Hypoxia-inducible Factor 1α (HIF-1α) Is a Non-heme Iron Protein

IMPLICATIONS FOR OXYGEN SENSING*

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The hypoxia-inducible factor 1 complex (HIF-1) is involved in the transcriptional activation of several genes, like erythropoietin and vascular endothelial growth factor, that are responsive to the lack of oxygen. The HIF-1 complex is composed of two b-HLH proteins: HIF-1 b, that is constitutively expressed, and HIF-1 a, that is present only in hypoxic cells. The HIF-1 a subunit is continuously synthesized and degraded by the ubiquitin-proteasome under aerobic conditions. Hypoxia, transition metals, iron chelators, and several antioxidants stabilize the HIF-1 a protein, allowing the formation of a transcriptionally active HIF-1 complex. HIF-1 a stabilization is due to the protection of a heme protein. To date, the precise oxygen sensor are unknown. (7) hypothesized the iron center to be a potent stimulator of HIF-1 a stabilization. The inhibitory effect of CO has not been found in all cell types. (8) proposed that heme oxygen sensing involving redox enzymes is a non-heme iron-binding protein and propose that oxygen sensing occurs by direct interaction of O 2 with this iron center.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—Hep3B and B-1 cells were grown in nucleoside-free α minimal essential medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 2.5 mg/ml fungizone (Life Technologies, Inc.), 100 units/ml penicillin (Life Technologies, Inc.), 100 μg/ml streptomycin (Life Technologies, Inc.). Cell lines were maintained in a well humidified incubator at 37 °C in 5% CO 2, 95% air. For hypoxic stimulation, the culture plates were incubated in a modular incubator chamber (Billups-Rothenburg, Del Mar, CA) and flushed with a gas mixture containing 1% O 2, 5% CO 2, and nitrogen balanced. For CO treatment, the cells were flushed with a gas mixture containing either 20% (normoxia) or 1% (hypoxia) O 2, 6% CO, 5% CO 2 and balanced with N 2. Cells were stimulated with a gas mixture containing either 20% (normoxia) or 1% (hypoxia) O 2, 6% CO, 5% CO 2 and balanced with N 2. Cells were stimulated with a gas mixture containing either 20% (normoxia) or 1% (hypoxia) O 2, 6% CO, 5% CO 2 and balanced with N 2. Cells were stimulated with a gas mixture containing either 20% (normoxia) or 1% (hypoxia) O 2, 6% CO, 5% CO 2 and balanced with N 2.

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The abbreviations used are: Epo, erythropoietin; HIF-1, hypoxiainducible factor 1 complex; ARNT, aryl hydrocarbon nuclear receptor translocator; Dfx, desferrioxamine; PCR, polymerase chain reaction; GST, glutathione S-transferase; ADA, dioxoheptanoic acid; IPTG, isopropyl-β-D-thiogalactopyranoside.
ulated with cobalt chloride or desferrioxamine (Dfx) (Sigma) at a final concentration of 100 or 130 μM, respectively. The B-1 cells are a Hep 3B derived cell line that was stably transfected with an expression vector containing a luciferase cDNA under the control of a minimal Epo promoter (330-base pair 5′-AN-XhoIII fragment) and the hypoxia-responsive enhancer from the human Epo gene (150-base pair Apal-Pat fragment). The response of this cell line to hypoxia, cobalt, desferrioxamine, and antioxidants has been reported (6). Inhibition of heme synthesis was studied by incubating B-1 cells with 2 mM 4,6-dioxoheptanoic acid (DHA, Sigma) for 8–32 h before exposure to 8 h of hypoxia. Inhibition of heme synthesis was evaluated by measurements of 59Fe incorporation into heme as described by Beru et al. (11). The Luciferase assay—Luciferase expression was assayed using a commercially available kit (Luciferase Assay System, Promega, Madison, WI). Briefly, cells were washed three times with cold phosphate-buffered saline prior to lysis with a 1 N HCl solution of the concentrated assay reagent in a TD 20/20 luminometer (Promega). The results are expressed as relative light units per μg of total protein. Protein concentrations were measured by the method of Bradford using a Bio-Rad kit (Bio-Rad, Hercules, CA), with bovine serum albumin (Sigma) as the standard.

Expression Vectors and Protein Production—All the HIF-1 expression plasmids were generated using the glutathione S-transferase expression system (pGEX-4T-1 expression vector) from Amersham Pharmacia Biotech. The HIF-1α inserts were generated using polymerase chain reaction (PCR) and a human HIF-1α cDNA as a template. The following PCR primer sequences were synthesized at the Jefferson Nucleic Acid Facility:

- 5′-GGATCCGAATTCAAGTTG-3′
- 5′-GTCGACTCGAGTCATCAGCTTG-3′

PCR reactions were carried out in a Perkin-Elmer (DNA-thermal cycler 2.1) PCR machine for 30 cycles. The reaction products were digested with EcoRI and SallI and ligated into the backbone of the 26-kDa GST protein cDNA, into pGEX-4T-1 vector. The effect of CO on hypoxia, cobalt, and desferrioxamine (Dfx) for another 8 h. The results are expressed as ratios of luciferase response between DHA-treated and untreated cells. The above results indicate that 6% CO might be exerting a minimal stimulatory effect. Similar results were obtained when 5% CO was utilized (not shown).

Effect of Heme Synthesis Inhibitors on Hypoxia Response—The effect of the heme synthesis inhibitor 4,6-dioxoheptanoic acid (DHA) (15) on the hypoxia, cobalt, and desferrioxamine responses was studied in B-1 cells. These cells were derived from Hep 3B cells by stable transfection with a luciferase reporter under the control of a minimal Epo promoter and the hypoxia-responsive enhancer. As described previously (6), B-1 cells respond to hypoxia, cobalt chloride, and iron chelators, in a time- and concentration-dependent way, by increasing luciferase expression. B-1 cells were incubated with 2 mM AHA for various periods and then exposed to hypoxia, cobalt, or Dfx for another 8 h. The results are expressed as ratios of luciferase response between DHA-treated and untreated cells.

Inhibition of heme synthesis was evaluated by measurements of 59Fe incorporation into heme, as described previously (6). B-1 cells were exposed to hypoxia, cobalt, or desferrioxamine (Dfx) for another 8 h. The results are expressed as ratios of luciferase response between DHA-treated and untreated cells. The above results indicate that 6% CO might be exerting a minimal stimulatory effect. Similar results were obtained when 5% CO was utilized (not shown).

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expressed in identical conditions as the HIF-1α peptides, showed almost complete absence of iron, as shown in Table I. The presence of iron in the HIF-1α fragments was confirmed by graphite furnace atomic absorption spectroscopy, as measured by an independent laboratory (Galbraith Laboratories Inc.). No heme was detected in the recombinant proteins (Drakking reagent, Fisher). Protein was measured by the Bradford method and by a micro-Kjeldahl nitrogen assay at Galbraith Laboratories Inc. The iron/protein molar ratio appears to be between 1:1 to 2:1. These estimates are based on six independent protein preparations.

Effect of Transition Metals and Iron Chelators on the Iron Content of HIF-1α Fragments. Fe(II) and Fe(III) are the transition metals that are commonly found in peptide fragments, so metallospearsment were incubated with Dfx (100 μM) or transition metals cobalt, manganese, and nickel (500 μM) for 30 min in the presence of IPTG. The results were confirmed by atomic absorption spectroscopy. Although manganese did not significantly affect the iron content in those samples, it produced a large increase over 2.1 in the deoxy form. A 1.3 fold increase in the deoxy form was observed in the samples treated with manganese. Iron was observed in the samples treated with nickel.

Removal of Iron by Chelating Agents. To determine the effect of chelating agents on the iron content of heme, we incubated the recombinant 529–658 fragment with Fe(II) chelater 1,10-bathophenanthroline or the Fe(III) chelator desferrioxamine and tiron. Both Fe(II) and Fe(III) chelators completely removed the iron from the peptide, whereas the Fe(III) chelator had little effect. The finding that chelators remove iron from the proteins explains the absence of detectable iron in the glutathione-treated peptides, because glutathione is a potent iron chelator (29).

**DISCUSSION**

The hypoxic activation of several genes is mediated by the hypoxia-responsive enhancer, first described in the 3’ end of the Epo gene (16–18). A hypoxia-inducible protein complex, termed HIF-1β, binds to the enhancer and stimulates transcription. Of the two proteins that form the complex, HIF-1β (ARNT) is constitutively expressed, whereas HIF-1α is only present in hypoxic cells (5). Recent evidence has shown that HIF-1α protein is continually synthesized, but rapidly de-
survival is unclear. Although hydrogen peroxide can react with Fe$^{2+}$, by way of the Fenton reaction generating hydroxyl radicals (OH·), these radicals are so reactive that it is difficult to conceive their role as specific signal transducers. Furthermore, studies using iodonium compounds, potent inhibitors of NADPH oxidases (22), have failed to show a stimulatory effect on HIF-1α expression (23).

The finding that HIF-1α is an iron-containing protein provides an alternative mechanism for oxygen sensing: localized Fenton reactions could occur in the core of the protein itself, leading to oxidative in situ modification of critical amino acid residues or changing the conformation of the protein, thus targeting it for proteasomal degradation. In this model, iron chelators would act by either decreasing the availability of a labile iron pool or by directly removing iron from HIF-1α. Transition metals may compete for iron for the metal binding site in the protein, as was shown for the case of manganese in our bacterial studies, and antioxidants may inhibit the localized redox reactions.

Although the exact sequences involved in the normoxic degradation of HIF-1α have not been completely defined, early work by Jiang et al. (24) indicated that the C-terminal end of HIF-1α contained the putative degradation domain. Further work by Pugh et al. (25) provided evidence suggesting that fragment amino acids 530–634 were involved in the oxygen-regulated degradation of the protein. Our finding of an iron binding site in fragment 529–658 provides a plausible mechanism for this oxygen-regulated degradation.

Because mitochondria, one of the main sources of H$_2$O$_2$ in cells, could be easily removed by chelators, both in vitro and in vivo, an unusual in di-iron centers, which usually have both irons an-

is unlikely. A possible structure may be a di-iron center, similar to the ones found in the oxygen carrying protein hemerythrin and in the enzymes diribonucleotide reductase and methane mono-oxynase (28). These proteins have the common property of binding oxygen, by way of oxidizing the Fe$^{2+}$ to Fe$^{3+}$.

Preliminary studies using electron paramagnetic resonance spectroscopy (EPR) analysis at low temperature (10 K), conducted in Dr. P. L. Dutton’s laboratory (University of Pennsylvania), showed no paramagnetic signal. However, this is not unusual in di-iron centers, which usually have both iron antiparamagnetically coupled (28). In summary, we provide evidence that suggests that oxygen sensing is mediated by an iron binding site(s) in the HIF-1α protein. The interaction of the iron center, either with H$_2$O$_2$ or directly with oxygen, may provide a signal for the ubiquitin-proteasomal degradation of the protein, thus controlling the transcriptional activation of hypoxia-responsive genes.

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