EFFECT OF α-METHYL ORNITHINE ON ORNITHINE DECARBOXYLASE ACTIVITY OF RAT HEPATOMA CELLS IN CULTURE

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SUMMARY

DL-α-methyl ornithine (α-MeOrn), a potent competitive inhibitor of ornithine decarboxylase (L-ornithine carboxy-lyase EC 4.1.1.7) (ODC), caused an increase of ODC activity of rat hepatoma tissue culture cells cultured in the presence of the compound. The effect was seen only when there was newly synthesized ODC present. The α-MeOrn mediated increase of ODC activity was independent of RNA synthesis and was expressed at a post-transcriptional level. ODC half-life, measured after cycloheximide treatment, was increased by a factor of three in the presence of α-MeOrn, suggesting the compound slowed enzyme degradation perhaps by its ability to enter the active site.

INTRODUCTION

In many cell systems cultured *in vitro* and tumor systems *in vivo* increases in levels of polyamines are associated with cell proliferation (1,2,3,4). One approach to the study of the effects of polyamines on cell growth processes has been to use inhibitors of ornithine decarboxylase (ODC)[1] and S-adenosylmethionine decarboxylase to block putrescine and spermidine synthesis (5). Methylglyoxal-bis (guanylhydrazone), a potent inhibitor of S-adenosylmethionine decarboxylase (6), inhibited spermidine synthesis in rat liver and kidney (7) and in lymphocytes (8). This was accompanied by inhibition of DNA replication (9,10). Specific increases of S-adenosylmethionine decarboxylase in the presence of methylglyoxal-bis(guanylhydrazone) were noted in lymphocytes (11) and in rat tissues (12) and were ascribed to decreases in the rate of degradation.

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[1] Abbreviations used are: α-MeOrn, D,L-α-methyl ornithine; HTC, hepatoma tissue culture; ODC, ornithine decarboxylase.

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A competitive inhibitor of ODC, α-hydrazino ornithine (13) blocked putrescine accumulation in HTC cells, in regenerating rat liver (14,15) and in mouse parotid glands (16). A dramatic increase was found in ODC activity measured in sonicates of HTC cells, regenerating rat liver, and mouse parotid glands treated with α-hydrazino ornithine (14,16). Enzyme turnover, as measured in the presence of cycloheximide, was considerably slowed in all three cases.

α-Methyl ornithine, a competitive inhibitor of ornithine decarboxylase (2,17,18,22), has been shown recently to deplete putrescine and spermidine in HTC cells and slow down DNA synthesis and cell division (19). We wish to report in the present communication that α-MeOrn when added to HTC cells causes ODC to increase to a steady state level above control, apparently acting by slowing the rate of ODC degradation.

MATERIALS AND METHODS

Cells. HTC cells were grown in spinner culture in Swim's S-77 medium to a high density (9 x 10^5/ml) and diluted into fresh medium containing 0.1% bovine serum albumin to a final concentration of 1.5 x 10^5/ml as previously described (4). After 24 hours, calf serum was added to a final concentration of 10% to start cell proliferation. α-Methyl ornithine hydrochloride (19) and cycloheximide dissolved in phosphate-buffered saline were added before and after addition of calf-serum as described in Results and Discussion.

Enzyme Assay. Aliquots of cells in medium (1.5 x 10^6 to 3.0 x 10^6 cells) were washed by centrifugation twice with phosphate-buffered saline at 4°C, sonicated and then assayed for ODC by the method described previously (4). Enzyme activity varied from experiment to experiment. Controls were always carried out using cells from the same spinner culture that was subjected to experimental manipulations. Cell protein was measured by the fluorescamine method (20).

Rates of Enzyme Disappearance. Data for the variation on ODC activity were plotted semi-logarithmically as a function of time after addition of cycloheximide to cell cultures. Half-life of enzyme activity was calculated from the slope obtained by linear least-squares regression using that portion of the curve where activity was decreasing as previously described (21).

RESULTS AND DISCUSSION

We found that ODC activity measured in washed, inhibitor-free sonicates of HTC cells cultured in the presence of 5 mM α-MeOrn increased to reach a new steady state level that was three times above control (Fig. 1). Dialysis of these sonicates did not modify this increased activity. When HTC cells were diluted into medium in the absence of

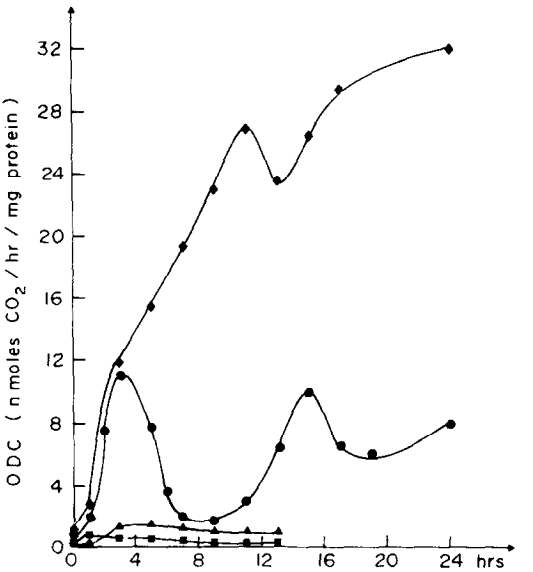

Figure 1. Enzyme activity in growing HTC cells after initiation of cell proliferation by dilution into fresh medium at time zero as specified in Materials and Methods. Aliquots of cells were taken and assayed for ODC at the indicated times. Activities are averaged from at least three experiments using different cultures. Substances being investigated were dissolved in phosphate-buffered saline and brought to pH 7.2 before addition to spinner cultures. Control (●); addition of 5 mM α-methyl ornithine at time zero (◆); dilution into medium without serum (0.1% bovine serum albumin) (■); dilution into medium without serum but with 5 mM α-methyl ornithine (▲).

serum only a low basal ODC activity was present after 24 hours. Addition of α-MeOrn did not cause an increase of that basal ODC activity (Fig. 1). Therefore it seems likely that α-MeOrn only has an effect on newly synthesized ODC.

To determine whether this effect was at the level of RNA synthesis or was post-transcriptional, we reinvestigated the effects of blocking RNA synthesis on the expression of ODC in HTC cells. Stimulation of HTC cell proliferation results in a biphasic increase of ODC during each cell doubling period (4). This fluctuation in ODC activity is illustrated in Fig. 2. If Actinomycin D (1 μg/ml) was added at the peak of initial ODC activity (3 hours), the second increase was not observed. When added during the decay of the first peak of ODC activity (5 hours), Actinomycin D progressively blocked enzyme activity but some activity was still present 8 hours later. If Actinomycin D was added 7 hours after dilution, the second increase of ODC was abbreviated

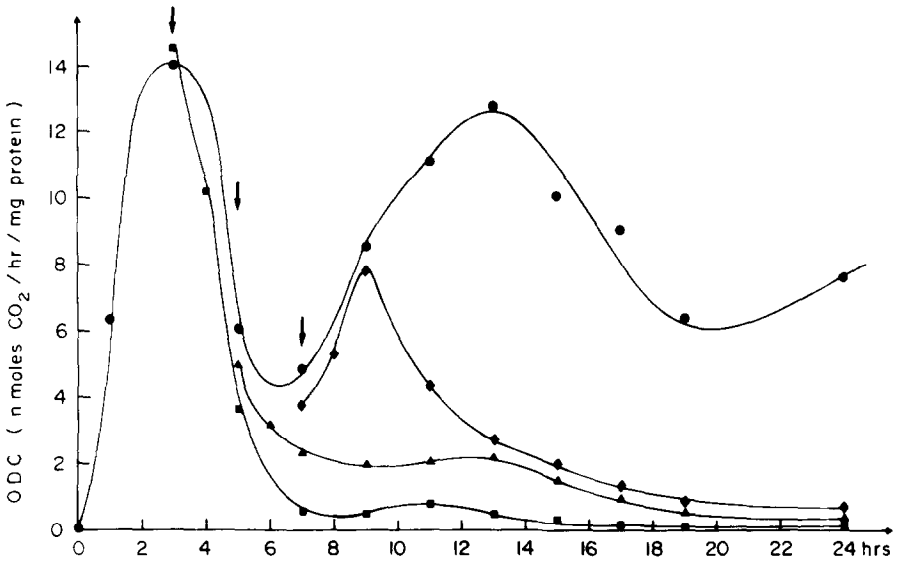

Figure 2. Effects of Actinomycin D on enzyme activity in growing HTC cells after induction of cell proliferation by dilution into fresh medium with 10% calf-serum (time zero). Aliquots of cells were assayed for ODC at the indicated times. Enzyme activities are averages from at least three experiments on different cultures. Actinomycin D (1 µg/ml) was dissolved in phosphate-buffered saline and brought to pH 7.2 before addition to spinner cultures at times indicated by the arrows. Control (●); addition of Actinomycin D 3 hours after dilution (■); addition of Actinomycin D 5 hours after dilution (▲); addition of Actinomycin D 7 hours after dilution (◆).

and largely suppressed. Actinomycin D added at 13 hours had no effect (not shown). These results suggested that biphasic increases of ODC were dependent on de novo synthesis of RNA.

We then used Actinomycin D in combination with α-MeOrn as shown in Fig. 3. Actinomycin D (1 µg/ml) added two hours after initiation of cell proliferation did not block the effect of α-MeOrn (Fig. 3A) as ODC activity in HTC cells continued to increase after one hour. Actinomycin D (1 µg/ml) added 13 hours after initiation of cell proliferation of α-MeOrn treated cells did not modify the effect of α-MeOrn (Fig. 3B). These results indicated that it is likely that the α-MeOrn mediated increase of ODC activity was independent of RNA synthesis, but in order to see the maximal effect of α-MeOrn it is probably necessary to have continued ODC synthesis.

Cycloheximide (50 µg/ml) added to cells at the time of dilution into fresh medium containing α-MeOrn (time zero) (Fig. 3A) or 13 hours later

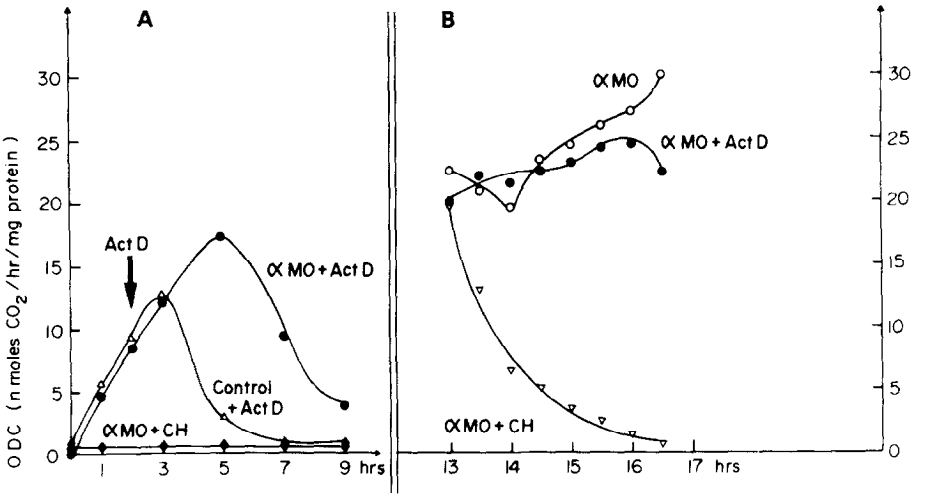

Figure 3. Enzyme activity in growing cells after the start of cell proliferation by dilution into fresh medium with 10% calf-serum. Activities are averages from at least three experiments using different cultures.
(A) Addition of 5 mM α-methyl ornithine and 50 μg/ml cycloheximide (time zero) (◆), addition of 5 mM α-methyl ornithine (time zero), and of 1 μg/ml Actinomycin D (●) (two hours); addition of 1 μg/ml Actinomycin D (Δ) (two hours).
(B) Additions thirteen hours after dilution into 5 mM α-methyl ornithine: Control (α-MeOrn) (○); 1 μg/ml Actinomycin D (●); 50 μg/ml cycloheximide (▽).

(Fig. 3B) did block the effect of α-MeOrn. We conclude that the effect of α-MeOrn on increasing latent ODC activity in HTC cells must be expressed at the post-transcriptional level. α-Methyl ornithine may act by increasing the number of enzyme molecules either by slowing down their degradation or by increasing the rate of enzyme synthesis. The possibility that α-MeOrn might cause activation of pre-existing enzyme was considered less likely since kinetic parameters of α-MeOrn-induced ODC were found to be identical with those previously reported for the enzyme in normally growing HTC cells (C. Danzin, personal communication).

When cycloheximide was added to cells cultured in the presence of α-MeOrn, the half-life of cellular ODC was observed to be 57 minutes. In the absence of α-MeOrn, half-life is 19 minutes (Fig. 4). Therefore, α-MeOrn appeared to decrease the decay of enzyme activity by a factor of three. The decrease in degradation might be due to the ability of the competitive inhibitor to enter the active site of ODC and thus block or slow its degradation by proteolytic enzymes (11,12,14,23). α-MeOrn

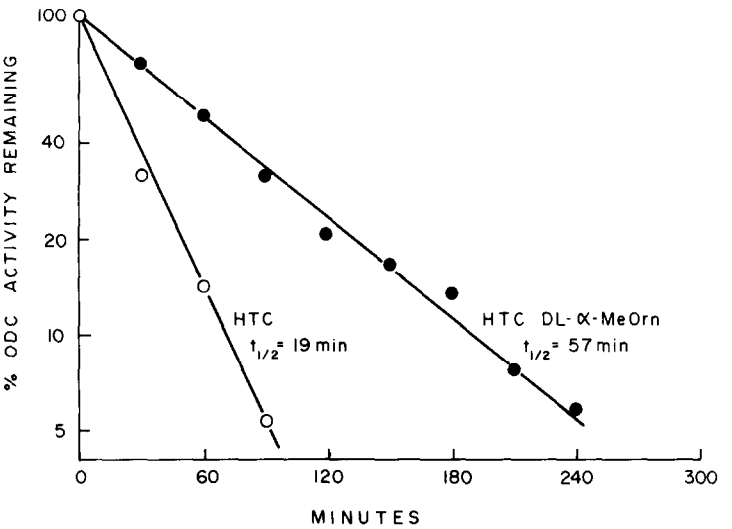

Figure 4. Half-life determinations of ODC activity after addition of cycloheximide in the presence or absence of α-MeOrn. Maximal enzyme activity (100% activity) was taken as the beginning of the phase of linear reduction of ODC activity.
Additions thirteen hours after dilution into fresh medium with 10% calf-serum: 50 μg/ml cycloheximide (○); 5 mM α-methylornithine and 50 μg/ml cycloheximide (●).

had no effect on the basal level of ODC activity in cells not stimulated to proliferate, in contrast to serum-induced cells (Fig. 1). This suggests that the stability of ODC may differ under varying conditions. Clark (24) reported that ODC half-life was longer in quiescent 3T3 cells than in growing cells. A change in half-life of ODC was also suggested by observations of Hogan et al. (25) on HTC cells cultured in the presence or absence of serum.

It is clear then that α-MeOrn causes a stabilization of ODC and it slows enzyme degradation. But the question remains as to why de novo synthesis of ODC is required to have expression of the α-MeOrn effect. It may be possible that α-MeOrn could also play another role in the regulation of ODC. A clone of HTC cells has been isolated where the half-life of ODC has been found to be stabilized ($t_{1/2}$ ≈ 6 hours) without regard to the presence or absence of α-MeOrn. However, the presence of this compound still causes the level of ODC activity to increase four fold (P.S. Mamont, to be published). This is suggestive perhaps that α-MeOrn might have a direct or indirect effect on ODC synthesis.

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