Acta Astronautica 129 (2016) 186–192

Contents lists available at ScienceDirect

Acta Astronautica

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

Simulated microgravity alters the expression of cytoskeleton- and ATP-binding-related genes in MLO-Y4 osteocytes

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ARTICLE INFO

Keywords:
Simulated microgravity
Random position machine (RPM)
Gene expression profile
Osteocyte
ATP-binding
Cytoskeleton

ABSTRACT

Bone undergoes dynamic modelling and remodelling processes, and it requires gravity-mediated mechanical stimulation for the maintenance of mineral content and structure. Osteocytes are the most commonly found cells in the mature bone, and they are sensitive to mechanical changes. The purpose of this study was to investigate the effects of microgravity simulated with a random position machine (RPM) on the gene expression profile of osteocytes. Genes sensitive to RPM treatment were sorted on the basis of biological processes, interactions and signalling pathways. Overall, 504 differentially expressed genes (DEGs) in osteocytes cultured under RPM conditions were found. The DEGs were further analysed using bioinformatics tools such as DAVID and iReport. A total of 15 ATP-binding and cytoskeleton-related genes were further confirmed by quantitative real-time PCR (qRT-PCR). Our findings demonstrate that the RPM affected the expression of genes involved in cytoskeleton remodelling and the energy-transfer process in osteocytes. The identification of mechanosensitive genes may enhance our understanding of the roles of osteocytes in mechanosensation and may provide some potential targets for preventing and treating bone-related diseases.

1. Introduction

Gravity, the most common type of mechanical stimulation, is necessary for all living organisms. In humans, the balance of the bone remodelling process, which is maintained by gravity, is altered after long-term space missions, and the imbalance results in bone mass loss that endangers the health and safety of astronauts [1,2]. Osteocytes are the predominant cell type in the mature bone [3]. Recent evidence has shown that osteocytes are involved in mechanotransduction and are the potential mechanosensors in bone tissue [4–6], including microgravity mechanotransduction [7]. Therefore, it is believed that osteocytes account for most of the bone mass lost during space flight [8].

There are currently a few ground-based methods available to simulate microgravity. Due to the cost and the limited opportunities for spaceflight experiments, it is necessary to develop ground-based models that simulate microgravity. At present, ground-based methods such as clinorotation [9,10], the NASA rotating wall vessel bioreactors [11] and diamagnetic levitation [12–16] have been employed to simulate the space microgravity environment and its effects on bone cells. The random position machine (RPM), a type of widely used clinorotation device, rotates biological samples along two independent axes to change their orientation spatially and to eliminate the effects of gravity [17]. We previously investigated the effects of RPM on both osteoblasts [18] and osteocytes [19], and the results showed that osteoblast differentiation and the levels of surface-biotinylated connexin43 in osteocytes were significantly decreased.

Although recent studies have shown which biological processes in osteocytes were altered under simulated microgravity, the mechanism of how osteocytes respond to microgravity is unclear. In this study, we compared the gene expression profile of osteocytes cultured under RPM or under static control conditions and found that the expression of cytoskeleton- and ATP-binding-related genes was sensitive to simulated microgravity, and these results were verified by qRT-PCR. The results can provide new insight into how microgravity alters the physiological state of osteocytes and an in-depth understanding of the molecular mechanism of bone loss during spaceflight.

2. Materials and methods

2.1. Random positioning machine (RPM)

As previously described, a desktop random positioning machine

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http://dx.doi.org/10.1016/j.actaastro.2016.09.017
Received 6 July 2016; Accepted 1 September 2016
Available online 16 September 2016

Fig. 1. The schematics of (A) the random positioning machine (RPM) and (B) the culture flask for the RPM.

(RPM) (the Center for Space Science and Applied Research of Chinese Academy of Sciences) was used to simulate microgravity conditions (Fig. 1A) [18–20]. The RPM contains outer and inner frames that are fixed to two axes separately and can rotate independently in random directions and speeds when controlled by a micro-computer system. The machine was operated in random mode with speeds ranging from 0.1 to 10 revolutions per minute and with random changes in direction for both the inner and outer frames [21]. The time-averaged gravitational vector acting on the samples was reduced to ~2×10^{-3} g at the center of the frame [22]. Custom-made glass culture flasks were manufactured by a local company for culturing cells inside the machine. The dimensions of the culture flask were 35.0 mm (length)×30.0 mm (width)×20.0 mm (height) (Fig. 1B) and a volume capacity of 15 ml..

2.2. Cell culture under simulated microgravity using the RPM

The murine MLO-Y4 osteocyte-like cells were a gift from Prof. Linda F. Bonewald of the University of Texas Health Science Center. Cells were cultured in α-MEM medium (Gibco, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 5% foetal bovine serum (FBS) and 5% bovine calf serum (BCS) (Gibco, Carlsbad, CA, USA) at 37 °C, 5% CO_2 in a humidified atmosphere.

To culture cells under RPM conditions, MLO-Y4 cells were detached by trypsinization, inoculated at a density of 4×10^4 cells/cm^2 onto carrier slides (pretreated with collagen), and cultured in a 37 °C, 5% CO_2 incubator until the cells attached to the substrate. The carrier slides were then carefully removed and transferred into custom-made culture flasks filled with culture medium while avoiding air bubbles. The flasks were then tightly capped and fixed to the inner frame of the RPM machine, and the machine was placed in a 37 °C, 5% CO_2 incubator for 48 h. A static control group was placed in the same incubator and cultured for 48 h. Each group had three samples.

2.3. Total RNA isolation and microarray preparation

Total RNA was extracted from MLO-Y4 cells cultured under the RPM or under control conditions for 48 h using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was purified using the RNeasy Micro Kit (Qiagen, GmBH, Germany) and RNase-Free DNase Set (Qiagen, GmBH, Germany), and then the purity of RNA was determined by measuring the 260/280 absorbance ratio. Gene expression was profiled by using the Affymetrix GeneChip mouse ST 1.0 (Affymetrix, Santa Clara, CA, USA). Briefly, cDNA was prepared using the One-Cycle cDNA Synthesis Kit and GeneChip IVT Labeling Kit (Affymetrix). Then, it was hybridized by Genechip Hybridization (Affymetrix) and then automatically washed and stained in an Affymetrix Genechip Fluidics Station 450. The fluorescence intensities of the microarray data were scanned by a GeneArrayTM scanner 3000 (Affymetrix).

2.4. Microarray data analysis

The Robust Multi-Chip Average (RMA) algorithm was used to normalize the raw data acquired by gene chip. Differentially expressed genes (DEGs) were identified based on statistically fold change(FC) at FC > 1.5. Generally, hierarchical clustering was selected as the clustering algorithm. Gene Ontology (GO) analysis provided a common vocabulary for the functional description of DEGs and gene products and consisted of three sub-ontology categories: Molecular Function (MF), Biological Process (BP) and Cellular Component (CC). DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/) were used to annotate the genes, and the functional annotation cluster tool and gene function classification tool were used to analyse the DEGs. To clarify the functions of DEGs, gene annotation and pathway analysis were conducted. The Ingenuity iReport system (http://www.ingenuity.com/products/ireport) was used to obtain the quantity control information on both the raw data and the normalized data. The DEGs of the cells cultured under RPM vs. the static control cells were filtered depending on the iReport system. The interactions of these genes with diseases, biological processes, and signalling pathways that have significant connections with these genes were then mined by the iReport system as well.

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from MLO-Y4 cells cultured under RPM or under static control conditions for 48 h using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was reverse-transcribed using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). Gene expression was then examined through qRT-PCR with a SYBR Premix Ex Taq II kit (TaKaRa, Dalian, China), and qRT-PCR analysis was performed using the Thermal Cycler C-1000 Touch system (BIO-RAD CFX Manager, USA). All amplifications were normalized to 18S rRNA. Data were analysed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) and expressed as fold change compared to control. Each group had three replicates.

2.6. Statistical analysis

All numerical data were expressed as the mean ± SD. Statistically significant differences were analysed using Student's *T*-test analysis in the statistical software Prism (GraphPad Software Inc, La Jolla, CA,