Desferrioxamine Induces Erythropoietin Gene Expression and Hypoxia-Inducible Factor 1 DNA-Binding Activity: Implications for Models of Hypoxia Signal Transduction

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Erythropoietin (EPO) gene transcription is activated in kidney cells in vivo and in Hep3B cells exposed to hypoxia or cobalt chloride. Hypoxia-inducible factor 1 (HIF-1) is a nuclear factor that binds to the hypoxia-inducible enhancer of the EPO gene at a site that is required for transcriptional activation. HIF-1 DNA-binding activity is induced by hypoxia or cobalt chloride treatment of Hep3B cells. We report that treatment of Hep3B cells with desferrioxamine (DFX) induced HIF-1 activity and EPO RNA expression with kinetics similar to the induction of HIF-1 by hypoxia or cobalt chloride. Induction by each of these stimuli was inhibited by cycloheximide, indicating a requirement for de novo protein synthesis. DFX appears to induce HIF-1 by chelating iron as induction was inhibited by coadministration of ferrous ammonium sulfate. DFX administration to mice transiently increased EPO RNA levels in the kidney. As previously shown for hypoxia and cobalt treatment, DFX also induced HIF-1 activity in non-EPO-producing cells, suggesting the existence of a common hypoxia signal-transduction pathway leading to HIF-1 induction in different cell types.

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vate, 2 mmol/L L-glutamine, and 1 mmol/L nonessential amino acids. Chinese hamster ovary (CHO) cells were grown in α-modified minimal essential medium. Media were supplemented with 10% heat-inactivated fetal calf serum, 50 μM/mL penicillin, and 50 μg/mL streptomycin (GBHC Grand Island, NY). Hypoxia was induced by placing cells in a modular incubator chamber flushed with a gas mixture consisting of 1% O2/5% CO2/balance N2. CoCl2, CHX, desferrioxamine mesylate, and ferrous ammonium sulfate (FAS; Sigma) were prepared as aqueous solutions. Hemin (US Biochemicals, Cleveland, OH) was dissolved in 0.5 M NaOH, neutralized with 1 mol/L Tris HCl (pH 7.8), and diluted with water.25 Cells were harvested, nuclear extracts and DNA probe were prepared, and EMSA was performed as described.16 Sequences of the double-stranded oligonucleotide used as probe in the EMSA are 5'-agtGCGTACGTACGTACG-3' in which EPO sequences are in lowercase type and artificial cloning sequences are in lowercase type.

RNA isolation and analysis. Cytoplasmic RNA was isolated28 from cells used for nuclear extract preparation. Human EPO RNA was quantitated by ribonuclease protection assay using plasmid mEP02 (provided by S. Schuster, Jefferson Medical College, Philadelphia, PA), which contains a 462-bp mouse EPO cDNA fragment and when transcribed by T7 RNA polymerase, generates a 482-bp antisense RNA probe. Hybridization was performed in 80% formamide/40 mmol/L PIPES (pH 6.7)/0.4 mol/L NaCl/1 mmol/L EDTA for 3 hours at 65°C. The reaction products were digested with ribonuclease A and T1 and analyzed by 8 mol/L urea/8% polyacrylamide gel electrophoresis.29

Animal studies. Six-month-old outbred female mice were studied. DFX mesylate, dissolved in phosphate-buffered saline, was administered by intraperitoneal injection at a dose of 200 mg/kg. Hypoxia was induced by exposing cells to 130 pmol/L DFX for 24 hours. The cells were maintained in 20% O2 (lane 3) nor did it affect induction of HIF-1 in 20% O2 (lane 4). Treatment of cells with 130 μmol/L DFX alone induced HIF-1 DNA-binding activity (Fig 1B, lane 3), generating a complex with electrophoretic mobility similar to the HIF-1 activity detected in nuclear extracts from CoCl2-treated cells. Moreover, the effect of 1% O2 + DFX (lane 4) was no greater than DFX alone. HIF-1 DNA-binding activity induced by treatment of cells with DFX showed the same sequence specificity as HIF-1 induced by hypoxia (data not shown). There was no effect on HIF-1 activity detected by EMSA when nuclear extracts from hypoxic or nonhypoxic Hep3B cells were incubated with probe in the presence of DFX at concentrations ranging from 13 μmol/L to 13 mmol/L (data not shown), indicating that DFX does not act directly on HIF-1.

Treatment with 130 μmol/L DFX also induced EPO RNA in the same cells (Fig 2B, lane 3) and the effect of 1% O2 + DFX (lane 4) was no greater than 1% O2 alone. In fact, the level of EPO RNA in cells treated with 1% O2 + DFX (lane 4) was lower than in cells exposed to 1% O2 alone (lane 2), suggesting that prolonged exposure to DFX may have had adverse effects on cellular RNA metabolism. This hypothesis is supported by the following observations: (1) EPO RNA expression was greater in cells treated with DFX for 6 hours (lane 6) compared with cells treated with DFX for 24 hours (lane 3). Note that the results shown are not directly comparable because different amounts of total RNA were analyzed (8 and 12 μg in lanes 3 and 6, respectively). However, when the results were corrected for the difference in RNA loaded, there was still a reduction in the amount of EPO RNA detected at 24 hours compared with that detected at 6 hours. (2) Recovery of cytoplasmic RNA from cells treated with DFX for 24 hours was reduced by 60% compared with untreated cells incubated in 1% or 20% O2. Ribonuclease protection analysis of exon 1 (not shown) indicated that EPO RNA induced by DFX was initiated at the same site as EPO RNA induced by 1% O2 or CoCl2.

To determine whether HIF-1 activity is regulated directly by intracellular heme concentrations, Hep3B cells were exposed to 40 μmol/L hemin for 6 hours in the presence of 20% or 1% O2 (Fig 1C). Hemin did not induce HIF-1 activity in 20% O2 (lane 3) nor did it affect induction of HIF-1 in 1% O2 (lane 4). EPO RNA levels in the same cells were unaffected by 40 μmol/L hemin (Fig 2A, lanes 5 through 8). Hemin also had no effect on HIF-1 activity when added directly to nuclear extracts from hypoxic or nonhypoxic cells before EMSA (data not shown).

The kinetics of HIF-1 induction by DFX were investigated by exposing cells to 130 μmol/L DFX for 15 minutes,
1 hour, or 4 hours before nuclear extract preparation (Fig 1D). HIF-1 activity increased progressively through 4 hours, at which time HIF-1 activity was similar to that seen in extracts from cells treated with DFX for 24 hours (compare Fig 1D, lane 4 and Fig 1B, lane 3). The kinetics of HIF-1 induction by DFX appear identical to the kinetics of HIF-1 induction by \( \alpha \% \) \( O_2 \) and by 75 \( \mu \)mol/L \( CoCl_2 \) as previously reported.\(^{22,30}\)

To show that the effects of DFX on EPO RNA levels and HIF-1 DNA-binding activity in Hep3B cells were mediated by chelation of iron, Hep3B cells were exposed to 130 \( \mu \)mol/L DFX in the presence of 300 \( \mu \)mol/L FAS (Fig 1E). FAS blocked the DFX-mediated induction of HIF-1 (compare lane 4 with lane 2). In contrast, FAS had no effect when added directly to nuclear extracts immediately before EMSA, at concentrations ranging from 10 \( \mu \)mol/L to 10 mmol/L (data not shown).

The data presented thus far are consistent with the hypothesis that DFX induces HIF-1 activity and EPO RNA by the same signal transduction pathway activated by \( \alpha \% \) \( O_2 \) and \( CoCl_2 \). To provide further evidence for this hypothesis, Hep3B cells were pretreated with 100 \( \mu \)mol/L CHX for 2 hours and then exposed to 130 \( \mu \)mol/L DFX or solvent alone for 4 hours in the continued presence of 100 \( \mu \)mol/L CHX. The induction of HIF-1 activity (Fig 1F) and EPO RNA (Fig 2B, lanes 5 through 8) by DFX was blocked by treatment of cells with CHX. These results suggest that HIF-1 induction by DFX requires de novo protein synthesis, as previously shown for induction of HIF-1 by \( \alpha \% \) \( O_2 \) and by \( CoCl_2 \).

Because DFX is used to treat iron overload resulting from transfusion therapy for \( \beta \)-thalassemia major, we were interested in determining whether DFX treatment induced EPO RNA in vivo. A mouse was administered a 200 mg/kg intraperitoneal injection of DFX and killed 22 hours later. Total RNA was isolated from kidney along with similar preparations from an untreated control mouse and a mouse made hypoxic by exposure to 5\% \( O_2 \) for 6 hours. Mouse EPO RNA was quantitated by ribonuclease protection assay using an antisense RNA probe complementary to 462 nt of mouse EPO RNA (Fig 3). Barely detectable levels of EPO RNA were present in the control mouse kidney (lane 1), whereas EPO RNA was readily detectable in samples from the hypoxic (lane 2) and DFX-treated (lane 3) mice. DFX was also administered to a mouse daily for 5 days. EPO RNA levels in the kidneys of this mouse (lane 4) were similar to those in the untreated control mouse (lane 1), suggesting that high-level induction of EPO RNA by DFX is transient.

We have shown that \( \alpha \% \) \( O_2 \) and 75 \( \mu \)mol/L \( CoCl_2 \) each induce HIF-1 DNA-binding activity in non-EPO-producing cells.\(^{22}\) To determine whether DFX is also a general inducer of HIF-1, we analyzed extracts prepared from Hep3B and CHO cells exposed to 130 \( \mu \)mol/L DFX for 6 hours in the presence of 20\% or 1\% \( O_2 \) (Fig 4). HIF-1 activity was also induced by DFX in CHO cells (lane 7) and the effect of \( 1\% O_2 + DFX \) (lane 8) was no greater than \( 1\% O_2 \) alone (lane 5). Similar results were obtained with nuclear extracts from Hep3B cells treated in parallel (lanes 1 through 4).

**DISCUSSION**

Further evidence for involvement of HIF-1 in the activation of EPO gene transcription. Several observations\(^{16,22,30}\)
INDUCTION OF EPO AND HIF-1 BY DFX

Fig 2. EPO RNA expression in Hep3B cells. Cells were treated as described below, and cytoplasmic RNA was isolated and analyzed by ribonuclease protection assay. The protected fragment corresponding to exon 2 of the human EPO gene is shown. (A) Cells were incubated in 20% O₂ (~ hypoxia) or 1% O₂ for the last 4 hours of treatment (~ hypoxia) in the presence (~) or absence (~) of 75 μmol/L CoCl₂ for 4 hours (lanes 1 through 4) or 40 μmol/L hemin for 6 hours (lanes 5 through 8). Fifteen micrograms (lanes 1 through 4) or 20 μg (lanes 5 through 8) of total RNA was analyzed. (B) Cells were incubated in 20% O₂ (~ hypoxia) or 1% O₂ for the last 4 hours of treatment (~ hypoxia) in the presence (~) or absence (~) of 130 μmol/L DFX for 24 hours (lanes 1 thru 4) or were incubated in 20% O₂ in the presence (~) or absence (~) of 130 μmol/L DFX and 100 μmol/L CHX for 6 hours (lanes 5 through 8). Eight micrograms (lanes 1 through 4) or 12 μg (lanes 5 through 8) of total RNA was analyzed.

Fig 3. EPO RNA expression in mouse kidney. Mice were treated as follows: lane 1, untreated control (C); lane 2, 5% O₂ for 6 hours as hypoxic stimulus (H); lane 3, one dose of DFX (22 hours before analysis); lane 4, 6 daily doses of DFX (last dose 8 hours before analysis). Total RNA isolated from kidney, 150-μg aliquots, was analyzed by ribonuclease protection assay.

DFX was reported to decrease EPO production by Hep3B cells cultured in 1% O₂ and the inhibitory effect of DFX was reduced by coaddition of DFX + FAS significantly, the effect of DFX alone (ie, in 20% O₂) was not reported. In those experiments, Hep3B cells were treated required for hypoxia-inducible enhancer function and the binding site is conserved in the mouse EPO enhancer; and (5) inducers of EPO transcription (1% O₂ and CoCl₂) induce HIF-1 activity with similar kinetics.

In this report we have shown that DFX also induces EPO RNA expression and HIF-1 DNA-binding activity. The kinetics of HIF-1 induction by DFX is similar to induction by CoCl₂ or 1% O₂. Induction of EPO RNA and HIF-1 activity mediated by DFX was inhibited by CHX, indicating a requirement for de novo protein synthesis, as was shown for CoCl₂ and 1% O₂.  Although we have shown that DFX treatment increases EPO RNA levels, we have not directly shown increased EPO transcription, as was determined for 1% O₂ and CoCl₂ by nuclear runoff experiments. However, induction by DFX occurs with the same kinetics and the same sensitivity to CHX as induction by 1% O₂ and CoCl₂, and all three stimuli result in HIF-1 induction, strongly suggesting that DFX also activates EPO transcription. The observation that each of the inducers and inhibitors of EPO transcription has an identical effect on HIF-1 activity is consistent with the proposed role of HIF-1 as an essential activator of EPO transcription.
with 130 μmol/L DFX for 24 hours and then exposed to 20% or 1% O₂ for an additional 24 hours before measurement of EPO protein secreted into the medium. Our quantitation of EPO RNA paralleled the measurements of EPO protein previously reported, as EPO RNA levels in cells treated with 1% O₂ + DFX were less than in cells treated with 1% O₂ alone. However, DFX alone induced EPO RNA expression (as well as HIF-1 activity), indicating that DFX did not act as an inhibitor of EPO gene expression. One interpretation of these data is that prolonged treatment with DFX resulted in adverse effects on cellular metabolism. Although the recovery of mRNA must be reduced preferentially (relative to total RNA) to explain the observed results, it is noteworthy that less total RNA was recovered from DFX-treated than from untreated cells. We were unable to analyze expression of other mRNAs because of limited amounts of RNA recovered.

Treatment with 2 mmol/L 4,6-dioxoheptanoic acid (DHA), an inhibitor of heme biosynthesis, was also reported to reduce EPO production by Hep3B cells exposed to 1% O₂. DHA treatment, 2 mmol/L, did not induce HIF-1 activity in 20% O₂, nor did it inhibit HIF-1 induction in 1% O₂ (Wang and Semenza, unpublished data, January 1993). In our experiments, cells were exposed to DHA for 24 hours compared with 48 hours in the previously published study. Depending on the half-life of the putative heme protein, the 24-hour exposure to DHA may have been insufficient to decrease the level of functional heme protein. Certain differences between our results and those previously reported by others may also reflect different effects of DFX or DHA on EPO transcription compared to EPO translation. Because our investigations have focused on the transcriptional regulation of the EPO gene, we did not measure EPO protein production.

DFX transiently induces EPO RNA expression in vivo. The administration of 200 mg/kg of DFX to a mouse resulted in increased EPO RNA levels in the kidneys 22 hours later, but the effects of DFX on steady-state EPO RNA levels were not sustained after a 5-day treatment. These results are consistent with published data indicating that mice administered DFX at this dose 5 days per week for 12 weeks showed no significant change in mean red blood cell counts compared with controls. Our data are also in agreement with other published studies of EPO expression in vivo. In rats subjected to 7.5% O₂, EPO RNA levels in kidney were highly induced at 8 hours but the degree of induction was markedly reduced after 36 hours of hypoxia. Our results suggest that DFX and hypoxia stimulate EPO expression via a common pathway.

Implications for models of oxygen sensing and hypoxia signal transduction. Hep3B cells and EPO-producing cells in vivo sense O₂ tension, reduction of which initiates transduction of intracellular signals that result in activation of EPO transcription. The only O₂ sensor characterized at the molecular level is a bacterial heme protein kinase. Indirect evidence is consistent with the involvement of a heme protein in the regulation of EPO gene expression. Data presented in this report provide additional indirect evidence supporting this hypothesis. In contrast to previous studies, in which secreted EPO protein was measured, we have analyzed EPO RNA levels and HIF-1 DNA-binding activity, representing more proximal steps in the hypoxia signal-transduction pathway.

We first analyzed the effects of CoCl₂ and 1% O₂ on Hep3B cells because of conflicting reports as to whether these stimuli have additive effects. We found that 1% O₂ + CoCl₂ did not have a greater effect than 1% O₂ alone, both with respect to EPO RNA levels and HIF-1 DNA-binding activity. Treatment with 1% O₂ + DFX also had no greater effect than either stimulus alone, for both EPO RNA and HIF-1 activity. These results are consistent with the hypothesis that 1% O₂, CoCl₂, and DFX induce EPO RNA and HIF-1 via a common pathway. Treatment of Hep3B cells with DFX or CoCl₂ resulted in the detection by EMSA of a HIF-1 complex with subtle differences from the complex induced by 1% O₂. These results suggest that DFX and CoCl₂ may function by displacing ferrous iron from the porphyrin ring of the putative heme protein O₂ sensor, which may induce different signaling properties than when the intact sensor adopts a deoxy conformation in response to 1% O₂. The observation that FAS blocks HIF-1 induction by DFX is consistent with DFX acting via chelation of ferrous iron.

We performed additional experiments to investigate the possible role of a heme protein in the hypoxia signal-transduction pathway leading to HIF-1 induction and EPO transcription. Treatment of intact cells with hemin and treatment of nuclear extracts with hemin, DFX, or FAS had no stimulatory or inhibitory effect on EPO transcription or HIF-1 induction, suggesting that heme does not directly modulate HIF-1 activity. These data can be contrasted with studies of yeast systems in which heme appears to directly interact with transcription factors to influence the expression of genes that are regulated by O₂ tension. Our data are most consistent with involvement of a heme protein at a more proximal step in the pathway, such as the initial sensing of O₂ tension, but the details of this pathway remain to be established. Although our conclusions regarding the effects of DFX on this pathway conflict with those based on earlier studies, our data are not inconsistent with the general model previously proposed. Purification of HIF-1 and isolation of cDNA sequences encoding its subunits will provide an opportunity to examine this pathway in greater detail.

General involvement of HIF-1 in transcriptional responses to hypoxia. HIF-1 activity was induced when cells in which EPO is not produced were exposed to 1% O₂. The intact HIF-1 DNA-binding complex, isolated HIF-1 DNA-binding subunit, and mechanism of HIF-1 induction were indistinguishable in EPO-producing and nonproducing cells. When reporter genes carrying the 50-nt EPO enhancer were transfected into non–EPO-producing CHO cells, transcription was induced by 1% O₂, and mutations in the HIF-1 binding site eliminated induction. These results provided evidence that HIF-1 and its recognition sequence are common components of a general mammalian response to cellular hypoxia. We have now extended these observations by showing that DFX induced HIF-1 activity in CHO
cells and that induction of HIF-1 by DFX in CHO cells also required de novo protein synthesis. These results suggest that the hypoxia signal-transduction pathway used in Hep3B cells also induces HIF-1 activity in other cell types. It will be of great interest to identify other genes that are transcriptionally regulated by O$_2$ tension and use the same signal transduction pathway that leads from the O$_2$ sensor to HIF-1.

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