcell delivered a force of 10,000 με at 0.05 Hz for 10 min. Neither PKC down-regulation nor COX 1/2 inhibition altered the mechanical strain–dependent increase in Egr-1 expression or translocation of NF-κB (*51*); these findings suggest that these effects depended on alternate pathways not mediated through PKC and PGE_2. Inhibition of the Src kinases blocked translocation of NF-κB. The translocation of NF-κB is likely a common pathway in other types of stress, because studies by Chen *et al*. showed that FSS caused NF-κB translocation within hours of mechanical stress mediated through a fluid flow–dependent release of intracellular calcium (*13*), which in turn stimulated *cox-2* expression.

In studies of the activator protein-1 (AP-1) family (c-Fos– or c-Jun–like molecules) on the Flexcell, Granet *et al*. (*52*) found that mechanical stress increased expression of the AP-1 family of molecules as others had seen with FSS (*14*, *25*, *53*–*58*) or gravitational forces (*34*–*36*, *59*). Expression of AP-1 molecules was not affected by inhibition of ERK 1/2 or Src kinases. Inhibition of ERK 1/2 or Src kinases blocked nuclear translocation of almost all the AP-1 members except Fra-1 and JunD.

The Flexcell (10,000 με for 10 min) also caused an increase in pFAK and pERK-2 within 4 hours in ROS 17/2.8 osteoblasts (*60*). This is in contrast to findings by Hatton *et al*. (*7*) that found no increase in pFAK in MC3T3-E1 osteoblasts after a 15-min pulse of 120 με in a g-vector stress model, which suggests that strain greater than 1000 με may be required for FAK phosphorylation. Both reports noted an increase in pERK within 30 min after mechanical stress, as have other investigators (*29*–*31*, *61*–*63*) with different stress models. In addition to ERK activation, Matsuda *et al*. showed that JNK was activated in mechanically stretched cells (*64*). Although specifically analyzed, JNK activation was not seen in fluid flow (*31*) or gravity compression (*7*), most likely because of the lower levels of strain delivered by those methods.

Carvalho *et al*. (*65*) applied mechanical strain with 20 kPa of vacuum intermittently at 0.05 Hz for periods of 0.5, 1, 5, 10, and 30 min and 1, 3, and 7 days using the Flexcell system. The concentrations of cAMP and IP_3, measured by radioimmunoassay as well as by kinase assay systems that measured PKC activity, increased with mechanical strain. The increase in IP_3 (0.5 min) peaked before the increase in PKC activity (5 min), which in turn peaked before the increase in cAMP (10 min). Immunolocalization of the cytoskeleton proteins vimentin and α-actinin, the focal contact protein vinculin, and PKC showed a marked difference between strained and nonstrained cells. Staining suggested that PKC participates actively in the transduction of mechanical signals to the cell through focal adhesions and the cytoskeleton; PKC content only seemed to change with short time periods of strain. In conclusion, osteoblasts responded to mechanical strain initially through increases in IP_3 and enhanced PKC activity, and later through increases in cAMP, much like the signaling mechanisms described by Duncan's group (*13*, *14*, *25*, *66*) using FSS.

Other Stress Techniques

The involvement of the cytoskeleton in mechanotransduction has also been reported by others (*14*, *67*, *68*). Single-cell mechanotransduction by atomic force (*69*, *70*) demonstrated that the microtubular and vimentin networks—as well as calcium stretch channels and intracellular calcium release—were key in transduction. Microbead twist or pulling (1333 με) with tripeptide Arg-Gly-Asp (RGD) on coated beads increased endothelin gene expression more than 100% (*71*). In contrast, nonspecific twisting did not induce ET-1 expression. Disrupting actin microfilaments, blocking stretch channels, or inhibiting tyrosine kinases blocked the induction of ET-1, suggesting multiple pathways in mechanotransduction (*71*). Others using the RGD peptide–coated microbeads demonstrated that multiple signaling molecules (e.g., pp60c-src, pp125FAK, phosphatidylinositol 3-kinase, PLC-γ, and Na^+/H^+ antiporter) involved in both integrin and growth factor receptor signaling pathways became associated with the C-terminal Src kinase (CSK) framework of the FAK within 15 min after binding to beads coated with integrin ligands. FAK may thus represent a major site for signal integration between regulatory pathways (*72*).

Vibration has also been used as a method to induce osteoblast gene expression. Tjandrawinata *et al*. (*73*) showed that 10 min of vibration significantly increased induction of c-*fos* and c-*myc* in osteoblasts ($P < 0.05$). Later studies by Rubin *et al*. showed that low-frequency vibration can cause modest increases in bone in postmenopausal women (*74*, *75*).

In studies by Sawada and Sheetz, Triton X-100 was used to isolate the intact cytoskeleton from the cytoplasm and cell membrane (*76*). The Triton X-100–insoluble cytoskeletons were then washed and stretched for 3 min by Flexcell. Biotinylated cytoplasmic protein was added back for 2 min, the unbound cytoplasmic proteins were removed by washing, and the remaining cytoskeleton and its bound labeled cytoplasmic proteins were analyzed by two-dimensional gel electrophoresis. Sawada and Sheetz found stretch-dependent binding of more than 10 biotinylated proteins to the isolated cytoskeleton, including paxillin, focal adhesion kinases, p130Cas, and PKB/Akt, whereas attachment of vinculin was unchanged. These data suggest that transduction of matrix forces occurs through force-dependent conformational changes in the intact cytoskeleton (*76*). In studies using shockwaves applied to human osteoblasts, Wang *et al*. (*77*, *78*) demonstrated Ras induction of superoxide production and ERK activation. In addition, their later studies showed that this stimulation induced hypoxia inducible factor–1α (HIF-1α) and vascular endothelial growth factor–A (VEGF-A) through Ras activation. This is in agreement with studies of stress where ERK phosphorylation was one of the early mechanotransduction responses (*7*, *30*, *39*, *52*, *79*), regardless of the method used to generate mechanical stress. It is of interest to note that many osteoblast growth factors are made by the osteoblasts themselves; these include TGF-β, fibroblast growth factor–2 (FGF-2), and insulin-like growth factors (ILGFs). The transcripts and proteins of these three autocrine factors are induced in the osteoblast within minutes to hours by mechanical stress (*7*, *80*, *81*). In addition, expression of the epidermal growth factor receptor (EGFR) is also increased by fluid flow (*82*) (Fig. 1).

It has been argued that the cell's cytoskeletal matrix is responsible for all mechanotransduction cellular responses (*83*–*89*). However, the cytoskeleton alone cannot completely account for all of the mechanisms involved in the mechanotransduction process of osteoblasts. In addition to the cytoskeleton, many other signaling pathway components are stimulated by mechanical stress, including calcium channels, adenylate cyclase, GTPases, PLC, IP_3, PKA, ERK kinases, and translocation of NF-κB, PGE_2, PGI_2, or NO. In fact, inhibition of the

Mechanical strain	Calculated strain	Response measured	Signaling molecules and pathways implicated	Reference
FSS	<800 $\mu\varepsilon$	c-*fos, cox-2, actin cytoskeleton,* cAMP, Ca^{2+}	PLC-IP_3, release of intracellular Ca^{2+}, Rho, NF-κB translocation, MAPK	(*12–14*, *22*, *25*, *29*)
FSS	>800 $\mu\varepsilon$	Ca^{2+}, cAMP, NO, Cox-2, pERK	IP_3, PKA, adenylate cyclase, MAPK, calcium channels, PGE_2 receptors	(*22*, *27*, *28*, *30*, *48*)
PFF or OFF	500 to 1,300 $\mu\varepsilon$	OPN, pERK, PGE_2	IP_3, Ca^{2+} channels and intracellular Ca^{2+}, p38 MAPK	(*11*, *31*, *32*)
Gravity compression	30 to 500 $\mu\varepsilon$	DNA synthesis, c-*fos*, pERK, *fgf-2*, *cox-2,* and proliferation	PKA, MAPK	(*7*, *33-37*)
Four-point bending	3,400 to 10,000 $\mu\varepsilon$	c-*fos*, pERK, and proliferation	pERK-1: IP_3, NO, Ca^{2+} channel; pERK-2: PGI_2, NO	(*39–43*, *48*)
Substrate stretch	1,000 to 10,000 $\mu\varepsilon$	c-*fos*, *egr-1*, NF-κB translocation, cAMP, IP_3	Src kinases, MAPK, JNK, FAK, and PKC	(*51–54*, *56*, *58-60*, *64*, *90–92*)
Magnetic bead RGB, twisting or pulling, atomic force or vibration	1,000 to 2,000 $\mu\varepsilon$	ET-1	pp60-src, pFAK, IP_3, PLC, PKA, calcium channels, Akt	(*67*, *69–71*, *73*, *89*, *93*, *94*)

Table 1. Comparison of mechanical strain technique and probable mediating pathway.

small GTPase Rho (*14*), PLC (*25*), or PKA (*22*, *37*) can completely inhibit mechanotransduction, indicating that several redundant pathways mediate mechanical stress signaling. That said, the cytoskeleton and its associated proteins play a major role in mechanotransduction. Pavalko *et al*. (*14*, *25*) showed that inhibition of actin stress fiber development by treatment of cells with cytochalasin D, by expression of a dominant negative form of Rho, or by inhibition of PLC blocked the shear-induced gene expression in osteoblasts. They concluded that fluid shear–induced mechanical signaling in osteoblasts leads to increased expression of *cox-2* and c-*fos* through a mechanism that involves Rho-mediated stress fiber formation and PLC activation, which in turn may promote fluid shear–induced metabolic changes in bone cells (*14*). Taken together, the majority of the literature shows that physiological levels of mechanical strain initiate mechanotransduction through coordinated activation of adenylate cyclases, GTPases, ERK and PKA activation, PLC activation of IP_3 (which causes release of intracellular calcium), translocation of NF-κB, and/or release of autocrine factors such as PGE_2, PGI_2, TGF-β, FGF-2, ILGFs, and NO (Table 1). The end result of most of these responses is induction of gene expression and proliferation or differentiation. In mechanotransduction, these multiple signal pathways work in concert with cytoskeletal elements, resulting in a complete osteoblast response to mechanical stress.

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