Zinc is required for the expression of ornithine decarboxylase in a difluoromethylornithine-resistant cell line

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Dilution of quiescent L1210-DFMO^r (difluoromethylornithine-resistant) cells in fresh medium containing serum led to the induction of ornithine decarboxylase (ODC) and to the expression of its mRNA, as determined by a sensitive solution-hybridization-RNAase-protection assay. Addition of the chelating agent diethylenetriaminepenta-acetic acid (DTPA) at seeding time caused an inhibition of the induction of ODC activity by up to 90 %, and only Zn²⁺ of the bivalent metal ions

tested was effective in reversing this effect. The inhibition of the induction of ODC activity was accompanied by a marked decrease, prevented by Zn²⁺ supplementation, of the accumulation of immunoreactive ODC protein and ODC mRNA. DTPA treatment also caused a slight acceleration of ODC turnover. These results indicate that a restricted Zn²⁺ availability in L1210-DFMO^r cells impairs ODC induction remarkably, mainly by affecting the expression of the messenger.

INTRODUCTION

The content and metabolism of zinc (Zn²⁺), an essential trace element for animals and humans, vary with age and in several pathological states [1–3]. Zn²⁺ deficiency limits growth in infants and children and has been regarded as the most important single factor governing birthweight. In cell cultures, Zn²⁺ has been shown to be required for DNA synthesis and proliferation of several cell types, although not of transformed cell lines [1,4].

The biochemical basis for the growth failure associated with Zn²⁺ deficiency is not clear: Zn²⁺ is known to be an essential cofactor for a variety of metalloenzymes, but alterations in the function of these enzymes do not appear to correlate with the manifestations of Zn²⁺ deficiency [5,6]. Other mechanisms have been proposed, and, in particular, a role has been hypothesized for Zn²⁺ in the regulation of transcription of specific genes which may be important for cell growth and differentiation [5,6]. The control of these genes by Zn²⁺ may be mediated by 'Zn finger' transcription factors and by 'metal regulatory elements'. Actually, the expression of specific proteins appears to be altered in Zn²⁺-deficient cells generally, but not always in a negative fashion [5–8].

Ornithine decarboxylase (ODC), the key enzyme in polyamine biosynthesis, is essential for cell proliferation and can be induced following growth stimuli [9,10]. It has been shown that ODC activity can be enhanced by Zn²⁺ supplementation in certain rat tissues or in cultured thymocytes [11,12]. In the present study we have investigated the effect of restricted Zn²⁺ availability on ODC induction and expression in L1210-DFMO^r cells [13], an ODC-overproducing cell line which has been selected for resistance to the inhibitor difluoromethylornithine (DFMO). These cells were treated with the chelating agent diethylenetriaminepenta-acetic acid (DTPA), which has been used successfully to ascertain a requirement of Zn²⁺ for growth, differentiation and gene expression of some cell types in culture [1,14,15].

EXPERIMENTAL

Materials

An *EcoRI*/*SphI* fragment of mouse ODC cDNA was subcloned from the plasmid pGEM3zf(–)ODC [16], generously provided by Professor A. E. Pegg (Pennsylvania State University, Hershey, PA, U.S.A.), into a pGEM3 plasmid (Promega, Madison, WI, U.S.A.). L1210-DFMO^r cells [13] and antibodies against mouse ODC [17] were generously given by Dr. L. Persson (University of Lund, Lund, Sweden). DFMO was kindly provided by the Merrell Dow Research Institute (Strasbourg, France). L-[1-¹⁴C]Ornithine was purchased from ICN Biomedicals (Irvine, CA, U.S.A.), DL-[3,4-³H]DFMO from du Pont–New England Nuclear (Boston, MA, U.S.A.) and [α -³²P]CTP from Amersham International (Amersham, Bucks., U.K.). SP6 and T7 RNA polymerase, T4 DNA lygase, *EcoRI*, *SphI*, *HindIII*, RNAase A, proteinase K and other molecular-biology products were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.), Gibco-BRL (Gaithersburg, MD, U.S.A.) or Promega. Fetal-calf serum was from Biological Industries (Kibbutz beth Haemek, Israel), and antibiotics and media for cell culture were from Gibco-BRL or Whittaker Bioproducts (Walkerville, MD, U.S.A.). Concanavalin A, dithiothreitol, β -mercaptoethanol and DTPA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DTPA was prepared as a 30 mM neutralized solution. L-Ornithine, pyridoxal 5'-phosphate, ZnSO₄, FeSO₄, other bivalent-metal salts and other chemicals were products of E. Merck (Darmstadt, Germany).

Cell culture and treatment

Mouse L1210-DFMO^r cells were routinely grown in the presence of 20 mM DFMO in RPMI 1640 medium containing fetal-calf serum, antibiotics and β -mercaptoethanol, as described by Persson et al. [13]. For experiments, cells were shifted to, and maintained in, a DFMO-free medium for 15–60 days. Then, 4-day-old cell cultures (cell density $\geq 2 \times 10^6$ /ml) were seeded at 2×10^5 cells/ml in fresh medium containing 10 % serum and

supplemented with DTPA, FeSO_4 , ZnSO_4 or other bivalent metal salts as indicated. Wild-type L1210 cells were utilized under the same experimental conditions. Thymocytes were isolated from rat thymus glands as previously described [12], diluted in RPMI 1640 medium as described above, but at a density of $10^7/\text{ml}$ and treated with $10 \mu\text{g}/\text{ml}$ concanavalin A for 4 h. DTPA (0.6 mM) and ZnSO_4 (0.4 mM) were added 15 min before the mitogen. At the time indicated after treatment, cells were harvested and washed with PBS.

Determination of ODC activity and immunoreactive protein content

Cell pellets were extracted with 50 mM Tris/HCl (pH 7.2)/5 mM dithiothreitol/0.1 mM EDTA/0.05 mM pyridoxal 5'-phosphate/0.5% Triton X-100. The cell lysates were centrifuged in a micro-centrifuge at 12000 *g* for 2 min and the resulting supernatants used for assay of ODC activity and immunoreactive content. As for ODC activity, 0.42 mM L-ornithine (final concn.; including 0.05 μCi of L-[1- ^{14}C]ornithine) was added to 40 μl of supernatant to a final volume of 50 μl ; after 1 h incubation at 37 °C, the release of $^{14}\text{CO}_2$ was measured [18]. ODC activity is expressed as units/mg of protein, where 1 unit corresponds to 1 nmol of CO_2/h incubation. DTPA did not affect ODC activity significantly when added directly to the assay mixture at the concentration of 0.6 mM. The content of immunoreactive ODC protein was determined by radioimmunoassay by using [^3H]DFMO-labelled ODC, as described by Seely and Pegg [19]. As for preparation of [^3H]DFMO-labelled ODC, the enzyme was partially purified from kidney of testosterone-treated mice [19], labelled with radioactive DFMO and further purified by Bio-Gel TSK DEAE-SPW chromatography (Bio-Rad, Richmond, CA, U.S.A.). This step also removed unbound [^3H]DFMO. Protein content was determined in cell extracts as described by Bradford [20].

Determination of ODC mRNA content

Total RNA was isolated from L1210-DFMO^r cells by using the acid guanidinium thiocyanate method [21]. ODC mRNA levels were determined by a solution-hybridization-RNAase-protection assay. A 750 bp *EcoRI/SphI* fragment of ODC cDNA (ranging from exon 2 to exon 8) was inserted into pGEM3, a RNA transcription vector containing the SP6 and T7 promoters on opposite sides of a multicloning region. Transcription of the plasmid linearized with *HindIII* generates a sense strand of ODC mRNA to construct a standard curve ranging from 2 to 250 pg of RNA, whereas transcription of the plasmid linearized with *EcoRI* in the presence of [^{32}P]CTP (800 Ci/nmol) gives an antisense strand (about 10^9 c.p.m./ μg) used to hybridize cellular ODC mRNA [22]. Samples were processed essentially as described by Krause et al. [23]. Briefly, total RNA extracted from L1210-DFMO^r cells (10 μg) or known amounts of *in-vitro*-synthesized sense RNA were hybridized to a ^{32}P -labelled antisense RNA probe (10^6 c.p.m.) at 55 °C for 18 h, followed by treatment with ribonuclease A to hydrolyse non-annealed nucleic acids and proteinase K [23]. Protected fragments were recovered after phenol/chloroform extraction and electrophoretically separated in a 4% non-denaturing polyacrylamide gel. Autoradiographic exposure was performed for 24–48 h. The individual bands were excised and counted for radioactivity by liquid-scintillation spectrometry and c.p.m. values were translated on the correlated standard curve and expressed as pg of mRNA/ μg of total RNA.

Total poly(A) RNA was purified from cell pellets by using PolyATtract System 1000 (Promega, Madison, WI) according to the manufacturers' instructions.

RESULTS

Effect of DTPA on ODC activity

Dilution of high-density L1210-DFMO^r cells in fresh medium containing serum induced ODC activity, which reached the highest values after 16–24 h (Figure 1). Treatment with the chelating agent DTPA at seeding time inhibited the induction markedly. However if Zn^{2+} was added to DTPA-treated cells, ODC activity could reach the level of control cells. In the experiment shown in Figure 1, Zn^{2+} was administered 1 h after DTPA, but it proved effective even when added after 8 h of treatment with the chelator. Figure 2(a) shows how the effect of DTPA was dose-dependent, with a strong inhibition at concentrations above 0.05 mM. The reversal of the inhibition of ODC induction by Zn^{2+} also showed a dose dependence, and a full recovery was reached by addition of 0.4 mM Zn^{2+} in the presence of 0.6 mM DTPA (Figure 2b). Treatment with other bivalent-metal ions, i.e. Fe^{2+} , Cd^{2+} , Ni^{2+} , Co^{2+} , Mn^{2+} , Cu^{2+} and Ca^{2+} , allowed only a modest protection (Table 1, Expt. a). Hg^{2+} was also ineffective (ODC activity was only 4% of control), and clearly exerted a toxic effect on the cells.

The effect of the simultaneous presence of exogenously added

Figure 1 Time course of the induction of ODC activity in the presence of DTPA and DTPA plus Zn^{2+}

High-density L1210-DFMO^r cells were diluted in fresh medium containing serum and immediately supplemented with 0.6 mM DTPA. After 1 h, ZnSO_4 was added at a concentration of 0.4 mM. ○, Control; ●, DTPA; △, DTPA plus Zn^{2+} . Results are means of three separate determinations with S.D. values within 10%.

Figure 2 Dose-dependent inhibition of the induction of ODC activity by DTPA and its reversal by Zn^{2+}

(a) L1210-DFMO^r cells were treated with different concentrations of DTPA and harvested 16 h later. (b) L1210-DFMO^r cells were treated with 0.6 mM DTPA and, 1 h later, with different concentrations of ZnSO_4 . Cells were harvested 16 h after DTPA treatment. Results represent means $\pm$ S.D. of three determinations.

Table 1 Effect of various bivalent-metal ions on the induction of ODC activity in DTPA-treated cells

(a) L1210-DFMO⁺ cells were treated with 0.6 mM DTPA and, 1 h later, with various bivalent-metal ions at the concentration of 0.4 mM. (b) L1210-DFMO⁺ cells were treated with 0.6 mM DTPA and FeSO₄ and/or ZnSO₄ at the concentration of 0.4 mM each unless indicated otherwise. Cells were harvested after 16 h of treatment. Results represent means $\pm$ S.D. of three separate determinations.

Metal ion added	ODC activity (% of control)
Expt. (a)	
None	9.3 $\pm$ 1.0
Zn ²⁺	106.0 $\pm$ 3.9
Fe ²⁺	17.4 $\pm$ 0.9
Cu ²⁺	24.2 $\pm$ 0.5
Ni ²⁺	21.0 $\pm$ 1.8
Co ²⁺	19.0 $\pm$ 1.2
Mn ²⁺	20.8 $\pm$ 0.8
Cd ²⁺	33.8 $\pm$ 5.0
Ca ²⁺	15.9 $\pm$ 1.0
Expt. (b)	
None	12.3 $\pm$ 1.2
Fe ²⁺	24.6 $\pm$ 0.6
Zn ²⁺	102.2 $\pm$ 6.1
Fe ²⁺ + Zn ²⁺	82.1 $\pm$ 1.8
Zn ²⁺ (0.6 mM)	51.6 $\pm$ 5.2

Fe²⁺ and Zn²⁺ ions in DTPA-treated cells was also investigated and the results are shown in Table 1 (Expt. b): ODC activity in cells treated with DTPA (0.6 mM), Fe²⁺ (0.4 mM) and Zn²⁺ (0.4 mM) at seeding time was significantly higher with respect to cells supplemented with DTPA and Fe²⁺ only, but appeared to be slightly reduced compared with control cells. This latter finding may be due to an excess of Zn²⁺ present in cells and not chelated by DTPA because of competition with Fe²⁺. In fact, addition of a higher concentration of Zn²⁺ (0.6 mM) to DTPA-treated cells resulted in an inhibition of ODC induction by about 50% (Table 1, Expt. b).

It should be noted that DTPA treatment inhibited cell growth (Figure 3), an effect which was fully reversed by Zn²⁺ addition, whereas Fe²⁺ was ineffective. However, cells treated with DTPA for 16 h were viable and could resume growth at a normal rate as Zn²⁺ was added.

Figure 3 Effect of DTPA and DTPA plus Fe²⁺ and/or Zn²⁺ on growth curves of L1210-DFMO⁺ cells

Cells were diluted and treated as follows: ○, control; ●, DTPA; △, DTPA plus Zn²⁺ 1 h later; ▲, DTPA plus Zn²⁺ 16 h later; □, DTPA plus Fe²⁺; ■, DTPA plus Fe²⁺ and Zn²⁺. DTPA was added at a concentration of 0.6 mM; FeSO₄ and ZnSO₄ were at 0.4 mM each.

A progressive decline in ODC activity was observed even when 0.6 mM DTPA was added 16 h after seeding to cells containing high levels of the enzyme: ODC activity was reduced to about 50% of control following 5 h of DTPA treatment and, again, addition of 0.4 mM Zn²⁺ restored ODC activity to control values within the following 5 h (results not shown).

Wild-type L1210 cells also showed the DTPA inhibiting effect on ODC induction, although to a lesser extent than L1210-DFMO⁺ cells, and the Zn²⁺ reversal effect. In fact ODC activity was 12.2 $\pm$ 1.6 units/mg of protein 16 h after cell dilution, 4.6 $\pm$ 0.3 units/mg of protein following treatment with 0.6 mM DTPA, and 11.4 $\pm$ 0.9 units/mg of protein after 0.6 mM DTPA plus 0.4 mM Zn²⁺. Finally, in order to assess the requirement for Zn²⁺ in cells other than L1210, the effect of DTPA on ODC activity was investigated in isolated rat thymocytes. Treatment with the mitogen concanavalin A for 4 h raised ODC activity from undetectable values to 9.8 $\pm$ 0.2 units/mg of protein, whereas ODC activity following pretreatment with DTPA or DTPA plus Zn²⁺ was 3.5 $\pm$ 0.13 and 7.9 $\pm$ 0.54 units/mg of protein respectively.

Effect of DTPA on ODC content, turnover and mRNA level

The inhibition of the induction of ODC activity in L1210-DFMO⁺ cells treated with DTPA was accompanied by a proportional decrease in the accumulation of immunoreactive ODC protein (Table 2), indicating an impairment in the synthesis and/or stability of the enzyme. Figure 4 shows that only a slight increase in ODC decay following cycloheximide was observed in

Table 2 Effect of DTPA and DTPA plus Zn²⁺ on ODC activity and immunoreactive ODC protein content in L1210-DFMO⁺ cells

Cells were treated as described in the legend to Figure 1. After 16 h of treatment, cells were extracted and assayed for ODC activity and immunoreactive ODC protein. Results are means $\pm$ S.D. of three separate determinations.

Treatment	ODC activity (units/mg of protein)	ODC protein (ng/mg of protein)
Control	269 $\pm$ 12	238 $\pm$ 30
DTPA	35 $\pm$ 3	33 $\pm$ 6
DTPA + Zn ²⁺	288 $\pm$ 20	274 $\pm$ 38

Figure 4 Effect of DTPA and DTPA plus Zn²⁺ on the rate of ODC decay

L1210-DFMO⁺ cells were treated as described in the legend to Figure 1. After 16 h, 0.2 mM cycloheximide was added and cell samples were removed at 30 min intervals. ○, Control; ●, DTPA; △, DTPA plus Zn²⁺. The values of ODC activity at zero time were 208, 31 and 202 units/mg of protein for control cells, DTPA-treated cells and cells treated with DTPA plus Zn²⁺ respectively.

Figure 5 Effect of DTPA and DTPA plus Zn^{2+} on the expression of ODC mRNA

L1210-DFMO^r cells were treated as described in the legend of Figure 1 and analysed for ODC mRNA content. Shown above is a representative autoradiogram of the solution-RNAase protection assay. The arrow indicates the migration of a 750 bp radiolabelled DNA marker. Lanes 1–3, quiescent cells; lanes 4–6, control cells (16 h after dilution); lanes 7–9, DTPA-treated cells; lanes 10–12, cells treated with DTPA plus Zn^{2+} . ODC mRNA levels (mean $\pm$ S.E.M. for three separate samples), determined by comparison with a standard curve run in parallel, were: 1.23 ± 0.59 pg/ μ g of total RNA in quiescent cells; 16.93 ± 3.64 pg/ μ g of total RNA in control cells; 2.33 ± 0.92 pg/ μ g of total RNA in DTPA-treated cells ($P < 0.05$ versus control cells by one-way analysis of variance, followed by the Newmann–Keuls test); 14.87 ± 5.78 pg/ μ g of total RNA in cells treated with DTPA and Zn^{2+} .

cells treated with the chelator (half-life decreased by about 75%), suggesting a major effect of DTPA at the level of ODC synthesis.

In order to evaluate the possibility that Zn^{2+} chelation by DTPA may inhibit gene expression of ODC, the levels of ODC mRNA were determined by using a sensitive RNAase-protection assay [23]. The level of the messenger, detectable in quiescent high-density L1210-DFMO^r cells, increased remarkably after dilution of the cells (Figure 5). Treatment of cells with the chelating agent prevented the accumulation of ODC mRNA, which was 14% of control, whereas Zn^{2+} supplementation resulted in a recovery of the ODC mRNA level (Figure 5). It should be noted that DTPA treatment did not modify appreciably the content of poly(A) RNA with respect to control cells (approx. 0.4μ g of poly(A) RNA/ 10^6 cells under both conditions).

DISCUSSION

The present study indicates that Zn^{2+} is required for the induction and maintenance of ODC activity in L1210-DFMO^r cells. In fact, treatment of the cells with the chelator DTPA inhibited ODC induction, although not completely, and the inhibition was reversed by Zn^{2+} , whereas other bivalent-metal ions were scarcely effective. Restricted Zn^{2+} availability has been reported to inhibit or, more rarely, to enhance, the expression of particular proteins in various cell types [5–8]. In particular, DTPA was found to prevent myoblast differentiation along with the increase in creatine kinase activity and mRNA, determined in isolated myoblasts after a few days of treatment, and these effects were prevented by Zn^{2+} addition [15]. In 3T3 cells stimulated to growth by serum, Chesters et al. [4] demonstrated a marked inhibition of DNA synthesis following DTPA treatment, but a complete prevention of the inhibition was obtained only by the addition of both Zn^{2+} and Fe^{2+} ions, Zn^{2+} alone allowing DNA synthesis at a suboptimal level [4]; further study by the same group [14] failed to show a significant difference between the values of ODC activity of control 3T3 cells, cells treated with DTPA and Fe^{2+} and cells treated with DTPA, Fe^{2+} and Zn^{2+} . On the other hand, in the present work Zn^{2+} alone allowed a full reversal of the inhibition of both ODC induction and growth rate

of L1210-DFMO^r cells; cells treated with DTPA, Fe^{2+} and Zn^{2+} did not show a higher growth rate with respect to cells treated with DTPA and Zn^{2+} and their ODC activity was still significantly higher than that of cells treated with DTPA and Fe^{2+} . Therefore it is possible that differences may exist in the requirement of metal ions, and particularly of Zn^{2+} , not only for growth [4,24], but also for the induction of ODC in different cell types. However, the finding of an impairment of ODC activity by DTPA even in parental L1210 cells and in isolated thymocytes suggests that the Zn^{2+} requirement for ODC induction may not be limited to L1210-DFMO^r cells, i.e. a particular cell line overproducing ODC.

The possibility that lack of Zn^{2+} may suppress ODC gene expression was evaluated by determining the content of ODC mRNA with a sensitive hybridization-solution-RNAase protection assay. So it has been feasible to ascertain that induction of ODC activity was largely sustained by an increase in ODC mRNA content, and that DTPA treatment prevented this increase almost completely. Thus the impairment of ODC activity and content observed in DTPA-treated cells with respect to the control appears mainly to be due to the lack of enhancement of the ODC mRNA level, although a slight increase in the turnover of the enzyme may contribute to a small extent. In its turn, a decreased content of ODC mRNA may result from alterations of transcription or stability and, actually, regulation of this messenger may occur at either level [9,10]. However, it may be of particular interest to consider the possibility that the promoter region of mammalian ODC genes, including that of mouse, contains multiple GC boxes, which are supposed to be important for ODC gene transcription and which represent binding sites for the Zn-finger-containing transcription factor Sp1 [25,26].

Furthermore, removal of Zn^{2+} from Sp1 by synthetic or natural occurring chelators such as thionein results in a suppression of DNA binding and transcriptional activation [27,28]. Interestingly thionein biosynthesis and level are influenced by the developmental state of tissues and by various hormones and signal molecules [28–30], which may also regulate ODC expression. In conclusion, these results not only indicate that ODC expression may be impaired in states of Zn^{2+} deficiency, but also suggest the possibility that physiological chelators may be involved in the regulation of this enzyme.

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