Inhibitory effect of zinc on stimulated erythropoietin synthesis in HepG2 cells

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The effect of zinc on erythropoietin (EPO) synthesis in HepG2 cells was investigated. The increase in EPO synthesis induced by Co^{2+} (50 μM), Ni^{2+} (300 μM) or oxygen (1 % O_2) was inhibited by the presence of ZnCl_2 (50–150 μM) in the tissue-culture medium, whereas basal EPO synthesis was unaffected. The effect was reflected by corresponding changes in the EPO mRNA level. These effects of zinc on EPO synthesis could not be mimicked by CdCl_2 ($\leq 2 \mu\text{M}$). Addition of FeCl_3 to the medium appeared to decrease the inhibitory effect of zinc on hypoxia-induced EPO synthesis, implying that zinc may interfere with an iron-dependent step in EPO regulation.

INTRODUCTION

The human hepatoma cell lines HepG2 and Hep3B can be utilized to study the regulation of the glyco-hormone erythropoietin (EPO). As in whole animals, EPO expression in these cells can be positively modulated by exposure to low oxygen partial pressure or by treatment with CoCl_2 (Goldberg *et al.*, 1987; Ueno *et al.*, 1989). Studies with Hep3B cells revealed that, in addition to Co^{2+} , Ni^{2+} and Mn^{2+} also can stimulate EPO synthesis, whereas Zn^{2+} was ineffective (Goldberg *et al.*, 1988). The importance of iron and haem for the oxygen-dependent regulation of EPO suggests a haem protein to be involved in this process (Goldberg *et al.*, 1988). Co^{2+} , Ni^{2+} and Mn^{2+} are supposed to stimulate EPO synthesis by substituting for the haem iron and, hence, locking this protein in its deoxygenated state. The latter is assumed because of results obtained from an analysis of the oxygen-binding activity of metallo-hybrid haemoglobins. It showed that Co^{2+} , Ni^{2+} or Mn^{2+} -protoporphyrin IX in such hybrid haemoglobins binds oxygen only with low affinity (Shibayama *et al.*, 1986a). However, this study also demonstrated a similar low affinity of Zn^{2+} - Fe^{2+} hybrid haemoglobins to oxygen (Shibayama *et al.*, 1986b). Therefore the failure of Zn^{2+} to induce EPO synthesis seems to be obscure. Possibly the function of the metal ion in the putative haem protein is more complex than in haemoglobin. For example, the metal ion might have to undergo a change of its oxidation state in order to initiate the alteration of the protein conformation. In contrast with its neighbouring transition metals, zinc is known to be inert to oxidoreductions (Vallee & Auld, 1990). There are also several examples that show that the action of zinc on proteins may differ from that of, e.g., cobalt, such as the 'zinc-finger'-containing aryl hydrocarbon receptor that was unable to bind to its corresponding DNA sequence when Zn^{2+} was displaced by Co^{2+} (Saatgioglu *et al.*, 1990) or the ferritin repressor protein that was inactivatable by Co^{3+} or Fe^{3+} , but not by Zn^{2+} -protoporphyrin (Lin *et al.*, 1990). Moreover, other examples even reveal that Co^{2+} and Zn^{2+} can have opposite effects on proteins. These are enzymes such as the α -mannosidase from rat epididymis (Dutta & Majumder, 1984), the alkaline phosphatase from bovine polymorphonuclear neutrophils (Yasuura *et al.*, 1985) or the hepatic UDP-glucuronosyltransferase (Aitio *et al.*, 1980), whose activities could be stimulated by Co^{2+} , but were inhibited by

Zn^{2+} . Therefore it is possible not only that does zinc have no stimulating effect on EPO synthesis, but also that it may inhibit the stimulation of EPO synthesis by other metal ions or hypoxia. To study this we analysed EPO synthesis in HepG2 cells in the presence of Zn^{2+} . It could be shown that (i) in HepG2 cells too, Zn^{2+} does not stimulate EPO synthesis and that (ii) zinc strongly decreases hypoxia and Co^{2+} and Ni^{2+} -dependent EPO synthesis.

EXPERIMENTAL

Cell culture

HepG2 cells were cultured in an antibiotic- and fungicide-free medium consisting of F12 medium/Dulbecco's modified Eagle medium (1:1, v/v; Flow) supplemented with 10% (v/v) fetal-calf serum (GIBCO-BRL). To reduce the tendency of HepG2 cells to form large three-dimensional aggregates, the cell suspension was vigorously shaken after each trypsin treatment. Then monolayers were preferentially formed.

Determination of immunoreactive EPO

At about 50% confluence, cells were trypsin-treated and suspended in culture medium. Cell concentration was adjusted to $3 \times 10^6/\text{ml}$. To each 75 cm^2 culture flask (Falcon) 0.5 ml of cell suspension and 10 ml of medium (if required, supplemented with metal ions) were added. After 3 days of incubation under normoxia ($\text{O}_2/\text{CO}_2/\text{N}_2$, 21:5:74) or hypoxia ($\text{O}_2/\text{CO}_2/\text{N}_2$, 1:5:94) the supernatant was removed and EPO secreted by the cells determined by radioimmunoassay (r.i.a.) as described by Eckardt *et al.* (1988). The cells of each flask were trypsin-treated and resuspended in culture medium. The cell number was measured by using a Neubauer chamber. EPO synthesis per cell was calculated from the immunoreactive EPO secreted and from the average cell number, which was estimated from the initial and final cell numbers.

RNA extraction and blotting

Total RNA was isolated as described by Birnboim (1988) with some modifications. Cells were seeded as described above. After 24 h of incubation under normoxia or hypoxia the medium was decanted and the cell layer of each flask washed with 5 ml of phosphate-buffered saline (PBS; 10.1 mM- $\text{Na}_2\text{HPO}_4/1.8 \text{ mM-KH}_2\text{PO}_4/136.9 \text{ mM-NaCl}/2.6 \text{ mM-KCl}$, pH 7.4, without Ca^{2+}

Abbreviations used: EPO, erythropoietin; PBS, phosphate-buffered saline; GTC, guanidinium thiocyanate; SSC, saline/sodium citrate; r.i.a., radioimmunoassay.

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Table 1. Effect of Zn²⁺ on EPO synthesis

Values are the fold inductions of EPO synthesis after 3 days of incubation relative to control conditions (normoxia, no metal-ion treatment; Zn²⁺, 150 μ M-ZnCl₂; Co²⁺, 50 μ M-CoCl₂; Ni²⁺, 300 μ M-NiCl₂; hypoxia, 1 % O₂; normoxia, 21 % O₂). EPO was measured by r.i.a. * Significantly different from corresponding control value, i.e. in the absence of Zn²⁺ (*t*-test; *P* < 0.005).

Normoxia					Hypoxia	
Zn ²⁺	Co ²⁺	Co ²⁺ + Zn ²⁺	Ni ²⁺	Ni ²⁺ + Zn ²⁺	None	Zn ²⁺
1.00 ± 0.05 (n = 8)	1.73 ± 0.12 (n = 23)	1.03* ± 0.05 (n = 24)	1.51 ± 0.08 (n = 8)	1.14* ± 0.05 (n = 8)	2.45 ± 0.48 (n = 14)	1.39* ± 0.24 (n = 14)

Table 2. Dose-dependent inhibition of hypoxia- and Co²⁺-induced EPO synthesis by Zn²⁺

Cells were grown for 3 days, either under normoxia or under hypoxia, and treated with 50 μ M-CoCl₂ and/or various concentrations of ZnCl₂ as indicated. EPO synthesis was determined by r.i.a. Values are fold inductions of EPO synthesis. * Significantly different from corresponding control value (*t* test; *P* < 0.005; n = 4).

Normoxia						Hypoxia			
Co ²⁺	Co ²⁺ + 10 μ M-Zn ²⁺	Co ²⁺ + 50 μ M-Zn ²⁺	Co ²⁺ + 100 μ M-Zn ²⁺	Co ²⁺ + 150 μ M-Zn ²⁺	150 μ M-Zn ²⁺	None	50 μ M-Zn ²⁺	100 μ M-Zn ²⁺	150 μ M-Zn ²⁺
1.68 ± 0.04	1.64 ± 0.05	1.36* ± 0.07	1.21* ± 0.03	0.89* ± 0.04	0.93 ± 0.08	2.75 ± 0.15	2.37* ± 0.07	2.20* ± 0.17	1.53* ± 0.12

and Mg²⁺). PBS was completely removed and the cells were homogenized by gentle shaking in a guanidium thiocyanate (GTC) solution (as described by Chirgwin *et al.*, 1979). A 6 ml-portion of GTC solution was sufficient to remove cells completely from three or four flasks. The cell homogenate was pipetted into a 15 ml Corex tube. RNA was precipitated by adding 126 μ l of 1 M-acetic acid and 4.6 ml of ethanol, followed by an incubation for 30 min at -20 °C and pelleted by centrifugation in an SS34 rotor for 20 min at 10000 rev./min. The pellet was washed with 3 × 1 ml of ethanol/0.1 M-NaCl (4:1, v/v) and resolved in 500 μ l of RES (0.5 M-LiCl/1 M-urea/0.25 % SDS/20 mM-sodium citrate/2.5 mM-EDTA, pH 6.8). After pipetting the solution into an Eppendorf tube, the RNA solution was extracted by 100 μ l of phenol/chloroform (10 g of phenol dissolved in 10 ml of chloroform) and 100 μ l of chloroform. The RNA was then precipitated by adding 7.5 μ l of 1 M-acetic acid and 500 μ l of 5 M-LiCl/ethanol (3:2, v/v) and incubating in ice/water for at least 2 h. The RNA was pelleted, washed in ethanol/NaCl as described above and dissolved in aqua dest. The RNA concentration was determined spectrophotometrically. RNA was precipitated by ethanol and stored at -70 °C.

Northern-blot analysis of EPO RNA was carried out as described by Eckardt *et al.* (1990), except that (1), for electrophoresis, 10–20 μ g of total RNA was loaded per lane, that (2) electrophoresis was carried out at 100 V for 1.5 h, that (3) the plasmid e49f (kindly provided by C. B. Shoemaker), containing a 1.2 kb human EPO cDNA insert, was used as an EPO-specific probe, which was labelled by ³²P to 2 × 10⁹ c.p.m./ μ g of DNA, and that (4) post-hybridization washing was carried out for 2 × 15 min in 0.1 × saline/sodium citrate (SSC; 1 × SSC is 0.15 M-NaCl/0.15 M-sodium citrate) at 54 °C. As a control the blot was rehybridized with α -tubulin cDNA.

RESULTS

EPO synthesis in presence of Zn²⁺

No stimulatory effect of Zn²⁺ on EPO synthesis in HepG2 cells could be detected, as determined by r.i.a. (Table 1). This is in

Fig. 1. Effect of Zn²⁺ on the level of EPO-specific RNA

Total RNA was isolated from HepG2 cells and probed for EPO-specific and for α -tubulin (α -TUB)-specific RNA by Northern-blot analysis. A 20 μ g sample of RNA was applied per lane. From right to left: lane 5, RNA from control cells grown under normoxia; 4, from cells treated with 50 μ M-CoCl₂; 3, from cells treated with 50 μ M-CoCl₂ plus 150 μ M-ZnCl₂; 2, from cells under hypoxia; and 1, from cells under hypoxia in the presence of 150 μ M-ZnCl₂.

agreement with results obtained with Hep3B cells (Goldberg *et al.*, 1988). However, Zn²⁺ was found to affect EPO synthesis that was stimulated by other metal ions or by hypoxia. In presence of 150 μ M-ZnCl₂ the increase in EPO synthesis induced by 50 μ M-CoCl₂ was completely abrogated. The increases induced by 300 μ M-NiCl₂ or hypoxia (1 % O₂) were inhibited by 73 or 73% respectively (Table 1). Even 50 μ M-ZnCl₂ was sufficient to cause a significant reduction of Co²⁺ and hypoxia-dependent EPO synthesis (Table 2). The decrease in EPO synthesis in the presence of Zn²⁺ was accompanied by a similar decrease in EPO mRNA as determined by Northern-blot analysis (Fig. 1).

These zinc-induced changes in EPO synthesis cannot be attributed to cytotoxic effects of this metal ion. This is indicated by the finding that ZnCl₂ did not adversely affect proliferation of HepG2 cells at concentrations up to 150 μ M (results not shown). Moreover, under hypoxia, where the proliferation rate was found to be strongly reduced, zinc even promoted cell growth. It was found that the addition of 75–150 μ M-ZnCl₂ led to a

Table 3. Effect of Cd²⁺ on hypoxia- and Co²⁺-induced EPO synthesis

Cells were grown for 3 days, either under normoxia or under hypoxia, and treated with 50 μM -CoCl₂ and/or various concentrations of CdCl₂ as indicated. As a positive control, cells were also incubated in presence of 100 μM -ZnCl₂. EPO synthesis was determined by r.i.a. Values are fold inductions of EPO synthesis. *Significantly different from corresponding control value (*t* test; *P* < 0.005; *n* = 4).

Normoxia							Hypoxia		
Co ²⁺	Co ²⁺ + 1.0 μM -Cd ²⁺	Co ²⁺ + 1.5 μM -Cd ²⁺	Co ²⁺ + 2.0 μM -Cd ²⁺	1.5 μM -Cd ²⁺	2.0 μM -Cd ²⁺	Co ²⁺ + 100 μM -Zn ²⁺	None	2.0 μM -Cd ²⁺	100 μM -Zn ²⁺
1.96 ± 0.06	1.92 ± 0.16	1.88 ± 0.13	1.84 ± 0.04	0.96 ± 0.08	0.92 ± 0.05	0.98* ± 0.07	3.15 ± 0.12	3.29 ± 0.15	1.83* ± 0.17

Table 4. Effect of iron on hypoxia-induced EPO synthesis in presence or absence of zinc

Cells were incubated for 3 days under hypoxia either in presence or absence of 150 μM -ZnCl₂ and treated with FeCl₃ ($\mu\text{g}/\text{ml}$ of medium) as indicated. EPO synthesis was determined by r.i.a. *Significantly different from corresponding control value (*t* test; *P* < 0.005; *n* = 4).

150 μM -ZnCl ₂	FeCl ₃ ...	Fold induction of EPO synthesis			
		0	11	22	33
Present		1.47 ± 0.06	1.73* ± 0.07	1.90* ± 0.06	2.05* ± 0.08
Absent		2.07 ± 0.07	3.27* ± 0.14	3.61* ± 0.22	3.19* ± 0.13

Table 5. Effect of iron on Co²⁺-induced EPO synthesis

After addition of 33 μg of FeCl₃ to each ml of culture medium, cells were grown for 3 days under normoxia in presence or absence of Co²⁺. EPO synthesis was determined by r.i.a. and the fold induction calculated. As controls, cells were also treated with Co²⁺ or Co²⁺ plus Zn²⁺. *Significantly different from value obtained with cobalt alone (*t* test; *P* < 0.005; *n* = 4).

50 μM -Co ²⁺	Fold induction of EPO synthesis		
	50 μM -Co ²⁺ + FeCl ₃	FeCl ₃	50 μM -Co ²⁺ + 150 μM -Zn ²⁺
1.78 ± 0.10	1.36* ± 0.04	1.15 ± 0.05	1.18* ± 0.05

significant increase of the final cell number by about 20% (*P* < 0.05; *t* test) when cells were grown for 3 days under hypoxia (not shown).

EPO synthesis in the presence of Cd²⁺

Because of similar properties cadmium is able to mimic the effect of Zn²⁺ on some biological processes. For example, HepG2 can be treated with 2 μM -CdCl₂ in place of 100 μM -ZnCl₂ to yield a similar fold induction of metallothionein-gene expression (Gedamu *et al.*, 1987). Therefore Cd²⁺ was tested for its ability to influence EPO synthesis in a Zn²⁺-like manner. However, CdCl₂ at concentrations of up to 2 μM failed to affect EPO synthesis under hypoxia and only slightly reduced EPO synthesis in Co²⁺-treated cells (Table 3). Cd²⁺ was not tested at higher concentrations because cell growth was then negatively affected.

EPO synthesis in the presence of iron

Iron seems to be essential for oxygen-dependent EPO regulation (Goldberg *et al.*, 1988). Zn²⁺ may interfere with the iron-dependent step. To study this, FeCl₃ was added to the culture

medium. Supporting the hypothesis, it resulted in a dose-dependent reduction of the Zn²⁺-dependent inhibition of hypoxia-induced EPO synthesis (Table 4). However, since iron was synergistic with hypoxia in inducing EPO synthesis (Table 4), it cannot be excluded that the effects of zinc and iron on EPO synthesis were independent of each other. On the other hand, addition of FeCl₃ also reduced the stimulatory effect of Co²⁺ (Table 5). This result and the finding that Zn²⁺ completely inhibited Co²⁺-induced EPO synthesis implies that the reduced effect of Zn²⁺ in the presence of iron more likely reflected interference of iron with the effect of zinc.

The bioavailable form of iron here may have been transferrin-bound iron or iron chelated by amino acids or albumin or the more soluble Fe²⁺ ion derived from Fe³⁺ by reduction. The Fe²⁺ concentration may vary depending on the oxygen concentration, possibly leading to significant differences in the effective iron concentration. Hence, the effects of iron on EPO synthesis can only be compared when the results were obtained under the same oxygen pressure, i.e. in the presence versus in the absence of Co²⁺ under normoxia and in the presence versus in the absence of Zn²⁺ under hypoxia.

DISCUSSION

The studies presented here demonstrate that, in the presence of Zn²⁺, hypoxia-, Co²⁺- and Ni²⁺-stimulated EPO synthesis in HepG2 cells was strongly reduced or abolished, whereas basal EPO synthesis remained unaffected. Like Co²⁺ and Ni²⁺ (Goldberg *et al.*, 1987, 1988), Zn²⁺ altered EPO synthesis mainly by affecting the level of EPO-specific mRNA. Since EPO synthesis seems to be mainly regulated on the RNA level by modulation of both the transcriptional activity (Goldberg *et al.*, 1989; Beru *et al.*, 1990; Costa-Giome *et al.*, 1990; Semenza *et al.*, 1991) and the mRNA stability (Rondon *et al.*, 1991), it implies that Zn²⁺ may have acted directly on the EPO regulatory mechanism. If so, the finding that Zn²⁺ also affected Co²⁺- and Ni²⁺-induced EPO synthesis may suggest that Zn²⁺, Co²⁺ and Ni²⁺ attack the same target, which may be the iron-binding site of a putative oxygen-sensing haem protein (Goldberg *et al.*, 1988). Consistent with this view we found that addition of iron reduced the Co²⁺-induced EPO synthesis and appeared also to reduce the inhibitory effect of Zn²⁺ on hypoxia-induced EPO synthesis.

The effect of iron on EPO synthesis was unexpected, since the intracellular content of iron is normally kept constant through a transferrin-receptor-regulatory mechanism (Owen & Kuhn, 1987; Casey *et al.*, 1988). Therefore cells might have been iron-depleted or, alternatively, they might have taken up iron by a transferrin-independent iron-transport system that is independent of cellular iron requirements (Sturrock *et al.*, 1990; Kaplan *et al.*, 1991).

Zn²⁺ might also have influenced stimulated EPO synthesis indirectly by acting on other cellular processes, such as metallo-

thionein synthesis, that are known to be inducible by Zn^{2+} . Higher levels of metallothionein might influence the availability of some metal ions for other cellular processes. However, Cd^{2+} , which also stimulated metallothionein synthesis, failed to inhibit EPO synthesis. Therefore it is unlikely that enhanced synthesis of this protein is mainly responsible for the observed effects of Zn^{2+} on EPO synthesis.

The inhibition of the hypoxia-induced EPO synthesis by Zn^{2+} might have been correlated with some changes of cellular metabolism due to hypoxic stress. Hypoxic stress might reduce the resistance of HepG2 cells to potentially toxic agents such as metal ions. However, at the concentrations used here, Zn^{2+} was found to have a rather positive effect on cells under hypoxia as indicated by enhanced cell proliferation in presence of Zn^{2+} . In addition, even low concentrations of Zn^{2+} were effective in reducing EPO synthesis.

An inhibition of haem biosynthesis would also lead to a strong reduction of EPO synthesis under hypoxia as found with Hep3B cells (Goldberg *et al.*, 1988). Some metal ions, including Zn^{2+} , Ni^{2+} and Co^{2+} , are known to be potential inhibitors of aminolaevulinate synthase (Iwasa *et al.*, 1989), the rate-limiting enzyme in the hepatic haem-biosynthetic pathway. However, *in vivo*, only Co^{2+} was capable of inhibiting haem biosynthesis in rat liver (De Matteis & Gibbs, 1977; Sinclair *et al.*, 1979).

Co^{2+} - and Ni^{2+} -induced EPO synthesis might have been indirectly affected by zinc in such a way that zinc interfered with the transport of these metal ions into the cell. In hepatocytes, to which HepG2 cells are closely related, two bivalent-cation inflow systems have been found, a basal one and a receptor-activated one, each of which has a broad range of specificity for metal ions, including Zn^{2+} , Ni^{2+} , Co^{2+} and Cd^{2+} (Crofts & Barritt, 1990). However, it is not yet known whether these transport systems allow one metal ion to interfere with the transport of another one. In contrast, such an interference could be demonstrated for the transferrin-independent ion ($\text{Fe}^{2+}/\text{Fe}^{3+}$)-transport system, which also seems to be capable of transporting other transition metal ions, such as Cd^{2+} , into the cell (Sturrock *et al.*, 1990). It was found that the non-transferrin iron uptake can be partially blocked by Cd^{2+} , by Mn^{2+} (Sturrock *et al.*, 1990) or by Zn^{2+} (Wright *et al.*, 1986) and that the Cd^{2+} import can be partially blocked by Fe^{3+} , Ni^{2+} or Mn^{2+} (Sturrock *et al.*, 1990). However, in neither case was a complete inhibition of the transport of one metal ion by another metal ion observed. Thus an inhibition of Co^{2+} or Ni^{2+} uptake may partially, but not fully, account for the effect of Zn^{3+} on Co^{2+} or Ni^{2+} -dependent EPO synthesis.

In conclusion, it seems that zinc affected stimulated EPO synthesis by acting directly on the EPO regulatory pathway rather than indirectly by influencing other cellular processes. As mentioned above, the present data suggest that Zn^{2+} , Co^{2+} and Ni^{2+} attack the same target involved in EPO regulation. If this target is the postulated haem protein, it would be interesting to clarify why, in contrast with Co^{2+} and Ni^{2+} , Zn^{2+} obviously inhibits this protein. As discussed above, it might have something to do with oxidoreductions in which the metal ion may be involved. Alternatively, Zn^{2+} might differ from the stimulating metal ions by the ligands to which it will preferentially bind in the

target protein, leading to a conformation that inactivates the protein.

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