Ornithine Decarboxylase Induction in Cells Stimulated to Proliferate Differs from that in Continuously Dividing Cells

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(Received 8 October 1979)

Ornithine decarboxylase activity increases at least 4–5-fold before DNA synthesis both in synchronous cycling cells and in quiescent cells stimulated to proliferate. The purpose of our experiments was to test whether the transient peaks of ornithine decarboxylase activity in both growth situations were biochemically regulated in a similar manner. We found that the regulation of this particular enzyme activity is distinct in two ways. Firstly, the addition of 2mM-hydroxyurea will block the induction of ornithine decarboxylase in continuously dividing Chinese-hamster ovary cells, while having no effect on ornithine decarboxylase induction in stimulated quiescent cells. Hydroxyurea added after the induction occurs has no effect on the enzyme activity. The apparent half-life of the enzyme is not altered in cells treated with hydroxyurea. Hydroxyurea does not affect the enzyme directly, since incubation of cell homogenates with this drug results in no loss of measurable ornithine decarboxylase activity and hydroxyurea does not markedly alter general RNA- or protein-synthesis rates. The inactivation of ornithine decarboxylase activity by hydroxyurea does not resemble the loss of activity observed with a 90min treatment with spermidine. Thiourea, a less potent inhibitor of ribonucleoside diphosphate reductase, will also inhibit ornithine decarboxylase activity, but to a lesser extent. Secondly, the expression of ornithine decarboxylase in quiescent cells stimulated to proliferate is biphasic as these cells traverse G₁ and enter S phase, whereas only one peak of activity is apparent in synchronous cycling G₁-phase cells. The time interval between the first peak of ornithine decarboxylase activity and the onset of DNA synthesis is approx. 5h longer in non-dividing cells stimulated to proliferate than in continuously dividing cells. The results suggest that the regulation of ornithine decarboxylase activity is different in the two growth systems in that the induction of ornithine decarboxylase in continuously dividing cells occurs closer in time to DNA synthesis and is dependent on deoxyribonucleoside triphosphates.

Ornithine decarboxylase (EC 4.1.1.17) activity has been extensively investigated because of its purported regulatory role in cell proliferation (Tabor & Tabor, 1976). In many tissues, ornithine decarboxylase activity increases dramatically in response to various growth stimuli (Cohen, 1971; Tabor & Tabor, 1972). By using synchronous Chinese-hamster ovary cells in culture, ornithine decarboxylase has been shown to increase in late G₁ and in the G₂ phases of the cell cycle as well as increasing when quiescent cells are stimulated to proliferate (Fuller *et al.*, 1977; Heby *et al.*, 1976; Hogan *et al.*, 1973). In the present paper, we have investigated whether the elevation of ornithine

decarboxylase activity at the G₁/S-phase boundary in continuously dividing cells is biochemically different from the increase observed after cells are stimulated from quiescence to enter the cell cycle. The uniqueness of this work lies in the revelation that the expression of an identical enzyme activity can be regulated differently in a cell-cycle-phase-dependent manner. In our case, the regulation of ornithine decarboxylase induction appears to be dependent on the signal, even when the endpoint (DNA synthesis) is identical. This concept differs from a classical view of the cell cycle, where specific molecular events have delineated the cell cycle phase (Tobey *et al.*, 1974). Further, the data in the present

report suggest that in continuously dividing cells the induction of ornithine decarboxylase depends on deoxyribonucleoside triphosphates.

Experimental

Cell culture techniques

Chinese-hamster ovary cells were grown as monolayer cultures as previously described (Cress & Gerner, 1977). Under these conditions, the cell culture doubling time was 13–14 h. Synchronous cell populations were obtained by selective detachment of mitotic cells (Tobey *et al.*, 1967). The mitotic index was 90% or more for all experiments. Plateau-phase cultures were used as a model for quiescent cells, as described by Hahn & Little (1972), and are defined as cells grown to a high density (25 000 cells/cm²), having a labelling and mitotic index less than 10% and a plating efficiency of 80% or more as previously described (Gerner *et al.*, 1976). Plateau-released cells are plateau-phase cultures which are harvested by trypsin and replated into fresh prewarmed McCoy's 5A medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 20% foetal calf serum.

Measurement of ornithine decarboxylase activity

This was measured by the liberation of ¹⁴CO₂ from DL-[1-¹⁴C]ornithine (New England Nuclear, Boston, MA, U.S.A.; 40–60 Ci/mmol). The specific radioactivity was changed to 5.2 mCi/mmol by the addition of L-ornithine. Cells were sonicated (E/MC Corp., ultrasonic cell disrupter, 4.5 in probe; 10⁶ cells/0.1 ml of buffer for 20 s) in 0.05 M-sodium/potassium phosphate buffer, pH 7.2, containing 0.1 mM-EDTA, 1.0 mM-dithiothreitol, 20 μ M-pyridoxal phosphate and 100 μ M-phenylmethanesulphonyl fluoride and centrifuged at 8730 g for 1.5 min at 4°C. The 200 μ l of the cell supernatant was incubated for 30 min 37°C in the presence of 0.5 μ Ci of L-[1-¹⁴C]-ornithine. Minimum cells numbers were 5.0 $\times$ 10⁶ cells per assay. Enzyme activity is expressed as pmol of ¹⁴CO₂ released/h per 10⁶ cells.

Measurement of RNA- and protein-synthesis rates

RNA-synthesis rates were determined by incubating cells with [³H]uridine (5.0 μ Ci/ml; 8.0 Ci/mmol, Schwarz–Mann) for 15 min. Protein-synthesis rates were measured by incubating cells with [³H]leucine (2.5 μ Ci/ml; 15 Ci/mmol) for 15 min in McCoy's 5A medium, modified to contain 10% of the normal leucine concentration and supplemented with 20% foetal calf serum.

Cells were harvested by scraping, and the amount of trichloroacetic acid-insoluble radioactivity was measured (Gerner *et al.*, 1976). Each sample point represents triplicate determinations of at least 2.0 $\times$ 10⁶ cells.

Measurement of DNA replication

The DNA-replication kinetics represent semi-conservative synthesis measured by CsCl-buoyant-density-gradient analysis as previously described (Cress & Gerner, 1977).

Results

Prevention of ornithine decarboxylase induction by hydroxyurea

Fig. 1(a) illustrates a 6-fold increase in ornithine decarboxylase activity 4 h after plateau release. This peak of activity is transient in that the values are decreased to less than 20 pmol/h per 10⁶ cells by 2 h. In addition, ornithine decarboxylase activity begins to increase again 6 h after plateau release, suggesting that the activity is biphasic as quiescent cells traverse G₁ to S phase. Incubation of these cells in 2 mM-hydroxyurea during replating has no effect on the expression of ornithine decarboxylase activity. DNA synthesis in these cells begins at 10 h, approx.

Fig. 1. Ornithine decarboxylase activity in cells after release from plateau and in continuously dividing cells after mitosis

(a) Ornithine decarboxylase activity during the hours after cells were released from plateau. Determinations were done in the presence (○) or absence (●) of 2 mM-hydroxyurea. In addition, DNA replication was measured (■) in the absence of 2 mM-hydroxyurea. (b) Ornithine decarboxylase activity during the hours after cells were selected in mitosis in the absence (●) and presence (○) of 2 mM-hydroxyurea. DNA replication (■) is also shown in cells proceeding from mitosis without 2 mM-hydroxyurea.

6h after the first peak of ornithine decarboxylase activity; 90% of the DNA is replicated by 18h. In plateau-released cultures, DNA synthesis is prevented by hydroxyurea (results not shown). In contrast, Fig. 1(b) shows that the induction observed 5–6h after mitosis in synchronous cells is remarkably sensitive to 2mM-hydroxyurea treatment. We have previously shown that hydroxyurea inhibits DNA synthesis under these conditions (Cress & Gerner, 1977). Ornithine decarboxylase activity increases to maximum values before the onset of DNA synthesis in untreated cultures, and then decreases as cells replicate their DNA. It became important then to ask if hydroxyurea can inhibit ornithine decarboxylase induction within 1h of treatment, i.e. 2mM-hydroxyurea given at 4h after mitosis will also completely inhibit the induction of the enzyme (Cress & Gerner, 1979). In addition, Fig. 2 shows that when 2mM-hydroxyurea is added at 5h after mitosis, when ornithine decarboxylase induction has already occurred, the drug has no effect on this enzyme activity. These data further suggest that hydroxyurea prevents the process of ornithine decarboxylase induction.

Normal translation in the presence of hydroxyurea

If hydroxyurea blocks only the induction of ornithine decarboxylase in synchronous cells, then normal translation, as probed by puromycin, should be observed in cells treated 5h after mitosis. Fig.

Fig. 2. Ornithine decarboxylase induction in the presence of hydroxyurea

Ornithine decarboxylase is measured here without hydroxyurea in cells selected in mitosis (○), and also in cells in which 2mM-hydroxyurea was added at 5h (arrow) after mitosis (●).

3(a) shows that the addition of puromycin (50 μg/ml) and 2mM-hydroxyurea at 5h after mitosis results in a comparable half-life of the enzyme activity independent of whether hydroxyurea is present. Fig. 3(b) shows that in plateau-released cells puromycin added during the increase of ornithine decarboxylase activity results in a similar biological half-life to that seen in continuously dividing cells (Fig. 3a). The data in Fig. 3(b) also show a similarity between the fall in ornithine decarboxylase activity normally beginning at 5h after plateau release and that induced by the translation inhibitor puromycin. Under these conditions, puromycin (50 μg/ml) decreased incorporation of radiolabelled leucine into acid-insoluble material by greater than 90% within 10min (results not shown).

Fig. 3. Effect of puromycin on ornithine decarboxylase activity with and without hydroxyurea

(a) Ornithine decarboxylase activity measured after cells are selected in mitosis (●) and in cells treated with puromycin (50 μg/ml) at 5h after mitosis (○). In addition, ornithine decarboxylase activity is shown in cells treated with 2mM-hydroxyurea and puromycin (50 μg/ml) at 5h after mitosis (*). (b) Ornithine decarboxylase activity after cells are stimulated from plateau phase (●) and when puromycin (50 μg/ml) was added at 4h after stimulation (○). In both parts, vertical arrow indicates time of drug addition.

Effect of hydroxyurea on protein- and RNA-synthesis rates

Previous experiments have shown that the treatment with 2 mM-hydroxyurea for up to 13 h of Chinese-hamster-ovary cells, selected in mitosis and allowed to traverse G_1 phase, will not result in cell death (Cress & Gerner, 1979). This indicates that the prevention of ornithine decarboxylase by hydroxyurea is not due to non-specific cytotoxicity. Table 1 illustrates that synchronous Chinese-hamster ovary cells incubated with 2 mM-hydroxyurea do not have appreciably altered RNA-synthesis rates.

Enzymes induction generally implies a dependence on protein synthesis, as well as RNA synthesis. However, Table 2 indicates that general protein-synthesis rates are unaffected by 2 mM-hydroxyurea, even though this particular enzyme induction is blocked. In addition, Table 3 shows that hydroxyurea added to cell homogenates has no direct effect on the enzyme activity.

Hydroxyurea does not act as a diamine

Our next task was to rule out the possibility that hydroxyurea may be acting similarly to diamines in decreasing ornithine decarboxylase activity (Heller *et al.*, 1976). Fig. 4 shows that treatment of asynchronous Chinese-hamster ovary cells with various concentrations of spermidine for 90 min will result in up to a 75% decrease in the measurable ornithine decarboxylase activity. In contrast, similar concentrations of hydroxyurea do not affect this enzyme activity.

Thiourea and ornithine decarboxylase activity

Thiourea is a structural analogue of hydroxyurea which is a less efficient inhibitor of ribonucleoside diphosphate reductase (Timson, 1975). If the

Table 1. Effect of 2 mM-hydroxyurea on RNA-synthesis rates in cells traversing G_1 into S phase

Chinese-hamster ovary cells were incubated with [3 H]uridine for 15 min at the times indicated. The cultures were then harvested and the amount of acid-insoluble material was determined as described in the Experimental section. Values represent the means of triplicate determinations $\pm$ S.E.M.

Time after mitosis (h)	$10^{-3} \times [^3\text{H}]$ Uridine incorporation (c.p.m./ 10^6 cells)	
	Control	Hydroxyurea-treated
2	7.01 $\pm$ 0.20	5.40 $\pm$ 0.24
4	4.82 $\pm$ 0.14	5.82 $\pm$ 0.38
6	7.05 $\pm$ 0.24	9.34 $\pm$ 0.06
8	10.97 $\pm$ 1.23	13.48 $\pm$ 0.27

Table 2. Effect of 2 mM-hydroxyurea on protein-synthesis rates in cells traversing G_1 into S phase

Chinese-hamster ovary cells were selected in mitosis and then incubated at the indicated times with [3 H]leucine. Acid-insoluble radioactivity was determined as described in the Experimental section. Values represent the means of duplicate determinations $\pm$ S.E.M.

Time after mitosis (h)	$10^{-3} \times [^3\text{H}]$ Leucine incorporation (c.p.m./ 10^6 cells)	
	Control	Hydroxyurea-treated
3	10.72 $\pm$ 0.21	10.34 $\pm$ 1.10
5	10.22 $\pm$ 0.50	12.57 $\pm$ 0.16
7	18.96 $\pm$ 0.20	15.30 $\pm$ 0.10
9	15.85 $\pm$ 1.15	16.49 $\pm$ 1.07

Table 3. Effect of hydroxyurea on ornithine decarboxylase activity *in vitro*

Various concentrations of hydroxyurea were added to the enzyme reaction mixture directly and ornithine decarboxylase activity was measured. Values represent the means of triplicate determinations $\pm$ S.E.M.

Concn. of hydroxyurea (mM)	Ornithine decarboxylase activity (pmol of $^{14}\text{CO}_2$ released/h per 10^6 cells)
0	133.7 $\pm$ 1.0
0.001	141.3 $\pm$ 0.7
0.01	149.0 $\pm$ 0.9
1.0	145.0 $\pm$ 1.9
2.0	146.7 $\pm$ 1.2

Fig. 4. Effect of various concentrations of spermidine and hydroxyurea

Ornithine decarboxylase activity is measured in asynchronous Chinese-hamster ovary cells treated for 90 min with different concentrations of hydroxyurea (●) or spermidine (○).

decrease in ornithine decarboxylase activity is due simply to the structure of hydroxyurea, then the inactivation of ornithine decarboxylase with the same concentrations of hydroxyurea and thiourea should be identical. If, however, the decrease in ornithine decarboxylase is more related to ribonucleoside diphosphate reductase activity, then thiourea should have a less profound effect on ornithine decarboxylase than does hydroxyurea. Fig. 5 shows that incubation of asynchronous Chinese-hamster ovary cells with 2 mM-thiourea results in approx. 30% more ornithine decarboxylase activity than if the cells are treated with 2 mM-hydroxyurea.

Discussion

The major general implication of this work is that the identical enzyme activities expressed in different growth states may be differentially regulated. We have found that the induction of ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis, is inhibited by hydroxyurea in synchronous populations, but not in cells stimulated out of quiescence to proliferate. This finding suggests that the preparation phase for DNA synthesis (G_1) in continuously dividing cells is biochemically different from the G_1 phase of cells stimulated out of quiescence (G_0) with respect to the regulation of this enzyme induction. In addition, these data suggest that the difference in ornithine decarboxylase activity regulation involves a deoxyribonucleoside triphosphate-dependent step in continuously dividing cells, but not in quiescent cells stimulated to proliferate. Our results indicate that the regulation of

the induction of ornithine decarboxylase, which occurs in G_0 as well as G_1 phase, is dependent on the growth state, since there is a differential sensitivity to hydroxyurea, depending on whether the cells are stimulated toward DNA synthesis from quiescence or from mitosis. This implies that the regulation of an event apparently common to G_0 and G_1 phases is in fact biochemically different. Others have shown a biochemical difference in the cell-cycle phases by the apparent preferential synthesis of a particular component such as actin during the transit from G_0 to S phase (Riddle *et al.*, 1979).

A second, more specific, implication is that either separate induction mechanisms or different forms of ornithine decarboxylase exist in mammalian cells. If different forms of ornithine decarboxylase exist, then the suggested dependence on deoxyribonucleoside triphosphates may be important for differential purification. In order to elucidate the possible differences in the enzyme activities, it became important to identify how hydroxyurea could possibly inhibit the induction of ornithine decarboxylase in continuously dividing cells. Our experiments show that hydroxyurea has no direct effect on the enzyme activity (Table 3), is not cytotoxic (Cress & Gerner, 1979), and has no effect on general RNA- and protein-synthesis rates (Tables 1 and 2). In addition, we have studied the effects of hydroxyurea on the cyclic-AMP-dependent protein kinase ratio (the ratio of enzyme activity measured *in vitro* in the absence and presence of saturating concentrations of cyclic AMP). This activity ratio, which is another cell-cycle event generally increasing in late G_1 phase (Costa *et al.*, 1976), is not impaired in the presence of hydroxyurea (results not shown).

Another consideration was whether hydroxyurea could possibly be stabilizing the biological half-life of ornithine decarboxylase in plateau-released cells. Alternatively, hydroxyurea could be interfering with translation and preventing induction of ornithine decarboxylase at the G_1 /S-phase boundary. In Fig. 3(a) the half-life of the enzyme was measured by using puromycin in the presence of hydroxyurea at 5 h after mitosis. Approximately the same half-life of ornithine decarboxylase is observed (Fig. 3b) for plateau-released cells. These data show that normal translation occurs in the presence of hydroxyurea and suggests that in plateau-released cells hydroxyurea is not simply stabilizing the half-life of the enzyme.

One well-known mechanism of inhibiting ornithine decarboxylase activity is through the action of the antizyme, whose production is dependent on protein synthesis and is stimulated by exogenous diamines (Heller *et al.*, 1976; Fong *et al.*, 1976). The possibility existed that the G_0 -phase cells were deficient in production of the ornithine decarboxylase antizyme. However, our data show that

Fig. 5. Effect of thiourea and hydroxyurea on ornithine decarboxylase activity

This Figure illustrates ornithine decarboxylase activity in asynchronous Chinese-hamster ovary cells treated with either 2 mM-thiourea (○) or 2 mM-hydroxyurea (●) for up to 6 h.

hydroxyurea does not resemble the inactivation of ornithine decarboxylase that can be observed when cells are treated with exogenous spermidine (Fig. 4). In addition, we have shown that inhibition of the induction can occur within 1 h (Cress & Gerner, 1979), and Fig. 2 shows that hydroxyurea added after the induction has no effect on the enzyme activity. We conclude from these data that hydroxyurea does not act as a diamine in preventing the ornithine decarboxylase induction normally seen as cells progress from mitosis toward DNA synthesis.

A major consequence of hydroxyurea is to limit primarily dATP and dCTP pools through the inhibition of ribonucleoside diphosphate reductase (Lewis & Wright, 1974; Walters *et al.*, 1973; Bjursell & Reichard, 1973). We suspected that the initiation of the induction of ornithine decarboxylase at the G₁/S-phase boundary is dependent on a full complement of DNA purine and pyrimidine nucleoside triphosphates, since the induction is sensitive to hydroxyurea. Therefore we used thiourea to test whether a similar inactivation of ornithine decarboxylase could be observed with an agent other than hydroxyurea. Thiourea is known to inhibit ribonucleoside diphosphate reductase but to a lesser extent than does hydroxyurea. In the presence of 2 mM-thiourea, 25% of the total DNA is able to replicate, whereas only 1–5% of the DNA can be synthesized in the presence of 2 mM-hydroxyurea (Timson, 1975). In Fig. 5, we show that thiourea will also decrease ornithine decarboxylase activity similarly to the inactivation seen with hydroxyurea. This strengthens our view that ornithine decarboxylase induction at the G₁/S-phase boundary is probably dependent on deoxyribonucleoside triphosphates. In addition, we have previously shown that the effect of hydroxyurea on ornithine decarboxylase and DNA synthesis is concentration-dependent. Another minor consequence of hydroxyurea is to chelate Fe²⁺ ions (Moore, 1969). However, attempts to add back Fe²⁺ by several methods and rescue ornithine decarboxylase activity have not been completely successful (results not shown).

The results in the present paper suggested to us that ornithine decarboxylase induction in G₀-phase cells is regulated differently from the induction seen in G₁ phase, since an apparent differential requirement exists for deoxyribonucleotide synthesis. This difference in regulation may reflect a difference in the synthesis of the enzyme or in the expression of

the enzyme activity itself. Our present working hypothesis is that two different forms of ornithine decarboxylase exist, depending on whether the cells are continuously dividing or stimulated out of plateau. The ornithine decarboxylase activity at the G₁/S-phase boundary probably requires deoxyribonucleotides or free transition metals, since these are the factors affected by hydroxyurea.

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