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Research Article

The Influence of Simulated Microgravity on Purinergic Signaling Is Different between Individual Culture and Endothelial and Smooth Muscle Cell Coculture

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Exposure to microgravity conditions causes cardiovascular deconditioning in astronauts during spaceflight. Until now, no specific drugs are available for countermeasure, since the underlying mechanism is largely unknown. Endothelial cells (ECs) and smooth muscle cells (SMCs) play key roles in various vascular functions, many of which are regulated by purinergic 2 (P2) receptors. However, their function in ECs and SMCs under microgravity conditions is still unclear. In this study, primary ECs and SMCs were isolated from bovine aorta and verified with specific markers. We show for the first time that the P2 receptor expression pattern is altered in ECs and SMCs after 24 h exposure to simulated microgravity using a clinostat. However, conditioned medium compensates this change in specific P2 receptors, for example, P2X7. Notably, P2 receptors such as P2X7 might be the important players during the paracrine interaction. Additionally, ECs and SMCs secreted different cytokines under simulated microgravity, leading into a pathogenic proliferation and migration. In conclusion, our data indicate P2 receptors might be important players responding to gravity changes in ECs and SMCs. Since some artificial P2 receptor ligands are applied as drugs, it is reasonable to assume that they might be promising candidates against cardiovascular deconditioning in the future.

1. Introduction

Exposure to microgravity conditions during space missions induces a variety of health issues in astronauts, including bone loss, muscle atrophy, decreased immune activity, and cardiovascular deconditioning [1–3]. The cardiovascular deconditioning is very likely caused by the dysfunction of the major vascular cells: endothelial cells (ECs) and smooth muscle cells (SMCs). ECs build up the monolayer coating inner surface of blood vessels. Layers of SMCs arranged in fibers support the EC monolayer by providing contraction and relaxation of vessels [4]. Importantly, the interaction between ECs and SMCs has been shown to be a key player in human cardiovascular physiology [5]. ECs are sensitive to mechanical stress, and they secret cytokines inhibiting SMC proliferation [6]. Purinergic receptors can bind extracellular nucleotides such as ATP [7, 8] and they are crucial players in regulating a series of physiological and pathological cardiovascular processes such as atherosclerosis, hypertension, and vascular pain [9, 10]. Purinergic receptors are divided into P1 receptors and P2 receptors [11]. P2 receptors can be subdivided into P2X receptors that are ion channels and P2Y receptors that are G protein-coupled receptors [12]. Until now, seven P2X (P2X1-7) and eight P2Y (P2Y1, 2, 4, 6, 11, 12, 13, and 14) have been characterized. However, the role of extracellular nucleotides on vascular cell function under microgravity condition is still unknown.

Recent publications have shown that cytoskeleton arrangement, gene expression of extracellular matrix, and cell surface adhesion molecules in ECs were altered after 22 seconds and 24 h exposure to microgravity [13–16]. ECs formed tubes after culturing for longer term (7 days)

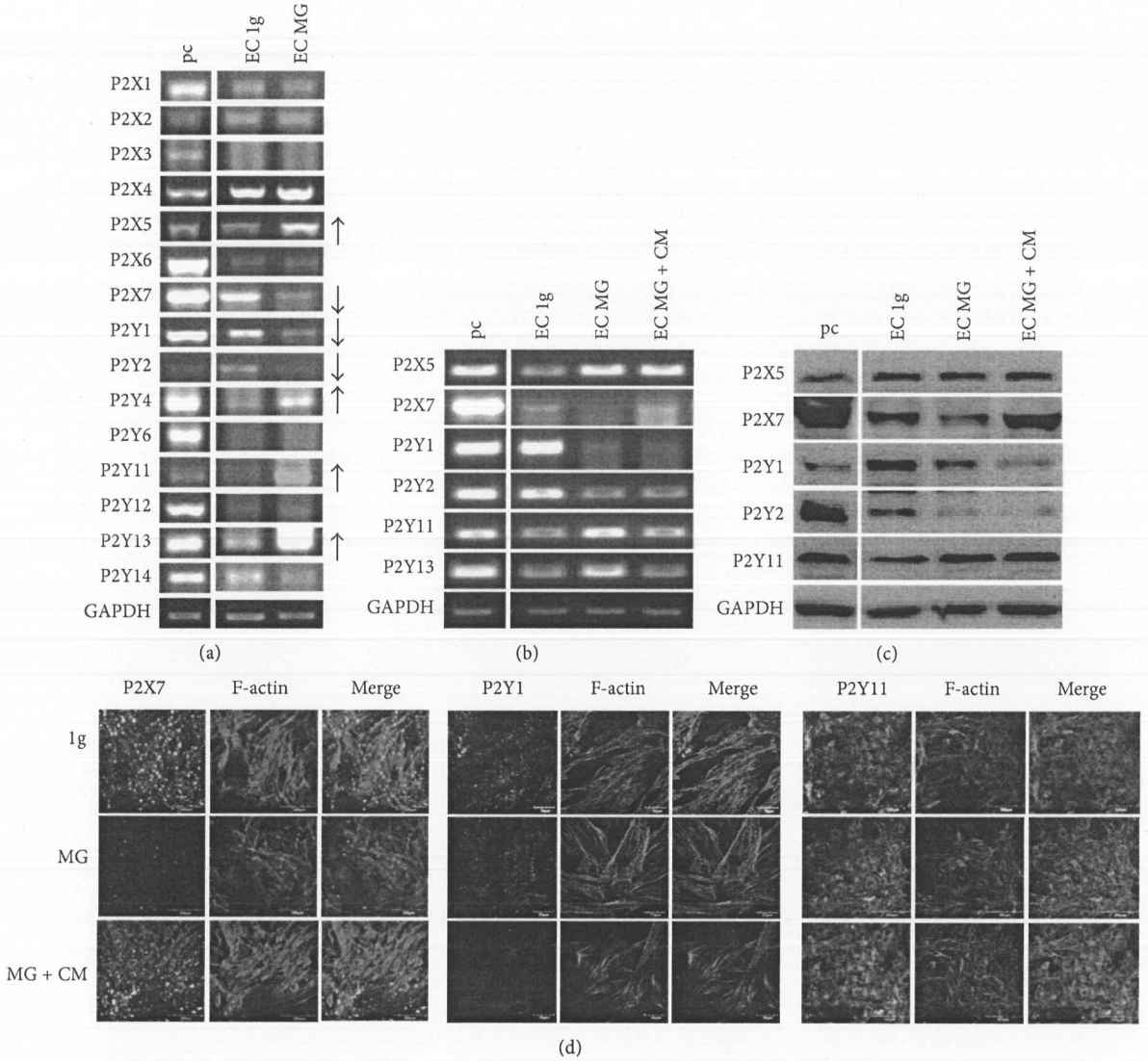

FIGURE 3: P2 receptor expression in endothelial cells after 24 h under normal gravity and simulated microgravity. All cells on the surface of flasks were isolated for RT-PCR. P2X5, P2Y4, P2Y11, and P2Y13 were upregulated and P2X7, P2Y1, and P2Y2 were downregulated in the ECs after 24 h in the clinostat (a). Only cells grown within 6 mm of the center had the optimal simulated microgravity condition and were therefore isolated to confirm the above P2 receptor alteration on the RNA (b) and protein (c) level after 24 h simulated microgravity with and without SMC-conditioned medium collected under normal gravity. P2X5 and P2Y11 were upregulated in ECs but P2X5 upregulation was not significant on protein level. P2X7, P2Y1, and P2Y2 were downregulated on both gene and protein level. The SMC-conditioned medium can compensate the decrease of P2X7 expression but cause no significant effect on the alteration of P2Y1, P2Y2, and P2Y11. The fluorescent staining confirmed the protein change of P2X7, P2Y1, and P2Y11 (d).

set and performed with SMCs as well. All experiments were performed with samples from three cows.

2.5. RNA Isolation and Semiquantitative PCR. RNA was extracted after clinorotation using a Ribozol RNA reagent (Amresco, OH, USA). cDNAs were synthesized from 2.0 μg total RNA by using Revert Aid Reverse Transcriptase and oligo-dT primer (Thermo Fisher Scientific, MA, USA). Primers for P2 receptors, EC, and SMC specific markers in the human and bovine system were designed and shown in the supplementary data available at http://dx.doi.org/10.1155/2014/413708. The RT-PCR conditions such as annealing temperature and magnesium concentration are given in the supplementary data as well. 1% of agarose gels were set up to evaluate the RT-PCR products. As positive control, RNA extracts from the cell lines HMEC-1, MG-63, C2C12, and U-87 MG were used for respective P2 receptor subtypes given in the supplementary data.

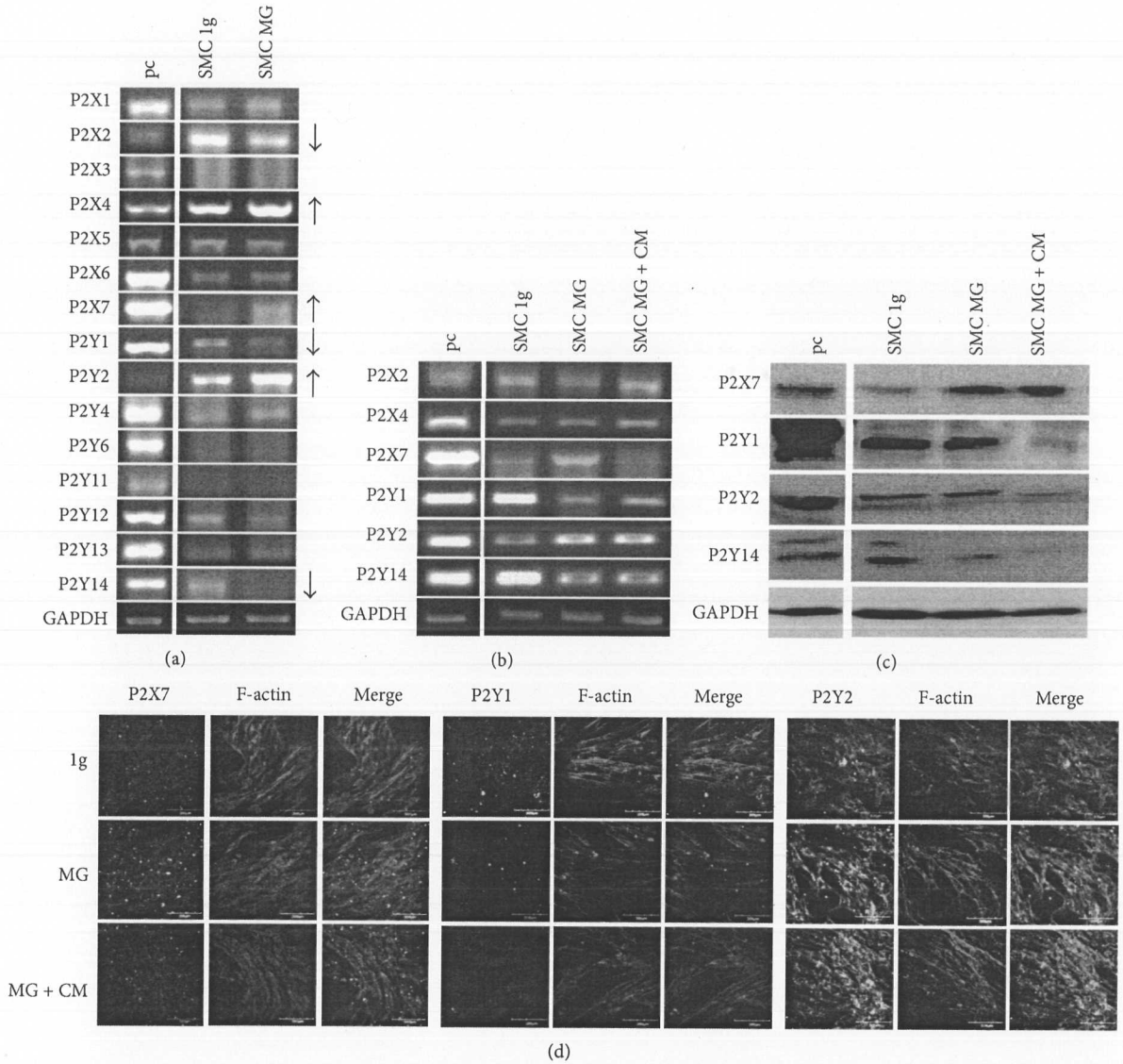

FIGURE 4: P2 receptor expression in smooth muscle cells after 24 h under normal gravity and simulated microgravity. The experiments for SMC were performed similarly to those for the endothelial cells above. All cells on the surface of flasks were isolated for RT-PCR. P2X4, P2X7, and P2Y2 were upregulated, whereas P2X2, P2Y1, and P2Y14 were downregulated in the SMCs after 24 h clinorotation (a). Only cells grown within 6 mm of the center had the optimal simulated microgravity condition and were thus isolated to confirm the above P2 receptor alteration on both RNA (b) and protein (c) level after 24 h clinorotation with and without EC-conditioned medium from normal gravity. P2X2 and P2X7 showed no significant change. P2X7 and P2Y2 were upregulated; P2Y1 and P2Y14 were downregulated. The EC-conditioned medium can compensate for the increase of P2X7 expression, but no significant effect was observed on P2Y1, P2Y2, and P2Y14 (d).

2.6. Western Blot Analysis. The proteins were extracted from the cells in a protein lysis buffer (Cell Signaling Technology, MA, USA) and subsequently centrifuged at 22,000 g for 5 min at 4°C to remove cellular debris. After boiling for 5 min, the lysate samples were separated by a 12% SDS-PAGE electrophoresis and electrotransferred to a PVDF membrane. The membrane was blocked in TBST containing 5% BSA and incubated with anti-P2X7, P2Y1, P2Y2, P2Y11, VEGFR2, VE-cadherin, PECAM-1, calponin, SMA-α, MYH-11 (1 : 500), or GAPDH antibodies (1 : 5,000) (Santa Cruz Biotechnology, CA, USA) overnight at 4°C. The membranes were washed three times with TBST and incubated with the secondary antibodies (1 : 5,000) (CALBIOCHEM, CA, USA) for 60 min at RT. After washing with TBST, immune-detection was accomplished by using the Luminata Forte Western HRP substrate (Merck Millipore, MA, USA) and images were taken using Bio-Rad Chemidoc system.

2.7. Immunofluorescence. The cells were fixed in 4% paraformaldehyde for 15 min. Cells were incubated with

under simulated microgravity conditions using a Random Positioning Machine (RPM) [17]. On the other hand SMCs showed suppressed proliferation and an enhanced rate of apoptosis after 72 h exposure to simulated microgravity using a rotating wall vessel (RWV) [18]. However, these findings were encountered when single cell type such as endothelial or smooth muscle cells was cultured under real or simulated microgravity. Considering ECs already showed to secrete different cytokines under simulated microgravity using RPM [19], the important interactions between ECs and SMCs under microgravity condition should be evaluated and thus require investigations.

In this study, an indirect cell coculture model was established by culturing SMCs with EC-conditioned medium and vice versa. The P2 receptor expression pattern was analyzed and compared under three conditions: normal gravity control (1 g), simulated microgravity (MG), and simulated microgravity with conditioned medium. For the simulation of microgravity a fast rotating clinostat was used, in which the cells were quickly rotated around one axis perpendicular to the direction of gravity [20]. The influence of conditioned medium collected from normal gravity and simulated microgravity on cell proliferation and migration was also investigated.

2. Methods

2.1. Isolation and Characterization of Bovine Aortic Endothelial and Smooth Muscle Cells. Bovine aorta was cut longitudinal into 5 cm sections and divided again into rectangles after removing residual and connective tissues. The cut aorta into type I collagenase (10 mg/mL in PBS) coated cell culture dishes, with the inner layer (endothelium) attached to the collagenase, and incubated for 60 min at 37°C. The aortic endothelial cells were slightly scraped with a cell scraper and put onto gelatin (in PBS (1% v/v)) coated culture plates [21]. Medium was added to the freshly scraped cells and the plates were then incubated at 37°C, 5% CO_2 under humidified conditions. Aortic smooth muscle cells were isolated by obtaining the media layer through removal of the outer layer and scraping off the endothelial cells. The media layer was cut into 2 mm × 2 mm sections and put into cell culture dish for 2 h without medium to allow these sections adhering tightly to the surface [22]. The medium was added and the pieces were incubated at 37°C, 5% CO_2 under humidified conditions for up to a week to let SMCs migrate and proliferate from the tissue pieces to the surface of culture dish.

2.2. Cell Culture. The cells were cultured in DMEM medium (Merck Millipore, Berlin, Germany) supplied with 10% FCS and 1% penicillin/streptomycin. ECs and SMCs were split and seeded at a density of 5000 cells/cm^2, after they reached a level of 80–90% confluence. ECs and SMCs with passage number 2–4 were used. The cell line human microvascular endothelial cell-1 (HMEC-1), C2C12 (ATCC number: CRL-1772) and MG-63 (ATCC number: CRL-1427), and U-87 MG (ATCC number: HTB-14) were cultured in DMEM medium and subsequently used as positive control.

2.3. Clinostat Experiments. The fast-rotating 2D clinostat used in this study was originally developed by the Institute of Aerospace Medicine, German Aerospace Center (DLR) (see Figure 1(a)). It has 6 parallel horizontal axes, each for fixation for up to 4 slide flasks. ECs and SMCs were seeded at a density of 10,000 cells/cm^2 onto 9 cm^2 cell culture slide flasks (Nunc, Thermo Fisher Scientific, Langenselbold, Germany). When they reached a confluence level of 60%–70%, the culture flasks were filled up completely with DMEM medium. To avoid shear stress and thus the induction of respective metabolic changes in signal transduction pathways, for example, apoptosis, air bubbles were removed carefully. The flasks were inserted on the clinostat and rotated at 60 rpm for 24 h in the CO_2 incubator at 37°C. Controls were also filled with medium and placed simultaneously under normal gravity.

Cells from the whole flask were first used to analyze the P2 receptor expression pattern that altered subtypes could be distinguished from unaffected. Later, according to the clinostat principle, only cells exposed to minimal low-g-forces were taken for further analysis. This means that only cells from the middle of the flask were taken (see Figure 1(b)). Under a defined constant speed of 60 rpm the maximal residual acceleration at an area of 6 mm provided an optimal quality of simulated microgravity (≤ 0.0121 g) [23]. Thus, only cells within this 6 mm area were isolated to evaluate altered P2 subtypes for both gene and protein expression in detail. To maintain the cells accurately and consistently in the center, a special chamber consisting of two cover plates, attached to a bottom plate (developed by DLR), was used to allow slide insertion without wiping off the cell layer. A corresponding cell scraper was used to scratch the cells from the specific 6 mm width area in the center (Figure 1(c)).

2.4. Conditioned Medium. To investigate a possible paracrine influence on P2 receptor expression, ECs and SMCs were seeded in a density of 2500 cells/cm^2. Cell growth medium was collected when they were 80%–90% confluent. The conditioned medium (CM) was composed out of cell growth medium and normal DMEM medium in a ratio of 1 : 2 on respective cell type. The SMC-conditioned medium was subsequently fully added into the culture slide with ECs and set of a 24 h clinorotation as a group of EC MG + CM. The ECs in normal gravity group (EC 1 g) and in clinorotation but filled with normal DMEM medium (EC MG) were set simultaneously. The similar experiments were set for SMCs with normal gravity (SMC 1 g), clinorotation (SMC MG), and clinorotation filled with EC-conditioned medium (SMC MG + CM).

To evaluate a possible paracrine effect on cell proliferation and migration, cell growth medium was collected from cells cultured 24 h in normal gravity and cultured 24 h under clinorotation, respectively. The used conditioned medium (CM) was composed out of cell growth medium and normal DMEM medium in a ratio of 1 : 2 on respective cell type. The ECs were subsequently treated with normal DMEM medium, SMC-conditioned medium from normal gravity (CM SMC + 1 g), and clinorotation (CM SMC + MG) separately for proliferation or migration assays. Similar experiments were