Alteration of Adenylyl Cyclase Type 6 Expression in Human Astrocytoma Cells After Exposure to Simulated Microgravity

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Although many physiological changes after space flight have been reported, it is not clear how microgravity influences our bodies. The focus of the present study was to clarify the changes in G-protein-coupled receptor-mediated intracellular signaling, especially G_s-adenylyl cyclase (AC)-adenosine 3′, 5′-cyclic monophosphate (cyclic AMP) pathway, under simulated microgravity. Human astrocytoma 1321N1 cells were cultivated under vector-averaged microgravity conditions generated by clinostat rotation (20 rpm) for 24 hr. Isoproterenol, a β-adrenergic agonist and forskolin, a direct AC stimulant, increased intracellular cyclic AMP level in concentration dependent manners, however, both of which response were decreased in cells cultivated in clinostat rotation. While the level of $G\alpha_s$ or intracellular ATP, a substrate for AC, was not changed, the AC activity was significantly low in the membranes of clinostat-rotated cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that AC type 3 (AC3), AC6, and AC9 and to a lesser extent AC7 and AC8 were expressed in 1321N1 cells. Among them, the expression of AC6 mRNA was significantly decreased by clinostat rotation. These results indicate that intracellular cyclic AMP production by agonists may be decreased via a reduction in AC6 expression under simulated microgravity conditions.

Key words —— simulated microgravity, adenylyl cyclase, adenosine 3′, 5′-cyclic monophosphate

INTRODUCTION

Various physiological changes have been reported in astronauts following space flight, including loss of bone mass, muscle atrophy and heart failure.[1–3] It is also known that immune responses are altered by changes in lymphocyte proliferation and cytokine production during space flight conditions.[4] These physiological changes are thought to be the results of exposure to microgravity. Clinostat rotation is assumed to be an experimental model of simulated microgravity conditions on the ground, as it cancels the cumulative gravity vector.[5–8] Therefore, vector-averaged clinostat rotation is widely used to examine the molecular and cellular mechanisms responsible for the various physiological changes observed under microgravity. For example, tumor necrosis factor-α (TNF-α)-dependent activation of nuclear factor-κB (NF-κB) is repressed in human osteoblastic HOS-TE85 cells under clinostat rotation.[9] In rat luteal cells, clinostat rotation induced apoptosis and suppression of progesterone production.[10] In glial cells, clinostat rotation caused cytoskeletal changes[11] and apoptosis.[12]

A variety of neurotransmitters, hormones and growth factors causes changes in intracellular second messengers which regulate various enzymes and gene transcription. G-protein-coupled receptors are one of the common cell receptors which belong to a huge receptor family and are crucial in converting extracellular stimuli into intracellular signals in all kinds of cells.[13] The trimetric G-protein family consists of G_s, G_i, G_q and G_{12}.[14] Among them, G_s and G_i regulate intracellular adenosine 3′, 5′-cyclic monophosphate (cyclic AMP) level by activation and inhibition of adenylyl cyclase (AC) activity, respectively.[15] Cyclic AMP regulates various

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cellular functions including metabolism, cell shape and gene transcription via several cyclic AMP binding proteins in response to external stimuli. While the cyclic AMP-protein kinase A (PKA) pathway is most well known and triggers cellular responses, cyclic AMP itself also regulates Epac (cyclic AMP-activated guanine nucleotide exchange factor) to activate monomeric G-protein Rap,[16] and cyclic nucleotide-gated channels involved in transduction of olfactory and visual signals.[17] Thus, cyclic AMP is ubiquitous and indispensable second messengers which mediate intracellular signals and thereby trigger physiological responses by numerous external stimuli including various neurotransmitters, hormones and growth factors. It is therefore critical to verify the effects of simulated microgravity on cyclic AMP level in order to predict the physiological and functional changes in our bodies during space flight. In the present study, we determined intracellular cyclic AMP induced by the G_s-coupled receptor stimuli (*i.e.* β-adrenergic receptor) and by the direct AC activation (*i.e.* forskolin) in stationary control and clinostat-rotated cells. Here, we provide evidence that the physiological changes observed in astronauts during space flight might be related to alterations in intracellular cyclic AMP signaling.

MATERIALS AND METHODS

Materials —— Dulbecco's modified Eagle's medium (DMEM) and histamine were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan) and Wako Pure Chemicals (Osaka, Japan), respectively. Fetal bovine serum (FBS), isoproterenol, forskolin and 4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-one (Ro20-1724) were from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). All other chemicals used were of reagent grade or the highest quality available.

Cell Culture —— Human astrocytoma 1321N1 cells were cultured in DMEM supplemented with 5% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

Clinostat Rotation —— Cells were cultured until almost confluent in appropriate sized, filter-attached aerated culture flasks. The culture medium was then removed and fresh DMEM containing 1% FBS was added to the flask until full to avoid air bubbles and reduce turbulence and shear forces during clinostat rotation. Cells were then subjected to two experimental conditions; the stationary condition (control) and the clinostat-rotated condition (simulated microgravity) in which the cultures were rotated around the horizontal axis. Rotation was performed at 20 rpm in a humidified incubator supplemented with 95% air/5% CO_2 for 24 hr at 37°C. Cells in the stationary control were cultured in the same incubator. Since it has been suggested that clinostat rotation increases apoptosis in several cultured cell types,[8, 10, 12, 18, 19] we first determined whether apoptosis was induced by our clinostat rotation (20 rpm, 24 hr). 1321N1 cells did not show any increase in apoptosis during clinostat rotation and the time course of cell growth tended to be slower in clinostat-rotated cells (data not shown). Cell shapes were similar in clinostat-rotated cells and stationary control cells (data not shown).

Radioimmunoassay (RIA) for Measuring Cyclic AMP —— Cells were grown on round-shaped cover glasses (13 mm diameter) until confluent in 24-well multi dishes. Cells on the cover glass were immobilized into the chamber, which was originally made from the sealed 15 ml culture tubes. The chamber was filled with DMEM containing 1% FBS without any air bubbles, then settled in the clinostat and rotated (20 rpm) for 24 hr in the incubator. Control cells were settled for 24 hr in the same incubator.

Drug application was performed as described below. Clinostat-rotated cells or stationary control cells were quickly removed from the incubator, washed in modified Tyrode solution twice and placed into pre-warmed modified Tyrode solution (270 μl) in 24-well multi dishes containing the desired concentration of isoproterenol or forskolin and 100 μM Ro20-1724. Cells were incubated with drugs for 5 min at 37°C in the stationary condition. Reactions were stopped by the addition of 2.5% perchloric acid (PCA), and acid extracts were neutralized by 1/10 volume of 4.2 N KOH. After sedimentation of potassium chlorate, cyclic AMP in the supernatant was succinylated and determined using a commercially available radioimmunoassay kit (^{125}I-cyclic AMP assay kit Yamasa; Yamasa Corp., Tokyo, Japan).

Measurement of Intracellular ATP —— Intracellular ATP level was measured using the luciferin-luciferase bioluminescence assay. Cells in filter-attached aerated 25 cm^2 culture flasks were cultured in stationary or clinostat-rotated conditions for 24 hr at 37°C in the incubator. Each culture medium was then removed from the flask, and cells were fixed

by the addition of 1% trichloroacetic acid (TCA; 500 μl) after washing the adherent cells twice with modified Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.18 mM $CaCl_2$, 5.6 mM glucose, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), pH 7.4). The acid extracts were used for analysis of intracellular ATP level after TCA in the samples was neutralized. Intracellular ATP levels were analyzed using a commercially available kit (Promega Co., Madison, WI, U.S.A.), according to the manufacturer's protocol. Cell lysates were dissolved in 1 N NaOH (500 μl), and the protein content in each flask was determined using the DC protein assay kit (Bio Rad Laboratories, Inc., Hercules, CA, U.S.A.). Each intracellular ATP level was normalized by the total protein content, and expressed as ATP (nmol/mg protein).

Crude Membrane Isolation and AC Assay —— Cells in filter-attached aerated 175 cm^2 culture flasks were cultured in stationary or clinostat-rotated conditions for 24 hr at 37°C in the incubator, then adherent cells were washed with modified Tyrode solution three times after aspirating the medium. Ice-cold homogenizing buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml antipain and 0.1 mM phenylmethanesulfonyl fluoride) was added, and incubated for 2 hr at 4°C. Cell lysates were centrifuged at $15000 \times g$ for 10 min at 4°C. Pellets were washed twice with the homogenizing buffer, and finally suspended in membrane buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM dithiothreitol).

The reaction mixture for the AC assay contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM ATP, 5 mM creatine phosphate, 60 units/ml creatine phosphokinase and the test drugs. Ro20-1724 (100 μM), a phosphodiesterase (PDE) inhibitor, was also included in the reaction mixture. Reactions were started by the addition of crude membranes (20 μg/each tube) and incubated for 15 min at 30°C. Thereafter, the reactions were terminated by PCA to make a final concentration of 2.5%. After centrifugation at $15000 \times g$ for 5 min at 4°C, supernatants were collected into fresh tubes and neutralized by adding 1/10 volume of 4.2 N KOH. After sedimentation with potassium perchlorate, the cyclic AMP in the supernatants was succinylated and determined using the radioimmunoassay kit described above.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) —— Total RNA was isolated from cells placed in the stationary or the rotated condition for 24 hr using ISOGEN reagent (Nippon gene Co. Ltd., Tokyo, Japan) according to the manufacturer's protocol. Complementary DNA (cDNA) produced from total RNA (1 μg) was diluted by 10 times with sterilized H_2O. PCR reaction was performed using each AC subtype-specific primer pair as summarized in Table 1. For analysis of the expression pattern of all AC subtypes (Fig. 4), PCR was performed under the following conditions: 94°C for 2 min; 94°C for 1 min, 56°C for 30 sec, 72°C for 2 min, 35 cycles; 72°C for 10 min. For semi-quantitative PCR analyses of each AC subtype (Fig. 5), PCR conditions were set as follows: AC type 3 (AC3, 94°C for 2 min; 94°C for 1 min, 56°C for 30 sec, 72°C for 2 min, 28 cycles; 72°C for 10 min), AC6 (94°C for 2 min; 94°C for 30 sec, 50°C for 15 sec, 72°C for 1 min, 29 cycles; 72°C for 10 min), AC7 (94°C for 2 min; 94°C for 30 sec, 56°C for 30 sec, 72°C for 2 min, 35 cycles; 72°C for 10 min), AC8 (94°C for 2 min; 94°C for 30 sec, 56°C for 30 sec, 72°C for 2 min, 35 cycles; 72°C for 10 min), AC9 (94°C for 2 min; 94°C for 1 min, 56°C for 30 sec, 72°C for 2 min, 32 cycles; 72°C for 10 min) and β-actin (94°C for 2 min; 94°C for 30 sec, 56°C for 30 sec, 72°C for 2 min, 16 cycles; 72°C for 10 min). PCR products underwent electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. NIH images (Macintosh, version 1.55) were used for analyzing the density of each band.

Western Blotting —— Cell lysates from stationary or clinostat-rotated cells were dissolved in sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) sample buffer. Samples containing equal amounts of protein were separated on 11% SDS-PAGE gels. Proteins in the gels were electrically transferred to polyvinylidene difluoride (PVDF) membranes (Hybond P, GE Healthcare) by the semidry blotting method. PVDF membrane was blocked in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl, 100 mM NaCl, 0.05% Tween 20, pH 7.4) containing 5% non-fat dry milk for 2 hr at room temperature. Blot was then incubated with a polyclonal rabbit anti-$G\alpha_s$ subunit antibody (EMD Biosciences, Inc., San Diego, CA, U.S.A.) diluted 1 : 100 in TBST containing 2% non-fat dry milk for 2 hr at room temperature. Membrane was washed 5 times for 5 min with TBST and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG diluted 1 : 4000 in TBST for 1 hr at room temperature. After washing the membrane with TBST, the proteins were detected

using the enhanced chemiluminescence (ECL) kit (GE Healthcare).

Data Analysis —— Data are expressed as the mean ± SEM, and statistical analysis was performed using the Student's *t*-test.

RESULTS

Cyclic AMP Accumulation Stimulated by Isoproterenol or Forskolin

To determine whether exposure of cells to simulated microgravity affects the cyclic AMP-mediated signaling pathway, we compared cyclic AMP accumulation in cells cultured either under stationary or clinostat-rotated conditions for 24 hr (Fig. 1). Isoproterenol, a β-adrenergic receptor agonist, is known to elevate intracellular cyclic AMP level by activating the G_s-AC signaling pathway,[20] while forskolin is thought to activate AC directly.[21] Ro20-1724, a PDE inhibitor, was included in the incubation medium to eliminate any differences in PDE activity between control and clinostat-rotated cells.

Both isoproterenol (Fig. 1A) and forskolin (Fig. 1B) elevated intracellular cyclic AMP level in both control and clinostat-rotated cells in a concentration-dependent manner. In contrast to the responses observed in stationary control cells, significant reductions in cyclic AMP accumulation induced by both isoproterenol and forskolin were observed in clinostat-rotated cells. As shown in Fig. 1, the increases in cyclic AMP levels were reduced by 19.8% and 39.4% in response to 10 μM isoproterenol and forskolin, respectively.

We next compared $G\alpha_s$ subunit levels in cell lysates from control and clinostat-rotated cells (Fig. 1C). While $G\alpha_s$ subunit was detected at around 45 kDa as expected, there was no difference in $G\alpha_s$ protein level between stationary control and clinostat-rotated cells.

Intracellular ATP Level

One possible reason for the downregulation of cyclic AMP in response to clinostat rotation, is the reduction in intracellular ATP levels, as ATP acts as a substrate for AC in the production of cyclic AMP. We therefore compared the intracellular ATP levels in control and clinostat-rotated cells, but no differences were observed (Fig. 2).

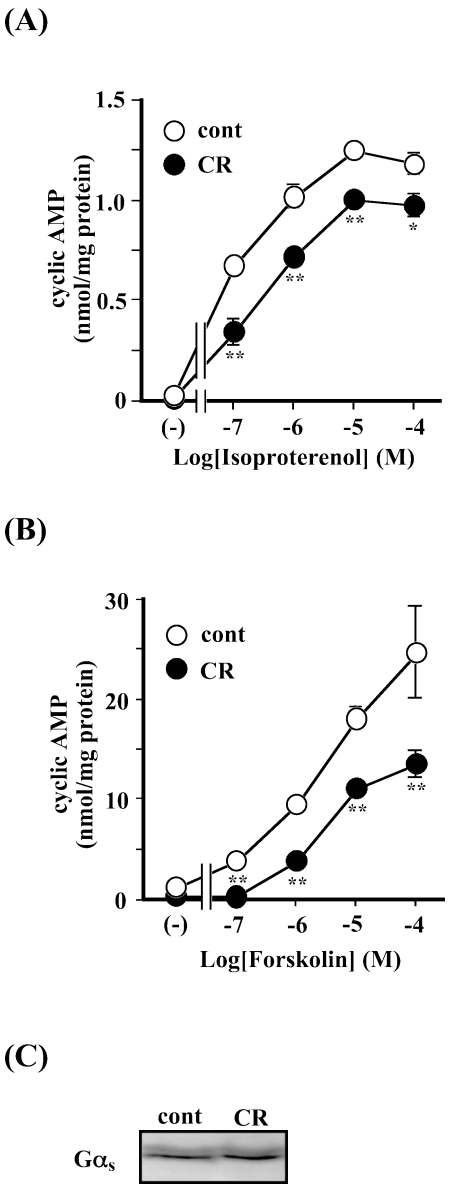

Fig. 1. Concentration-response Relationships of Cyclic AMP Accumulation for Isoproterenol or Forskolin in Stationary or Clinostat-rotated Cells

(A), (B) Human astrocytoma 1321N1 cells on cover glasses were cultured under either stationary (cont) or clinostat-rotated (CR) condition for 24 hr. Cells were washed quickly and then incubated with isoproterenol (A, $n = 9$) or forskolin (B, $n = 3$) in the presence of Ro20-1724 (100 μM) for 5 min at 37°C. The reaction was stopped by 2.5% PCA. Cyclic AMP level in the acid extracts was measured as described in Materials and Methods. Results were normalized by total cell protein levels, and expressed as the mean ± SEM ($n = 3$–9). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control. (C) The level of $G\alpha_s$ subunit in stationary (cont) or clinostat-rotated (CR) cells were analyzed by Western blotting. Representative data are shown from three independent results.

AC Activity in Cell Membranes

The cyclic AMP accumulation induced by forskolin as well as isoproterenol was decreased

Table 1. Primers for RT-PCR Used in This Study

	Accession No.	Primer Sequences	Target	Expected size (bp)
AC1	L05500	ATTAGCCACGGAGAACCACT	173- 192 (S)	381
		CTGTTCCTCTCATGTCCGTA	553- 534 (AS)	
AC2	AF410885	AGAAAAAGAGTACCGGGCCA	1790-1809 (S)	517
		CTCATAGTTTACCCGCAGGA	2306-2287 (AS)	
AC3	AF033861	TTTGACTGCTACGTGGTGGT	409- 428 (S)	488
		TTGCGGTCAGCCATGTAGTA	896- 877 (AS)	
AC4	AF497516	AACAGTGGAAGCAGTCGAAG	1661-1680 (S)	345
		CCAAGGCTATTCTCAGTCCT	2005-1986 (AS)	
AC5	AF497517	ACTACCTGAATGGGGACTAC	736- 755 (S)	591
		TCACAGACACAAACACCACC	1326-1307 (AS)	
AC6	AF250226	AAGGAGCAGCACATTGAGAC	2321-2340 (S)	421
		TGAAGCAGAAGACCAACAGG	2741-2722 (AS)	
AC7	D25538	CTTGGACTGCCTATGGAAGA	2731-2750 (S)	625
		TTTCCCAAGTTCTCCAGTGC	3355-3336 (AS)	
AC8	Z35309	CCCTAGCAACTCGGATTTCT	2499-2518 (S)	276
		CAGATCACTACCTCAATGCC	2774-2755 (AS)	
AC9	AF036927	CTCTTGTTGGTCTGGTTCCT	2984-3003 (S)	233
		AAGTTGACGATGCTGGCGAA	3216-3197 (AS)	
β-actin	NM031144	AGGGAAATCGTGCGTGACAT	616- 635 (S)	467
		TCCTGCTTGCTGATCCACAT	1082-1063 (AS)	

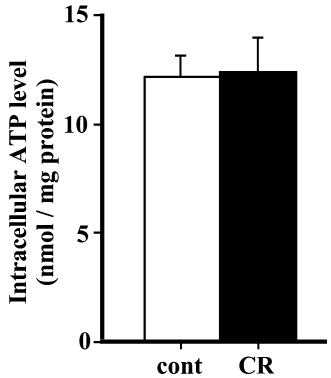

Fig. 2. Comparison of Intracellular ATP Levels between Control and Clinostat-rotated Cells

Cells were cultured in 25 cm^2 flasks filled with culture medium under either stationary (cont) or clinostat-rotated (CR) conditions for 24 hr. Culture medium was then removed and adherent cells were fixed with 1% TCA. Acid extracts were neutralized, and ATP levels in extracts were measured by the Luciferin-luciferase bioluminescence assay as described in Materials and Methods. Data are expressed as the mean ± SEM (n = 3).

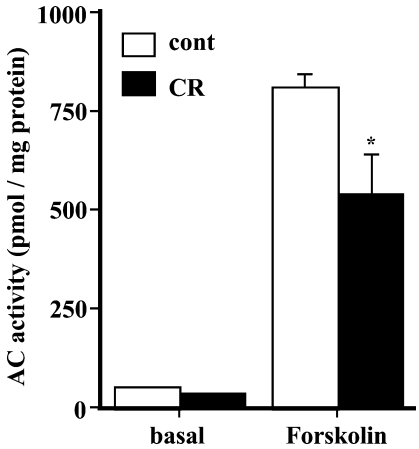

Fig. 3. AC Activity in Cell Membrane Preparations

Cell membranes from stationary (cont) or clinostat-rotated (CR) cells were obtained as described in Materials and Methods. Cell membranes (20 μg) were incubated with forskolin (10 μM) in the presence of Ro20-1724 (100 μM) for 15 min at 30°C. Reaction was stopped by 2.5% PCA, and acid-extracted cyclic AMP was measured by radioimmunoassay as described in Materials and Methods. Data are expressed as the mean ± SEM (n = 9). $^*p < 0.05$ compared with forskolin-stimulated AC activity in control cell membranes.

significantly in clinostat-rotated cells; nonetheless $G\alpha_s$ protein level was unchanged. Therefore, we next examined AC activity in the membrane fractions prepared from stationary or clinostat-rotated cells. AC activity stimulated by 10 μM forskolin was analyzed in the presence of 100 μM Ro20-1724. As shown in Fig. 3, membranes from clinostat-rotated cells showed a 32.4% reduction in forskolin-induced AC activation, suggesting that simulated microgravity might reduce the expression levels of AC.

Expression of AC Subtypes in Human Astrocytoma Cells

To date, nine human AC subtypes have been identified and characterized by their distinct regu-

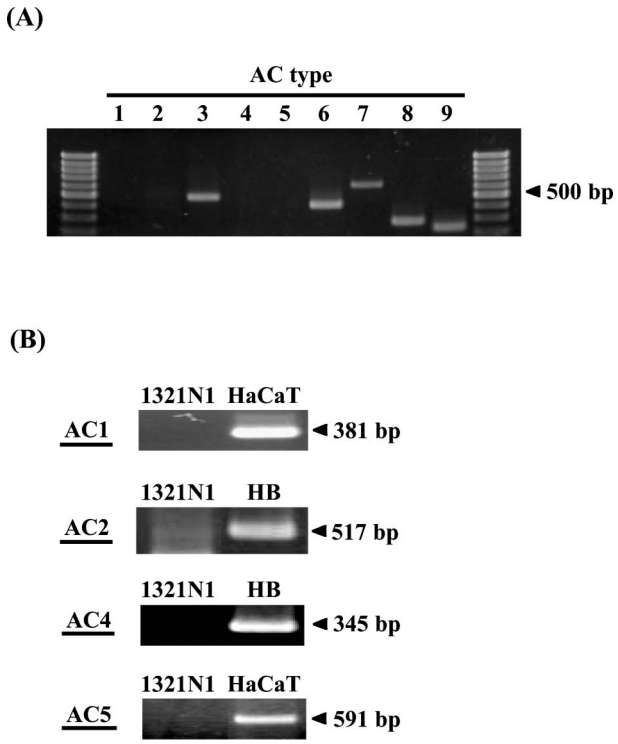

Fig. 4. RT-PCR Analysis of AC Subtype mRNA Expressed in 1321N1 Cells

(A) Expression of AC subtype mRNA in 1321N1 cells. RT-PCR was performed by using a specific primer pair for each AC subtype. (B) AC1 and AC5 were expressed in human keratinocyte-derived HaCaT cells but not in 1321N1 cells. Neither AC2 or AC4 was expressed in 1321N1 cells, in contrast to the positive signals obtained from human brain-derived cDNA library (HB).

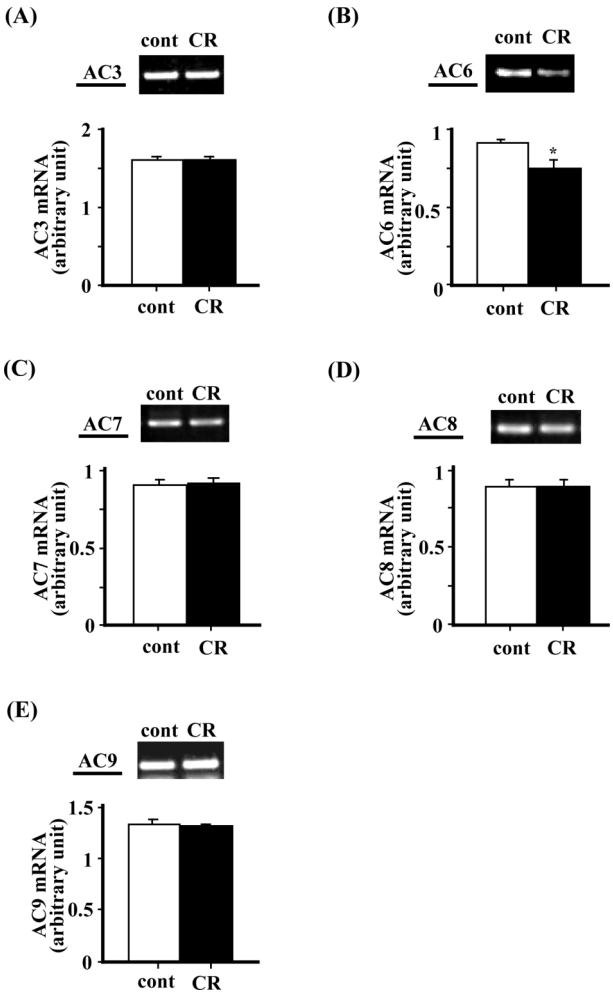

Fig. 5. Comparison of AC mRNA Level by semi-quantitative RT-PCR

The mRNA level of each AC subtype, AC3 (A), AC6 (B), AC7 (C), AC8 (D) and AC9 (E) in stationary (cont) or clinostat-rotated (CR) cells was compared by semi-quantitative RT-PCR. Data was normalized by the β-actin mRNA level, and expressed as the mean ± SEM ($n = 4$). $^{*}p < 0.05$ compared with control.

latory properties.[21] To examine the AC subtype expression in 1321N1 cells, we investigated mRNA expression of nine AC subtypes using RT-PCR. Nine AC primers were carefully designed not to bind to the different AC subtypes (Table 1). We identified AC3, AC6, AC7, AC8 and AC9 as AC subtypes expressed in 1321N1 cells (Fig. 4A). AC1, AC4 and AC5 were not expressed in 1321N1 cells, whereas the positive bands for these AC subtypes could be detected in keratinocyte-derived HaCaT cells and in human brain-derived cDNA library (Fig. 4B). We also concluded that AC2 mRNA seemed to be little expressed in 1321N1 cells, as we could not detect a positive band in several PCR conditions tested. In contrast, a clear band from human brain-derived cDNA library was observed (Fig. 4B). We tested several PCR conditions to determine which AC subtypes were abundantly expressed, and confirmed that AC3, AC6 and AC9 were the major subtypes expressed in 1321N1 cells (data not shown). We next performed semi-quantitative PCR to compare the mRNA levels of AC3, AC6, AC7, AC8 and AC9 in control and clinostat-rotated cells (Fig. 5). Only AC6 mRNA expression level was significantly decreased to about 80% by clinostat rotation, while the other major AC subtypes AC3 and AC9 were unaffected. The mRNA expression levels of AC7 and AC8 in clinostat-rotated cells were also similar to those in stationary control cells, suggesting that the decrease in AC activity in clinostat-rotated cells might be caused by the suppression of AC6 mRNA level abundantly expressed in 1321N1 human astrocytoma cells.

DISCUSSION

The aim of the present study was to determine whether the exposure of 1321N1 human astrocytoma cells to simulated microgravity affects the G_s-AC-cyclic AMP signaling pathways. Our findings showed that: significantly decreased cyclic AMP production induced by isoproterenol or forskolin; markedly inhibited AC activity in the cell membranes; reduced the expression of AC6 mRNA, but did not influence other AC subtypes (AC3, AC7, AC8 and AC9). This is the first demonstration that simulated microgravity influences the cyclic AMP signaling pathway via the reduction of AC6 mRNA level.

Cyclic AMP production induced by isoproterenol or forskolin was inhibited by clinostat rotation. Neither $G\alpha_s$-protein level (Fig. 1C) or intracellular ATP level (Fig. 2) was changed after clinostat rotation. In contrast, a significant depression in AC activity was observed in membranes derived from the clinostat-rotated cells (Fig. 3), suggesting that there may be two possibilities to explain this phenomenon; the enzymatic activity of AC was modulated by certain mechanisms, and expression of AC was decreased under simulated microgravity.

To expand on the first possibility as the cause of reduced AC activity, AC6 and AC8 have been reported to exist in caveolae or caveolae-related microdomains (also referred to as lipid rafts) on cell membranes,[22, 23] which are formed by cholesterol and sphingolipids and act as a signaling platform due to the accumulation of many signaling molecules including trimetric G-proteins and their effectors.[24] Caveolins and flotillins are usually used as the lipid raft marker proteins, as they are selectively collected in the lipid raft fractions prepared using sucrose-density ultracentrifugation.[25] We therefore investigated the localization of these proteins and ganglioside GM_1 to lipid raft fractions obtained from stationary or clinostat-rotated cell lysates, as the disruption of lipid rafts by clinostat rotation might influence AC activity due to irregular localizations of signaling molecules including receptors, G_s and AC. However, clinostat rotation changed neither localizations nor the amounts of GM_1 or lipid raft marker proteins such as flotillin-1, flotillin-2 and caveolin-1, suggesting that lipid rafts are not affected by simulated microgravity (Ohkubo, S., unpublished observations).

To support the second possibility as the cause of reduced AC activity, a decrease in AC6 mRNA was observed in cells cultured under clinostat rotation (Fig. 5). We also demonstrated the existence of AC3, AC6, AC7, AC8 and AC9 mRNA in 1321N1 cells (Fig. 4). In the preliminary experiments, we confirmed that AC3, AC6 and AC9 were major AC subtypes expressed in 1321N1 human astrocytoma cells at the mRNA level. Among these subtypes, forskolin has been shown not to activate AC9, but activate other subtypes AC1–8.[26] Since clinostat rotation of 1321N1 cells tended to suppress forskolin-induced cyclic AMP formation more potently compared with the response induced by isoproterenol (Fig. 1), AC9 may not be responsible for cyclic AMP production in 1321N1 cells. Furthermore, AC6 has been reported as a predominant form in C6-2B rat glioma cells,[27] suggesting that AC6 would be abundantly expressed in glia-derived cell lines including 1321N1 cells. Therefore, the decrease in AC6 mRNA seems to be related to the depression of AC activity in clinostat-rotated cell membranes. In a preliminary experiment, we tried to detect AC6 protein level in 1321N1 cells cultivated in control or clinostat rotation, but the quality of anti-AC6 antibody was inadequate for detection of AC6 by Western blotting (Matsuoka, R., unpublished observation). It will be necessary in the future studies to confirm whether an alteration of AC6 mRNA expression relates to the decrease in AC6 protein expression, resulting in cyclic AMP production by the clinostat rotation.

It is unclear how the gravitational change alters the AC6 mRNA level. Furthermore, it is totally unknown how AC6 mRNA expression is regulated under normal conditions. In general, mRNA levels are regulated at the transcriptional and post-transcriptional levels. At the transcriptional level, promoter analysis of the human AC6 gene has not been carried out until now. On the other hand, microgravity might affect AC6 expression at the post-transcriptional level. Recently, the (in)stability of mRNA has been shown to be regulated through RNA-binding proteins such as AU-rich element binding proteins, which have been shown to regulate mRNA stability through binding to the AU-rich element which exist in the 3′-untranslated region (UTR) of mRNA.[28] There are no reports which analyze the regulation of AC6 mRNA mediated by 3′-UTR and RNA-binding proteins. Therefore, future studies to clarify the regulation of AC6 mRNA both at the transcriptional and the post-transcriptional level will provide useful knowledge in understanding how microgravity affects AC6 mRNA expres-

sion as presented in this study.

We performed both intracellular cyclic AMP assays and AC assays in the presence of the PDE inhibitor Ro20-1724 in the present study. Ro20-1724 has been shown to be a specific inhibitor of PDE type 4 (PDE4).[29] PDE4 has been reported as an abundantly expressed isozyme in glial cells such as LRM55 astroglial cells[30] or cultured rat astrocytes,[31] suggesting that glia-derived 1321N1 cells also possess PDE4 as a main PDE isozyme and that Ro20-1724 used in the present study would inhibit PDE activity in 1321N1 cells effectively. However, recent reports suggest that any single cell type can express several different PDEs,[32] implicating that it will be also necessary whether the change in other PDE(s) expression might be relevant to the inhibition of cyclic AMP accumulation by clinostat rotation in the future study.

In the present study, we showed that the cyclic AMP pathway may be influenced by simulated microgravity via a reduction in AC6 mRNA level. Interestingly, AC6 is ubiquitously expressed throughout the body, and is an essential AC subtype in the heart,[21] and AC6 expression was indeed decreased in pacing-induced heart failure in dogs[33] and in infarcted heart in rats,[34] suggesting a relationship between AC6 expression and heart function. Furthermore, a significant decrease in Ca^{2+} sensitivity was observed in cardiac myocytes from tail-suspended rats,[35] an animal model simulating bone turnover and muscle changes seen in spaceflight.[36] In space shuttle crewmembers, spaceflight induces significant changes in heart volume affecting left ventricular function.[1] Further detailed experiments are needed to demonstrate the relevance of the observed decrease in AC6 mRNA and the numerous physiological changes including heart failure occurring under microgravity conditions.

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