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A HUMAN NEUROBLASTOMA CELL LINE WITH A STABLE ORNITHINE DECARBOXYLASE IN VIVO AND IN VITRO

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A human neuroblastoma cell line (Paju) grew in 10 mM difluoromethylornithine, which at this concentration normally stops the growth of all mammalian cells. Ornithine decarboxylase from Paju was resistant to inhibition in vitro by difluoromethylornithine, and required 10 uM of the compound for 50% inhibition, whereas ornithine decarboxylase from SH-SY5Y cells (another human neuroblastoma) and from rat liver needed only 0.5 uM difluoromethylornithine. Paju ornithine decarboxylase also exhibited a long half-life (over eight hours) in vivo. The half-life of immunoreactive protein was significantly longer than that of the activity. The long half-life of ornithine decarboxylase in Paju cells leads to its accumulation to a specific activity of 2000 nmol/mg of protein per 30 min during rapid growth (the corresponding activity in SH-SY5Y cells was about 2.5). When partially purified ornithine decarboxylase from Paju cells was incubated with rat liver microsomes it was inactivated with a half-life of 75 min. This inactivation was accompanied by a fall in the amount of immunoreactive protein. In the same inactivating system partially purified SH-SY5Y ornithine decarboxylase had a half-life of 38 min and its half-life in vivo was 50 min. The corresponding values for rat liver ornithine decarboxylase were 45 min and 40 min, respectively. Rat liver microsomes also inactivated rat liver adenosylmethionine decarboxylase. These results suggest that Paju ornithine decarboxylase has an altered molecular conformation, rendering it resistant to (i) difluoromethylornithine and (ii) proteolytic degradation both in vivo and in vitro.

Ornithine decarboxylase (ODC; L-ornithine-carboxylyase; EC 4.1.1.17) is the first enzyme in the biosynthetic pathway of polyamines in eukaryotes (1-3). This enzyme has been intensively studied because in mammalian cells it has the shortest half-life reported for any enzyme (4). The reason for the rapid decay of ODC is unknown, but it may be an important regulatory mechanism, because ODC activity in mammalian cells seems to be modulated mainly by changing the amount of the enzyme

Abbreviations used: ODC; Ornithine decarboxylase, ADC; Adenosylmethionine decarboxylase, DFMO; Difluoromethylornithine, $T_{1/2}$, Half-life

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present (2,3,5-7). Thus, the most important factor operating to increase ODC-activity when required may act by increasing its rate of synthesis, or decreasing its rate of degradation, or both (5-10).

In this paper we report a form of ODC which is very insensitive to difluoromethylornithine (DFMO; an enzyme-activated irreversible inhibitor of ODC; 11), and which exhibits a greatly reduced degradation rate both in vitro and in vivo leading to the elevation of the enzyme activity within the cell to a specific activity higher than any previously reported.

MATERIALS AND METHODS

Chemicals: L-(1-^{14}C)Ornithine (62 mCi/mmol) and S-Adenosyl-L-(carboxy-^{14}C) methionine (62 mCi/mmol) were purchased from Amersham (Buck., U.K.). DFMO (RMI 71781) was a generous gift from Merrel-Dow Research Center (Cincinnati , OH., U.S.A.) . All other biochemicals were from Sigma Chemical Co.,(St. Louis, MO., U.S.A.).

Neuroblastoma cell lines: The Paju cell line was established from the pleural fluid of a sixteen year old girl who had a wide spread metastatic tumor. She received several cytostatic drugs, which did not inhibit the progress of the tumor. Cells have now been cultivated for eleven months and they grow mainly in suspension cultures with a few adherent cells. The addition of retinoic acid and of neural growth factor causes a differentation characterized by surface adherence and the acquisition of ganglion cell morphology with slender dendritic processes. This differentiation and other cytobiological properties will be reported in detail elsewhere. Paju cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum. SH-SY5Y neuroblastoma cell line was kindly provided by Dr. S. Påhlman (Department of Pathology, University of Uppsala, Sweden) and it was cultivated as described in (12).

Animals and their treatments: Rats of Spraque-Dawley strain (weighing 150 g) were used in this study. Partial hepatectomy was performed under light ether anaesthesia by the method of Higgins and Anderson (13).Thioacetamide was injected intraperitoneally to induce ODC, as in (14).

ODC-purifications: Paju ODC was obtained from 5.8 g of cells (wet weight) grown in a volume of 6.2 l of RPMI medium. ODC was purified as described earlier (15) and the preparation used in this study was from the DEAE-cellulose step. ODC isolated from SH-SY5Y neuroblastoma cells (650 mg wet weight grown in 1.5 l of medium) and from rat liver after thioacetamide treatment was partially purified as in (15)(through the ammonium sulphate fractionation) and used for inactivation studies in vitro.

Inactivation of ODCs by microsomal suspension: A microsomal inactivation system from rat liver was prepared as described earlier (16). The microsomal inactivating system and partially purified Paju-ODC, SH-SY5Y-ODC and rat liver ODC or rat liver adenosylmethionine decarboxylase (ADC; EC 4.1.1.50) were incubated together in a total volume of 250 ul for times shown in the figures. The radioactive substrates were then added to initiate the enzyme assays. The control enzyme activities were determined from incubations in the absence of the microsomal inactivating system.

Immunotitrations: ODC from the kidneys of 150 mice was purified as described earlier (15). Antiserum against kidney ODC was produced in rabbits as in (6,7) and this antibody was used for immunotitrations which were performed as in (17).

Analytical methods: The activities of ODC (15) and ADC (18) were assayed by published methods. Protein was determined by the method of Bradford using bovine serum albumin as standard (19).

RESULTS AND DISCUSSION

Paju cells continued to grow in 10 mM DFMO, which is usually enough to stop the growth of all mammalian cells (20). Table 1 shows that partially purified ODC from Paju cells was very resistant to DFMO, because 10 uM DFMO was needed to cause 50% inactivation whereas only 0.5 uM DFMO was needed for SH-SY5Y and rat liver ODC, as shown earlier for the liver enzyme (5,6,10). An other peculiar feature of Paju-ODC was its high intracellular level (up to 2000 nmol/30 min per mg of protein) during growth of the cells. This is the highest intracellular level yet reported for a mammalian ODC (3,5,15,20). Fig. 1 shows a possible cause of the high level of ODC in Paju cells: after cycloheximide treatment ODC activity had a half-life of 8 h in Paju cells but only 50 min in SH-SY5H cells and 45 min in regenerating rat liver. Fig 1 shows that the decay of immunoreactive protein was only a little slower than that of catalytic activity, consistent with the idea that inactivation is followed by a more complete degradation of ODC (21).

The stability of Paju-ODC would be expected to lead to the accumulation of large amounts of active enzyme in the cells, when the rate of synthesis is increased indicating that a decreased rate of ODC-degradation can be an important way to increase ODC activity during rapid growth.

Table 1

Effect of DFMO on the inactivation of Paju, SH-SY5Y and rat liver ODCs

DFMO-concentration (uM)	% ODC-activity remainig after 60 min		
	Rat liver ODC	Paju-ODC	SH-SY5Y-ODC
0	100	100	100
0.2	78	95	75
0.5	47	91	51
1.0	23	76	28
2.0	7	71	5
5.0	3	66	2
10.0	2	51	2
20.0	2	12	2

The ODCs were partially purified as described in Materials and Methods and then treated with different concentrations of DFMO as described earlier (15).

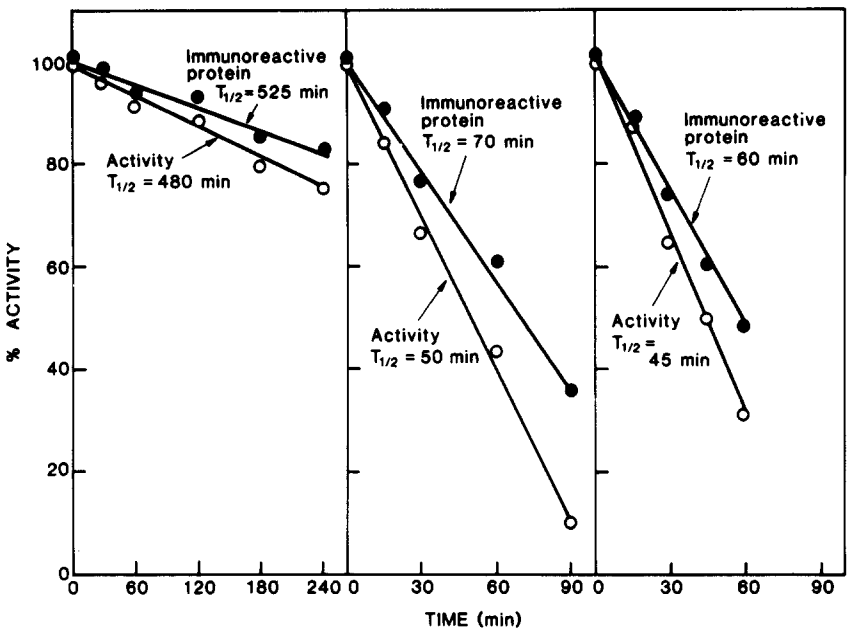

Figure 1: Decay of ODC-activity and immunoreactive protein in (A) Paju cells, (B) SH-SY5Y cells and (C) regenerating rat liver following administration of cycloheximide (A and B 20 ug/ml; C 1.5 mg/100 g of body weight). Neuroblastoma cells were grown to maximum density in suspension culture and then diluted to $5x10^5$ cells/ml 18 h before the addition of cycloheximide at zero time. At the indicated times samples ($5x10^6$ cells) were taken for the determination of ODC-activity and immunoreactive protein. The specific activity of Paju-ODC at the start of the experiment was 1200 nmol/mg of protein per 30 min and that of SH-SY5Y-ODC 1.5 nmol/mg of protein per 30 min. Rats were partially hepatectomized and 24 h later were injected intraperitoneally with cycloheximide at zero time. The initial specific activity of ODC in the regenerating livers was 0.8 nmol/mg of protein per 30 min. (○) ODC-activity and (●) Immunoreactive protein. First order plots were fitted to the data by the least-squares method.

Since this is the first mammalian ODC reported to be either insensitive to DFMO (Table 1) or stabilized against intracellular degradation (Fig. 1), it seemed possible that it might have an altered structure, which makes it resistant both to DFMO and to intracellular degradation. To study this idea further the inactivation *in vitro* of ODC from rat liver and Paju and SH-SY5Y cells was investigated by using a recently described microsomal system (16,22,23). Fig. 2 shows that partially purified Paju-ODC (apparent half-life about 75 min) was inactivated by rat liver microsomes more slowly than either rat liver ODC ($T_{1/2}$ = 40 min) or SH-SY5Y ODC ($T_{1/2}$ = 38 min). The amounts of immunoreactive protein decreased, rather more slowly than did the enzyme activities, resembling the situation *in vivo* (Fig. 1).

Fig. 3 shows that rat liver ADC (which also has a short half-life *in vivo* 5,24) could also be inactivated *in vitro* by this microsomal

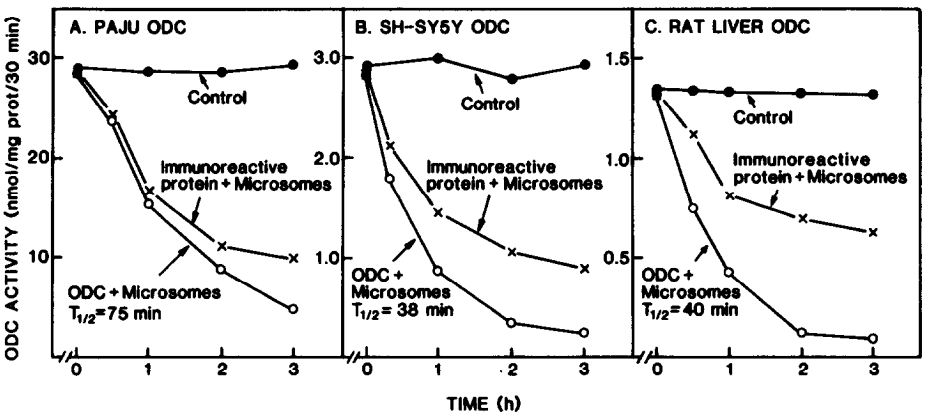

Figure 2: Inactivation by rat liver microsomes of ODCs from rat liver or Paju or SH-SY5Y cells. ODCs were partially purified and then incubated with rat liver microsomes as described in Materials and Methods. After incubation times shown in the Figure the remaining ODC-activity or immunoreactive protein was determined as described in Materials and Methods. (●) Activity without microsomes; (○) Activity with microsomes; (×) Immunoreactive protein with microsomes.

inactivating system from rat liver. This suggests that several rapidly decaying proteins, perhaps with a common structural feature (possibly similar to the methionine-rich region shown for a pool of rat liver non-lysosomal degraded protein; 25), are recognized by the degradation mechanism *in vivo* and *in vitro*.

The physiological significance of the microsomal ODC-inactivating system is an open question, as discussed earlier (16). However, our present results show that an ODC with a long half-life *in vivo* also has

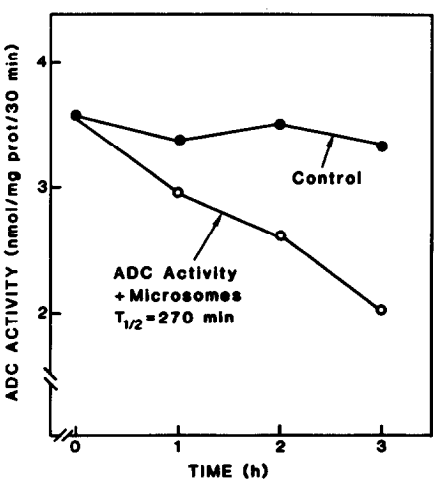

Figure 3: Inactivation of rat liver ADC by rat liver microsomes. ADC used was obtained from 100000$_g$ supernatant of rat liver homogenate. Other details as in Figure 2. (●) Activity without microsomes and (○) Activity with microsomes.

a long half-life in vitro. The decay of ODC-activity proceeds much faster in vitro than in vivo, which suggests that there may be some stabilizing factor(s) in the cell cytosol. Our results also suggest that the structure of Paju-ODC may differ from that of most mammalian ODCs in a way which makes it (i) resistant to DFMO; (ii) resistant to the rapid intracellular degradation that normally affects mammalian ODCs and (iii) resistant to the microsomal inactivating system.

Paju-ODC offers an interesting model for the study of the molecular mechanism(s) involved in the degradation of short-lived proteins. Experiments are in progress to investigate in detail the putative change(s) in the molecular structure of Paju-ODC responsible for the striking stability of the enzyme.

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