Microgravity Modifies Protein Kinase C Isoform Translocation in the Human Monocytic Cell Line U937 and Human Peripheral Blood T-Cells

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Abstract

Individual protein kinase C (PKC) isoforms fulfill distinct roles in the regulation of the commitment to differentiation, cell cycle arrest, and apoptosis in both monocytes and T-cells. The human monocyte like cell line U937 and T-cells were exposed to microgravity, during spaceflight and the translocation (a critical step in PKC signaling) of several PKC isoforms to the particulate fraction examined. PKC activating phorbol esters induced a rapid translocation of several PKC isoforms to the particulate fraction of U937 monocytes under terrestrial gravity (1g) conditions in the laboratory. In microgravity, the translocation of PKC βII, δ, and ε in response to phorbol esters was reduced in microgravity compared to 1g, but was enhanced in weak hypergravity (1.4g). All isoforms showed a net increase in particulate PKC following phorbol ester stimulation, except PKC δ which showed a net decrease in microgravity. In T-cells, phorbol ester induced translocation of PKC δ was reduced in microgravity, compared to 1g, while PKC βII translocation was not significantly different at the two g-levels. These data show that microgravity differentially alters the translocation of individual PKC isoforms in monocytes and T-cells, thus providing a partial explanation for the modifications previously observed in the activation of these cell types under microgravity. J. Cell. Biochem. 87: 39–50, 2002. © 2002 Wiley-Liss, Inc.

Key words: monocyte; microgravity; signal transduction; protein kinase C

Experiments performed in orbiting spacecraft have shown that the growth, differentiation, and cell cycle of mammalian cells are altered under low gravity or “microgravity” conditions. [Hughes-Fulford and Lewis, 1996; Cogoli and Cogoli-Greuter, 1997; Hashemi et al., 1999; Lewis et al., 2001]. Signal transduction processes appear sensitive to microgravity, particularly signaling by protein kinase C (PKC) isoforms [de Groot et al., 1991; Schmitt et al., 1996; Hatton et al., 1999]. Since gene expression is modulated by signal transduction processes and cytoskeleton reorganization, microgravity induced modifications in these processes could have important consequences for cell fate.

PKC isoforms [Mochly-Rosen and Kauvar, 2000], a family of serine threonine kinases, play a critical role in the regulation of monocyte differentiation, as well as the initiation of apoptosis (Fig. 1). Phorbol esters, which activate most PKC isoforms, are potent inducers of differentiation in the monocytic cell line U937, and sustained PKC activation is necessary for differentiation since this induces c-jun upregulation and c-myc downregulation proceeding...
growth arrest which signals commitment to differentiation [Ways et al., 1994]. Additionally, cell cycle arrest can be induced by phorbol esters which promote induction of the cyclin inhibitor p21CIP/WAF1 and downregulation of cdc25 [Vrana et al., 1998]. Individual PKC isoforms appear to play distinct roles in regulation of monocyte differentiation. In U937 cells, PKC βII regulates CD11b integrin translocation to the cell surface (necessary for cell adhesion) and early gene expression controlling the commitment to differentiate to a macrophage like phenotype and cell cycle arrest. PKC ε appears to regulate the expression of anti-apoptotic genes. PKC δ is also involved in the commitment to differentiation following phorbol ester stimulation. However, apoptosis inducing agents such as TNF-α activate caspase 3, which in turn cleaves PKC δ into a constitutively active catalytic fragment (PKM). The PKM translocates to the mitochondria where it mediates cytochrome c release, a key step in the induction of apoptosis.

Previously, we reported that during spaceflight the proportion of PKC in the nuclear fraction of both U937 and Jurkat cells (a T-cell...
line) decreased with increasing g-level between microgravity and 1.4g, while PKC in the cytosolic fraction increased with g-level, suggesting a gravity dependant redistribution of PKC between these cellular fractions [Schmitt et al., 1996]. Additionally, IL-1β synthesis by U937 cells following phorbol ester stimulation was significantly decreased in microgravity compared to 1g samples. Furthermore, the kinetics of PKC translocation in U937 cells were modified during spaceflight compared to 1g ground conditions, while total PKC content of spaceflown cells was elevated relative to cells cultured on the ground [Hatton et al., 1999]. Given the apparent sensitivity of PKC translocation to microgravity and the diverse roles of individual PKC isoforms in both U937 and T-cells, we investigated the effect of microgravity culture on the translocation of selected PKC isoforms during the STS-81 space shuttle mission. Here, we show that the phorbol ester translocation of PKC βII, δ, and ε in U937 cells was inhibited in microgravity and enhanced in 1.4g hypergravity, compared to 1g conditions, resulting in different relative levels of each isoform at each g-level. In peripheral blood T-cells, PKC βII and δ showed differential sensitivities to the microgravity environment depending upon the method used to activate PKC. Given the importance of PKC isoforms in monocyte and T-cell signal transduction, these data provide a partial explanation for the alterations in gene expression and cell function which have been reported to occur in these cell types when exposed to microgravity or hypergravity.

MATERIALS AND METHODS

Materials

Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Lymphoprep 1.077 medium (ficoll solution), penicillin, streptomycin sulphate, l-glutamine, and penicillin-streptomycin-neomycin antibiotic mix were from Gibco-BRL (Paisley, UK). Rabbit polyclonal antibodies against PKC βII, PKC δ, PKC ε were from Santa Cruz biotechnology (Santa Cruz, CA). Tween 20 was from Biorad.
Nitrocellulose membranes (0.45 μm pore size) were obtained from Pierce (Rockford, IL). Anti-CD14, -CD19, and IgG2 antibody coated magnetic beads were from Dynal France (Compiegne, France). The Bichro-2m ML-glutamine, 1% (v/v) penicillin-streptomycin containing 10% fetal bovine serum (FBS), medium was changed every 2 days, with cell concentration adjusted to 5 × 10⁵ cells/ml at each medium change.

Human T-cells were purified from fresh peripheral blood of healthy volunteers. Approximately 150 ml of whole peripheral blood was subject to a 20 min centrifugation at 400g on a ficoll gradient to separate leukocytes from erythrocytes. After separation, the leukocyte fraction was washed twice in RPMI-1640 medium with supplements before being incubated with Dynal magnetic beads coated with anti-CD14, -CD19, and -CD56 antibodies for 2 × 30 min at 4°C to eliminate monocytes, B-cells, and natural killer cells. This treatment yielded better than 98% pure human T-cells, as determined by cytofluorimetry. After purification the T-cells were resuspended in medium at 2 × 10⁶ cells/ml.

For the space shuttle experiments, U937 and T-cells were loaded into special GCAK-1 and -2 culture modules (described in detail in [Hatton et al., 1998]) which permit cell culture manipulations in microgravity. Prior to loading into GCAK-1 cassettes, cell cultures were transferred to RPMI-1640 medium supplemented to permit culture in the absence of CO₂: RPMI-1640 at pH 7.4, 25 mM HEPES, 12 mM sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% (v/v) heat inactivated FBS, 1% (v/v) penicillin-streptomycin-neomycin antibiotic mix. For experiments evaluating PKC isoform translocation, cells were cultured in the GCAK-2 modules and stimulated with agonists as indicated in the figures. At the end of the incubation period, samples were fixed with a digitonin-based inhibitor mix (0.5 mg/ml digitonin, 50 mM MOPS, pH 7.2, 5 mM NaF, 5 mM EDTA, 10 mM EGTA, 2 mM sodium ortho-vanadate, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin final concentration with cells). This mixture permeabilizes the cell to release the cytosol and preserve the translocation state of PKC to the particulate fraction of the cell [Hatton et al., 1999]. Samples were maintained at −20°C until post flight analysis. In experiments evaluating surface marker expression, cells were cultured in GCAK-1 cassettes. Samples were fixed in-flight with 3.7% paraformaldehyde, washed once with PBS, then maintained at +4°C until postflight analysis.

### Space Shuttle Flight

The experiments on PKC translocation and surface receptor expression in U937 and T-cells during microgravity were performed aboard the Space Shuttle Atlantis during the STS-81 mission, launched from the Kennedy Space Center on 12 January 1997 for a 10 day mission, which included several days docked to the Russian Mir space station. The g-level aboard during the mission was generally better than 10⁻³g. Cell cultures in GCAK modules were loaded into the Space Shuttle ~17 h prior to launch and were maintained at ambient temperature (21.4–24.9°C) until 24 h after launch when they were transferred to the ESA Biorack facility which provides a 37°C incubator containing on-board 1g centrifuge as well as a glovebox. For each experimental condition, one cassette was placed in the static (microgravity) racks of the incubator while an identical cassette was placed on a centrifuge within the incubator exposing samples to 1g (1g in flight control). The cassettes containing U937 or T-cell cultures were incubated for 17 h at 37°C until the start of experimental manipulations. Experiment cassettes were transferred briefly to the
Biorack glovebox where a crew member injected activators into the cell culture as described in the results section. Cultures were incubated for 0', 10', or 60' at 37°C in the Biorack incubator. At the end of the incubation period, cassettes were transferred back to the glovebox where the digitonin-based inhibitor mix (described above) was added to the cell cultures, before the samples were transferred to −20°C. A near identical experiment was performed in parallel on the ground at the NASA LSSF, delayed 2 h with respect to the flight experiment, with static rack (1g ground) and centrifuge (1.4g) samples.

Cells for cytofluorimetric analysis were incubated for 17 h after launch at ambient temperature, then fixed with paraformaldehyde as described above. An identical experiment was performed on the ground at the NASA LSSF, delayed 2 h with respect to the flight experiment.

**Western Blot**

Samples stored at −20°C were unfrozen and vortexed followed by centrifugation at 14,000 g for 30 min at 4°C. The supernatant which contained the cytosolic fraction of the cells as well as culture medium was transferred to a separate tube. The pellet, containing the particulate fraction of the cell, was washed once in MOPS buffer with inhibitors (50 mM MOPS, pH 7.2, 5 mM NaF, 5 mM EDTA, 10 mM EGTA, 2 mM sodium ortho-vanadate, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin final concentration with cells), then resuspended in 250 μl of buffer. The particulate fraction was sonicated twice for 5 s on ice, followed by addition of 20% SDS to give a final concentration of 1% w/v. A small aliquot of particulate fraction was taken for determination of protein concentration using a commercial bichromatic acid assay kit (Pierce, Rockford, IL). Reducing buffer (5 × concentration; 10% SDS, 25% β-mercaptoethanol, 25% glycerol) was added to particulate fraction samples, which were then heated at 70°C for 5 min.

Equal quantities of protein from each sample were loaded on a SDS-PAGE minigel, migrated, then transferred onto nitrocellulose membranes (Miniprotein electrophoresis kit, Biorad, Ivry-sur-seine, France). Reference samples of constant PKC concentration were run on the same gel to act as a standard for band intensity quantification. Membranes were blocked for 1 h in 10% Genosys blocking buffer, 0.05% Tween 20 in Tris-buffered saline (TBS; 16 mM Tris-HCl, 3 mM Tris, 150 mM NaCl, pH 7.5) followed by 2 × 5 min washes in 0.5% Tween 20/TBS before incubation with the primary antibody. Rabbit polyclonal antibodies directed against PKC βII, PKC δ, PKC ε were used at between 0.1 and 0.25 μg/ml concentrations. Membranes were incubated with primary antibodies for 1.5 h in 4% blocking buffer, 0.05% Tween 20 in TBS, washed three times in 0.5% Tween 20/TBS, then incubated for 1.5 h with peroxidase coupled goat anti-rabbit antibodies, in the same buffer used for the primary antibodies. The membranes were then washed in 0.5% Tween 20 before ECL detection by Supersignal or Supersignal ultra reagents (Pierce, Rockford, IL) according to the manufacturer’s instructions. The intensity of the bands recorded on film was quantified by a computer image analysis program (Biocom image analysis system, Les Ulis, France).

**Cytofluorimetry**

Samples previously fixed and washed as described above were centrifuged at 400g for 5 min then resuspended in 500 μl of PBS. Each sample was split into five separate aliquots for labeling. Five μl of FITC or PE directly labeled antibodies (CD11b, CD11c, CD16, CD25, CD64, CD71) were incubated with 100 μl aliquots of sample for 30 min, followed by 400g centrifugation and resuspension of the sample in 250 μl of PBS. Five μl of unlabelled antibodies (CD13, CD71) were incubated for 30 min with sample, followed by 400g centrifugation. The sample was then resuspended in 100 μl of buffer to which 1 μl of FITC labeled secondary antibody was added. The sample was incubated for 30 min then centrifuged at 400g, followed by resuspension in 250 μl of PBS. Labeled samples were analyzed using a Becton Dickinson FACScan.

**RESULTS**

We have previously reported that the intracellular distribution and translocation of phorbol ester binding PKC is sensitive to microgravity. In standard 1g laboratory conditions phorbol ester treatment of U937 cells induced a rapid translocation of PKC βI, βII, and ε from the cytosol to particulate fraction of the cell (Fig. 3). Little or no PKC βI, βII, or ε was associated with the particulate fraction of unstimulated cells, but within 5 min of stimulation with 100 ng/ml
PDBu a large fraction of PKC \( \beta_I \) and all of PKC \( \beta_{II} \) and \( \varepsilon \) were translocated to the particulate fraction.

U937 and T-cells were exposed to microgravity during a space shuttle flight and stimulated with phorbol esters. Post flight the intracellular distribution of PKC isoforms was assessed by Western blot. In unstimulated U937 cells, neither PKC \( \beta_{II} \) nor \( \varepsilon \) were detectable in the particulate fraction at any g-level. In contrast, PKC \( \delta \) was present in the particulate fraction at all g-levels in unstimulated U937 cells (Fig. 4a). Stimulation of the cells with phorbol ester for 10 min induced the translocation of PKC \( \beta_{II} \) and \( \varepsilon \) to the particulate fraction at all g-levels (Fig. 4b). For both isoforms, the level of translocation was lowest in the microgravity and 1g flight samples and highest in the 1.4g ground sample. Likewise, quantity of particulate PKC \( \delta \) increased with g-level, being lowest in the microgravity sample and highest in the 1.4g sample. However, in contrast to the other isoforms, PKC \( \delta \) showed a net decrease in the particulate fraction of the microgravity sample following phorbol ester stimulation (Fig. 5). After 10 min of PDBu stimulation, the quantity of PKC \( \delta \) in the 1g flight samples did not change significantly from that in unstimulated cells, while the ground 1 and 1.4g samples showed a net increase. By 60 min after PDBu stimulation, no significant differences in particulate PKC \( \beta_{II}, \delta, \) and \( \varepsilon \) were detectable between g-levels.

T-cells were treated with either PDBu/Ionomycin, anti-CD3 beads, or medium (unstimulated control) for 60 min. PDBu/Ionomycin is a potent PKC activator, while anti-CD3 beads
simulate activation of the T-cell by an antigen presenting cell. Both PKC\(\beta\)II and \(\delta\) were detectable in the particulate fraction of unstimulated T-cells at all g-levels (Fig. 6A,C). PDBu/I stimulated a significant increase in particulate PKC\(\beta\)II and \(\delta\) compared to unstimulated cells at all g-levels (Fig. 6A). Anti-CD3 bead stimulated cells showed comparable or lower levels of particulate PKC to unstimulated cells (Fig. 6C,F). This is likely because the 60 min timepoint is after the peak in PKC translocation. No statistically significant differences in particulate PKC\(\beta\)II were detectable between any of the g-levels for each activation condition. However, the data suggests a trend of increasing particulate PKC\(\beta\)II with increasing g-level for PDBu/I stimulation, but decreasing particulate PKC\(\beta\)II with increasing g-level for unstimulated and anti-CD3 stimulated cells. In contrast, particulate PKC\(\delta\) levels were significantly higher in the 1g samples than microgravity samples for unstimulated and PDBu/I stimulated samples. No significant difference in particulate PKC\(\delta\) could be detected between g-levels for anti-CD3 bead stimulated cells, although the data suggests an increase in this isoform in the particulate fraction of the cell with increasing g-level. Taken together, these data indicate that microgravity has a differential effect on PKC\(\beta\)II and \(\delta\) isoform activation in T-cells.

Expression of selected surface activation in markers in unstimulated U937 cells exposed to 17 h of microgravity was compared to markers in 1g ground cells by cytofluorimetry. The relative abundance of CD 11b (aL subunit of integrin LFA-1, inducible expression), CD11c (aM subunit of integrin CR3, inducible expression), CD13 (aminopeptidase N, myeloid cell marker), CD16 (Fc\(\gamma\)RIII), CD64 (Fc\(\gamma\)RI receptor, inducible expression), CD71 (Transferrin receptor) were assessed. A significant upregulation of CD11b, CD11c, CD13, and CD64 was observed in microgravity (Table I). No significant changes were observed for CD16 and CD71. These data suggest that microgravity may induce a weak activation of the unstimulated cell. However, the level of upregulation is small in comparison to that which occurs when U937 cells are treated with differentiation inducers such as \(\gamma\)-interferon (Fig. 7).

**DISCUSSION**

PKC isoforms play crucial roles in monocyte differentiation and T-cell activation. Translocation of PKC isoforms to the plasma membrane and other cellular compartments is a key step in signal transduction by this family of kinases, as experiments inhibiting the translocation of individual isoforms have shown [Mochly-Rosen and Kauvar, 2000]. Our data shows that PKC isoform translocation is generally reduced in microgravity, suggesting microgravity impairs PKC translocation and thus signal transduction through these kinases. Furthermore, the pattern of PKC isoform translocation varies with g-level. The isoforms we examined in the space shuttle experiment, play key roles in U937 cell differentiation, while those in T-cells are believed to play important roles in cell activation following stimulation of the T-cell receptor. PKC\(\beta\)II activation plays a critical role in U937 differentiation including CD11b vesicle translocation to the cell surface [Kiley and Parker, 1997], MAP kinase activation [Kaneki et al., 1999], and early gene expression [Hass et al., 1993]. In T-cells, PKC\(\beta\) appears to regulate the secretion of IL-2 [Long et al., 2001], probably through a regulation of the microtubule organizing center (MTOC), which PKC\(\beta\) associates and is also necessary for regulation of cell polarization during locomotion following stimulation of LFA-1. Therefore, it is possible that the changes, we observed in PKC translocation, could have important consequences for the responsiveness of these cell types to mitogens.
In T-cells, other investigators have found functional changes in microgravity, which could be a result of a perturbation of PKC signaling. IL-2 synthesis and secretion by T-cells stimulated by mitogen is inhibited [Pippia et al., 1996] a processes which is believed to be regulated by PKC θ and PKC β [Altman et al., 2000, Long et al., 2001]. The polarization of the microtubule...
organizing center (MTOC) in the T-cell towards the site of contact with antigen presenting cell (APC) is necessary for cytokine containing vesicles to be targeted to the APC [Kupfer and Dennert, 1984]. The localization of the MTOC in human peripheral blood T-cells stimulated with anti-CD3 bead, which simulate the APC, was examined in microgravity under very similar conditions to those used in our experiment (same experiment hardware and activation conditions). Polarization of the MTOC towards the anti-CD3 beads was found to be completely inhibited in microgravity, while in the 1g flight samples polarization was found to be normal [Hashemi et al., 1998]. Both PKC\(\beta\) and PKC\(\theta\) are believed to play roles in the regulation of MTOC organization [Monks et al., 1997; Volkov et al., 2001].

PKC\(\delta\) is implicated in the transduction of pro-apoptotic signals in both U937 and T-cells. Generally, PKC activation appears to be anti-apoptotic [Kaneko et al., 1999], while apoptosis promoting agents often induce cleavage of PKC isoforms at the hinge region, to yield a constitutively active PKM fragment [Mizuno et al., 1997]. In T-cells, PKC\(\delta\) translocates to the nucleus during apoptosis, a translocation which can be reversed by anti-apoptotic factors such as IFN-\(\beta\) [Scheel-Toellner et al., 1999]. In U937 cells, pro-apoptotic agents such as TNF-\(\alpha\) induces PKC\(\delta\) translocation to the mitochondria, which mediates cytochrome \(c\) release from mitochondria, an important step in apoptosis [Majumder et al., 2000]. During apoptosis, caspase 3 is activated which in turn cleaves PKC\(\delta\) to a PKM fragment [Koriyama et al., 1999]. In contrast, PKC\(\varepsilon\) activation blocks TNF-\(\alpha\) induced apoptosis in U937 cells [Mayne and Murray, 1998]. Therefore, the apparent reversal of PKC\(\delta\) translocation, from particulate to cytosolic fraction, in U937 cells under microgravity (Fig. 5) as well as the significant differences in PKC\(\delta\) and \(\varepsilon\) isoforms present in the cell particulate fraction could result in either the induction or suppression of apoptosis. Unfortunately, the analysis methods used for the spaceflight experiment do not allow us to differentiate between translocation to specific cellular compartments such as the mitochondria or nucleus, nor the presence of PKM fragments. However, evidence that apoptosis is increased in microgravity has been provided by Lewis et al. [Cubano and Lewis, 2000] who reported that Jurkat cells (a T-cell line) exposed to microgravity in three different spaceflights have higher levels of apoptosis and FAS-L expression than 1g cultured cells. This report is particularly interesting since PKC activation and FAS mediated apoptosis are antagonistic processes [Mizuno et al., 1997].

The translocation of some PKC isoforms in microgravity is similar to that of the 1g in-flight control, but significantly different to the 1g control (Fig. 4), while in other cases the response of the 1g flight sample is intermediate.
between that of the microgravity and 1g controls (Fig. 6). We observed a similar phenomena in our previous spaceflight experiments [Schmitt et al., 1996, Hatton et al., 1999]. The most likely explanation for this discrepancy between flight and ground 1g controls is that all flight samples were exposed to microgravity after launch until loading into Biorack at least 24 h later, when the 1g flight samples were placed on the centrifuge. During this time, it is possible that the cells were sensitive to microgravity and that subsequent culture at 1g did not reverse changes which may have occurred in the cell. Additionally, it was necessary to remove the experiment cassettes from the 1g centrifuge for manipulation, hence the cell cultures were exposed to microgravity for a brief period immediately before and after addition of activator.

The mechanisms by which microgravity affects PKC isoform translocation is unknown. However, the fact that individual isoforms show distinct differences in sensitivity which may vary depending on the activation conditions provides some clue to the mechanism. The U937 and T-cells in our experiments were exposed to microgravity for an extended period prior to stimulation, hence modifications in cell architecture may have occurred. Although PKC isoforms translocation is diffusion limited [Schaefer et al., 2001], changes in expression of RACK's or other PKC binding sites could affect PKC translocation. Many PKC isoforms associate with cytoskeleton, both prior to and after activation [Keenan and Kelleher, 1998]. Since cytoskeleton organization has been reported to be modified in both immune and bone cells exposed to microgravity [Hughes-Fulford and Lewis, 1996; Lewis et al., 1998], it is possible that changes in cytoskeleton organizations could cause modifications to PKC translocation. In turn, many cytoskeletal proteins are PKC substrates, so inhibited PKC signaling could result in altered cytoskeleton reorganization following cell stimulation. The recent observation by Tabony’s group [Papaseit et al., 2000] that self organization of microtubule polymerization in vitro is drastically reduced in microgravity provides a potential mechanism by which gravity, or absence of gravity, could be perceived directly by the cell. Altered microtubule architecture and microtubule disruption under microgravity conditions has been observed in several different cell types including Jurkat, HL-60 and human breast cancer cells [Piepmeier et al., 1997; Lewis et al., 1998; Vassy et al., 2001]. Microtubule disruption is believed to activate several different signaling pathways, including PKC, which induce apoptosis [Wang et al., 1999]. Microtubule disruption and mitochondrial abnormalities occurred in Jurkat cells exposed to microgravity with these events preceding apoptosis [Schatten et al., 2001]. Therefore, it is possible that modifications in the microtubule cytoskeleton induced by microgravity could be responsible for changes in PKC translocation that we observed. However, further experiments will be necessary to determine how the changes in cytoskeleton, signal transduction and apoptosis that have been observed in microgravity are related. Ultimately, information from these experiments will help in identifying and understanding the underlying mechanisms by which mammalian cells perceive gravity.

ACKNOWLEDGMENTS

We thank the following for help in the development of this research project: The ESA Biorack team for management and interfacing of the experiment with the Biorack facility, the NASA Life Science Support Facility (LSSF) staff at Cape Canaveral Air Force Station for use of experiment facilities during the preflight and immediate postflight preparation of the experiments, the crew of Atlantis Shuttle mission STS-81 for operation of the experiment in flight. Additionally, we thank Dr Millie Hughes-Fulford for critically reviewing this manuscript prior to submission to this journal.

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