Epolones induce erythropoietin expression via hypoxia-inducible factor-1α activation

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Introduction

The glycoprotein hormone erythropoietin (Epo), produced by the embryonic liver and the adult kidney, is the main stimulator of erythropoiesis. Recombinant Epo is widely used to treat patients suffering from anemia. However, recombinant Epo is expensive and must be administered intravenously or subcutaneously. Thus, an orally active, small molecular weight compound that induces endogenous Epo production would be an alternative for the treatment of anemia not caused by deficient renal Epo production. Therefore, fungal products were screened for their ability to induce a reporter gene under the control of 6-kilobase (kb) 5′ and 0.3-kb 3′ flanking sequences derived from the Epo gene. Several compounds (the 3 sesquiterpene tropolones: pycnidione, epolone A and epolone B, and 8-methyl-pyridoxatin) were isolated, which in a reporter cell line (termed HRCHOS) was constructed containing a stably integrated luciferase gene under the control of triplicated heterologous HREs. Among various agents tested, we identified a class of substances called epolones, which induced HRE-dependent reporter gene activity in HRCHOS cells. Epolones are fungal products known to induce Epo expression in hepatoma cells. We found that epolones (optimal concentration 4-8 μmol/L) potently induce HIF-1α protein accumulation and nuclear translocation as well as HIF-1 DNA binding and reporter gene transactivation. Interestingly, the activity of a compound related to the fungal epolones, ciclopirox olamine (CPX), was blocked after addition of ferrous iron. This suggests that CPX might interfere with the putative heme oxygen sensor, as has been proposed for the iron chelator deferoxamine mesylate (DFX). However, about 10-fold higher concentrations of DFX (50-100 μmol/L) than CPX were required to maximally induce reporter gene activity in HRCHOS cells. Moreover, structural, functional, and spectrophotometric data imply a chelator:iron stoichiometry of 1:1 for DFX but 3:1 for CPX. Because the iron concentration in the cell culture medium was determined to be 16 μmol/L, DFX but not CPX function can be explained by complete chelation of medium iron. These results suggest that the lipophilic epolones might induce HIF-1α by intracellular iron chelation. (Blood. 2000; 96:1558-1565)
activation, and unraveled the mechanism by which epolones activate Epo expression under normoxic conditions.

Materials and methods

Reagents

DFX, 2,2′-dipyridyl, CPX, neocuproine, ferrozine, luminal, and coumaric acid were purchased from Sigma (Buchs, Switzerland) and ferrous ethylendiammonium sulfate from Fluka (Buchs, Switzerland). Pycnidione and 8-methyl-pyridoxin were isolated as described previously.2,3 Stock solutions (50 mmol/L) of the epolones (CPX, pycnidione, and 8-methyl-pyridoxin) were prepared in methanol and stored at −30°C. Immediately before use, they were diluted in water to a working concentration of 1 mmol/L.

Cell culture and transfection

Chinese hamster ovary (CHO) cells were a kind gift of Peter J. Nielsen (Freiburg, Germany), and human hepatoma HepG2 cells were purchased from the American Type Culture Collection (HB-8065S). Both cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; high glucose, Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS; Boehringer-Mannheim, Basel, Switzerland), 100-U/mL penicillin, 100-μg/mL streptomycin, 1 × nonessential amino acids, and 1-mmol/L Na-pyruvate (all purchased from Life Technologies, Basel, Switzerland) in a humidified atmosphere containing 5% CO2 at 37°C. Oxygen partial pressures in the incubator (Forma Scientific, Illkirch, France) were either 140 mmHg (20% O2 vol/vol, normoxia) or 7 mmHg (1% O2 vol/vol, hypoxia). For transfection, 0.5 mg of DNA in 50 μg of DNA in 50 μL of 10-mmol/L Tris-HCl (pH 7.4), and 1-mmol/L ethylenediaminetetraacetic acid and electroporated at 250 V and 960 μF (GenePulser, Bio-Rad, Glattbrugg, Switzerland).

Hypoxia-reporter assays

A firefly luciferase reporter gene plasmid (pH3SVL) containing a total of 6 HBSSs derived from the transferrin HRE was constructed by inserting 2 copies of the oligonucleotide TiHBSsw into the Smal site of the plasmid pGLTHBSsw.12 For stable transfection, pH3SVL was linearized with XmnI, mixed with the EcoRI-linearized neomycin expression vector pSV2neo at a molar ratio of 100:1, and coelectroporated into CHO cells. Following limited dilution and selection in 2-mg/mL G418 (Alexis, Wisselchen, Switzerland), a hypoxia-reporter cell line (termed HRCHOS) was chosen based on the efficiency of hypoxic reporter gene induction. For transient transfection assays, pGLHIF1.3 containing 3 copies of the HBS derived from the Epo 3’ HRE13 was coelectroporated into HepG2 cells together with the β-galactosidase reference vector pCMVlacZ.12 The cells were split and incubated for 43 hours under normoxic or hypoxic conditions. Following stimulation, stably transfected HRCHOS cells and transiently transfected HepG2 cells were lysed in reporter lysis buffer (Promega), and luciferase and β-galactosidase activities were determined according to the manufacturer’s instructions (Promega, Catalys, Wallisellen, Switzerland) using a Lumat LB9501 luminometer (EG&G Berthold, Regensdorf, Switzerland) and a DigiScan 96-well plate photometer (ASYS, BioBlock, Illkirch, France), respectively. Differences in the transfection efficiency and extract preparation were corrected by normalization to the corresponding protein contents (Bradford assay, Bio-Rad) or β-galactosidase activities.

Immunoblot and immunofluorescence analysis

Following stimulation, cells were harvested and nuclear extracts were prepared as described previously,13 except that the cells were lysed with 0.02% Nonidet P-40 instead of dounce homogenization. For immunoblot assays, aliquots (30 μg) of nuclear extracts were fractionated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Rieden, Switzerland). Staining with ProteinA (Sigma) confirmed equal loading and blotting efficiency. After blocking nonspecific binding sites with 4% defatted milk powder in phosphate-buffered saline (PBS), the blot was probed with the affinity-purified anti–HIF-1α monoclonal antibody mgc3 described previously14 (Affinity BioReagents, Lausen, Switzerland), followed by a horseradish peroxidase–coupled secondary goat antimouse antibody (Pierce, Socochim, Lausanne, Switzerland). Chemiluminescence detection was performed by incubation of the membrane with 100-mmol/L Tris-HCl (pH 8.5), 2.65-mmol/L H2O2, 0.45-mmol/L luminal, and 0.625-mmol/L coumaric acid for 1 minute, followed by exposure to x-ray films (SuperRX, Fuji, Dielsdorf, Switzerland). For immunofluorescence analysis, adherent cells were fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 10 minutes, washed with PBS, permeabilized with 0.5% Triton X-100 for 5 minutes, and rinsed again with PBS. After blocking nonspecific binding sites with 10% FCS in PBS for 30 minutes, the cells were incubated overnight with the anti–HIF-1α antibody mgc3 diluted 1:10 with 3% BSA in PBS, followed by a fluorescein isothiocyanate–coupled secondary donkey antimouse antibody diluted 1:100 with 3% BSA in PBS (Jackson, Milan Analytica, La Roche, Switzerland). After extensive washings in PBS and mounting in Mowiol (Calbiochem, Stethelin, Basel, Switzerland), the cells were analyzed by fluorescence microscopy.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were carried out as described previously.15 A double-stranded, HBS-containing oligonucleotide derived from the Epo 3’ enhancer was used as probe. For supershift analysis, the anti–HIF-1α monoclonal antibody mgc3 was added to the completed DNA-protein binding reaction mixture and incubated for 16 hours at 4°C prior to loading.

Iron determinations

Iron concentrations were determined by a colorimetric assay according to Fish.16 Briefly, iron was released from 100-μL samples by treatment with 50 μL of 142-mmol/L K MnO4 and 600-mmol/L HCl at 60°C for 2 hours. Thereafter, 10 μL of 5-mol/L ammonium acetate, 2-mol/L ascorbic acid, 13.1-mmol/L neocuproine, and 6.5-mmol/L ferrozine were added and incubated at room temperature for 30 minutes. Iron was determined by measuring the absorption at 562 nm in a DU-65 spectrophotometer (Beckmann). Iron standards were prepared by dissolving ferrous ethylenediammonium sulfate in 10-mmol/L HCl. The detection limit of this assay was 2 ng of iron, and the standard curve was linear up to 800 ng of iron. It has been validated by demonstrating a 2-fold molar ratio of iron to protein in 10-μg samples of iron-saturated purified transferrin (Life Technologies).

Results

Generation of the HRCHOS hypoxia-reporter cell line

To facilitate the screening for novel agonists/antagonists of the oxygen-regulated signaling pathway, CHO cells were stably transfected with the hypoxia-dependent reporter gene shown in Figure 1A. This construct contains the firefly luciferase cDNA under the control of the SV40 promoter and 3 HREs derived from the hypoxia-responsive transferrin 5’ enhancer.12 Of note, the transferrin HRE contains 2 HBSSs and the whole construct, hence, a total hypoxia-responsive transferrin 5’ enhancer. To facilitate the screening for novel agonists/antagonists of the oxygen-regulated signaling pathway, CHO cells were stably transfected with the hypoxia-dependent reporter gene shown in Figure 1A. This construct contains the firefly luciferase cDNA under the control of the SV40 promoter and 3 HREs derived from the hypoxia-responsive transferrin 5’ enhancer. Of note, the transferrin HRE contains 2 HBSSs and the whole construct, hence, a total hypoxia-responsive transferrin 5’ enhancer.
mM (CoCl₂) and 100 mM (NiCl₂), respectively. Luciferase activity dropped again at higher concentrations, presumably because these agents are cytotoxic. Similar results were obtained with the iron chelator DFX, which maximally induced normoxic luciferase activity at concentrations of 50 to 200 mM (16 mM for CPX), luciferase activity decreased again at higher doses. Hypoxic exposure of HRCHO5 cells (Figure 2, bottom graph) had additive effects, but the decrease in reporter gene activity occurred already at lower epolone concentrations than under normoxic conditions.

**The epolone CPX activates reporter gene expression via the HRE**

We next analyzed whether epolones activated luciferase expression specifically via the HRE or whether other cis-regulatory elements were involved, as could be expected from the fact that hypoxia and epolones had additive effects. Therefore, luciferase reporter gene constructs containing 3 copies of the Epo 3′ HBS, either wild type (pGLHIF1.3) or mutant (pGLHIF1mt.3), were transiently transfected into HepG2 cells, which were split and exposed to hypoxia and/or CPX. As shown in Figure 3, CPX increased reporter gene activity also in this reporter gene–cell line combination under both normoxic and hypoxic conditions. However, the mutant HBSs

μM (CoCl₂) and 100 μM (NiCl₂), respectively. Luciferase activity dropped again at higher concentrations, presumably because these agents are cytotoxic. Similar results were obtained with the iron chelator DFX, which maximally induced normoxic luciferase activity at concentrations of 50 to 200 μM (Figure 1B, top graph). Under hypoxic conditions, CoCl₂ and DFX additionally induced reporter gene activity with a similar dosage dependence as found under normoxic conditions. In contrast, NiCl₂ had no additional effects under hypoxic conditions (Figure 1B, bottom graph).

**Epolones induce reporter gene activity in HRCHO5 cells under normoxic and hypoxic conditions**

Epolones have previously been identified as a family of fungal products capable of inducing Epo expression under normoxic conditions. Because the reporter gene used for Epo-inducing drug screening contained the 3′ HRE derived from the Epo gene,
conferred neither CPX nor hypoxic induction of reporter gene expression, suggesting that both stimuli activate reporter gene expression via similar mechanisms.

Epolones induce HIF-1α protein stability, nuclear translocation, and DNA binding activity

The binding of HIF-1 to an HBS is obligatory for the activation of an HRE and subsequent gene expression. The sequential steps in the formation of the HIF-1 complex include the stabilization of its α subunit followed by nuclear translocation and heterodimerization with ARNT to form a functional DNA-binding transcription factor complex. We thus investigated whether this pathway could be mimicked by epolones.

First, HIF-1α protein levels were determined by immunoblotting in HepG2 cells. As shown in the left part of Figure 4, all 3 epolones efficiently induced HIF-1α protein expression in normoxic HepG2 cells to the same extent as CoCl2. Hypoxic conditions (Figure 4, right part) also induced HIF-1α protein, which could be further enhanced by adding 8-methyl-pyridoxatin or CPX but not pycnidione or CoCl2. Next, as shown by immunofluorescence, hypoxia as well as the epolone CPX induced nuclear accumulation of HIF-1α in HepG2 cells (Figure 5). Finally, as shown by EMSA, CPX also activated DNA binding of HIF-1 to an oligonucleotide probe containing an HBS derived from the Epo HRE (Figure 6). This induction was slightly more pronounced than with hypoxia or DFX or the combination of hypoxia with CPX. Supershift analysis using the monoclonal anti-HIF-1α antibody mgc3 confirmed that (most of) the HIF complexes present in HepG2 nuclear extracts contained the HIF-1α subunit.

Iron blocks DFX- and CPX-mediated induction of HIF-1α-dependent gene activation

Having established that epolone-dependent reporter gene activation followed HIF-1α induction, we addressed the question of how epolones might induce HIF-1α. Based on functional (Figures 1 and 2) and structural similarities between DFX and CPX, we followed the hypothesis that CPX also might act as an iron chelator. Feroxamine contains 1 atom of ferrous iron in the center of a hexadentate cluster formed by 3 hydroxamic acid groups.17 The epolone CPX contains a single hydroxamic acid group, suggesting a bidentate structure theoretically requiring 3 mol of CPX to chelate 1 mol of iron. We thus titrated the concentration of iron necessary to block DFX- and CPX-induced reporter gene activation in HRCHO5 cells. As shown in Figure 7A, efficient inhibition of luciferase activity under normoxic and hypoxic conditions occurred at an iron:DFX molar ratio of 1:1, consistent with the ability of 1 molecule of DFX to stoichiometrically chelate 1 molecule of iron. Interestingly, CPX-induced luciferase activity was abolished at an iron:CPX ratio of 1:2 but not at 1:4, supporting the idea that CPX functions as an iron chelator with the expected stoichiometry of 3 mol of CPX per 1 mol of iron.

As shown in Figure 7B, the iron chelation–dependent induction of reporter gene activity in HRCHO5 cells can also be blocked by addition of AlCl3. However, while inhibition of DFX activity again

Figure 3. Activation of reporter gene expression in transiently transfected HepG2 hepatoma cells by CPX. HepG2 cells were cotransfected with the indicated luciferase reporter gene constructs together with a β-galactosidase control expression vector. Following splitting and stimulation with hypoxia (1% oxygen) and/or CPX (8 μmol/L) for 43 hours, reporter gene activities were determined and expressed as a ratio between luciferase and β-galactosidase activities. The luciferase constructs contained 3 wild-type HBSs (pGLHIF1.3) or 3 mutant HBSs (pGLHIF1mt.3) as described previously.13 The empty parental vector pGL3 promoter was included as control. Means ± SD of 3 independent experiments.

Figure 4. Immunoblot analysis of HIF-1α protein expression in HepG2 cells. HepG2 cells were treated with the epolones pycnidione, 8-methyl-pyridoxatin, and CPX (8 μmol/L) as well as with CoCl2 (100 μmol/L) for 4 hours under normoxic (20% oxygen) or hypoxic (1% oxygen) conditions. HIF-1α protein was detected by immunoblotting of nuclear extracts using the monoclonal anti-HIF-1α antibody mgc3.14

Figure 5. Immunofluorescence analysis of HIF-1α expression in HepG2 cells. HepG2 cells were treated with hypoxia (1% oxygen) and/or CPX (8 μmol/L) for 4 hours, and HIF-1α was detected using the monoclonal antibody mgc3 followed by a fluorescein isothiocyanate–conjugated secondary antibody.
show iron-dependent (but not aluminium-dependent) absorption maxima at 430 nm and 421 nm, respectively, allowing the direct estimation of the iron chelation stoichiometry. Whereas DFX iron saturation was found to be completed at a 1:1 molar ratio, CPX was saturated with iron at a molar ratio of 1:3 (Figure 9B), thus confirming the functional results obtained in the HRCHO5 cell line.

In this context, it would be important to know the iron required stoichiometric concentrations of AlCl3, the activity of CPX was already reduced by approximately 50% at a molar aluminium:CPX ratio of 1:8. This cannot be attributed to a higher toxicity of AlCl3 compared with FeCl2 because the actual AlCl3 concentration inhibiting CPX function was only 1 μmol/L, whereas 50-μmol/L AlCl3 had no significant effect on DFX-induced reporter gene activity. Thus, these data suggest that CPX-mediated activation of luciferase expression in HRCHO5 cells might be due to metal chelation.

To exclude the possibility of an inhibitory function of iron downstream of HIF-1α induction, we analyzed HIF-1α protein expression directly by immunoblotting. As shown in Figure 8, addition of iron inhibited HIF-1α induction by CPX as well as by the established activators DFX and 2,2'-dipyridyl.

**Stoichiometry of iron chelation by DFX and CPX and its relation to the cell culture medium iron concentration**

The iron chelation stoichiometry suggested by the titration experiments shown in Figure 7 might be compromised by the (unknown) concentration of iron in the cell culture medium. To confirm our data, the iron saturation curves of DFX and CPX were determined by spectrophotometry. As shown in Figure 9A, DFX and CPX

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**Figure 6. EMSA of HepG2 nuclear extracts.** HepG2 cells were treated for 4 hours with DFX (100 μmol/L) or CPX (8 μmol/L) under normoxic (20% oxygen) or hypoxic (1% oxygen) conditions. Nuclear extracts were incubated with a radioactively labeled oligonucleotide probe derived from the Epo 3' HRE and separated by native polyacrylamide gel electrophoresis. Specific HIF-1 DNA binding was confirmed by supershift analysis using the monoclonal antibody mgc3.

**Figure 7. Iron and aluminium block the DFX- and CPX-mediated reporter gene induction in HRCHO5 cells.** The cells were stimulated with the optimal DFX (100 μmol/L) and CPX (8 μmol/L) concentrations, as determined in Figures 1 and 2, respectively, for 18 hours under normoxic (20% oxygen) and hypoxic (1% oxygen) conditions. The indicated concentrations of ferrous ethylenediammonium sulfate (A) and AlCl3 (B) were added at the beginning of the experiment. After preparation of cell extracts and determination of the luciferase activities and protein contents, the results were expressed as luciferase activities in relative light units per microgram of cellular protein. Means ± SD of 3 independent experiments.

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[Graphs showing iron and aluminium block the DFX- and CPX-mediated reporter gene induction in HRCHO5 cells.]

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concentration in the cell culture medium. Using a colorimetric assay with a lower iron detection limit of 0.4 μmol/L (see “Materials and methods”), we determined an iron concentration in the FCS of 148 ± 19 μmol/L, whereas the iron concentration in the DMEM itself was 1.4 ± 0.35 μmol/L and, hence, close to the detection limit. Therefore, the medium containing 10% FCS contains 16-μmol/L iron. In conclusion, iron in the cell culture medium can be completely chelated with DFX but not CPX at their respective optimal concentrations, especially considering the fact that 3 mol of CPX are necessary to chelate 1 mol of iron, whereas 1 mol of DFX is sufficient for the same purpose.

**Discussion**

In this study, we describe the construction of the HRCHO5 hypoxia-reporter cell line, which contains a stably integrated hypoxia-responsive luciferase reporter gene under the control of 6 HBBSs. This allows easy and rapid monitoring of the activity of HIF-1. However, it cannot be formally excluded that one of the other HIF-α family members, HIF-2α,20-23 or HIF-3α,24 might also be involved in the activation of reporter gene expression, because they display features very similar to HIF-1α.25,26 Originally thought to be expressed specifically in endothelial cells, HIF-2α has recently been found in many other cell lines of nonendothelial origin.24 Thus, CHO cells might also express HIF-2α or even HIF-3α, the expression pattern of the latter not being known yet. We therefore specified our results by detecting HIF-1α in a human cell line (HepG2) using the monoclonal antihuman HIF-1α antibody mcg3 that does not cross-react with the other family members.14

The functioning of the HRCHO5 cell line has been validated by stimulation with hypoxia as well as the known hypoxia-mimetics Co2+, Ni2+, and DFX.11,27 Interestingly, Co2+ and DFX, but not Ni2+, showed additive effects on HRCHO5 reporter gene activity when combined with hypoxia. We previously reported that hypoxia and Co2+ also had additive effects on Epo secretion in hepatoma cell lines,28 which is not in agreement with other reports.3 Our results imply that Co2+ and DFX do not only interfere with the putative oxygen sensor but might have additional positive effects on the oxygen signaling pathway, for example, associated with reactive oxygen species production by a localized Fenton reaction probably involved in oxygen sensing and signaling.29,30

Using the HRCHO5 cell line, we identified the 2 fungal epolones,2-3 pycnidione and 8-methyl-pyridoxatin, as well as the related pyridone, CPX, as potent inducers of HIF-1α activation. Most of the studies in this work were performed with the epolones, CPX, which has been chosen because of its relatively simple molecular structure and because it is commercially available at a low price. Clinically, CPX is used as an antimycoticum in dermatologic and vaginal creams.31 The mechanisms of its antimicrobial action have not been completely resolved, but CPX seems to interfere with membrane integrity, a probable HIF-1α-independent effect. Apart from the epolones, several other substances were tested in the HRCHO5 cell line, which we suspected to influence HIF-1α activity: insulin and insulin-like growth factors I and II,23-34 interleukin-1α, interleukin-1β, tumor necrosis factor-α,32 lipopolysaccharide,36 tumor growth factor-β,37,38 ferrous ethylenediammonium sulfate, FeCl2, FeCl3,28,39 ZnCl2,40 the angiogenesis inhibitor epigallocatechingallate found in drinking tea41; and taurin.42 These agents have been reported either to be induced by hypoxia or to interfere with the expression of hypoxia-inducible genes. However, none of these agents affected HIF-1α-dependent reporter gene activity in normoxic or hypoxic HRCHO5 cells (data not shown).

The epolones attenuated luciferase activity in HRCHO5 cells when added at concentrations above 16 μmol/L under normoxic conditions and above 4 μmol/L under hypoxic conditions. We attribute this effect to a putative cytotoxicity of the epolones, which might increase when combined with the additional stress of exposure to hypoxia. Consistent with this notion, we observed a
detachment of the cells with a concomitant decrease of cellular reporter gene activity after treatment with higher doses of epolones (data not shown). In support of this idea, it has been reported that CPX (as well as DFX) can block the cell cycle at the G1/S phase boundary,3,14 HIF-1α protein induction in HepG2 cells by hypoxia and epolones (Figure 4) was diminished compared with the corresponding luciferase activities in HRCHO5 cells (Figure 2). The reason for this finding is not completely clear but might be related to the presence of other members of the HIF family that contribute to reporter gene activation or be related to a higher sensitivity of HepG2 cells to hypoxic cell culture conditions than CHO cells.

Interestingly, the function of both the established iron chelator DFX, a sideramine obtained from Streptomyces pilosus,17 and of CPX could be blocked dosage dependently by adding ferrous iron salts. Ferrous iron is rapidly oxidized by ambient oxygen yielding the DFX-chelatable ferric iron. We hence do not know whether ferrous or ferric iron preferentially inhibits CPX-mediated HIF-1α activation. Because we discovered that CPX is an iron-dependent chromophore, we could directly determine the iron chelation stoichiometry to be 1:3 iron:CPX. This is in agreement with the presence of 1 hydroxamic acid group in CPX (bidentate) compared with 3 such groups in DFX (hexadentate), the latter chelating iron at a 1:1 stoichiometry.

The well-characterized, HIF-1α–inducing iron chelators DFX1 and 2,2′-dipyridyl19 are thought to mimic hypoxia by displacing iron from the porphyrin ring of the putative heme oxygen sensor5,39 or by removing iron from the reactive oxygen species-generating Fenton reaction.29,30 Therefore, CPX could induce HIF–1-dependent gene expression via similar mechanisms. The finding that AICl3 also blocked CPX-induced reporter gene activity suggests that epolones (as well as DFX) potentially might induce HIF-1α by chelation of a metal other than iron. This would have important implications for the nature of the oxygen sensor, but further experiments will be required to establish such a mechanism.

While 50- to 100-mmol/L DFX is necessary to maximally induce HIF-1α–dependent reporter gene expression, the epolones already show maximal activation at 4 to 8 μmol/L. Because we found an iron concentration in the cell culture medium of 16 μmol/L, the concentration of DFX, but not of CPX, would be sufficient to completely chelate total iron in the medium. Considering that a 3-fold higher molarity of the bidentate CPX than of the hexadentate DFX is required to chelate the same quantity of iron, and regarding the fact that 8-methyl-pyridoxatin is even 10 times more potent than CPX (5-fold induction of Epo gene expression at 0.3 μmol/L compared with 3-μmol/L CPX),17 we conclude that the epolones cannot activate HIF-1α simply by chelating all iron in the medium as might be the case for DFX.

The hexadentate iron chelator DFX has been reported to be ineffective as an intracellular iron chelator (at concentrations similar to those used in our study), whereas the lipophilic bidentate hydroxypyridinone class of iron chelators efficiently chelated intracellular iron.45 In analogy, differences between the cellular permeability of DFX and epolones could explain their different concentration optima. Indeed, the bidentate iron chelator 1,2-diethyl-3-hydroxy pyridin-4-1 (CP-94) induced VEGF messenger RNA (mRNA) expression in Hep3B hepatoma cells already at 10 μmol/L.37 However, CP-94 did not induce Epo mRNA at 10 μmol/L, and the highest VEGF and Epo mRNA induction was found at 200 μmol/L, which is clearly different from our results with the epolones.

In conclusion, fundamental differences appear to exist between CPX and other known hypoxia-mimicking iron chelators with respect to their structure; metal ion preference; iron chelation stoichiometry, affinity, and kinetics; cellular uptake; intracellular stability; and ability to chelate the intracellular labile iron pool. Therefore, differences might also exist in their interaction with the putative oxygen sensor iron center as well as their interference with the oxygen signaling pathway. Better understanding of these differences probably will help in the elucidation of the different steps involved in the regulation of oxygen-dependent gene expression.

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