Journal of Cellular Biochemistry 98:1271–1283 (2006)

Fluid Flow Induces Rankl Expression in Primary Murine Calvarial Osteoblasts

Meenal Mehrotra,[1] Masatomo Saegusa,[1] Sunil Wadhwa,[2] Olga Voznesensky,[1] Donald Peterson,[1] and Carol Pilbeam[1]*

[1]Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut 06030
[2]Department of Orthodontics, University of Connecticut Health Center, Farmington, Connecticut 06030

Abstract Mechanical loading of bone generates fluid flow within the mineralized matrix that exerts fluid shear stress (FSS) on cells. We examined effects of FSS on receptor activator of nuclear factor κ B ligand (RANKL), a critical factor for osteoclast formation. Primary murine osteoblasts were subjected to pulsatile FSS (5 Hz, 10 dynes/cm^2) for 1 h and then returned to static culture for varying times (post-FSS). Protein levels were measured by Western analysis and mRNA by Northern analysis, RT-PCR and quantitative PCR. There were 20- to 40-fold increases in RANKL mRNA at 2–4 h post-FSS. RANKL protein was induced by 2 h post-FSS and remained elevated for at least 8 h. Effects were independent of cyclooxygenase-2 activity. Small increases (up to three-fold) in mRNA of the decoy receptor for RANKL, osteoprotegerin, were seen. Five min of FSS, followed by static culture, was as effective in stimulating RANKL mRNA as 4 h of continuous FSS. FSS induced cAMP activity, and H-89, a protein kinase A (PKA) inhibitor, blocked the FSS induction of RANKL. H-89 also inhibited the PKC pathway, but specific PKC inhibitors, GF109203X and Go6983, did not inhibit FSS-induced RANKL. FSS induced phosphorylation of ERK1/2, and PD98059, an inhibitor of the ERK pathway, inhibited the FSS induction of RANKL mRNA 60%–90%. Thus, brief exposure to FSS resulted in sustained induction of RANKL expression after stopping FSS, and this induction was dependent on PKA and ERK signaling pathways. Increased RANKL after mechanical loading may play a role in initiating bone remodeling. J. Cell. Biochem. 98: 1271–1283, 2006.

Key words: mechanical loading; protein kinase A; protein kinase C; osteoprotegerin; ERK

Interactions between signals controlling bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts) are ultimately responsible for the adaptive response of the skeleton to its environment. Bone remodeling, cycles of resorption followed by formation in focal remodeling units, occurs throughout life, and the net result of these cycles is an important determinant of whether bone is lost or not. Although remodeling is initiated by resorption, it is dependent on signals from cells of the osteoblast lineage. The discovery of receptor activator of nuclear factor-κB ligand (RANKL) elucidated the mechanism by which osteoblastic cells can regulate osteoclast differentiation [Wong et al., 1997; Lacey et al., 1998; Yasuda et al., 1998a]. Osteoblastic cells express RANKL in response to factors that stimulate resorption, and RANKL can bind to its receptor RANK, on osteoclast precursors, causing them to differentiate into multinuclear, bone-resorbing osteoclasts [Suda et al., 1999]. The RANK/RANKL interaction can occur via membrane-bound RANKL (cell-to-cell) or via soluble RANKL. The actions of RANKL are inhibited by osteoprotegerin (OPG), a decoy receptor for RANKL [Yasuda et al., 1998b]. The ratio of RANKL to OPG expression by osteoblasts is believed to be a key determinant of osteoclastogenic activity.

Mechanical loading of bone is essential for maintaining bone mass and integrity. Bone is a dynamic tissue that adapts to mechanical stress with changes in mass and structure to achieve a better balance between stress and load bearing capacity. External forces applied to bone result in small deformations, or strains, in the bone

Grant sponsor: NIH; Grant number: RO1AR47673.

*Correspondence to: Carol Pilbeam, Room AM047, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030.
E-mail: pilbeam@nso.uchc.edu

Received 30 November 2005; Accepted 9 January 2006
DOI 10.1002/jcb.20864

matrix that are converted into intracellular signals in mechanosensitive cells, and these signals are communicated to non-mechanosensitive cells in order to produce a coordinated response [Turner and Pavalko, 1998]. How mechanical loading is coupled to cellular responses in bone and which cells are involved is still debated. However, there is general agreement that interstitial fluid flow in the interconnecting network of osteocytes, end stage cells in the osteoblastic differentiation pathway that are trapped within the mineralized matrix, is likely to play an important role [Hillsley and Frangos, 1993; Turner et al., 1994; Knothe et al., 1998; Burger and Klein-Nulend, 1999; Srinivasan and Gross, 2000; Smit et al., 2002]. Fluid flow generates fluid shear stress (FSS) that deforms cell surfaces and is modeled in vitro by subjecting cells plated on fixed surfaces to cyclic laminar fluid flow. Another possibility is that lining osteoblasts residing on endosteal and periosteal bone surfaces are deformed as these surfaces undergo deformation during the bending of long bones. This is modeled by growing cells on flexible substrates and subjecting the substrates to cyclic stretching. Hence, mechanical loading in vivo may subject osteoblastic cells at different differentiation stages to different types of cellular deformation and stimulate different signaling pathways [Mullender et al., 2004].

RANKL expression in bone cells has been shown to be regulated by deforming the substrate on which the cells are grown. Several studies in murine marrow cultures, which contain precursors for both osteoblasts and osteoclasts, and in murine marrow stromal cells, which contain largely osteoblastic precursors, have reported that cyclic stretching of the substrate can decrease the ability of a RANKL agonist, $1,25(OH)_2D_3$, to stimulate RANKL expression and to stimulate osteoclast formation. [Rubin et al., 2000, 2002, 2003]. This effect was dependent on ERK phosphorylation. Basal RANKL mRNA expression was found to be decreased by cyclic tensile strain in human osteoblasts [Kusumi et al., 2005]. On the other hand, there was a trend for RANKL protein levels to increase when human osteosarcoma MG-63 cells were loaded by cyclic 3-point bending of their substrate [Saunders et al., 2005].

We examined the effect of pulsatile FSS on basal RANKL expression in primary murine osteoblasts plated on collagen-coated slides. Because in vivo studies suggest that only a short duration of mechanical loading is necessary to initiate an adaptive response [Umemura et al., 1997;Turner, 1998; Rubin et al., 2001], we examined the response in cells replaced in static culture after brief application of FSS. FSS markedly elevated RANKL expression. In contrast to the large effects on RANKL, there were only small increases in mRNA for OPG, which can oppose the effects of RANKL. The induction of RANKL was dependent on both the PKA and ERK signaling pathways. Increased RANKL after mechanical loading may play a role in initiating bone remodeling.

MATERIALS AND METHODS

Materials

Murine RANKL and OPG cDNA was a kind gift from Dr. D Anderson (Amgen, Inc., Seattle, WA). Murine COX-2 cDNA was the kind gift of Dr. Harvey Herschman (University of California, Los Angeles, CA). cDNA for glyceraldehyde phosphate dehydrogenase (GAPDH) was obtained by PCR using a control amplifier set from Clontech (Palto Alto, CA). GF109203X, H-89, HA-1004, KT-5720, and PD98059 were purchased from BIOMOL (Plymouth Meeting, PA). All other chemicals were from Sigma (St. Louis, MO). The anti-rabbit RANKL antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Total ERK 1/2 and phosphorylated ERK 1/2 antibodies were from Cell Signaling Technology (Beverly, MA).

Fluid Flow Chamber

The parallel plate fluid chamber was designed by M.A.F. Epstein and D.R. Peterson [Epstein et al., 1995; Peterson, 1995] and its use has been previously described [Wadhwa et al., 2002a]. This chamber generates a uniform flow field in which magnitude and direction of the velocity vector are constant under steady flow conditions. The apparatus has a test area of 13.5 cm by 8.5 cm, which can accommodate four standard microscope slides (75 mm $\times$ 25 mm). The flow medium was Dulbecco's modified Eagle's medium without phenol red (DMEM, Sigma) containing 1% heat-inactivated fetal calf serum (FCS, Gibco BRL, Grand Island, NY). The flow medium was maintained at 37°C. A gassing system saturated the medium with 5% CO_2.

Cell Culture

To make primary osteoblastic cells, calvariae were excised from neonatal mice, dissected free of loose connective tissue and washed with phosphate-buffered saline (PBS). All animal protocols were approved by the Animal Care and Use Committees of the University of Connecticut Health Center. Calvariae were then treated with crude collagenase P (Roche Diagnostic Corporation, Indianapolis, IN) and trypsin at 37°C for 10 min to release fractions 1–4 and for 60 min to release fraction 5. Released cells were removed and the reaction was stopped with DMEM + 10% FCS. A single cell suspension was formed by filtering the cells through a nitex membrane. Populations 2–5 were pooled and cells were grown to confluence in phenol-red free DMEM with 10% heat-inactivated FCS, penicillin (100 U/ml) and streptomycin (50 μg/ml) before replating for experiments. For experiments, cells were plated on Type I collagen coated (rat-tail collagen; Collaborative Biomedical Products, Bedford, MA) glass slides at 5,000/cm^2 in the same medium as above and grown to near confluence over 4–6 days at 37°C in a humidified atmosphere of 5% CO_2 in air. Slides were coated according to the manufacturer's instructions with 5 μg/cm^2 collagen. To avoid the effects of fresh serum on gene induction, the medium used in the FSS apparatus and for post-FSS culture had reduced FCS (1% FCS). Cells were changed to this medium 30 min before FSS. All experiments after the initial one were performed in the presence of NS398 (0.1 μM), a selective inhibitor of COX-2 activity. Cells were pretreated with NS-398 or other inhibitors for 30 min. NS-398 and the other inhibitors were present during FSS and throughout the post-FSS culture period. Concentration of the vehicle for the inhibitors was 0.1% or less in both treated and control cultures.

Extraction of RNA and Northern Blot Analysis

Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Twenty micrograms of total RNA was run on a 1% agarose, 2.2 M formaldehyde gel, transferred to a nylon membrane by capillary pressure and fixed to the membrane by UV irradiation. After 4 h of prehybridization in a 50% formamide solution at 42°C, filters were hybridized overnight in a similar solution in rotating cylinders at the same temperature with a random [^{32}P]deoxycytocine triphosphate (dCTP)-labeled cDNA probes. Filters were washed once in a 1× SSC, 1% sodium dodecyl sulfate (SDS) solution at room temperature, once in 0.1× SSC, 0.1% SDS solution at 65°C and three more times in the latter solution at room temperature. After washing, the filters were exposed to Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY) at −70°C. Bands were scanned into the computer and the band intensity was quantitated by the National Institutes of Health (NIH) Image 1.61 software (free software available from the NIH).

RT-PCR Analysis

Two-five μg of total RNA was converted to cDNA by M-MLV reverse transcriptase and random hexamer (Invitrogen, Carlsbad, CA) following company recommended protocol. Aliquots of RT mixture were used for PCR, which was done in a thermal cycler (GeneAmp PCR System, Perkin Elmer, Wellesley, MA) using gene-specific PCR primers and *Taq* polymerase (Invitrogen). The temperature cycling was as follows: initial denaturation at 94°C for 3 min; denaturation at 94°C for 45 s, primer annealing at 65°C for 45 s, and extension at 72°C for 2 min for 10 cycles. In subsequent cycles, the primer annealing temperature was decreased stepwise (step-down method), by 5°C, every 5 cycles. After the last cycle, the mixture was incubated at 72°C for 5 min. For RANKL and OPG the number of cycles was 30 while for 18S it was 26. Specific primer sets were designed from published cDNA sequences: murine RANKL: antisense 5′GGGAATTACAAAGTGCACCAG3′; sense 5′GGTCGGG-CAATT CTGAATT3′), murine OPG: antisense 5′TCAAGTGCTTGAGGGCA-TAC3′; sense 5′TGGAGATCGAATTCTGCTT-G3′), murine 18S: antisense 5′CATGTGGTGT-TGAGGAAAG3′; sense 5′GCCCAGAGACTC-ATTTCTT3′. The amplified samples were run in a 1.5% agarose gel and photographed under UV illumination by Syngene Bioimaging, Gene-Flash (Atkinson, NH).

Quantitative Real-Time PCR (qPCR) Analysis

qPCR was performed for different genes in separate wells (singleplex assay) of 96-well plate in a reaction volume of 20 μl. 18S or GAPDH served as the endogenous control for all

experiments. Three replicates of each sample were amplified using assays-on-demand gene expression (Applied Biosystems, Foster City, CA) to give one measurement for a sample. Predesigned unlabeled gene-specific PCR primers and TaqMan dye-labeled probes were purchased from Applied Biosystems. Primers were tested for equal efficiency over a range of target gene concentration before use. All primers were designed to cross exon–exon boundaries. The PCR reaction mixture (including 2X TaqMan Universal PCR Master Mix, 20X assays-on-demand gene expression assay mix, 100 ng of cDNA) was run in Applied Biosystems ABI Prism 7500 Sequence Detection System instrument utilizing universal thermal cycling parameters. The relative quantification of target gene expression in a test sample to a control calibrator sample ($\Delta\Delta C_t$ Method) was used for data analysis.

Intracellular cyclic AMP Measurement

Primary osteoblastic cells were treated with 0.5 mM of isobutyl methyl xanthine (IBMX) to block phosphodiesterase activity for 30 min. They were then subjected to FSS for 5 and 15 min. After FSS, medium was removed and cells were scraped in 500 μl of ice-cold ethanol. The ethanolic cell suspension was collected in tubes and centrifuged at 1,500*g* for 10 min at 4°C. Supernatants were collected and evaporated to dryness. The dried residue was dissolved in EIA buffer (Cayman Chemicals, Ann Arbor, MI) and cAMP was measured according to the manufacturer's instructions. The cAMP activity was normalized to the total protein in the sample.

Western Blot Analysis

Cells were washed once and scraped in ice-cold PBS and collected into a microcentrifuge tube. They were centrifuged for 5 min at 4°C at 5,000 rpm. The supernatant was discarded and the cells were lysed in 1× cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM β-glycerophosphate, 1 μg/ml leupeptin, and 1 mM PMSF (Cell Signaling Technology). After lysing the cells for 20 min on ice, the lysates were centrifuged at 10,000*g* for 20 min at 4°C. Equal amounts of supernatant proteins (50 μg), determined by BCA protein assay kit (Pierce Chemical Co., Rockford, IL), were separated on 12% SDS–PAGE and electrotransferred onto nitrocellulose membrane. Membranes were washed with Tris-buffered saline with Tween-20 (TBST, pH 7.6), blocked with blocking buffer (1X TBS, 0.1% Tween-20 with 5% wt/vol nonfat dry milk), and incubated with primary antibody (1:200 for anti RANKL and 1:500 for total and phosphorylated ERK 1/2) in primary antibody buffer (5% BSA in TBST) at 4°C overnight. After washing with TBS containing 0.1% Tween-20 (TBST), the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000) in the blocking buffer for 1 h at room temperature. After washing again with TBST, the signals were detected by Lumi GLO chemiluminescence reagent (Cell Signaling Technology).

Statistical Analysis

Statistical significance of differences among means was determined by analysis of variance (ANOVA) with post-hoc comparison of more than two means by the Bonferroni method using SigmaStat (Jandel Scientific, San Rafael, CA).

RESULTS

FSS Induced RANKL mRNA and Protein Expression

Primary murine calvarial osteoblasts were subjected to pulsatile fluid flow (5 Hz), equivalent to FSS of 10 dynes/cm^2, for 1 h and then returned to static culture for varying time periods. Constitutive RANKL mRNA was often not seen on Northern analysis (Fig. 1A), but with more sensitive methods of detection, like RT-PCR or qPCR, constitutive RANKL mRNA expression was evident (Fig. 1B). RANKL mRNA was not elevated immediately after 1 h of FSS but was elevated in static culture 2 h after FSS (Fig. 1A). RANKL mRNA levels remained elevated at least 8 h after stopping FSS (Fig. 2B) and had almost returned to baseline at 24 h (Fig. 1B).

The induction of COX-2 mRNA expression under these conditions can be seen immediately on stopping FSS, and COX-2 levels continue to increase in post-FSS culture (Fig. 2A). Since COX-2 produced prostaglandins can induce RANKL mRNA [Kanzaki et al., 2002; Udagawa et al., 1999; Li et al., 2002], we examined the effects of NS-398 (0.1 μM), at a dose shown to inhibit COX-2 activity in osteoblasts [Pilbeam

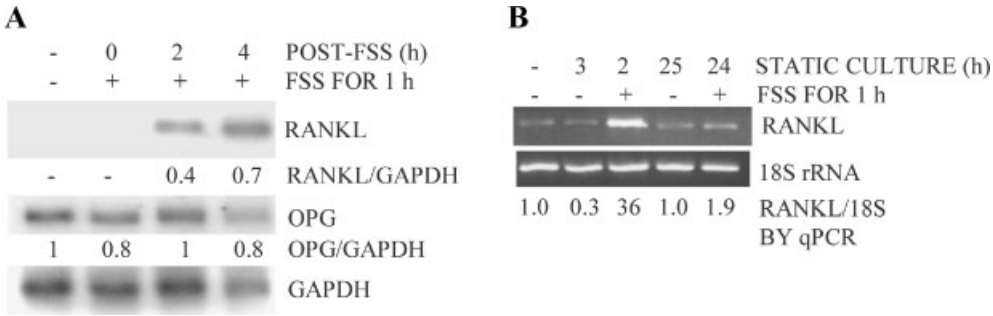

Fig. 1. Time course for FSS induction of RANKL mRNA expression in primary osteoblasts. Cells were subjected to FSS for 1 h and extracted immediately or returned to static culture (post-FSS) for the times indicated. **A**: Northern blot analysis for RANKL, OPG and GAPDH mRNA. The ratio of RANKL or OPG mRNA band density to GAPDH mRNA band density is indicated below the bands. **B**: RT-PCR analysis for RANKL and 18S mRNA. No-flow controls, changed to new dishes at the same time other cells were put in the fluid flow apparatus, were taken down at the same times as the treated samples. The fold induction of RANKL mRNA relative to a 0 time point control was calculated by quantitative PCR (qPCR) and is indicated below each band.

et al., 1997], on the FSS induction of RANKL. On Northern analysis, NS-398 did not reduce the FSS induction of RANKL mRNA (Fig. 2B). Nevertheless, all further experiments were done in the presence of NS-398 (0.1 μM), added 30 min prior to FSS and continued throughout FSS and post-FSS culture, to avoid any effects of FSS induced COX-2.

To examine RANKL protein expression, primary osteoblasts were subjected to FSS for 1 h and returned to static culture for 2, 4, and 8 h post-FSS. On Western analysis, RANKL protein was barely detectable in the static controls (Fig. 3). RANKL protein expression was increased 2 h after FSS and remained elevated at 8 h post-FSS, paralleling the changes seen in RANKL mRNA expression.

Since it is the ratio of RANKL to its decoy receptor, OPG, that may ultimately regulate bone resorption, we also examined OPG mRNA levels. Constitutive OPG expression was easily detectable on Northern analysis (Figs. 1A, 2B). Effects of FSS on OPG mRNA levels were small, and it was difficult to determine a trend on Northern analysis.

In our fluid flow chamber, four slides of cells can be subjected simultaneously to FSS. For Northern analysis, we pooled RNA extracted from all four slides to obtain sufficient RNA at each time point. To quantify the results for RANKL and OPG mRNA, we performed quantitative real time PCR (qPCR) on these samples from multiple independent experiments and calculated mean values (Table I). RANKL

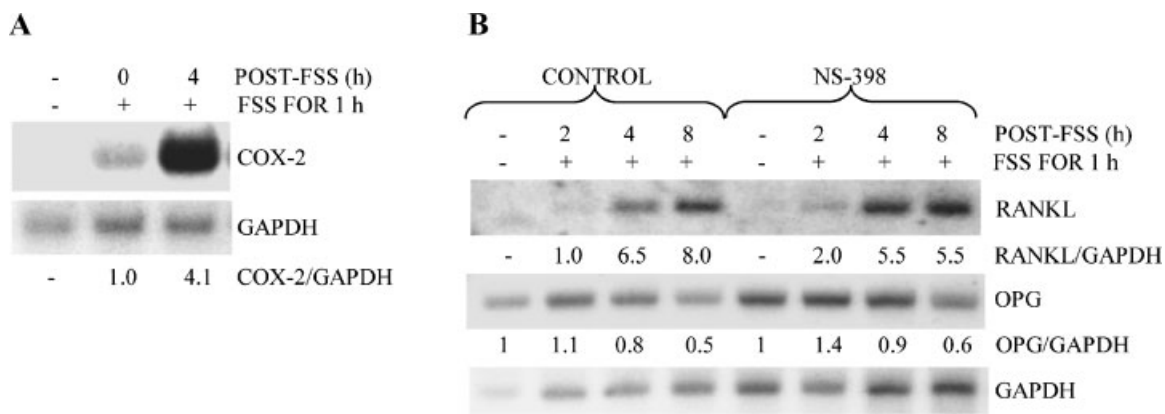

Fig. 2. Effect of inhibiting COX-2 activity on the FSS induction of RANKL mRNA. Cells were subjected to FSS for 1 h and extracted immediately or returned to static culture (post-FSS) for the times indicated. **A**: Northern blot analysis for COX-2. The ratio of COX-2/GAPDH mRNA is shown below the bands. **B**: Northern blot analysis for RANKL and OPG. Cultures were treated throughout with NS398 (0.1 μM) or vehicle (control). The relative band intensities for RANKL and OPG mRNA to GAPDH mRNA are indicated below the bands.

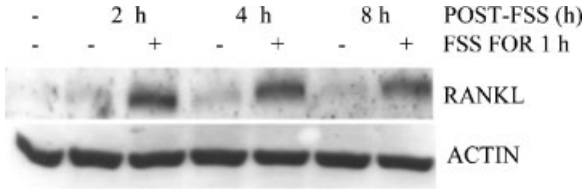

Fig. 3. Western blot analysis for RANKL protein. Cells were left in static culture (control) or subjected to FSS for 1 h and the returned to post-FSS static culture for the times indicated. Each time point had a static control taken down at the same time as the FSS treated samples. Equal loading was confirmed by probing for actin.

mRNA levels tended ($P = 0.07$) to be decreased immediately after stopping FSS but were increased 20 to 40-fold at 2 to 4 h in post-FSS static culture. There were small but statistically significant increases in OPG mRNA levels, with the greatest increase of 2.7-fold seen immediately after stopping FSS.

Comparison of Different FSS Durations

Five min of FSS was sufficient to cause an increase in RANKL levels on Northern analysis at 4 h of post-FSS culture (Fig. 4A). Levels of RANKL mRNA appeared lower immediately following 4 h of FSS than following 5 min FSS plus 4 h of post-FSS culture (Fig. 4A). To test the possibility that continuous FSS might suppress the maximal stimulation of RANKL mRNA expression, we compared the effects of FSS for 1 h plus 3 h of post-FSS culture with FSS for 4 h using qPCR (Fig. 4B). Three slides were analyzed per group, each in triplicate. Two controls, both cultured for 4 h, were used: (1) static control slides were changed to new media each time the slides subjected to FSS were changed, and (2) sham control slides were placed inside the FSS apparatus with media, and the apparatus closed briefly. There was no significant difference between the static controls and the sham controls. RANKL mRNA was increased 5-fold after FSS for 1 h plus 3 h of static culture, compared to 3.3-fold after 4 h of FSS.

Role of the PKA and PKC Signaling Pathways in the FSS Induction of RANKL

Both PKA and PKC pathways have been implicated in the regulation of RANKL expression. Five min of FSS was sufficient to induce a 3.5-fold increase in cAMP in primary calvarial osteoblasts (Fig. 5A). Treatment with H-89 (30 μM), a PKA inhibitor [Chijiwa et al., 1990], decreased the FSS induction of RANKL at 2 h post-FSS by 97% (Fig. 5B). While these results might seem to confirm the involvement of the PKA pathway, we previously found H-89 used at any dose that effectively inhibited the PKA pathway also inhibited the PKC pathway [Wadhwa et al., 2002b]. To test the degree of specificity under the current experimental conditions, we treated cells for 3 h under stationary conditions with forskokin (10 μM), a PKA agonist, and phorbol myristate acetate (PMA, 1 μM), a PKC agonist, with and without H-89 (30 μM). In two experiments, one analyzed by both RT-PCR and qPCR, with only 1 sample for each group (Fig. 5B), and the other analyzed by qPCR, with three replicates per group (Fig. 6A), forskolin and PMA were both potent inducers of RANKL. H-89 decreased the forskolin and the PMA induction of RANKL 95%–99% and 87%–99%, respectively. In fact, there was no significant stimulation of RANKL by PMA in the presence of H-89. We tried to find more specific PKA inhibitors. For HA-1004, we could not find a dose that would inhibit effects of forskolin

TABLE I. Changes in RANKL and OPG mRNA Levels After FSS Analyzed by qPCR

Post-FSS		RANKL		OPG		
Culture (h)	N	Control	FSS	Control	FSS	RANKL/OPG
0	3	1.0 ± 0.4	0.5 ± 0.2	1.0 ± 0.1	2.7 ± 0.3[a]	0.2
2	12	1.0 ± 0.2	22.8 ± 4.9[a]	1.0 ± 0.1	1.6 ± 0.2[b]	14
4	6	1.0 ± 0.3	39.0 ± 15.7[b]	1.0 ± 0.1	1.3 ± 0.1[b]	30

Cells were subjected to fluid shear stress (FSS) for 1 h and immediately extracted or returned to static culture (post-FSS) for 2 and 4 h. Static controls were taken down at the same times as the FSS treated samples. Each RQ value for RANKL and OPG was calculated relative to its static control at that time point. Single data values were pooled from N independent experiments. The 4 h data come from a subset of the 2 h experiments while the 0 time data are from a different set of experiments.
The data are the mean ± SEM values from N independent experiments. Values were normalized to the control mean for that post-FSS time point before being pooled.
[a]Significantly different from control, $P < 0.01$.
[b]$P < 0.05$.

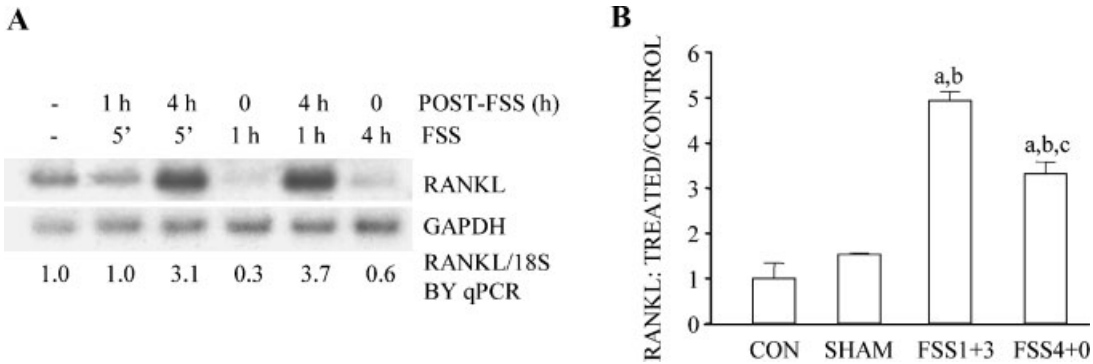

Fig. 4. Comparison of different FSS durations. **A**: Northern blot analysis for RANKL mRNA. Cells were left in static culture or subjected to FSS and post-FSS culture for times as indicated. The fold increase for RANKL/18S mRNA, relative to control, for each sample run on the gel was calculated by quantitative PCR (qPCR) and is indicated below the bands. **B**: qPCR for RANKL mRNA. Cells (three slides per treatment group) were subjected to 1 h of FSS plus 3 h of post-FSS culture (FSS 1+3) or 4 h of FSS with no post-FSS culture (FSS 4+0). Control slides were left in static culture but put into new dishes (with the same medium as used in flow apparatus) when FSS slides were put into apparatus and when they were taken out. Sham slides were put into the flow apparatus, which was briefly closed, and replaced in static culture. The relative quantification (RQ) values were calculated by the $\Delta\Delta C_t$ method using a reference sample. From these RQ values, the treated over control fold inductions were calculated. [a]Significant effect of FSS versus Control, $P < 0.01$. [b]Significant effect of FSS versus Sham, $P < 0.01$. [c]Significant difference between FSS 1+3 and FSS 4+0, $P < 0.01$; [d]$P < 0.05$.

(data not shown). For KT5720, we found inhibition of both the forskolin and PMA induction of RANKL mRNA at all the doses tested from 0.1 to 10 μM (data not shown).

Since H-89 inhibited both PKA and PKC pathways, we treated with specific PKC inhibitors to assess the contribution of the PKC pathway. GF109203X is a potent PKC inhibitor [Toullec et al., 1991]. To examine specificity under our experimental conditions, we treated cells in static culture with forskokin (10 μM) and PMA (1 μM) for 3 h, with and without GF109203X (0.25 μg/ml). Treatment with GF109203X blocked the PMA induction of RANKL without inhibiting the forskolin induction of RANKL (Fig. 6A). Similar results were seen in a second experiment. In the same experiment, H-89 blocked the FSS induction of RANKL mRNA, while GF109203X had no effect on the FSS induction of RANKL (Fig. 6B).

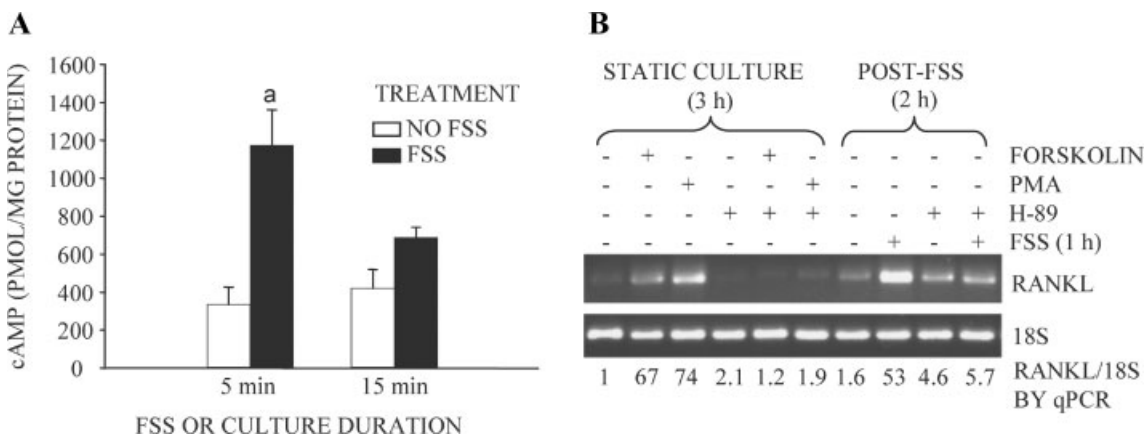

Fig. 5. Involvement of the PKA pathway in the FSS induction of RANKL. **A**: Measurement of cAMP production. Cultures were subjected to FSS for 5 and 15 min. Controls were transferred to another dish at start and end of FSS and taken down along with the FSS treated samples. cAMP levels were normalized to total protein. [a]Significant effect of FSS, $P < 0.05$. **B**: Effect of H-89. Cultures were treated with H89 (30 μM) for 30 min prior to FSS, during FSS and in post-FSS culture. Cells were subjected to FSS for 1 h and returned to static culture for 2 h or treated with forskolin (10 μM) and PMA (1 μM) for 3 h in static culture. Controls were transferred to another dish at start and end of FSS and taken down along with the FSS treated samples. RANKL mRNA expression was analyzed by RT-PCR. The fold induction of RANKL/18S mRNA relative to control was calculated by quantitative PCR (qPCR) and is indicated below the gel.

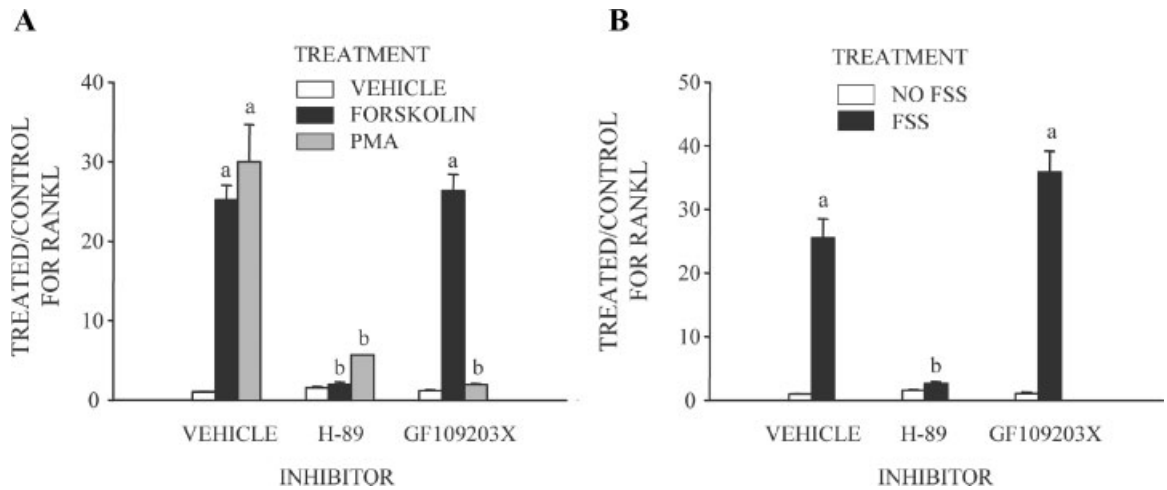

Fig. 6. Comparison of the effects of PKA and PKC agonists and inhibitors on RANKL mRNA levels. Cultures were treated with or without H-89 (30 μM) or GF109203X (0.25 μg/ml) beginning 30 min prior to FSS and continuing throughout FSS and post-FSS culture. Controls were transferred to another dish at start and end of FSS and taken down along with the FSS treated samples. Analysis was by quantitative PCR. RQ values were calculated by ΔΔct method using a reference sample from which the treated over control values were calculated. **A**: Effects of agonists and inhibitors in static culture. Cells (three slides per group) were treated with forskolin (10 μM) or PMA (1 μM) for 3 h. **B**: Effects of inhibitors on the FSS induction of RANKL. Cells (three slides per group) were subjected to FSS for 1 h and returned to static culture for 2 h. [a]Significant effect of agonist or FSS, $P < 0.01$. [b]Significant inhibition by inhibitor, $P < 0.01$.

As additional confirmation that the PKC pathway was not involved, we examined the effects of another PKC inhibitor, Go6983, on FSS induced RANKL expression by qPCR. Go6983 (1 μM) had no effect on the forskolin induction of RANKL mRNA, while inhibiting the PMA induction of RANKL by 85% (data not shown). FSS caused a seven-fold increase in the RANKL mRNA expression, which was unaffected by Go6983 (data not shown).

Role of the ERK Pathway in the FSS Induction of RANKL

FSS induced a rapid and transient phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 in primary calvarial osteoblasts (Fig. 7A). Phosphorylation peaked at 5 min of FSS and returned to baseline by 30 min of FSS. We then examined the effects of PD98059 (50 μM) on the FSS induction of RANKL (Fig. 7B).

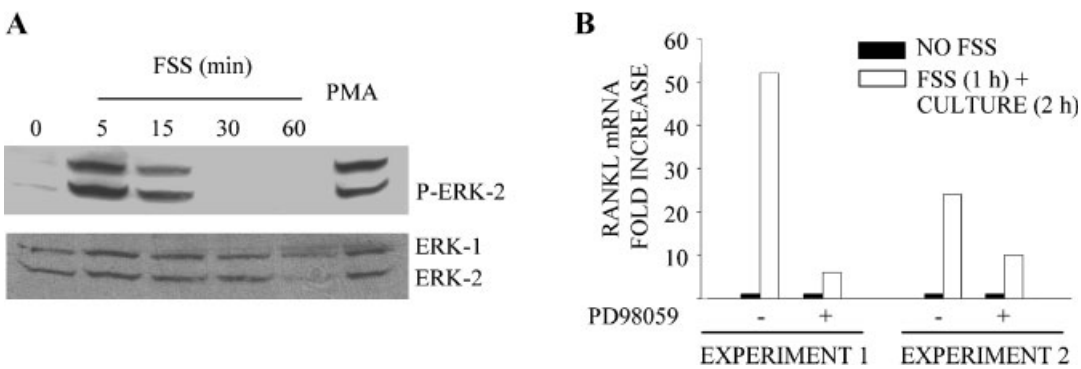

Fig. 7. Involvement of the ERK pathway in the FSS induction of RANKL. **A**: Western analysis for ERK-1/2 phosphorylation. Cells were subjected to FSS from 5 to 60 min and analyzed immediately. PMA (1 μM, 30 min) was used as a positive control. Antibodies were specific for phosphorylated ERK (p-ERK) or total ERK. **B**: Effect of PD98059 (50 μM) on the FSS induction of RANKL. After 1 h of FSS cells were returned to static culture for 2 h. Controls were transferred to another dish at the start and end of FSS. Quantitative PCR was done to analyze RANKL mRNA expression. Each RQ value was calculated relative to its corresponding static control. Results are from two independent experiments.

In two independent experiments, PD98059 inhibited the FSS induction of RANKL mRNA expression by 60%–90% at 2 h of post-FSS static culture.

DISCUSSION

A number of studies have shown that only a brief period of loading is necessary to generate anabolic effects in bone [Rubin et al., 2001; Robling and Turner, 2002; Lee et al., 2003], that increased duration of loading does not increase bone formation [Umemura et al., 1997], and that the anabolic response to loading declines soon after loading is initiated and mechanosensitivity is increased by the insertion of rest periods [Robling et al., 2001, 2002; Srinivasan et al., 2002; Umemura et al., 2002]. Hence, it is possible that what happens after mechanical loading is stopped might be as important as effects occurring during loading. For this reason we examined the development of gene expression after stopping briefly applied FSS. We found that 5 min or 1 h of FSS, followed by static culture, was as effective in stimulating RANKL mRNA expression as 4 h of continuous FSS.

Skeletal adaptation to mechanical loading is thought to occur through regulation of modeling and remodeling [Lanyon and Skerry, 2001]. Although modeling, in which new bone formation is stimulated on quiescent surfaces without preceding resorption, is thought to occur largely during skeletal growth, renewed modeling can occur on periosteal surfaces in response to loading [Pead et al., 1988; Forwood et al., 1996; Chow et al., 1998; Boyde, 2003]. Remodeling, in which bone resorption is coupled to bone formation in focal remodeling units, occurs throughout life, and some regulation of remodeling would seem to be necessary for adaptive changes in bone architecture in response to mechanical loading. An imbalance in remodeling, such that resorption exceeds formation, causes bone loss and can lead to osteoporosis. Loading has been proposed to be antiresorptive [Huiskes et al., 2000; Lanyon and Skerry, 2001] and there are models in which loading decreases osteoclast number or activity [Hillam and Skerry, 1995; Hagihara et al., 2005]. It has been suggested that age-related osteoporosis results from a decline in the level of physical activity that loads bone, combined with a failure of the appropriate osteogenic response of cells in the remodeling units to load bearing, thereby leading to increased sites of resorption activation, each with negative remodeling balance [Forwood et al., 1996].

In the current study, brief exposure to FSS increased basal expression of RANKL, which is required for the differentiation and activity of osteoclasts, with less stimulatory effect on OPG, which opposes the action of RANKL. It might seem contradictory that a system designed to model mechanical loading, which should ultimately result in increased bone formation, would initially increase RANKL, which is essential for bone resorption. However, there are potent stimulators of RANKL expression and bone resorption, such as PGE_2 and PTH, which have anabolic actions in vivo [Reeve et al., 1980; Hock et al., 1988; Jee and Ma, 1997; Qin et al., 2004]. Moreover, both PTH and PGE_2 have been shown to enhance the anabolic effects of mechanical loading [Tang et al., 1997; Li et al., 2003]. Through mechanisms not yet understood, PTH can activate remodeling but stimulate formation more than resorption, although it is not clear if resorption is necessary for the anabolic effects of PTH [Martin, 2004]. There is also an in vivo study suggesting that mechanical loading can upregulate RANKL expression. Mice were subjected to catabolic signals (unloading by hind limb suspension) and anabolic signals (platform vibration for 10 min/day, 5 days a week) for 4 or 21 days [Judex et al., 2005]. As expected, the catabolic regimen decreased, and the anabolic regimen increased, bone formation rates and mineralization surface in the tibiae. Whole tibiae were extracted for mRNA analysis. RANKL mRNA levels in the tibiae were not significantly affected by unloading at either time point but were significantly increased by loading after 21 days.

RANKL expression in bone cells can be regulated by deforming the substrate on which the cells are grown. In studies of murine bone marrow and bone marrow stromal cells on flexible membranes, 6–24 h of equibiaxial mechanical strain at 10 Hz, ranging from 0.25% to 2% strain, inhibited $1,25(OH)_2$ vitamin D_3-stimulated RANKL expression and osteoclastogenesis [Rubin et al., 2000, 2002, 2003]. This effect was dependent on ERK phosphorylation. The authors noted that the strain system used in this work did not impose any biologically relevant fluid shear stress on the cells. No effect on basal RANKL expression was reported. In human osteoblasts, tensile strain

(7% strain, 0.25 Hz) applied 4 h a day for 3 days decreased basal RANKL mRNA expression, an effect mediated by the p38 MAP kinase pathway [Kusumi et al., 2005]. On the other hand, there was a trend for basal RANKL protein levels to increase when human osteosarcoma MG-63 cells were loaded by cyclic 3-point bending (0.35% strain, 0.9 Hz) of their substrate for 2 h followed by 1 h incubation [Saunders et al., 2005]. Substrate deformation studies have also been done on cells from other tissues. Although large cyclic tensile strains (20%) decreased interleukin-1 stimulated RANKL expression in fibrochondrocytes, there was no effect on basal RANKL expression [Deschner et al., 2005]. Similarly, cyclic tensile strains of 20% had no effect on basal RANKL expression in human periodontal ligament cells [Tsuji et al., 2004]. Our experiments differed from previous experiments in cell type (primary murine osteoblasts) and loading protocol (briefly applied fluid shear followed by up to 24 h of static culture).

It seems likely that effects on gene expression will depend not only on the cell type but also on the type of loading. Several studies have reported differences in the effects of fluid flow and substrate loading on gene expression. For example, a variety of osteoblastic cells, including rat calvarial cells, MC3T3-E1 cells, and UMR-106 cells, were subjected to unidirectional linear strains up to 0.5% or to pulsatile laminar fluid flow [Smalt et al., 1997]. Low levels of fluid flow (0.1 dynes/cm^2) induced both NO and PGE_2 production but neither was detected after substrate deformation. Similarly, another side-by-side comparison study in human bone cells found that pulsating fluid flow but not cyclic strain applied through the substrate increased PGE_2 [Mullender et al., 2004]. Hence, different types of loading, established to model different in vivo loading environments, may generate different signaling pathways.

Signal transduction pathways regulating RANKL expression in osteoblasts/stromal cells fall into at least four categories: vitamin D receptor, PKA, calcium/PKC, and gp130 mediated signals [Suda et al., 1995; Takami et al., 2000]. We found both PKA and PKC agonists, forskolin and PMA, to be potent stimulators of RANKL mRNA in primary osteoblasts. Five min of FSS caused a significant increase in cAMP production. The FSS induction of cAMP in osteoblasts has been reported previously and said to be prostaglandin dependent since it was prevented by a non-specific inhibitor of cyclooxygenase activity [Reich et al., 1990]. Our experiments were done in the presence of a selective COX-2 inhibitor, which does not rule out a rapid release of prostaglandin secondary to COX-1 activity. H-89, frequently used as a PKA inhibitor, blocked the FSS induction of RANKL but also blocked the PMA induction of RANKL. However, two specific inhibitors of the PMA induction of RANKL had no effect on the FSS induction of RANKL, and we concluded that the FSS induction was primarily dependent on the PKA pathway. PTH also stimulates RANKL mRNA expression through the PKA pathway [Lee and Lorenzo, 2002; Kondo et al., 2002].

Another pathway that may be involved in the FSS induction of RANKL is the protein kinase D (PKD) pathway, formerly called PKC mu (PKCμ) [Lint et al., 2002]. PKD is a serine/threonine kinase that can be activated by phorbol esters. We have shown that 10 min of FSS phosphorylates Ser744/748, transphosphorylation sites located in the activation loop of PKD, and Ser916, an autophosphorylation site located at the C-terminal of PKD [Mehrotra et al., 2004]. Although PKC inhibitors can inhibit PKD activity, GF109203X, at the dose used in this study (0.25 μg/ml), and Go6983 are not predicted to inhibit PKD [Gschwendt et al., 1996; Shapiro et al., 2002]. Since H-89 has been predicted to have a higher sensitivity for PKD than for members of the PKC family, some of its effect may be due to inhibiting the PKD pathway [Johannes et al., 1995]. Therefore, along with PKA, the PKD pathway may be involved in the FSS induction of RANKL.

The ERK signaling pathway is involved in the regulation of several genes by mechanical loading [Hughes-Fulford, 2004]. The decrease of $1,25(OH)_2$ vitamin D_3-stimulated RANKL expression in marrow stromal cells by substrate deformation was mediated by ERK phosphorylation [Rubin et al., 2002, 2003]. We previously showed that FSS induced phosphorylation of ERK in MC3T3-E1 osteoblastic cells, derived from mouse calvariae, with peak effects at 5 min [Wadhwa et al., 2002a]. Inhibiting ERK phosphorylation with the specific inhibitor PD98059 inhibited the FSS induction of COX-2 mRNA by 55%–70% in MC3T3-E1 cells. In the current study, FSS induced phosphorylation of ERK in primary murine calvarial osteoblastic cells,

with peak effects at 5 min and PD98059 suppressed the FSS induction of RANKL mRNA by 60%–90%. Hence, both stimulatory and inhibitory effects on RANKL of different mechanical loading regimens are dependent on the ERK pathway. Both the PKA and PKC pathways have been implicated as being upstream of the activation of ERK by mechanical signals [Traub et al., 1997; Jessop et al., 2001]. Thus, ERK appears to represent a common distal pathway where multiple mechanical signals converge.

Greater understanding of the signaling pathways that are activated by FSS might lead to new pharmacological therapies for preventing and treating bone loss of aging and osteoporosis. Purely antiresorptive therapies may suppress the bone formation response that would normally be coupled to resorption and thus have limited ability to build new bone [Seeman, 2003]. The best therapeutic agents would seem to be those that have the ability to activate remodeling by stimulating resorption, but tip the balance of remodeling cycles in favor of formation. It is possible that mechanical loading could also generate anabolic effects via stimulation of such unbalanced remodeling cycles.

ACKNOWLEDGMENTS

The authors thank Cindy Alander for performing sequential digestions to obtain the primary calvarial osteoblasts and Dr. Lawrence Raisz for reading the manuscript and making helpful comments. Funding for this study came from NIH RO1AR47673 (PI: Pilbeam).

REFERENCES

Boyde A. 2003. The real response of bone to exercise. J Anat 203:173–189.

Burger EH, Klein-Nulend J. 1999. Mechanotransduction in bone–role of the lacuno-canalicular network. FASEB J 13 (Suppl):S101–S112.

Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H. 1990. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem 265:5267–5272.

Chow JW, Wilson AJ, Chambers TJ, Fox SW. 1998. Mechanical loading stimulates bone formation by reactivation of bone lining cells in 13-week-old rats. J Bone Miner Res 13:1760–1767.

Deschner J, Wypasek E, Ferretti M, Rath B, Anghelina M, Agarwal S. 2005. Regulation of RANKL by biomechanical loading in fibrochondrocytes of meniscus. J Biomech (Epub ahead of print).

Epstein MAF, Peterson DR, Bennett JC. 1995. Design and validation of a large scale flow chamber for exposure of cells to controlled shear stress conditions. Ann Biomed Eng 23 (Suppl 2):S53.

Forwood MR, Owan I, Takano Y, Turner CH. 1996. Increased bone formation in rat tibiae after a single short period of dynamic loading in vivo. Am J Physiol 270:E419–E423.

Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller H-J, Johannes F-J. 1996. Inhibition of protein kinase C μ by various inhibitors. Differentiation from protein kinase C isoenzymes. FEBS Letts 392:77–80.

Hagihara Y, Fukuda S, Goto S, Iida H, Yamazaki M, Moriya H. 2005. How many days per week should rats undergo running exercise to increase BMD? J Bone Miner Metab 23:289–294.

Hillam RA, Skerry TM. 1995. Inhibition of bone resorption and stimulation of formation by mechanical loading of the modeling rat ulna in vivo. J Bone Miner Res 10:683–689.

Hillsley MV, Frangos JA. 1993. Review: Bone tissue engineering: The role of interstitial fluid flow. Biotechnol Bioeng 43:573–581.

Hock JM, Gera I, Fonseca J, Raisz LG. 1988. Human parathyroid hormone-(1-34) increases bone mass in ovariectomized and orchidectomized rats. Endocrinology 122:2899–2904.

Hughes-Fulford M. 2004. Signal transduction and mechanical stress. Sci STKE 2004:RE12.

Huiskes R, Ruimerman R, van Lenthe GH, Janssen JD. 2000. Effects of mechanical forces on maintenance and adaptation of form in trabecular bone. Nature 405: 704–706.

Jee WS, Ma YF. 1997. The in vivo anabolic actions of prostaglandins in bone. Bone 21:297–304.

Jessop HL, Sjoberg M, Cheng MZ, Zaman G, Wheeler-Jones CP, Lanyon LE. 2001. Mechanical strain and estrogen activate estrogen receptor alpha in bone cells. J Bone Miner Res 16:1045–1055.

Johannes F-J, Prestle J, Dieterich S, Oberhagemann P, Link G, Pfizenmaier K. 1995. Characterization of activators and inhibitors of protein kinase C mu. Eur J Biochem 227:303–307.

Judex S, Zhong N, Squire ME, Ye K, Donahue LR, Hadjiargyrou M, Rubin CT. 2005. Mechanical modulation of molecular signals which regulate anabolic and catabolic activity in bone tissue. J Cell Biochem 94: 982–994.

Kanzaki H, Chiba M, Shimizu Y, Mitani H. 2002. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res 17(2):210–220.

Knothe Tate ML, Knothe U, Niederer P. 1998. Experimental elucidation of mechanical load-induced fluid flow and its potential role in bone metabolism and functional adaptation. Am J Med Sci 316:189–195.

Kondo H, Guo J, Bringhurst FR. 2002. T Cyclic adenosine monophosphate/protein kinase A mediates parathyroid hormone/parathyroid hormone-related protein receptor regulation of osteoclastogenesis and expression of RANKL and osteoprotegerin mRNAs by marrow stromal cells. J Bone Miner Res 17:1667–1679.

Kusumi A, Sakaki H, Kusumi T, Oda M, Narita K, Nakagawa H, Kubota K, Satoh H, Kimura H. 2005. Regulation of synthesis of osteoprotegerin and soluble receptor activator of nuclear factor-κB ligand in normal human osteoblastsvia the p38 mitogen-activated protein kinase pathway by application of cyclic tensile strain. J Bone Miner Metab 23:373–381.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176.

Lanyon L, Skerry T. 2001. Postmenopausal osteoporosis as a failure of bone's adaptation to functional loading: A hypothesis. J Bone Miner Res 16:1937–1947.

Lee SK, Lorenzo JA. 2002. Regulation of receptor activator of nuclear factor-kappa B ligand and osteoprotegerin mRNA expression by parathyroid hormone is predominantly mediated by the protein kinase A pathway in murine bone marrow cultures. Bone 31:252–259.

Lee K, Jessop H, Suswillo R, Zaman G, Lanyon L. 2003. Endocrinology: Bone adaptation requires oestrogen receptor-alpha. Nature 424:389.

Li X, Pilbeam CC, Pan L, Breyer RM, Raisz LG. 2002. Effects of prostaglandin E_2 on gene expression in primary osteoblastic cells from prostaglandin receptor knockout mice. Bone 30:567–573.

Li J, Duncan RL, Burr DB, Gattone VH, Turner CH. 2003. Parathyroid hormone enhances mechanically induced bone formation, possibly involving L-type voltage-sensitive calcium channels. Endocrinology 144:1226–1233.

Lint JV, Rykx A, Vantus T, Vandenheede JR. 2002. Getting to know protein kinase D. Int J Biochem Cell Biol 34: 577–581.

Martin TJ. 2004. Does bone resorption inhibition affect the anabolic response to parathyroid hormone? Trends Endocrinol Metab 15:49–50.

Mehrotra M, Alander CB, Voznesensky O, Raisz LG, Pilbeam CC. 2004. Fluid shear stress induces cyclooxygenase-2 via activation of protein kinase D in murine primary osteoblasts. J Miner Res 19(S1):S390.

Mullender M, El Haj AJ, Yang Y, van Duin MA, Burger EH, Klein-Nulend J. 2004. Mechanotransduction of bone cells in vitro: Mechanobiology of bone tissue. Med Biol Eng Comput 42:14–21.

Pead MJ, Skerry TM, Lanyon LE. 1988. Direct transformation from quiescence to bone formation in the adult periosteum following a single brief period of bone loading. J Bone Miner Res 3:647–656.

Peterson DR. 1995. Development of a parallel plate flow chamber to study the fluid shear effects on living cells. Thesis Dissertation, Mechanical Engineering, U Conn.

Pilbeam CC, Fall PM, Alander CB, Raisz LG. 1997. Differential effects of nonsteroidal anti-inflammatory drugs on constitutive and inducible prostaglandin G/H synthase in cultured bone cells. J Bone Miner Res 12: 1198–1203.

Qin L, Raggatt LJ, Partridge NC. 2004. Parathyroid hormone: A double-edged sword for bone metabolism. Trends Endocrinol Metab 15:60–65.

Reeve J, Meunier PJ, Parsons JA, Bernat M, Bijvoet OL, Courpron P, Edouard C, Klenerman L, Neer RM, Renier JC, Slovik D, Vismans FJ, Potts JT, Jr. 1980. Anabolic effect of human parathyroid hormone fragment on trabecular bone in involutional osteoporosis: A multicentre trial. Br Med J 280:1340–1344.

Reich KM, Gay CV, Frangos JA. 1990. Fluid shear stress as a mediator of osteoblast cyclic adenosine monophosphate production. J Cell Physiol 143:100–104.

Robling AG, Turner CH. 2002. Mechanotransduction in bone: Genetic effects on mechanosensitivity in mice. Bone 31:562–569.

Robling AG, Burr DB, Turner CH. 2001. Recovery periods restore mechanosensitivity to dynamically loaded bone. J Exp Biol 204:3389–3399.

Robling AG, Hinant FM, Burr DB, Turner CH. 2002. Improved bone structure and strength after long-term mechanical loading is greatest if loading is separated into short bouts. J Bone Miner Res 17:1545–1554.

Rubin J, Murphy T, Nanes MS, Fan X. 2000. Mechanical strain inhibits expression of osteoclast differentiation factor by murine stromal cells. Am J Physiol Cell Physiol 278:C1126–C1132.

Rubin C, Xu G, Judex S. 2001. The anabolic activity of bone tissue, suppressed by disuse, is normalized by brief exposure to extremely low-magnitude mechanical stimuli. FASEB J 15:2225–2229.

Rubin J, Murphy TC, Fan X, Goldschmidt M, Taylor WR. 2002. Activation of extracellular signal-regulated kinase is involved in mechanical strain inhibition of RANKL expression in bone stromal cells. J Bone Miner Res 17: 1452–1460.

Rubin J, Murphy TC, Zhu L, Roy E, Nanes MS, Fan X. 2003. Mechanical strain differentially regulates endothelial nitric-oxide synthase and receptor activator of nuclear kappa B ligand expression via ERK1/2 MAPK. J Biol Chem 278:34018–34025.

Saunders MM, Taylor AF, Du C, Zhou Z, Pellegrini VD, Jr., Hj D. 2005. Mechanical stimulation effects on functional end effectors in osteoblastic MG-63 cells. J Biomech (Epub ahead of print).

Seeman E. 2003. The structural and biomechanical basis of the gain and loss of bone strength in women and men. Endocrinol Metab Clin North Am 32:25–38.

Shapiro BA, Ray S, Jung E, Allred WT, Bollag WB. 2002. Putative conventional protein kinase C inhibitor Godecke 6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole] stimulates transglutaminase activity in primary mouse epidermal keratinocytes. J Pharmacol Exp Ther 302: 352–358.

Smalt R, Mitchell FT, Howard RL, Chambers TJ. 1997. Induction of NO and prostaglandin E2 in osteoblasts by wall-shear stress but not mechanical strain. Am J Physiol 273:E751–E758.

Smit TH, Burger EH, Huyghe JM. 2002. A case for strain-induced fluid flow as a regulator of BMU-coupling and osteonal alignment. J Bone Miner Res 17:2021–2029.

Srinivasan S, Gross TS. 2000. Canalicular fluid flow induced by bending of a long bone. Med Eng Phys 22: 127–133.

Srinivasan S, Weimer DA, Agans SC, Bain SD, Gross TS. 2002. Low-magnitude mechanical loading becomes osteogenic when rest is inserted between each load cycle. J Bone Miner Res 17:1613–1620.

Suda T, Udagawa N, Nakamura I, Miyaura C, Takahashi N. 1995. Modulation of osteoclast differentiation by local factors. Bone 17:87S–91S.

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. 1999. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20:345–357.

Takami M, Takahashi N, Udagawa N, Miyaura C, Suda K, Woo JT, Martin TJ, Nagai K, Suda T. 2000. Intracellular calcium and protein kinase C mediate expression of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in osteoblasts. Endocrinology 141:4711–4719.

Tang LY, Cullen DM, Yee JA, Jee WS, Kimmel DB. 1997. Prostaglandin E2 increases the skeletal response to mechanical loading. J Bone Miner Res 12:276–282.

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15781.

Traub O, Monia BP, Dean NM, Berk BC. 1997. PKC-epsilon is required for mechano-sensitive activation of ERK1/2 in endothelial cells. J Biol Chem 272:31251–31257.

Tsuji K, Uno K, Zhang GX, Tamura M. 2004. Periodontal ligament cells under intermittent tensile stress regulate mRNA expression of osteoprotegerin and tissue inhibitor of matrix metalloprotease-1 and -2. J Bone Miner Metab 22:94–103.

Turner CH. 1998. Three rules for bone adaptation to mechanical stimuli. Bone 23:399–407.

Turner CH, Pavalko FM. 1998. Mechanotransduction and functional response of the skeleton to physical stress: The mechanisms and mechanics of bone adaptation. J Orthop Sci 3:346–355.

Turner CH, Forwood MR, Otter MW. 1994. Mechanotransduction in bone: Do bone cells act as sensors of fluid flow? FASEB J 8:875–878.

Udagawa N, Takahashi N, Jimi E, Matsuzaki K, Tsurukai T, Itoh K, Nakagawa N, Yasuda H, Goto M, Tsuda E, Higashio K, Gillespie MT, Martin TJ, Suda T. 1999. Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: Receptor activator of NF-kappa B ligand. Bone 25:517–523.

Umemura Y, Ishiko T, Yamauchi T, Kurono M, Mashiko S. 1997. Five jumps per day increase bone mass and breaking force in rats. J Bone Miner Res 12:1480–1485.

Umemura Y, Sogo N, Honda A. 2002. Effects of intervals between jumps or bouts on osteogenic response to loading. J Appl Physiol 93:1345–1348.

Wadhwa S, Godwin SL, Peterson DR, Epstein MA, Raisz LG, Pilbeam CC. 2002a. Fluid flow induction of cyclo-oxygenase 2 gene expression in osteoblasts is dependent on an extracellular signal-regulated kinase signaling pathway. J Bone Miner Res 17:266–274.

Wadhwa S, Choudhary S, Voznesensky M, Epstein M, Raisz L, Pilbeam C. 2002b. Fluid flow induces COX-2 expression in MC3T3-E1 osteoblasts via a PKA signaling pathway. Biochem Biophys Res Commun 297:46–51.

Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett FS, Frankel WN, Lee SY, Choi Y. 1997. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 272:25190–25194.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. 1998a. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602.

Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, Sato Y, Goto M, Yamaguchi K, Kuriyama M, Kanno T, Murakami A, Tsuda E, Morinaga T, Higashio K. 1998b. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): A mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology 139:1329–1337.