Altered Gravity Conditions Affect Early EGF-Induced Signal Transduction in Human Epidermal A431 Cells

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ABSTRACT

Epidermal growth factor (EGF) activates a well-characterized signal transduction cascade in human A431 epidermal carcinoma cells. Among the early responses evoked by EGF are receptor clustering, cell rounding, and early gene expression. These processes have been studied under various gravity conditions. In addition, we have investigated signalling pathways as induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), forskolin, and A23187 that bypass the EGF receptor, but mimic the partial activation of signal transduction pathways. Hypergravity, simulated microgravity, and real microgravity have been obtained by means of centrifuge, fast-rotating clinostat, and sounding rocket, respectively.

EGF-induced c-fos gene expression is suppressed in simulated microgravity (clinostatting) and even more so in real microgravity, while hypergravity increases early gene expression. This indicates that gravity inhibits early EGF-induced signal transduction. However, neither microgravity nor clinostatting affect EGF-induced EGF receptor clustering, suggesting that inhibition of EGF-induced signal transduction by microgravity and clinostatting is independent of EGF receptor clustering. EGF-induced cell rounding is enhanced under clinostatting, while hypergravity does not significantly influence this process. Furthermore, both under clinostatting and real microgravity, EGF- and TPA-induced c-fos expression is decreased, while forskolin and A23187-induced c-fos expression remains unaltered. These observations demonstrate that gravity affects specific components in the EGF-induced signal transduction pathway, in particular the protein kinase C pathway which is common to EGF and TPA activated intracellular signalling.

INTRODUCTION

A number of studies have indicated that gravity affects mammalian cell growth and differentiation (for a review, see Gmünder and Cogoli, 1988). Most noticeable is the severe depression in activation of human lymphocytes by the artificial mitogen concanavalin A (Con A), both under simulated and real microgravity conditions (Cogoli et al., 1980; Cogoli et al., 1984; Bechler and Cogoli, 1986). Hypergravity, on the other hand, has been observed to enhance stimulation of lymphocytes by concanavalin A and to promote proliferation of mammalian cells (Lorenzi et al., 1986; Tschopp and Cogoli, 1983; Kumei et al., 1989). Although these observations may be taken as indicative for an effect of gravity on well-conserved regulatory mechanisms in cell proliferation, the molecular mechanisms involved have remained unidentified.

Mitogenic activation of human lymphocytes with concanavalin A resembles the mechanism by which several growth factors induce cell proliferation in mammalian cells (Becker, 1988; Yarden and Ullrich, 1988). Regulation of cell proliferation and differentiation by growth factors is mediated by transmembrane receptors with inducible protein tyrosine kinase activity. Epidermal growth factor (EGF) exerts its effects through oligomerization of EGF receptors and enhancement of activity of the EGF receptor tyrosine kinase (Carpenter and Cohen, 1990; Schlessinger, 1988; Spaargaren et al., 1991; Yarden and Ullrich, 1988). This process is followed by intermolecular tyrosine phosphorylation of the oligomerized receptors, which is required for EGF-induced interaction with phospholipase Cγ(PLCγ) and other EGF receptor tyrosine kinase substrate proteins (Honegger et al., 1989; Yarden and Schlessinger, 1987; Ullrich and Schlessinger, 1990). Receptor phosphorylation is obligatory for phosphorylation and activation of PLCγ and for efficient signal transmission (Anderson et al., 1989; Koch et al., 1991; Magni et al., 1991; Margolis et al., 1990). Activation of PLCγ in its turn leads to the release of 1,2-diacylglycerol and inositol 1,4,5-trisphosphate, resulting in activation of protein kinase C (PKC) and in an increase in intracellular free calcium concentration, respectively (Carpenter and Cohen, 1990).

These events participate in triggering signalling pathways that lead, within minutes, to rapid cell morphology changes (Chinkers et al., 1979), rapid reorganization of the actin microfilament system (Rijken et al., 1991a), and elevated expression of specific genes, including the nuclear proto-oncogenes c-fos and c-jun (for a schematic representation of the signal transduction pathways evoked by EGF, see Figure 1). The expression

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of these genes and subsequent formation of Jun and Fos proteins has been implicated in cell growth control (Verma and Sassone-Corsi, 1987) and has recently been demonstrated to be required for normal cell cycle progression (Kovary and Bravo, 1991). Ultimately, the continuous presence of EGF induces DNA synthesis in target cells (Carpenter and Cohen, 1990; Koch et al., 1991; Polet, 1990).

In this chapter, we compare the effects of various gravity conditions, as created by means of centrifuge, fast-rotating clinostat, and sounding rocket, on the cellular responses to EGF. Based on the results obtained so far, it is concluded that gravity affects specific components of early EGF-induced signal transduction. In addition, we conclude that both sounding rockets and clinostats provide useful tools to establish the sensitivity of cellular processes to gravity.

Figure 1. Schematic representation of early cellular responses to epidermal growth factor (EGF). Binding of EGF to the EGF receptor (EGFR) leads to activation of the EGF receptor kinase, which phosphorylates several proteins on tyrosine residues, including phospholipase Cγ (PLC). Activated PLC hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2), thereby producing inositol triphospholipids including inositol triphosphate (IP3), and diacylglycerol (DAG). IP3 stimulates the release of calcium ions (Ca^{2+}) from intracellular stores into the cytoplasm, whereas DAG activates protein kinase C (PKC), a protein serine/threonine kinase. In addition, growth factors may induce a rise in intracellular cyclic AMP (cAMP) levels through activation of protein kinase A (PKA). These responses induce the activation of early gene expression, including the c-fos and c-jun gene. The EGF-induced response can be mimicked by agents that partially activate signal transduction pathways, i.e., TPA, A23187, and forskolin, as indicated.

RESULTS AND DISCUSSION

Epidermal growth factor (EGF) activates a well-characterized signal transduction cascade in human A431 epidermal carcinoma cells. Among the early responses evoked by EGF are receptor clustering, cell rounding, and early gene expression. In this chapter, the effects of hypergravity, simulated microgravity, and microgravity on these particular processes are compared to the normal gravity reference conditions. In addition, we have investigated the effect of altered gravity conditions on signaling pathways as induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), forskolin, and A23187 that bypass the EGF receptor, but mimic the partial activation of signal transduction pathways (see also Figure 1).

Sounding rockets have been used to investigate whether EGF-induced c-fos gene expression is affected by microgravity. During sounding rocket flight, a six minute period of microgravity (g <2 x 10^{-4}) can be obtained. A431 cells were mounted in plunge box units (de Groot et al., 1990) and subsequently placed in the MASER-3 and MASER-4 sounding rockets, allowing automated experimentation. As soon as microgravity was reached, cells were activated with EGF (100 ng/ml), TPA (100 ng/ml), A23187 (2.5 μM), or forskolin (10 μM). After a six minute incubation time, cells were lysed and prepared for Northern blot analysis. To detect launch effects, some samples were lysed at the start of the microgravity phase or, alternatively, after six minutes, to determine the influence of microgravity on unactivated cells.

Both EGF- and TPA-induced c-fos expression is decreased when cells are exposed for six minutes to microgravity (Figure 2a). Quantification of these data demonstrated that c-fos expression is decreased by ~50% under these conditions. However, forskolin- and A23187-induced c-fos expression is comparable to the normal gravity samples (de Groot et al., 1991b). Unstimulated cells did not show any significant c-fos expression, irrespective of the gravity condition. Furthermore, the expression of genes that are constantly expressed in these cells and that are not affected by the agents used, e.g., β-2 microglobulin, is not affected by microgravity. This demonstrates that only a limited set of signal transduction pathways is affected by gravity.

Similar experiments have been performed under simulated microgravity, produced by a fast-rotating clinostat. The action of the clinostat is based on randomization, rather than reduction of the gravity field, as obtained in sounding rockets (Block and Briegel, 1986; Silver, 1976). A431 cells were rotated for two hours (60 rpm), prior to the addition of EGF (100 ng/ml), TPA (100 ng/ml), A23187 (2.5 μM), or forskolin (10 μM). After a 15 minute incubation time, cells were lysed and Northern blotting was performed. In simulated microgravity, EGF- and TPA-induced c-fos expression was decreased by ~25% under otherwise similar conditions (de Groot et al., 1990; Figure 2b). Also in simulated microgravity, A23187- and forskolin- induced gene expression proceeded normally, while unstimulated cells showed no significant c-fos expression and β-2 microglobulin expression remained constant (Figure 2b).

As microgravity and clinostating both reduce early gene expression, we were interested in the effect of
increased g-values, as produced by a centrifuge, on early gene expression. Quantification of the ratio of c-fos expression at 10 g over 1 g, or at µg over 1 g, demonstrates that under hypergravity (~10 g), EGF-induced c-fos expression is enhanced by ~18% (de Groot et al., 1991a; Figure 2c). These results clearly show that only a subset of EGF-induced signal transduction pathways that lead to the induction of c-fos expression is sensitive to gravity. Furthermore, these data indicate that gravity may affect signalling pathways mediated by protein kinase C. The results obtained in real microgravity are consistent with those obtained in simulated microgravity, although a less pronounced response was monitored under the latter condition.

In contrast to the results obtained under low-gravity conditions, hypergravity promotes EGF-induced c-fos expression. Lorenzi and coworkers have shown that Con A-induced DNA replication is depressed both under simulated microgravity and hypergravity (Lorenzi et al., 1988).

One straightforward explanation for decreased EGF-induced c-fos expression in microgravity would be that it results from a reduction in EGF-induced EGF receptor oligomerization, leading to inhibition of receptor autophosphorylation and subsequent inhibition of receptor mediated signalling. The importance of EGF receptor redistribution in the plasma membrane is indicated by observations that receptor oligomerization is crucial for EGF receptor tyrosine kinase activation and interaction with substrate proteins (Yarden and Schlessinger, 1987; Ullrich and Schlessinger, 1990; Anderson et al., 1989; Koch et al., 1991; Margolis et al., 1990; Spaargaren et al., 1991). Using label-fracture and electron microscopy, the lateral distribution of plasma membrane localized proteins can be directly visualized at high resolution (Pinto da Silva and Kan, 1984).
To determine the position of EGF receptors in the plasma membrane under control conditions, cells were fixed in formaldehyde, immunogold-labeled, and prepared for electron microscopy. As shown in Figure 3a, receptor distribution (as represented by electron dense 10 nm gold particles) of control cells appears more or less random. Within five minutes following the addition of EGF (100 ng/ml), drastic receptor redistribution is induced, leading to receptor clustering (Figure 3b). As demonstrated earlier, maximal clustering occurs after 10 to 15 minutes of treatment with EGF, after which the receptor distribution returns to control levels within another two hours in the continuous presence of EGF.

To investigate the influence of gravity alterations on this plasma membrane localized process, A431 cells were mounted in plunger box units and placed in the MASER-3 and MASER-4 sounding rockets. At the start of the microgravity phase, cells were treated with EGF (100 ng/ml), or buffer alone, for five minutes and fixed. After recovery, cells were prepared for electron microscopy. The EGF receptor distribution was quantified using computer aided image analysis. As depicted in Figure 3b microgravity (µg + 5) does not affect EGF-induced EGF receptor clustering, as compared to the normal gravity control samples (1 g + 5), suggesting that inhibition of EGF-induced signal transduction by microgravity and simulated microgravity is independent of EGF receptor clustering (Rijken et al., manuscript submitted). Importantly, the receptor distribution of cells fixed at the start of the microgravity phase (µg - 0) was comparable to the normal gravity control (1 g - 0), indicating that launch effects did not occur. Five minutes of microgravity also did not significantly affect the receptor distribution (compare µg - 5 and 1 g - 5). Similar experiments were performed in the fast-rotating clinostat (60 rpm). Here also, EGF-induced receptor clustering in simulated microgravity progressed in a similar fashion to that under normal gravity conditions (not shown). We therefore conclude that the inhibition of signal transduction by microgravity is independent of epidermal growth factor-induced EGF receptor clustering. This suggests that microgravity influences EGF-induced signal transduction downstream of EGF binding and EGF receptor oligomerization. Also in this case, the results produced by microgravity and clinostatting are similar.

Taken together, these findings indicate that gravity does not act on plasma membrane localized processes, but rather affects processes occurring elsewhere in the cell. To further investigate this hypothesis, we studied EGF-induced cell rounding under simulated microgravity.
and hypergravity, as produced by the fast-rotating clinostat and centrifuge, respectively (Rijken et al., 1991b). This change in cell morphology occurs within 10 to 20 minutes (Figure 4A, 4B, 4C) and can be easily monitored by phase-contrast microscopy. Cell rounding is an actin microfilament mediated process (Chinkers et al., 1979) and as such is an excellent marker for the detection of gravity effects on a process localized in the cytoplasm. Interestingly, under simulated microgravity cell rounding is significantly enhanced (Rijken et al., 1991b; Figure 4), indicating that gravity may specifically interact with this filament system. Under hypergravity, no significant effects were observed, as judged by the Student-t significance test.

![Figure 4](image-url)

Figure 4. Simulated microgravity enhances EGF-induced cell rounding. A431 cells were treated with EGF (100 ng/ml) or buffer (A) and representative phase-contrast images were taken after 10 minutes (B) and 20 minutes (C). N and R indicate nucleus and rounded cell, respectively. (D) EGF-induced cell rounding is determined as the percentage rounded cells of the total number of cells and was investigated under clinostatting (60 rpm; μg), normal gravity (1 g) and hypergravity (10 g). Data represent means of 5 independent experiments. Bars represent standard error of the mean.

In conclusion, these observations indicate that gravity affects EGF-induced signal transduction through a gravity sensitive component downstream of the EGF receptor, possibly via a component in the cellular cytoskeleton or through protein kinase C mediated signal transduction pathways. In this respect, it is of interest to note that the actin polymerization inhibitory reagent cytochalasin D strongly inhibited EGF + insulin-induced c-fos and β-actin expression, while c-N-ras expression was unaffected (Rebillard et al., 1987). In addition, our results indicate that the fast-rotating clinostat is a useful tool to establish the sensitivity of cellular processes to gravity, although the simulated microgravity produced by clinostats evokes less pronounced responses in cells when compared to real microgravity.

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