A Short Pulse of Mechanical Force Induces Gene Expression and Growth in MC3T3-E1 Osteoblasts via an ERK 1/2 Pathway

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ABSTRACT

Physiological mechanical loading is crucial for maintenance of bone integrity and architecture. We have calculated the strain caused by gravity stress on osteoblasts and found that 4–30 g corresponds to physiological levels of 40–300 μstrain. Short-term gravity loading (15 minutes) induced a 15-fold increase in expression of growth-related immediate early gene c-fos, a 5-fold increase in egr-1, and a 3-fold increase in autocrine bFGF. The non–growth-related genes EP-1, TGF-β, and 18s were unaffected by gravity loading. Short-term physiological loading induced extracellular signal-regulated kinase (ERK 1/2) phosphorylation in a dose-dependent manner with maximum phosphorylation saturating at mechanical loading levels of 12 g (p < 0.001) with no effect on total ERK. The phosphorylation of focal adhesion kinase (FAK) was unaffected by mechanical force. 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 MEK1/2 inhibitor U0126 (p < 0.001) but was not affected by MEK1 or p38 MAPK-specific inhibitors. The long-term consequence of a single 15-minute 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 induce immediate early gene expression and growth in MC3T3-E1 osteoblasts primarily through an ERK 1/2-mediated pathway. (J Bone Miner Res 2003;18:58–66)

Key words: MAP kinase, mechanical stress, signal transduction, gravity, mechanotransduction

INTRODUCTION

OSTEOBLASTS ARE key effectors and regulators of bone remodeling in response to mechanical strain.(1) It is estimated that bone growth and structure can be maintained with as little as 15 minutes of gravity loading per day. (2) In vitro, gravity loading has been shown to induce DNA synthesis.(3) However, the underlying mechanisms by which osteoblasts sense gravity loading, activate signaling, and induce cell proliferation are poorly characterized.

We have previously shown that physiological g-loading (3g) induces c-fos expression in MC3T3-E1 osteoblasts.(4) Higher, superphysiological levels of mechanical stimulation (287g) induced a 10-fold increase in c-fos in MC3T3-E1 cells, which could be blocked by the protein kinase A (PKA) inhibitor H-89, but not by protein kinase C (PKC) downregulation,(5) showing that high levels of gravity loading induce changes in gene expression and that specific signaling pathways are involved in the transduction of the mechanical signal.

The MAP kinase extracellular signal-regulated kinase (ERK) plays an important role in osteoblast function, including the regulation of osteoblast-specific gene expression, proliferation, matrix mineralization, and adhesion in both human osteoblasts(6) and MC3T3-E1 cells.(7,8)

In this study we show that a pulse of physiological gravity stress significantly induced c-fos expression in MC3T3-E1 osteoblasts mediated by ERK activation. ERK 1/2 is phosphorylated in a load-dose-dependant manner over a physiological range of g-stress. Moreover, only 15 minutes of

The authors have no conflict of interest.
12 g loading was sufficient to induce significant proliferation of osteoblasts 24 h after the g-pulse. Finally, focal adhesion kinase (FAK), which has been implicated as a component of the mechanoperception system in some cell types, was not activated by gravity loading. These studies suggest that even brief periods of mechanical force at levels encountered during normal exercise are sufficient for activation of anabolic pathways in bone.

**MATERIALS AND METHODS**

Minimum essential media alpha (αMEM) was purchased from Fisher Scientific (Pittsburgh, PA, USA). U0126 and PD98059 were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Taq DNA polymerase, RNase inhibitor, and MuLV reverse transcriptase were purchased from Perkin Elmer (Branchburg, NJ, USA). Oligonucleotides were purchased from Operon Technologies Inc. (Alameda, CA, USA). Phospho-p42/44 MAP kinase (ERK) and phospho-p42 MAP (ERK) kinase antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Phospho-FAK (phospho-Tyr397) and FAK antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Supersignal pico-enhanced chemiluminescence kits were purchased from Pierce (Rockford, IL, USA) and exposed on autoradiography film. Films were scanned and bands quantified by the Kodak EDAS 290 image analysis system (Eastman Kodak, Rochester, NY, USA).

**RNA isolation, reverse transcription, and polymerase chain reaction**

RNA was isolated from cells using Tri-reagent (Sigma-Aldrich). Reverse transcriptase (RT) reaction and polymerase chain reaction (PCR) protocol and primers have been previously described.\(^{(10)}\) Membranes were probed with either anti-phospho p42/p44 MAPK (ERK), anti-p42/p44 MAPK (ERK) rabbit polyclonal antibody, anti-phospho FAK, or anti-FAK antibodies. Proteins were detected by enhanced chemiluminescence (Supersignal Pico kit; Pierce, Rockford, IL, USA) and exposed on autoradiography film. Films were scanned and bands quantified by the Kodak EDAS 290 image analysis system (Eastman Kodak, Rochester, NY, USA).

**Immunofluorescence analysis**

Cells on coverslips were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) and maintained at 4°C until subsequent processing. Primary antibodies were incubated in PBS for 30 minutes and then incubated for 30 minutes with goat-anti-rabbit Alexa Fluor 488 antibodies in PBS. Cell-specific proteins were imaged using a Zeiss Axioskop Fluorescent Microscope (Carl Zeiss, Jena, Germany) and an Orca-ER CCD camera (Hamamatsu Corp., Bridgewater, NJ, USA).

**Cell proliferation assay**

Cells were plated on 96-well plates in αMEM medium containing 10% fetal calf serum (FCS). Once attached, they were grown in αMEM medium containing 4% FCS for 65 h. The cells were then treated with various agents (see figure legends), loaded for 15 minutes at 12 g, and then incubated for 24 h in the 37°C incubator with 5% CO₂. The cells were frozen, and cell number was quantitated using the CyQuant Kit according to the manufacturer’s protocol.
RESULTS

Gravity loading model as a method for mechanical stimulation of osteoblasts

It has been shown that mild exercise is sufficient to maintain bone mass, and in this study we used gravity force to simulate compression forces found in exercise. To establish physiological loading from G-forces, we have mathematically modeled the osteoblast. Gravitational loading causes a change in cell geometry, making it a useful method for mechanical stimulation of osteoblasts. The osteoblast cell can be considered an elastic solid disc with radius \( R_{\text{cell}} \) and height or length \( L_{\text{cell}} \) that flattens with increasing radial acceleration or G-forces during centrifugation. As a result, the cell will be exposed to both strain (stretching) and shear (pressure) forces. Deformation of the cell is approximated that osteoblasts have similar mechanical properties as chondrocytes and are behaving as a uniform solid disc. The total change in height \( \delta \) of the cell is calculated by integration through the axis of the cell from the culture plate interface (\( L = 0 \)) to the top of the cell (\( L = L_{\text{cell}} \); Eqs. 4–7). The calculation of strain follows is shown in Eq. 8.

\[
\varepsilon = \frac{\sigma}{E} \tag{1}
\]

\[
\varepsilon = \frac{\delta}{L} \tag{2}
\]

where \( \delta \) is the change in total length \( L \).

\[
\sigma = \frac{\text{force/area}}{\text{area}} \tag{3}
\]

\[
d\delta/dL = \frac{\sigma}{E} \tag{4}
\]

\[
\sigma = (L_{\text{cell}} - L)(R_{\text{cell}})^2 \pi g(\rho_{\text{cell}} - \rho_{\text{media}})/(R_{\text{cell}})^2 \pi, \quad \text{(at } L \text{ distance from bottom of cell)} \tag{5}
\]

\[
d\delta = \int_{0}^{L_{\text{cell}} - L}(R_{\text{cell}})^2 \pi g(\rho_{\text{cell}} - \rho_{\text{media}})/E \, dL \tag{6}
\]

\[
\delta = (L_{\text{cell}})^2 g(\rho_{\text{cell}} - \rho_{\text{media}})/2E \tag{7}
\]

\[
\varepsilon = L_{\text{cell}} g(\rho_{\text{cell}} - \rho_{\text{media}})/2E \tag{8}
\]

\( \varepsilon = 10 \mu \text{strain at } 1g \), given that cell density is approximately 1100 kg/m\(^3\), media density is 1000 kg/m\(^3\), cell thickness is \( L_{\text{cell}} = 10^{-3} \) m, \( g \) is 9.8 m/s\(^2\), and \( E \) is 500 N/m\(^2\). With centrifugation, osteoblast strain increases linearly with \( g \) such that at 4g, \( \varepsilon = 40 \mu \text{strain} \); 10g, \( \varepsilon = 100 \mu \text{strain} \); and 30g, \( \varepsilon = 300 \mu \text{strain} \). In our experiments, the cells are exposed to strains that are similar to those observed in human tibia during walking and light exercise. It is likely that embedded osteoblasts are exposed to similar forces.

Shear is a function of the magnitude of flattening the osteoblasts in the centrifuge in proportion to cell height. Because flattening is greater near the culture dish and less near the top of the cell, shear forces develop across the cell and also at the cell culture dish interface. The shear at the culture dish interface is in the range of \((V)g(\rho_{\text{cell}} - \rho_{\text{media}})/A\), where \( A = \text{cross-sectional area of cell height and } V = \text{volume of cell} \). Given a typical osteoblast of 30 \( \mu \text{m} \) in diameter and a height of 10 \( \mu \text{m} \), shear at 1g is approximately 0.01 N/m\(^2\); at 10g, it is 0.1 N/m\(^2\); and at 30g, it is 0.3 N/m\(^2\). It has been shown that 4 h of fluid flow shear as low as 0.14 dynes/cm\(^2\) (0.014 N/m\(^2\)) can induce \( \text{cox-2} \) expression in MC3T3-E1 osteoblasts.

Hydrostatic pressure depends on the gravity force and the height of the liquid column above the sample. For our samples in a 6-well plate with 3 ml of media (3.3 mm fluid column height), hydrostatic pressure at the bottom of the well can be calculated by \( P = \sigma gh \), where \( P = \text{hydrostatic pressure, } \sigma = \text{cell culture medium density (1.01g/cm}^3\), \( g \) = g-level (1g = 9.81 m/s\(^2\)), and \( h = \text{fluid column height (3.3 mm)} \). In an experimental system, hydrostatic pressure on the cell well floor at 1g is 0.033 KPa and at 12g is 0.392 Kpa (1 KPa = 1000 N/m\(^2\)). However, because the cell behaves as a fluid-filled incompressible solid, it is unlikely to be compressed as a result of mild hydrostatic pressure. Although other investigators have shown that some cell types respond to hydrostatic pressure with increases in proliferation and cyclic adenosine monophosphate (cAMP) turnover, they used hydrostatic pressures in the 7–10 Kpa range, which is 25 times greater than the pressure generated in our experimental system. Therefore, it is unlikely that hydrostatic pressure is a significant contributor to the generation of shear forces in our cell system.

Gravity stress induces early gene expression primarily through an ERK-dependent, p38 MAPK-independent mechanism

To determine the role of the MAP kinases in g-loading induction of \( \text{c-fos} \) expression, we preincubated MC3T3-E1 osteoblasts with the following inhibitors: the MEK inhibitors U0126 (10 \( \mu \text{M} \)) and PD98059 (10 \( \mu \text{M} \)), and the p38MAPK inhibitor SB203580 (10 \( \mu \text{M} \)). Osteoblasts were exposed to 12g for 15 minutes and harvested at the same time as unloaded controls (\( t = 30 \) minutes). Semiquantitative RT-PCR analysis showed that 12g loading induced a significant 15-fold increase in \( \text{c-fos} \) messenger RNA (mRNA) expression relative to 1g samples (Fig. 1). No differences were noted in \( \text{c-fos} \) expression after 12g centrifugation between the vibration-damped CGA and an undamped centrifuge, indicating that g-level rather than vibration caused \( \text{c-fos} \) induction in these studies (data not shown). All subsequent experiments were performed with the CGA. Gravity-induced \( \text{c-fos} \) was unaffected by
SB203580. U0126 inhibited c-fos expression by 74% (p < 0.01), whereas PD98059 made a small but significant inhibition (16%) of c-fos expression (p < 0.05). In addition, mRNA expression of early growth response-1 (egr-1), osteocalcin, and basic fibroblast growth factor (bFGF) was significantly increased in 12g-loaded osteoblasts compared with unloaded samples, whereas cytosolic phospholipase A2 (cPLA2), transforming growth factor beta (TGF-β), and prostaglandin E1 receptor (EP1) mRNA expression was unaltered by gravity loading (Fig. 2).

**g-Loading induces ERK activation above a threshold g-level**

To determine if physiological g-loading induced ERK activation and to confirm the action of the inhibitors, we examined ERK 1/2 phosphorylation as a marker of kinase activation. Fifteen minutes after the end of a 27g 1-minute load, samples were collected for western blot analysis. Using anti-phospho Thr202/phospho-Tyr204 p42/p44 MAPK (ERK) antibody, we observed that gravity induced a significant phosphorylation of both ERK 1 and ERK 2, comparable with the level of phosphorylation induced by the application of 5% FCS to osteoblasts (Fig. 3). No phosphorylation of either ERK isoform was detectable in the 1g controls, indicating that there is negligible ERK activity in quiescent cells. Furthermore, loading did not induce phosphorylation of either JNK or p38MAPK (data not shown). Essentially, all induced ERK phosphorylation was abolished by U0126, whereas PD98059 only partially inhibited ERK 2 phosphorylation.

To further explore ERK mediation of gravity mechanotransduction, MC3T3-E1 cells were loaded for 15 minutes at 2, 4, 8, 12, and 27g, because these g-levels correspond to the range of forces that can be encountered during exercise. Striking stimulation of ERK phosphorylation in loaded samples occurred somewhere between 4g and 8g, with phosphorylation reaching saturation at 12g (p < 0.001; Fig. 4).

**Focal adhesion kinase is not activated by gravity loading**

To determine if focal adhesion kinase (FAK) is activated by gravity loading, MC3T3-E1 osteoblasts were subject to a
12g, 15 minute loading as described in the Materials and Methods section. FAK was phosphorylated in unstimulated cells and the phosphorylation state was not significantly activated by gravity loading (Fig. 5). Likewise, total FAK levels in the 1g and 12g samples did not change and were comparable (data not shown).

Phosphorylated ERK is localized to the perinuclear region and nucleus after loading

The subcellular localization of phospho-ERK 1/2 localization was investigated further over a period of 120 minutes after a 15-minute pulse of 12g. In unloaded 1g cells, a weak generalized staining of phospho-ERK 1/2 was seen throughout the cell. A significant increase in phosphorylated ERK was detected in the perinuclear region of MC3T3-E1 cells immediately after a 15-minute 12g pulse (Fig. 6). By 30 minutes, phosphorylated ERK was detectable in some cell nuclei, and at 60 minutes, most of the phosphorylated ERK was within the nuclei. At 120 minutes, phosphorylated ERK was almost undetectable in the cell.

Pulse g-loading induces osteoblast growth activation in part through an ERK-dependant pathway

To determine whether gravity loading induced growth activation, we exposed MC3T3-E1 osteoblasts to 12g for 15 minutes, and then incubated the cells at 37°C for 24 h before determining cell number by CyQuant assay. Loading induced a 64% increase in cell number (Table 1). There were no significant changes in cell numbers in unloaded samples, indicating that these cells were quiescent. The g-load induction of cell proliferation was significantly reduced by 50% by the MEK inhibitor U0126 (p < 0.001). This suggests that while ERK 1/2 mediates one-half the growth induction by mechanical stress, other independent signaling pathways contribute as well.

A significant increase in the number of dividing cells in g-loaded samples was also observed by microscopy (Fig. 7). After 24 h, increases in cell number were apparent in addition to increased mitotic index and enlarged G2 phase nuclei as shown in Fig. 7B. In contrast, the non-loaded osteoblasts were slow growing in the absence of mechanical stimulation (Fig. 7A).

DISCUSSION

We calculated the physiological strain caused by gravity loading and applied physiological levels of loading on osteoblasts to study the mechanisms by which bone cells sense physiological mechanical stress. A brief exposure to physiological stress induced the expression of immediate-early genes in MC3T3-E1 osteoblasts, including c-fos, egr-1, and...
osteocalcin and bFGF. Cell cycle progression and proliferation have been shown to be regulated by c-fos expression in some cell types, including osteoblasts. The egr-1 transcription factor is induced by both EGF and PGE2 in osteoblasts. Furthermore, cyclic strain forces induced transient egr-1 in MC3T3-E1 osteoblasts. Basic FGF induces expression of prostaglandin H/H synthase-2 (PGH-2), an important enzyme involved in prostaglandin synthesis. Therefore, gravity loading induces the expression of genes that promote osteoblast proliferation.

The induction of c-fos was inhibited 74% by U0126 but was only inhibited 16% by PD98059. At the concentrations used in the experiment, PD98059 primarily inhibits MEK1, whereas U0126 inhibits MEK1 and MEK2 in turn preventing activation of both ERK1 and ERK2. This suggests that a significant fraction of the gravity signal can pass through ERK2. In contrast, neither p38 MAPK nor c-jun N-terminal kinase (JNK) were phosphorylated by g-force stress. In unloaded samples, a small, but significant, increase in basal c-fos levels was noted in SB203580-treated cultures. This compound also augments c-fos levels in rat fibroblasts, suggesting that p38MAPK activity may normally inhibit c-fos expression in unloaded cells. The presence of SB203580 did not significantly affect the upregulation of c-fos by gravity.

ERK has previously been reported as a key regulator of osteoblast gene expression associated with growth and other important cellular processes. Prolonged 13g centrifugation has previously been reported to induce collagen synthesis in mineralizing osteoblasts through ERK activation. Although these authors observed ERK activation within 5 minutes of the start of g-loading, induction of collagen I required a 24-h continuous stimulation. In contrast, our results indicate that a very brief (15 minutes) mechanical stimulus was sufficient to induce early gene expression as a result of ERK activation. Phosphorylation of ERK by gravity stress was dose-dependent, with the mechanotransduction threshold occurring between 4g and 8g and saturating at 12g. These data suggest that mechanotransduction in MC3T3-E1 osteoblasts is coupled to ERK activation, which is triggered within a narrow range of loads independent of FAK. The mathematical models of gravity forces on the cell correspond closely to our experimental data and to the forces created in bone during normal walking. These forces are comparable with fluid shear forces that induce early gene expression in MC3T3-E1 osteoblasts.

Hydrostatic pressures in the 10 Kpa (KN/m2) range have been reported to induce MAP kinase activation and proliferation in mesangial cells. However, the mechanisms by which mild hydrostatic pressure acts on cells is unclear, because the cell behaves as an incompressible solid. In our system, the hydrostatic pressure generated by 12g centrifugation was 0.39 Kpa, approximately 25-fold less than the 10-Kpa pressures reported to be biologically significant. Furthermore, we did not observe any differences in the induction of ERK phosphorylation between experiments performed with 1.5 and 3 ml of medium, corresponding to 0.196 and 0.392 Kpa at 12g, respectively (data not shown).

Gravity loading by centrifugation exerts both a continuous strain and minor shear force on osteoblasts. The effects of continuous and cyclic mechanical strain (stretching) and fluid shear on signaling and gene induction have been assessed by other investigators. Continuous mechanical strain of peridontal ligament cells induced transient expression of both c-fos and c-jun after initial application of strain. Inhibitor studies suggested that ERK, p38MAPK, and Rho kinase may regulate activation of these genes. Similarly, pulsed fluid flow induced cox-2 mRNA expression through ERK activation in MC3T3-E1 osteoblasts. In rat osteosarcoma cells, mechanical strain induced egr-1 expression. These reports show a pattern of response similar to our data, suggesting that initiation of early gene expression by fluid shear, continuous stress, and gravity loading may act through similar mechanisms. However, in MC3T3-E1 cells, oscillatory fluid flow induced both ERK and p38MAPK activation, which in turn regulated osteopontin mRNA expression 24 h after stimulation. Further, high-frequency pulsed fluid flows were more effective at stimulating Ca2+ fluxes than continuous flow in human embryonic osteoblasts, whereas oscillatory (back and forth) fluid flow was only a weak stimulus. While the different methods of mechanical stimulation probably activate the same signaling pathways, there may be subtle differences in both the efficiency of activation and secondary signaling pathways as a result of extended exposure to mechanical stress.

Signal transduction through ERK 1/2 involves translocation of the phosphorylated kinase to the nucleus, where it phosphorylates and activates transcription factors. Mutant PC12 cells with defective ERK nuclear translocation failed to induce c-fos expression via growth factor anabolic pathways. In Chinese hamster ovary (CHO) cells, nu-
clear translocation of phosphorylated ERK 2 is a key step in cell cycle progression.\textsuperscript{34} The translocation of activated ERK to the nucleus plays a critical step in signal transduction. We observed a significant increase in phosphorylated ERK in the perinuclear region and nucleus in MC3T3-E1 osteoblasts after gravity loading, suggesting that ERK may mediate transduction of the mechanostimulation signal directly to the nucleus. One possible function of ERK translocation into the nucleus is to phosphorylate transcription factors, such as \textit{elk}.\textsuperscript{35} Phosphorylated ERK was initially perinuclear, but by 60 minutes after the start of the gravity pulse, it was localized primarily in the nucleus. It became almost undetectable at 120 minutes. The dephosphorylation of ERK is mediated by phosphatases and is probably necessary for timely attenuation of the signal.\textsuperscript{32}

Focal adhesions have been proposed as a site for cellular perception of external mechanical forces.\textsuperscript{36} In fibroblasts, FAK phosphorylation on Tyr-397 is a necessary step in cell migration signaling,\textsuperscript{37} whereas in MC3T3-E1 osteoblasts, cell binding to collagen I activates FAK and ERK.\textsuperscript{38} Furthermore, in human epithelial cells, ERK2 is activated by cyclic stretch through a FAK-dependant mechanism.\textsuperscript{39} Our data showed that while FAK was phosphorylated on Tyr-397 in unloaded osteoblasts, the phosphorylation state of FAK was unaltered after loading. This indicates that FAK is not directly involved in the transduction of mechanical force sensing in our model system.

We observed that 15 minutes of \( g \)-loading is sufficient to cause a significant 64\% increase in osteoblast growth 24\,h after stress. This is consistent with the estimation that 15 minutes of exercise can maintain in vivo bone growth,\textsuperscript{2} and other in vivo studies show that only 10 minutes of normal loading per day restored 50\% of the bone lost in hind limb unloaded animals during a 28-day period.\textsuperscript{12} Furthermore, our data expands on a previous report showing an increase in DNA synthesis in MC3T3-E1 osteoblasts after a 30-minute pulse of centrifugation.\textsuperscript{3}

We found that at least one-half of the gravity-induced growth is mediated by an ERK signal transduction that does not involve p38, JNK, or FAK. In conclusion, this is the first data to show that a single brief pulse of physiological strain is sufficient to induce immediate early gene expression, stimulate bone proliferation, and initiate anabolic signal transduction of ERK at levels comparable with serum activation.

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**FIG. 6.** Gravity loading induced a transient nuclear localization of ERK 1/2. Cells were cultured on coverslips as described in the Materials and Methods section, and then exposed to 12\( g \) for 15 minutes in the CGA. Samples were fixed at 0 (1\( g \) control), 15, 30, 60, and 120 minutes after the start of gravity loading. Coverslips were stained for phosho-ERK (phospho-Thr202/ phospho-Tyr204), and then imaged. A fixed exposure was used for each stain to permit comparison of treatments. (A) 1\( g \) unloaded (0 minute); (B–E) 12\( g \) 15-minute loaded at 15, 30, 60, and 120 minutes after start of \( g \)-loading.

**TABLE 1. FIFTEEN MINUTES OF 12\textit{g}-LOADING INDUCES GROWTH IN MC3T3-E1 OSTEOBLASTS**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number per well ± SD</th>
<th>Percentage increase in cell number relative to unloaded cells, 24-h after 12( g ) pulse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unloaded sample</td>
<td>7461 ± 1052</td>
<td>—</td>
</tr>
<tr>
<td>Unloaded sample, U0126 treated</td>
<td>7973 ± 1382</td>
<td>—</td>
</tr>
<tr>
<td>12( g )-loaded sample</td>
<td>12203 ± 1946*</td>
<td>64% (( p &lt; 0.0001 ))</td>
</tr>
<tr>
<td>12( g )-loaded sample, U0126 treated</td>
<td>10354 ± 1138*</td>
<td>30% (( p &lt; 0.005 ))</td>
</tr>
</tbody>
</table>

\* These samples were statistically significantly different (Student’s \( t \)-test, 2-tailed) from the unloaded sample at the \( p < 0.0001 \) level.

\† These samples were statistically significantly different (Student’s \( t \)-test, 2-tailed) from the 12\( g \)-loaded sample at the \( p < 0.005 \) level. For each condition \( n = 6 \).
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