Biochemical and Biophysical Research Communications 463 (2015) 928–933

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hypergravity-induced enrichment of β1 integrin on the cell membranes of osteoblast-like cells via caveolae-dependent endocytosis

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ARTICLE INFO

Article history:
Received 18 March 2015
Accepted 5 June 2015
Available online 9 June 2015

Keywords:
Hypergravity
Osteoblasts
Integrin
Caveolae
Mechanotransduction
Gravity sensors

ABSTRACT

In bone cells, integrins on the cellular surface are the primary sensors of their mechanical environment. Although gravitational changes are known to affect the adhesion and functions of bone cells, whether integrins respond to hypergravity in osteoblasts remains unclear. In this work, we demonstrate that exposure to a hypergravitational environment (20 × *g* via centrifugation) resulted in the concentration of β1, but not β3, integrin on the cell membrane of osteoblast-like (MC3T3-E1) cells. Notably, the total expression of both integrins was unaffected by the hypergravitational environment. In addition, caveolin-dependent endocytosis was discovered to be involved in the regulation of the enrichment of β1 integrin on the cell surface after stimulation by hypergravity. These findings could aid in the improvement of our understanding of the mechanisms underlying the effects of different gravitational forces on the human body.

1. Introduction

Not only the chemical but also the mechanical environment can regulate cellular properties including the cell cycle, shape, differentiation, and/or motility. The transduction of mechanical stimuli in bone cells is crucial to bone generation, resorption, and regeneration [1–6]. Hypergravity, accomplished with centrifugation, is a convenient approach to forge mechanical stress on cultured cells and has been found to impact osteogenesis. In addition, as an inevitable consequence of acceleration in space flight, the effects of hypergravity on bone cells have been investigated [7,8]. These recent findings suggest that hypergravity triggers certain mechanotransduction pathways and results in altered cell adhesion and the post-translational modification of extracellular matrix (ECM) proteins in bone cells [7,8]. However, the mechanisms underlying the effects of gravity on osteoblasts have yet to be clearly defined.

Of the various mediators involved in mechanotransduction, integrins play a crucial role in the response of osteoblasts to mechanical forces, and are positioned at the start of most mechanical sensing pathways. By assembling into obligate αβ heterodimers, integrins couple the ECM outside a cell to the actin bundles inside the cell. Upon activation by mechanical stimuli, integrins undergo tension-dependent conformational changes that affect kinase activity, phosphorylation site availability, intracellular localization, and/or ligand affinity [9,10]. Osteoblasts express a variety of integrin protein subunits, including β1, β3, α1, α2, α3, α4, α5, α6, and αv [2,11]. Different types of integrins recognize and bind diverse ECM proteins and mediate mechanotransduction independently or cooperatively.

Osteoblasts primarily express the integrin β subunits β1 and β3. The interaction of β1 integrin with fibronectin is essential for the survival and proliferation of osteoblasts [12,13], while the interaction of β1 integrin with collagen is important for the activity of the Runx2 transcription factor [14,15]. However, by binding to vitronectin and fibronectin, β3 integrin negatively modulates bone mineralization and osteoblast differentiation [16]. Therefore, these two subunits were examined in the present work to evaluate their role in hypergravity-sensing processes.

By employing centrifugation to apply a hypergravitational condition of 20 × *g* for 24 h to a well-established osteoblast-like cell line (MC3T3-E1), we determined the total expression of both β1 and β3 integrin and their individual contribution to the cell membrane protein content during hypergravity. In addition, we probed the possible mechanisms involved in the resulting β1

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http://dx.doi.org/10.1016/j.bbrc.2015.06.037
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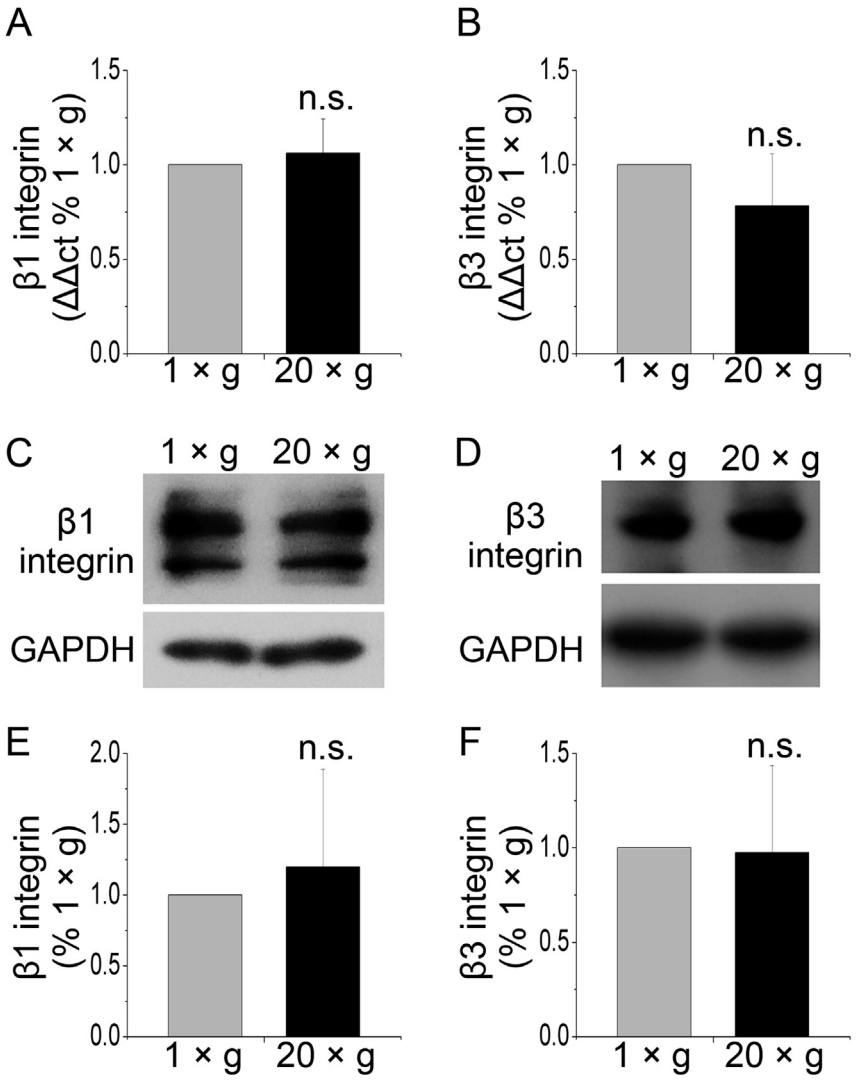

Fig. 1. The effects of gravity on the total expression of β1 and β3 integrins in MC3T3-E1 osteoblast-like cells. The mRNA and protein expression levels of β1 and β3 integrins after 24 h at either 1 × *g* or 20 × *g* are shown. (A) Comparative CT quantitation of real-time PCR results of β1 expression (mean ± SEM; n = 4). (B) Comparative CT quantitation of real-time PCR results of β3 expression (mean ± SEM; n = 4). (C) Western blots probed with anti-β1 integrin antibody, with anti-GAPDH as a loading control, in cells. (D) Western blotting results with anti-β3 integrin antibody, with anti-GAPDH as a loading control, in cells. (E) Statistical analyses of results from C. The means are shown as a percentage of the control values ± SEM from four experiments. (F) Statistical analyses of results from D. Means are represented as a percentage of the 1 × *g* values (mean ± SEM; n = 6). n.s. indicates no statistical difference between 1 × *g* and 20 × *g*.

integrin concentration on the cell membrane of MC3T3-E1 cells due to the hypergravitational environment.

2. Materials and methods

2.1. Reagents

Primaquine (PQ), monodansylcadaverine (MDC), and methyl-β-cyclodextrin (MBCD) were purchased from Sigma–Aldrich.

2.2. Cell culture

Osteoblast-like MC3T3-E1 cells were purchased from the cell center of the School of Basic Medicine of Peking Union Medical College. The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 1% penicillin–streptomycin at 37 °C in an atmosphere containing 5% CO_2. After reaching confluence, the cells were trypsinized; 1 × 10^5 cells were seeded for 2 h in flasks, and then exposed to 20 × *g* hypergravity for 24 h in a 37 °C incubator. PH of the culture medium was adjusted by adding 15 mM Hepes. In some experiments, MC3T3-E1 cells were incubated with the specific inhibitors PQ (60 μM), MDC (200 μM), or MBCD (10 mM) during exposure to hypergravity. The control cells were subjected to the same conditions as the experimental cells in terms of timing, incubation media, and other procedures, with the exception of the gravity condition; for this, control cells were incubated at 1 × *g* for 24 h in the 37 °C incubator as that used for the experimental cells.

2.3. RNA isolation and RT-PCR

The total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA, USA) and converted to cDNA by using a reverse transcription kit (Tiangen Biotech, Beijing, China), according to the manufacturer's instructions. The primers were designed using the Primer3Web software [17]:

β1 integrin, (sense, 5′-GCCAGGGCTGGTTATACAGA-3′; antisense, 5′-TCACAATGGCACACAGGTTT-3′), *β3 integrin*, (sense, 5′- GCTCA TTGGCCTTGCTACTC-3′; antisense, 5′-TAATGGCAGAGAGTCCC ACG-3′), GAPDH, (sense, 5′-TGCACCACCAACTGCTTAG-3′; antisense, 5′-GGATGCAGGGATGATGTTC-3′).

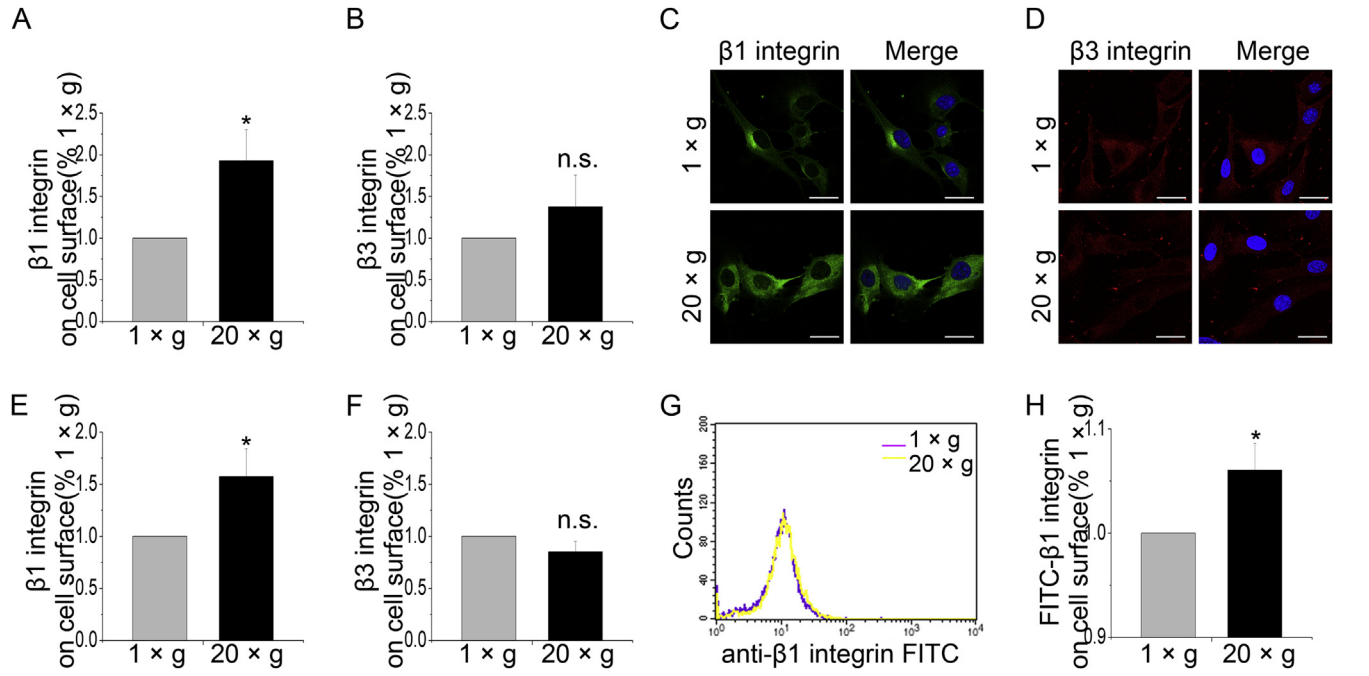

Fig. 2. The effects of gravity on the expression of β1 and β3 integrin on the surface of the osteoblast-like cells. (A and B) β1 integrin (A) and β3 integrin (B) on the cell surface analyzed by biotin labeling and capture ELISA (mean ± SEM; n = 6). (C and D) β1 integrin (C) and β3 integrin (D) on the cell surface were analyzed by immunofluorescence staining without cell permeation. (Scale bar = 25 μm). (E) Statistical analysis of results in C (mean ± SEM; cell count = 200) (F) Statistical analysis of results in D (mean ± SEM; cell count = 90) (G) Flow cytometry results of MC3T3-E1 cells freshly harvested and analyzed for cell surface expression of β1 integrin. (H) Statistical analysis of results in G (mean ± SEM; n = 7). n.s. indicates no statistical difference between 1 × *g* and 20 × *g*. *$P < 0.05$.

SYBR® Green Real-time PCR Master Mix (Toyobo Co., Osaka, Japan) was used in real-time PCR analysis. The samples were amplified with specific primers using the following cycling parameters: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, using a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany). The comparative CT method was used for gene quantitation with the housekeeping gene GAPDH employed as the internal standard.

2.4. Biotin labeling of cell surface proteins

Biotin labeling of cell surface integrins was performed according to the previously reported protocol [18]. Briefly, MC3T3-E1 cells were washed and incubated twice with 1 mg/mL sulfo-NHS-LC-biotin (Pierce Biotechnology, Rockford, IL, USA) for 20 min at 4 °C. Next, the cells were lysed with RIPA lysis buffer (Applygen Technologies, Inc., Beijing, China), and the whole cell lysate was collected. The supernatant was subjected to capture ELISA for detection of biotinylated β1 or β3 integrin. Antibodies for β1 integrin (1:100; Abcam, Cambridge, UK) and β3 integrin (1:100; Abcam) were coated onto 96-well plates (Nunc™ MaxiSorp™). After washing with 0.05% Tween-20 in PBS (PBST), the wells were incubated with 5% BSA for 1 h at room temperature (RT), and the cell lysate samples were incubated for 1 h at RT. After washing with PBST, the wells were incubated with streptavidin-conjugated horseradish peroxidase (HRP, Sigma–Aldrich, St. Louis, MO, USA) for 1 h at 4 °C. Finally, biotinylated integrin was detected by reaction with the chromogenic HRP substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma–Aldrich).

2.5. Western blot analysis

MC3T3-E1 cells were lysed with RIPA lysis buffer, and the total cell lysate was separated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes, blocked with 5% non-fat milk, and incubated with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:10,000) or anti-rabbit IgG (1:10,000) (Applygen Technologies, Inc.). Anti-GAPDH was used as a loading control. Finally, the membranes were developed with enhanced chemiluminescence reagents (EMD Millipore, Billerica, MA, USA) and exposed to an X-ray film (Eastman–Kodak, Rochester, NY, USA).

2.6. Immunofluorescence staining

MC3T3-E1 cells were fixed with 4% paraformaldehyde. Then, the fixed cells were incubated for 5 min with 0.1% Triton X-100 followed by 5% BSA for 30 min at RT for blocking. Primary antibodies for β1 integrin (1:200, Abcam), β3 integrin (1:200, Abcam), caveolin-1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and clathrin (1:200, Santa Cruz Biotechnology) were applied overnight at 4 °C. A secondary DyLight 488-conjugated antibody (1:200, EarthOx LLC, Millbrae, CA, USA) or DyLight 594-conjugated antibody (1:200, EarthOx) was applied for 1 h and DAPI stained (1:10,000, Invitrogen) for 10 min at RT. The samples were imaged using a Leica TCS SP5 confocal microscopy system (Leica Microsystems, Wetzlar, Germany).

2.7. Flow cytometry analysis

MC3T3-E1 cells were collected and blocked with 5% BSA. Cell surface β1 integrins were stained with FITC-conjugated β1 integrin antibody (0.5 μg/10^6 cells) for 30 min at RT. The fluorescence intensity was determined using a BD FACSCalibur™ system (BD Biosciences, Franklin Lakes, New Jersey).

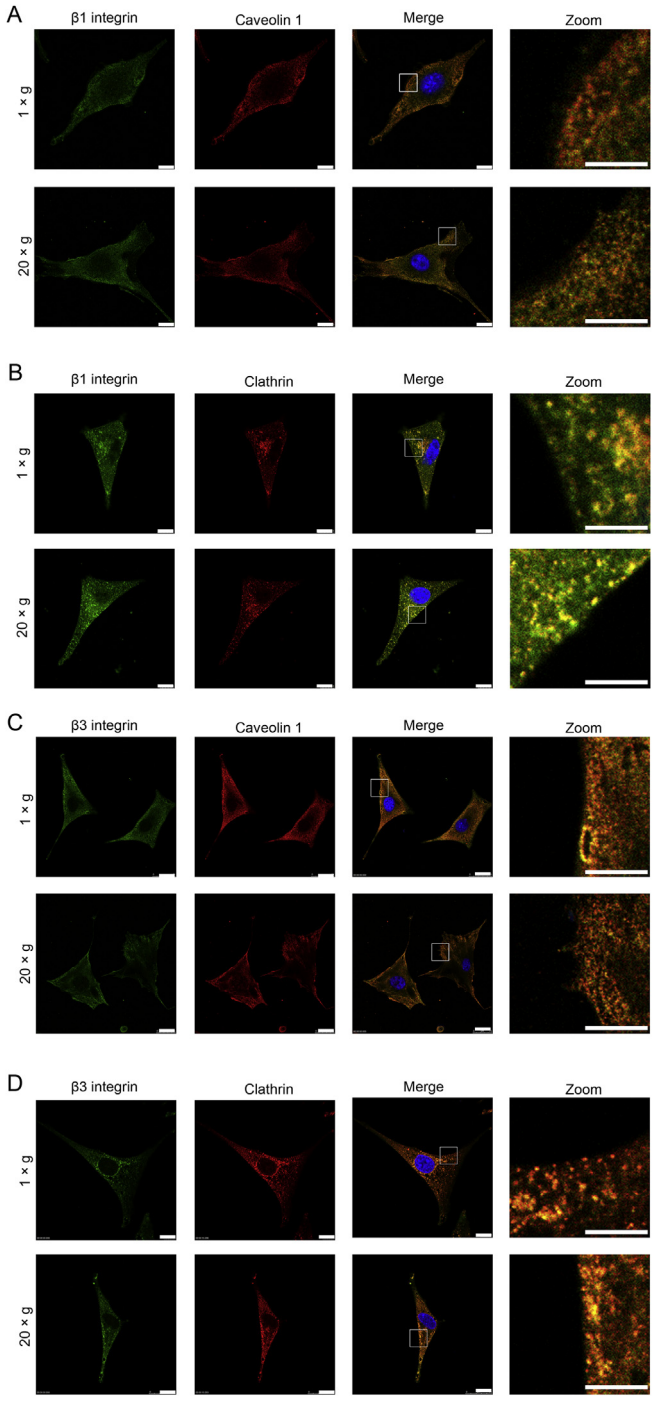

Fig. 3. The co-localization of β1 or β3 integrin with caveolin-1 and clathrin. MC3T3-E1 cells were im munofluorescently stained for the presence of β1 or β3 integrin with caveolin-1 or clathrin after exposure to 1 × *g* and 20 × *g* conditions. Panels show individual immunostains for the integrin and either caveolin-1 or clathrin, an overlay of the two, and a magnified view of the merge. (A) β1 integrin (green) and caveolin-1 (red). (B) β1 integrin (green) and clathrin (red). (C) β3 integrin (green) and caveolin-1 (red). (D) β3 integrin (green) and clathrin (red). (Scale bars = 7.5 μm).

2.8. Statistical analysis

The results were expressed as mean ± SE. All experiments were performed at least in triplicate. Student's *t*-test was used to compare the differences between the two experimental groups. A *P*-value <0.05 was considered significant.

3. Results

3.1. Effects of hypergravity on total β1 and β3 integrin expression in MC3T3-E1 cells

We first examined the hypergravitational effects on the total expression of β1 and β3 integrins in MC3T3-E1 cells. MC3T3-E1 cells were subjected to hypergravity by centrifugation at 20 × *g*, or kept at 1 × *g* for controls, for 24 h. Subsequently, the expression of β1 and β3 integrins was determined by RT-PCR and western blots. Our results revealed that the mRNA and protein levels of β1 and β3 integrin were not affected by hypergravity in MC3T3-E1 cells (Fig. 1).

3.2. Effects of hypergravity on the levels of β1 and β3 integrins on the cell membrane

We assayed the levels of β1 and β3 integrins on the cell surface by labeling those on the cell surface with biotin and detecting them with ELISA. Upon hypergravity stimulation, the level of β1 integrin on cell membrane increased, while the level of β3 integrin remained unchanged, compared to the controls (Fig. 2, panels A and B). Immunofluorescent staining of cell surface proteins showed similar results (Fig. 2, panels C–F). Moreover, hypergravity-enrichment of β1 integrin on the cell surface was confirmed by flow cytometry assays (Fig. 2, panels G and H). These results indicated that 20 × *g* hypergravity enhanced β1 integrin concentration on the cell membrane, but not β3 integrin concentration.

3.3. Correlation of the localization of β1 integrin with caveolin-dependent endocytosis

To investigate the contribution of endocytosis on β1 and β3 integrin cell surface distribution under hypergravity, MC3T3-E1 cells were double-labeled with antibodies recognizing β integrins and caveolin-1 or clathrin. Both β1 and β3 integrins co-localized with caveolin-1 and clathrin in the presence or absence of hypergravity (Fig. 3). Addition of MBCD, a well-documented inhibitor of caveolae-mediated endocytosis, increased the concentration of both β1 and β3 integrin on cell surface in controls, while inhibiting the hypergravitational effects of the surface distribution of β1 integrin (Fig. 4 panels A and B). MDC, an inhibitor of clathrin-mediated endocytosis, however did not contribute to the hypergravity-induced surface distribution of β1 or β3 integrins (Fig. 4 panels C and D). Together, these data suggest that caveolin-dependent endocytosis was required for the hypergravitational enrichment of β1 integrin on the cell surface.

3.4. Contribution of integrin recycling to hypergravity-induced accumulation of β1 integrin on the cell surface

We used PQ, a membrane-trafficking inhibitor, to evaluate the effects of cell recycling on the hypergravity-induced β1 integrin accumulation on the cell surface. PQ did not affect the level of β1 integrin on cell surface in control groups, and no significant differences were observed under conditions of hypergravity (Fig. 4E). These results suggest that the process of recycling integrin back to the membrane was not involved in the hypergravitational enrichment of β1 integrin distribution on the cell surface. Therefore, the effects of a hypergravity environment on the distribution of β1 integrin were associated with caveolin-dependent endocytosis.

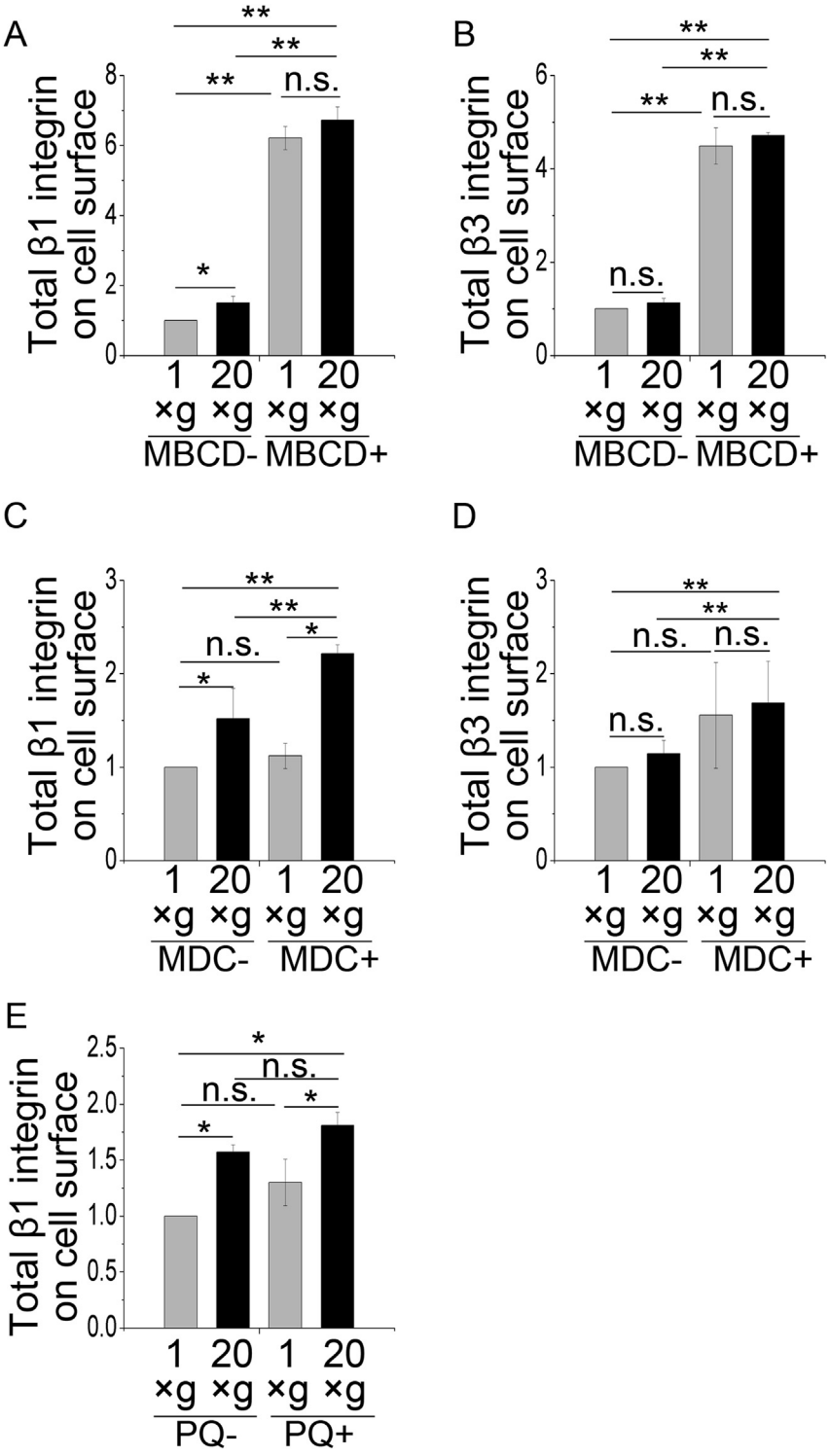

Fig. 4. The expression of β1 and β3 integrins on the cell surface after the inhibition of endocytosis and membrane trafficking. The levels of β1 integrin (A and C) and β3 integrin (B and D) on the MC3T3-E1 cell surface were analyzed by biotin labeling and capture ELISA after exposure to 1 × *g* and 20 × *g* for 24 h. (A and B) The cells were treated with 10 mM MBCD or culture medium during hypergravity stimulation (mean ± SEM; n = 3). (C and D) The cells were treated with 200 μM MDC or culture medium during hypergravity stimulation (mean ± SEM; n = 4). (F) The level of β1 integrin on the cell surface was analyzed by biotin labeling and capture ELISA. The cells were treated with 60 μM PQ or medium at 1 × *g* and 20 × *g* for 24 h (mean ± SEM; n = 5). n.s. indicates no statistical difference between 1 × *g* and 20 × *g*. $^{*}P < 0.05$, $^{**}P < 0.01$.

4. Discussion

The effects of hypergravity on the expression of integrins have been investigated in several types of cells. The expression of β1 integrin in MG-63 osteosarcoma cells exposed to centrifugal loading at 209 × *g* for 10 min rapidly increased after 30 min, followed by reduction to levels similar to unloaded groups after 12 h [19]. In contrast, centrifugal force at 25 × *g* for 24 h did not change the mRNA expression of β1 integrin in rat dermal fibroblasts [20]. In our study, we found that MC3T3-E1 human osteoblast-like cells

exposed to hypergravity at 20 × g for 24 h did not exhibit a significant difference in the expression of β1 or β3 integrins at either mRNA or protein levels, compared to 1 × g controls.

Unlike the total expression of integrins, the subcellular distribution of integrins after stimulation with hypergravity was previously undetermined. However, studies have demonstrated that integrin was crucial for cellular adhesion under altered gravity. It was reported that microgravity affected integrin-mediated ROS17/2.8 osteoblast adhesion during space flight [21], and hypergravity altered the number and distribution of focal adhesions in osteoblasts [7]. We, therefore, suspected that hypergravity might regulate integrin distribution on the cell membrane, and found that hypergravity enriches β1, but not β3, integrin on the cell membrane. Different integrin subtypes have distinct functions; for example, α5β1 integrin determines cell adhesion, while αvβ3 integrin contributes to mechanotransduction in fibroblasts [22]. It has also been observed that the activation of β1, but not β3, integrin promotes the traction force in mouse embryonic fibroblasts [23]. The present results suggest that β1-specific signal pathways under hypergravity are worth further investigation.

The increased distribution of β1 integrin on the cell surface could be modulated through several possible mechanisms. The changes in the distribution of β1 integrin on the cell surface could be attributed to changes in their recycling or endocytosis [24,25]. As the β subunits of integrins can be engulfed in a clathrin-dependent manner or follow the caveolae internalization route [24–26], we analyzed whether hypergravity has effects on integrin internalization via these pathways. We found that caveolin-dependent endocytosis was required for hypergravity-enhanced β1 integrin distribution on the cell membrane. Evidence from other groups and our previous study have shown that caveolin-mediated endocytosis is considerably involved in mechanotransduction [27,28], and plays crucial roles in altered cellular properties. Therefore, we propose that β1 integrin and caveolae are co-candidate gravity-sensors in osteoblasts; an intensive study of the possible upstream elements and the downstream effect on caveolin-dependent endocytosis under hypergravity would be of significant interest. Similarly, an investigation of osteoblasts at earlier stages of hypergravitational exposure would advance the search for other potential gravity sensors.

In summary, the data presented here suggest that hypergravity increases the distribution of β1 integrin on the cell membrane in a caveolin-dependent manner. Examination of the relationship between β1 integrin and caveolae for gravity sensing will aid in elucidating the mechanisms underlying the cellular effects of hypergravity.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (Nos. 31170885 and 31370939), Tsinghua University (2011Z02175). We would like to thank Editage (www.editage.com) for English language editing.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.037.

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