Insulin does not reverse neither exalt the effects of modeled gravity on the metabolism of normal and transformed cultured cells

R. Coinu[1], G. Palumbo[2]

[1] Dipartimento di Scienze Fisiologiche Biochimiche e Cellulari, Università di Sassari
[2] Dipartimento di Patologia e Biologia Cellulare e Molecolare/IEOS CNR, Università Federico II, Napoli

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Abstract

In this paper we report the behavior of normal rat vascular smooth muscle cells (VSMC) and transformed human breast cancer cells (MCF7) under normal versus simulated microgravity conditions. In this regard, we have report comparative observations on cell proliferation, cell cycling and protein expression. We conclude that cells perceive the condition of reduced gravity and respond reducing the rate of their metabolism. We do not yet know if this effect is transient or long lasting.

Introduction

Glycogen synthesis, amino acid uptake, protein synthesis, triglyceride storage, increase in glucose transport and cell proliferation are the most prominent biological effects of insulin. Indeed, it is well known that main target organs of this hormone are skeletal muscle, adipose tissue, and brain. Experiments in flight and after flight and ground-based bed-rest studies have associated microgravity and its experimental paradigms with manifestations similar to those of diabetes, physical inactivity, and aging. The in flight observations collected during these years on astronauts, animals and cell lines, however are limited in number and, understandably hampered by the heterogeneity of the "*at latere*" conditions including the flight duration and conditions, stresses other than microgravity, environment, delayed analysis, etc. Obviously, the experiments performed in simulated conditions (on humans, animals and cellular systems) are more numerous and apparently may take advantage by more controlled experimental conditions. Nevertheless the approaches adopted by the various Authors to simulate microgravity vary very largely. In this regard the most popular techniques take advantage from the "Prolonged bed rest" conditions for humans, the "Hindlimb" (or Hindquarter) suspension has been used as a model of simulated weightlessness (SW) for ground based study of the effects of microgravity on small animals. Prolonged bed rest has long been utilized as a paradigm for insulin resistance and glucose intolerance; it also reflects metabolic alterations observed in microgravity. Human bed rest studies demonstrate increased peripheral tissue insulin resistance and reduced glucose tolerance. Similarly, postflight investigations of cosmonauts after free-fall orbit demonstrate increased plasma glucose and decreased glucose tolerance. Other spaceflight investigations illustrate altered C-peptide excretion and insulin resistance during earth orbit. These observations and others suggest that bed rest and in-flight experiments support changes similar to those observed in the glucose intolerance of prediabetes, aging, physical inactivity, or obesity. Take together these data indicated that alterations of the metabolism affected by insulin occur in microgravity (Brian et al., 2002).

In this paper we set to study comparatively the specific metabolic effects and response caused by microgravity stress on insulin induced cell proliferation, glucose transport, amino acid uptake and incorporation into proteins, into cultured cells. To this purpose, we have selected the neoplastic human MCF-7 cells and normal murine VSMC. Both cell types, indeed, presents operational insulin receptors on their external membranes suggesting normal insulin-dependent metabolic activities (glucose transport, glycogen synthesis, amino acid uptake, protein synthesis, cell proliferation, etc).

As all transformed cells, MCF-7 depends upon constant refurbishment to meet their energy requirements so they are particularly dependent for growth on glucose metabolism.

VSMCs are a major constituent of blood vessel walls responsible for the maintenance of vascular tone. Accelerated VSMC growth, hypertrophy, and abnormal vascular tone play a central role in the development of atherosclerosis. Alterations in insulin action due to hyperglycemia and hyper-insulinemia have been proposed to contribute to atherosclerosis and the regulation of vascular tone. Despite the clinical importance of atherosclerosis its dependence on VSMC proliferation and its association with diabetes, little is know about mechanisms and factors modulating glucose transport in VSMC (Begum et al., 2000).

Material and methods

Cell cultures - Human breast cancer MCF-7 cells were routinely grown at 37°C in a humidified atmosphere composed of 95% air and 5% CO2 Dulbecco's modified Eagle's medium (DMEM) supplemented with Phenol Red, L-Glutamate (2mM), antibiotics. 20 mM Hepes buffer and 5% fetal calf serum (FCS). Cells were provided with fresh medium every 3 days.

Vascular smooth muscle cells (VSMC) were harvested from an explant from aortas of young male rats (Sprague Dawley) by the media explant technique (Owens et al., 1986) and were cultured in DMEM containing 10% FCS. VSMC prepared from these rats were not contaminated with fibroblasts or endothelial cells as evidenced by a >99% positive immuno-staining of smooth muscle-actin with FITC-conjugated -actin antibody (data not shown). Subcultures of VSMC at passages 4-8 were used in all experiments.

Modeled microgravity (Mg): Normally six (MCF-7) or (VSMC) P10 flasks containing 1.5 x10^5 cells at time 0 were filled completely with FCS-containing media and capped. Three (MCF-7) or (VSMC) flasks, were transferred in a temperature-controlled room (37°C) on a running Random Positioning Machine (RPM); the remaining were used as controls (see below). Both controls and samples in modeled gravity were let to grow for 48 hours.

Normal gravity (Ng): Cells grown on the RPM machine are subjected to both changes in gravity (modeled gravity) and mechanical stress due to the vibration of the RPM machine. For this reason the cells that we have used as controls were kept in filled flasks fastened on the edges of the RPM Machine. All the control cells considered thereafter are those subjected to mechanical stress only.

DNA synthesis - The assay method was performed essentially as previously described by Igarashi et al. (2000). The number of cells (MCF-7 or VSMC) employed in the various experiments was 1.5-2.0-x 10^5 cells (P10 flask); cells growth in Ng and Mg for 48 ours were subsequently incubated with 1 mCi/ml of 3H-Thymidine (Amersham, Milan, Italy) for further 8 ours in the presence or in the absence of 1 μM insulin. The incorporation experiments were terminated by aspiration of media, cell washings with cold PBS and addition of 10% Trichloroacetic acid at 4° C for 60 min. The precipitate was rinsed with absolute ethanol, dried by evaporation and finally solubilized with 1N NaOH. The resulting solution was neutralized with HCl and counted by liquid scintillation. When necessary, protein concentrations were measured by the method of Bradford (1976). Thymidine counts were expressed as a fraction of counts found in controls.

Glucose uptake assay - Cells were plated at a density of 1.5 x 10^5 cells per P10 flask and grown in DMEM containing 10% (VSMC) or 5% (MCF-7) FCS. The cell layers were let to grow for about 24 hours and transferred in a temperature-controlled room (37°C) on a running Random Positioning Machine (RPM, Dutch-Space) where they were let in modeled microgravity for 48 hours. Cells were washed and starved for 4 hours with modified Krebs Ringer phosphate solution. MCF-7 and VSMC cells were divided in two subsets (with or without 1 mM insulin) and incubated for an extra hour. Incubation at 37°C was prolonged of extra 30 min after the addition of 2-deoxy-d-[^{14}C]-Glucose in the presence of 0.17 mM glucose as carrier (Crescenzi et al., 2004). Finally cells were washed and solubilized in NaOH as above and counted. [^{14}C]-Glucose counts were expressed as a fraction of counts found in controls.

Methionine uptake assay and incorporation into neo-synthesized proteins - Amino acid uptake was determined by measuring the influx of [35S]-Methionine in normal or modeled gravity. After 36 h of simulated microgravity the MCF-7 and VSMC were removed from the RPM, washed, repositioned on the RPM and starved for additional 12 hours with modified Krebs Ringer phosphate solution in the presence of small amounts of albumin. MCF-7 and VSMC cells were divided in two subsets (with or without 1 μM insulin) and incubated for an extra hour. Incubation at 37°C was prolonged of 30 min after the addition [^{35}S]-Methionine (12,5 μCi/flask). Total radioactivity (free plus incorporated into proteins) was determined by counting solubilized cells. Protein-associated radioactivity was measured by TCA precipitable [^{35}S]-Methionine counts (Consiglio et al., 1998).

Flow cytometry - The cell layers to be analyzed cytofluorimetrically were handled as described by Crescenzi et al. (2004). For each sample, at least 20,000 events were collected. Samples were routinely run in quadruplicates in a Becton Dickinson (Mountain View, CA) Excalibur Cytofluorimeter.

Statistical analysis - Results from Thymidine incorporation, Methionine incorporation and 2-deoxy-d-[^{14}C]-Glucose transport were expressed as mean ±SE of at least three experiments performed in triplicate. Statistical significance was estimated by one-way analysis of variance (ANOVA) for comparison of several groups, and the differences were designated to be significant at $p < 0.05$.

Results and Discussion

Cell growth. - Cytofluorimetric analysis of both cell lines grown in Mg conditions for about 48 hs, indicated a sensible accumulation of cells in the G2M phase (Table I). These data are largely coincident with those previously obtained by Lewis et al. (1998). At variance with the cell system used by Lewis et al., who studied free-floating Jurkat cells, we made our observations with two adherent cell lines: the "normal" VSMC and the "malignant" MCF-7. In both cases the S phase was partially depleted in favor of the G2/M phase, although to different extents. This partial remodeling of the cell cycle profile accompanies a sensible reduction of cell proliferation.

Cell proliferation. The proliferation rate of cells kept in Mg conditions appeared substantially reduced in respect to those grown in Ng conditions. This information was directly obtained by [^{3}H]-Thymidine incorporation (indicative of DNA synthesis) experiments. Indeed the incorporation in Mg conditions was much lower (40%, $p < 0.05$) in Mg in respect to "ground" conditions (Fig. 1, upper

panel). Irrespective of cell line, insulin stimulation did not fully reverse the incorporation lessening induced by Mg (Fig. 1, lower panel).

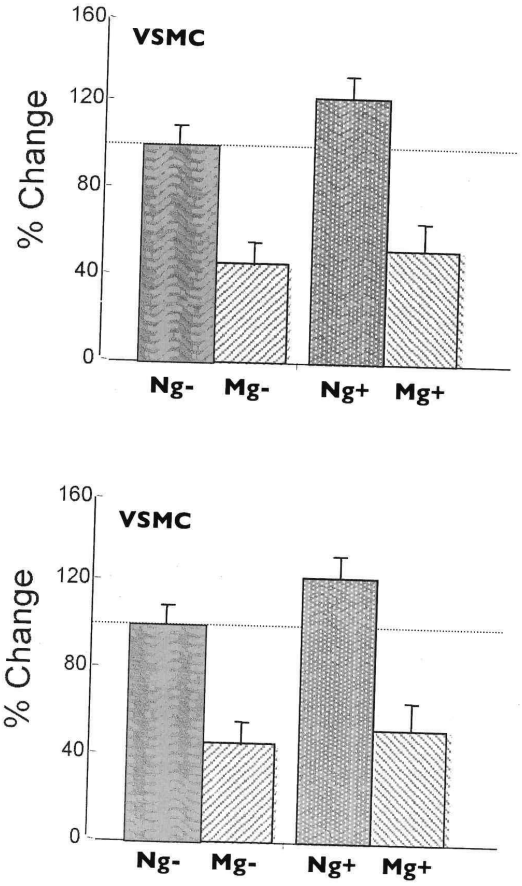

Fig 1 - [3H] -Thymidine incorporation in VSMC and MCF-7 cells in modeled gravity (Mg) versus normal gravity (Ng) (a). Modeled gravity induced 60% reduction (P<0,05) in incorporation in both cell types and was not visibly modified by insulin stimulation (1 μM).
Results are means of 4 experiments each performed in duplicate. Data are expressed as fractions of Ng controls (set at 100).

Cellular metabolic activity – We analyzed the effects of modeled gravity on three major metabolic activities affected by insulin in both malignant MCF-7 and normal VSMC cells. To this purpose we investigated 1) the uptake of deoxyglucose, 2) the transport of methionine and 3) its incorporation into TCA-precipitable materials (indicative of protein synthesis). All these metabolic activities were evaluated in N and Mg conditions in basal versus insulin stimulated conditions.
In basal conditions Mg induced a significant reduction of deoxy-glucose uptake in VSMC cells and in MCF-7 (Fig. 2).

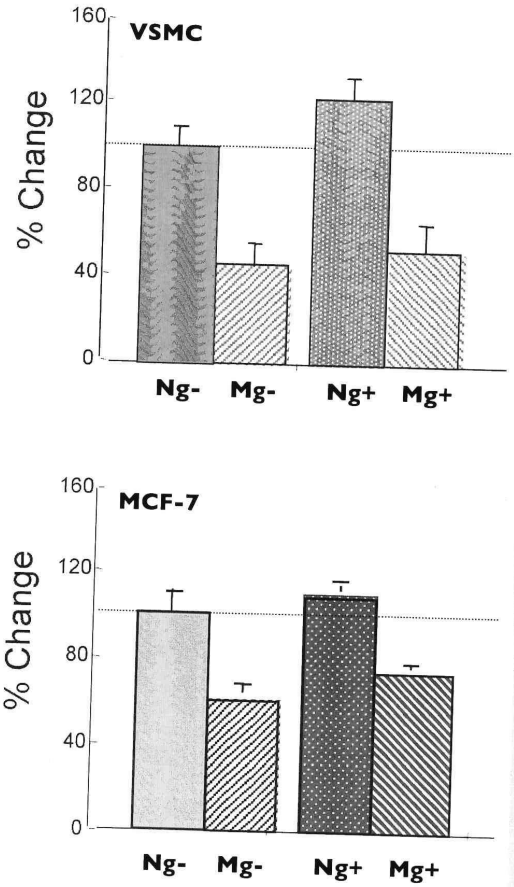

Fig 2 - [14 C]-2-Deoxyglucose uptake in VSMC (left) and MCF-7 cells (right) exposed to Ng and Mg in basal (-) and insulin stimulated (+) conditions. In respect to Ng, Mg induces a significant reduction (P<0,005) of deoxy-glucose uptake in both cell types. Expectedly, insulin stimulation improved glucose influx but the amount of glucose gained by cells in Mg was in any case lower than in Ng.

In addition, it appears that in either MCF-7 or VSMC cells, the glucose influx in normal gravity largely exceeded the glucose influx in Mg (>60%) (Fig 2). Insulin stimulation improved glucose influx but the amount of glucose gained by cells in Mg was in any case lower than in Ng.
A similar behavior was observed studying Methionine transport and utilization (protein synthesis). In modeled gravity, Methionine transport and protein incorporation matched in full being in both cases and in both cell lines 70% lower than that observed in the respective normal gravity controls. This reduction was statistically significant (P<0,005). Insulin stimulation in the range 100 nM - 1 μM, indeed enhanced Methionine transport and protein incorporation, but the such increase was not influenced by insulin addition (Fig. 3).

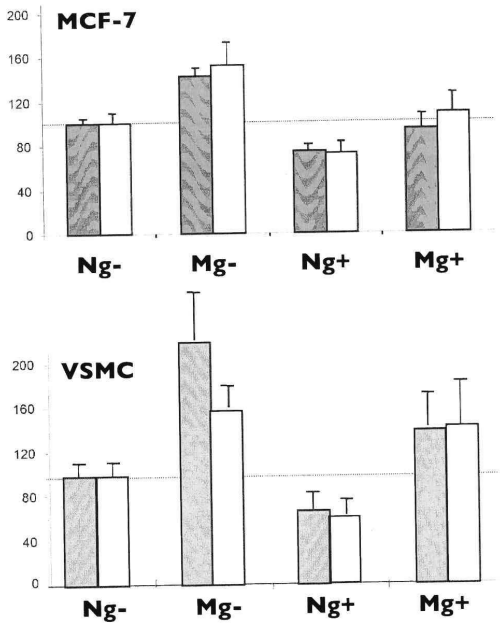

Fig 3 - [35S]-Methionine uptake and TCA-precipitable (protein synthesis) in MCF-7 and VSMC cells exposed to Ng and Mg in basal and insulin stimulated conditions. In basal conditions(-) Mg causes significantreduction (P<0,05) of Methionine uptake. Protein synthesis is lessened as well. Expectedly, insulin stimulation (+) increases Methionine transport and protein synthesis but the enhancement is smaller than those observed in Ng controls.

The body of these preliminary data suggests that insulin stimulation is unbiased by the modeled gravity conditions. On the contrary the modeled gravity conditions affect the basal metabolic conditions of both normal and neoplastic cells. Our results indicate that cells do not adjust their specific responsivity to insulin, but, rather is the microgravity conditions that compels cells to exit the original active metabolic state and enter a sort of cell cycle stand. We do not know if this quiescent state, observed in both normal and/or transformed cells in Mg, endures as long as the Mg conditions persist or cells recover their cycling ability within short time because adaptation. Certainly these aspects deserve further investigation.

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	MCF-7		VSMC	
	Normal gravity	*Modeled gravity*	*Normal gravity*	*Modeled gravity*
G_1	63.4	64.4	69.4	68.7
S	27.7	18.4	24.7	16.2
G_2/M	8.9	**17.2**	5.86	**15.1**

Tab. 1 - *Effects on cell cycle of MCF-7 and VSMC cells caused by 24 hours of exposure to modeled microgravity on RPM*