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SHORT NOTE

Gravitational Loading of a Simulated Launch Alters mRNA Expression in Osteoblasts

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Serum-deprived mouse osteoblastic cells (MC3T3-E1a) were centrifuged under a regime designed to simulate a space shuttle launch (maximum of 3*g*). Messenger RNA levels for eight genes involved in bone growth and maintenance were determined using RT-PCR. Following 30 min of centrifugation, mRNA level for early response gene *c-fos* was significantly increased 89% ($P < 0.05$). The *c-fos* induction was transient and returned to control levels after 3 h. The mRNA level for the mineralization marker gene osteocalcin was significantly decreased to 44% of control level ($P < 0.005$) 3 h after centrifugation. No changes in mRNA levels were detected for *c-myc*, TGFβ1, TGFβ2, cyclophilin A, or actin. No basal mRNA level for TGFβ3 was detected. In addition, no change in the steady-state synthesis of prostaglandin E_2 was detected, possibly due to lack of lipid substrates in serum-deprived cells, suggesting that the increase in *c-fos* mRNA in response to gravitational loading is a result of mechanical stimulation. These results indicate that a small magnitude mechanical loading, such as that experienced during a shuttle launch, can alter mRNA levels in quiescent osteoblastic cells.

INTRODUCTION

The mechanisms by which mammalian cells respond to gravitational signals are unknown. Several reports have described gravity-specific changes in mRNA levels following exposure of cultured cells and whole animals to varying periods of microgravity in experiments performed in spaceflight [1, 2], sounding rockets [7], and clinostats [8]. However, few studies have examined the effect of increased gravity or hypergravity on gene expression. Previous studies demonstrate that changes in gene expression can occur in cells subject to extreme gravitational conditions. Changes detected in HeLa cells in response to 35*g* hypergravity include elevated *c-myc* mRNA, IP_3, and total phosphorylation levels; a reduction in G1-phase duration; and a reduction in cellular cAMP levels [16, 17]. Nose and Shibanuma [20] demonstrated that hypergravity induced *c-fos* (at 900*g*) and another early response gene, *egr-1* (at 50*g*), with little effect on *c-jun* mRNA in cultured mouse MC3T3-E1a osteoblastic cells.

Since there are no reports documenting the effects of physiological stress caused by a space shuttle launch on gene expression, we measured changes in mRNA levels due to a gravitational launch simulation. In this report, we examined quiescent cells for gravity-dependent changes in mRNA levels for eight genes involved in bone cell growth and maturation. We show that *c-fos* and osteocalcin mRNA levels are altered in comparison to noncentrifuged controls. These data are consistent with those of other investigators who show that mechanical loading can alter mRNA levels of key genes involved in bone cell growth and differentiation [13, 23, 27].

MATERIALS AND METHODS

Experimental hardware. Experimental flight hardware was designed according to ESA specifications for use in the BIORACK facility in SpaceHab on the space shuttle and constructed by Centrum voor Constructie en Mechatronica (Nuenen, Netherlands). Each type HM $\frac{2}{3}$ container has two cell chambers each holding two coverslips. RNA was isolated from each chamber and the medium was collected and assayed for prostaglandin E_2 (PGE_2). Material recovered from each chamber (two coverslips) represents one data point and is the contribution of approximately 160,000 cells.

Cell culture. MC3T3-E1a cell line is clonally derived from embryonic mouse calvaria [28]. For each experiment, 80,000 cells were plated onto each 22 × 11-mm glass coverslip (Thomas Scientific, Swedesboro, NJ), placed into six-well multiwells, and grown as described previously [11]. The following day, the coverslips were loaded into flight hardware in media containing 1% fetal calf serum (FCS) and incubated overnight at room temperature.

Centrifugation. At the time of centrifugation, the cells were quiescent since they had been incubating for 18–22 h at room tempera-

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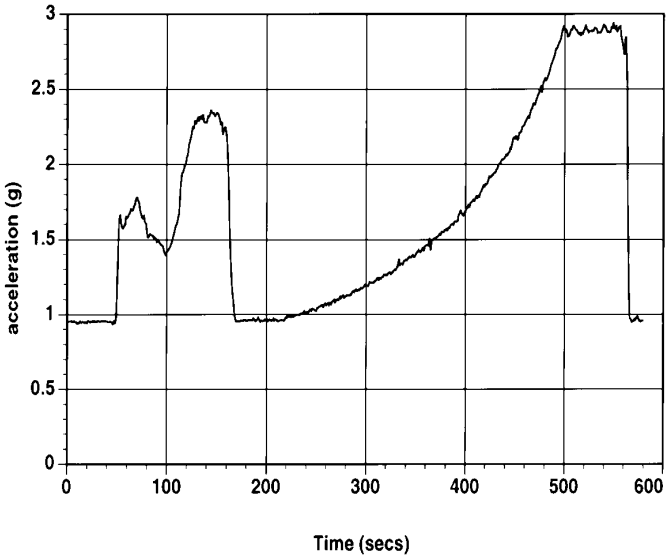

FIG. 1. Gravitational profile for centrifuged samples.

ture in medium containing 1% FCS, in parallel with our BIORACK space shuttle flight protocol. This incubation step effectively depletes the medium of growth factors and ensures that alterations in mRNA levels are due to the mechanical loading induced by centrifugation and not to components in the medium. The cells were centrifuged in the 20*g* centrifuge at NASA Ames (Mountain View, CA). Rotational speed of the centrifuge was varied to simulate the gravitational forces of a space shuttle launch (Fig. 1). The gravitational forces reach a maximum of 2.94*g* 515 s after launch. The cells were fixed at 36 and 186 min after the start of centrifugation. Each centrifugation run had four experimental replicates and two controls at both 36 and 186 min. Two separate centrifugation runs were performed 4 weeks apart. Since there was no difference between the 36- and the 186-min noncentrifuged control mRNA levels, they were pooled and treated as one control in subsequent analysis.

RNA isolation, reverse transcription, and PCR. RNA from cultured MC3T3-E1a osteoblasts was isolated using a modified guanidinium thiocyanate method based on the protocol previously described by Chomczynski and Sacchi [4] (manuscript in preparation). RT and PCRs were performed and PCR products analyzed as previously described [10]. Oligonucleotide primers were designed to span at least one intron in order to detect any contaminating genomic DNA carried over from the RNA isolation step. The actin (ACT) primers (actin-F 5′-CCG CAA ATG CTT CTA GGC-3′, actin-R 5′-GGT CTC ACG TCA GTG TAC GG-3′) amplified both β- and x-actin in MC3T3-E1a cells resulting in a double of bands of 656 and 620 bp. The *c-fos* and *c-myc* primer sequences were from Irving *et al.* [14], and TGFβ genes were from Birch *et al.* [3]. Other primer sequences were designed from Genebank sequences by M.H.F.: osteocalcin, OC-F 5′-TCC TCC TGG TTC ATT TCT TTG-3′ and OC-R 5′-CCT TAT TGC CCT CCT GCT TGG-3′; cyclophilin, CPH1-F 5′-CGT CTC CTT TGA GCT GTT TGC AGA C-3′ and CPH1-R 5′-CAT AAT CAT AAA CTT AAC TCT GCA ATC CAG C-3′. Oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA). Conditions were established so that the amplification reaction was stopped in the linear range so the reaction products could be accurately quantified and compared. To correct for small variations between experiments, each PCR product was compared to either the ACT or the CPH1 PCR products derived from the same RT reaction. ACT was used as the standard for all data analysis; however, the same results were obtained when CPH1 was used as the control. PCR products were electrophoresed on 2.5% agarose gels and photographed on Polaroid 667 film using a Polaroid DS-34 camera. Photographs were then scanned and digitized using the Lacie Silverscan III at 360 dpi into Adobe Photoshop v3.0 program. Density analysis was performed using the public domain NIH Image 1.58 program.

PGE_2 analysis. The exogenous PGE_2 levels were quantitated using the PGE_2 monoclonal enzyme immunoassay kit (Cayman Chemical; Ann Arbor, MI) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

In order to determine whether increased gravitational and acceleration forces of a space shuttle launch affect gene activation, mRNA levels from MC3T3-E1a cells that were centrifuged under a regime designed to simulate the gravity profile of a launch were compared to mRNA levels from noncentrifuged cells. Following centrifugation, mRNA levels for eight genes involved in osteoblast cell growth and differentiation were examined: proto-oncogenes *c-fos* and *c-myc;* osteocalcin (OC); cytokine transforming growth factors β 1, 2, and 3 (TGFβ1, TGFβ2, TGFβ3). In addition, mRNA levels for chaperone cyclophilin A and the cytoskeletal proteins β- and x-actin were examined.

A representative gel from RT-PCR analysis is shown in Fig. 2 and a summary of four to six experiments from two centrifugation runs is shown in Fig. 3. The level of *c-fos* mRNA significantly increased 89% above control levels 30 min after centrifugation ($P < 0.05$).

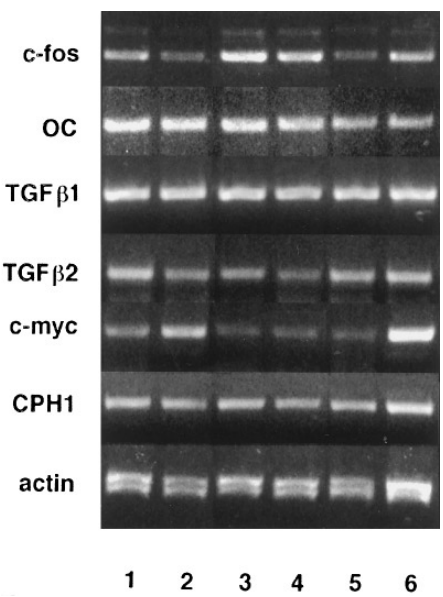

FIG. 2. Effect of gravitational launch profile on mRNA levels in MC3T3-E1a osteoblasts. RT and PCRs were performed as described under Materials and Methods. Lanes 1 and 2 were noncentrifuged controls, lanes 3 and 4 were fixed 36 min after centrifugation, lanes 5 and 6 were fixed 186 min after the start of centrifugation. OC, osteocalcin; TGF, transforming growth factor; and CPH1, cyclophilin A.

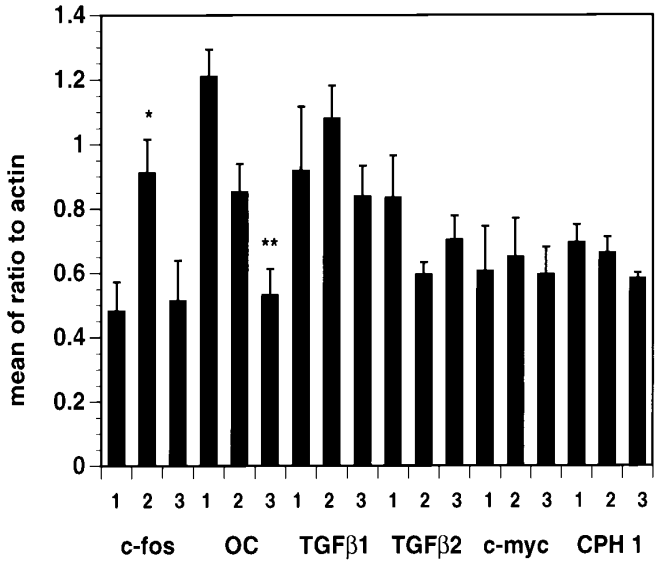

FIG. 3. Summary of alterations in mRNA levels due to centrifugation. Quantitation of PCR band intensity for each gene and time point was generated from four to six data points from two separate centrifugation runs. Values shown were calculated by taking the ratio of the band intensity for each gene to the band intensity of the actin signal amplified from the same RT reaction. Lane 1, samples not subject to centrifugation. Lane 2, cells fixed 36 min after centrifugation. Lane 3, cells fixed 186 min after centrifugation. Errors are ± SEM. *, $P < 0.05$ (Student's t test) compared to mean of *c-fos* control; **, $P < 0.005$ compared to mean of OC control.

c-fos mRNA levels returned to control levels after 3 h. In contrast to this rapid rise in *c-fos* mRNA, OC mRNA levels were reduced to 70% of noncentrifuged controls 30 min after centrifugation. This trend continued at the 3-h time point where the OC mRNA levels were significantly reduced to 44% of control levels ($P < 0.005$). Changes in mRNA levels for TGFβ1, TGFβ2, *c-myc*, or CPH1 were not detected when compared to the controls. We noted that the *c-myc* mRNA levels exhibited some variation although no consistent change was observed. No basal mRNA level for TGFβ3 was detected.

Expression of the proto-oncogene *c-fos* is associated with osteoblast proliferation and differentiation in bones and in the repair of fractures [5, 21]. High gravitational forces have previously been shown to upregulate early response genes *c-myc* in HeLa cells [17] and *egr-1* and *c-fos* in MC3T3-E1a cells [20]. Nose and Shibanuma [20] found that a transient *c-fos* mRNA increase occurred following a 5-min centrifugation at 900*g*. This increase was maximal 30 min after centrifugation and returned to control levels 2 h later. We detected an 89% *c-fos* induction compared to >10-fold induction in the samples centrifuged at 900*g*. Thus, our result is consistent with these reports except for the magnitude of induction which may be accounted for by the difference in centrifugation speeds. These data indicate that low-speed centrifugation can alter gene expression.

The forces experienced by the cells are due to a combination of both increased gravity and the forces of acceleration as the centrifuge rotates. With the current experimental design it was not possible to separate the effects of these two forces. Taken together, the gravitational and acceleration forces can be described as mechanical loading. Previous experiments have shown that *in vitro* mechanical loading transiently stimulates *c-fos* expression in condylar tissue [5], cardiac myocytes [24], caudal vertebra [13], and tibial periosteum [23]. In studies on bone tissue, the time of onset of *c-fos* upregulation was 1 to 2 h following mechanical loading [13, 23]. Our results are consistent with these studies in that a transient increase in *c-fos* mRNA is detected in cells exposed to mechanical stress.

One effect of chronic mechanical stimulation in osteoblast-like cells (UMR-106.01) is to activate and upregulate mechano-sensitive calcium channels [9]. Rapid transcription of the *c-fos* gene is under the control of different signal transduction pathways, several of which depend upon calcium-dependent protein kinase. Therefore, it is possible that *c-fos* is transcribed in response to increased intracellular calcium caused by activation of stretch-activated calcium channels.

High levels of mechanical strain have been shown to stimulate PGE_2 secretion in osteoblasts and osteoblastic cell lines [15, 25]. In addition, in spaceflight under conditions of microgravity, steady-state synthesis of PGE_2 is reduced in cultured MC3T3-E1a cells [11]. Therefore, PGE_2 may act as a general mechanical or gravitational sensing factor whereby under conditions of increased mechanical loading it is synthesized and released but under conditions of very little loading its synthesis is downregulated. However, in this study no differences in PGE_2 levels were detected in the medium of cells exposed to gravitational loading compared to control cells (control, 162.1 ± 3.6 ng/ml; 36 min postcentrifugation, 158.9 ± 3.8 ng/ml; 186 min postcentrifugation, 156.6 ± 4.4 ng/ml). One possible explanation for this is that in quiescent cells, there is a reduction in media lipids and fatty acids available for use as substrates for the arachidonic acid pathway. Downregulation of arachidonic acid metabolism would limit the synthesis and release of PGE_2 and may explain why changes in COX-2 mRNA, which is regulated by PGE_2, were not observed in these experiments. It was also of interest to determine whether PGE_2 synthesis was increased since it has been shown to specifically increase *c-fos* mRNA [6, 10, 26]. Since there is no change on PGE_2 it is unlikely to be responsible for the transient *c-fos* induction. Therefore, these data suggest that the transient *c-fos* increase induced by gravitational loading is not due to chemical stimulation of the arachidonic acid pathway but due to mechanical stimulation.

OC protein is associated with a mature osteoblast phenotype and is expressed prior to the onset of mineralization [12, 18, 22]. In our studies, OC mRNA levels in osteoblasts subjected to a gravitational launch profile were reduced to 44% of control levels (Fig. 2). Previous studies on osteocytic cells have shown that mechanical strain does not alter mRNA OC levels [13, 19]. However, mechanical deformation of MC3T3-E1a osteoblasts increased cell proliferation and decreased OC expression compared to unstrained controls [27]. The differences in OC expression between osteocytic and osteoblastic cells is probably related to function. Osteoblasts participate directly in the mineralization process and are more likely to regulate expression of bone matrix proteins such as OC than osteocytes which only continue to secrete a small amount of matrix.

Our data suggest that a mechanical stress activates some growth-related responses in the absence of PGE_2 release. The magnitude of loading (approximately 3*g*) is similar to the loading experienced during mild exercise such as walking. Thus the changes in gene expression described here may also occur during exercise.

It has been suggested that the events responsible for cellular proliferation and those responsible for mineralization are mutually exclusive [22, 29]. Therefore, changes in response to mechanical stress may indicate which of these states predominates. The data presented here show that the cellular responses to mild transient 3*g* hypergravity are an increase in *c-fos* mRNA and a decrease in OC mRNA levels, suggesting that mechanical stress may reduce differentiation.

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