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Nuclear Responses to Protein Kinase C Signal Transduction Are Sensitive to Gravity Changes

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A number of studies have suggested that gravity changes may influence mammalian cell growth and differentiation. To obtain insight in the molecular mechanisms underlying these effects, we have studied immediate early gene expression in response to activation of cytoplasmic signal transduction under microgravity conditions. In this paper we show that epidermal growth factor (EGF)- and 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced expression of the c-*fos* and c-*jun* protooncogenes is decreased in microgravity, while no effect of gravity changes was observed on A23187- and forskolin-induced expression of these genes. These decrease in c-*fos* expression was not due to delayed kinetics under microgravity. These results demonstrate that gravity differentially modulates distinctive signal transduction pathways.

INTRODUCTION

Since the start of biological experiments in space in the early 1970s a number of studies have suggested that microgravity may have profound effects on cell growth and differentiation of both prokaryotic as well as eukaryotic cellular systems (for a review, see Ref. [1]). The most extensive study of gravity effects on mammalian cells was performed by Cogoli and co-workers, who have shown that mitogenic stimulation of human lymphocytes by the plant lectin concanavalin A (Con A) is almost completely abolished under microgravity conditions [2, 3]. Furthermore, they have shown that hypergravity enhances Con A-induced lymphocyte proliferation [4] as well as the proliferation of other mammalian cells [5].

Although the mechanism of gravity sensation in plant cells is relatively well understood [6], the molecular events underlying the effects of gravity alterations on mammalian cells are still largely unknown. However, recent studies have shown that expression of growth regulatory genes is modulated by gravity changes. In HeLa cells it was found that hypergravity enhances the expression of the c-myc protooncogene [7], a gene whose product is known to play an important role in cellular proliferation [8]. Moreover, we have shown that EGF-induced expression of the c-*fos* and c-*jun* genes in human A431 epidermoid carcinoma cells is decreased under simulated [9] and real [10] microgravity conditions, while enhanced by hypergravity [9]. These results show that gravity exerts its effect already at the early stages of the signal transduction cascade evoked by EGF.

The products of the c-*fos* and c-*jun* gene family are components of transcription factor AP-1 and play an important role in cell proliferation (for reviews see [11, 12]) and differentiation [13, 14]. The expression of these genes is rapidly induced by a wide variety of growth factors and other agents that selectively activate some components of signal transduction pathways (reviewed in [15, 16]). To further investigate the effects of microgravity on the expression of these genes, we performed an experiment in the CIS-2 module on the MASER-4 sounding rocket. Here we show that c-*fos* and c-*jun* induction by EGF and TPA was decreased under microgravity conditions, while no effect was found on c-*fos* and c-*jun* induction by forskolin and the Ca^{2+} ionophore A23817. Furthermore we show that the decrease in EGF-induced c-*fos* expression is not due to a delay in the kinetics of c-*fos* expression.

METHODS

Cells. Human A431 epidermoid carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal calf serum (FCS). One to four hours prior to stimulation, the medium was replaced for DMEM-Hepes without serum.

RNA isolation and RNase protection analysis. Total cellular RNA was isolated by the guanidine isothiocyanate–caesium chloride method [17]. RNase protection analysis was performed according to Melton *et al.* [18]. Total cellular RNA (1–2 μg) was hybridized to ^{32}P-labeled complementary RNA probes derived from the human c-

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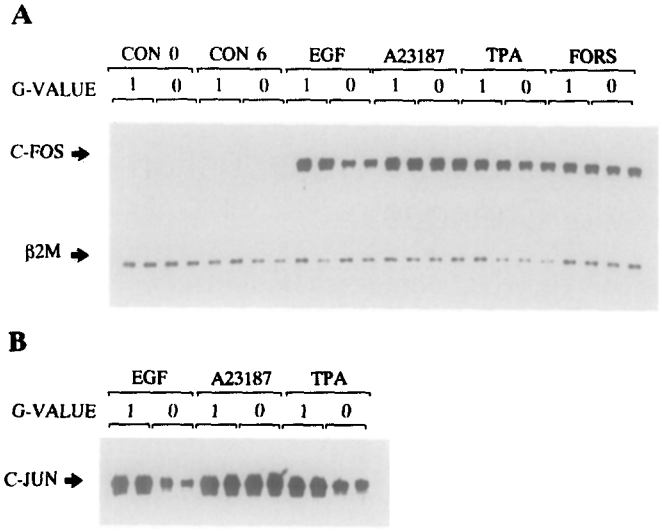

FIG. 1. Gravity alterations differentially modulate distinctive signal transduction pathways. (A) A431 epidermoid carcinoma cells cultured in the sounding rocket (0*G*) or in the 1G reference setup (1*G*) were treated for 6 min with EGF (100 ng/ml), A23187 (2.5 μM), TPA (100 ng/ml), forskolin (10 μM), or medium alone (CON 6), after which the cells were lyzed. As control to determine the effect of high G levels reached during the launch of the rocket, cells were lyzed directly after reaching microgravity in the rocket (CON 0). All experiments were performed in duplicate. RNA was isolated and analyzed for c-*fos* and β-2-microglobulin (β2M) expression by RNase protection. (B) A subset of RNA samples from A were analyzed for c-*jun* expression.

fos gene [19]), the human c-*jun* gene [20], and the human β-2-microglobulin gene [21]. After RNase digestion of the single strand transcripts, protected fragments of 110, 155, and 80 nucleotides are indicative for expression of c-*fos*, c-*jun*, and β-2-microglobulin, respectively.

Clinostat experiments. For clinostat experiments, a portable fast rotating clinostat developed in cooperation with CCM (Centre for Construction and Mechanization, Nuenen, The Netherlands) was used. All experiments were performed at 60 rotations per minute at 37°C (see also Refs. [9, 10, 32]).

MASER-4 sounding rocket experiment. A431 epidermoid carcinoma cells were cultured on coverslips and mounted into the CIS-2 (Cells In Space) plunger box experiment units (CCM, Nuenen) as described previously [10]. The experiment units were assembled in boxes and loaded in the CIS-2 module (Fokker Space and Systems, Amsterdam, The Netherlands) in the payload of the rocket. The temperature of the experiment units remained 37°C during the whole experiment due to active temperature control of the experiment boxes. After microgravity was reached in the rocket, the cells were stimulated with EGF (100 ng/ml), A23187 (2.5 μM), TPA (100 ng/ml), forskolin (10 μM), or medium alone by activation of a plunger (see Ref. [10]). After 6 min, the cells were lyzed in guanidine isothiocyanate by activation of another plunger. After recovery of the payload, RNA was isolated as described above. RNA recovery from the different treatment groups randomly varied between 7 and 9 μg.

RESULTS

We have previously shown that EGF-induced expression of the c-*fos* and c-*jun* protooncogenes was decreased under simulated and real microgravity conditions [9, 10]. To examine whether other signaling pathways leading to the expression of these genes were also sensitive to gravity changes, we performed an experiment on the MASER-4 sounding rocket. A431 epidermoid carcinoma cells were cultured on thermanox coverslips in "plunger box units" [10]. After microgravity was reached in the rocket, cells were automatically stimulated with EGF, TPA, the Ca^{2+} ionophore A23187, forskolin, or medium alone for 6 min, after which the cells were lyzed. RNA was isolated after recovery of the rocket and analyzed for the expression of c-*fos* and c-*jun*. A 1*G* reference experiment was performed in parallel on the ground using identical hardware. Figure 1A shows that EGF- and TPA-induced c-*fos* expression was decreased under microgravity conditions (47 and 26%, respectively; Table 1), while no effects of microgravity were observed on c-*fos* expression induced by A23187 or forskolin. No c-*fos* expression was detected in untreated cells (Con 0 and 6). The expression of the β-2-microglobulin gene, that is not influenced by signal transduction, was not significantly modulated by microgravity (Fig. 1A and Table 1), indicating that the observed effects of gravity changes on c-*fos* expression result from specific modulations of the signal transduction processes induced by EGF and TPA. Although these experiments were only performed in duplicate due to space limitations in the rocket, they are in agreement with previous results from experiments under simulated microgravity conditions reached in a fast rotating clinostat [9, 10] as well as under real microgravity [10]. When the expression of c-*jun* was studied, similar results were obtained. Figure 1B shows that EGF- and TPA-induced c-*jun* expression was decreased by microgravity (56 and 51%, respectively; Table 1), while A23187-induced c-*jun* expression was not altered significantly (forskolin was

TABLE 1

Microgravity Effects on c-*fos* and c-*jun* Expression

	1G/0G Ratio		
Stimulus	c-*fos* I–II	c-*jun* I–II	β2M I–II
Con 0	NC	NC	0.98–1.12
Con 6	NC	NC	1.04–1.26
EGF	2.01–1.79	2.06–2.44	1.01–0.89
A23187	1.08–1.02	0.89–1.01	1.08–1.02
TPA	1.38–1.32	1.94–2.16	1.04–0.86
Forskolin	0.99–0.91	ND	1.03–1.07

Note. Protected fragments from Fig. 1 were cut out of the gel and counted in a liquid scintillation counter. The ratio of gene expression in 1*G* and 0*G* was calculated by dividing the 1*G* samples (I and II) by the 0*G* samples (I and II), respectively. β2M, β-2-microglobulin; NC, not calculated, since no expression of these genes was observed under these conditions; ND, not done; Con 0 and 6, untreated cells lyzed 0 and 6 min after the start of microgravity, respectively.

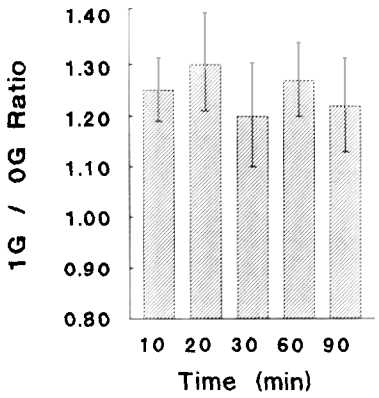

FIG. 2. Simulated microgravity does not influence the kinetics of c-*fos* expression. A431 epidermoid carcinoma cells cultured in a fast-rotating clinostat (0*G*) or in the 1*G* reference setup were treated for the indicated times with EGF. RNA was isolated and analyzed for c-*fos* expression. Protected fragments were cut out of the gel and counted in a liquid scintillation counter. Bars represent the 1*G*/0*G* ratio of c-*fos* expression at the indicated time points after EGF addition and are the mean of six independent experiments. Error bars represent the standard deviation.

not tested, since it does not induce c-*jun* expression in these cells). These results indicate that the effects of gravity on immediate-early gene expression do not result from a general cellular stress response, but are more likely caused by specific modulations of distinctive signal transduction pathways.

Since these experiments did not address the question of whether under microgravity conditions the induction of c-*fos* and c-*jun* was decreased or was delayed compared to the 1*G* reference, we performed time-course experiments under simulated microgravity in the fast rotating clinostat [32]. Our previous studies have shown that the effects of simulated microgravity c-*fos* and c-*jun* induction is qualitatively comparable to the effects of real microgravity obtained in a rocket experiment [9, 10]. Therefore, A431 epidermoid carcinoma cells cultured on thermanox coverslips were prerotated for 2 h in a fast rotating clinostat at 60 rpm, after which EGF was added for 10 to 90 min (maximum levels of c-*fos* expression are reached after 30 min, while after 90 min c-*fos* expression is decreased to about 20% of its maximal value), after which RNA was isolated and analyzed for c-*fos* expression by RNase protection analysis. As shown in Fig. 2, the ratio of c-*fos* expression under 1*G* conditions and under simulated microgravity (0*G*) conditions was significantly larger than 1 for all the time points tested, indicating that simulated microgravity does not delay the kinetics of EGF-induced c-*fos* expression, but decreases the level of expression of this gene.

DISCUSSION

EGF exerts its effect through binding to its plasma-membrane-located receptor, followed by a rapid activation of an intracellular signal transduction cascade (reviewed in [22]). Since activation of protein kinase C (PKC), the natural receptor for TPA, is one of the key events in this signal transduction cascade, our results suggest that PKC or a PKC-modulated protein may be one of the cell targets for gravity alterations. By contrast, the rise in intracellular Ca^{2+} concentration induced by both EGF and A23187 is not likely to be modulated by gravity changes. Forskolin-induced changes in gene expression are mediated by protein kinase A (PKA), which does not share common second messengers with PKC in its signal transduction cascade. Interestingly, recent experiments by Limouse *et al.* (33) show that the production of interleukins (IL-1 and IL-2) by T lymphocytes and monocytes in response to TPA is almost completely decreased under long-duration microgravity conditions (12 h), while IL production induced by cell-to-cell contacts was not gravity-dependent (accompanying paper). Combined with our data, these results strongly suggest PKC or one of its down-stream targets in gravity-dependent modulations of mammalian signal transduction, although we did not observe a complete inhibition of TPA-induced gene expression, probably due to the short exposure to microgravity (6 min).

At present, we can only speculate about the gravity-sensitive component in the PKC signaling pathway. However, we have previously shown that the activity of the c-*fos* serum-response element (SRE) is decreased under simulated microgravity conditions [10]. Interestingly, this element mediates c-*fos* induction by EGF as well as by TPA [23–26]. By contrast, A23187 and forskolin induce c-*fos* expression through regulatory sequences distinct from the SRE [27–28]. The SRE binds at least three different proteins, of which p67-SRF seems to be responsible for activating c-*fos* expression in response to EGF [29]. Treatment of cells with EGF or serum leads to a rapid phosphorylation of SRF, which is suggested to be of major importance for transcriptional activation of c-*fos* [30]. Since our results clearly demonstrate that SRE-dependent c-*fos* induction is sensitive to gravity alterations, it seems likely that PKC-activated events leading to phosphorylation of SRF might be one of the processes influenced by altered gravity conditions.

The products of the c-*fos* and c-*jun* genes are implicated in the regulation of mammalian cell proliferation and differentiation [11–14]. A number of previous studies have demonstrated effects of gravity changes on cellular proliferation and differentiation [2–4, 31]. It is therefore tempting to speculate that gravity-dependent modulations of c-*fos* and c-*jun* expression may play a role in these processes. In this respect it is noteworthy to mention that stimulation of lymphocyte proliferation by Con A, a process that is highly sensitive to gravity changes [2–4], is preceded by the induction of c-*fos* and

c-myc (R. P. de Groot, unpublished results). More experiments are, however, needed to further elucidate the mechanism by which gravity influences early gene expression responses to signal transduction and to determine whether these changes are critical for the cellular response to gravitational stress.

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