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Hypergravity Stimulates Collagen Synthesis in Human Osteoblast-Like Cells: Evidence for the Involvement of p44/42 MAP-Kinases (ERK 1/2)[1]

Jens Gebken,[†] Barbara Lüders,[†] Holger Notbohm,[†] Harald H. Klein,[*] Jürgen Brinckmann,[‡] Peter K. Müller,[†] and Boris Bätge[*,2]

[*]*Medizinische Klinik I,* [†]*Institut für Medizinische Molekularbiologie, and* [‡]*Klinik für Dermatologie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany*

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The formation and organization of skeletal tissue is strongly influenced by mechanical stimulation. There is increasing evidence that gravitational stress has an impact on the expression of early response genes in mammalian cells and may play a role in the formation of extracellular matrix. In particular, osteoblasts may be unique in their response to gravitational stimuli since in these cells microgravity has been reported to reduce collagen synthesis, while in fibroblasts the opposite effect was observed. Here, we have investigated the influence of hypergravity induced by centrifugation on the collagen synthesis of human osteoblast-like cells (hOB) and studied the possible involvement of the mitogen-activated protein (MAP) kinase signaling cascade. Collagen synthesis was significantly increased by $42 \pm 16\%$ under hypergravity at $13\times g$, an effect paralleled by the enhanced expression of the collagen I alpha 2 (COL1A2) mRNA. No difference was seen in the proportion of collagen types I, III, and V synthesized by hOB. Hypergravity induced a markedly elevated phosphorylation of the p44/42 MAP kinases (ERK 1/2). The inhibition of this pathway suppressed the hypergravity-induced stimulation of both collagen synthesis as well as COL1A2 mRNA expression by about 50%. Our results show that the collagen synthesis of non-transformed hOB is stimulated under hypergravitational conditions. This response appears to be partially mediated by the MAP kinase pathway.

Key words: collagen synthesis, hypergravity, osteoblasts, mechanical stress, signal transduction.

It has been well known for more than a century that the formation and organization of skeletal tissue is influenced by mechanical stimulation (*1, 2*). Several *in vivo* as well as *in vitro* studies have demonstrated that applied mechanical forces can affect both bone matrix synthesis and mineralization (*3*). Different types of mechanical stimulation have been used in these studies, *e.g.* continuous or intermittent hydrostatic pressure, pulsatile fluid shear stress and vibrational force (*4-6*). In addition, there is increasing evidence that various forms of gravitational stress influence cellular functions by altering transcriptional activity, matrix organization or cell matrix adhesion. Fitzgerald *et al.* reported an increased c-*fos* mRNA level within 30 min that was paralleled by a decrease in osteocalcin mRNA levels in a mouse osteoblastic cell line centrifuged at a maximum of $3\times g$ (*7*). ROS 17/2.8 osteosarcoma cells were shown to modify their shape and focal adhesions when submitted to small switches of gravity during of 3-h parabolic flight conditions (*8*). Furthermore, human dermal fibroblasts exposed to hypergravity over a period of 8 days show increases in the activities of various enzymes involved in the remodeling of the extracellular matrix (*9*). Surprisingly little is known about the influence of hypergravity on the synthesis of the most abundant bone matrix protein, collagen I, by human osteoblasts. Various mechanisms have been suggested to be involved in the cellular response to mechanical stimuli, including force transduction *via* integrins, stretch-activation of cation channels, and the activation of various intracellular signal transduction pathways (*10*). In hypergravity, signal transduction may involve inositol 1,4,5-triphosphate and adenosine 3′,5′-cyclic monophosphate as second messengers (*11*). Little is known, however, about the role in hypergravity signal transduction of intracytoplasmatic protein phosphorylation and the activation of mitogen-activated protein (MAP) kinase, which have been shown to be important in the integrin signaling system (*12*). Furthermore, it is still unclear by which cascades signals are transmitted to the nuclei to induce matrix alterations in response to gravitational stress. In the present study on human non-transformed osteoblast-like cells, we investigate the influence of hypergravity on collagen synthesis and study the involvement of the MAP-kinase pathway.

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[2] To whom correspondence should be addressed. Phone: +49-451-500-4080, Fax: +49-451-500-3637, E-mail: Baetge@molbio.mu-luebeck.de

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MATERIALS AND METHODS

All chemicals used were of analytical grade and purchased from Merck (Darmstadt, FRG), Sigma (Deisenhofen, FRG), or Serva (Heidelberg, FRG). Cell culture reagents were from Biochrom (Berlin, FRG).

Human Bone Cell Culture—Human bone cells (hOB) were isolated from the trabecular bone of adult femoral head samples obtained by informed consent during routine hip replacement surgery in the orthopedic clinic of the Medical University of Lübeck. The fragments were seeded as explants into tissue culture flasks and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Culture medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.7 g/liter $NaHCO_3$ 50 μg/ml L-ascorbate, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum (FCS), pH 7.2. Cells were subcultured at second or third passage at a density of 10^4 cells/cm^2. Cells were characterized as osteoblast-like cells by the determination of osteoblast markers as described in detail previously (*13*). Briefly, cytochemical staining for alkaline phosphatase (ALP) could be demonstrated on 60–80% of the cells and was negative on control fibroblasts; the ALP activity was stimulated 3.7-fold by 1,25di(OH) vitamin D3 (50 nM), and the osteocalcin concentration increased 18-fold under the same conditions; incubation with 90 nM hPTH(1-34) stimulated cAMP 4-fold and *in vitro* mineralization was demonstrable (*13*).

Analysis of Collagen Types—Cells growing on the bottom of 25 cm^2 tissue culture flasks were preincubated for 24 h (DMEM, 100 U/ml penicillin, 50 μg/ml L-ascorbate, 2 mM L-glutamine, 1% FCS). Incubation with 370 kBq/ml L-[2,3-^{3}H]proline (1.6 GBq/mmol; Amersham, Freiburg, FRG) in incubation medium I (DMEM, 50 μg/ml L-ascorbate, 100 U/ml penicillin, 0.15 mg/ml β-aminoproprionitrile, 1% FCS, 25 mM HEPES, 15 mM MOPS, pH 7.4) was carried out for 24 h with centrifugation or under normal conditions. Medium and cells were pooled and samples digested with 0.1 mg/ml pepsin (Boehringer, Mannheim, FRG) in 0.05% acetic acid, pH 1, for 16 h at 4°C. Collagen extracts were concentrated and washed with Microsep ultrafiltration units with a cut off of 10 kDa (Filtron, Karlsruhe, FRG) to remove unbound radioactivity. After direct resuspension in sample buffer for electrophoresis, the α-chains were separated by SDS-PAGE using conditions of delayed reduction (*14*). After separation and visualization of individual bands with Coomassie Brilliant Blue, the separated chains were cut out and the activity per α-chain was determined in a liquid scintillation counter. Additionally, individual bands of newly synthesized collagen were visualized by fluorography.

For quantitative collagen analysis, medium and cells were analyzed separately. The samples were concentrated and washed by ultrafiltration as described above, resuspended in 6 N HCl, hydrolyzed (110°C, 24 h) and dried in a desiccator. After resuspension in amino-acid sample-dilution buffer (Beckmann, Munich, Germany), the amounts of [^{3}H]proline and [^{3}H]hydroxylproline were determined by amino-acid analysis using ion exchange chromatography with a solid phase scintillation detector. Quantitative collagen synthesis was expressed as hydroxyproline counts per cell. The secretion factor was calculated as percent [^{3}H]-hydroxyproline found in the medium. Since previous studies revealed no evidence for an altered degree of prolin residue hydroxylation in collagen I synthesized by mesenchymal cells under different gravitational conditions, the [^{3}H]hydroxyproline counts per cell directly reflected the quantity of newly synthesized collagen normalized to cell number (*15*).

Tritiated Thymidine Incorporation Assay—Cells growing on the bottom of 25 cm^2 tissue culture flasks were treated according to the protocol used for the analysis of collagen types. The incubation medium I contained 37 kBq/ml [methyl-^{3}H]thymidine (925 GBq/mmol, Amersham, Freiburg, FRG) instead of [^{3}H]proline. After 24 h of incubation, unincorporated label was washed of with three gentle washes in phosphate-buffered salt solution (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.3). The cells were incubated in 5% trichloroacetic acid (TCA) for 20 min on ice. TCA was removed by two gentle washes with ice cold ethanol. Residues were dissolved for 1-2 h in 1 ml 0.1 M NaOH/2% Na_2CO_3 at 37°C. Triplicate aliquots of 300 μl were neutralized with 100 μl 1 M HCl and counted in a scintillation counter (*16*).

Incubation Conditions—Hypergravity conditions were achieved in a specially constructed swinging bucket hyperfuge incubator (Diport AG, Uster, Switzerland). Cells growing on the bottom of the culture flasks were centrifuged for 24 h generating a constant force of 13 g, which was found to be the best compromise to produce a maximal effect with minimal cellular damage. The hypergravity was calculated as follows: $a_r = 4\pi^2 v^2 r$ ($[r] = m$, radial distance of the centrifuge; $[v] = s^{-1}$, rotation frequency; $[a_r] = g$; 1 $g = 9.81$ ms^{-2}, earth's gravitational force). Both centrifuged and control cells were treated identically until the start of the centrifugation in a closed system, so that differences in the specific activities of the cellular proline pools were unlikely. The temperature inside and outside the centrifuge was monitored throughout the experiments and kept precisely at 37°C. Immediately after the end of the incubation, the pH of the decanted medium was measured to ascertain that the experiments were conducted under identical conditions. The cell number in each flask was determined at the end of the assay. Quantitative collagen analysis in both centrifuged and control cells was performed as described above. To determine collagen synthesis in the presence of p44/42 MAP-kinases, incubation medium containing 50 μM of the MEK1 inhibitor PD98059 was used.

Analysis of Collagen α2 (I) Gene Expression by RT-PCR—For semi-quantitative analysis, collagen α2 (I) (COL1A2) gene expression was studied by RT-PCR in relation to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a coamplified internal standard (*17*). Total RNA from human osteoblast-like cells was isolated using an RNeasy kit (Qiagen, Hilden, FRG). Samples of 1 μg were reverse-transcribed with Oligo dT Primer (Gibco, Berlin, FRG) and amplified using sequence-specific oligo nucleotide primers for both the COL1A2 and GAPDH genes included in the same reactions. To exclude contamination by genomic DNA as a source for amplified products, each reaction was additionally carried out without reverse transcriptase. Sequences of the the antisense and sense primers were as follows: GAPDH: 5′-GCA ACT GTG AGG AGG GGA GAT

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TCA G-3', 5'-CCG CAT CTT CTT TTG CGT CGC-3'; COL1A2: 5'-GGT GGT TAT GAC TTT GGT TAC-3', 5'-CAG GCG TGA TGG CTT ATT TGT-3'. PCR was performed on 1/20 of the reverse-transcription reaction using Vent-Polymerase (Biolabs, Schwalbach, FRG) following the protocols supplied by the manufacturers (each cycle consisted of 35 s of denaturation at 95°C, 35 s annealing at 55°C, and 60 s of elongation at 72°C). Amplification of both, GAPDH and COL1A2 were found to be in the linear range when 28 cycles of amplification were used. The influence of hypergravity on the expression of COL1A2 mRNA was assessed after centrifuging the human osteoblasts for 48 h as described above.

Immunoblot Analysis of Tyrosine Phosphorylated Proteins and Phosphorylated p44/42 MAP-Kinases—Cells were covered with incubation medium II [DMEM, 0.25 mM Na-ascorbate, 2 mM glutamine, 100 I.E. penicillin, 100 mg/liter streptomycin, 1% (v/v) FCS dialyzed against PBS, 45 mM $NaHCO_3$, pH 7.2, 300 μM Na_3VO_4, 3 mM H_2O_2] and subjected to hypergravity for 0, 5, 10, 20, or 30 min. After centrifugation, cells were briefly rinsed with ice-cold detachment buffer (250 mM saccharose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, supplemented with a protease inhibitor cocktail, Boehringer Mannheim, FRG) and then scraped off in the presence of 0.5 ml detachment buffer with a disposable scraper. The wells were rinsed with a second aliquot of detachment buffer. Cells were pelleted by centrifugation at $20,000 \times g$ for 2 min at 4°C in 2-ml microcentrifuge tubes and the supernatant was discarded. Cell pellets were resuspended by repeated pipetting with 60 μl solubilization buffer [125 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0, 1% (v/v) Triton X-100, supplemented with an inhibitor cocktail] and incubated for 40 min at 4°C with-end-over-end mixing. Insoluble cell debris was removed by centrifugation at $20,000 \times g$ at 4°C for 15 min. The protein content of the lysate was determined according to Lowry (Lowry, DC Protein Assay kit, Bio-Rad, Hercules, CA, USA). The supernatant was mixed with an equal volume of electrophoresis sample buffer and heated at 90°C for 2 min; equal amounts of protein were then subjected to SDS-PAGE on 10% polyacrylamide gels and blotted onto a nitrocellulose membrane. The blots were incubated with antibodies against phosphorylated tyrosine residues (Sigma) and visualized by enhanced chemiluminescence (Amersham, Germany). The phosphorylation of the MAP kinases p44/42 were determined using specific antibodies directed against the tyrosine-phosphorylated form of these kinases (Biolabs, Germany).

Inhibition of p44/42 Phosphorylation—For the inhibition of MAP-kinase p44/42 phosphorylation, cells were treated with 50 μM of the MAPK kinase (MEK) inhibitor PD98059 (New England Biolabs, Schwalbach) prior to incubation under hypergravity. For this purpose incubation medium II containing 50 μM PD98059 was used. Previous studies have shown both the specifity of this inhibitor as well as its stability and effectiveness *in vitro* over a culture period of 48 h (*18, 19*).

Statistical Analysis—Calculation of means and standard deviations was performed on data derived from four different human osteoblast populations, each with three independent determinations. The effect of the different conditions tested was analyzed by one way analysis of variance (ANOVA).

RESULTS

Cell Morphology and Cell Proliferation—Light microscopic examination revealed no morphological differences in human osteoblast-like cells after 24 h incubation under hypergravity at $13 \times g$ (Fig. 1). In contrast there was a slight reduction ($21 \pm 8\%$) in cell number after 24 h of hypergravity compared to control samples maintained at 1 g. This effect could not be explained by the inhibition of cell proliferation, since [^{3}H]thymidine incorporation was low (600 cpm; MG63 cells under the same conditions 8.77×10^5 cpm) and only marginally reduced by $4 \pm 15\%$.

Collagen Synthesis under Hypergravity—The effect of gravitational stress on the quantity of newly synthesized collagen by human osteoblast-like cells is shown in Fig. 2. In all four experiments hypergravity ($13 \times g$) resulted in a significant increase in collagen synthesis per cell by 42% on average. In an additional experiment, collagen synthesis was studied within 24 h after the termination of exposure to hypergravity. The hypergravity-induced stimulation of collagen synthesis was temporary and reversible, since there were no significant differences in the proline and hydroxyproline counts in osteoblasts with or without prior exposure to hypergravity (100 *vs.* $93 \pm 22\%$). The propor-

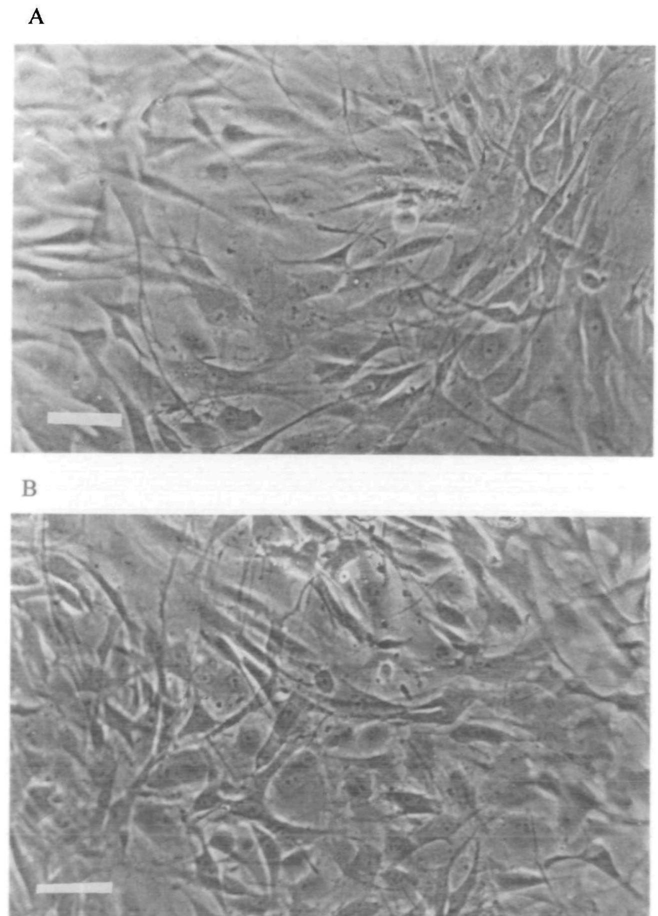

Fig. 1. **Osteoblast morphology under hypergravity.** Human osteoblast-like cells after centrifugation (24 h, $13 \times g$, A) and control cells ($1 \times g$, B). Light microscopic examination revealed no difference between the two groups. Bars: 100 μm.

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tion of collagen secreted into the medium was 94±0.47%, elevated compared to $1\times g$ controls (82±8.34%; $p<0.05$). The mRNA levels of COL1A2 increased by 7±4% under 24 h (not shown) and by 35±11% under 48 h exposure to hypergravity (see below; Fig. 8). The mean relative proportions of α1(I), α2(I), α1(III), and α1(V) proteins were 54.1, 26.3, 15.1, and 4.6%, respectively, and these relative protein amounts remained unchanged after 24 h incubation at $13\times g$ as determined in three separate experiments (Fig. 3).

Immunoblot Analyses—In order to detect hypergravity-induced phosphorylation of proteins that might play a role in stress induced signaling, immunoblot analysis was carried out. When antibodies against phosphorylated tyrosine residues were used, no differences in the electrophoretic banding pattern between controls and centrifuged human osteoblast-like cells could be observed (Fig. 4). In contrast, immunoblotting with specific antibodies to the tyrosine-phosphorylated (activated) form of p44/42 (ERK-1/2) revealed a gradual enhancement of phosphorylation with increasing time of exposure to hypergravity (Fig. 5). Under the same conditions, the addition of the inhibitor PD 98059 compeletely blocked the activation of both MAP kinases, as shown for the 5 min centrifugation in Fig. 6, and also observed after 30 min centrifugation.

Collagen Synthesis and Inhibition of p44/42 (ERK-1/2) Phosphorylation—In order to find out whether hypergravi-

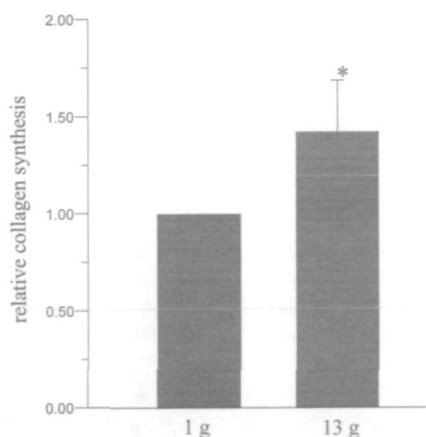

Fig. 2. **Effect of hypergravity on osteoblastic collagen synthesis.** Collagen synthesis in human osteoblast-like cells under hypergravity at $13\times g$ (centrifugation for 24 h, right) and control cells at $1\times g$ (left). Quantitative collagen synthesis (hydroxyproline counts per cell) in four separate experiments was normalized to control values (* = $p<0.05$).

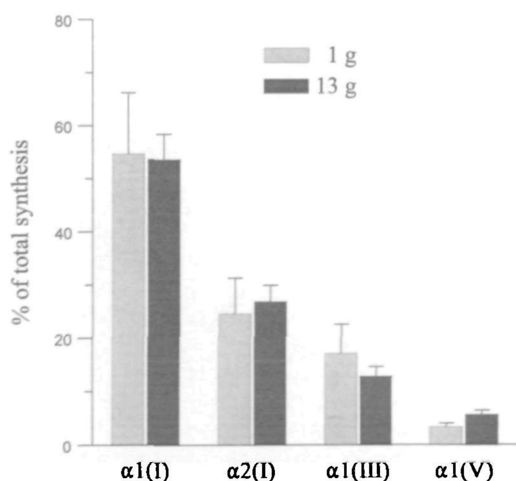

Fig. 3. **Synthesis of different collagen types under hypergravity.** Qualitative collagen synthesis in human osteoblast-like cells as visualized by fluorography of cells and medium (left). Relative amounts of collagens I, III, and V expressed as % of total collagen synthesized by human osteoblast-like cells under hypergravity at $13\times g$ (black bars) as compared to controls (grey bars) (right: means±SD of three separate experiments).

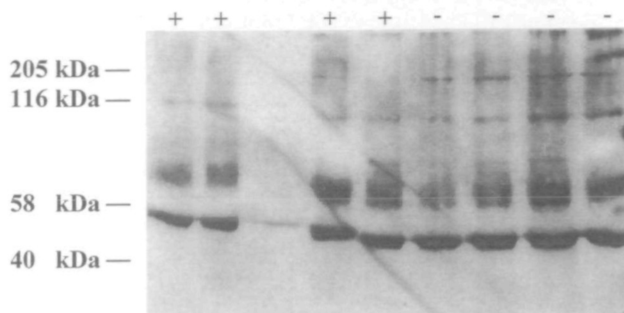

Fig. 4. **Tyrosine-phosphorylated proteins under hypergravity.** Immunoblotting analysis of tyrosine-phosphorylated proteins from 4 different human osteoblast-like cell population lysates with (+) and without (−) preincubation under hypergravity at $13\times g$ for 30 min.

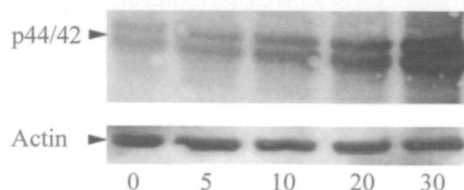

Fig. 5. **Effect of hypergravity on the phosphorylation of p44/42 MAP kinases.** Immunoblotting analysis of phosphorylated p44- and p42-MAP kinases (ERK-1 and ERK-2) in human osteoblast-like cells under hypergravity at $13\times g$ for 5, 10, 20, and 30 min, and control cells ($1\times g=0$ min at $13\times g$). Values were normalized for actin immunoblot staining of the respective samples (bottom).

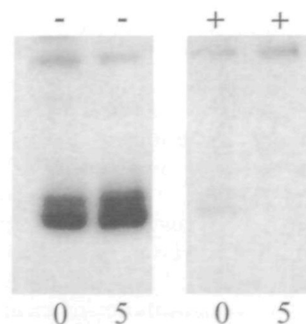

Fig. 6. **Inhibition of p44- and p42-MAP kinase (ERK-1 and ERK-2) phosphorylation by the specific inhibitor PD98059.** Immunoblotting analysis of phosphorylated p44- and p42-MAP kinases (ERK-1 and ERK-2) in human osteoblast-like cells under hypergravity at $13\times g$ for 0 and 5 min (+) or control cells (−).

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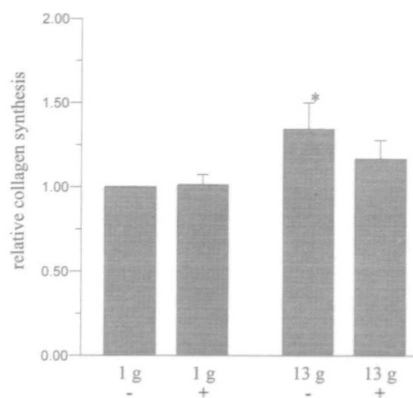

Fig. 7. **Effect of hypergravity on osteoblastic collagen synthesis after inhibition of p44/42 MAP kinases.** Reduced stimulatory effect of hypergravity ($13 \times g$ for 24 h) on collagen synthesis in human osteoblast-like cells after the inhibition of p44- and p42-MAP kinase (ERK-1 and ERK-2) phoshorylation by the specific inhibitor PD98059 (+). Quantitative collagen synthesis (hydroxyproline counts per cell) in four separate experiments was normalized to control values at $1 \times g$ without (−) inhibitor. After the addition of the inhibitor, the hypergravity-induced increase in collagen synthesis was reduced by about 50%.

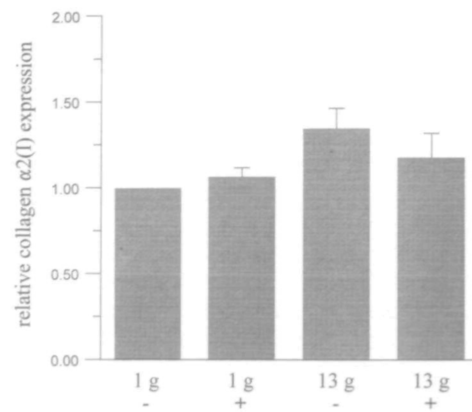

Fig. 8. **Effect of hypergravity on COL1A2 mRNA expression with or without inhibition of p44/42 MAP kinases.** Reduced stimulatory effect of hypergravity ($13 \times g$ for 48 h) on COL1A2 mRNA expression in human osteoblast-like cells after the inhibition of p44- and p42-MAP kinase (ERK-1 and ERK-2) phosphorylation by the specific inhibitor PD98059 (+). Values were obtained by RT-PCR using GAPDH as a coamplified internal standard.

ty-induced phosphorylation of p44/42 (ERK-1/2) can be implicated in the increase in collagen synthesis, the collagen synthesis of human osteoblast-like cells was measured with and without the addition of the specific inhibitor of MAPK kinase (MEK) PD 98059. The specifity of this inhibitor, as well as its stability and effectiveness over a 48 h culture period, has been extensively studied in previous investigations (*18, 19*). As shown in Fig. 7, the increase in collagen synthesis induced by hypergravity was reduced by about 50% (mean stimulation of 17%), while no influence of the inhibitor was seen under 1 *g* conditions. Likewise, the hypergravity-induced increase in COL1A2 mRNA expression was reduced by about 50% when the inhibitor was added (Fig. 8).

DISCUSSION

It is well documented that skeletal tissues are able to adapt bone mass and tissue architecture to changing mechanical demands. Animal models of skeletal loading and unloading show altered osteoblast activity and recruitment as a response to mechanical stimuli (*20*). Since the synthesis and deposition of organic matrix is the initial step in bone tissue formation, providing the organic scaffold for the subsequent deposition of mineral, new bone mass can only be acquired by increased matrix synthesis. In order to determine whether elevated gravitational forces affect bone matrix formation, we measured the amount of collagen synthesized by human osteoblast-like cells centrifuged for 24 h at $13 \times g$, and compared the results with control cells maintained at $1 \times g$. In all osteoblastic populations tested, this standardized mechanical stimulation consistently led to a significant increase in both total collagen synthesis and COL1A2 mRNA expression. The data imply that the initial stimulation of collagen production occurs mainly at the posttranscriptional level while higher steady state levels of COL1A2 mRNA are found later. The relative proportions of different collagen types were not affected. It is interesting to note that microgravity has recently been shown to reduce collagen I alpha 1 (COL1A1) gene expression in MG63 osteosarcoma cells (*21*). Thus, gravitational loading appears to have an important stimulatory role in the differentiated functions of osteoblasts, *e.g.* matrix synthesis, whereas microgravity seems to have the opposite effect. Moreover, the observed cellular responses might be characteristic for osteoblasts, since human dermal fibroblasts show a significant decrease in collagen synthesis under gravitational conditions similar to those applied in our experiments (*15*). Accordingly, there is recent evidence that fibroblasts, in contrast to osteoblasts, do not show enhanced COL1A1 gene expression under mechanical stress (*22*).

In osteoblastic cells, most studies involving gravitational forces use rather short incubation periods. The induction of early response genes has been shown to occur after a few minutes of exposure to hypergravity and has been shown to be transient (*7, 23*). Our results show that the synthesis of collagen is the initial step in bone formation and, as a prerequisite for the subsequent stabilizing mineralization, is increased under exposure to chronic hypergravity for 24 h.

It cannot be excluded that in addition to pure gravitational stress other mechanical stimuli would be effective in our experimental model. Accelerational and vibrational forces have been reported to affect osteoblasts under similar experimental conditions (*6, 7*). However, while these forces are likely to be present at the start of the centrifugation, their relative importance over a 24 h period of hypergravity should be rather small with respect to late cellular responses, *e.g.* matrix production.

The mechanisms by which mammalian cells respond to gravitational stress are still largely unknown. While among others nitric oxide and protein kinase C might act as early mediators of non-gravitational mechanical stimulation (*24, 25*), only a few studies have investigated signaling pathways in hypergravity. In ROS 17/2.8 osteosarcoma cells, prostaglandin E2 appears to be involved in the cell shape changes observed during parabolic flight (*26*). However, in

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the medium of mouse osteoblastic MC3T3 cells exposed to gravitational loading, no differences in the PGE2 levels were detected, suggesting distinct responses in different cell lines (*7*). Inositol 1,4,5-trisphosphate and cAMP have been found to act as second messengers in hypergravity signal transduction in HeLa cells, which also show enhanced phosphorylation of distinct microtubule-associated proteins (*11*). The present investigation in primary human osteoblast-like cells reveals an enhanced phosphorylation of the p44 and p42 MAP kinases (ERK-1 and ERK-2) in response to $13\times g$ hypergravity. Interestingly, a recent study using non-gravitational magnetomechanical stimulation of the β1-integrin subunit reported the phosphorylation of two proteins in the region of 40 kDa in osteoblasts but not in fibroblasts, but no phosphorylation of p44/p42 MAP kinases (ERK-1/2) (*27*). These results may reflect distinct mechanosignaling pathways in different forms of mechanical stimulation. Since the ERK 1/2 pathway has been shown to be activated by certain forms of mechanical stimulation also in fibroblasts, the differences observed between osteoblasts and fibroblasts might be due to differences upstream or downstream of the MAP kinase signaling cascade (*28*).

The MAP kinases are proline-directed serine/threonine kinases that are activated in response to a wide array of extracellular stimuli; among other kinases they serve to connect the plasma membrane with cytoplasmic and nuclear events (*29, 30*). The p44 and p42 MAP kinases (ERK-1 and ERK-2) have been shown to be activated by integrin-dependent cell-matrix interaction and thus to be cell shape-dependent (*12, 31, 32*). The hypergravity-induced increase in osteoblastic collagen synthesis under the experimental conditions applied seems to be partially mediated by the MAP kinase pathway, because the inhibition of the p44/p42 (ERK-1/2)-pathway reduced the magnitude of this response without completely blocking it. Based on reports from other investigators, the MAP kinase-induced regulation of the transcription factor AP-1 may provide one explanation for the association of collagen synthesis and MAP kinase activation, since AP-1 is implicated in collagen I gene expression (*33-35*).

In summary, collagen synthesis and COL1A2 mRNA expression are increased in human, non-transformed osteoblast-like cells submitted to chronic hypergravity at $13\times g$. The elevated phosphorylation of the p44/42 kinase reflects the activation of the p44/42 (ERK-1/2) MAP kinase pathway under the gravitational loading conditions applied in the study. The data obtained in experiments using a specific inhibitor of the p44/42 (ERK-1/2) MAP kinase suggest that this MAP kinase pathway plays a role in the hypergravity-induced increase in collagen synthesis in human osteoblast-like cells.

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