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Effects of Transition Metals on the Expression of the Erythropoietin Gene: Further Evidence That the Oxygen Sensor Is a Heme Protein

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Both *in vivo* and in Hep3B cells, expression of the erythropoietin gene is induced by hypoxia as well as by certain transition metals (cobalt and nickel) and by iron chelation. When Hep3B cells were incubated in an iron deficient medium, Epo mRNA expression was enhanced 4-fold compared to Hep3B cells in iron enriched medium. The increased Epo expression in iron deficient medium was abolished when Fe_2-transferrin complex was added. Epo induction by cobalt was also affected by iron concentration. In iron enriched medium, erythropoietin expression in Hep3B cells was maximally induced at $CoCl_2$ concentrations between 100 to 200 μM. In contrast, in iron poor medium, a high level of induction was obtained at a $CoCl_2$ concentration of only 50 μM, indicating competition between iron and cobalt. Under hyperbaric oxygen, cobalt induction of erythropoietin mRNA was modestly suppressed while nickel induction was markedly enhanced. These observations support the proposal that the oxygen sensor is a heme protein in which cobalt and nickel can substitute for iron in the porphyrin ring.

A number of physiologically important genes are induced by hypoxia [reviewed in Bunn and Poyton (1)]. A wealth of recent evidence strongly suggests that oxygen sensing and chemical signaling occur via a common pathway, leading to the activation of a hypoxia-inducible transcription factor, HIF-1 (2, 3). The most thoroughly studied of these hypoxia inducible genes is erythropoietin (Epo) which encodes the hormone that regulates red blood cell production (4). Epo expression is markedly up-regulated by certain transition metals: cobalt, nickel and manganese (5), which, like iron, can be incorporated into protoporphyrin IX. Goldberg et al (5) have presented evidence that the oxygen sensor which regulates Epo synthesis is a heme protein, which binds oxygen. Hypoxic signaling is mimicked by replacing iron with one of these other transition metals.

This model predicts that iron should play an important role in the O_2 sensing mechanism and regulation of the Epo gene. Iron can be expected to compete with cobalt and nickel for incorporation in the heme moiety and to antagonize the stimulatory effects of these metals on the Epo gene. Furthermore, since iron catalyzes the conversion of peroxide to reactive oxygen intermediates via the Fenton reaction, it may significantly alter levels of chemical messengers in the oxygen sensing pathway. Wang and Semenza (6) have shown that when Hep3B cells were treated with the iron chelating agent, desferrioxamine, HIF-1 DNA binding activity was activated and Epo mRNA expression was induced. These effects were abolished by the co-administration of ferrous ammonium sulfate.

In this study, the effect of iron concentration and its impact on cobalt stimulation were analyzed by quantitation of Epo mRNA levels in Hep3B cells. To further investigate the proposal that the oxygen sensor is a metal-containing heme protein, we investigated the effect of hyperbaric oxygen on induction of Epo mRNA by cobalt and nickel. Cobalt protoporphyrin binds oxygen with low affinity whereas nickel protoporphyrin cannot bind oxygen even at high pO_2. Therefore, we

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predicted that, in comparison to nickel induction, cobalt induction of Epo mRNA would be attenuated under hyperbaric oxygen.

MATERIALS AND METHODS

Low iron culture medium was prepared by adding 50 ml bovine calf serum [HyClone] (average iron concentration = 25 μg/dl) to 450 ml αMEM culture medium, yielding a final iron concentration of 2.5 μg/dl or 0.43 μM. High iron culture medium was prepared by adding 50 ml iron enriched calf serum (average iron concentration = 600 μg/dl) to 450 ml αMEM, yielding a final iron concentration of 60 μg/dl or 10 μM. Human apo-transferrin (apoTF) was obtained from CalBiochem. 100 mg lyophilized apo-transferrin was dissolved in 5.0 ml sterile filtered 0.9% NaCl and dialyzed against αMEM medium. Cobalt complexes of transferrin were prepared by a method modified from Aisen et al (7). A 6-fold molar excess of cobalt citrate (50 mM $CoCl_2$ in 0.01 M citric acid pH 5.0) was combined with apoTF (see above) in 0.04 M $NaHCO_3$ followed by addition of 3 μl 30% H_2O_2. The cobalt transferrin complex, which has an absorbance peak of 405 nm, was isolated on Sephadex G-25 in αMEM. Saturation of transferrin with Fe-Nitrilotriacetate was achieved by a modification of the method of Zaman et al (8). After preparing a solution of 12.5 mM ferric chloride in 100 mM disodium nitrilotriacetate (NTA), a 5-fold molar excess of Fe-NTA solution was combined with apo-transferrin prepared above and incubated in 37°C for 30 minutes. The red complex was isolated on Sephadex G25. The final concentration of Fe-transferrin was determined by multiplying the OD280 by 0.95 mg/ml.

In order to perform experiments at elevated oxygen tension, we modified a one cubic foot reinforced steel autoclave equipped with a pressure proof door, sealed by a rubber gasket. A pressure gauge was installed at the air inlet valve. A tank of gas composed of 98.3% O_2 and 1.7% CO_2 was connected to the inlet port. The entire chamber was placed inside an incubator which was maintained at a temperature of 37°C. At 3 Atm pressure, the pCO_2 was $\sim$38 mmHg (0.017 × 3 × 760 mmHg) maintaining the pH in the bicarbonate buffered medium nearly constant at $\sim$7.4.

Hep3B cells were incubated in these media, generally at 21% O_2, 5% CO_2, after which RNA was prepared and Epo mRNA was measured by ribonuclease protection analysis as previously described (9). Constructs expressing Epo riboprobes were prepared as described previously (9). Autoradiograms were prepared by exposure to XO-mat film. Epo mRNA was quantified by scanning the dried gel with a Phosphorlmager (Molecular Dynamics) and the respective bands quantitated by ImageQuant software.

RESULTS

As shown in Figure 1, Hep3B cells incubated for 48 hours at 21% O_2 in low iron medium (total iron concentration = 2.5 μg/dl = 0.43 μM) (Lane 1) had a 4-fold increase in Epo expression (quantified by Phosphorlmaging,) compared to cells in high iron media (total iron concentration = 60 μg/dl = 10.25 μM) (Lane 2). When 2 μM iron-transferrin complex, or 4 μEq/l of Fe (each transferrin molecule binds 2 iron atoms), was added to this low iron medium, the stimulatory effect was abolished (Lane 5) and basal expression was comparable to that observed in iron enriched media (Lane 2). When Hep3B cells were incubated in αMEM supplemented with 10% albumin instead of serum (Lane 3), no Epo induction was observed, probably because the viability and function of these cells depends upon lipids, growth factors, and other components normally present in serum. Although the induction with low iron demonstrated here is less robust than caused by hypoxia (Lane 12), cobalt (Lane 9) or 150 μM desferrioxamine (Lane 4), the results clearly demonstrate that iron deficiency increases Epo gene expression under normoxic incubation.

Competition between iron and cobalt is shown in Figures 2A and B. In culture medium containing a high total iron content (10 μM), maximum stimulation of Epo expression was observed at a cobalt chloride concentration of 200 μM. In contrast, in low iron medium, maximum induction was achieved at only 50 μM cobalt chloride. These results reveal increased sensitivity to cobalt under low iron conditions (Figure 2B). It is also interesting to note that in low iron media even without cobalt (Figure 2A, Lane 2), Epo expression was higher than the high iron lanes (Figure 2A, Lanes 7 and 8) containing low doses of cobalt, again illustrating the stimulatory effect of iron deficiency. To test whether increased sensitivity to cobalt was indeed caused by a lack of iron, iron-transferrin (Fe_2-TF) was added into the low iron media. In this experiment, shown in Figure 1, a monotonic dose dependent response was observed with small doses of $CoCl_2$ (Lanes 7–9) in low iron media; however, when the medium was supplemented with 2 μM Fe_2-TF, the cobalt response was completely abolished (lane 11). A similar result was observed with Hep3B cells

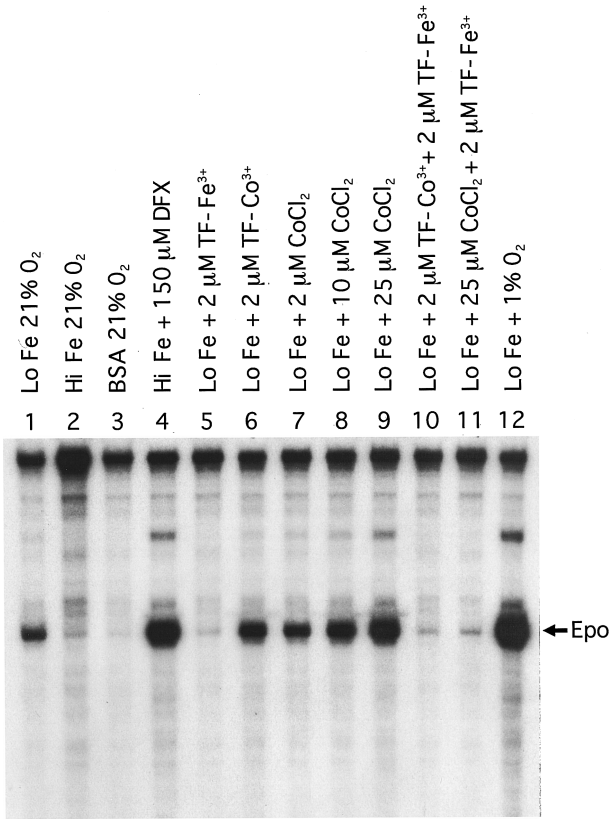

FIG. 1. Effect of iron on Epo mRNA expression in the absence and presence of cobalt induction. Hep3B cells were incubated in high iron (HiFe), [total iron = 10.25 μM], or low iron (LoFe), [total iron = 0.43 μM]. DFX = desferrioxamine; TF-Co^{3+} = cobalt transferrin complex; TF-Fe^{3+} = iron transferrin complex. In experiment BSA (lane 3), 10% bovine serum albumin was substituted for calf serum in the media

induced by cobalt-transferrin complex (Co_2-TF) instead of cobalt chloride. When Hep3B cells were treated with 2 μM Co_2-TF (equivalent to 4 microequivalents of cobalt), induction similar to that of 10 μM $CoCl_2$ was observed (Figure 1, Lane 6). When 2 μM Fe_2-TF was added to this mixture, the induction was completely nullified (Figure 1, Lane 10). Addition of uncomplexed apotransferrin alone had no effect on low iron or cobalt induction (data not shown). This set of results suggests that transferrin enables efficient uptake of cobalt into Hep3B cells and that iron competes directly with cobalt and interferes with cobalt's ability to induce the Epo gene, probably at the level of the heme protein oxygen sensor.

Figure 3 shows the effect of high oxygen pressure on the expression of Epo mRNA in Hep3B cells after 8 hours incubation with cobalt or nickel. As expected, when neither metal was present, there was no induction at 1 or 3 atmospheres. At 1 Atm, cobalt gave the expected robust induction. When the cells were subjected to hyperbaric O_2, cobalt induction was only slightly diminished. In contrast, at 1 Atm the induction of Epo mRNA by 300 μM $NiCl_2$ was less than that by 100 μM $CoCl_2$, but was markedly enhanced at 3 Atm O_2. These results were consistently reproducible in three subsequent experiments.

DISCUSSION

Although the impact of iron in Epo expression was suggested from recent studies utilizing iron chelators (10,11), the direct effect of altering iron levels has not been demonstrated until the present

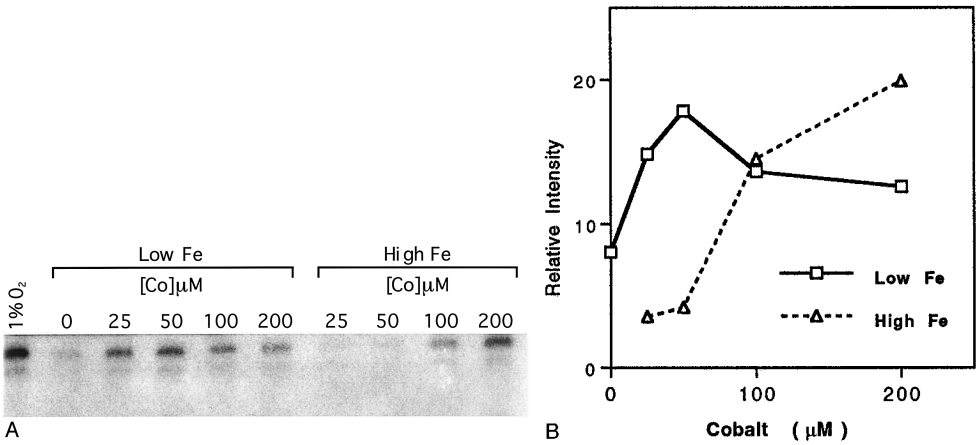

FIG. 2. A. Effect of increasing concentrations of $CoCl_2$ on Epo expression in Hep3B cells incubated overnight in low and high iron media. **B.** Quantitation of data in Figure 2A. Epo mRNA signal intensity was measured by Phosphorlmager analysis of ribonuclease protection gel.

study. The increased Epo expression in Hep3B cells exposed to low levels of iron reported here further supports the proposal that iron plays an important role in the hypoxia sensing pathway.

The oxygen sensor has been proposed to be a NAD(P)H oxidase, which, under normoxic conditions, binds free O_2 and converts it to H_2O_2 (1,12) (Figure 4). Hydrogen peroxide is subsequently converted to hydroxyl radicals ($OH\cdot$) and hydroxide (OH^-) through the iron-dependent Fenton reaction. These reactive oxygen intermediates could then act as chemical messengers which suppress expression of genes induced by hypoxia. A deficiency of iron may lower production of

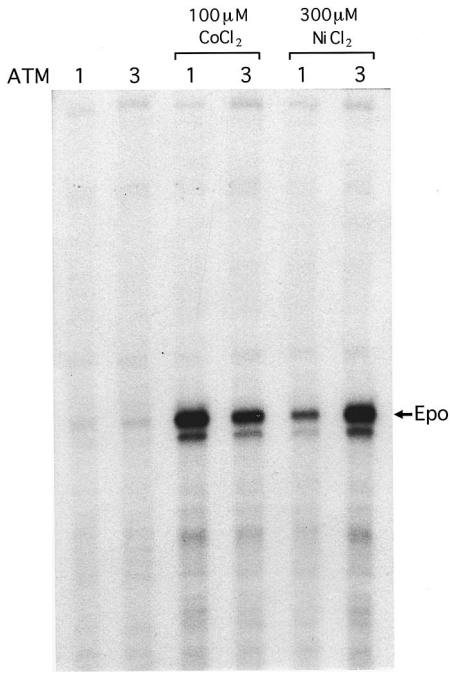

FIG. 3. Cobalt and nickel induction of Epo mRNA in normoxic and hyperoxic incubation. Hep3B cells were treated with 100 μM $CoCl_2$ or 300 μM $NiCl_2$ and incubated for 8 hours in 1 Atm (760 mmHg, 21% O_2) or 3 Atm (2280 mmHg, 98.3% O_2). Two left-hand lanes, Hep3B cells not treated with metals.

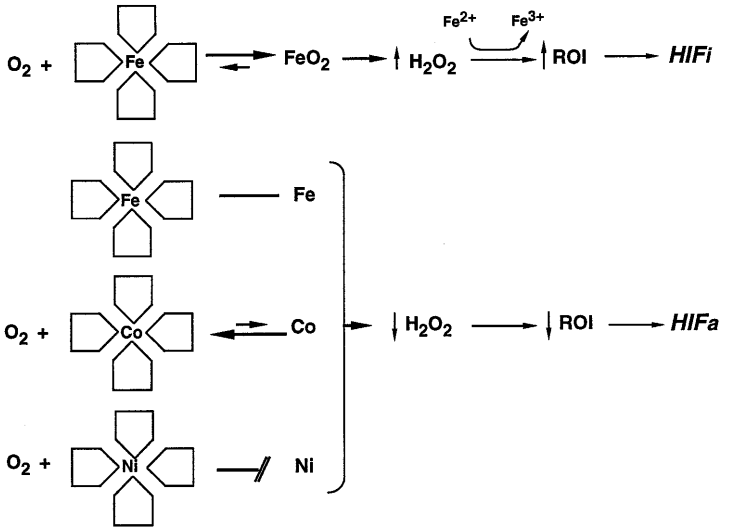

FIG. 4. Model for hypoxia sensing and signaling pathway. Cobalt protoporphyrin binds O_2 with very low affinity whereas nickel protoporphyrin does not bind O_2. ROI = reactive oxygen intermediates; HIFa = activated hypoxia inducible factor; HIFi = inactivated hypoxia inducible factor.

these reactive oxygen intermediates, thereby mimicking a hypoxic environment. The drastic reduction in intracellular iron could impair biosynthesis of the putative cytochrome b NAD(P)H oxidase necessary for superoxide production. More likely, it would retard the Fenton reaction which requires free iron as a catalyst, thereby reducing the formation of hydroxyl radicals. A third possibility, although unlikely, is that reduced intracellular levels of iron may favor the incorporation of cobalt, manganese and/or nickel, which normally exist in trace amounts, into the porphyrin ring of the oxygen sensor.

In the model depicted in Figure 4, cobalt leads to the induction of HIF-1 and hypoxia responsive genes through its incorporation into the porphyrin ring of the heme oxygen sensor. Since cobalt heme binds O_2 with extremely low affinity, the oxygen sensor containing cobalt can not catalyze the formation of reactive oxygen intermediates. As demonstrated by Görlach et al (13), this leads to a decrease in reactive oxygen intermediates, mimicking a hypoxic environment and activating HIF-1.

Since cobaltous heme can bind oxygen, albeit with low affinity whereas nickel heme cannot bind oxygen at all, we investigated the effects of cobalt and nickel under normal versus high oxygen tension. The induction of Epo mRNA by cobalt was modestly attenuated under hyperoxic incubation. Consistent with this result, Necas and Neuwirt (14) previously demonstrated that when rats were treated with cobalt and then subjected to a 3 atmosphere O_2 hyperbaric chamber, induction of serum Epo levels was suppressed. In contrast, we found that Epo mRNA induction by nickel was consistently increased by high oxygen tension, a result that cannot be explained by the model depicted in Figure 4.

The findings reported here may have relevant clinical implications. The high serum Epo levels in patients with iron deficiency anemia decline rapidly after the administration of iron, before any improvement in oxygen delivery or increase in reticulocyte count is detected (15,16). The *in vitro* studies reported here complement these clinical observations well and suggest that the enhanced Epo response in iron deficient patients results from direct stimulation of the Epo gene by low iron, an effect which is abolished upon iron supplementation.

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