Multiple regulation of ornithine decarboxylase in enzyme-overproducing cells

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We have isolated from mouse FM3A cells a variant cell line, termed EXOD-1, that overproduces ornithine decarboxylase (ODC). The cells were resistant to α -difluoromethylornithine, an irreversible inhibitor of the enzyme, and produced the enzyme protein to the extent of approx. 3–6% of total cytosolic protein. The rate of ODC synthesis in this cell line accounted for 25–50% of the rate of total protein synthesis. The amounts of the ODC gene and its mRNA in the variant cells were both about 60 times as much as those in wild-type FM3A cells. Upon removal of the inhibitor, the growth of the ODC-overproducing cells was stimulated approx. 2-fold. Under these conditions, the rate of ODC synthesis increased about 4-fold on day 1 and then decreased to near the original level by day 3. The amount of ODC mRNA increased about 1.7-fold on day 1 and 2.5-fold on day 3. No correlation was observed between changes in ODC

synthesis rate and in ODC mRNA content, suggesting a translational repression of ODC mRNA due to accumulation of polyamines. In fact, the cellular contents of putrescine and spermidine markedly increased and that of spermine inversely decreased during the same period. Pulse-chase experiments showed that the accumulation of putrescine and spermidine also elicited a rapid degradation of ODC. Excess amounts of newly synthesized putrescine and cadaverine were excreted into the medium, whereas spermidine, spermine and acetylated polyamines were undetectable there. We conclude that ODC regulation upon removal of the inhibitor is dependent on at least three steps, namely the level of mRNA, the translational efficiency of mRNA and the stability of the enzyme, the last two of which are involved in cellular polyamines.

INTRODUCTION

Polyamines, namely putrescine, spermidine and spermine, are essential for cellular growth. Polyamine biosynthesis in most eukaryotic cells is regulated by two key enzymes, ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC) [1–3]. These enzyme levels are regulated not only by various growth stimuli but also by polyamines themselves via complex mechanisms affecting gene transcription [4,5], mRNA stability [6–8], mRNA translation [9–12], enzyme degradation [13,14], enzyme modification [15–17] and processing of precursor AdoMetDC [18]. It has been reported by Kameji and Pegg that the translation of mRNAs for polyamine-synthesizing enzymes is more sensitive to polyamines than is that of other mRNAs [19,20]. Similar results were reported by Persson et al. [11]. Recently it was demonstrated that a part of the 5'-untranslated region of ODC mRNA was important for the translational regulation [21–24]. To obtain further information about the regulatory mechanisms of ODC, we recently isolated an ODC-overproducing variant cell line from mouse FM3A cells by selecting cells resistant to α -difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC. Several cell lines, which overproduced ODC due to gene amplification have been reported previously [11,25–29]. A mouse lymphoma cell mutant [26] synthesized ODC protein most abundantly among those cell lines, to an amount equivalent to about 15% of total protein synthesis. Our mutant synthesized ODC protein preferentially, corresponding to 25–50% of total protein synthesis. Using the mutant, we showed that ODC regulation in

these cells was mainly associated with alterations in mRNA levels, translational efficiency of mRNA, and the rate of enzyme degradation.

EXPERIMENTAL

Materials

DFMO was kindly provided by Dr. P. P. McCann (Marion Merrell Dow Research Institute, Cincinnati, OH, U.S.A.). Protein A was purchased from Calbiochem Corp. Multiprime DNA-labelling system was from Amersham. Deoxycytidine 5'-[³²P]triphosphate (3000 Ci/mmol) and L-[1-¹⁴C]ornithine (50–60 mCi/mmol) were from ICN Biochemicals. L-[³⁵S]-Methionine (800 Ci/mmol) was from American Radiolabeled Chemicals. The plasmid containing a mouse ODC cDNA was generously provided by Dr. C. Kahana (Weizman Institute of Science, Israel). ES medium [30] was from Nissui Pharmaceutical Co., and heat-inactivated fetal-calf serum (FCS) was from GIBCO/BRL. Other biochemical reagents were of analytical grade.

General methods

Protein was measured by the method of Lowry et al. [31], with BSA as a standard. ODC activity was assayed by using a microassay system as described previously [32]. One unit of enzyme is defined as the amount releasing 1 nmol of CO₂/h at 37 °C under the specified conditions.

Abbreviations used: DFMO, α -difluoromethylornithine; ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; FCS, fetal-calf serum.

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Isolation of ODC-overproducing FM3A cells

The FM3A cells were kindly supplied by Dr. H. Matsuzaki (Saitama University, Japan). The cells (1×10^4 /ml) were cultured in ES medium, supplemented with 50 units/ml streptomycin, 100 units/ml penicillin G and 2% heat-inactivated fetal-calf serum (FCS), for 3 days at 37 °C under an atmosphere of 5% CO₂ in air. The cells (2.5×10^5 /ml) were then treated with 0.6 µg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for 2 h at 37 °C to facilitate the ODC gene amplification [33]. After MNNG was removed by washing the cells with the medium, the cells (2.5×10^5 /ml) were cultured further in the above medium for 4 days. Then ODC-overproducing variant cells were enriched by incubating them with step-wise increases (1, 3, 10 and 20 mM) in DFMO in the medium for a total of 8 months. The concentration of the drug was increased as the cell growth rate became faster. Finally, an ODC-overproducing clone was obtained from the plate containing medium supplemented with 5% FCS, 20 mM DFMO and 0.5% agar.

Immunoblotting of ODC

Proteins in cell extracts were separated by SDS/PAGE on 11% acrylamide gel as described by Lugtenberg et al. [34]. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membrane was incubated with Tris-buffered saline (TBS; 20 mM Tris/HCl, pH 7.5, and 0.15 M NaCl) containing 5% skim milk and then with 5 ml of TBS containing 5% skim milk and 5 µl of anti-ODC antiserum for 1 h. After washing with TBS containing 0.05% Tween 20 (TTBS), the membrane was exposed to goat anti-rabbit IgG-alkaline phosphatase conjugate in TTBS containing 0.1% BSA for 1 h. The membrane was washed three times with TTBS, once with 0.1 M Tris/HCl (pH 9.5)/0.1 M NaCl/50 mM MgCl₂ and then developed with phosphatase substrate.

Cell extracts

Cells (2.5×10^5 /ml) cultured in ES medium with or without 20 mM DFMO were harvested by low-speed centrifugation and then homogenized with 0.5 ml of ice-cold 0.25 M sucrose containing 1 mM dithiothreitol by using a tight-fitting Dounce-type all-glass homogenizer. The homogenate was centrifuged at 20000 *g* for 30 min and the supernatant was used for the assays.

Determination of polyamines

Cell homogenates were treated with 2% HClO₄ and the extracts were analysed for polyamines by h.p.l.c. with a Shimadzu ISC-05/S0504 cation-exchange system as described previously [35].

Determination of the rates of ODC synthesis and degradation

ODC synthesis and degradation rates were determined by measuring the amount of [³⁵S]methionine in the enzyme. Cells grown in ES medium/2% FCS containing 20 mM DFMO were harvested by low-speed centrifugation and reseeded (2.5×10^5 /ml) in fresh medium/2% FCS without DFMO every 24 h. For measurement of the ODC synthesis rate, 50 µCi of [³⁵S]methionine was added in the presence of 100 µM unlabelled methionine (normal concentration of methionine in ES medium) and cells were incubated at 37 °C for 30 min. The cells were collected by centrifugation and cell extracts were prepared as described above. Portions of the supernatants containing 3.5 µg of protein were treated with anti-ODC antiserum at room temperature for 20 min. The incorporated [³⁵S]methionine/3.5 µg

of protein in each sample did not change significantly. Immunoprecipitated ODC was analysed by SDS/PAGE on 11% acrylamide gel. The band of ODC was detected by fluorography and quantified by densitometry. For measurement of the ODC degradation rate, cells labelled with [³⁵S]methionine for 30 min were collected by centrifugation, washed quickly with the same medium supplemented with 10 mM L-methionine and cultured in that medium. Cells were harvested at given intervals, and radioactivity in immunoreactive ODC protein was determined as described above.

Determination of ODC mRNA contents

Total RNA was extracted from cells by the guanidinium/hot-phenol method [36]. ODC mRNA contents were then analysed by Northern-blot analysis [37] using a ³²P-labelled cDNA probe prepared as described previously [10]. The relative amounts of ODC mRNA were quantified by scanning with a densitometer.

Analysis of genomic DNA

Mouse genomic DNA was extracted from wild-type and DFMO-resistant cells and digested with *Pst*I restriction endonuclease at 37 °C overnight. DNA fragments were fractionated on 1% agar, transferred to GeneScreen and hybridized with a ³²P-labelled cDNA probe.

RESULTS

Selection of ODC-overproducing cells

We used an irreversible inhibitor of ODC, DFMO, for the selection of variant cells overproducing ODC. FM3A cells treated with MNNG were propagated in increasing concentrations of DFMO. Figure 1 shows that a cell line, termed EXOD-1, had adapted to grow in high concentrations of the inhibitor. Growth of wild-type cells was inhibited by 50% by 6 µM DFMO, whereas 10 mM DFMO was required to produce comparable growth inhibition in the DFMO-resistant cells.

Characterization of ODC-overproducing cells

An extract from the DFMO-resistant EXOD-1 cells cultured in the presence of 20 mM DFMO was directly fractionated by

Figure 1 Relative growth of the parental and variant cells in the presence of various concentrations of DFMO

Cells (5×10^3 /ml) were cultured in ES medium containing various concentrations of DFMO; 3 days later the cell number of each culture was counted. The number of cells grown in ES medium in the absence of DFMO was expressed as 100%. Doubling times of the parental and DFMO-resistant cells were both 12 h. ○, Parental cells; ●, DFMO-resistant cells.

Figure 2 Synthesis of ODC in wild-type and DFMO-resistant cells

Cells were incubated for 30 min in 5 ml of ES medium/2% FCS supplemented with 50 μ Ci of [35 S]methionine. Proteins (3.5 μ g) labelled with [35 S]methionine were analysed by SDS/PAGE followed by fluorography. (a) Immunoprecipitate of cytosol with anti-ODC antiserum. Wild-type cells were cultured for 1 day with (lane 2) or without (lane 1) 0.05 mM DFMO. Resistant cells were cultured for 1 day with (lane 4) or without (lane 3) 20 mM DFMO. (b) Total cytosolic protein synthesized in DFMO-resistant cells cultured for 1 day with DFMO. Arrows indicate ODC protein. Molecular-size markers are shown to the left.

Figure 3 Southern (a) and Northern (b) blot analysis of the ODC gene and its mRNA

(a) Total genomic DNA (12 μ g) was digested with *Pst*I, fractionated on 1.0% agarose gel and then hybridized to a radioactive probe after transfer to GeneScreen. Lane 1, wild-type cells cultured for 1 day with 0.5 mM DFMO; lane 2, resistant cells cultured for 1 day with 20 mM DFMO. (b) Total RNA (1.4 μ g) from wild-type (lane 1) and resistant (lane 2) cells was subjected to electrophoresis on 2% agarose gel and hybridized to a radioactive probe after transfer to nitrocellulose filter. Arrows indicate the position of ODC gene digested by *Pst*I (a) and ODC mRNA (b).

SDS/PAGE, and the proteins were stained with Coomassie Blue. The band of ODC protein in the resistant cells was visible, corresponding to approx. 3–6% of the total cytosolic proteins by densitometric analysis (results not shown). In parental cells the band of ODC protein was undetectable, in spite of the application of 30 times as much cytosolic protein. Since parental cells contained about 6 ng of ODC protein/mg of total cytosolic protein when estimated by Western-blot analysis, the ODC

Figure 4 ODC activity and the content of ODC protein during the growth of resistant cells in the presence or absence of 20 mM DFMO

Cells cultured with 20 mM DFMO (day 0) were transferred to fresh ES medium/2% FCS without DFMO. Thereafter 20 mM DFMO was added to the resistant cell cultures on day 3. At various time points, cells were harvested and used for determination of ODC activity (●) and Western-blot analysis of ODC protein (○). The amount of ODC protein was expressed as a relative value compared with the value at day 0. Values are mean $\pm$ S.E.M. ($n = 3$). Horizontal bar indicates the period of culture without DFMO.

content in the resistant cells was roughly 5000 times that in parental cells. The synthesis rate of ODC was determined in the DFMO-resistant cells and wild-type cells by labelling for 30 min with [35 S]methionine. As shown in Figure 2(a), the intensity of the band of labelled ODC was far greater in the resistant cells than in the parental cells when the same amount (3.5 μ g) of protein was treated with anti-ODC antiserum. Densitometric analysis of the fluorogram showed that ODC synthesis accounted for 25–50% of the total protein synthesis in the resistant cells cultured with DFMO (Figure 2b). The relative rate of ODC synthesis in the resistant cells was greater in the cells cultured for 1 day in the absence of DFMO than in the presence of 20 mM DFMO (Figure 2a, lane 3 versus lane 4).

Judging from the overproduction of the enzyme in the resistant cells, we presumed that these cells had amplified ODC genes associated with a high level of ODC mRNA. We examined ODC mRNA and its genomic DNA contents in both wild-type and resistant cells. Restriction-endonuclease analysis of genomic DNA exhibited a similar hybridization pattern to that described by Radford et al. [38]. When DNA from the resistant cells was compared with that from the parental cells, the intensity of the 1.8 kb *Pst*I fragment increased at least 60-fold in the resistant cells compared with the parental cells (Figure 3a). Northern-blot analysis of ODC mRNA from both parental and resistant cells showed a single predominant ODC mRNA of about 2.2 kb. Densitometric scanning of the ODC mRNA band indicated that approx. 60-fold more ODC mRNA was present in the resistant cells than in the wild-type cells (Figure 3b).

The enzyme was shown by several criteria to be normal in every respect: the specific activity of purified enzyme (1.5×10^6 units/mg of protein), the degree of inhibition by antizyme [39] and DFMO, the optimal pH and substrate specificity of enzyme, the acceleration of the enzyme degradation by exogenous polyamines (results not shown).

Changes in ODC synthesis rate and polyamine contents in the resistant cells upon removal and re-addition of DFMO

The resistant cells exposed chronically to DFMO exhibited about 30 times higher ODC activity than the parental cells when

Figure 5 Changes in the relative rate of ODC synthesis and the amount of ODC mRNA in DFMO-resistant cells

Cells were treated as described in Figure 4. For determination of the rate of ODC synthesis (○), labelling of ODC with [³⁵S]methionine was carried out for 30 min starting at the indicated times, and the radioactivity incorporated into immunoreactive ODC was determined by densitometry of the SDS/PAGE gel fluorograms. For the amount of ODC mRNA (●), total RNA was isolated at the indicated times as described in the text. The content of ODC mRNA was determined by Northern-blot analysis. Values are means ± S.E.M. (*n* = 3) and are expressed relative to that at day 0. Horizontal bar indicates the period of culture without DFMO.

the activity was measured in dialysed cell extract. Removal of the inhibitor from the medium led to a further increase in enzyme activity, about 30-fold on day 2 and 150-fold on day 3 compared with that on day 0. On the other hand, the amount of ODC protein determined by Western-blot analysis increased about 1.6-fold on day 1, and then decreased gradually thereafter (Figure 4). These results indicated that large amounts of ODC protein in the cells were made inactive by the binding of the inhibitor and that only certain portions of the newly synthesized ODC protein were active. As shown in Figure 5, the amount of ODC mRNA gradually increased to about 1.7-fold on day 1 and 2.5-fold on day 3 after removal of the inhibitor, whereas the rate of ODC

synthesis increased 4-fold on day 1 and decreased gradually thereafter. The results suggest that the stimulation of ODC synthesis was due to changes in the efficiency of ODC mRNA translation as well as in the amounts of ODC mRNA. Involvement of the accumulation of mRNA in the stimulation of ODC synthesis may be minor, since no correlation between them was observed.

The contents of intracellular putrescine and spermidine in the resistant cells were 64 % and 21 %, respectively, of those found in the parental cells (Figure 6, day 0), whereas that of spermine was slightly higher in the resistant cells than in the parental cells. Removal of the inhibitor from the medium led to a dramatic accumulation of putrescine and spermidine, owing to the increase in active ODC (Figure 6a). The accumulated putrescine and spermidine were likely to have caused the suppression of the ODC synthesis rate on days 2 and 3, in spite of continuous increase in the amount of ODC mRNA. On the contrary, spermine content decreased concomitantly with the increase of spermidine. Excess putrescine and cadaverine, an unusual diamine formed from lysine by the action of ODC, were excreted into the medium from the resistant cells (Figure 6b), whereas spermidine, spermine and acetylated polyamines were undetectable there (results not shown). The results were consistent with those of the DFMO-resistant L1210 cells reported by Pegg et al. [40].

The results shown in this section were accompanied by changes in the rate of cell growth and in polyamines. Doubling times of the DFMO-resistant cells cultured in the absence and presence of 20 mM DFMO were 12 and 26 h respectively, indicating that the cells were not totally resistant to 20 mM DFMO (see Figure 1). Similar results were obtained either when medium containing 20 mM DFMO was changed to that containing 2 mM DFMO or less, or when medium containing 7 mM DFMO was changed to that without DFMO (results not shown).

Changes in ODC degradation after removal of DFMO

The degradation rate of ODC was measured by pulse-chase experiments. In medium containing 20 mM DFMO, ODC protein

Figure 6 Time courses of polyamine contents in DFMO-resistant cells grown in the absence or presence of 20 mM DFMO

Cells were grown as described in Figure 4. Intracellular (a) and extracellular (b) polyamine contents were determined by using h.p.l.c. Each point represents the mean of duplicate determinations. Resistant-cell/wild-type-cell ratios for intracellular polyamine contents on day 0 were 0.64 (Put), 0.21 (Spd) and 1.2 (Spm). Horizontal bars indicate the period of culture without DFMO. Abbreviations: Cad, cadaverine; Put, putrescine; Spd, spermidine; Spm, spermine.

Figure 7 Effect of removal of DFMO from medium on the degradation rate of immunoreactive ODC

Cells cultured in the presence of 20 mM DFMO were transferred to medium without DFMO and cultured for 0 (a), 1 (b), 2 (c) or 3 (d) days and then labelled for 30 min with 50 μ Ci of [35 S]methionine in fresh ES medium/2% FCS. The medium was then replaced with the same medium containing 10 mM unlabelled methionine and the culture was continued. Cells were harvested at the indicated times and analysed for labelled immunoreactive ODC protein obtained by immunoprecipitation of an equal amount of soluble protein (20 μ g). Enzyme half-lives were calculated from the densitometric data obtained from the fluorogram. Values are means $\pm$ S.E.M. ($n = 3$).

was extremely stable. When cells were transferred to medium lacking the inhibitor, the half-life of the enzyme was gradually shortened from > 340 min on day 0, to 130 min on day 1, 60 min on day 2 and 25 min on day 3 (Figure 7). No ODC protein of a different molecular mass was observed under these conditions. The induction of the rapid degradation of ODC was likely to be due to the accumulation of intracellular putrescine and spermidine, since it has been reported that DFMO does not affect the stability of ODC [41], and the addition of 100 μ M spermidine and 500 μ M aminoguanidine (an inhibitor of amine oxidase) to the medium containing 20 mM DFMO caused rapid degradation of ODC (results not shown).

DISCUSSION

Polyamine biosynthesis is highly regulated in eukaryotic cells. In order to study the regulation of ODC at the molecular level, we selected mouse mammary tumour cell variants resistant to DFMO and obtained a cell line in which the content of DFMO-bound ODC protein accounted for 3–6% of the total cytosolic proteins. When grown in the presence of DFMO, spermidine content of the variant cells was about 20% of that in the parental cells grown in the absence of DFMO, whereas spermine content was slightly more in the variant cells than in the parental cells. Both growth rate and spermidine content of the variant cells increased upon transfer to the medium lacking DFMO. This suggests that the low level of spermidine was the rate-limiting factor for cellular growth.

Our cell line was the highest ODC overproducer among similar cells thus far reported [11,25–29]. The rate of ODC

synthesis assessed by labelling with [35 S]methionine for 30 min represented 25–50% of the rate for the total soluble proteins. Several mechanisms were involved in the ODC overproduction: (a) 60-fold increase in ODC mRNA content, owing to 60-fold increase in the copy number of the ODC gene; (b) stimulation of the translation of ODC mRNA, owing to decreases in cellular polyamine contents; (c) more than 14-fold increase in stability of ODC protein. Assuming that ODC content in the variant cells is 5000 times that in parental cells, the extent of translational stimulation in the variant cells was calculated to be approx. 5-fold. It remains to be clarified why our variant cells synthesized large amounts of ODC compared with those in the other DFMO-resistant cells. It may be assumed to be one of the reasons why parental cells exhibited a high ODC activity.

Replacement of the medium with fresh medium not containing DFMO induced a large rise in ODC activity and a large increase in the contents of putrescine and spermidine on day 3. This was not observed when the fresh medium contained 20 mM DFMO. Thus this was probably caused by the stimulation of cell growth by the removal of DFMO. As there is a time lag between the maximal increase in ODC synthesis seen on day 1 and the maximal ODC activity observed on day 3 after the removal of DFMO, a portion of DFMO, enough to inhibit newly synthesized ODC, may still have existed in the cells on day 1.

Previous reports from Pegg and co-workers [19,20] and Persson et al. [11] showed that, in translation systems *in vitro*, low concentrations of spermidine and/or spermine stimulated translation of ODC and AdoMetDC mRNAs, but at higher concentrations they were strongly inhibitory. Putrescine was less effective than spermidine and spermine, even when used at a 10-fold higher concentration. Recently, polyamine-responsive element(s) was found to be present in the 5'-untranslated region of ODC mRNA [23,24]. Taken together, our results suggest that after removal of the inhibitor the stimulation of ODC synthesis on day 1 was caused, at least in part, by the low level of cellular putrescine and spermidine and by the decrease in cellular spermine. The suppression of ODC synthesis after day 2 was presumably due to translational repression caused by the large accumulation of cellular spermidine.

The accumulation of putrescine and spermidine progressively induced a rapid degradation of ODC. Several lines of evidence indicate that induction of antizyme is involved in the polyamine-induced acceleration of ODC decay [39,42,43]. Many other mechanisms have also been proposed for the rapid degradation of ODC [44–48]. These include covalent modifications and interconversion of isoforms. It has also been proposed that the degradation of rapidly turning-over proteins is dependent on the intramolecular peptide region called the PEST sequence, which consists of proline, glutamic acid, serine and threonine [49]. ODC contains two PEST sequences, and the truncated form of ODC lacking the C-terminal PEST sequence was reported to be more stable than normal ODC in the absence of exogenous polyamines [50]. The determination of just how the rapid degradation of such a large amount of ODC is induced in the variant cells is awaited with considerable interest.

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