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Biochimica et Biophysica Acta 1495 (2000) 231–236

BIOCHIMICA ET BIOPHYSICA ACTA

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Erythropoietin induction in Hep3B cells is not affected by inhibition of heme biosynthesis

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Received 5 October 1999; received in revised form 21 December 1999; accepted 23 December 1999

Abstract

Erythropoietin (Epo) is one of the physiologically important genes whose transcription is up-regulated by hypoxia. Our laboratory previously proposed that the sensor of this event is a heme protein which turns over rapidly. We have investigated the effects of four inhibitors of heme synthesis (4,6-dioxoheptanoic acid (DHA), isoniazid (INH), *N*-methyl protoporphyrin IX (MPP), and deferoxamine mesylate (DSF)) on hypoxia-, cobalt-, and DSF-induced erythropoietin (Epo) mRNA expression, heme biosynthesis, and cell viability in Hep3B cells. DHA (0.1–1.0 mM) inhibited heme biosynthesis more than 85%, but did not suppress Epo mRNA expression. Epo mRNA expression was inhibited only at higher concentrations of DHA (2, 4 mM) which also inhibited cell viability. No suppression of Epo mRNA expression by INH was observed at doses known to inhibit heme biosynthesis. MPP did not suppress Epo mRNA expression although it showed an inhibitory effect on heme biosynthesis without any decreased cell viability. 130 μM DSF, a dose which inhibited heme biosynthesis without cell toxicity, suppressed hypoxia-induced Epo mRNA expression, but enhanced cobalt-induced Epo mRNA expression. These results show that although the oxygen sensor is probably a heme protein it does not turn over rapidly. Therefore, cobalt is unlikely to act by substituting for heme iron.

Keywords: Erythropoietin; Heme; Hypoxia; Oxygen sensing

1. Introduction

Almost all living organisms from bacteria to human beings utilize oxygen in order to acquire efficient chemical energy. Therefore, the survival of cells and organisms depends upon some type of sensitive oxygen sensing system. In fact a number of physiologically important genes are induced by hypoxia. These include erythropoietin (Epo), tyrosine hydroxylase, vascular endothelial growth factor, and genes involved in glucose metabolism [1]. Currently there is considerable interest in the mechanism of oxygen sensing and transduction of signal that effects expression of these and other oxygen responsive genes.

Epo, a glycoprotein hormone produced in the kidney and liver, regulates erythrocyte production. Epo production is strongly induced not only by hypoxia, but also by certain divalent metals including cobalt (Co^{2+}), nickel, and manganese. The expression of

Abbreviations: DHA, 4,6-dioxoheptanoic acid; INH, isoniazid; DSF, deferoxamine mesylate; MPP, *N*-methyl protoporphyrin IX; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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0167-4889/00/$ – see front matter
PII: S0167-4889(99)00169-X

Epo in human hepatoma cell lines such as Hep3B cells or HepG2 cells [2] has frequently been used as an effective model for studying the oxygen sensor. Goldberg et al. [3] have proposed that oxygen is sensed by binding to a heme protein with a resulting change in conformation. In oxygenated cells, the oxy form does not stimulate Epo induction whereas, at low oxygen tension, the deoxy form triggers increased expression of the Epo gene. In this model, the ability of cobalt to simulate hypoxia was explained by the substitution of Co^{2+} for ferrous iron in the porphyrin ring, thereby locking the heme protein in the deoxy conformation just as at low oxygen tension.

This heme protein model was supported by evidence that two heme synthesis inhibitors, 4,6-dioxoheptanoic acid (DHA, succinylacetone) and deferoxamine mesylate (DSF), reduced hypoxia- or Co^{2+}-induced Epo induction in Hep3B cells [3]. DHA is a potent and specific inhibitor of δ-aminolevulinic acid (ALA) dehydratase, the enzyme which converts two molecules of ALA to porphobilinogen. However, the concentration of DHA that was used (2 mM) is considerably higher than that previously shown to inhibit ALA dehydratase activity and heme biosynthesis [4–6]. Therefore, the reduced Epo production in Hep3B cells treated with DHA reported by Goldberg et al. might be due to a toxic effect on cells rather than its inhibitory effect on heme biosynthesis. In fact, Srinivas et al. [7] recently reported that DHA, at a concentration sufficient to inhibit heme synthesis, failed to blunt hypoxic induction of a reporter gene. In order to resolve this issue it is necessary to develop dose–response relationships to determine the impact of heme synthesis inhibitors on Epo induction versus cell viability. In this paper we tested not only DHA but also three additional heme synthesis inhibitors: isoniazid (INH), *N*-methyl protoporphyrin IX (MPP) and DSF, and have monitored the effects of these inhibitors on Epo mRNA expression, heme biosynthesis and cell viability in Hep3B cells.

2. Materials and methods

2.1. Cell culture

Hep3B cells were maintained in α-modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) at 37°C. At the initiation of each experiment, cells were resuspended in the medium at a density of 2.5×10^5/ml and after preincubation for 24 h, heme synthesis inhibitors, DHA, INH, DSF and MPP were added and the incubation was continued for another 24 h. DHA, INH and DSF were purchased from Sigma, and MPP was obtained from Porphyrin Products. Cells were cultured either under normoxic conditions (95% air and 5% CO_2), or, in some experiments, were exposed to hypoxia (1% O_2, 94% N_2, and 5% CO_2) in an Espec BNP-210 incubator.

2.2. Ribonuclease protection analysis

We used a previously described [8] construct (pGRm) in which a 203 bp long DNA fragment of genomic human Epo (GenBank accession number M11319, nucleotides 2565–2767) was inserted into pGEM3Zf(+). The Epo gene in this construct has been 'marked' by site-directed mutagenesis at three positions in the coding region of Exon 5 [8]. As a result, after hybridization of this marked riboprobe to endogenous Epo mRNA, RNase digestion yields two major fragments of 75 bp and 61 bp. RNA was purified from Hep3B cells by using TRI ZOL Reagent (Gibco BRL). The concentrations of total RNA was calculated by measuring the absorbance at 260 nm and 280 nm. The intactness of each RNA sample was confirmed by visualization on a UV transilluminator after running small aliquots on a 1% agarose gel. Sixty μg of each RNA sample was mixed with Epo riboprobe labeled by α-[^{32}P]CTP (NEN) and hybridized at 55°C overnight. The hybridized RNAs were digested by RNase and electrophoresed in 10% polyacrylamide gel as described previously [8]. The dried gel was analyzed by a phosphoimager (Molecular Dynamics).

2.3. Assay of heme biosynthesis

The newly synthesized heme in Hep3B cells was measured as described by Shedlofsky et al. [9]. After preincubation in 3.5-cm culture dishes, Hep3B cells were further incubated for 24 h with the heme precursor δ-[^{14}C]ALA (NEN) (0.2 μCi/dish). Cold δ-ALA (20 μM) was also added to the culture medium as the carrier for δ-[^{14}C]ALA. At the end of incu-

bation, the total heme was extracted from the cells by using acetone–HCl and diethyl ether. With the exception of experiments testing the effects of INH, the incorporation of radioactivity into the extracted heme provided a measure of heme biosynthesis.

2.4. Cell viability assay

We used a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay for evaluating cell viability [10]. One-tenth volume of MTT solution (Sigma) was added to the media of cultured cells and incubated at 37°C for 1 h. At the end of incubation period the media was removed from the culture plate and the converted dye was solubilized with DMSO. Cell viability was determined from the absorbance of the dye at 570 nm with background subtraction at 630 nm.

3. Results

We treated Hep3B cells for 24 h in a normoxic atmosphere with four types of heme synthesis inhibitors, DHA, INH, MPP and DSF. We then cultured the cells under either hypoxia, or in the presence of 100 μM Co^{2+}, or 130 μM DSF for 6 h. We determined Epo mRNA expression in these cells by ribonuclease protection assay (RPA) and measured heme biosynthesis and viability of the cells.

3.1. DHA

As shown in Fig. 1A, 100 μM and 1 mM of DHA inhibited heme biosynthesis in Hep3B cells more than 85%, consistent with previous reports [4–6]. However, 1 mM of DHA suppressed hypoxia-, Co^{2+}- and DSF-induced Epo mRNA expression by no more than 20% (Fig. 1B). We observed strong diminution of Epo mRNA expression by DHA only at higher concentrations (2 and 4 mM). This result is also consistent with previously reported results on the inhibition of Epo production, assayed by RIA, at 2 mM DHA [3]. However, cell viability was clearly decreased at these higher doses of DHA by morphologic observation (not shown) as well as by MTT assay (Fig. 1C). These results indicate that the suppression of Epo mRNA induction by DHA at

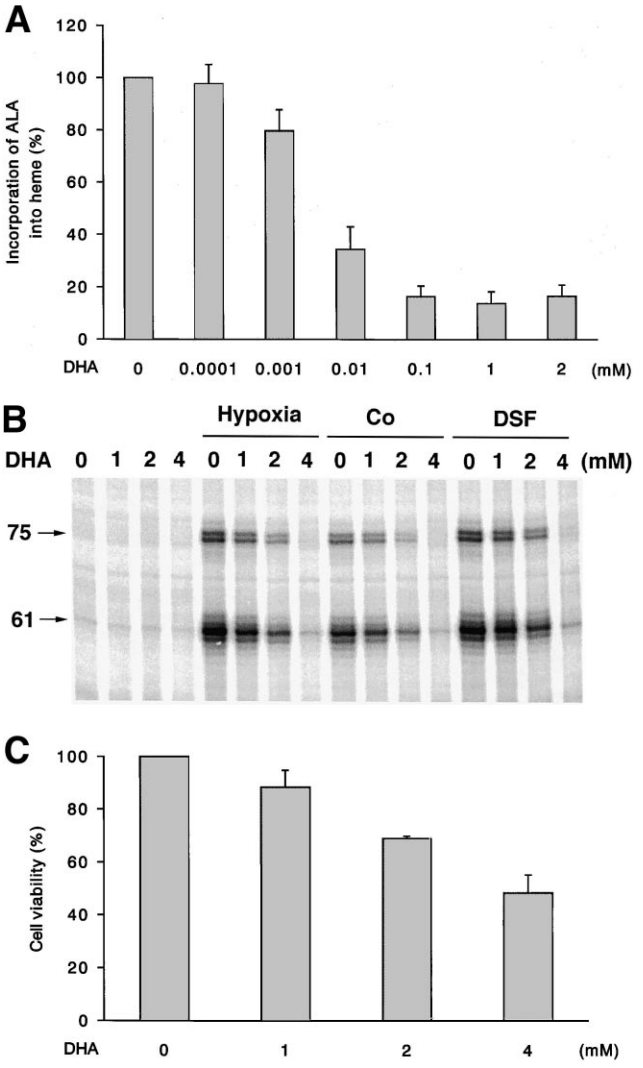

Fig. 1. Effect of DHA on heme biosynthesis (A), Epo mRNA induction (B) and cell viability (C). Hep3B cells were treated with 4,6-dioxoheptanoic acid (DHA) at the indicated doses of DHA for 24 h followed by a 6-h exposure to either hypoxia (1% O_2), 100 mM cobalt (Co^{2+}), or 130 mM deferoxamine mesylate (DSF). Heme synthesis was measured by incorporation of [^{14}C]ALA into heme. RNase protection assay was performed to show the two bands of protected Epo mRNA at 75 bp and 61 bp. Results in panel B are representative of three independent experiments. The data in panels A and C are expressed as mean ± S.D. of three independent experiments.

2 mM was not due to inhibition of heme biosynthesis, but rather to cell toxicity.

3.2. INH

INH inhibits pyridoxal phosphate, an essential co-

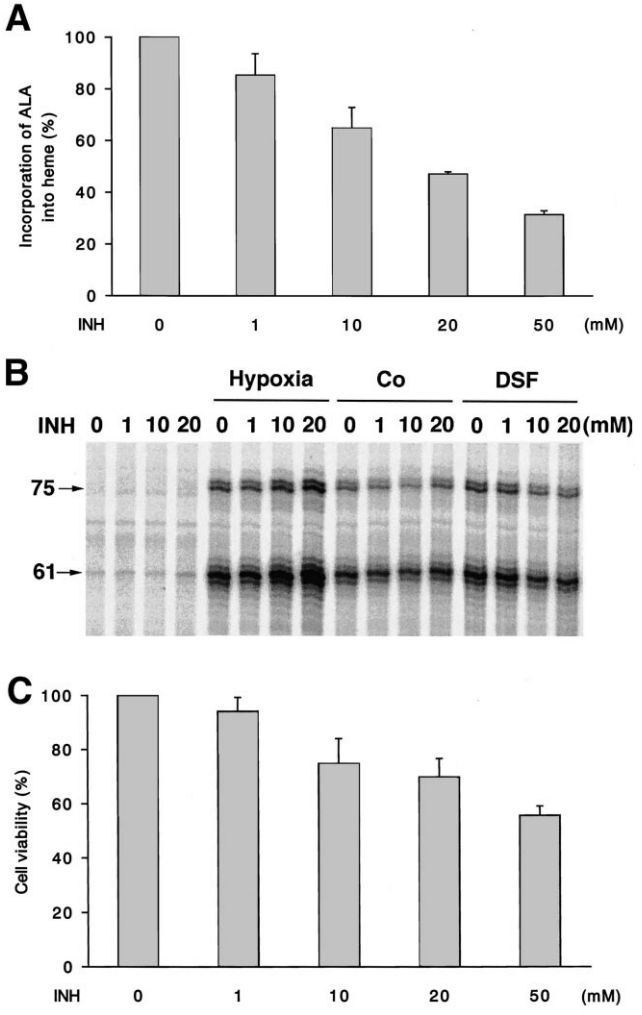

Fig. 2. Effect of INH on the incorporation of [^{14}C]ALA into heme (A), Epo mRNA induction (B) and cell viability (C). Cells were treated with isoniazid at the indicated doses for 24 h. Experimental conditions were as described in the legend to Fig. 1.

factor of ALA synthase. In rabbit reticulocytes, 1 mM INH causes a 70% suppression of incorporation of ^{59}Fe into heme [11]. In Hep3B cells, 1–20 mM INH had no effect on the induction of Epo mRNA by hypoxia, cobalt or DSF (Fig. 2B). Because of its site of inhibition, INH would not be expected to affect the incorporation of [^{14}C]ALA into heme. The modest suppression which we observed at high concentrations of INH (10, 20, 50 mM) (Fig. 2A) is probably a non-specific toxic effect since cell viability was also impaired to about the same extent (Fig. 2C).

3.3. MPP

MPP is a powerful inhibitor of ferrochelatase, the final enzyme in the heme biosynthetic pathway, catalyzing the insertion of ferrous iron into protoporphyrin IX to form protoheme [12,13]. As shown in Fig. 3, in Hep3B cells, MPP, at concentrations ranging from 0.05 μg/ml up to 5 μg/ml (0.09–9 μM), had a dose-dependent inhibitory effect on heme biosynthesis without any cell toxicity. Even at 80% inhibition observed with the highest dose (5 μg/ml), MPP had no effect on cell viability or on induction of Epo

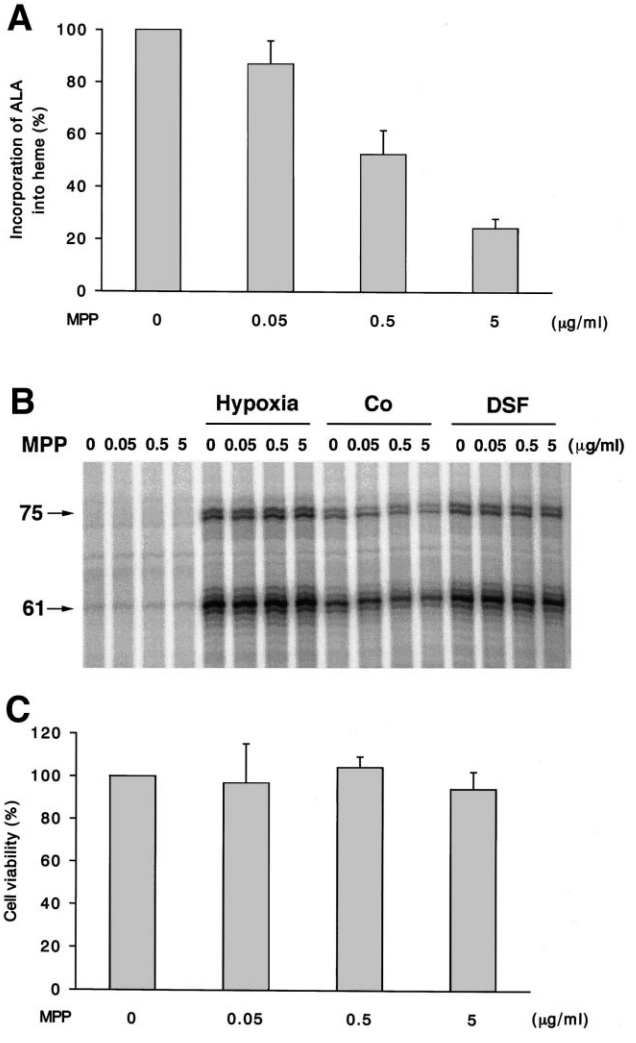

Fig. 3. Effect of MPP on heme biosynthesis (A), Epo mRNA induction (B) and cell viability (C). Cells were treated with MPP at the indicated doses for 24 h. Experimental conditions were as described in the legend to Fig. 1.

mRNA by hypoxia, Co^{2+} or DSF. These results again show that Epo can be robustly induced despite strong inhibition of heme biosynthesis.

3.4. DSF

DSF, an iron-chelating agent, is an inducer of Epo [14]. DSF is also an inhibitor of heme synthesis, preventing protoporphyrin from being converted to heme by removing the iron that is normally incorporated into the porphyrin ring [15,16]. DSF showed dose-dependent inhibition of heme biosynthesis (Fig. 4A) without obvious cell toxicity (Fig. 4C). At the highest concentration tested (130 μM), DSF inhibited hypoxia-induced Epo mRNA expression by about 80% (Fig. 4B), consistent with earlier measurements of Epo protein [2]. However, DSF had no effect on the induction of Epo mRNA by cobalt.

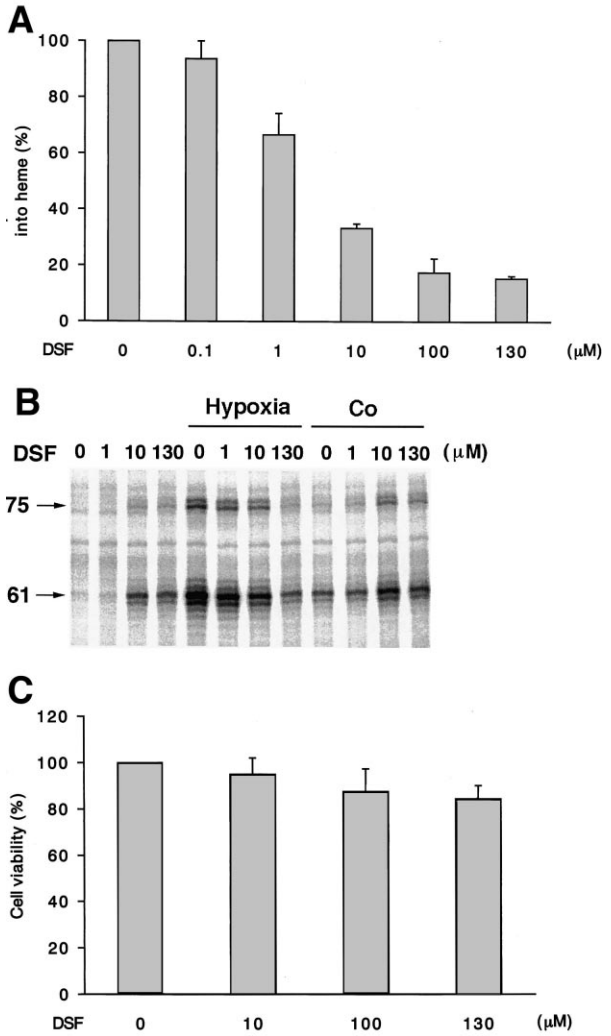

Fig. 4. Effect of DSF on heme biosynthesis (A), Epo mRNA induction (B) and cell viability (C). Cells were treated with DSF at the indicated doses for 24 h. Experimental conditions were as described in the legend to Fig. 1.

4. Discussion

In these experiments, Epo mRNA was monitored as an index of cellular response to hypoxia. This induction of Epo mRNA depends on an intact and functional oxygen sensing and signaling pathway. We have investigated the role of heme synthesis in this process by using four compounds having different inhibitory mechanisms. We could not observe any relationship between suppression of Epo induction and heme biosynthesis inhibition. We previously reported that a heme synthesis inhibitor, DHA, reduced hypoxia- and Co^{2+}-induced Epo production in Hep3B cells [3]. However, in Fig. 1 we present evidence that DHA suppresses Epo induction by virtue of cell toxicity rather than through inhibition of heme synthesis.

Among the four heme inhibitors, only DSF showed a dose-dependent suppressive effect on hypoxia-induced Epo mRNA expression with intact cell viability. In contrast, MPP, which, like DSF, blocks conversion of protoporphyrin to heme, had no effect on Epo induction. Because DSF has contradictory effects on Epo induction by hypoxia versus cobalt, interpretation of this result is difficult. Through chelating iron, DSF can affect not only heme synthesis but also Fenton chemistry which is thought to play an important role in hypoxic signaling, upon which Epo induction depends [1]. Our finding that DSF suppresses hypoxic induction of Epo but has no effect on Co^{2+} induction is difficult to reconcile with recent models of oxygen sensing and signal transduction.

In 1988, Goldberg et al. proposed that Co^{2+} and other transition metals (Ni^{2+} and Mn^{2+}) induced Epo expression by substituting for the iron atom in a heme protein which they proposed to be the oxygen sensor. This suggestion was based on the fact that these substituted metalloporphyrins either have a very low affinity for oxygen (Co^{2+}-heme) or fails to bind oxygen at all (Ni^{2+}-heme, Mn^{2+}-heme). This

proposed mechanism for the induction of Epo by these transition metals implies, and, in fact, requires, that the heme protein oxygen sensor turns over very quickly to enable the Co^{2+}-heme (or Ni^{2+}-heme and Mn^{2+}-heme) to fully replace the Fe^{2+}-heme. The fact that inhibition of heme synthesis fails to suppress Epo induction argues strongly that the postulated heme protein oxygen sensor turns over relatively slowly and therefore Co^{2+}, Ni^{2+} and Mn^{2+} do not function by substituting for the heme iron. It is far more likely that these transition metals act by suppressing O_2 and Fe^{2+} catalyzed oxidation of a protein involved in the signaling pathway ([17,18]; W. Willmore, H.F. Bunn, unpublished observations).

Our results provide insight into the mechanism by which certain transition metals such as Co^{2+} mimic hypoxia, but they in no way negate the contention that the oxygen sensor is a heme protein. The fact that nitric oxide [19–21] and carbon monoxide, at high concentration [3,20,21], suppresses hypoxic induction of Epo and the hypoxia-inducible transcription factor HIF-1 is consistent with the oxygen sensor being a heme protein.

Acknowledgements

We thank Drs. Peter Sinclair and Prem Ponka for their helpful suggestions.

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