BIPHASIC INDUCTION OF ORNITHINE DECARBOXYLASE AND PUTRESCINE LEVELS IN GROWING HTC CELLS

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SUMMARY

A biphasic induction of ornithine decarboxylase with concomitant increase of intracellular putrescine was seen in growing rat hepatoma cells during each generation period. In non-growing HTC cells no coordinate accumulation of intracellular putrescine followed the unique induction of ODC by dilution into fresh serum-deprived medium. The data together suggest that the biphasic increases of ODC activity occur just before and after DNA synthesis and that a growing HTC cell has a finely regulated cycle of ODC activity. Finally, ODC activity may not always correlate with the intracellular putrescine levels.

INTRODUCTION

The polyamines, putrescine, spermidine and spermine, have been linked to many biochemical processes crucial for cell growth, division, and differentiation (1). However as yet no definitive evidence has been reported as to the precise role that the polyamines play in the early events (e.g. protein, DNA and RNA synthesis) leading to cell division which have been referred to as the pleiotypic response (2). The most extensively used systems are the regenerating rat liver (3,4) and lymphocytes in culture (5,6) where the regulation of ornithine decarboxylase (ODC), the putrescine biosynthetic enzyme and also the rate limiting enzyme in polyamine biosynthesis, have been studied. Interestingly it was noted that during the regeneration of rat liver, as well as in several dividing cell culture systems, there are biphasic increases of ODC activity (4,7,8,9,10). However several other systems (e.g. intact rat liver) are known where several different environmental stimuli such as hor-

mones (11), can trigger a rise in ODC activity which may or may not lead to cell division.

These facts have not given a clear and unambiguous role for the polyamines in the control of cell growth and led us to study more closely the relationship between the polyamines, their biosynthetic enzymes, and cell division, using as our model system the previously described cell line of rat hepatoma cells (HTC cells).

MATERIALS AND METHODS

HTC cells with a doubling time of approximately 24 hours were grown to a high density (about $9x10^5$ ml^{-1}) in spinner culture as previously described (12). Cells were collected by centrifugation, washed three times with phosphate-buffered saline, and diluted with fresh growth medium (10 % calf serum in Swim's 77 medium supplemented with 50 mM Tricine, 2 gm/litre glucose, and 2mM glutamine) to a final concentration of about $1.2x10^5$ ml^{-1}. Ornithine decarboxylase was analyzed by measuring (^{14}C)CO_2 released from (^{14}C)DL-ornithine in cell extracts. Aliquots (approximately $1.5x10^7$ to $3x10^7$ cells) were collected by centrifugation, washed twice at 4°C with 5 ml phosphate-buffered-saline, disrupted by sonication for 30 seconds in 1 ml 0.1 mM EDTA, 5 mM DTT and 50 mM Tris-HCl (pH 7.2) and the sonicate centrifuged at 100,000xg for 1hour. 200 µl of sonicate was incubated with an assay mixture containing 5 mM DTT and 50 mM Tris-HCl as above with 0.2 mM L-ornithine, 0.1 mM pyridoxal phosphate and 0.4 µ Ci (^{14}C)DL-ornithine (58mCi/m mol, Amersham) in a final volume of 1 ml. (^{14}C)CO_2 release was measured after one hour of incubation at 37°C essentially as described (13). Results are expressed in nmol CO_2 released per hour per mg cell protein, determined on aliquots of cell sonicate. Protein was measured by the method of Lowry et al (14).

Putrescine was analyzed by automated ion exchange chromatography in cell extracts. Aliquots (approximately $6x10^7$ to $12x10^7$ cells) were collected as above, and the polyamines were extracted in 2 ml of 10 % CCl_3COOH. The protein was precipitated at 4°C and putrescine was assayed in the supernatant fluid by the method of Tabor et al (15) on a Beckman multichrome amino acid analyzer. Results are expressed in nmol putrescine per mg. cell weight.

To measure (^3H)thymidine incorporation into HTC cells for 24 hours, HTC cells were collected and diluted with fresh growth medium with and without 10 % calf serum as described above. Aliquots of 5 ml ($6.0x10^5$ to $12.0x10^5$ cells) were incubated for one half hour in the presence of 5 µC (^3H)thymidine per ml. The

cells were washed three times at 4°C with phosphate-buffered saline and dissolved in 1 ml of 0.1 % sodium dodecyl sulfate. 100 µl of the samples were dried on Whatman glass filters and the filters placed in 10 % CCl_3COOH at 4°C for 1 hour. Each filter was then washed five times in 0.1 N HCl and three times in 95 % ethyl alcohol at 4°C. The filters were dried and the radioactivities determined by scintillation counting. (^{3}H)thymidine incorporation is expressed as c.p.m. incorporated $x10^{-3}$ per 10^5 cells.

RESULTS AND DISCUSSION

HTC cells in resting phase were diluted with fresh growth medium (Swim's S-77 + 10 % calf serum) to a density of not more than $1.2x10^5$ ml^{-1}. It has been shown that diluting HTC cells with fresh medium thereby triggering cell growth, results in an early and dramatic rise of ODC activity (16,17).

In our studies ODC activities were first followed through two generations of HTC cells (48 hours). Generations were measured by cell counting. Two distinct peaks of ODC activity were noted during the first 24 hours with the maximum activities found at 5-7 hours and 14-15 hours (Fig. 1a). The second peak has not been previously observed (17). Putrescine was measured from cell extracts taken at the same time points and was found to correlate with rises in ODC activities (Fig. 1c). The same pattern of ODC activity was also seen during the second generation period (24-48 hours).

To try to relate these biphasic increases of ODC activity to cell division, ODC was measured in diluted HTC cells but in the absence of serum (Swim's S-77 + 0.5 % bovine serum albumin). In these conditions the cells survive for at least three days but do not grow or divide. One broad peak of ODC activity similar to the one seen in previous studies after stimulation by amino acids (16) was found over the first 24 hour period (Fig. 1b-1). Putrescine measured in these cells at the same time points showed no net increase above the basal level (Fig. 1c). Also in contrast to what was observed in growing HTC cells, there was no significant peak of ODC activity during the second period, from 24-48 hours (Fig. 1b-2). This result suggests that in growing HTC cells biphasic increases of ODC activity do not simply result from stimulation by amino acids and glucose.

An experiment was done to confirm that the biphasic response of ODC activity in the growing cells was a direct consequence of growth factors in the serum. HTC cells were deprived of serum for 24 hours and then divided into two parts ;

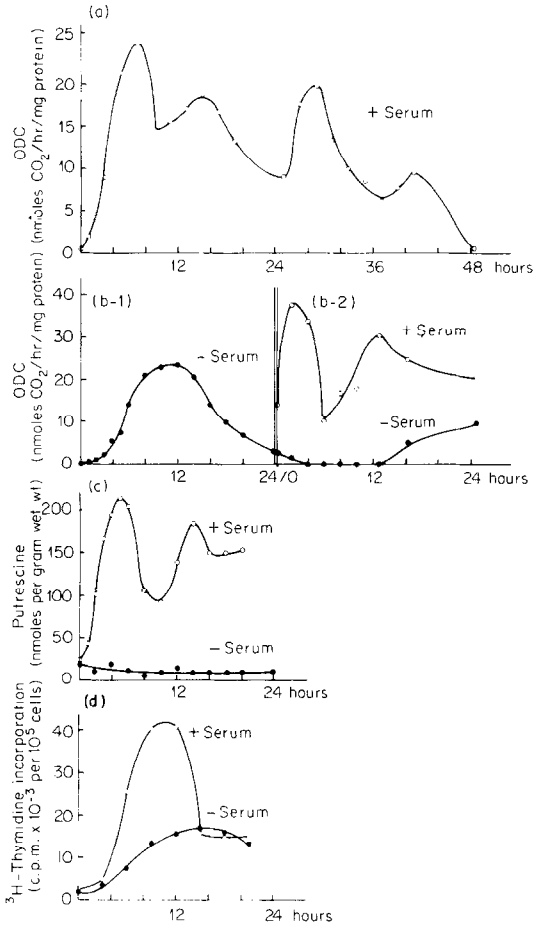

Figure 1a Activity of ornithine decarboxylase in HTC cells grown for 24 and 48 hours in the presence of serum as described in Materials and Methods.

1b 1. Activity of ODC in HTC cells diluted with fresh medium containing 0.5 % bovine serum albumin, otherwise methods are as above.

2. HTC cells were suspended in fresh medium without serum as above and after 24 hours one half of the cells received dialyzed calf serum (final concentration 10 %) -o-o- while the other half served as a control -●-●-. Aliquots of cells were collected and ODC measured as above.

1c Levels of putrescine in HTC cells in the presence and absence of serum measured as described in Materials and Methods. -o-o- cells with 10 % calf serum, -●-●- cells without calf serum in 0.5 % BSA.

1d Incorporation of [^{3}H] thymidine into HTC cells during a 24 period as described in Materials and Methods.

dialyzed calf serum (final concentration 10 %) was added back to one part, while the other part was used as a control with no addition of serum. ODC was then measured again over a 24 hour period. There was an immediate burst of ODC activity in the serum stimulated cells (Fig. 1b-2) and the same typical biphasic pattern as reported above, was observed. The cells without added serum showed no specific burst of ODC activity during the same period. Therefore it would seem that the one broad peak of ODC activity seen in non-growing cells after dilution into medium without serum is a response of the cells to amino acids and glucose, while growing HTC cells would always seem to have a biphasic induction of ODC activity per cell generation time. The non accumulation of intra-cellular putrescine in non growing cells (Fig. 1c) indicates that an increase in ODC activity in a non-growing cell may not necessarily be synonymous with an increase in putrescine. This is an important observation and suggests for example that the regulation of putrescine biosynthesis in HTC cells might also depend on substrate availability.

Incorporation of (^{3}H)thymidine into DNA was measured in growing and non-growing HTC cells over a 24 hour period in order to estimate the degree of synchrony in the growing cells after dilution into fresh medium. Maximal incorporation into DNA was at 10-11 hours (Fig. 1d). Previous studies of (^{3}H)thymidine incorporation into colcemid synchronized HTC cells (18) showed maximal incorporation at 18 hours.

The HTC cell S phase has been estimated to be nine hours, G_1 ten hours, G_2 four hours, and mitosis one hour (14). Therefore this would suggest that before dilution our stationary cells were primarily blocked somewhere in the G_1 phase which seems to be a general phenomena for quiescent mammalian cells (19). Thus, the two bursts of ODC activity came in late G_1 and G_2, just before and after the S phase of DNA synthesis. This essentially agrees with studies done with synchronized Don C cells (8).

Our data indicate that while ODC activity can be induced in non-growing cells, only growing and dividing HTC cells show biphasic induction and corresponding increased levels of putrescine per generation time. This suggests that ODC activity may not necessarily always be indicative of the intracellular situation with regard to putrescine concentration, and secondly that there is a finely modulated regulation of ODC activity in coordination with the putrescine levels during cell division.

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