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The requirement of Zn^{2+} for the increase in ornithine decarboxylase induced by insulin and epidermal growth factor in primary cultured rat hepatocytes

Teruyo Nakatani, Kimiko Ohtani, Yoshihisa Yano,* Shuzo Otani,* and Isao Matsui-Yuasa

*Department of Food and Nutrition, Faculty of Human Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku Osaka 558, and *Department of Biochemistry, Osaka City University Medical School, 1-4-54 Asahimachi Abeno-ku, Osaka 545. Japan*

The effect of restricted Zn^{2+} on ornithine decarboxylase (ODC) was studied in primary cultured adult rat hepatocytes. ODC activity was increased 4 hr after adding insulin and epidermal growth factor. The maximal activity was maintained for 12 hr, it then decreased and returned to the control level by 24 hr. When the chelator, diethylenetriamine penta-acetic acid (DTPA) was added to insulin and epidermal growth factor, the enzyme activity was decreased by 50% and only the Zn^{2+} of the bivalent metal ions tested was effective in reversing this effect. The level of the ODC messenger RNA did not change with addition of the chelator. The half-life of the ODC activity was decreased 2-fold by the chelator. Exposing the hepatocytes to DTPA reduced the incorporation of [^{3}H]thymidine into acid insoluble fraction. The inhibition of DNA synthesis was fully reversed by the addition of 600 μM Zn^{2+}. These results suggest that a lack of Zn^{2+} in hepatocytes impairs ODC activity mainly by destabilizing the enzyme resulting in the inhibition of DNA synthesis. (J. Nutr. Biochem. 7:386–391, 1996.)

Keywords: zinc; ornithine decarboxylase; insulin; epidermal growth factor; diethylenetriamine penta-acetic acid; hepatocytes

Introduction

Zinc is necessary for growth and DNA synthesis in animals[1,2] and a reduction in thymidine kinase and DNA polymerase activity occurs in fetuses from Zn-deficient rats.[3,4] Furthermore, the content and metabolism of Zn^{2+} varies with age. In cell culture, zinc is required for DNA synthesis and the proliferation of several cell types.[5,6] In several of these systems, the reduction in DNA synthesis was accompanied by a closely similar decrease in DNA polymerase and thymidine kinase activities. These results suggest that Zn^{2+} is required for the expression of the group of enzymes that must be induced in normal cells before their entry into S phase. The biochemical bases for the requirement of Zn^{2+} are not clear.

The polyamines putrescine, spermidine, and spermine are present in many cells, and evidence indicates that polyamines play an essential role in cell growth and differentiation.[7,8] Eukaryotic cell proliferation proceeds by an increase in the activity of ornithine decarboxylase (ODC) and by polyamine biosynthesis. Studies in animal tissues have indicated changes in the polyamine content with increasing age of the animal.[9] Duffy and Kremzner[10] have reported that the onset of the in vitro cellular senescence of WI-38 human fibroblasts is associated with reduced ODC activity in response to fresh culture medium. The content and metabolism of zinc varies with age.[11] Polyamine synthesis is required for regeneration in several tissues, including the liver.[12] During the cell cycle in synchronized cells, ODC and polyamine levels are highest at the end of the G_1 phase.[13,14] We recently reported that an increased cellular

Address reprint requests to Dr. Isao Matsui-Yuasa at Department of Food and Nutrition, Faculty of Human Life Science, Osaka City University. 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558. Japan.

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level of spermidine or spermine was essential for DNA synthesis in primary cultured rat hepatocytes.[15] In hepatocytes, insulin and epidermal growth factor are potent mitogens, which significantly enhance ODC activity. The combined exposure to insulin and EGF enhanced the enzyme activity additively at the level of posttranscription.[16]

We therefore examined the effect of a zinc deficiency on ODC activity in primary cultured rat hepatocytes and found that it destabilized ODC.

Methods and materials

Materials

Insulin, epidermal growth factor (EGF) and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase (type I) and diethylenetriamine penta-acetic acid were from Wako Pure Chemicals (Osaka, Japan). L-[1-^{14}C] Ornithine (56mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA). [6-^{3}H]Thymidine (31 Ci/mmol) were purchased from Amersham International (Buckinghamshire, England).

Hepatocyte preparation and culture

The protocol of the experiment was approved by the Animal Research Committee of Osaka City University, and care of the animals was in accordance with the standards of this institution (Guide for Animal Experimentation, Osaka City University, Japan). Hepatocytes were isolated from male Sprague Dawley rats weighing 200 to 250 gm by collagenase perfusion.[17] The viability of the isolated hepatocytes was over 90% as judged by trypan blue exclusion. The cells were plated in 35-mm plastic dishes at a density of 2.5×10^5/ml in Williams' medium E supplemented with 10% FCS, 1×10^{-7} mol/L insulin and 1×10^{-6} mol/L dexamethasone. The cells were cultured at 37°C for 24 hr in a humidified atmosphere of 5% CO_2 and 95% air. After the medium was replaced with fresh medium, the cells were cultured in the absence of hormones for another 24 hr. This medium was exchanged for that with inducer (10^{-7} mol/L insulin, 10 ng/ml EGF) and/or diethylenetriaminepenta-acetic acid (DTPA) at a concentration of 600 μM. One hour after adding the chelator, cultures were supplemented with $ZnSO_4$ to a final concentration of 600 μM. At the indicated time, the cells were harvested to measure the ODC activity.

Assay for ODC activity

The ODC activity was assayed as described.[18] In brief, cells were washed three times with PBS and harvested with a rubber policeman in 300 μl of 50 mmol/L Tris (pH 7.5) containing 200 μmol/L pyridoxal phosphate, 0.1 mmol/L EDTA and 2.5 mmol/L dithiothreitol. Cell integrity was disrupted by three cycles of freezing and thawing and centrifuged at 30,000 g for 20 min at 4°C. Supernatant (90 μl) was added to a glass tube containing 0.25 μCi L-[1-^{14}C] ornithine (5 μl) and 80 nmol L-ornithine (5 μl). After 1 hr incubation at 37°C, the release of $^{14}CO_2$ from [^{14}C] ornithine was measured.[19] ODC activity is expressed as nmol/mg of protein. The protein concentration was measured by a Bio-Rad protein assay with BSA as the standard.

Zinc analysis

Total cell zinc concentration was measured by flame atomic absorption spectrophotometry using a single-slot burner head (Hitachi 180-30, Japan). For each total cellular zinc measurement, hepatocytes from three culture dishes (1.5×10^6 cells) were washed with PBS and harvested into glass distilled-deionized H_2O(ddH_2O) using a rubber policeman and pooled. Part of the suspension (1.0×10^6 cells) was removed for ashing (at 500°C, 20 hr) and the remainder was used for the measurement of protein concentration. After ashing, the samples were digested with nitric acid and perchloric acid (1:1), followed by a 1:9 dilution with 1N HCl, and the zinc concentration was measured. Recovery was 65% as determined by analyzing a substance with known zinc concentration. For the measurement of protein concentration, the hepatocytes were solubilized in 0.5N NaOH. A portion of this solution was retained to measure the protein concentration by the Bio-Rad Protein Assay.[20]

Measurement of ODC mRNA level

Total RNA was isolated from cultured cells using acid guanidium phenol-chloroform.[21] The RNA concentration was measured by absorption at 260 nm with a Hitachi U-2000 spectrometer (Tokyo, Japan). RNA hybridization proceeded as described.[22] Total RNA (15 μg) was denatured by heating in 20% formaldehyde/50% formamide at 60°C for 15 min before gel electrophoresis. Samples were fractionated by electrophoresis on 1% agarose gels containing 2.2 mol/L formaldehyde and transferred to a nylon membrane. The membrane were prehybridized at 42°C for 2 hr in 0.5% dextran sulfate, 5 × standard saline phosphate EDTA, 0.5 × Denhardts' solution and 50% formamide, then hybridized in the same mixture for 24 hr with ODC complementary DNA (cDNA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA labeled with [^{32}P]dCTP using the Multiprime 1-Labelling System (Amersham). The plasmid sp64, containing ODC cDNA was supplied by Dr. C. Kahana (Weizmann Institute of Science, Rehovot, Israel).[23] To normalize the amounts of RNA applied to the gel, the relative amounts of ODC and GAPDH messenger RNA were calculated by densitometrically scanning the autoradiogram films (LKB Ultrascan XL laser densitometer).

Measurement of [^{3}H]Thymidine incorporation into acid-insoluble fraction

[^{3}H]Thymidine (1 μCi/dish) was added to the cell culture 4 hr before the cells were harvested. The incorporation of radioactivity into the acid-insoluble fraction was measured as described previously.[24] The incorporation was expressed as counts per minute per microgram of DNA. The amount of DNA was determined by the method of Burton.[25,26] Salmon Spermary DNA (WAKO Pure Chemical Industries, Ltd.) was used as a standard.

Results and discussion

The ODC activity was significantly increased with time after adding insulin and EGF, as shown in *Figure 1*. The enzyme activity increased 4 hr after adding the mitogens. The maximal activity was maintained until 12 hr, then it decreased and returned to the control level by 24 hr. The simultaneous addition of the chelating agent DTPA with mitogens inhibited ODC induction. The enzyme activity was affected dose dependently by the chelator up to 600 μM. DTPA (600 μM) reduced the enzyme activity to about 50% of that in control cells. A higher concentration of DTPA did not cause any further reduction (data not shown). *Table 1* shows that 600 μM DTPA inhibited ODC induction, which was fully reversed by 600 μM Zn^{2+}. Fe^{2+} modestly protected the enzyme activity. However, the other divalent metal ions, Ca^{2+}, Mn^{2+}, and Mg^{2+} were ineffective.

To determine whether the changes in ODC activity are

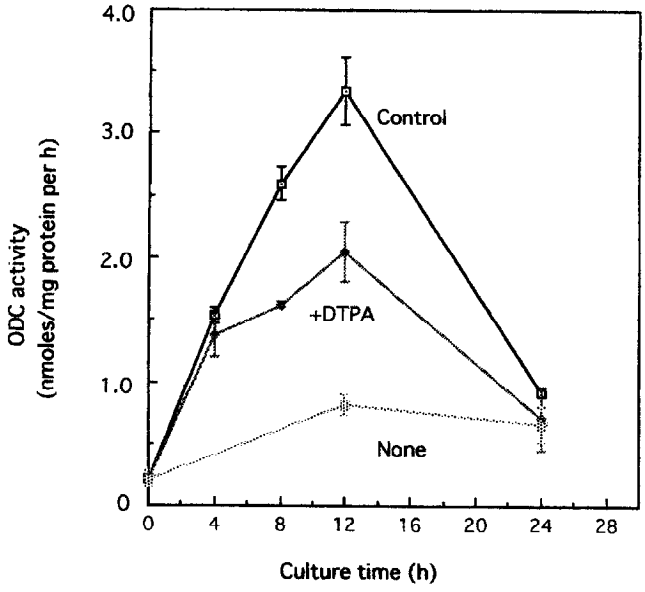

Figure 1 Effect of DTPA on ODC activity in primary cultured hepatocytes. Cells were cultured as described under Methods and materials. The medium was replaced with or without that containing insulin (10^{-7} mol/L), EGF (10 ng/ml) and/or DTPA (600 μM), and the cells were cultured for the indicated periods. Results are the means ± S.E. of three experiments.

linked to cellular zinc content, we examined the cellular zinc content after the addition of insulin and EGF in the absence or presence of 600 μM DTPA. The zinc content of cells treated with DTPA for 10 and 12 hr were 108% and 88% of the DTPA-non-treated (control) cells, respectively (the levels in control cells for 10 and 12 hr were 2.6 nmol Zn/mg protein and 2.5 nmol Zn/mg protein, respectively. The zinc content of cells, therefore, was not significantly altered by treatment with DTPA. On the role of zinc, Bettger and O'Dell have argued that membrane zinc is important for regulation of cell function.[27,28] Additional evidence

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

Treatment	ODC activity (nmol/mg protein per h)	% of control
control	1.92 ± 0.19	100
+DTPA	0.93 ± 0.06	48.5
+DTPA + Zn^{2+} (400 μM)	1.40 ± 0.04	72.6
+DTPA + Zn^{2+} (600 μM)	1.85 ± 0.09	96.0
+DTPA + Fe^{2+} (400 μM)	1.22 ± 0.07	63.3
+DTPA + Fe^{2+} (600 μM)	1.16 ± 0.18	60.6
+DTPA + Ca^{2+} (400 μM)	0.98 ± 0.12	51.0
+DTPA + Ca^{2+} (600 μM)	0.97 ± 0.04	50.7
+DTPA + Mn^{2+} (400 μM)	0.79 ± 0.07	41.2
+DTPA + Mn^{2+} (600 μM)	0.40 ± 0.08	20.6
+DTPA + Mg^{2+} (400 μM)	0.88 ± 0.05	45.5
+DTPA + Mg^{2+} (600 μM)	0.84 ± 0.02	43.6

The medium was replaced with that containing insulin and EGF in the absence or presence of DTPA. Various bivalent-metal ions were added after 1hr and the cells were cultured for a further 13 hr. Results are mean ± S.E. of three experiments.

Table 2 Effect of DTPA on ODC activity in vitro

Concentration of DTPA (μM)	ODC activity (nmol/mg Protein per h)	(% of control)
0	1.47 ± 0.09	100.0
50	1.51 ± 0.06	102.9
100	1.79 ± 0.10	121.6
200	1.60 ± 0.05	108.7
400	1.75 ± 0.04	119.0
600	1.73 ± 0.12	117.8

The medium was replaced with that containing insulin and EGF in the absence of DTPA for 12 hr. ODC activity in the cell lysate was assayed in vitro in the presence of various concentrations of DTPA. The results are mean ± S.E. of three assays.

shows that zinc is an effective stabilizer of cells and cell membranes.[29,30] In addition to its role as a membrane stabilizer, zinc affects as a modulator of cell signaling.[31–37] Furthermore, Forbes et al. have shown that Zinc greatly augmented binding of phorbol dibutyrate ([^{3}H]PDBu) to protein kinase C (PKC) in cell homogenates and intact B lymphocytes. The heavy-metal chelating agent 1,10-phenanthroline completely reversed the increased [^{3}H]PDBu binding in cells pretreated with ^{65}Zn and ionophore and this was associated with a decline of about 17.6% in cell-associated ^{65}Zn, suggesting that a relatively small pool of intracellular Zn acts on PKC.[38] Others have shown that cells contain a small pool of metabolically active Zn.[39] Thus, our results and these observations suggest that the chelator slightly affects the levels of Zn associated with membrane and/or causes a change in the levels of the metabolically active pool of the metal in the cell and only a relatively small pool of chelatable Zn is involved in the regulation of ODC activity.

To rule out the possibility that DTPA acts directly on the enzyme to suppress its activity, ODC activity was assayed in vitro in the presence of DTPA at various concentrations up to 600 μM. The enzyme was not inhibited at all the concentrations of DTPA used (*Table 2*).

To examine when Zn^{2+} is required to maintain ODC activity, Zn^{2+} was added at various times after exposure to DTPA. When Zn^{2+} was added for 6 hr after DTPA, ODC activity was maintained to the same levels found in DTPA-non-treated (control) cells (*Figure 2*). These results suggested that Zn^{2+} was required between 6 and 8 hr after the addition of the two inducers and DTPA.

To determine whether the decrease of ODC activity was due to the level of transcription, the ODC mRNA level was measured by means of Northern-blotting. *Figure 3* shows the levels of ODC and GAPDH mRNA 9 hr after adding insulin, EGF, and DTPA. DTPA did not affect the relative amount of ODC mRNA to GAPDH mRNA in the cells treated with inducers and DTPA and in the cells treated with inducers. Furthermore, the amounts in the cells with DTPA and Zn^{2+} were similar to those seen in the cells with inducers alone. This indicated that Zn^{2+} deficiency affected ODC activity at the level of posttranscription.

To examine the rate of ODC turnover, cells that were cultured for 14 hr after the addition of inducers, DTPA, or Zn^{2+} were incubated with cycloheximide, and ODC activi-

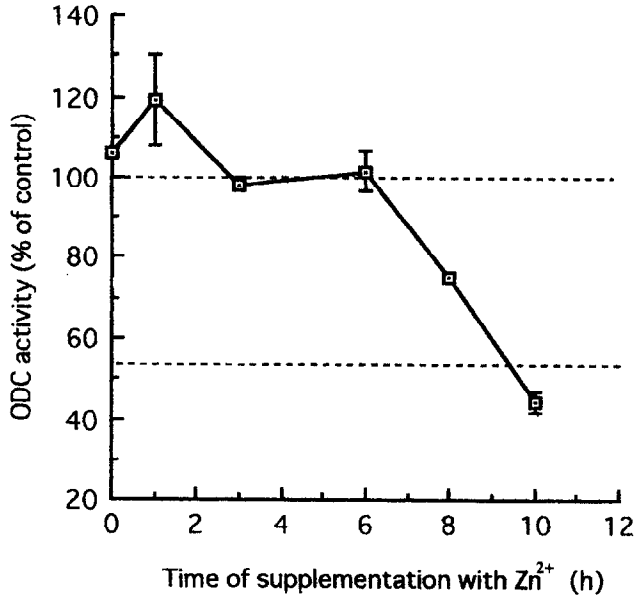

Figure 2 Effect of time of supplementation with Zn^{2+} on ODC activity in primary cultured hepatocytes with and without DTPA. The medium was replaced with that containing insulin and EGF in the absence or presence of DTPA. Zn^{2+} was added at the indicated times, then the cells were cultured for 12 hr. Results are the means ± S.E. of three experiments.

Table 3 Half life of ODC activity in primary culture hepatocytes

Treatment	Half life of ODC ($t_{1/2}$)
None	15 min
Insulin + EGF	30
Insulin + EGF + DTPA	14
Insulin + EGF + DTPA + Zn^{2+}	27

Culture medium was replaced with new medium supplemented with or without insulin and EGF. DTPA and Zn^{2+} were added 0 and 1 hr later, respectively and the cells were cultured for a further 13 hr. The cells were then exposed to cycloheximide (20 μg/ml) for 0, 10, 20, and 30 min.

ties were monitored (*Table 3*). The biological half-times of ODC in the cells incubated with and without insulin and EGF were 30 and 15 mins, respectively. The increase in ODC decay caused by the chelator was recovered by adding Zn^{2+}. These results suggested that Zn^{2+} is required to stabilize ODC in primary cultured adult rat hapatocytes. In cells incubated with insulin and EGF, the biological half-life of ODC was longer than that in control cells. The stabilization of the enzyme with insulin and EGF was not maintained in the presence of the chelator DTPA. The supplementation of Zn^{2+} fully recovered the stabilization.

To determine whether the change in ODC activity with deficiency of Zn^{2+} are linked to DNA synthesis or not, we examined the effect of DTPA on the DNA synthesis. The DNA synthesis was measured 24 hr after the addition of insulin and EGF in the absence or presence of 600 μM

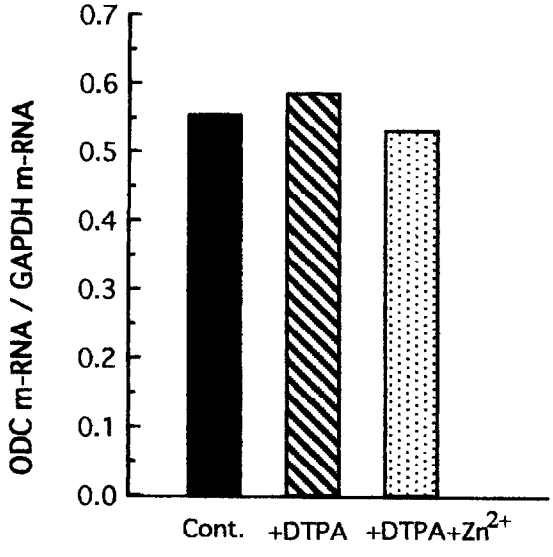

Figure 3 Effect of DTPA on ODC mRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels in primary cultured hepatocytes. The medium was replaced with that containing insulin (10^{-7} mol/L), EGF (10 ng/ml) and/or DTPA (600 μM), and cells were cultured for 9 hr. Zn^{2+} was added 1 hr after adding the two inducers and/or DTPA. Total RNA (15 μg) was fractionated in agarose gels containing formaldehyde, blotted onto a nylon membrane and hybridized to ^{32}P-labeled probes. To normalize the amounts of RNA applied to the gel, the relative amounts of ODC and GAPDH mRNAs were found by densitometrically scanning the autoradiogram films.

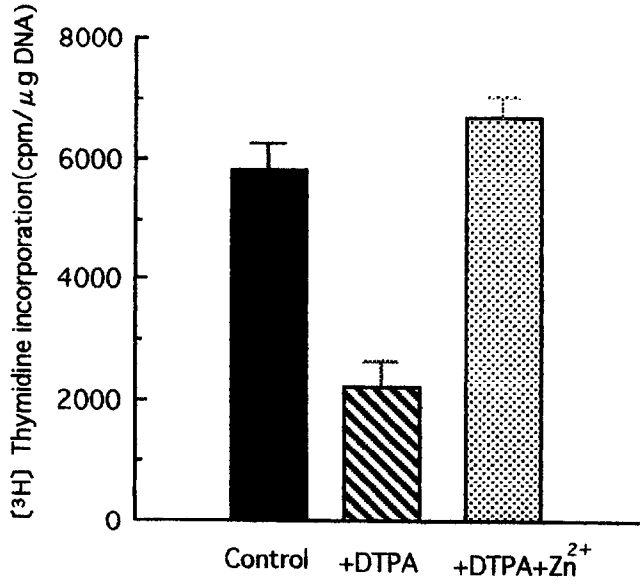

Figure 4 Effect of DTPA on DNA synthesis in primary cultured rat hepatocytes. Culture medium was replaced with new medium supplemented with insulin and EGF. DTPA and Zn^{2+} were added 0 and 1 hr later, respectively. The cells were cultured for a further 20 hr. The cells then labelled with [^{3}H]thymidine (1 μCi/dish) for 4 hr. Results are mean ± S.E. of five experiments.

DTPA. As shown in *Figure 4,* exposing the hepatocyte to DTPA reduced the incorporation of [^{3}H]thymidine into acid-insoluble fraction to about 44% that in control cells. The inhibition of the incorporation of [^{3}H]thymidine was fully reversed by 600 μM Zn^{2+}.

Ornithine decarboxylase is not a metalloenzyme and Zn^{2+} is not an essential cofactor for the enzyme.[7,8] The levels of ODC mRNA were not changed. These results suggest that restricted Zn^{2+} availability in hepatocytes impairs ODC activity, mainly by affecting the stabilization of the enzyme. The relationship between zinc deficiency and ornithine decarboxylase activity has been described. Flamigni et al.[40] have reported that a restricted Zn^{2+} availability in L1210 DFMO^r cells remarkably impairs ODC induction, mainly by affecting the expression of the messenger RNA. In hepatocytes restricted Zn^{2+} impairs ODC activity, mainly by affecting the enzyme stability. We have previously shown that the amounts of ODC mRNA were almost the same as those seen in the control not treated with mitogen.[16] These results indicated that the mitogen enhances ODC activity at the level of posttranscription. Therefore, there may be differences in the expression of the ODC messenger between L1210 DFMO^r and hepatocytes.

Here we have shown that Zn^{2+} is required between 6 and 8 hr after that adding insulin, EGF, and DTPA, suggesting that a factor(s) that stabilizes ODC enzyme is formed during this period or that Zn^{2+} inhibits production or activity of ODC antizyme that stimulate ODC degradation.[41,42] The precise mechanism of the destabilization of ODC activity with a zinc deficiency remains to be investigated.

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