The Effect of Acute Microgravity On Mechanically-Induced Membrane Damage and Membrane-Membrane Fusion Events

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ABSTRACT

Although it is unclear how a living cell senses gravitational forces there is no doubt that perturbation of the gravitational environment results in profound alterations in cellular function. In the present study, we have focused our attention on how acute microgravity exposure during parabolic flight affects the skeletal muscle cell plasma membrane (i.e. sarcolemma), with specific reference to a mechanically-reactive signaling mechanism known as mechanically-induced membrane disruption or "wounding". This response is characterized by both membrane rupture and membrane resealing events mediated by membrane-membrane fusion. We here present experimental evidence that acute microgravity exposure can inhibit membrane-membrane fusion events essential for the resealing of sarcolemmal wounds in individual human myoblasts. Additional evidence to support this contention comes from experimental studies that demonstrate acute microgravity exposure also inhibits secretagogue-stimulated intracellular vesicle fusion with the plasma membrane in HL-60 cells. Based on our own observations and those of other investigators in a variety of ground-based models of membrane wounding and membrane-membrane fusion, we suggest that the disruption in the membrane resealing process observed during acute microgravity is consistent with a microgravity-induced decrease in membrane order.

Key Words: microgravity, membrane wounding, membrane fusion, membrane order

INTRODUCTION

Mechanically-induced membrane wounding is a phenomenon known to occur in a variety of different tissues within the body as a consequence of the mechanical forces generated during normal day-to-day activities, both voluntary and involuntary. Mechanically-induced membrane wounding *in vivo* has been detected in skeletal (10, 34) and cardiac (8, 22) muscle, endothelial cells (55), auditory hair cells (38) and the epithelial layers of the gut (32) and skin (33), and has been demonstrated to be directly correlated with the level of mechanical force inflicted upon cells both *in vivo* (8) and *in vitro* (9). In its simplest form, the membrane wound response can be broken down into four major components: (1) plasma membrane disruption as a consequence of the imposition of mechanical force; (2) influx of calcium from the extracellular compartment into the cell cytoplasm; (3) calcium-dependent mobilization of internal membrane stores and (4) resealing of the plasma membrane disruption by both active (i.e. energy-dependent) and passive (i.e. entropy-driven) membrane-membrane fusion events. The time taken to complete this series of events in tissue cultured cells under terrestrial gravity is in the order of seconds (24, 35). Overall wound repair efficiency can be altered by a number of experimental manipulations which directly modulate the biophysical properties of the plasma membrane (e.g. membrane fluidity, elasticity and rigidity) (11, 16, 44, 51).

Recently, we reported that mechanical unloading during 14 days of 6°-head-down-tilt bedrest resulted in a significant reduction in the amount of mechanically-induced sarcolemmal disruption (i.e. myofiber wounding) in the skeletal muscle of human subjects (7). This reduction was paralleled by a concomitant reduction in the amount of

fibroblast growth factor (FGF) released from the myofiber sarcoplasm into the muscle micro-environment (7), a true mechanically-reactive signaling pathway demonstrated to be important in muscle anabolism *in vitro* (9). The reduction in myofiber wounding during bedrest was also paralleled by an increase in the susceptibility of atrophied myofibers to mechanically-induced wounding when the muscle was reloaded at the end of the bedrest period. A similar increase in mechanically-induced membrane damage has been reported in atrophied rat skeletal muscle as a consequence of reloading after both hindlimb suspension (28, 30) and space flight (45). These results indicate that myofiber membranes undergo remodeling during mechanical unloading-induced muscle atrophy and suggest that mechanical load has a direct effect on membrane structure and function.

The purpose of the present study was to investigate whether or not acute microgravity exposure *per se* is capable of disrupting the basic cellular mechanisms that occur at the plasma membrane level during mechanical load-induced membrane wounding. Utilizing both a human myoblast culture model (9) and an HL-60 cell membrane-membrane fusion model (36), coupled with a technique known as impact-mediated loading (IML) (13, 14), we have investigated the possibility that the removal of gravity during parabolic flight aboard NASA's KC-135 parabolic flight aircraft can disrupt the normal membrane wound response observed in terrestrial gravity.

METHODS AND MATERIALS

Cell Culture

Human Primary Myoblast Culture (HSkMC): HSkMC were obtained as frozen cultures from the Clonetics Corporation (Walkersville, MD) and stock cultures were grown in standard growth medium (SGM) as previously described (9). Myoblasts (passage 7 or below) were seeded on collagen Type 1-coated 24-well tissue culture plates (Sigma, St. Louis, MO) at a density of 10,000 cells/well and grown for a further 16 hr. After this time, the cells were washed three times with standard growth medium (SGM) containing 50 mM HEPES (pH 7.2) (hSGM) to remove any unattached cells and placed into a "Zip-Lock™" plastic bag containing approximately 100 ml of hSGM. Additional hSGM was then added to the partially sealed bag to ensure that all the wells of the 24-well plate were completely filled with medium. The remaining air was expelled from the bag by gentle squeezing and the bag was sealed. The cultures were then placed into an insulated chest that contained gel blocks, previously heated to 37°C, which served as a mobile tissue culture incubator during parabolic flight.

Differentiated HL-60 Cell Culture: HL-60 cells were obtained from ATTC and grown in RMPI 1640 medium containing 10% fetal calf serum (FCS) and standard antibiotics. Cells were then differentiated using 1% (v/v) DMSO for seven days (with medium changes every two days) in order to induce a granulocytic phenotype (36). On the day of the experiment, differentiated HL-60 cells were collected by centrifugation, washed once by centrifugation with fresh growth medium (10%FCS.RPMI 1640) containing 1% (v/v) DMSO, 50 mM HEPES but without phenol red and resuspended in the same medium at a cell density of 1 million cells/ml. Aliquots (1.5 ml) of this cell suspension were then

placed into sterile 2 ml polypropylene injection vials and sealed with a screw-on cap containing an integral teflon/silicone septum. A sterile 30-gauge hypodermic needle (1 inch long) was then inserted through the septum into the cell suspension. A second sterile 25-gauge hypodermic needle (1/2 inch long) was also inserted through the septum so that the tip remained in the air space above the cell suspension. Both needles were then taped in place so that they could not be dislodged from the septum during flight. This arrangement allowed injection of phorbol 12-myristate 13-acetate (PMA) solution or sodium azide solution directly into the cell suspension through the 30-gauge needle and allowed venting of displaced air from the injection vial through the 25-gauge needle. An added benefit of this arrangement was the efficient mixing of the injected liquid with the cell suspension even in microgravity. The vials were then placed into the 37°C mobile tissue culture incubator in preparation for flight.

Impact Mediated Loading (IML) Apparatus

The device described in Figure 1 is designed to provide reproducible levels of membrane wounding in adherent monolayers of tissue cultured cells. The device is based on the principle that impact of uniform-sized particles (i.e. 8 μm spherical glass beads) with the plasma membrane of living cells results in the production of membrane wounds of a reproducible size (13, 14). The particles are initially supported on a rupturable plastic membrane as an even, one-particle-thick layer (Figure 1A). When a predetermined volume of nitrogen gas maintained at a constant pressure (both volume and pressure being controlled by an electronic valve system) is released into the particle containment shell, the membrane ruptures and the particles are propelled directly at the cell monolayer attached to the base of each well of a 24-well plate (Figure 1). As both cell density and

particle number are kept constant, the extent and severity of membrane wounding within and between different cultures subjected to IML under these conditions are equivalent. Temperature is maintained at 37°C throughout the IML process utilizing an integral, solid-state, thermostat-controlled heating block (Figure 1B)

Experimental Procedures During Parabolic Flight

The parabolic flight profile of NASA's KC-135 parabolic flight aircraft produces periods of microgravity lasting approximately 25 - 30 sec and periods of hypergravity (~ 1.8 *xg*) for 35 – 40 sec. The standard flight pattern of the aircraft is to perform ten parabolic maneuvers followed by a period of approximately 5 min of normal level flight in order to return to the starting position. This flight pattern is repeated a total of four times producing a total of 40 periods of microgravity, interspersed with periods of hypergravity. All experiments were initiated at the beginning of the microgravity portion of each parabola and completed within 15 sec. Normal terrestrial gravity (i.e. 1*xg*) control experiments were carried out aboard the KC-135 aircraft during level flight.

Membrane Wounding During Microgravity: HSkMC cells were removed from their medium-filled plastic bags during level flight and were drained of excess medium by gentle tapping of the 24-well plate on a adsorbent pad. A 50 µl aliquot of 100 µM FITC-conjugated dextran (M*r* - 10,000, 50,000, 145,000 or 2,000,000 Daltons) (all FITC-linked dextrans had a FITC-glucose molar ratio of between 0.007 – 0.008 and were obtained from The Sigma Chemical Company, St. Louis, MO) was added to each well using an Eppendorf repeating pipette and mixed over the surface of the cell by gently swirling the plate. The plate was then secured in the IML device (Figure 1) and membrane wounding as a consequence of particle impact was initiated once the aircraft had entered the

microgravity phase. Sixteen of the 24 wells per plate were impacted with similar numbers of 8 μm particles in a pressure controlled blast of nitrogen gas (35 p.s.i.), 4 wells per plates were exposed to a nitrogen blast (35 p.s.i.) only, whereas the 4 remaining wells remained untouched. Impact-mediated loading was carried out within 15 sec of the beginning of microgravity leaving a minimum of 5 – 10 sec prior to gravity transition (i.e. microgravity to hypergravity). After IML, the 24-well plate was returned to its medium-filled plastic bag and checked so that all wells contained medium. Excess air was removed from the partially sealed bag by gentle squeezing, the bag completely sealed and then returned to the mobile 37°C tissue culture incubator. Control experiments were performed under identical conditions during level flight under normal terrestrial gravity conditions.

Primary Granule Fusion in Differentiated HL-60 Cells: Differentiated HL-60 cell suspensions contained within injection vials were removed from the mobile tissue culture incubator during level flight and gently shaken in order to evenly disperse the cells within the medium. At the beginning of microgravity, a pre-filled 1 ml syringe was used to rapidly inject 100 μl of warm serum-free RPMI-1640 containing 1% (v/v) DMSO and 1.6 mM PMA directly into the cell suspension (to final concentration of 100 μM PMA) in order to initiate primary granule fusion with the plasma membrane. After a period of 15 sec of microgravity, a second 1 ml syringe was used to rapidly inject 200 μl of warm phenol-red free RPMI-1640 medium containing 2M sodium azide into the cell suspension in order to inhibit any further energy-dependent membrane events. The injection vial was then replaced in the mobile tissue culture incubator until processed in the laboratory. Control samples (injected with sodium azide solution but not PMA solution at the end of

the flight) were also flown along with test samples in order to ascertain the effects of multiple gravity transitions on primary granule fusion with the plasma membrane.

Laboratory Processing of Parabolic Flight Samples

Impact-mediated Loaded Cultures: HSkMC cells which had been wounded by IML under normal and microgravity conditions aboard the KC-135 parabolic aircraft were returned to the laboratory within 2 hours of wounding. Each 24-well plate was removed from its medium bag, carefully dried off with an absorbent towel and the outside of the plate was swabbed down with 75% (v/v) alcohol. Each well was washed once with SGM (without HEPES) which was then replaced with 1 ml of fresh SGM. A clean sterile lid was placed on each 24-well plate and the cultures were incubated for a further 24 hr in a standard laboratory tissue culture incubator at 37°C. After 24 hr, each well of the 24-well plate was washed twice with warm phosphate-buffered saline containing 1 mM calcium and 1 mM magnesium (D-PBS) (pH 7.2) to remove any dead or unattached cells, trypsinized and the resulting cells in suspension were collected by centrifugation at 100 *xg*. Each cell pellet was resuspended in 1 ml of SGM from which a 400 µl aliquot was removed. The cells in the 400 ul aliquot were collected by centrifugation at 10,000 *xg*. The supernatant was completely removed from the cell pellet by aspiration and wicking of any residual liquid with clean tissue paper and frozen at –80°C in preparation for determination of DNA content (as a measure of cell number) using the commercially available CytoQuant DNA Assay (Molecular Probes, Eugene, OR). Percentage (%) cell survival (24 hr after IML) was calculated in the 16 individual wells per 24-well plate subjected to IML by comparison to the mean DNA value obtained from the remaining eight control wells per plate (i.e. both nitrogen blasted and untouched wells) at each gravity condition as no

significant differences in recoverable DNA were detected between those wells which had been exposed to a nitrogen blast alone and untouched control wells. The remaining cells were analyzed using a fluorescent flow cytometer to determine the (%) wounded cells (i.e. FDx positive cells) and the mean fluorescent value (MFV) of the wounded cells in the surviving population as previously described (11). A minimum of 5000 cells per sample were analyzed in this fashion.

Assessment of Primary Granule Fusion in Differentiated HL-60 Cells: The primary granules of differentiated HL-60 cells contain large quantities of the enzyme β-glucuronidase that is released into the surrounding medium when the primary granule fuses with the plasma membrane. This model has been widely used to study the underlying mechanisms involved in membrane-membrane fusion events (17, 23, 36). As such, the amount of β-glucuronidase released from the cells can be used as (1) a direct measure of primary granule fusion with the plasma membrane, and by extension, (2) an indirect measure of membrane-membrane fusion events. Differentiated HL-60 cells which had been exposed to PMA during normal gravity or microgravity to initiate primary granule fusion with the plasma membrane were removed from their injection vials and centrifuged at 10,000 *xg* for 10 min at 4°C to remove cellular material from the medium. A one ml aliquot of the supernatant was removed from each sample and assayed for β-glucuronidase using a commercially available assay kit (Sigma Chemical Company, St. Louis, MI). The amount of β-glucuronidase (and hence by extension primary granule fusion with the plasma membrane) present in the test sample is directly proportional to the absorbance of the reaction solution at 550 nm.

RESULTS

The results depicted in Figure 2 represent pooled data from a total of 62 individual experiments (N = 32 cultures in two, 24-well plates per gravitational condition) carried out on two separate KC-135 flights using the same batch of stock HSkMC cells and stock FDx (*Mr* – 10 kD) solution. No significant differences in recoverable DNA were detected between those wells which had been exposed to a nitrogen blast alone and untouched control wells. Percentage (%) cell survival (24 hr after IML) (and hence cell death) was calculated in the remaining 16 individual wells per 24-well plate subjected to IML by comparison to the mean DNA value obtained from the eight control wells per plate (i.e. both nitrogen blasted and untouched wells) at each gravity condition. Similar results with respect to cell survival of membrane wounding during parabolic flight have been obtained on a total of seven KC-135 flights over a period of seven months utilizing different batches of primary myoblast cultures and FDx solutions.

The IML procedure produces membrane disruptions in approximately 60% of the cells [i.e. (%) cell death + (%) wounded cells] in each individual culture regardless of gravitational condition (Figure 2). When membrane wounding was initiated by IML under normal gravity conditions, ~ 70% of the original cell population survived for 24 hr whereas if membrane wounding was initiated under microgravity conditions significantly ($p < 0.003$) fewer cells (~ 50% of the original cell population) survived (Figure 2A). In addition to cell survival, the mean fluorescent value (MFV) and (%) wounded cells in the surviving cell populations were analyzed by fluorescent flow cytometry as previously described (15). A significant ($p < 0.02$) decrease in the (%) wounded cells was detected when membrane wounding was initiated during microgravity as compared to normal

gravity (Figure 2B), whereas no significant difference in the MFV of the wounded cells was detected between microgravity and normal gravity utilizing FDx (M*r* –10 kD) (Figure 2C).

In a separate series of experiments using a different stock culture of HSkMC and FDx wound marker solutions containing dextrans of different sizes (i.e. M*r* – 50 kD, 145 kD and 2000 kD rather than 10kD), it was noted that if the FDx molar concentration was kept constant, but the molecular weight of the FDx wound marker was increased, a highly significant ($p < 0.001$) decrease in the mean fluorescent value (MFV) of the wounded cell population in normal gravity was observed (Figure 3A). Conversely, if IML was carried out in microgravity, there was no significant difference in the MFV of the wounded cell populations as the relative molecular weight of the FDx wound marker was increased (Figure 3A). In addition, unlike the results observed in normal gravity, there was a significant ($p < 0.03$) reduction in the (%) wounded cells in microgravity as the molecular weight of the FDx wound marker increased (Figure 3B).

As survival of a mechanically-induced plasma membrane disruption involves wound resealing (i.e. membrane-membrane fusion events), the potential affects of microgravity on membrane-membrane fusion processes was investigated. Utilizing a differentiated HL-60 model of membrane-membrane fusion events, primary granule fusion with the plasma membrane was initiated under normal and microgravity conditions by exposing the cells to PMA aboard the KC-135 parabolic flight aircraft. PMA-induced primary granule fusion with the plasma membrane of HL-60 cells was assessed by determination of the amount of the primary granule-associated enzyme, β-glucuronidase, released into the surrounding medium. As illustrated in Figure 4, PMA-induced primary

granule fusion with the plasma membrane was significantly ($p < 0.005$) reduced in microgravity from the levels observed during normal gravity. No detectable amounts of β-glucuronidase were found in the medium obtained from HL-60 cells exposed to multiple gravity transitions aboard the KC-135 aircraft but which had not been stimulated with PMA.

DISCUSSION

When a cell suffers a plasma membrane disruption as a consequence of particle impact one of two outcomes can occur. The first is that the membrane disruption created as a consequence of particle impact is lethal and the cell dies. The second is that the membrane disruption created by particle impact is resealed and the cell survives. The second of the two outcomes constitutes a *membrane wound*, an event and can be detected using a variety of experimental approaches (31). Our results aboard the KC-135 parabolic aircraft suggest that human primary skeletal muscle cells propagated under normal terrestrial gravity conditions in the laboratory are more susceptible to membrane wound damage induced during microgravity than in normal gravity.

One possible explanation for the decrease in cell survival observed in microgravity after IML is that the membrane wounds inflicted by particle impact in microgravity are larger than those that are produced under the same conditions in normal gravity. As such, these wounds may be sufficiently large to cause immediate cell death (i.e. mechanically-induced cell disruption) rather than a membrane wound (i.e. by definition, a survivable, resealable disruption of the plasma membrane). However, as membrane wound size is related to particle impact energy (i.e. particle velocity x particle mass, where particle size and velocity are kept constant) it is unlikely that microgravity conditions increase the energy with which a particle strikes a particular cell, rather the opposite is true (i.e. the particle will weigh less in microgravity).

A second potential explanation for the decrease in cell survival observed in microgravity is that once a membrane wound has been created by IML, plasma membrane resealing is less efficient compared to that occurring in normal gravity. Under normal

terrestrial gravity conditions, we previously have shown a relationship between wound marker size and the amount of the marker that enters the cell through membrane wounds created under identical conditions of mechanical disruption (11). In addition, we and others have also demonstrated that the survival rate of plasma membrane disruption in normal gravity is dependent on the biophysical properties of the membrane (11, 16, 44, 51), biophysical properties which can be described collectively by the composite descriptor term, membrane order. The membrane order (MO) parameter of a biological membrane reflects a number of separate but related membrane biophysical properties, such as fluidity (a negative correlation to MO), elasticity (a negative correlation to MO) and rigidity (a positive correlation to MO)(21, 39, 40, 46).

In addition, the relative concentration of the membrane ordering sterol, cholesterol (5, 53), or the addition of exogenous membrane-active agents (i.e. polaxamers, fatty acids, organic solvents) to cellular membranes can directly impact the MO parameter (16, 43, 44, 49), as well as the membrane wound resealing process (11, 13, 16). Furthermore, experimental modification of MO, utilizing either cholesterol enrichment/depletion or membrane-active agents, has been shown to affect the inherent fusogenic properties of the membrane with regard to exocytosis during synaptic transmission (12, 26, 27), endocytosis (6, 12, 20, 48) or the membrane fusion events which occur during viral infection (2, 18, 42, 54).

Based on these experimental observations, we have formulated a general model of membrane wounding which has seven central predictions: **Prediction (1)** - for a given amount of mechanical force applied to a cell membrane, a membrane wound of a given size is produced; **Prediction (2)** – there is a threshold value, dependent on both

membrane wound size and length of time the wound remains open, which if exceeded results in cell death; **Prediction (3)** - the amount of wound marker which enters the cell is dependent on its concentration gradient across the membrane; **Prediction (4)** - the amount of wound marker which enters the cell is dependent on its molecular size and radius of gyration; **Prediction (5)** - the amount of wound marker which enters the cell is dependent on the rate of wound resealing; **Prediction (6)** – if the membrane wound resealing rate remains constant and the wound marker size increases, the amount of marker which enters the cell is reduced and **Prediction (7)** – membrane resealing efficiency is dependent on inherent membrane fusogenicity.

In the case of membrane wounding caused by particle impact in normal gravity, the predicted results of this model are consistent with the observed experimental results, namely that as wound marker size increases then the average amount of fluorescent wound marker (i.e. MFV) present in the wounded cell population decreases (Figure 3A), whereas the overall number of wounded cells in the surviving population remains constant (Figure 3B). However, when this experiment was carried out on parallel cultures in microgravity, the observed results differed from the predicted results. Instead of decreasing as in normal gravity, the MFV of the wounded cell population remained constant (Figure 3A), whereas the (%) wounded cells decreased relative to membrane wound marker size (Figure 3B). As the physical size of the membrane disruption created in microgravity by IML is the same as that created in normal gravity then the decrease in cell survival observed in microgravity (Figure 2), and the decrease in (%) wounded cells relative to wound marker size (Figure 3B) without a concomitant decrease in the MFV of the wounded population (Figure 3A), can only be explained by a reduction in the rate or

efficiency of membrane wound resealing in microgravity compared to that occurring under normal terrestrial gravity.

If the underlying reason for the decrease in cell survival of membrane wounding observed in microgravity is in fact due to a reduction in membrane resealing rate/efficiency then it should be possible to demonstrate that other membrane-membrane fusion events can be altered by microgravity exposure. A model which has long been used to study the underlying cellular mechanisms involved in membrane-membrane fusion is PMA-induced primary granule fusion with the plasma membrane of HL-60 cells differentiated into a granulocytic phenotype (17, 23). PMA directly stimulates protein kinase C (PKC) resulting in the initiation of a cascade of events ultimately leading to primary granule fusion with the plasma membrane. Unlike for example, a more specific receptor-mediated degranulation response (e.g. an IgE receptor mediated response), PMA stimulation of these cells by-passes such a transmembrane signaling pathway and directly stimulates intracellular PKC. When primary granule fusion in these cells was induced in microgravity, a relatively small (~ -9%), but highly significant ($p < 0.005$) reduction in the amount of β-glucuronidase enzyme released by HL-60 cells was detected (Figure 4). This observation supports the concept that microgravity exposure may inhibit membrane-membrane fusion events involved in this response. However, our experiments with HL-60 cells do not definitively show which part of the membrane-membrane fusion event is inhibited during acute microgravity exposure.

Based on our experimental results in human skeletal muscle cells exposed to acute microgravity conditions, we suggest that the removal of normal terrestrial gravity from a cell is capable of disrupting the membrane-membrane fusion events essential for

membrane wound resealing after mechanical disruption of the plasma membrane. A central question is raised by this observation. How does exposure of individual cells to microgravity alter membrane-membrane fusion events? The ability of acute microgravity exposure to alter the mechanically-induced membrane wound response within such a short time frame (i.e. 15 sec aboard the KC-135 aircraft) supports a conclusion that microgravity exposure must some how alter the biophysical, rather than the biochemical, properties of the membrane.

In the past, we and others have demonstrated that alterations in membrane order (MO) resulted in modification of the membrane wound resealing response (11, 16, 44, 51). These experimental data suggest that there is an optimal level of MO, and by extension membrane fusogenicity, for efficient membrane resealing. As such, disruption of optimal MO by microgravity exposure could explain both the increase in susceptibility to mechanically-induced membrane wounding in human skeletal muscle cells (Figure 2) and the decrease in primary granule fusion of differentiated HL-60 cells observed during microgravity (Figure 4). It is interesting to note that cellular events involved in primary granule fusion (i.e. membrane docking, pore formation and membrane-membrane fusion) have also been shown to be susceptible to disruption by alterations in MO or membrane cholesterol content (which directly modulates MO) in a variety of other cell-based or synthetic membrane models of membrane fusion (2, 25, 29, 41, 42, 48, 52).

The concept of optimal MO with regard to correct functioning of a biological membrane is not new (4, 19, 49, 53). However, the possibility that acute microgravity exposure may disrupt the optimal level of MO actively maintained by living cells in normal gravity has important implications. Firstly, although mechanical loading is

reduced during space flight, the ability to reseal myofiber wounds inflicted as a consequence of physical effort may also be reduced in microgravity leading to excessive muscle membrane damage during or after space flight (7). Secondly, basic transmembrane signaling processes, such as growth factor signaling or ion channel activity, which have already been shown in normal gravity to be affected by alterations in MO mediated by membrane cholesterol content (reviewed in 1), may also be negatively affected by a microgravity-induced decrease in MO. Thirdly, the membrane-membrane fusion events essential for mechanically-induced membrane wound resealing (37, 50) have been shown to be very similar, if not identical, to that which occurs during synaptic transmission (3, 47), suggesting the possibility that microgravity exposure may lead to a disruption in neural function. Indeed, the inhibition of neurotransmitter release in tissue cultured neuronal cells and the dose-dependent decrements in rat learning and memory models due to the administration of a synthetic membrane ordering agent (12) indicate that the MO parameter of neuronal membranes is of great importance in neuronal function. Finally, as living cells maintain an optimal value of MO depending on their environment, and if microgravity perturbs this optimal value, then the cell would be expected to adapt by altering its MO value to the new optimum.

In conclusion, our experimental observations suggest that acute microgravity produced aboard NASA's KC-135 parabolic aircraft is capable of perturbing the biophysical properties of the living cell plasma membrane in a fashion which is consistent with a reduction in MO. This reduction in MO results in a decrease in the rate of mechanically-induced membrane wound resealing which may be explained by a disruption in normal membrane-membrane fusion events. In addition, a reduction in MO

as a consequence of microgravity exposure may also have negative effects on other transmembrane signaling events, such as growth factor receptor signaling and ion channel function. Furthermore, due to the adaptive nature of living systems, prolonged exposure to microgravity may lead to an adaptive response in these cells designed to regain optimal MO levels and normal transmembrane signaling. Such an adaptive response (e.g. an increase in membrane cholesterol content) to prolonged microgravity exposure may place the cell in a state that is not optimal for cellular function upon subsequent re-exposure to normal terrestrial gravity conditions, resulting in a further period of re-adaptation in order to restore normal cellular function.

FIGURE LEGENDS

Figure 1 Diagramatic Representation of the Impact-Mediated Loading (IML) Device. **(A)** Cells are plated onto a tissue culture substratum and covered with a loading solution containing dissolved FDx at a pre-determined concentration. Glass particles (8 μm diameter) are supported by a plastic membrane which is ruptured when a pressure-controlled blast of nitrogen gas is pumped into the particle containment shell (arrow). The particles are then propelled in the nitrogen blast and impact the cell layer beneath to create uniform membrane wounds. The FDx in the loading solution gains direct entry to the cell cytoplasm via the membrane wounds across a concentration gradient. **(B)** Multi-Sample IML Device illustrating the multiple sample configuration and the integral heating block which maintains the cells at 37°C during the IML procedure aboard the KC-135 parabolic flight aircraft.

Figure 2 The Effect of Microgravity On (A) Cell Survival, (B) (%) Wounded Cells In the Surviving Population and (C) Mean Fluorescent Value (MFV) of The Wounded Cells Utilizing A Fluorescently Labeled Dextran (FDx) (M*r* – 10kD) as a Wound Marker. **(A)** A significant (** - $p < 0.003$; $n = 32$ per condition, Student's *t*-test) reduction in cell survival was observed if membrane wounding was initiated during microgravity as compared to that observed in normal gravity. **(B)** A significant (* - $p < 0.02$; $n = 32$ per condition, Student's *t*-test decrease in (%) Wounded Cells was also observed in the same surviving cell populations. **(C)** No significant difference in the MFV of either cell population was detected.

Figure 3 The Effect of Microgravity On (A) Mean Fluorescent Value (MFV) of Wounded Cells and (B) (%) Wounded Cells In the Surviving Population Utilizing A Range of Fluorescently Labeled Dextrans (FDx) (M*r* – 50kD, 145 kD and 2000 kD) as Wound Markers. **(A)** As the wound marker size (but not its molar concentration) increased, highly significant (* - $p < 0.001$; $n = 16$ per condition; Two-Way ANOVA with post-hoc *t*-test) reductions in the MFV of wounded cells was observed if membrane wounding was initiated in normal gravity, unlike in microgravity where no significant differences in MFV were detected. **(B)** As the wound marker size (but not its molar concentration) increased, significant (* - $p < 0.03$; ** - $p < 0.01$; $n = 16$ per condition; Two-Way ANOVA with post-hoc *t*-test) reductions in the (%) Wounded Cells was observed if membrane wounding was initiated in microgravity, unlike in normal gravity where no significant differences in (%) Wounded Cells were detected.

Figure 4 The Effect of Microgravity On PMA-Induced Primary Granule Fusion in Differentiated HL-60 Cells. A highly significant reduction in the amount of the enzyme, β-glucuronidase, released into the medium after fusion of the primary granule and the plasma membrane was observed if the cells were stimulated with PMA during microgravity as compared to that released if stimulated in normal gravity. Stimulation was for a period of 15 sec after the initiation of microgavity after which time all active membrane processes were inhibited by addition of sodium azide to the system. Cells exposed to multiple microgravity-normal gravity-hypergravity transitions did not release any detectable amounts of β-glucuronidase (data not shown).

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Figure 1

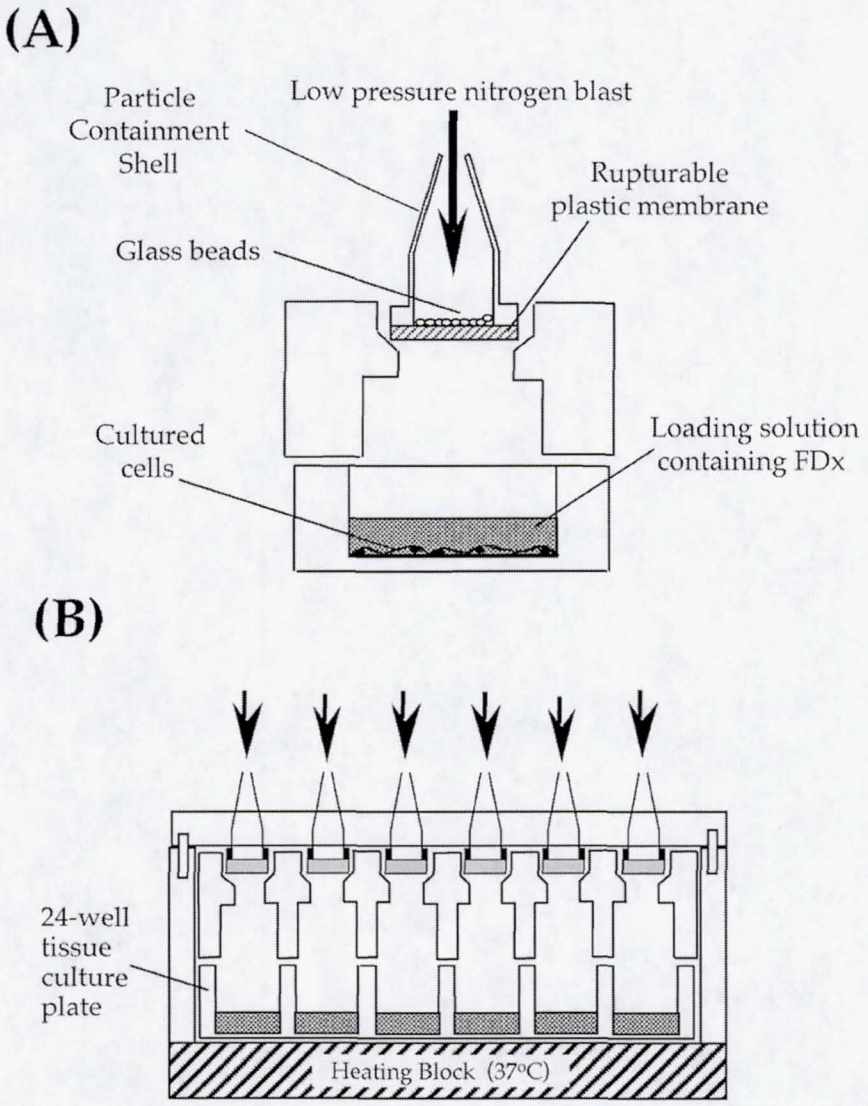

(A)
Particle Containment Shell
Low pressure nitrogen blast
Rupturable plastic membrane
Glass beads
Cultured cells
Loading solution containing FDx
(B)
24-well tissue culture plate
Heating Block (37°C)

Figure 2

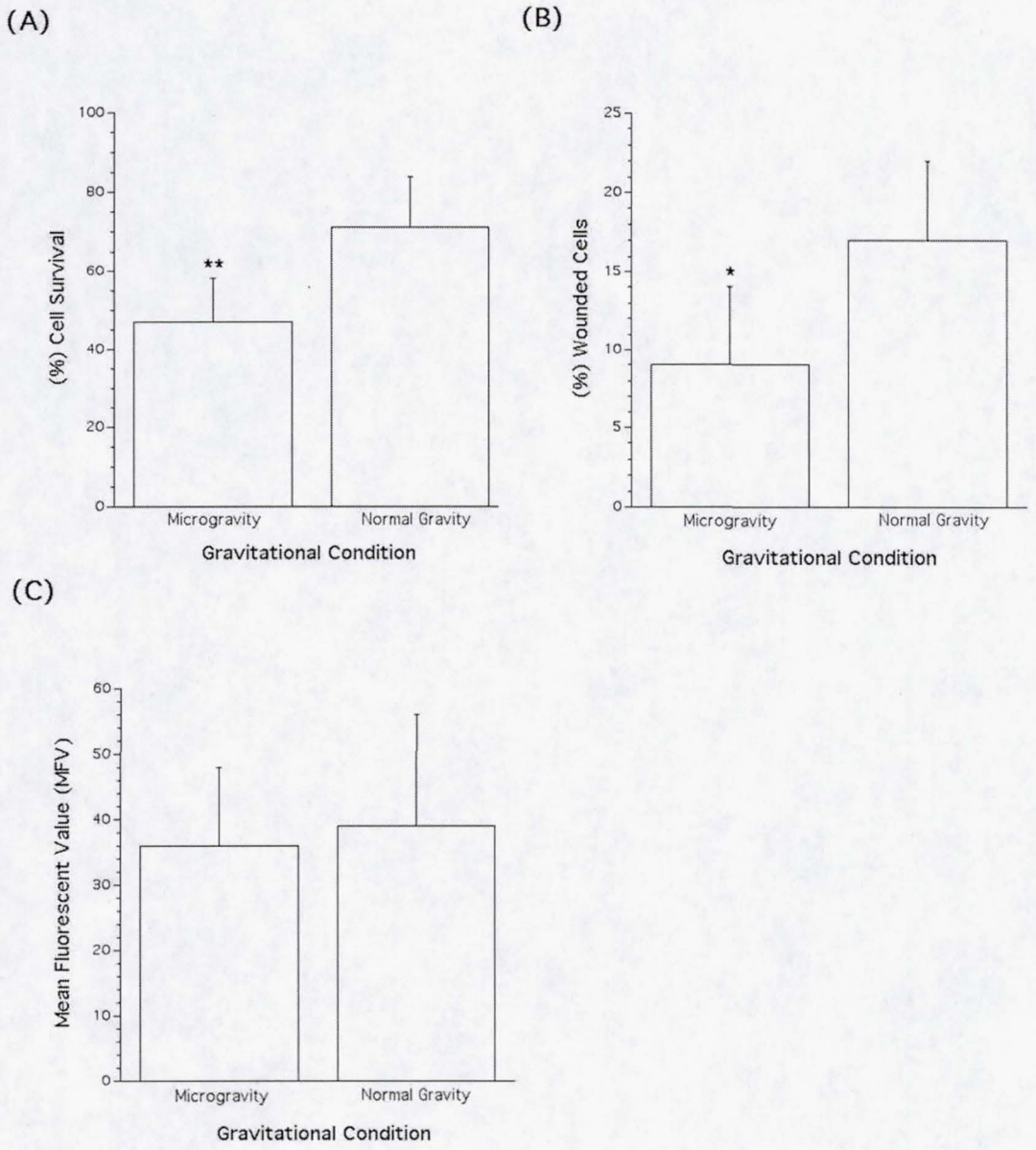
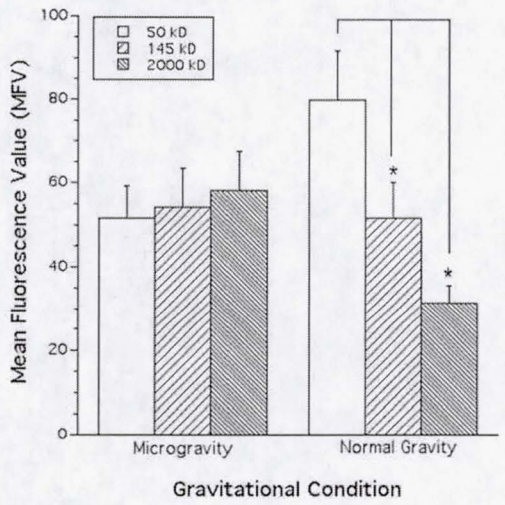

(A)
100
80
60
40
20
0
(%) Cell Survival
**
Microgravity
Normal Gravity
Gravitational Condition
(B)
25
20
15
10
5
0
(%) Wounded Cells
*
Microgravity
Normal Gravity
Gravitational Condition
(C)
60
50
40
30
20
10
0
Mean Fluorescent Value (MFV)
Microgravity
Normal Gravity
Gravitational Condition

Figure 3

(A)


50 kD
145 kD
2000 kD
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100
80
60
40
20
0
*
*
Microgravity
Normal Gravity
Gravitational Condition

(B)

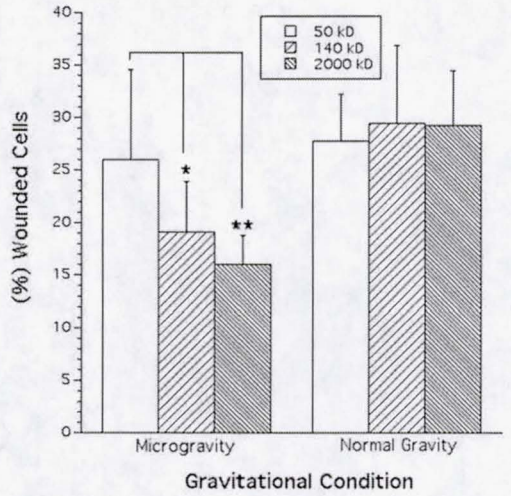
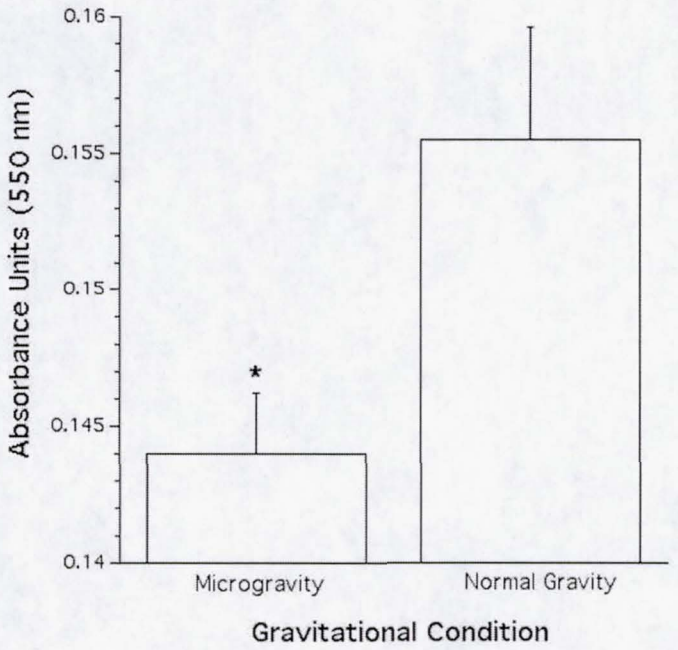

50 kD
140 kD
2000 kD
(%) Wounded Cells
40
35
30
25
20
15
10
5
0
*
**
Microgravity
Normal Gravity
Gravitational Condition

Figure 4


0.16
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0.15
0.145
0.14
Absorbance Units (550 nm)
*
Microgravity
Normal Gravity
Gravitational Condition