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Zinc can influence ornithine decarboxylase activity in rat thymus cells

C. Stefanelli, F. Ferrari, C. Rossoni, F. Flamigni, and **C. M. Caldarera**

Department of Biochemistry, University of Bologna, Bologna, Italy

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Summary. The thymus of young rats contained a high basal activity of ornithine decarboxylase (ODC). Treatment with zinc sulphate caused a slight increase of thymic ODC activity within 6 hours and a more marked enhancement (three-fold) in the spleen 24 h after treatment. In spite of the high activity of thymic ODC *in vivo*, ODC was not detectable in primary cultures of rat thymocytes, but was early and largely induced after treatment with Concanavalin A (Con A). The presence of 0.1 mM zinc in the medium increased the response of ODC to Con A. This effect of zinc in mitogen activated thymocytes may be due to the stabilization of ODC, which was found to decay with a half life of 65 min after the block of protein synthesis with cycloheximide. On the contrary in absence of zinc the half life of the enzyme was 40 min, as in the rat thymus in vivo.

Zinc alone, at 0.1 mM concentration, did not affect ODC activity in resting thymocytes during the early times, but the metal was able to cause an increase of the enzyme activity after 4–6 days of culture. Other heavy metals such as mercury, cadmium and copper provoked a late increase of ODC activity, but their action was evident only at dosages which were toxic for the cells.

Keywords: Amino acids – Ornithine decarboxylase – Thymocytes – Zinc – Metal ions

Introduction

Zinc is an essential trace element and its deficiency induces a complex of pathological signs in all animal studied. Zinc's role in metabolism has been studied at many different levels. It has been established that the metal is essential for cell replication, and growth failure is one of the earliest signs of experimental zinc deficiency (Prasad, 1979).

Beside the systemic effects of zinc, there are several evidences that the metal has a specific and critical role in immune function (Fraker et al., 1986). The mechanism whereby zinc exerts its immunomodulatory effects is not known. However it is well established that zinc stimulates in vitro DNA synthesis in

lymphoid cells of humans and rodents (Berger and Skinner, 1974; Hart, 1978; Reardon and Lucas, 1981; Kirchner and Solas, 1987; Reardon and Lucas, 1987a), and the metal is considered the only ubiquitous cellular component that can function as a lymphocyte mitogen.

The polyamines putrescine, spermidine and spermine are organic polycations widely distributed in living organisms and strictly related to cell proliferation (Pegg, 1988; Tabor and Tabor, 1984). Ornithine decarboxylase (ODC), the first and rate-limiting enzyme of polyamine biosynthesis, is induced in response to several stimuli promoting cell growth and proliferation (Pegg, 1988), such as hormones, mitogens and also toxics (Bachrach, 1984).

In spite of the large number of data reported on the factors which can affect ODC activity in several cells, only a few informations on the effects of metals on this enzyme are available. It is known that heavy metals, as cadmium, increase polyamines synthesis in several rat tissues as kidney, liver and lung (Kacew et al., 1976; Yoshida et al., 1984; Yoshida et al., 1986). Yoshida et al. (1986) also reported that intraperitoneal administration of $ZnCl_2$ caused the induction of ODC in rat kidney but not in liver. More recently a report indicate that zinc was not necessary for ODC induction in 3T3 cells (Chester et al., 1990). In preliminary experiments we have observed that zinc was able to provoke a "late" induction of ODC activity in thymus cells (Stefanelli et al., 1991). In the present research we have extended the studies about the effects of Zn^{2+} and other metal ions on the activity of ODC in primary cultures of rat thymocytes.

Material and methods

Chemicals

The chemicals for preparation of culture media were obtained from Gibco Laboratories. Biochemicals were from SIGMA Chemical Co. Zinc sulphate, the sulphate or chloride salts of other metal ions and laboratory chemicals were products of E. Merck.

Animals and cell cultures

Male Wistar rats were used. Thymocytes were obtained from thymuses of 4 to 10 week-old animals. Thymus glands were removed, washed in phosphate-buffered saline and immersed in standard medium containing RPMI 1640 with 10% fetal bovine serum, 2 mM glutamine, 25 mM HEPES, penicillin and streptomycin (100 units/ml). Thymuses were chopped into small pieces and disaggregated. The cell suspension was filtered and centrifuged at 800 g for 5 minutes. The pelleted cells were resuspended in 2 ml of standard medium, counted and diluted to about 5×10^6 cells/ml. Cell suspensions (5 ml) were incubated in sterile flasks in a humidified atmosphere of 5% CO_2 in air at 37 °C. Zinc concentration in the medium, measured by atomic adsorption spectrophotometry, ranged from 3 to 8 μM.

ODC activity

At the time indicated after treatment, the cells were harvested and washed with phosphate-buffered saline. The cell pellets were extracted with 0.5 ml of 50 mM Tris HCl pH 7.2, 5 mM dithiothreitol, 0.1 mM EDTA and 0.5% Triton × 100. The cell lysates were centrifuged at 10,000 g for 15 minutes. Rats thymus and spleen extracts were obtained as previously described (Stefanelli et al., 1987). ODC activity was assayed in the supernatants by measuring the amount of $^{14}CO_2$ formed: 0.04 ml of supernatant were assayed in a final volume of 0.05 ml

containing 0.4 mM ^{14}C ornithine (0.1 μCi), 2.5 mM dithiothreitol, 0.05 mM pyridoxal-5-phosphate, 50 mM Tris-HCl pH 7.2 and 0.1 mM EDTA. After incubation at 37 °C for 1 h, the reaction was stopped with 0.2 ml of 10% trichloroacetic acid. During a further incubation for 30 minutes the radioactivity of $^{14}CO_2$ was trapped in paper filters soaked with protosol (New England Nuclear) suspended over the reaction mixture. Hence the filters were counted for radioactivity in 5 ml of toluene containing 0.5% PPO. ODC-inhibiting activity and complexed ODC were measured as previously described (Flamigni et al., 1986). One unit of ODC or ODC inhibiting activity is defined as the amount that decarboxylates or inhibits the decarboxylation, respectively, of one nmol of ornithine in one hour.

Results

Effects of Zn^{2+} injection on ODC activity in rat thymus and spleen

It has been previously reported that the injection of $ZnCl_2$ caused ODC induction in rat kidney (Yoshida et al., 1986). We tested if ODC of lymphoid tissues could also be induced by the metal ion. In the present study, zinc was used as sulphate salt, to avoid the strong acidity of the solutions of the chloride salt. In Fig. 1 the time course of ODC activity in the thymus and spleen of rats treated with zinc sulphate (0.1 mmol/kg) is reported. In the thymus Zn^{2+} provoked a slight increase of ODC activity 4 h after the treatment. In the spleen ODC resulted unaffected during early times, but the enzyme activity was increased after 24 h and was returned to control levels at 48 h. No further effect was observed during the following days (not shown). It should be noted that in control rats treated with 0.9% NaCl or with 0.1 mmol/kg sodium sulphate in 0.9% NaCl, ODC activity was slighty decreased, probably as a response to Na^+.

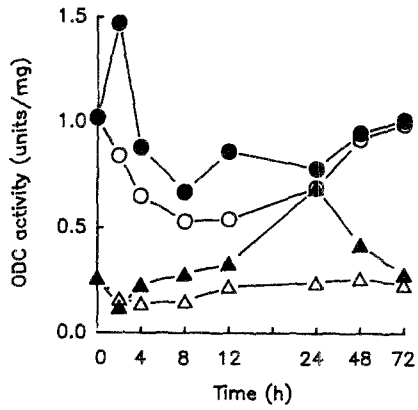

Fig. 1. Time course of ODC activity in thymus (○, ●) and spleen (△, ▲) of rats treated with Zn^{2+}. Six weeks-old male rats were injected i.p. with zinc sulphate (0.1 mmol/kg body weight) dissolved in 0.9% NaCl (filled symbols), or 0.1 mmol/kg sodium sulphate (open symbols). ODC activity was measured in tissue extracts at the indicated time. Results are means of duplicate determinations of three rats with standard deviations ranging from 4 to 23%

Effect of Zn^{2+} on ODC induction in mitogen-activated thymocytes

The thymus from young rats contained high levels of ODC, in part as a complexed, criptic form, releasable by an excess of DFMO-inactivated ODC added

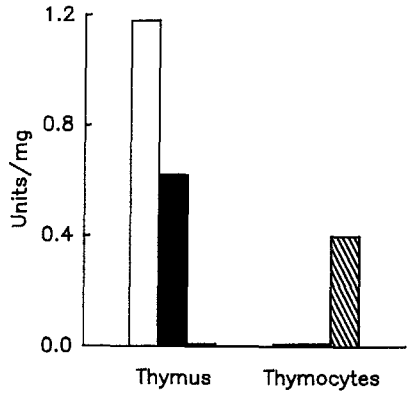

Fig. 2. ODC activity (□), complexed ODC (■) and ODC-inhibiting activity (▨) in rat thymus and in isolated thymocytes. Extracts were prepared from thymus or thymocytes as detailed in Material and methods. Results are means of duplicate determination of three flasks with standard deviations ranging from 3 to 11%

to the thymus extracts (Fig. 2), in accordance with a previous report of Peng et al. (1989). On the other hand, thymocytes obtained from thymus of untreated animals did not show detectable ODC, either free or complexed, despite they represent most of the cell population of the thymus. Furthermore, thymocyte lysates contained significant ODC inhibiting activity. However the activity of ODC could be early and largely induced in rat thymocytes by the mitogen Con A. In our experimental conditions ODC peaked after 4–6 h of cell exposure to 10 μg/ml of Con A (Fig. 3).

The addition of Zn^{2+} to the cell medium increased the induction of ODC in mitogen-activated thymocytes. When 0.02 mM Zn^{2+} was added to the medium, the activity of ODC induced after 4 h of Con A-treatment was further increased by about 35%, and the increase was more than 80% in presence of 0.1 mM Zn^{2+}

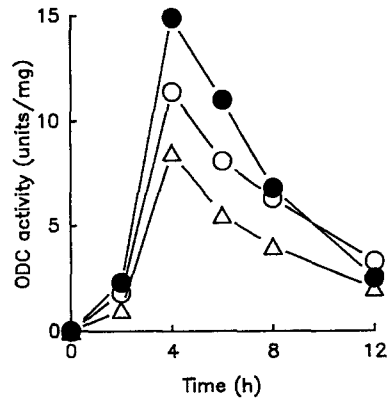

Fig. 3. Effects of Zn^{2+} on ODC activity in Con A-activated thymocytes. Rat thymocytes (5×10^6 cells/ml, 5 ml/flask) were treated with Con A (10 μg/ml) and incubated without exogenous Zn^{2+} (△) or with 0.02 mM (○) or 0.1 mM (●) $ZnSO_4$ added to the incubation medium. Cells were harvested at the indicated times and ODC activity was measured in cell extracts. Results are means of duplicate determinations of three flasks with standard deviations ranging from 2 to 18%

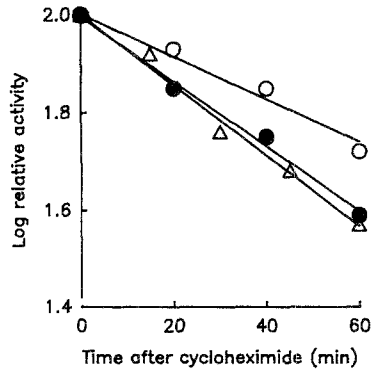

Fig. 4. ODC turnover in rat thymus *in vivo* (△) and in isolated thymocytes treated with Con A (10 μg/ml) for 4 h in the absence (●) or in the presence (○) of 0.1 mM zinc sulphate added to the cell medium. Cycloheximide was added to intact animals (20 mg/kg) or to the thymocytes medium (0.2 mM) in order to stop protein synthesis and measure the decay of ODC activity. Results are means of three determinations in duplicate. Standard deviations ranged from 5 to 12%

(Fig. 3). In Zn^{2+}-treated thymocytes, ODC activity resulted higher even after 6, 8 and 12 h of Con A treatment. During these early times, Zn^{2+} alone as well as sodium sulphate (0.1 mM) did not provoke any effect on ODC activity, which remained undetectable.

To test the possibility that the increased response of ODC to Con A in the presence of Zn^{2+} could be due to stabilization of the enzyme, the half life of ODC in mitogen-activated cells was studied. Cycloheximide (0.2 mM) was added 4 h after the addition of Con A (10 μg/ml), in order to stop protein synthesis (Fig. 4). The half life of ODC activity was about 40 minutes in the absence of zinc as in the rat thymus *in vivo*, but was increased to 65 minutes in the presence of 0.1 mM Zn^{2+} in the medium.

Effects of long-term treatment of rat thymocytes with Zn^{2+} and other metal ions

In preliminary experiments it was observed a late increase of ODC activity in thymocytes treated with Zn^{2+} (Stefanelli et al.,1991). We have now compared the effect of Zn^{2+} to that of other heavy metals. All the metals tested, i.e. Cd^{2+}, Cu^{2+} and Hg^{2+}, as well as Zn^{2+}, caused a late induction of ODC activity when added to the culture medium at a concentration of 0.1 mM. A tipical experiment is shown in Fig. 5. The time course was similar for all the metal ions: ODC was not detectable for about 3 days, then began to increase. ODC activity resulted maximal after six days. However a certain variability in the time courses was observed and in some experiments maximal ODC activity was reached after five days for all the metal ions. Cadmium was the stronger inducer of ODC activity followed by mercury. Zinc and copper were about 50% as effective as Cd^{2+} in inducing ODC. It should be observed, however, that the effect of the metal ions on ODC activity was very low in comparison with the inducing effect of the mitogen Con A (see Fig. 3).

Despite their relatively similar action on ODC activity, the metal ions displayed different toxicity toward thymocytes. In Table 1 is reported the cell

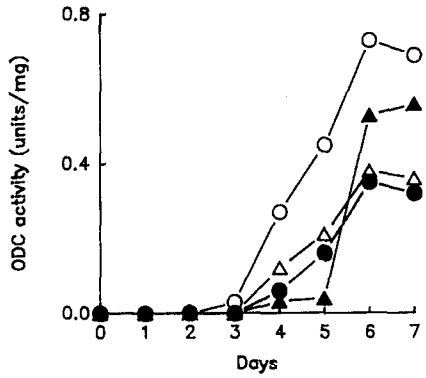

Fig. 5. Effect of heavy metal ions on ODC activity in rat thymocytes. Cell suspensions (5×10^6 cells/ml, 5 ml/flask) were incubated in the absence (controls) or in the presence of 0.1 mM of the following metals: Zn^{2+} (●), Cu^{2+} (△), Hg^{2+} (▲) and Cd^{2+} (○). Cells were collected at the indicated times for ODC assay. Results are means of three flasks. In untreated control cells, as well as in cell treated with 0.1 mM sodium sulphate, ODC activity always remained below detection limit. Results are means of duplicate determinations of three flasks with standard deviations ranging from 2 to 31%

Table 1. Effect of metal ions on thymocyte recovery. Thymocytes were initially suspended at a density of 5×10^6 cells/ml (100%) and incubated six days in the absence (controls) or in the presence of the indicated concentrations of metal ions. Results are means of five flasks ± SD

Metal ion added	Metal ion concentration (mM)	Cell recovery (%)
None	—	46 ± 5
Zn^{2+}	0.050	53 ± 4
Zn^{2+}	0.100	57 ± 4
Zn^{2+}	0.200	24 ± 2
Cu^{2+}	0.100	18 ± 3
Cd^{2+}	0.100	16 ± 5
Hg^{2+}	0.100	13 ± 6

recovery after six days of culture in the presence of metal ions. In the absence of metals about 50% of initial cells could be recovered. The presence of Zn^{2+} at 0.05 mM and 0.1 mM did not show any toxic effect and actually the recovery resulted higher in zinc-treated thymocytes than in controls. On the contrary Zn^{2+} at concentration of 0.2 mM elicited toxic effects and recovery was reduced to 50% of controls. All the other heavy metals were toxic at 0.1 mM concentration and the cell recovery was very low.

Discussion

Rat thymocytes do not express ODC when cultured in absence of a mitogen, and an ODC-inhibiting activity is present in their extracts. However the present

investigation has revealed that several metal ions, such as Cd^{2+}, Hg^{2+} and Cu^{2+} as well as Zn^{2+} are able to induce ODC activity after a few days of culture.

ODC is generally induced very rapidly following cell stimulation (Bachrach, 1984; Tabor and Tabor, 1984; Pegg, 1988) and in lymphoid cell dramatic increases are observed after mitogen treatment (Kay and Lindsay, 1973; Otani et al., 1980; Brand, 1987). In rat thymocytes ODC is induced by Concanavalin A and IL-2 (Brand, 1987) within a few hours treatment. Hence the "late" response of ODC to metal ions is an uncommon feature. On the other hand, zinc and other heavy metals actually do stimulate "late" (> Day 5) lymphoproliferation, similarly to proliferation induced by soluble antigens, in contrast to earlier peak responses for other mitogens such as Con A (Oppenheim and Rosenstreich, 1976; Warner and Lawrence, 1986; Kirchner and Solas, 1987; Reardon and Lucas, 1987a).

Generally an increased polyamine synthesis is related to cell proliferation and the induction of ODC is a common event in cell cycle progression (Bachrach, 1984; Tabor and Tabor, 1984; Pegg, 1988). However although Zn^{2+} and Hg^{+} are T-cell mitogens (Reardon and Lucas, 1981), other heavy metals which can induce ODC, such as cadmium and copper, cannot activate lymphocytes (Berger and Skinner, 1974; Reardon and Lucas, 1981; Reardon and Lucas, 1987b). Furthermore it has been reported that mouse thymocytes, differently from other T-cells, are not sensitive to Hg^{2+}, and that mitogenic effect of zinc on these cells requires the presence of 2-mercaptoethanol and bacterial lipopolysaccharide (Reardon and Lucas, 1987a). The culture media used in the present work did not contain either 2-mercaptoethanol or lipopolysaccharide, and actually the mitogenic effect of zinc in our experimental conditions was very low (Stefanelli et al., 1991). On the basis of these considerations it may be concluded that the induction of ODC by heavy metals is not related to cell proliferation processes. ODC may be induced by toxic stimuli, and the emzyme response to heavy metals could result from their cytotoxic effects on thymocytes. However, zinc is not toxic at a concentration which can cause ODC induction. Therefore, it is possible that zinc, which has several positive effects on immune system (Prasad, 1979; Fraker et al., 1986) acts on ODC with a mechanism different from the other metals.

Beside its late effect on ODC, zinc also increases the response of the enzyme to the mitogen Con A: in fact ODC induction in mitogen-stimulated thymocytes is much higher in the presence of 0.1 mM $ZnSO_4$ in the medium. Zinc appears to stabilize ODC protein against degradation and it is possible that the effect of the metal ion is due to an inhibition of specific proteases (Nakamura et al., 1991). Interestingly Cd^{2+}, Hg^{2+} and Cu^{2+} in non-toxic concentrations did not affect the induction of ODC by Con A (not shown) suggesting a specific role of zinc.

Zinc is known to modulate the proliferative response of lymphoid cells to mitogens. In fact zinc enhances the response to phytohemoagglutinin and Con A of peripheral lymphocytes from young but not old human donors (Rao et al., 1979) and is a potentiator of the response to lipopolysaccharide of spleen and lymphonode cells (Hart, 1978). Furthermore supplementation of zinc enhances the mitogenic response of blood lymphocytes from subjects in which low proliferative responses were observed (Duchateau et al., 1981) and of spleen cells from low responder mice (Gaworsky and Sharma, 1978; Malave and Benaim,

1984). It appears, therefore, that zinc may enhance the proliferation activity of several lymphoid cells and it is possible that this effect of zinc is mediated by its ability to influence ODC induction.

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Authors' address: Prof. C. M. Caldarera, Dipartimento di Biochimica, Via Irnerio 48, I-40126 Bologna, Italy.

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