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ORIGINAL ARTICLE

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Erythropoietin production: evidence for multiple oxygen sensing pathways

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Abstract The production of erythropoietin (Epo), the glycoprotein hormone which controls red blood cell formation, is regulated by feedback mechanisms sensing tissue oxygenation. The mechanism of the putative oxygen sensor has yet to be elucidated. There is evidence that at least two pathways participate in hypoxia signal transduction. One appears to involve a specific haem protein, and a second implicates reactive oxygen species (ROS). Iron catalyses the generation of intracellular ROS and therefore alters the cellular redox state. We have investigated the effect of modulating intracellular iron content on Epo production in Hep 3B cells. Iron chelation stimulates Epo production at 20% O_2 and enhances Epo production at 1% O_2, but it has no additive effect on cobalt-induced Epo production. Excess molar iron inhibited Epo production in response to hypoxia, desferrioxamine (DFO) and cobalt chloride and inhibited the DFO-enhancing effect of hypoxia-induced Epo production. We found that sulphydryl oxidising agents exert a differential inhibitory effect on hypoxia-induced versus DFO-induced Epo production, providing further evidence that multiple pathways of oxygen sensing exist.

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Introduction

Erythropoietin (Epo) is a prime regulator of the growth and differentiation of erythroid progenitor cells. It is produced in the fetal liver and adult kidneys, and its secretion is enhanced by hypoxia and suppressed by exposure to hyperoxia [3, 5] or by hypertransfusion [1]. Various other stimuli such as cobalt, nickel [10, 15] and the iron chelator desferrioxamine (DFO) [7, 8, 15, 18, 28] can induce Epo production independently of hypoxia.

Epo production is regulated via feedback mechanisms involving tissue oxygenation. The nature of the oxygen-sensing mechanism has been the subject of numerous investigations (for review, see [2]). Utilising the hepatoma cell line Hep 3B, which produces Epo in a regulated manner in response to hypoxia or cobalt, Goldberg and his colleagues [10] postulated that Epo production is regulated by pO_2-dependent conformational changes of a specific haem protein oxygen sensor. They proposed a model in which a common mechanism is invoked for the response to hypoxia and to cobaltous chloride [10]. Epo production is also sensitive to the intracellular redox state [6]. Under conditions of lowered oxygen tension the resulting decrease in hydrogen peroxide (H_2O_2) or related reactive oxygen species (ROS) levels would initiate a hypoxia signal transduction pathway [6, 26]. An H_2O_2-generating b-type cytochrome was postulated as a candidate oxygen sensor in Hep G2 cells with H_2O_2 as the signalling molecule [12].

Iron catalyses the generation of cellular ROS as predicted by the Fenton reaction and therefore influences the cellular redox state. We have investigated the effect of modulating intracellular iron content on Epo production in response to hypoxia and cobalt in Hep 3B cells. The effects of sulphydryl oxidising agents on hy-

poxia-induced and DFO-induced Epo production were also examined.

Materials and methods

Materials

Cell culture media, glutamine and foetal bovine serum were obtained from Gibco BRL, Paisley, Scotland, penicillin from Britannia Pharmaceuticals Ltd, Redhill, England and streptomycin from Evans Medical Ltd, Horsham, England. Cadmium chloride, diamide, N-ethylmaleimide (NEM) and sodium m-arsenite (SAR) were obtained from Sigma Chemical, Poole, England, ferrous ammonium sulphate (AnalaR grade) from BDH, Poole, England and cobaltous chloride from Aldrich Chemical Company, Gillingham, England. DFO was purchased from Ciba Laboratories, Horsham, England.

Cell culture

The hepatoma cell line was maintained by subculture in a-minimal Eagle's medium (MEM), supplemented with 10% (v/v) foetal bovine serum which had been heat-inactivated at 56 °C for 30 min, 10% nonessential amino acids, 2 m*M* l-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) in 25-cm^2 tissue culture flasks. The cells were maintained in a humidified incubator at 5% CO_2 at 37 °C. The cultures were split 1:6 once a week. The experiments were performed 3 days after the split, when cell cultures approached confluence. Fresh medium was added 24 h prior to each experiment. At the end of each experiment the cell count was checked in each flask and was generally in the range of $2.5–3.0 \times 10^5/cm^2$. Hypoxia was achieved by incubating Hep 3B cells in sealed boxes flushed for 30 min with a gas mixture of 1% O_2, 5% CO_2, and balance nitrogen (British Oxygen Company, Guildford, England). The boxes were maintained in an air incubator at 37 °C for 24 h.

In all experiments cellular toxicity was excluded by the absence of cell detachment and by the results of the trypan-blue exclusion test. Iron solutions up to 750 μ*M* are not cytotoxic to Hep 3B cells incubated for 24 h under both hypoxic and normoxic conditions [4]. Similarly, DFO is not cytotoxic at the concentrations used [7, 8, 15, 18, 28].

Radioimmunoassay for Epo

Epo was determined by radioimmunoassay [20] with some modifications. The method uses a disequilibrium system in which the addition of the labelled antigen is delayed for 3 days after the initial mixing of the test or standard Epo solutions with the anti-Epo antibody. Iodine 125-labelled recombinant human Epo with a specific activity of 300–900 Ci/mmol was obtained from Amersham International plc, Little Chalfont, England. Antibody to human Epo was obtained from Dr. Gerald Krystal, Terry Fox Laboratories, B.C. Cancer Research Center, Vancouver, B.C., Canada. The Multicalc program version 1.26 (Pharmacia Biotech Norden, Uppsala, Sweden) was used to generate a curve relating radioactivity in the precipitate to the concentration of Epo present in the standard samples and to calculate the Epo concentration in the test samples. The coefficient of variation for the assay is 8–10%.

Data analysis

Epo values are given as the mean ±SD. For each time point $n = 3–6$, as shown in the corresponding figures. Comparison between groups was performed using the analysis of variance T-test, utilising the SPSS for Windows Package, Release 6.1 (SPSS, Chicago, Ill., USA). The inhibition of stimulated Hep 3B cells is given as a percentage only when the inhibition is significant ($p < 0.05$).

Results

Experiments performed at 20% O_2

Incubation of Hep 3B cells in the presence of 130 μ*M* DFO resulted in a 16-fold increase in Epo production relative to the control, from 6 ± 2 mU/ml to 95 ± 13 mU/ml. The addition of 300 μ*M* ferrous ammonium sulphate inhibited the DFO stimulatory effect by 92 ± 4% (Fig. 1a).

Incubation of Hep 3B cells with 100 μ*M* $CoCl_2$ or 130 μ*M* DFO increased Epo levels approximately 10-to 12-fold, from 6 ± 1 mU/ml in the absence of either agent to 75 ± 13 mU/ml and 60 ± 6 mU/ml, respectively. Co-administration of 130 μ*M* DFO and 100 μ*M* $CoCl_2$ resulted in no additive effect, and the Epo level was 81 ± 24 mU/ml (Fig. 1b).

Experiments performed at 1% O_2

Hypoxia increased Epo production approximately 14-fold, from a value of 5 ± 2 mU/ml for the normoxic control to 72 ± 12 mU/ml at 1% O_2. Addition of 300 μ*M*

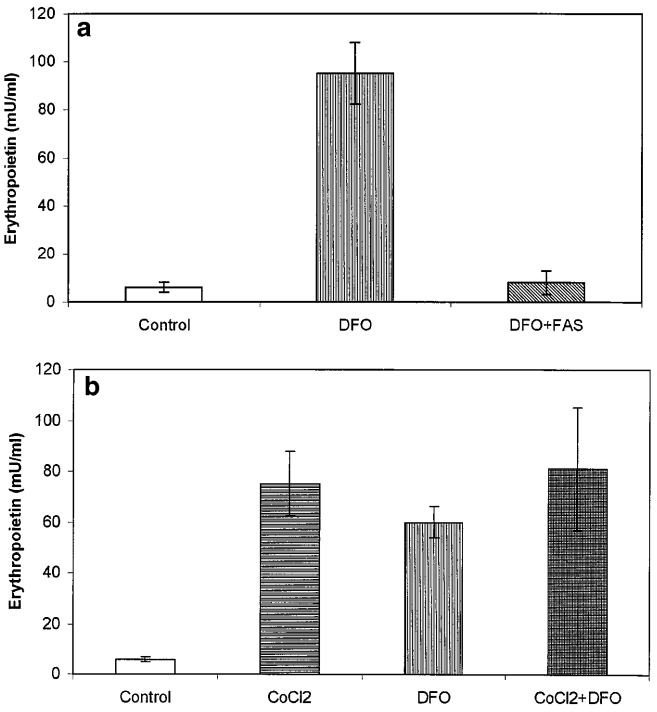

Fig. 1a,b. Lack of additive effect between desferrioxamine and cobalt. **a** DFO stimulates erythropoietin synthesis in Hep 3B cells. Cells were incubated for 24 h at 20% O_2 alone (control) or in the presence of 130 μ*M* DFO. The addition of 300 μ*M* FAS abrogated the stimulatory effect. Results are given as mean ±SD; for each value $n = 3$. **b** The stimulatory effects of cobalt and DFO on erythropoietin production in Hep 3B cells are not additive. Cells were incubated at 20% O_2 in the presence of 100 μ*M* cobaltous chloride ($CoCl_2$) or 130 μ*M* desferrioxamine (DFO) and in the presence of 100 μ*M* cobaltous chloride plus 130 μ*M* DFO. Results are given as mean ±SD; for each value $n = 3$

ferrous ammonium sulphate abrogated the hypoxia-induced Epo by 65 ± 11% (Fig. 2a).

The addition of 130 μ*M* of DFO to cells grown at 1% O_2 enhanced Epo production significantly ($p < 0.05$) compared with 1% O_2 alone. Epo levels increased by approximately 66%, from 85 ± 24 mU/ml at 1% O_2 alone to 141 ± 49 mU/ml at 1% O_2 plus 130 μ*M* DFO (Fig. 2). The addition of 300 μ*M* ferrous ammonium sulphate to cells incubated in the presence of 1% O_2 and 130 μ*M* DFO decreased Epo production significantly ($p < 0.05$) by 72 ± 13% (Fig. 2b).

The effect of sulphydryl reagents

The sulphydryl reagents diamide, SAR, NEM and cadmium chloride caused differential inhibition of hypoxia-induced versus DFO-induced Epo production in Hep 3B cells (see Table 1).

Discussion

These results show that altering the intracellular iron content of Hep 3B cells modulates Epo production.

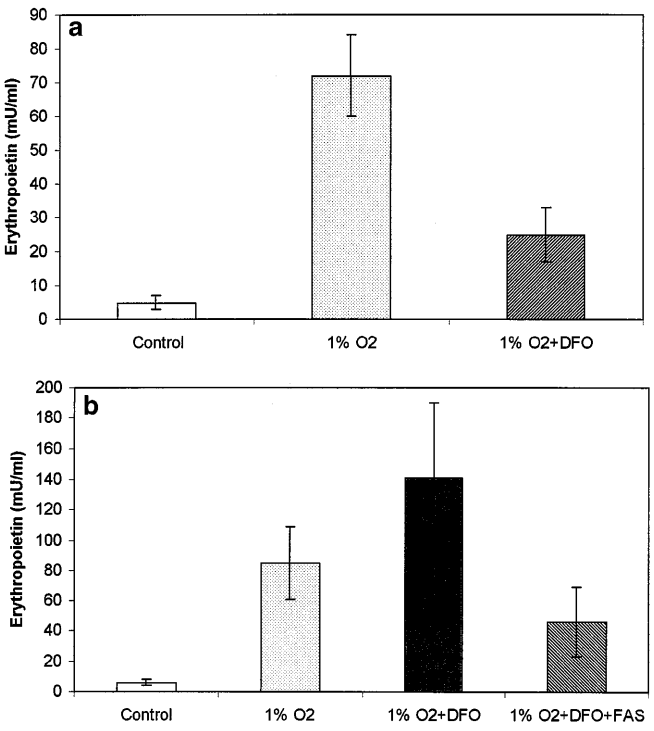

Fig. 2a,b. DFO enhances hypoxia-induced Epo production. **a** Hypoxia induces erythropoietin synthesis in Hep 3B cells. Cells were incubated for 24 h at 1% O_2 alone or in the presence of 130 μ*M* DFO. The addition of 300 μ*M* FAS abrogated the stimulatory effect. Results are given as mean ± SD; for each value $n = 3$. **b** Hypoxia-induced erythropoietin production is enhanced by DFO. Cells were incubated at 20% O_2 (control), or at 1% O_2 in the presence or absence of 130 μ*M* DFO. The addition of 300 μ*M* ferrous ammonium sulphate abrogated the DFO-enhancing effect. Results are given as mean ± SD; for each value $n = 6$

Table 1 The inhibitory effect of sulphydryl agents on hypoxia-induced versus desferrioxamine-induced Epo production in Hep 3B cells[a]

Additions	Percentage of inhibition[b]	
	1% O_2	Desferrioxamine
75 μ*M* diamide	77 ± 4	0
40 μ*M* SAR	92 ± 1	58 ± 11
50 μ*M* NEM	32 ± 15	0
75 μ*M* NEM	72 ± 6	44 ± 7
20 μ*M* $CdCl_2$	75 ± 5	54 ± 7
40 μ*M* $CdCl_2$	69 ± 12	44 ± 13

[a] Cells were incubated at 1% O_2 or with 130 μ*M* DFO in the presence or absence of sulphydryl modifying agents for 24 h. The Epo concentration was determined in the supernatant by radioimmunoassay and the percentage inhibition calculated.
[b] Results are given as mean ± SD; for each value $n = 3$.

Iron chelation stimulates Epo production at 20% O_2 and further enhances Epo production at 1% O_2, but it has no effect on cobalt-induced Epo production. Excess molar iron inhibits Epo production in response to hypoxia, DFO and cobalt and abrogates the DFO-enhancing effect on hypoxia-induced Epo production.

Iron readily crosses hepatoma cell membranes and raises non-haem cellular iron levels [4, 21, 22, 27], whereas DFO decreases cellular iron content by chelation [14, 22, 27]. Alterations in the intracellular iron content would be expected to cause parallel changes in intracellular ROS levels, according to the Fenton reaction, which in turn influences the intracellular redox state. Exogenous as well as increased levels of endogenous H_2O_2 inhibit EPO production in response to hypoxia [6]. We have found that various ROS-generating agents inhibit EPO production in response to hypoxia, cobalt and DFO (unpublished data). Recently, Fandrey and his colleagues [7] found that scavenging H_2O_2 by iron chelation stimulates Epo production, and they postulated that iron chelators reverse the inhibitory effect of H_2O_2 by interference with the Fenton reaction. Imagawa and his associates [17] have reported that H_2O_2 inhibits Epo gene expression by up-regulating the expression of the human GATA transcription factor. Other investigators have suggested that iron may exert its modulatory effect on EPO production by factors affecting Epo gene regulation [15, 18]. It is also possible that ROS inhibit Epo production by inactivating hypoxia-inducible factor 1 alpha (HIF-1α), presumably by the oxidation of sulphydryl groups [16]. The kinetics of HIF-1α induction paralleled the kinetics of Epo gene transcription in Hep 3B cells exposed to 1% O_2, $CoCl_2$ or DFO [28, 29, 30].

In the work presented here we have demonstrated that the sulphydryl oxidising agents SAR, which interacts with and blocks sulphydryl groups [25], and cadmium, a transition metal which perturbs redox-sensitive pathways [25], inhibited DFO-induced Epo production less markedly than hypoxia-induced Epo production (Table 1). When NEM, which alkylates and specifically

blocks sulphydryl groups [13], and the diazene carbonyl derivative diamide, which catalyses the oxidation of free sulphydryl groups [19], were utilised the differential inhibitory effect was more pronounced. Our data indicate that a thiol-sensitive factor is involved in the hypoxia-signal transduction pathway but is probably not involved in the mechanism related to iron deprivation. Such a factor could be involved in Epo mRNA turnover. Rondon and his associates [24] have demonstrated that sulphydryl oxidising and reducing agents modify the binding activity of a cytosolic Epo mRNA-binding protein which influences Epo mRNA turnover [23].

Consistent with other investigators [6, 7, 10, 11], we chose to assay the mass of the Epo protein produced rather than Epo mRNA levels. A subtle increase in Epo mRNA level which may not be readily detected by Northern blotting or ribonuclease protection assay analysis may still be translated into a several-fold increase in secreted hormone. Ultimately, in vivo the biological response to changes in cellular oxygenation is effected by the circulating Epo protein acting as the prime humoral regulator of erythropoiesis.

Our data support the view that alteration of the intracellular redox state influences Epo production. The additive effect of DFO on hypoxia-induced but not cobalt-induced Epo production and the differential inhibitory effect of sulphydryl oxidising agents on hypoxia versus DFO-induced Epo production, shown in this study, argue for the existence of multiple oxygen-sensing pathways. This conclusion is supported by the recent observations that iodonium compounds such as diphenylene iodonium exert differential inhibitory effects between hypoxia-induced and cobalt- or DFO-induced Epo production in Hep 3B cells [9, 11].

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