Vibrational force alters mRNA expression in osteoblasts

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ABSTRACT **Serum-deprived mouse osteoblastic (MC3T3E1) cells were subjected to a vibrational force modeled by NASA to simulate a space shuttle launch (7.83 G rms). The mRNA levels for eight genes were investigated to determine the effect of vibrational force on mRNA expression. The mRNA levels of two growth-related protooncogenes, c-fos and c-myc, were up-regulated significantly within 30 min after vibration, whereas those of osteocalcin as well as transforming growth factor-β1 were decreased significantly within 3 h after vibration. No changes were detected in the levels of β-actin, histone H4, or cytoplasmic phospholipase A2 after vibration. No basal levels of cyclooxygenase-2 expression were detected. In addition, the extracellular concentrations of prostaglandin E_2 (PGE_2), a potent autocrine/paracrine growth factor in bone, were not significantly altered after vibration most likely due to the serum deprivation state of the osteoblasts. In comparison with the gravitational launch profile, vibrational-induced changes in gene expression were greater both in magnitude and number of genes activated. Taken together, these data suggest that the changes in mRNA expression are due to a direct mechanical effect of the vibrational force on the osteoblast cells and not to changes in the local PGE_2 concentrations. The finding that launch forces induce gene expression is of utmost importance since many of the biological experiments do not dampen vibrational loads on experimental samples. This lack of dampening of vibrational forces may partially explain why 1-G onboard controls sometimes do not reflect 1-G ground controls. These data may also suggest that scientists use extra ground controls that are exposed to launch forces, have these forces dampened on launched samples, or use facilities such as Biorack that provide an onboard 1-G centrufuge in order to control for space shuttle launch forces.—Tjandrawinata, R. R., Vincent, V. L., Hughes-Fulford, M. Vibrational force alters mRNA expression in osteoblasts. *FASEB J.* 11, 493–497 (1997)**

Key Words: vibration · shear force · mRNA expression · mechanical stress

ALL TISSUES IN THE BODY ARE subjected to both passive and active forces originating either from tension created by cells themselves or forces from their environment (1). Bone continually adapts to a pattern of daily mechanical stress and is sensitive to changes in this stress pattern, altering its mass and geometry in response to the new forces (2). The mechanisms used by cells for sensing and responding to mechanical perturbations are not known. It has been proposed that mechanical forces can activate adenylate cyclase (3), phospholipase C (4), PGE_2 (5), and mechanosensitive ion channels (6).

We have found previously that gravitational force (3 *g*) mimicking that of a space shuttle launch can up-regulate c-fos mRNA expression concomitantly with a decrease in osteocalcin (OC)[2] mRNA expression (7). OC is a gene coding for a bone matrix protein that is expressed only during bone differentiation. We also reported that growth activation of osteoblasts in microgravity (during a spaceflight mission) resulted in reduced growth, glucose utilization, prostaglandin synthesis, and significantly altered actin cytoskeleton (8).

Various reports have described gravity-specific changes in mRNA levels after exposure of cells or animals to microgravity (9) or hypergravity (10). However, to our knowledge no studies have investigated the effect of the vibrational force on mRNA expression that occurs during space shuttle launch. The vibrational force originated from the NASA-designed amplifier-controlled shaker head that simulates vibrations of a space shuttle launch. The present studies were conducted in order to examine the effects of launch forces on biological flight samples by monitoring alterations in gene expression; we also wanted to investigate whether such alterations mimic either the cellular proliferation or differentiation in response to vibrational forces similar to that experienced during a space shuttle launch and whether launch forces should be controlled in flown biological samples. We report here that simulated launch vibrations significantly alter mRNA levels of genes involved in growth and differentiation of growth-arrested osteoblasts.

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[2] Abbreviations: OC, osteocalcin; COX-2, cyclooxygenase-2; $cPLA_2$, cytoplasmic phospholipase A_2; HisH4, histone H4; PGE_2, prostaglandin E_2.

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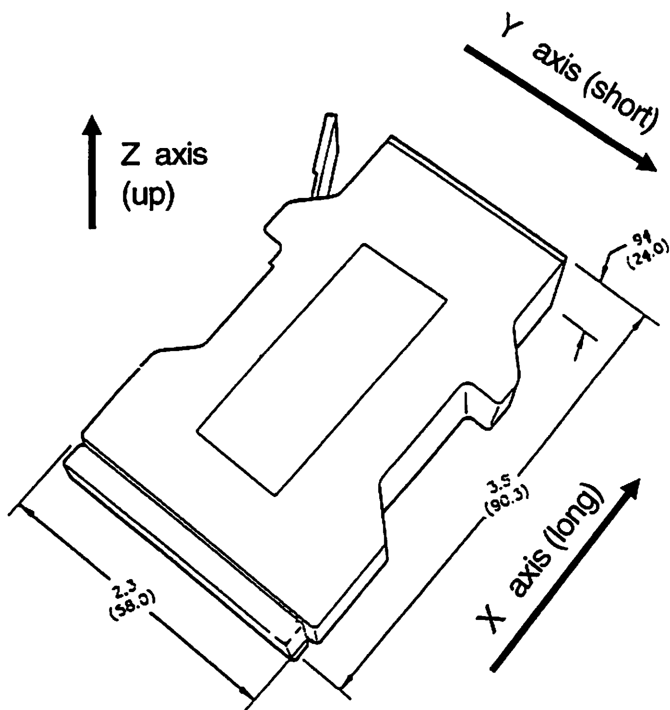

Figure 1. Axis definition for type I container. All dimensions are in inches and millimeters (mm).

MATERIALS AND METHODS

Materials

α-MEM medium was purchased from Fisher Scientific (Pittsburgh, Pa.). L-glutamine and HEPES were from UCSF Cell Culture Facility (San Francisco, Calif.); fetal bovine serum was from HyClone (Logan, Utah). Antibiotic-antimycotic solution (containing penicillin, streptomycin, and amphotericin B) were from Sigma Cell Culture (St. Louis, Mo.).

Experimental hardware

Experimental hardware was designed by Centrum voor Constructie en Mechatronica (Nuenen, the Netherlands) specifically for our experiment, "OSTEO," which is to be flown as part of the BIORACK payload on space shuttle flights STS-76, 81, and 84. The hardware consists of two different units: the plunger box unit type HM 2/3 and the type I experiment container. The plunger box unit contains two cell chambers, each holding two 22 × 11 mm glass coverslips on which the cells grow. The plunger box is placed into a type I container that provides a secondary containment for biological samples during the experiment.

Cell culture

Mouse osteoblastic MC3T3-E1 cells were grown in T-75 flasks with 10% fetal bovine serum-containing α-MEM medium supplemented with L-glutamine, HEPES, and antibiotic-antimycotic solution (containing penicillin, streptomycin, and amphotericin B). Cells were maintained at high density in a 37°C incubator with 5% CO2 and fed three times a week. For each experiment, 120,000 cells were plated onto each coverslip (Thomas Scientific, Swedesboro, N.J.), placed in 6-well plates, and grown in 10% serum-containing medium overnight. Cell-coated coverslips were transferred into the plunger box units in 1% fetal bovine serum-containing medium. The units were incubated for 17 h at room temperature before the experiment to ensure that changes in mRNA levels were due to the vibration and not to serum factors or other components in the culture medium after our Biorack space shuttle flight experimental protocol.

Vibration

The vibrational experiment was carried out at the NASA Ames facility. The type I containers (with cell-containing plunger boxes inside) were attached to a Shaker Head A300B made by Ling Electronics in a space shuttle launch configuration. The NASA-maintained vibrational testing facility exerted a vibrational profile using a shaker head that was controlled by a 35 kVA amplifier under the control of the GenRad 2511 Control System programmed by NASA engineers to mimic a shuttle launch vibrational profile. The frequency of the vibration driven by the amplifier was so subtle that the vibration itself was not apparent to the naked eye. Type I containers were exposed to vibration along the x-axis (as depicted in **Fig. 1**) at 7.838 G rms (vigorous vibration) for a 2 min period (initial launch forces, **Fig. 2*A***), followed by a vibration at 4.098 G rms (minimal vibration) for 6 min [mimics from the time of Max Q (maximum force on the shuttle) to MECO (main engine cutoff, Fig. 2*B*]. These levels were chosen to closely follow NASA's recorded vibration levels that are experienced by com-

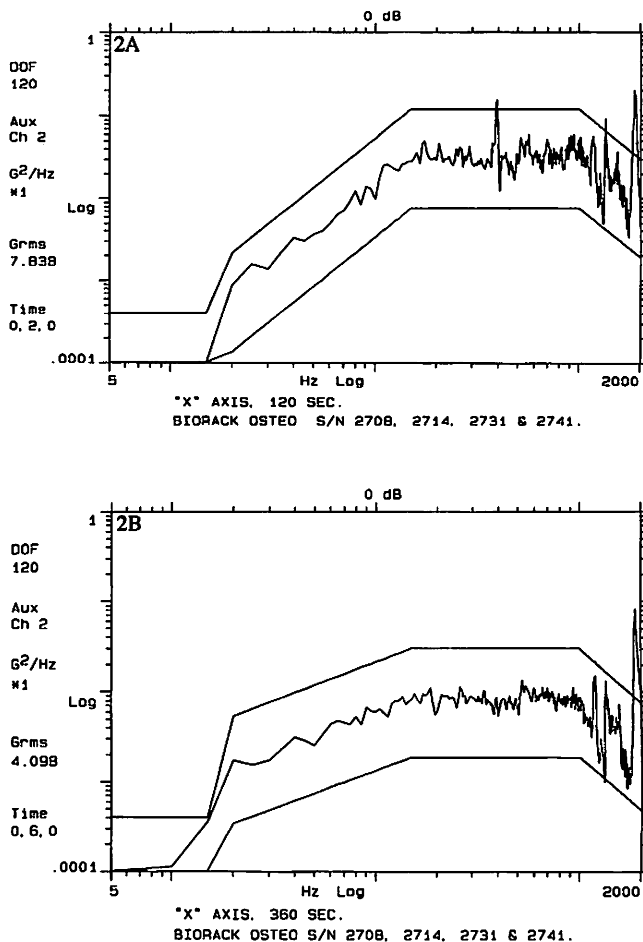

Figure 2. Vibrational profile of space shuttle launch. Pattern of a 2 min vigorous vibration at 7.383 G rms (*A*) and a 6 min minimal vibration at 4.098 G rms (*B*).

ponents in the space shuttle's middeck during launch. The cells were fixed at 32 min (representing the 30 min time point) and 182 min (representing the 3 h time point) after the start of vibration.

RNA isolation

RNA was extracted and purified by the acid guanidium thiocyanate/phenol/chloroform extraction method as described in ref 11, with a slight modification. Each RNA sample was isolated from the cell lysates of two coverslips and represents one data point. The experiment was repeated three times on three different dates. The data presented are representive of at least six data points (12 coverslips) ± SEM. In addition, each time point had its own controls that were not exposed to vibrational force; the data from the both control groups (30 min and 3 h) were combined as the control point.

RT-PCR analysis

The RT-PCR analysis was performed as described previously (12). The primers used for amplification of the c-fos and c-myc genes were taken from ref 13 and for the TGF-β1 gene from ref 14. Other primer sequences were designed from Genebank sequences by MHF: OC sense, 5-TCC TCC TGG TTC ATT TCT TTG, and anti-sense, 5-CCT TAT TGC CCT CCT GCT TGG; β-actin, sense, 5-CCG CAA ATG CTT CTA GGC, and anti-sense, 5-GGT CTC ACG TCA GTG TAC GG; cyclooxygenase-2 (COX-2), sense, 5-GTG CCT GGT CTG ATG ATG TAT GC, and anti-sense, 5-CCA TAA GTC CTT TCA AGG AGA ATG; cytoplasmic phospholipase A_2 ($cPLA_2$), sense, 5-GGA TTC TCT GGT GTG ATG AAG G, anti-sense, 5-CCC AAT CTG CAA ACA TCA GC, histone H4 (HisH4), sense, 5-GAG GAA AGG GCG GAA AAG GCT TAG GCA AAG, anti-sense 5-CAA AAA GGG CCT TTG GGA TCG AAA CGT GCA; and internal standard cyclophilin, sense 5-CGT CTC CTT TGA GCT GTT TGC AGA C, and anti-sense, 5-CAT AAT CAT AAA CTT AAC TCT GCA ATC CAG C. PCR bands were identified on a 2% agarose gel. Ethidium bromide bands were visualized with UV light and photographed with a direct screen instant camera DS-34 (Polaroid Corporation, Cambridge, Mass.). For quantification, the bands of interest were scanned at 400 dpi with LaCie Silver Scanner III (LaCie, Portland, Oreg.) and stored as Macintosh TIFF files. The peak areas and densities were determined using NIH Image 1.55 (an image analysis program written by Wayne Rasband at the U.S. National Institutes of Health, Bethesda, Md.). The data were represented as relative mRNA levels; these were the pixel densities of each gene after correction to the density of the internal standard cyclophilin (i.e., by dividing the pixel densities of each gene by those of cyclophilin).

PGE_2 analysis

The exogenous PGE_2 levels were measured using a PGE_2-Monoclonal Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, Mich.) according to the protocol recommended by the manufacturer. This kit assay system is highly specific for native PGE_2 and does not detect $dmPGE_2$ or any prostaglandin of other series (A, B, D, or F). The samples contained in the 96-well plate were read at 410 nm wavelength using the Dynatech MR5000 Microplate Reader (Dynatech Laboratories, Chantilly, Va.) and the data were analyzed with the BioLinx 2.0 Software (Dynatech Laboratories) run on an IBM-compatible PC.

RESULTS

Alterations in mRNA levels after vibrational launch profile

Messenger RNA expression levels from three separate experiments (N=6) were analyzed for genes involved in growth and/or differentiation of osteoblasts were analyzed after the vigorous vibration profile. The genes analyzed included c-fos, c-myc, OC, TGF-β1, HisH4, $cPLA_2$, COX-2, and the cytoskeletal protein β-actin. As shown in **Fig. 3**, the two proto-oncogenes c-fos and c-myc were up-regulated after vibration. The levels of c-fos and c-myc mRNA increased by four- ($P<0.05$) and twofold ($P<0.05$), respectively, over the control levels 30 min after the vibration profile. However, these levels returned to control levels by the 3 h time point. There were no significant changes in either the HisH4, $cPLA_2$, or cytoskeleton β-actin mRNA levels detected after vibration as compared to the controls. No basal level of COX-2 mRNA was detected.

We also determined the levels of OC and TGF-β1 mRNA. There was no alteration in the OC mRNA level seen at 30 min after vibration. However, the OC mRNA level was significantly decreased (fourfold, $P<0.001$) 3 h after vibration as compared to the controls. At both 30 min and 3 h time points, TGF-β1 mRNA levels were significantly lower (1.5-fold, $P=0.01$) than the controls.

Levels in PGE_2 released after vibration

We determined the levels of PGE_2 released into the culture medium as a result of the vibrational profile.

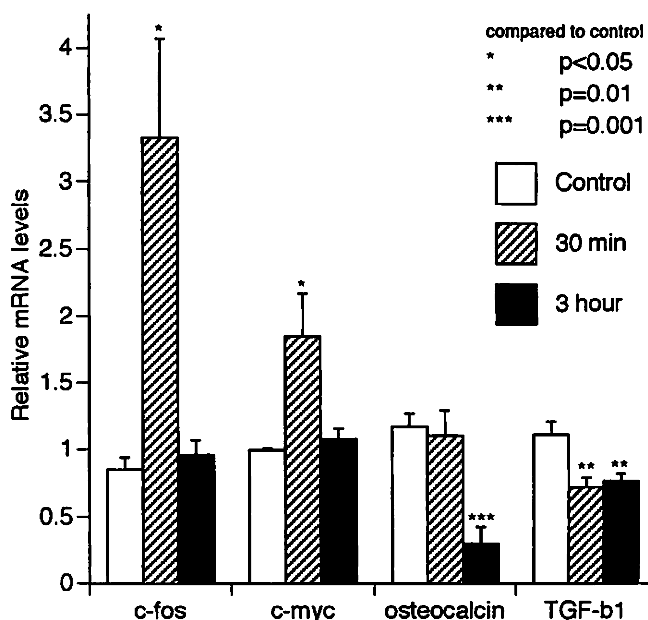

Figure 3. Alterations in mRNA levels in osteoblasts subjected to space shuttle launch vibrational profile. All measurements have been corrected to the internal standard cyclophilin for each data point ± SEM.

There were no significant changes in the levels of extracellular PGE_2 after vibration (**Fig. 4**).

DISCUSSION

Two major forces that could potentially alter gene expression during space shuttle launch are gravity and vibration. We have shown previously that the gravitational force incurred during space shuttle launch is of itself able to induce mRNA expression of the early intermediate gene c-fos as well as decrease mRNA levels of the bone differentiation marker, OC (7). Studies reported in this paper were carried out to determine the effect of the vigorous vibration that occurs during a space shuttle launch on mRNA expression. We found that in osteoblasts subjected to this vibrational profile, c-fos and c-myc mRNA levels were increased, OC and TGF-β1 mRNA levels were decreased, and HisH4 and $cPLA_2$ mRNA levels were not changed. It has previously been reported that induction of genes associated with osteoblast proliferation, c-fos and c-myc, can be reciprocally related to those associated with matrix maturation (TGF-β1) and mineralization (OC) (15). Therefore, the data presented here suggest that vigorous vibrational force induces a cellular response that favors proliferation and inhibits differentiation.

The changes in mRNA expression resulting from the vibrational force were much greater in both magnitude and effect than changes resulting from the gravitational launch profile. Gravitational forces altered mRNA expression of only two genes: c-fos and OC (7). c-fos mRNA increased by twofold ($P<0.05$) above the controls, whereas OC decreased by 2.4-fold ($P<0.005$) as compared to the controls. No profound changes were found in c-myc or TGF-β1 mRNA levels after gravitational stress. The data presented here show that vibrational force up-regulated mRNA levels of c-fos and c-myc by four- and twofold, respectively, over the control levels whereas those of OC and TGF-β1 significantly decreased fourfold and 1.5-fold, respectively, as compared to the controls. The typical 1-G gravity controls are in reality the ground controls, which do not experience launch force; they may not be accurate experimental controls and therefore may cause the differences in 1-G ground controls and onboard 1-G controls. Although the long-term effects of space shuttle launch forces are not yet known, the changes in RNA expression demonstrated in this study show that it is critical to dampen as much of the vibrational force as possible during launch and to have alternate ground controls exposed to launch forces or use facilities such as Biorack that have built-in onboard 1-G gravity controls.

We also studied other biological responders to mechanical stress. PGE_2 has been demonstrated to be a potent inducer of bone cell growth (16). COX-1 and COX-2 are constitutive and inducible enzymes, respectively, that regulate the conversion of arachidonic acid into PGE_2. $cPLA_2$ is a stress- and calcium-inducible enzyme responsible for releasing arachidonic acid from membrane phospholipids to serve as the substrate for COX-1 and COX-2. We did not observe significant changes in the levels of $cPLA_2$ mRNA at either 30 min or 3 h. In addition, basal level expression of COX-2 could not be detected. This indicates that serum deprivation limited the amount of media lipids and unsaturated fatty acid substrates for PGE_2 synthesis. Thus, the extracellular PGE_2 detected was most likely synthesized by the constitutive COX-1 enzyme. Taken together, the data suggest that the changes detected in mRNA expression were not mediated by the osteoblast autocrine/paracrine mediator PGE_2 and were conceivably due to a direct result of the vibrational profile acting through other mechanical stress mechanisms such as mechanosensitive calcium channels to increase mRNA levels.

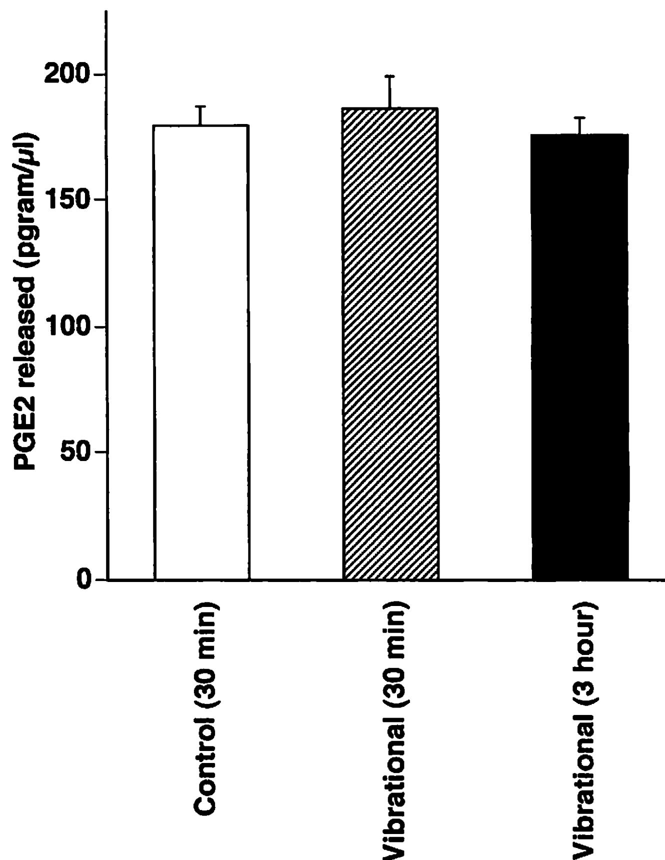

Figure 4. Concentrations of extracellular PGE_2 after vibrational force ± SEM.

The vibrational force along the x-axis (Fig. 1) causes a form of shearing force exerted on the cells by the culture medium in the cell chamber. It is believed that bones experience daily shear stress such as that exerted during vibrational force. Similarly, stress is experienced by endothelial cells in blood cells due to hemodynamic forces. The flow of blood

is associated with shear stress, which acts in the direction of the blood flow on the surface of the inner blood vessel wall (17). Studies indicate that flow-induced shear stress can stimulate the release of prostacyclin (18), hormones (19, 20), as well as growth factors (17). We currently do not know whether bones in the intact animals release similar factors when subjected to vibrational profile. The present study adds to the understanding of molecular mechanisms by which cells adapt themselves in response to external shearing perturbations. In addition, this study provides the first evidence that the vibration force component of the space shuttle launch can by itself change the mRNA expression of cultured cells. These changes in mRNA expression due to launch forces need to be controlled for in subsequent spaceflight experiments measuring the effects of microgravity on biological systems. FJ

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