transgenic mouse strain that displays many of the physical and histologic changes seen in human neonatal α_1-Pi disease. These animals are likely to be useful models for this human disease. In addition, the liver damage observed suggests that these animals may subsequently develop cirrhosis, and thus may also be useful in the study of this pathophysiologic state.

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Regulation of the Erythropoietin Gene: Evidence That the Oxygen Sensor Is a Heme Protein

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Erythropoietin (Epo), the hormone that stimulates red blood cell production, is synthesized in the kidney and liver in response to hypoxia. The human hepatoma cell line Hep3B regulates its production of Epo in a physiologic manner. Either hypoxia or cobalt chloride markedly increases expression of Epo mRNA as well as production of biologically active and immunologically distinct Epo protein. New protein synthesis is required before the induction of increased levels of hypoxia- or cobalt-induced Epo mRNA. Hypoxia, cobalt chloride, and nickel chloride appear to stimulate Epo production through a common pathway. The inhibition of Epo production at low partial pressures of oxygen by carbon monoxide provides evidence that a heme protein is integrally involved in the oxygen-sensing mechanism. This hypothesis is further supported by the finding that when heme synthesis is blocked, hypoxia-, cobalt-, and nickel-induced Epo production are all markedly inhibited. A model is proposed in which a ligand-dependent conformational change in a heme protein accounts for the mechanism by which hypoxia as well as cobalt and nickel stimulate the production of Epo.

ONE OF THE MAJOR UNANSWERED questions in the regulation of erythropoiesis is the mechanism by which hypoxia triggers the increased production of erythropoietin (Epo). In the fetus the liver is the primary site of Epo synthesis (*1*), whereas after birth, production shifts to the kidney (*2*). The lack of a cell culture system that produces readily measurable amounts of Epo in a regulated manner has hindered efforts to understand the intracellular and molecular events by which hypoxia induces increased production of Epo. We have reported that the human hepatoma cell line Hep3B increases its level of Epo mRNA and its production of Epo protein 20- to 50-fold in response to hypoxia and 6- to 12-fold in response to cobalt chloride (*3*). Using this cell culture system, we can now address questions regarding the nature of the oxygen sensor, which have been difficult to pursue with in vivo systems.

The existence of possible "second messengers" in the Epo regulatory pathway remains enigmatic. Androgens (*4*), prostaglandins (*5*), adenosine 3′,5′-monophosphate (cAMP) (*6*), and activated oxygen compounds (*7*) have all been proposed as intermediaries between stimulus and response. However, there is no consistent or convincing evidence that any of these substances act physiologically. Using the Hep3B cell culture system, we reported that prostaglandin E_2 had no effect on Epo production (*3*). We have now also investigated the effects of the calcium ionophore A23187 (0.1 to 5.0 μM), phorbol myristate acetate (10 to 100 nM), combinations of A23187 and phorbol myristate acetate, forskolin ($10^{-6}M$ to $10^{-4}M$), heat shock (42°C for 6 to 24 hours), hydrogen peroxide ($10^{-4}M$), triiodothyronine (1 to 10 nM), the potent calcium channel blocker lanthanum (1 mM), the androgens 5α-androstan-17β-ol-3-one ($10^{-7}M$ to $10^{-5}M$) and fluoxymesterone ($10^{-7}M$ to $10^{-5}M$), α-interferon (2×10^3 to 2×10^4 U/ml), adenosine ($10^{-8}M$ to $10^{-3}M$), and interleukin-1 (5 U/ml) on Epo production. None of these agents were active in inducing Epo protein synthesis in the Hep3B cell culture system.

Besides being stimulated by hypoxia, Epo production is reliably stimulated by the administration of cobalt. Cobalt increases the red cell mass in both man (*8*) and experimental animals (*9*). Experiments with intact animals (*9*) as well as perfused kidneys (*10*) have shown that cobalt stimulates erythropoiesis by increasing the production of Epo. However, the mechanism by which this response occurs is not understood. The suggestion (*11*) that cobalt acted through inhibition of cellular oxidative phosphorylation is not tenable, since more potent inhibitors of cell respiration, such as cyanide, have no effect on Epo production (*12*). Intrarenal injections of nickel also induce erythrocytosis (*13*). Hence, we incubated Hep3B cells for 24 hours in the presence of increasing concentrations of nickel chloride, and we observed a dose-dependent increase in Epo production to levels similar to those achieved with $CoCl_2$, albeit at a slightly higher molar concentration of nickel (Fig. 1). Subsequently, we studied the effects of manganese (50 to 600 μM), zinc (50 to 600 μM), iron (50 to 500 μM), cadmium (1 to 500 μM), and tin (10 to 100 μM) on Epo production by Hep3B cells. Of these elements only manganese induced measurable Epo levels, but these levels were less than those obtained by either cobalt or nickel (Fig. 1). Although cobalt, nickel, and manganese are not physiologic stimuli, information on their mechanism of action may provide insights into the regulation of Epo production.

To further delineate the nature of the oxygen-sensing mechanism in the Hep3B

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cell line, we asked whether protein synthesis was necessary before the hypoxia- or cobalt-induced increase in Epo mRNA. Experiments were performed in which Hep3B cells were incubated with cycloheximide in order to block protein synthesis and then exposed to either $CoCl_2$ or hypoxia. We used the RNA blot procedure to examine the total cellular RNA and probed with a radiolabeled Epo cDNA and actin cDNA. As shown in Fig. 2, although actin mRNA levels were unchanged by the presence of cycloheximide, there was a marked decrease in Epo mRNA in both $CoCl_2$- and hypoxia-treated cells that had been incubated in the presence of cycloheximide. This finding indicates that protein synthesis is necessary before the increase in Epo mRNA can be induced by either cobalt or hypoxia.

Many proteins that participate in reactions with molecular oxygen do so via a heme moiety. Indeed, the reversible binding of oxygen to ferroheme proteins affords a better means of "fine-tuning" than the consumption of oxygen in an irreversible chemical reaction. Hemoglobin is a classic example of such a heme protein. When the ferrous iron atom in the center of the porphyrin ring of heme binds oxygen, the hemoglobin molecule changes its conformation from the deoxy or tense (T) state to the oxy or relaxed (R) state (*14*). Furthermore, others have shown that cobalt, nickel, or manganese can be substituted for ferrous iron in the center of the porphyrin ring of heme (*15–17*). However, cobalt hemoglobin binds oxygen with low affinity (*15*), and nickel hemoglobin is unable to bind oxygen at all (*16*); hence, the resulting metal-substituted hemoglobin molecule is locked in the deoxy conformation. Furthermore, neither the cobalt- nor nickel-substituted porphyrin is able to bind carbon monoxide. Manganese(II) hemoglobin has properties similar to those of cobalt and nickel hemoglobin, but it is readily oxidized to the (III) state in which it becomes locked in the oxy conformation (*17*).

On the basis of these earlier findings and our results presented above, we propose the following hypothesis: The oxygen sensor for the regulation of Epo production is a heme protein that is dependent on the oxygen tension to which Epo-producing cells are exposed. When the oxygen tension is sufficiently low, this heme protein is in the deoxy conformation and it triggers increased expression of the erythropoietin gene. Conversely, when the oxygen tension is sufficiently high, the heme protein is in its inactive oxy conformation and does not stimulate Epo production. The results of the cycloheximide experiments described above suggest that this may be a rapidly turning over protein. When either cobalt or nickel is introduced into this environment, it can substitute for ferrous iron in the porphyrin ring. The resulting substituted heme protein is locked in the deoxy conformation and, like the native deoxygenated iron heme protein, acts to increase Epo expression. The manganese(II)-substituted heme protein would be expected to perform in a similar fashion to activate Epo production; however, when it is oxidized to the manganese(III) state it would assume an oxy conformation and hence become inactive. Although we have yet to show that cobalt chloride, nickel chloride, and manganese chloride are converted to their respective metalloporphyrins in Hep3B cells, it has been demonstrated that cobalt chloride is converted to cobalt protoporphyrin in the rat liver in vivo (*18*) and by chicken hepatocytes in culture (*19*).

Our hypothesis has been tested in the following ways:

1) According to the proposed model, hypoxia, cobalt, and nickel exert their effects through a common pathway. Hence, if the Hep3B cells were maximally stimulated by hypoxia to increase Epo expression, one would expect a much less than additive effect when the cells are grown in hypoxic conditions, plus either cobalt or nickel. Similarly, one would predict no additive effect when the Hep3B cells are grown in the presence of optimal amounts of cobalt and nickel in combination as opposed to either stimulus alone. We observed that the effects of the combination of hypoxia plus cobalt or hypoxia plus nickel were essentially the same as the effect of hypoxia alone (Fig. 3, A and B), implying that all of these stimuli exert their actions through the same pathway. In another series of experiments ($n = 3$), there was no additional increase in the production of Epo by Hep3B cells grown in the presence of 50 μM $CoCl_2$ plus 300 μM $NiCl_2$, as compared to either stimulus alone (*20*).

2) If this heme moiety is similar to that of hemoglobin, one would predict that carbon monoxide would mimic oxygen and that even in the presence of hypoxia, carbon monoxide would keep the heme protein in

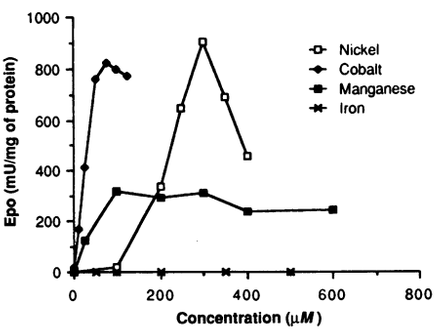

Fig. 1. Dose-response curves showing the production of Epo by Hep3B cells over 24 hours as a function of the concentration of cobalt, nickel, manganese, or iron. Hep3B cells were grown in 25-cm^2 tissue culture flasks (Corning no. 25020) in 5 ml of α minimal essential medium (Gibco) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum (Gibco). The culture medium was changed daily for 1 or 2 days before all experiments. Experiments were performed when the cells had approached confluence. The cells were grown in fresh culture medium containing the desired concentration of the indicated metal for the duration of each experiment (24 hours). At the conclusion of each experiment the culture medium was collected and frozen at −80°C until assayed for Epo. The cell pellet was also collected and was frozen at −20°C until the protein concentration was measured with the Bio-Rad protein assay. For both $CoCl_2$ and $NiCl_2$, at concentrations greater than the peak in the dose response, we observed increased cell toxicity as evidenced by increasing numbers of dead cells and a decrease in the amount of protein measured in the adherent cell pellets at the conclusion of the 24-hour incubation. This probably accounts for the dose-related decrease in Epo production at concentrations of $CoCl_2$ and $NiCl_2$ above that at which the peak effect was observed. Epo concentrations were determined in duplicate by radioimmunoassay as described (*3*). Comparable Epo concentrations have been obtained by bioassay (*3*).

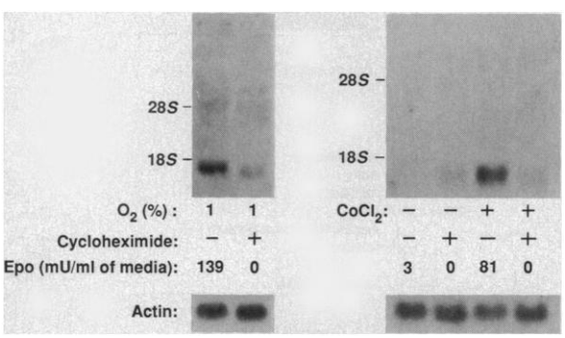

Fig. 2. Effect of cycloheximide on hypoxia- and cobalt-induced Epo mRNA levels. Hep3B cells were grown in 150-cm^2 tissue culture flasks containing 30 ml of medium in the presence or absence of 200 μM cycloheximide for 4 hours. $CoCl_2$ (100 μM) or a 1% oxygen-containing environment was then introduced as described (*3*), and the incubation was continued for an additional 24 hours (in the continued presence or absence of cycloheximide). Total cellular RNA was isolated and an RNA blot analysis was performed (*3*). Total cellular RNA (15 μg) was loaded in each lane. The results obtained when the RNA-containing filter was hybridized with ^{32}P-labeled Epo cDNA are shown. As a control, the hybridization to radiolabeled mouse β-actin is shown at the bottom. The corresponding Epo protein production by these cells is depicted as well.

the oxy conformation and thus prevent the hypoxia-induced increase in Epo expression. Carbon monoxide is a highly selective ligand with regard to its metal-binding site as well as the oxidation and spin states of the metal. Reduced heme proteins with accessible iron-binding sites are the only known targets for carbon monoxide interactions in biological systems (*21, 22*). Hence, the inhibition of hypoxia-induced Epo expression by carbon monoxide, if demonstrated, would strongly support the hypothesis that the oxygen sensor is a heme protein. Hep3B cells were therefore grown for 24 hours in an environment containing 1% oxygen plus 10% carbon monoxide. Under these conditions the cells expressed approximately one-fourth to one-third as much Epo as identical cells grown in the presence of 1% oxygen in the absence of carbon monoxide (Fig. 3, A and B). Heme proteins are known to differ in their relative affinities for oxygen and carbon monoxide (*22*). Since carbon monoxide did not totally abrogate the hypoxia-induced Epo production by Hep3B cells, the oxygen-sensing heme protein may not bind carbon monoxide quite as well as oxygen. This is a specific effect of carbon monoxide, presumably on a heme protein, and not a nonspecific toxic effect, as confirmed by the finding, also shown in Fig. 3, A and B, that Hep3B cells grown in 1% oxygen, 10% carbon monoxide, and either 100 μM $CoCl_2$ or 300 μM $NiCl_2$ make at least as much Epo as Hep3B cells grown in 100 μM $CoCl_2$ or 300 μM $NiCl_2$ alone.

3) Since cobalt protoporphyrin and nickel protoporphyrin cannot bind carbon monoxide, one would predict that carbon monoxide would not block the cobalt-induced or nickel-induced stimulation of Epo production. In fact, as shown in Fig. 3, A and B, we observed that Hep3B cells grown in the presence of 1% oxygen, 10% carbon monoxide, plus either 100 μM $CoCl_2$ or 300 μM $NiCl_2$ produced significantly more Epo than cells grown under identical conditions but in the absence of cobalt or nickel. To remove the confounding variable of hypoxia, an additional series of experiments was performed in which Hep3B cells were grown in medium containing either 100 μM $CoCl_2$ ($n = 6$) or 300 μM $NiCl_2$ ($n = 3$) in an atmosphere of 15% oxygen, 5% carbon dioxide, and either 80% carbon monoxide or 80% nitrogen. The difference in Epo expression in response to either cobalt or nickel stimulation in the carbon monoxide–containing atmosphere from Epo expression in the nitrogen-containing atmosphere was not statistically significant (*20*), again supporting our hypothesis.

4) Independent confirmation that oxygen sensing depends on a heme protein can be provided by blocking heme synthesis in Hep3B cells, thereby impairing the oxygen-sensing mechanism and demonstrating that hypoxia-, cobalt-, and nickel-induced Epo production are inhibited. Hep3B cells were therefore incubated for 24 hours in media containing either desferrioxamine, a potent iron chelator and inhibitor of heme synthesis (*23*), or 4,6-dioxoheptanoic acid, an inhibitor of aminolevulinate dehydratase (*24*), an enzyme in the heme synthetic pathway. The cells were then grown either in a 1% oxygen environment or in the presence of 75 μM $CoCl_2$ or 300 μM $NiCl_2$, and Epo production was measured after 24 hours. As depicted in Fig. 3C, 130 μM desferrioxamine caused approximately a fivefold reduction in hypoxia-induced Epo production, which was significantly ameliorated when 300 μM ferrous ammonium sulfate was also added. Similarly, 2 mM 4,6-dioxoheptanoic acid caused about a 2.3-fold reduction in hypoxia-induced Epo expression as well as about a 3.3-fold decrease in nickel-induced and approximately a 4-fold decrease in cobalt-induced Epo production. The somewhat less potent effect of the 4,6-dioxoheptanoic acid compared to desferrioxamine may reflect the inability of the former compound to completely inhibit heme synthesis and lower cellular heme content (*25*).

Incubation of the various cell preparations (in Fig. 3) with carrier-free [^{3}H]leucine showed that changes in Epo production could not be accounted for by differences in overall protein synthesis.

In summary, these results strongly support the hypothesis that the oxygen-sensing mechanism involves a specific oxygen receptor rather than a nonspecific block in oxidative phosphorylation. We present evidence that the oxygen sensor is a heme protein and that ligand binding to this heme protein influences Epo production and secretion.

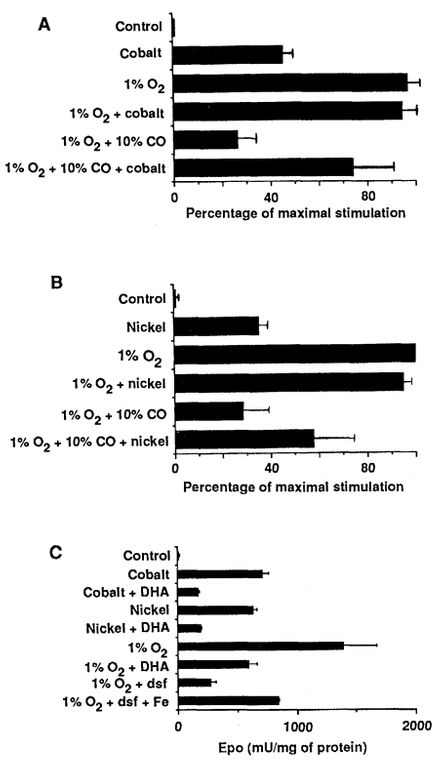

Fig. 3. (**A**) Effects of cobalt, hypoxia, and carbon monoxide on the Epo production by Hep3B cells. The Hep3B cells were cultured as detailed in the legend of Fig. 1. The atmosphere to which the cells were exposed was manipulated as described (*3*). All experiments were performed in an atmosphere containing 5% CO_2, with N_2 plus O_2 and CO at percentages indicated in the figure. All gas mixtures were obtained from Yankee Oxygen. When used, $CoCl_2$ was at a concentration of 100 μM. The results are expressed as the percentage of maximal Epo production for each individual experiment. Data shown are the mean of six sets of experiments, with error bars indicating the standard deviations. Epo concentrations were determined by radioimmunoassay (*3*), and Epo production for each experimental point was normalized to the milligrams of protein in the adherent Hep3B cell pellet at the conclusion of each experiment. The mean (±1.0 SD) maximal stimulation for the six experiments was 2198 ± 548 mU of Epo per milligram of cell pellet protein. (**B**) Effects of nickel, hypoxia, and carbon monoxide on Epo production by Hep3B cells. Experiments ($n = 3$) were performed exactly as detailed in Fig. 3A except that 300 μM nickel chloride was substituted for 100 μM cobalt chloride. (**C**) Effects of 4,6-dioxoheptanoic acid (DHA) on hypoxia-induced, cobalt-induced, and nickel-induced Epo production by Hep3B cells and of desferrioxamine (dsf) on hypoxia-induced Epo production. Cells were grown as stated above but were incubated for 24 hours with no additives, with 130 μM dsf or 2 mM DHA before exposure to a 1% oxygen environment, or with 2 mM DHA plus 75 μM $CoCl_2$ or 300 μM $NiCl_2$ for an additional 24 hours. When ferrous ammonium sulfate was added, it was at a final concentration of 300 μM. All experiments were performed three times except the 1% O_2 experiments ($n = 8$) and the experiments in which iron was added ($n = 2$).

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Human Insulin-Degrading Enzyme Shares Structural and Functional Homologies with *E. coli* Protease III

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A proteinase with high affinity for insulin has been proposed to play a role in the cellular processing of this hormone. A complementary DNA (cDNA) coding for this enzyme has been isolated and sequenced. The deduced amino acid sequence of the enzyme contained the sequences of 13 peptides derived from the isolated protein. The cDNA could be transcribed in vitro to yield a synthetic RNA that in cell-free translations produced a protein that coelectrophoresed with the native proteinase and could be immunoprecipitated with monoclonal antibodies to this enzyme. The deduced sequence of this proteinase did not contain the consensus sequences for any of the known classes of proteinases (that is, metallo, cysteine, aspartic, or serine), but it did show homology to an *Escherichia coli* proteinase (called protease III), which also cleaves insulin and is present in the periplasmic space. Thus, these two proteins may be members of a family of proteases that are involved in intercellular peptide signaling.

DESPITE EXTENSIVE STUDIES, THE mechanism by which insulin elicits its varied responses remains unknown. Recent data implicate the intrinsic tyrosine kinase activity of the insulin receptor in the initiation of many of the biological responses to insulin (*1*). However, other proposed pathways include the generation of a novel inositol phosphate glycan (*2*), effects via Ca^{2+} (*3*), or possibly a direct effect of insulin on subcellular organelles (*4*). Also controversial has been the subsequent processing of insulin by cells. Although it is known that the same cells that respond to insulin also degrade the hormone (*5*), the fate of the internalized insulin has been difficult to determine. For example, several reports have indicated that the internalized insulin is transported directly to lysosomes where it is degraded (*6*); others indicate that most of the insulin is first cleaved in a nonlysosomal pathway at several specific sites (*6*).

One enzyme that has been proposed to play a role in the cellular processing of insulin is a proteinase (called insulin-degrading enzyme or IDE) with high affinity for insulin ($K_d \simeq 100$ n*M*). Although this enzyme was first described over 40 years ago (*7*), its purification has proven very difficult and considerable uncertainty still exists as to its properties (size, specificity, and metal- and thiol-dependence) (*6*). Evidence that this proteinase participates in the intracellular degradation of insulin includes the following: (i) inhibitors of the purified proteinase inhibit insulin degradation in cells (*8*); (ii) the insulin cleavage sites observed in vitro with the purified proteinase are also observed in insulin extracted from cells (*9*); (iii) microinjection of monoclonal antibodies to this proteinase inhibits cellular insulin degradation (*10*); and (iv) insulin can be cross-linked in intact cells to this proteinase (*11*). However, since IDE is primarily cytosolic, it has remained unclear how the enzyme could come in contact with endocytosed insulin.

In the current studies, we have used an affinity column composed of monoclonal antibodies to IDE (*10*) to purify 200 μg of the enzyme from 12 liters of packed human red blood cells (*12*). The purified protein, which gave a single band on SDS gels of $M_r \simeq 110{,}000$, was found to have a blocked NH_2-terminus, a characteristic of many cytosolic proteins. The enzyme was therefore digested with trypsin, the resulting peptides were isolated by high-performance liquid chromatography (HPLC), and the sequences of 13 peptides were determined by automated Edman degradation (*12*). Six different synthetic oligonucleotides were designed on the basis of five of these peptide sequences and were used to screen various cDNA libraries (*12*). A partial clone was identified and used to obtain a full-length cDNA from a λgt10 human hepatoma library, and its insert was subcloned and sequenced (Fig. 1).

The sequence of this cDNA clone (3048 bp) contains a single open reading frame (ORF) of 952 amino acids with an initiator methionine codon at nucleotide 0 and a terminator codon at nucleotide 2855 (Fig. 1). The predicted polypeptide encoded by this cDNA has a calculated M_r of 110,453 and contains the sequences of all 13 previously determined tryptic peptides. Codon usage analysis of the ORF reveals a strong preference for the "least common" codons (*13*) at positions encoding highly hydrophilic amino acids (Glu, Asp, Lys, and Asn), which may decrease the efficiency of translation in vivo. This is consistent with the low levels of this protein observed in most cells. Sequence analysis reveals one possible glycosylation site (Asn-X-Thr) at amino acids 732 to 734. A Kyte-Doolittle hydrophobicity plot (*14*), however, shows no obvious NH_2-terminal signal peptide sequence, suggesting that the protein is not processed and therefore not glycosylated. Finally, blot analyses of total RNA from human lymphocytes (Fig. 2) and human hepatoma cells (HepG2) reveal message sizes of 3.1 and 3.5 kb; perhaps this is because of differences in the processing of the RNA, but possibly because of different isozymes.

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