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Inhibition by rapamycin of ornithine decarboxylase and epithelial cell proliferation in intestinal IEC-6 cells in culture

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1 Induction of the enzyme ornithine decarboxylase (ODC) appears to be controlled primarily at the level of ODC mRNA translation. The immunosuppressant drug, rapamycin, blocked the induction of ODC in response to serum by roughly 50% but was without effect on transport of putrescine into the intracellular space. The effect on ODC was specific for the intracellular signalling pathway leading to activation of $p70^{S6k}$, as the immunosuppressant FK 506 was without effect on ODC activity.

2 Exposure of IEC-6 duodenal epithelial cells to rapamycin inhibited cellular proliferation. The effect of rapamycin was cytostatic in that removal of the immunosuppressant from the medium resulted in renewed cell division. Conversely, addition of exogenous putrescine, the product of the ODC catalysed reaction, was unable to reverse the cytostatic effects of rapamycin.

3 At a concentration of 10 nM, rapamycin inhibited the induction of ODC by 50%, a level of inhibition which could not be enhanced by exposure cells to 1000 nM rapamycin. This observation suggests that other intracellular signalling pathways, in addition to the $p70^{S6k}$ cascade, might be involved in regulation of translation of ODC mRNA or that rapamycin does not completely inhibit $p70^{S6k.}$

Keywords: Rapamycin; ornithine decarboxylase; protein translation; cellular proliferation

Introduction

Ornithine decarboxylase (ODC), one of the rate limiting enzymes in the polyamine biosynthetic pathway, is transiently induced after exposure to a number growth factors or hormones. There is little or no constitutive expression of the enzyme in mammalian systems and enzyme induction is tightly regulated by a complex set of regulatory mechanisms. In the duodenal epithelial cell, IEC-6, the ODC protein may be induced 25 fold by exposure of G_0/G_1 arrested cells to growth promoting agents (Ginty *et al*., 1990). While protein levels increase by 25 fold, the ODC mRNA level increases by only two fold or less. Indeed, during treatment with putrescine, the product of the ODC catalyzed reaction, ODC mRNA levels are still relatively high although ODC protein falls to undetectable levels. Likewise, after induction, ODC protein stability increases by only a factor of three. Thus, control of induction appears to be primarily at the level of translation. The 5′-untranslated region (UTR) of the ODC message contains a 130 base GC-rich sequence predicted to form a stable stem-loop structure which is both necessary and sufficient for repression of ODC mRNA translation (Manzella & Blackshear, 1990; Grens & Scheffler, 1990).

The eukaryotic initiation factor eIF-4F participates in the melting of mRNA secondary structures. eIF-4F consists of three subunits, a high molecular weight protein, p220, eIF-4A (an RNA helicase) and eIF-4E which binds to the 5′ cap structure of mammalian mRNAs (Thach, 1992). Overexpression of eIF-4E increases the translational efficiency of mRNAs containing extensive secondary structure including ODC mRNA (Koromilas *et al*., 1992; Rousseau *et al*., 1996). The ability of the ODC 5′ UTR to repress translation has been demonstrated in a growth hormone reporter system. Plasmids constructed to include sequences of the ODC 5′ UTR and a growth hormone reporter gene demonstrated that insulin stimulation of growth hormone translation was inhibited by constructs containing either the complete ODC 5′ UTR or the 5′-most 115 bases which contain the stem loop structure. Equally important, the same experiments demonstrated that insulin also modulated the phosphorylation state of eIF-4E (Manzella *et al*., 1991) suggesting that eIF-4F might be in a signalling pathway by which insulin could regulate initiation of translation of the heavy secondary structure in the ODC 5′ UTR.

Regulation of the eIF-4F initiation factor complex has recently been described. PHAS I is an inhibitor of eIF-4E. PHAS I forms a heterodimer with the eIF-4E subunit which represses association of 4E with the eIF-4F complex. Phosphorylation of PHAS I induces dissociation of eIF-4E from PHAS I allowing 4E to reassociate with p220/eIF-4A and initiate transcriptional activity (Pause *et al*., 1994). Insulin-like growth factor I and platlet derived growth factor increase PHAS I phosphorylation and relieve the inhibition of translation produced by PHAS I on eIF-4E (Graves *et al*., 1995). These effects are abolished by exposure of cells to the immunosuppressant, rapamycin. Rapamycin binds the FK 506 binding protein and this complex inhibits the mammalian target of rapamycin (mTOR, also known as FRAP or RAFT). By an unknown mechanism, rapamycin inhibits PHAS I phosphorylation, an effect which maintains the PHAS-I/eIF-4E complex and thereby limits translation. The effect of rapamycin may be selective for repression of translation of an mRNA family characterized by a polypyrimidine-rich tract immediately following their N^7-methylquanosine cap (Jefferies *et al*., 1994). The human ODC mRNA contains a polypyrimidine rich tract between bases 39–68 of its 5′ UTR; 24 of 29 consecutive bases are pyrimidines. Because of the heavy secondary structure of the 5′ UTR of the ODC mRNA, its sensitivity to growth factors such as insulin and its polypyrimidine rich tract, we hypothesized the induction of ODC is mediated by the action of the eIF-4F eukaryotic initiation factor. If so, induction of ODC should be sensitive to rapamycin. The effect of rapamycin on activity of the ODC protein is described below and the results provide further evidence that the eIF-4F initiation factor may indeed be involved in the translational regulation of the ODC enzyme and thereby polyamine synthesis.

Methods

The IEC-6 cell was cultured in Dulbecco's Modified Eagles Medium (DMEM)/5% foetal bovine serum (FBS) at 37°C in 5% CO_2 as previously described (Ginty & Seidel, 1989). Cells

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were serum deprived for 24 h before the beginning of each experiment. Activity of ODC was assayed by determining the rate of $^{14}CO_2$ production (Ginty & Seidel, 1989) when $30{,}000 \times g$ supernatants of cell sonicates were incubated in the presence of [^{14}C]-ornithine (NEN, sp. act. 57.2 mCi mmol^{-1}). Incorporation of [3,4-^{3}H]-glutamine (NEN, 60 Ci mmol^{-1}) into trichloro-acetic acid (TCA) precipitable material was determined in IEC-6 cells incubated in glutamine-free DMEM as previously described (Ginty *et al*., 1989). Uptake of putrescine (NEN, 30.2 Ci mmol^{-1}) into the intracellular space was measured in subconfluent IEC-6 monolayers as previously described (Scemma *et al*., 1993). Protein was measured by the Bradford method (with gamma globulin as a standard) (Bradford, 1976).

The immunosuppressant compounds rapamycin (Wyeth-Ayerst, Philadelphia, PA), FK 506 (tacrolimus) and cyclosporin A (Biomol, Plymouth Meeting, PA) were dissolved in ethanol at a stock concentration of 1 mM and stored at $-80°$C. These compounds were diluted to working concentrations in DMEM on the day of use. Cycloheximide was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Rapamycin was an effective inhibitor of ODC activity in IEC-6 cells (Figure 1). Concentrations as low as 3 nM inhibited roughly 40% of the induction of ODC in response to 10% FBS (Figure 1). However, increasing the concentration of rapamycin to 1 μM produced only slightly more inhibition, 55% of control, than did the 3 nM concentration. The peak in ODC activity in the IEC-6 cell occurs about 4 h after the addition of serum. Whether rapamycin, 10 nM, was added 30 min before or 30 min after addition of 10% FBS made little difference; both treatment times resulted in an equivalent inhibition of ODC activity. When rapamycin was added 3 h after enzyme induction with FBS, the immunosuppressant was no longer effective (Figure 2). Of the three immunosuppressants employed, only rapamycin was an effective inhibitor of ODC (Figure 3). Neither FK-506 nor cyclosporin A blocked activity of the enzyme. It should be noted that concentrations of FK 506 from 0.01 to 1 μM and of cyclosporin A from 0.03 to 1.0 μM were tested and that all concentrations of the immunosuppressants were without effect on ODC activity. The inhibitor of protein synthesis cycloheximide, 10 μg ml^{-1}, produced a complete inhibition of ODC activity demonstrating the need for protein translation during induction of the enzyme.

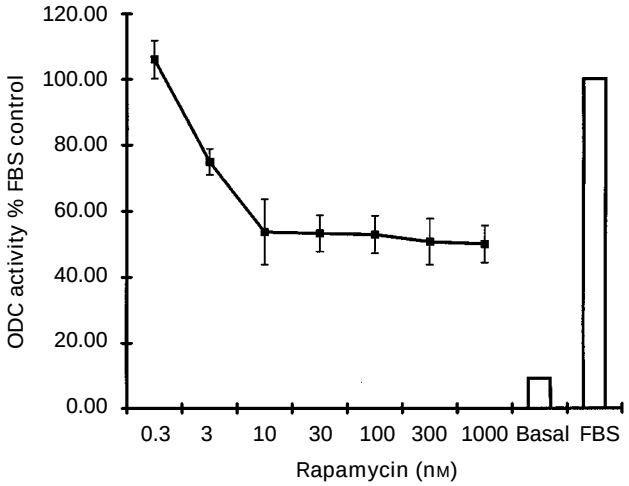

Figure 1 Concentrations of rapamycin required to inhibit induction of ornithine decarboxylase (ODC). Data are presented as % inhibition of the induction of ODC produced by treatment of cells with 10% foetal bovine serum (FBS) for 4 h. Each point represents the mean of 5 to 7 determinations; vertical lines show s.e.mean.

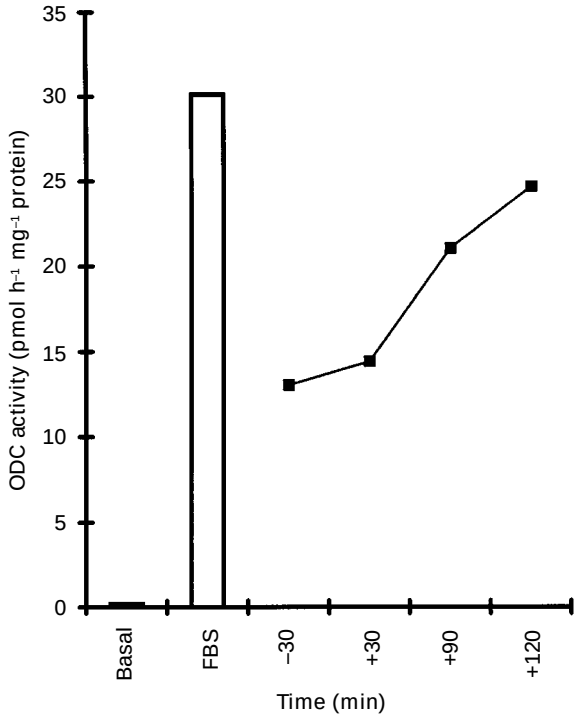

Figure 2 Time course of rapamycin action. Cells were serum deprived for 24 h (Basal) and treated with 10% foetal bovine serum (FBS). Rapamycin was added thirty min before or at various times after treatment with FBS. One representative experiment which was performed three times in duplicate with similar results.

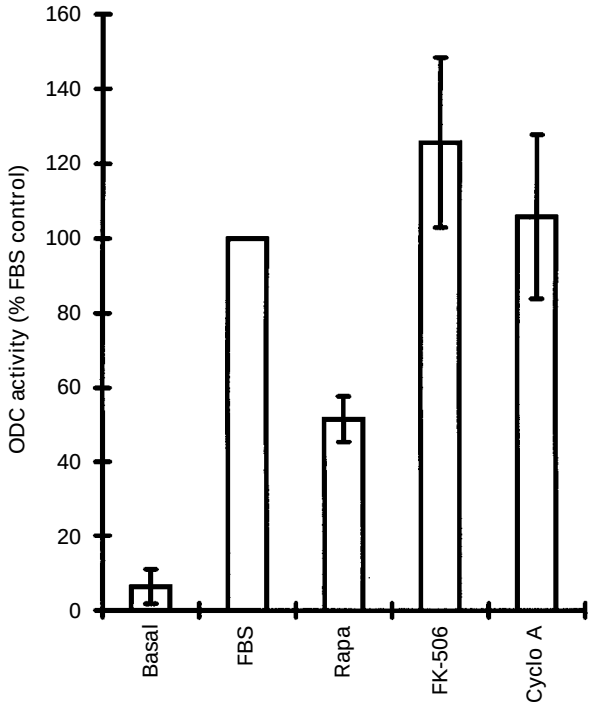

Figure 3 Effect of immunosuppressant pretreatment on the response of IEC-6 cells to foetal bovine serum (FBS) induction of ornithine decarboxylase activity (ODC). Cells were serum deprived for 24 h (Basal) followed by exposure to 10 nM rapamycin (Rapa), 1 μM FK-506 or 1 μM cyclosporin A (Cyclo A). Thirty minutes later 10% foetal bovine serum (FBS) was added to induce ODC enzyme. Data are presented as % FBS control and each point represents the mean $\pm$ s.e.mean response of 5–7 determinations.

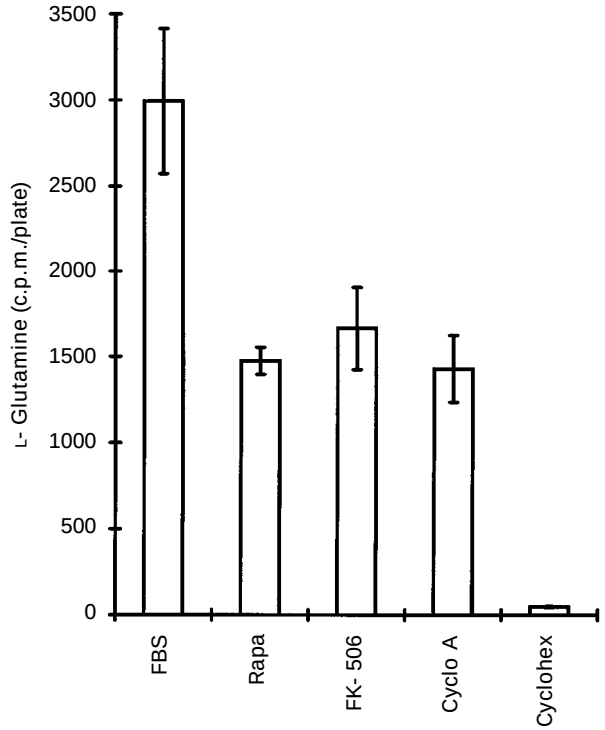

Figure 4 Effect of immunosuppressant treatment on incorporation of [3,4-^{3}H]-glutamine into acid precipitable material. Cells were treated with 10 nM rapamycin (Rapa), 1 μM FK-506, 1 μM cyclosporin A (Cyclo A), or 10 μg ml^{-1} cycloheximide (Cyclohex) 30 min before the addition of 10% foetal bovine serum (FBS). After 2 h 1 μCi ml^{-1} [3,4-^{3}H]-glutamine was added and 2 h later the incubation was stopped. Each point represents the mean $\pm$ s.e.mean response of six determinations.

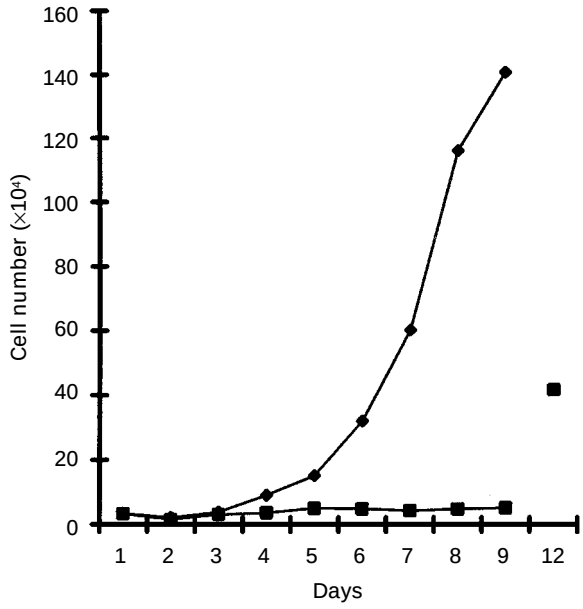

Figure 5 Effect of rapamycin on IEC-6 cell proliferation. Cells were plated in DMEM/5% foetal bovine serum at 40,000 cells/plate in the presence (■) or absence (◆) of 10 nM rapamycin and a cell count made daily for nine days. On days seven through nine, cells received 10 μM putrescine in addition to 10 nM rapamycin. After the cell count on day nine, medium containing rapamycin/putrescine was replaced with fresh DMEM/5% FBS containing neither agent for three days (single square on day 12). The results are from one representative experiment which was performed twice. Cell counts were performed daily in duplicate plates.

The specificity of the effect of rapamycin on the activity of ODC was demonstrated by determining the effects of the three immunosuppressants on protein synthesis. The rate of protein synthesis in serum stimulated cells was 3000 c.p.m. glutamine incorporated/plate but only 1500 c.p.m./plate after exposure of cells to 10 nM rapamycin (Figure 4). Both FK-506 and cyclosporin A were equally effective inhibitors of protein synthesis. In contrast, cycloheximide completely blocked the incorporation of glutamine into protein. Thus, while all three immunosuppressants blocked protein synthetic rate to the same degree, only rapamycin had an effect on ODC enzyme activity.

The effect of rapamycin on IEC-6 cell proliferation was also determined. Cells were plated at 40,000 cells/35 mm dish in the absence and presence of 10 nM rapamycin. Control cells followed a normal growth pattern but cells treated with rapamycin did not divide during the six days following rapamycin treatment (Figure 5). We have previously demonstrated that 10 μM putrescine, the product of the ODC catalysed reaction, will reverse the growth inhibition produced by treatment with the ODC inhibitor difluoromethylornithine (Ginty & Seidel, 1989). In addition, putrescine is as effective as 5% FBS in moving cells from G_0/G_1 into the DNA synthetic phase (Ginty *et al*., 1989). It was of interest to determine if addition of putrescine would reverse the inhibition of proliferation produced by rapamycin; thus, on the seventh day of the experiment 10 μM putrescine was added to the rapamycin containing medium. Putrescine had no effect on the proliferative status of the cells over the next three days in culture. On day 9 the rapamycin/putrescine containing medium was replaced with DMEM/5% FBS and cells maintained under these conditions until day 12 after plating. Removal of the rapamycin was followed by a vigorous proliferative response indicating the effect of rapamycin was cytostatic and that the cells were still fully capable of division. To insure that cells could still transport polyamines after rapamycin treatment, a final experiment was performed to determine the effect of rapamycin on putrescine uptake. Cells were cultured in the presence of 100 nM rapamycin for 84 h before measurement of uptake of exogenously supplied putrescine. Under control conditions the IEC-6 cell transported 2,254 $\pm$ 88 d.p.m. [^{3}H]-putrescine/10^6 cells-15 min ($n = 11$), whereas the rapamycin treated cells transported 2,408 $\pm$ 282 d.p.m. [^{3}H]-putrescine/10^6 cells-15 min ($n = 10$). Thus, rapamycin was without effect on putrescine transport and therefore the failure of putrescine to reverse the cytostatic effect of rapamycin was not due to the ability of the immunosuppressant to inhibit polyamine transport.

Discussion

These data support the hypothesis that the eIF-4F initiation factor complex is in the pathway regulating the translation of ODC mRNA. This intracellular signalling pathway leads to activation of p70 S6 kinase (p70^{S6k}) and phosphorylation of the S6 ribosomal protein, the small subunit (40S) of eukaryotic ribosomes (for review see Proud, 1996). S6 phosphorylation is enhanced by a number of trophic stimuli such as hormones and growth factors that stimulate protein synthesis. However, in addition to the regulation of protein synthesis, these agents modulate cellular proliferation. Overexpression of eIF-4E is mitogenic (Smith *et al*., 1990). It produces tumourogenic transformation (Lazarius-Karazas *et al*., 1990) in NIH3T3 cells and both enhanced rate of proliferation and morphological changes in HeLa cells (DeBenedetti & Rhodes, 1990).

Rapamycin inhibited proliferation of the IEC-6 cell. Similar effects have been observed in other cells including vascular smooth muscle (Marx *et al*., 1995), phagocytes (Cooper *et al*., 1994), mast cells (Tsai *et al*., 1993), hepatocytes (Francavilla *et al*., 1992). In the current experiments, when rapamycin was removed from the medium, cells began to proliferate normally suggesting the immunosuppressant has a cytostatic effect in this epithelial cell line. Inhibition

of ODC with difluoromethylornithine, a suicide substrate inhibitor of the enzyme, leads to depletion of intracellular polyamine stores and G_1 cell cycle arrest; the effect is reversed by addition of exogenous putrescine (Ginty & Seidel, 1989). Although rapamycin partially blocked ODC enzyme activity, an effect which may lead to polyamine depletion, the cytostatic effect was not reversed by addition of exogenous putrescine. Three possibilities can be presented: (1) either, in addition to ODC, rapamycin inhibits the synthesis of a number of proteins required for cell cycle progression or (2) the partial ODC block did not deplete the cell of polyamines or (3) the exogenously added putrescine was not transported to the intracellular space. The first possibility is likely to be true. For instance, eIF-4E increases nucleocytoplasmic transport of cyclin D1 mRNA (Rousseau *et al*., 1996), an effect which should be sensitive to rapamycin. The second option, inhibition of ODC by rapamycin blocked only 50% of enzyme activity and therefore was not accompanied by intracellular polyamine depletion, may also be true but awaits determination of the effect of rapamycin on intracellular polyamine levels. The third possibility, that rapamycin inhibits polyamine transport, is unlikely as rapamycin did not attenuate putrescine transport in the IEC-6 cell.

FK506, which binds to the same intracellular protein as rapamycin, FKBP 12, yet does not inhibit $p70^{S6k}$ phosphorylation, partially inhibited protein synthesis but was without effect on ODC activity, demonstrating the specificity of rapamycin inhibition of ODC. However, rapamycin, while specific, blocked only 50% of the induction of ODC enzyme, even though concentrations 100 times the effective concentration were tested. The effect of rapamycin on the translation of other messages including those for eEF-1 α, EF-2 and ribosomal protein S6 is also not complete (Jefferies *et al*., 1994). This observation suggests that either a second intracellular signalling pathway is involved in regulation of ODC mRNA translation or rapamycin is unable to inhibit completely the pathway leading to $p70^{S6k}$ phosphorylation. There is evidence to support the latter possibility. Serum stimulation of Swiss 3T3 cells results in phosphorylation of seven sites on $p70^{S6k}$. Rapamcyin blocks phosphorylation of four of these sites but in the presence of serum the remaining three sites continue to be phosphorylated (Han *et al*., 1996). Phosphorylation of these three sites in the presence of rapamycin may allow ODC mRNA translation to proceed at a reduced rate and account for the incomplete inhibition of enzyme activity observed in our experiments.

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