Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of \( O_2 \) tension

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Jiang, Bing-Hua, Gregg L. Semenza, Christian Bauer, and Hugo H. Marti. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of \( O_2 \) tension. Am. J. Physiol. 271 (Cell Physiol. 40): C1172–C1180, 1996.—Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric basic helix-loop-helix protein implicated in the transcriptional activation of genes encoding erythropoietin, glycolytic enzymes, and vascular endothelial growth factor in hypoxic mammalian cells. In this study, we have quantitated HIF-1 DNA-binding activity and protein levels of the HIF-1α and HIF-1β subunits in human HeLa cells exposed to \( O_2 \) concentrations ranging from 0 to 20% in the absence or presence of 1 mM KCN to inhibit oxidative phosphorylation and cellular \( O_2 \) consumption. HIF-1 DNA-binding activity, HIF-1α protein, and HIF-1β protein each increased exponentially as cells were subjected to decreasing \( O_2 \) concentrations, with a half-maximal response between 1.5 and 2% \( O_2 \) and a maximal response at 0.5% \( O_2 \), both in the presence and absence of KCN. The HIF-1 response was greatest over \( O_2 \) concentrations associated with ischemic/hypoxic events in vivo. These results provide evidence for the involvement of HIF-1 in \( O_2 \) homeostasis and represent a functional characterization of the putative \( O_2 \) sensor that initiates hypoxia signal transduction leading to HIF-1 expression.

cell hypoxia; gene expression; homeostasis; oxygen sensing; transcriptional regulation

WHETHER DUE TO A SYSTEMIC condition (e.g., anemia) or local process (e.g., vascular occlusion), hypoxia is a cellular phenomenon characterized by \( O_2 \) concentrations that are inadequate to meet metabolic demands. The degree of hypoxia experienced by a cell is determined by its proximity to the arterial blood supply and rates of \( O_2 \) consumption by the cell and its neighbors. The fundamental requirement for \( O_2 \) is supported by many systemic, local, and cellular homeostatic mechanisms. Examples include systemic erythropoiesis resulting from increased erythropoietin (EPO) production in response to hypobaric or anemic hypoxia (reviewed in Refs. 20 and 38), local neovascularization resulting from increased vascular endothelial growth factor production by ischemic cardiomyocytes (1, 51), and increased glycolytic enzyme synthesis in cells cultured at low \( O_2 \) concentrations (35). These adaptive responses serve to increase \( O_2 \) delivery or provide alternative metabolic pathways that do not require \( O_2 \). Indirect evidence is consistent with the role of hemoprotein(s) in \( O_2 \) sensing in human cells (18, 46). The only \( O_2 \) sensor that has been characterized is a bacterial hemoprotein with kinase activity that is inhibited by \( O_2 \) (17, 31, 32).

Many clinically significant forms of hypoxia are chronic disorders, and the homeostatic responses to these conditions involve changes in gene expression. Studies of animals, isolated perfused organs, and cultured cells have demonstrated that EPO production is regulated at the transcriptional level by cellular \( O_2 \) tension (reviewed in Ref. 38). An enhancer in the EPO gene 3′-flanking region is essential for modulation of transcription by cellular \( O_2 \) concentration (2, 3, 5, 28, 33, 40, 42). EPO gene transcriptional activation is associated with expression of hypoxia-inducible factor 1 (HIF-1), which binds to the enhancer at a site required for transcription in hypoxic cells (42). HIF-1 activity was induced when EPO-producing Hep 3B cells and non EPO producing mammalian cell lines (HeLa, 293, C3A, Ltk−, Chinese hamster ovary, and Rat-1) were exposed to 1% \( O_2 \) for 4 h (48). Analysis of genes encoding vascular endothelial growth factor (26, 27), the glycolytic enzymes aldolase A, lactate dehydrogenase A, and phosphoglycerate kinase 1 (14, 15, 41), glucose transporter 1 (10), and inducible nitric oxide synthase (30) has identified HIF-1 binding sites within promoter sequences that mediate hypoxia-inducible transcription. These results provide evidence that HIF-1 is a general mediator of transcriptional responses to hypoxia. Induction of HIF-1 activity leading to EPO gene expression is a specific response to hypoxia that cannot be elicited by exposure of cells to heat shock (18, 48) or inhibitors of oxidative phosphorylation (29, 33, 44).

Biochemical purification, protein microsequence analysis, and cDNA isolation demonstrated that HIF-1 is a heterodimer and that both subunits are basic helix-loop-helix (bHLH) proteins (45, 49). The HIF-1α and HIF-1β subunits also contain a PAS (PER-ARNT-SIM) domain that was originally defined by its presence in the period (PER)-aryl hydrocarbon receptor nuclear translocator (ARNT) (ARNT) (19), and single minded (SIM) (9) proteins. HIF-1α is a novel member of the bHLH-PAS family, whereas HIF-1β is a series of ARNT gene products (45). ARNT can heterodimerize with HIF-1α (45), ARNT (44), or SIM (43) and is thus a common subunit for bHLH-PAS transcription factors. The ARNT/ARNT heterodimer activates transcription of genes encoding cytochrome P-450 proteins that are involved in the metabolism of environmental toxins (13), whereas ARNT/SIM heterodimers activate genes involved in central nervous system development (16).

Expression of HIF-1α and HIF-1β (ARNT) was induced by exposure of human Hep 3B cells to 1% \( O_2 \), with peak mRNA and protein levels at 1–2 and 4–8 h of continuous hypoxia, respectively (45). HIF-1α and HIF-1β RNA and protein returned to basal levels within 15 min after cells were returned from 1 to 20% \( O_2 \) (45), as previously shown for HIF-1 DNA-binding...
activity (47), demonstrating that HIF-1 expression is tightly regulated by cellular O2 concentration. In these studies, cells were exposed to ambient gas mixtures containing 1 or 20% O2, but the actual O2 concentration in the culture medium and within cells was not measured or controlled directly, and expression of HIF-1 in cells exposed to intermediate O2 concentrations between 1 and 20% was not determined. In this paper, we have analyzed HIF-1α and HIF-1ß showed similar or different responses and to determine the critical range of O2 concentrations over which responses occurred for comparison with the range of O2 concentrations experienced by cells in vivo under physiological and pathophysiological conditions.

METHODS

Cell Culture and Treatment

Human cervical carcinoma HeLa S3 cells (ATCC CCL-2.2), which have been adapted to growth in suspension, were cultured in Ham’s F-12 medium with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, MEM nonessential amino acids, 2 mM l-glutamine, and 1 mM sodium pyruvate (GIBCO-BRL). Cells were grown in a humidified atmosphere containing 5% CO2 at 37°C to a density of 6 x 10^5 cells/ml and maintained by dilution to 10^5 cells/ml every third day. For treatment, 4 x 10^5 cells for each experimental condition were collected in 8 ml of fresh medium, with or without 1 mM KCN, and incubated in an IL237 tonometer (Instrumentation Laboratory, Milan, Italy) for 4 h at 37°C. The equilibration system consisted of a rotating water bath assembly that held the equilibration chamber and sample vessel. The equilibration chamber contained 25 ml of distilled water and received dry gas, at a flow rate of 500 ml/min, which underwent humidification and thoroughly permeated the chamber before exiting. At the same time, a thin film of medium containing the cells inside the sample vessel underwent alternating rotations and pauses during a stirring cycle. This allowed the medium to completely equilibrate with the incoming humidified gases, which contained 5% CO2 balanced with N2/O2 content <0.01%; referred to in the text as O2) or a preanalyzed mixture of 5% CO2 and 0.125-20% O2 balanced with N2 (PanGas, Luzern, Switzerland). Cells were incubated for 4 h and washed with ice-cold phosphate-buffered saline, and the cell pellet was frozen in liquid N2.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay

Frozen cell pellets were shipped from Zurich to Baltimore for nuclear extract preparation, analysis of HIF-1 DNA-binding activity by electrophoretic mobility shift assay (EMSA), and quantitation of HIF-1α and HIF-1β protein levels by immunoblot assays. Nuclear extracts were prepared from human HeLa S3 cells as described previously (42, 49). The cells were washed and resuspended in (in mM) 10 tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.6), 1.5 MgCl2, 10 KCl, 2 dithiothreitol (DTT), 0.4 phenylmethylsulfonyl fluoride (PMSF), and 1 mM Na3VO4 (buffer C). After the lysis of cells, nuclei were pelleted by centrifugation and resuspended in 0.42 M KCl, 20% glycerol, 1.5 mM MgCl2, 2 mM DTT, 0.4 mM PMSF, and 1 mM Na3VO4 (buffer C). Nuclear proteins were extracted in buffer C and dialyzed in 25 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, 20% glycerol, 2 mM DTT, 0.4 mM PMSF, 1 mM Na3VO4, and 100 mM KCl. Protein concentrations in the nuclear extracts were determined by the Bradford assay (6) with a commercial kit (Bio-Rad Laboratories, Hercules, CA) with the use of bovine serum albumin as standard. EMSA was performed as described using oligonucleotide probes W18 (42, 49). The coding strand W18 oligonucleotide sequence, corresponding to nucleotides 1-18 of the EPO enhancer, was 5'-GCCCTACGCTGTCTCA-3' (HIF-1 binding site underlined). Supershift assays were performed as previously described (45) by addition of 1 µl of antisera raised against HIF-1α or HIF-1β at 1:3 and 1:10 dilution, respectively. Competition assays were performed as described using double-stranded oligonucleotides W18 and M18 (42). The sense strand sequence of M18 was 5'-GCCCTAAAAGCTGCTTCTCA-3' (mutation underlined).

Affinity Purification of Antibodies Against HIF-1α and HIF-1β for Immunoblot Analysis

Production of glutathione S-transferase fusion proteins. Plasmids containing an HIF-1α cDNA fragment cloned into pGEX-3X and an HIF-1β cDNA fragment cloned into pGEX-2T were constructed as described in Ref. 45. Transformed Escherichia coli DH5α cells were cultured in 50 ml of Luria-Bertani (LB) medium with 50 µg/ml ampicillin at 37°C at 200 revolutions/min (rpm) overnight, inoculated into 1 liter of LB medium with 50 µg/ml ampicillin, and cultured at 37°C at 200 rpm until OD600 = 1.0. Glutathione S-transferase (GST/HIF-1α fusion protein synthesis was induced by adding isopropyl β-D-thiogalactopyranoside to 0.1 mM and shaking at 200 rpm at 30°C for 1 h. The fusion protein was isolated as described in Ref. 45, except that the elution of protein was in 20 mM reduced glutathione. GST/HIF-1β fusion protein was induced and isolated as described in Ref. 45. The affinity-purified fusion protein was analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantitated by the Bradford assay (6) as described above.

Fusion protein resin preparation. Purified fusion protein was coupled to hydrated CNBr-activated Sepharose-4B (Pharmacia Biotech) in 0.1 M NaHCO3 (pH 9.0)-0.5 M NaCl for 1 h at 22°C. The coupling efficiency was >95% as determined by analyzing unbound protein in the supernatant. Purified HIF-18 fusion protein (1.5 mg) was coupled to 2.5 ml of resin (0.71 g of freeze-dried powder), 0.82 mg of purified HIF-1α fusion protein was coupled to 1 ml of resin (0.3 g of freeze-dried powder), and 2.5 mg of GST were coupled to 2.5 ml of resin (0.71 g of freeze-dried powder). After blocking with 0.1 M Tris-HCl (pH 8.0) and washing with 0.1 M sodium acetate (pH 4.0), 0.5 M NaCl, and then with 0.1 M Tris-HCl (pH 8.0)-0.5 M NaCl, the gel was equilibrated and stored in Tris-buffered saline (TBS; in mM: 25 Tris-HCl [pH 8.0], 0.5 M NaCl, and 2.7 KCl) with 0.02% (wt/vol) NaN3 at 4°C.

HIF-18 antibody purification. A 1:10 mixture by volume of anti-GST/HIF-18 antiserum to TBS to bacterial lysate containing GST/HIF-1α was incubated at 22°C for 1 h to adsorb components of the crude antiserum that were found to nonspecifically cross-react with GST/HIF-18 fusion protein and with HeLa cell proteins. A volume of GST-coupled Sepharose-4B equal to the volume of antiserum was added and incubated at 4°C for 4 h. After centrifugation at 1,500 g for 3 min, the supernatant was collected with the volume of GST/HIF-18-coupled Sepharose-4B equal to the volume of antiserum and incubated at 4°C for 4 h. The resin was washed twice with 10 vol of 50 mM sodium phosphate (pH 7.6)-0.1% Triton X-100 and then washed twice with 10 vol of 10 mM sodium phosphate (pH 7.6). The adsorbed protein was eluted with 0.2 M glycine-HCl (pH 2.0), 0.1 M NaCl, and 0.1% Triton X-100 at 22°C for 15 min, collected in a tube containing 0.1 vol of 1 M

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Tris·HCl (pH 8.0), and then dialyzed twice at 4°C in 100 vol of TBS buffer.

**HIF-1α antibody purification.** Anti-GST/HIF-1α antiserum (1.2 ml) was incubated with 10.8 ml of TBS buffer and 1.2 ml of GST-coupled Sepharose-4B at 4°C for 4 h. After centrifugation at 1,500 g for 3 min, the supernatant was incubated with 1 ml of GST/HIF-1α-coupled Sepharose-4B at 4°C for 4 h. The resin was washed twice with 12 ml of 50 mM sodium phosphate (pH 7.6)-0.1% Triton X-100, once with 12 ml of 10 mM sodium phosphate (pH 7.6)-0.1% Triton, and once with 12 ml of 10 mM sodium phosphate (pH 7.6). The adsorbed protein was eluted and dialyzed.

**Immunoblot analysis.** Nuclear extracts (15 μg) were fractionated at 30 mA by SDS-PAGE in a 7.0% acrylamide-0.23% bisacylamide gel. Proteins were detected as described in Ref. 45, except that the first antibodies were affinity-purified HIF-1α or HIF-1β antibodies at 1:400 (vol/vol) dilution.

**Densitometry and Data Analysis**

Autoradiographic signals of gel shift assays and immunoblots were quantitated by scanning laser densitometry (Molecular Dynamics, Sunnyvale, CA). To control for variation between different sets of cell cultures and analytic procedures, all densitometric values from a single experiment were normalized by dividing the value obtained for cells cultured at 6% O2 in media containing 1 mM KCN, because this condition was in common between different experiments. To directly compare cells cultured over the range of 0.5-20% O2 in the absence or presence of KCN, all densitometric values obtained were divided by the value for cells cultured at 6% O2 in each set. The densitometric values for cells exposed to 0-2% O2 were normalized to the value for cells at 2% O2 in each experiment. For statistical analysis of data, mean and SE values were determined and Student’s t-test was performed using the StatWorks program (Cricket Software, Philadelphia, PA).

**RESULTS**

**Experimental Design and Data Presentation**

Experiments were performed with cells maintained in suspension culture in the absence or presence of 1 mM KCN to inhibit oxidative phosphorylation, reduce cellular O2 consumption, and eliminate intracellular and extracellular O2 gradients. Human HeLa S3 cells were utilized because of the ability to maintain these cells in suspension during the course of the experiment. Preliminary small-scale experiments were performed to explore the characteristics of the response of HeLa cells to reduced O2 concentrations as reflected in the level of HIF-1 DNA-binding activity. Initial characterization of HIF-1 DNA-binding activity in HeLa cells is presented in Fig. 1.

An extensive series of experiments was then performed to prepare all cell samples at the same time for analysis of HIF-1 DNA-binding activity and protein levels. The experimental sets are 1) 0, 0.5, 1% O2 in the presence and absence of KCN; experiment 2) 0.5, 2, 3, 4, 5, 6% O2 in the presence and absence of KCN; experiment 3) 6, 8, 10, 12, 14, 20% O2 in the presence and absence of KCN. (Multiplying these O2 concentrations by a factor of 7 will approximate the O2 tension in mmHg.) Experiments 1 and 3 were performed in duplicate, and each cell sample was assayed once, whereas experiment 2 was performed once and assayed in duplicate. This part of the study thus consisted of 30 experimental conditions, 48 cell samples, and 60 assays for each of the three parameters measured (HIF-1 activity and HIF-1α and HIF-1β protein). Representative EMSA and immunoblots from one of the duplicate sample sets that were analyzed in the experiment are presented in Figs. 2 and 3, respectively. All EMSA and immunoblot data were quantitated by scanning laser densitometry. The mean results for the duplicate data sets are presented graphically in Figs. 2 and 3, where densitometry data were normalized to the result obtained for nuclear extract from HeLa cells cultured in the presence of 1 mM KCN at 6% O2 (common to experiments 2 and 3). This normalization permitted comparison of cells cultured at the same O2 concentration in the presence and absence of KCN.

**Analysis of HIF-1 DNA-Binding Activity**

HIF-1 DNA-binding activity was assayed using probe W18 that contains an 18-base pair sequence from the human EPO gene enhancer that mediates hypoxia-inducible transcription (42). When incubated with nuclear extract prepared from HeLa cells exposed to 1% O2 for 4 h, this probe was bound by HIF-1 and also by a
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nuclear protein, the activity of which does not vary with O2 concentration (Fig. 1, lane 2). This latter, constitutive, binding activity, which migrates below HIF-1 in the EMSA and which has recently been shown to consist of the transcription factors ATF-1 and CREB (25), provided an internal control to demonstrate that equal amounts of nuclear extract were present in each binding reaction. Compared with HeLa cells exposed to 1% O2, the constitutive activity was present at similar levels in nuclear extracts prepared from cells exposed to 20% O2, whereas HIF-1 DNA-binding activity was markedly reduced (Fig. 1, lane 1).

To demonstrate that the DNA-binding activity induced by hypoxia was identical to the HIF-1 DNA-binding activity previously characterized in Hep 3B cells (42), we performed supershift and competition assays. Addition of antiserum raised against recombinant HIF-1α (Fig. 1, lane 4) or HIF-1β (lane 6) resulted in loss of the band representing HIF-1/probe complexes and appearance of a more slowly migrating band representing HIF-1/probe/antibody complexes. In contrast, the respective preimmune sera had no effect on the mobility of HIF-1/probe complexes (Fig. 1, lanes 3 and 5). These results indicate that the DNA-binding activity induced by exposure of HeLa cells to 1% O2 contains HIF-1α and HIF-1β. To determine the sequence specificity of binding, we added 50 ng (~500-fold molar excess) of unlabeled W18 oligonucleotide, binding activity previously characterized in Hep 3B cells (42), we performed supershift and competition assays. Addition of antiserum raised against recombinant HIF-1α (Fig. 1, lane 4) or HIF-1β (lane 6) resulted in loss of the band representing HIF-1/probe complexes and appearance of a more slowly migrating band representing HIF-1/probe/antibody complexes. In contrast, the respective preimmune sera had no effect on the mobility of HIF-1/probe complexes (Fig. 1, lanes 3 and 5). These results indicate that the DNA-binding activity induced by exposure of HeLa cells to 1% O2 contains HIF-1α and HIF-1β. To determine the sequence specificity of binding, we added 50 ng (~500-fold molar excess) of unlabeled W18 oligonucleotide,
which competed with the labeled probe for binding of HIF-1 and the constitutive binding activity (Fig. 1, lane 7). In contrast, 50 ng of unlabeled M18 oligonucleotide, which was identical to W18 except for a three-base-pair substitution within the HIF-1 binding site, had no effect on binding of these factors to the probe (Fig. 1, cf. lanes 8 and 9). Thus HIF-1 present in HeLa cells demonstrates the same sequence-specific binding as HIF-1 present in these two cell types.

We next assayed HIF-1 DNA-binding activity in HeLa cells as a function of O2 concentration. HIF-1 DNA-binding activity increased modestly between 20 and 6% O2 (EMSA results are shown in Fig. 2C and densitometry data are plotted in Fig. 2E). At these higher O2 concentrations, the presence of 1 mM KCN in the culture medium was associated with increased HIF-1 DNA-binding activity. Exposure of cells to O2 concentrations ranging from 6 to 0.5% was associated with a progressive increase in HIF-1 DNA-binding activity as O2 concentration declined, which was unaffected by KCN concentration (EMSA results are shown in Fig. 2B and densitometry data are plotted in Fig. 2D; note the difference in scale on the y-axis of Fig. 2B compared with Fig. 2E). When cells were exposed to 0, 0.5, or 1% O2, the maximal HIF-1 response occurred at an O2 concentration of 0.5% both in the presence and absence of KCN (Fig. 2A).

Analysis of HIF-1α and HIF-1β Protein Levels

To determine whether the changes in HIF-1 DNA-binding activity reflected changes in HIF-1α and HIF-1β nuclear protein levels, immunoblot assays were performed. Polyclonal antibodies were raised in rabbits against recombinant proteins consisting of GST fused to amino acids 329–531 of HIF-1α or amino acids 496–789 of HIF-1β (45). Affinity-purified antibodies were utilized for immunoblot assays of HIF-1α and HIF-1β. Purification of HIF-1 from HeLa and Hep 3B cells revealed that HIF-1α migrated on polyacrylamide gels as a broad band with an apparent molecular mass of ~120 kDa (49). Immunoblot analysis of HeLa nuclear extracts (Fig. 3) allowed resolution of HIF-1α into at least four isoforms with apparent molecular masses of ~113, 121, 123, and 129 kDa, based on the migration of protein standards on the same gels (not shown). There was no cross-reactivity between HIF-1α and HIF-1β antibodies.

The results of the immunoblot assay were in concordance with the results of the EMSA. Between 20 and 6% O2, there were modest increases in HIF-1α and HIF-1β protein levels as O2 concentration decreased (immunoblot results are shown in Fig. 3C and densitometry data are plotted in Fig. 3E). Protein levels at each O2 concentration were increased in the presence of KCN. HIF-1α and HIF-1β levels paralleled one another, and the response of each protein, in the presence and absence of KCN, also showed remarkable parallelism over this range of O2 concentrations. Between 6 and 0.5% O2, HIF-1β levels increased modestly, whereas those of HIF-1α rose dramatically in response to hypoxia (immunoblot results are shown in Fig. 3B and densitometry data are plotted in Fig. 3D; note the difference in scale on the y-axis of Fig. 3D compared with Fig. 3E), more closely reflecting the changes in DNA-binding activity (Fig. 2D). HIF-1α levels were maximal at 0.5% O2 (Fig. 3A), as was the case for HIF-1 DNA-binding activity (Fig. 2A), whereas HIF-1β levels appeared more similar at 0, 0.5, and 1% O2 (Fig. 3A), again demonstrating less sensitivity to changes in O2 concentration.

Construction of Continuous Dose-Response Curves

To generate HIF-1 response curves from 0.5 to 20% O2, data from cells cultured in the presence and absence of KCN (experiments 2 and 3; Figs. 2 and 3) were analyzed separately. Densitometric data for cells exposed to 0.5–6% and 6–20% O2 in the absence of KCN were normalized to the result obtained at 6% O2, the condition common to both experiments. Data for HIF-1 DNA-binding activity and HIF-1α and HIF-1β protein were analyzed on a single plot (Fig. 5A). The same procedure was followed with respect to data obtained from cells cultured in the presence of KCN (Fig. 5B). The plots representing data from cells cultured in the presence and absence of KCN are virtually superimposable. In both cases, HIF-1 DNA binding activity shows a response that is intermediate between that of HIF-1α and HIF-1β protein. The small increases in HIF-1 DNA-binding activity and protein levels that occurred as O2 concentration declined from 20 to 6% (Figs. 2E and 3E) appear minor when placed in the context of the full range of O2 concentrations. Because HIF-1 DNA-binding activity and HIF-1α and HIF-1β protein levels were all maximal at 0.5% O2, it was possible to determine the O2 concentration at which the response was half maximal. For each of the six independent data sets plotted in Fig. 5, the half-maximal response occurred between 1.5 and 2% O2.

DISCUSSION

In this paper we demonstrate that the levels of HIF-1 DNA-binding activity and HIF-1α and HIF-1β protein
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Fig. 4. Analysis of HIF-1 expression at low O₂ concentrations. Expression of HIF-1 DNA-binding activity (A), HIF-1α protein (B), and HIF-1β protein (C) was analyzed in nuclear extracts of HeLa cells that were exposed to 0, 0.125, 0.25, 0.5, 1, or 2% O₂ for 4 h in absence of KCN. Three independent experiments were performed and each extract was assayed twice. Left: representative results from 1 of 6 assays performed for each parameter. Autoradiographic signals were quantitated by laser densitometry and normalized to result obtained at 2% O₂ for each of the 6 data sets. Right: plots of mean ± SE values obtained at each O₂ concentration.

are inversely related to cellular O₂ concentration. The degree of hypoxic induction was greatest when levels of HIF-1α protein were measured. The observation that HIF-1 DNA-binding activity follows HIF-1α protein levels most closely suggests that the HIF-1β subunit may be in excess over HIF-1α. Both the higher degree of regulation of HIF-1α and the presence of HIF-1β in excess are consistent with the fact that the HIF-1α subunit is unique to HIF-1 (45), whereas the HIF-1β (ARNT) subunit is shared among other bHLH-PAS transcription factors (34, 43). It should be noted that in a previous study we showed differences in HIF-1 protein levels in Hep 3B and HeLa cells (45). In Hep 3B cells at 20% O₂ there was no detectable HIF-1α and only trace amounts of HIF-1β protein, whereas both HIF-1 proteins were detectable in HeLa cells at 20% O₂, in agreement with the results presented here. Different cell types may therefore vary with respect to their basal and/or induced levels of HIF-1 expression.

HeLa cells were exposed for 4 h to specific O₂ concentrations in the presence of 1 mM KCN to block oxidative phosphorylation, so that the intracellular and extracellular O₂ concentrations would be similar and any intracellular O₂ gradients (21, 22) would be eliminated to analyze HIF-1 expression as a function of intracellular O₂ concentration. Our expectation was that, at any given extracellular O₂ concentration, HIF-1 levels would be lower in the presence than in the absence of KCN. Instead, KCN increased basal HIF-1 levels (i.e., in cells at 20% O₂) by ~50%. The mechanism underlying the KCN effect is unknown, since this small molecule is likely to alter the activity of many proteins, including the putative hemoprotein O₂ sensor(s). The effect of KCN on HIF-1 was detectable at high but not at low O₂ concentrations, because in the latter case the stimulatory effect of hypoxia overshadowed the KCN effect. The very modest effects of KCN on HIF-1 expression are consistent with previous studies demonstrat-
HIF-1 levels are determined by cellular O2 tension

![Diagram](image)

Fig. 5. Continuous dose-response curves. Densitometric data from experiments 2 and 3 (see Figs. 2 and 3) were normalized to results obtained at 6% O2. A: expression of HIF-1 in HeLa cells in absence of KCN. B: expression of HIF-1 in HeLa cells in presence of KCN. Open symbols, -KCN; closed symbols, +KCN; square, HIF-1 DNA-binding activity; triangle, HIF-1P.

ing that inhibitors of oxidative phosphorylation neither induce EPO expression under nonhypoxic conditions nor block EPO expression under hypoxic conditions, both in isolated perfused kidneys (44) and in tissue cultures (29, 33). Despite the modest effect of KCN at high O2 concentrations, the composite response curves from untreated and KCN-treated cells (Fig. 5, A and B, respectively) are superimposable, suggesting that there are no intracellular O2 gradients that have a major effect on HIF-1 expression.

The results reported here provide a physiological characterization of the putative O2 sensor that initiates the signal transduction pathway(s) leading to HIF-1 expression and EPO transcriptional activation (38, 46). The half-maximal response occurred at 1.5–2% O2 (10–15 mmHg) could be viewed as the P50 for O2 binding or, alternatively, as the 50% inhibitory concentration for inhibition of catalytic function. HIF-1 DNA-binding activity and protein levels increased exponentially as O2 concentration declined, but, rather than reaching a plateau, the response actually declined at O2 concentrations <0.5%. These results are difficult to interpret, given the complex metabolic changes that are likely to occur under conditions of severe O2 limitation. In particular, the effects of very low O2 concentrations on overall rates of transcription and translation in HeLa cells have not been determined. However, it should be noted that, whereas HIF-1 DNA-binding activity declined at O2 concentrations <0.5%, the constitutive DNA-binding activity was unchanged. The cellular responses to hypoxia that we have demonstrated provide a framework for future studies designed to elucidate the nature of the mammalian O2 sensor(s).

The relationship between HIF-1 expression and O2 tension reported here is consistent with the exponential increase in EPO mRNA levels demonstrated in IIep 3B cells as the O2 tension in ambient gas mixtures was decreased from 160 to 7 mmHg (12). Expression of the human EPO gene in the liver of transgenic mice showed a graded response to anemic hypoxia (23). In contrast, when mice were made progressively more anemic, the number of renal peritubular interstitial cells expressing EPO mRNA, rather than the quantity of EPO mRNA per cell, was found to increase exponentially, suggesting a threshold rather than a graded response at the cellular level (24, 39). A possible explanation for these differences is that local or systemic responses to hypoxia, such as regional changes in renal blood flow, may affect the degree of cellular hypoxia and thus the response of individual cells in vivo, whereas in tissue culture all cells are subjected to a similar degree of hypoxia. In the kidney it is not possible to determine the relationship between EPO-producing cells and the vasculature. In the liver, however, in situ hybridization demonstrated that EPO mRNA was present at highest levels within perivenous hepatocytes, the liver cells that are subjected to the greatest relative hypoxia due to their distance from the arterial blood supply (23).

The most dramatic response with respect to HIF-1 expression occurred at physiologically relevant O2 concentrations. For example, in the liver, venous O2 concentrations range from 30 to 35 mmHg (4–5%) (52). In the renal cortex, O2 levels have been measured at 20–30 mmHg (3–4%) (11, 37). In the cerebral cortex, a mean O2 tension of 18 mmHg (2.5%) was measured at a depth of 1 mm (50). In the heart, epicardial microvascular and myocardial PO2 measurements of 17 and 12 mmHg, respectively, have been reported (4, 36). Occlusion of a distal branch of the left anterior descending artery for 1 min resulted in an O2 concentration of near zero in the ischemic core and a gradient of O2 concentrations as distance from the core increased (36). Thus, in all of these organs, any decrease in O2 concentration would occur along the steep portion of the HIF-1 response curve, assuming that results obtained for HeLa cells are applicable to cells in vivo. There are obvious caveats...
regarding the extent to which the data we have obtained from HeLa cells can be extrapolated to cellular responses that occur in vivo, and a challenge for the future will be to extend these results to in vivo studies. However, the use of cultured cells allowed us to study the response of a homogeneous population of cells to a uniform stimulus for a defined period of time, conditions that could not be achieved in vivo or by using primary cell cultures ex vivo. The results we have obtained provide evidence for a precise physiological mechanism, as yet undefined, by which human cells are able to respond to changes in O2 concentration by expression of a transcription factor capable of mediating adaptive cellular responses to hypoxia.

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