During hypoxia, hypoxia-inducible factor-1α (HIF-1α) is required for induction of a variety of genes including erythropoietin and vascular endothelial growth factor. Hypoxia increases mitochondrial reactive oxygen species (ROS) generation at Complex III, which causes accumulation of HIF-1α protein responsible for initiating expression of a luciferase reporter construct under the control of a hypoxic response element. This response is lost in cells depleted of mitochondrial DNA (ρ0 cells). Overexpression of catalase abolishes hypoxic response element-luciferase expression during hypoxia. Exogenous H2O2 stabilizes HIF-1α protein during normoxia and activates luciferase expression in wild-type and ρ0 cells. Isolated mitochondria increase ROS generation during hypoxia, as does the bacterium Paracoccus denitrificans. These findings reveal that mitochondria-derived ROS are both required and sufficient to initiate HIF-1α stabilization during hypoxia.

Hypoxia initiates transcription of a number of gene products that help to sustain the supply of O2 to tissues and to enhance cell survival during severe O2 deprivation. Gene products that augment O2 supply at the tissue level include erythropoietin (Epo) which increases the proliferation of erythrocytes, tyrosine hydroxylase which is necessary for the synthesis of the neurotransmitter dopamine in the carotid bodies, and the angiogenic factor VEGF which stimulates growth of new capillaries (1–3). At the cellular level, gene products that enhance survival during hypoxia include the glycolytic enzymes and the glucose transporters Glut1 and Glut3 (4). The induction of these genes is mediated by hypoxia-inducible factor-1 (HIF-1) (5–7), a heterodimeric transcription factor consisting of HIF-1α and the aryl hydrocarbon nuclear translocator (ARNT or HIF-1β) subunits (7–9). The significance of HIF-1α stabilization during hypoxia regulation was recently demonstrated by the marked decrease in mRNA expression of VEGF and glycolytic enzymes seen during hypoxia in HIF-1α- or ARNT-deficient murine embryonic stem cells (10–12).

The mechanism by which HIF-1α activation is initiated during hypoxia remains unclear. Both HIF-1α and ARNT mRNAs are constitutively expressed, indicating that functional activity of the HIF-1α-ARNT complex is regulated by post-transcriptional events. ARNT levels are not significantly affected by [O2], whereas HIF-1α protein is rapidly degraded under normoxic conditions by the ubiquitin-proteasome system (13, 14). Hypoxia enhances HIF-1α protein levels by inhibiting its degradation, thereby allowing it to accumulate, to dimerize with ARNT, and to bind to the hypoxia-responsive element (HRE) in the promoter or enhancer regions of various genes. Thus, the functional HIF-1α-ARNT complex is primarily regulated by the abundance of the HIF-1α subunit.

Although much has been learned about the role of HIF-1 in controlling the expression of hypoxia-responsive genes, the underlying mechanism by which cells detect the decrease in [O2] and initiate the stabilization of HIF-1α is not known. Presently, four diverse O2-sensing mechanisms have been proposed to mediate the transcriptional response to hypoxia (15). Two of these models postulate the involvement of an iron-containing unit in the form of either a heme group or an iron/sulfur cluster, which undergoes a change in activity during hypoxia that triggers the transcriptional response. These models are supported by the observation that cobaltous ions, or alternatively the iron chelator desferrioxamine (DFO), stabilize HIF-1α under normoxic conditions (16). However, no specific proteins with this role have been identified in mammalian systems. Two other models involve the generation of reactive oxygen species (ROS) by a flavoprotein-containing NAD(P)H oxidase or by mitochondria. The NAD(P)H oxidase theory postulates that a decrease in ROS production triggers the transcriptional response to hypoxia (17, 18). In support of that model, exogenous H2O2 was found to inhibit subsequent hypoxic stabilization of HIF-1α (19). However, diphenylene iodonium (DPI), a specific flavoprotein inhibitor that blocks ROS generation by NAD(P)H oxidase, abolishes the hypoxic induction of HIF-1-dependent genes (20), whereas the model would predict that DPI should activate the response during normoxia. We previously proposed that hypoxia partially inhibits mitochondrial electron transport, producing redox changes in the electron carriers that increase the generation of ROS. These oxidants then enter the cytosol and function as second messengers in the signaling pathway leading to stabilization of HIF-1α (21). In support of this model, hypoxia failed to increase ROS production or the expression of EPO, VEGF, and glycolytic enzymes in ρ0 cells, which lack mitochondrial DNA and electron transport activity. Also, the...
response to hypoxia was abolished by the DPI, which abrogates mitochondrial ROS generation by inhibiting electron transport at the flavin site in mitochondrial Complex I (22).

Our previous study tested whether ROS are required for the DNA binding of HIF-1 and the subsequent mRNA expression of Epo, VEGF, and glycolytic enzymes during hypoxia. However, protein levels of HIF-1α were not measured, so that study did not reveal whether mitochondrial ROS were required to trigger stabilization of HIF-1α during hypoxia. Moreover, although ROS were found to be necessary for the transcriptional response to hypoxia, it was not clear whether ROS by themselves were sufficient to initiate HIF-1α stabilization. Accordingly, the present study tested the following: (a) whether mitochondrial ROS are required for HIF-1α stabilization during hypoxia, coxalt treatment, or DFO; (b) whether cytosolic ROS are sufficient to trigger HIF-1α stabilization; and (c) whether mitochondrial cytochrome c oxidase serves as the O2 sensor responsible for the redox changes underlying the increase in ROS generation during hypoxia.

EXPERIMENTAL PROCEDURES

Cell Culture—Human hepatoma 3B (Hep3B) cells and the human kidney 293 cells were cultured in α-minimum essential medium and Dulbecco’s modified essential medium, respectively, to subconfluent conditions. Rat hepatocytes were isolated as described previously (23) and maintained in Dulbecco’s modified essential medium. Media were supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum. The p-B-293 and p-B-Hep3B cells were generated by incubating wild-type cells in medium containing etidium bromide (25 ng/ml), sodium pyruvate (1 mM), and uridine (50 μg/ml) (24). Hypoxic conditions were achieved as described previously (21).

Measurement of ROS—Intracellular ROS generation was assessed using 2',7’-dichlorofluorescein diacetate (Molecular Probes). ROS in the cells cause oxidation of DCFH, yielding the fluorescent product 2’,7’-dichlorofluorescein (DCF). Cells were plated on Petri dishes and incubated with DCFH-DA (10 μM) under various conditions. The media were then removed, cells were lysed and centrifuged to remove debris, and the fluorescence in the supernatant was measured using a spectrofluorometer (excitation, 500 nm; emission, 530 nm). Data were normalized to values obtained from normoxic, untreated controls.

Preparation of Nuclear Extracts—Nuclear extracts were prepared by lysing cells in a hypotonic solution (KCl (10 mM), HEPES (10 mM), protease mixture inhibitors (Roche Molecular Biochemicals), phenylmethylsulfonyl fluoride (1 mM), freshly added, pH 7.8) for 30 min. The disrupted cells were then centrifuged at 14,000 rpm, and the pellet was resuspended in a solution containing KCl (400 mM), HEPES (20 mM), glycerol (25%, v/v), EDTA (0.2 mM), and MgCl2 (1.5 mM) at pH 7.8 in order to lyse the nuclear membrane. The suspension was then centrifuged, and the proteins in the supernatant were quantified by Bradford reagent (Sigma).

Immunoblotting—Nuclear protein extract (30 μg) was mixed with equal volume of electrophoresis buffer (1.0 ml of glycerol, 0.5 ml of β-mercaptoethanol, 3.0 ml of 10% SDS, 1.25 ml of Tris-HCl (1.0 mM), pH 6.7, and 1–2 mg of bromphenol blue). After heating, the protein was resolved on an SDS-7.5% polyacrylamide gel and transferred to a hybrid ECL nitrocellulose paper (Amersham Pharmacia Biotech). After transfer, the gel was stained with Ponceau stain to verify uniform loading and transfer. Membranes were blocked with 5% milk in TBS-T (Tris-HCl (10 mM), NaCl (150 mM), Tween 20 (0.1%), pH 8.0) overnight and subsequently incubated with the HIF-1α antibody (Novus Biological Sciences) for 3 h at room temperature. The membrane was washed with TBS-T three times and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. Subsequently, the membrane was washed three times with TBS-T and analyzed by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Transfection and Reporter Gene Assays—Transfections of 293 and p-B cells were carried out on cells plated on 35-mm Petri dishes at 60–80% confluence using Transfast-LTI (Panvera) according to the manufacturer’s protocol. A typical transfection was performed by using 0.5 μg of the luciferase reporter driven by a trimer of murine Epo 3' enhancer and the Glut-1 promoter (pEpo3’/Glut1-Luc) and 0.5 μg of the pSV-β-galactosidase (Promega) as a control vector for monitoring transfection efficiencies. In some experiments, 0.5 μg of empty vector (pBIC/CMV2) or human catalase gene driven by SV40 enhancer-promoter (pZeoSVCAT) was co-transfected with the luciferase and β-galactosidase. The DNA/LipofectAMINE mixture was incubated with plated cells for 24 h. Subsequently, the media were replaced and cells exposed to various conditions. Cell lysis was performed using the reporter gene lysis buffer (Promega). Duplicate transfection experiments were performed with four independent transfections. Luciferase assays were performed using Luciferase Assay System (Promega). β-Galactosidase assays were performed using the luminescent β-galactosidase genetic reporter system II (CLONTECH).

Determination of H2O2—The concentration of H2O2 or t-buty1 hydroperoxide was assessed in media of Hep3B cells using the xylene orange assay (25). Cells were incubated in serum-free α-medium without phenol red (Life Technologies, Inc.) with H2O2 (25 or 40 μM) for 30 min with samples (900 μl) taken every 5 min (Fig. 4A), or with t-buty1 hydroperoxide (25 or 40 μM) for 120 min with samples (900 μl) taken every 30 min (Fig. 4B). The aliquots of media were incubated at 25 °C for 45 min with 100 μl of a 10× xylene orange stock solution (xylene orange (10 μM), Fe(NO3)3, H2O2 (2.5 μM), H2SO4 (25 μM), Sigma) after which the absorbance was read at 590 nm (25). The concentration of H2O2 or t-buty1 hydroperoxide was determined from a standard curve (0, 25, and 40 μM). Fresh stock solutions of H2O2 (10 mM) and t-buty1 hydroperoxide were prepared immediately prior to each trial.

Isolation of Mitochondria—Rat liver mitochondria were isolated by differential centrifugation from the livers of adult rats. The liver homogenate was prepared using a Dounce homogenizer at 4 °C in buffer containing mannitol (200 mM), sucrose (70 mM), HEPES (25 mM), (Sigma) for 3 h at room temperature. The membrane was washed with TBS-T three times and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. Subsequently, the mitochondrial aliquot (100 μl) was lysed with 1 ml of H2O2, and the sample fluorescence was measured using a spectrofluorometer. The head space of the spinner flasks was gassed with various O2 concentrations as described previously (26).

Isolation of Porous Anaerobic Flavin-containing Systems—Wild-type P. denitrificans (ATCC, Rockville MD) were grown aerobically with succinate as the carbon source as described previously (49). Bacteria were harvested in mid-logarithmic growth phase and washed twice with KH2PO4 (50 mM). Cells were suspended in Krebs-Henseleit buffer containing KCl (150 mM), NaCl (135 mM), MgSO4 (1 mM), KH2PO4 (50 mM), MgCl2 (1.5 mM), EGTA (1 mM), pH 7.2 at a protein concentration of 0.1 mg/ml at 25 °C and were incubated with succinate (5 mM), antimycin (1 μg/ml), rotenone (1 μg/ml), and DCFH-DA (10 μM) for 30 min in spinner flasks. Subsequently, a mitochondrial aliquot (100 μl) was lysed with 1 ml of H2O2, and the sample fluorescence was measured using a spectrofluorometer. The head space of the spinner flasks was gassed with various O2 concentrations. Subsequently, fluorescence was measured from an aliquot of medium.

RESULTS

Hypoxia and Cobalt, but Not Desferrioxamine, Increase ROS Production—Intracellular ROS generation was examined in human Hep3B cells in response to hypoxia, cobalt chloride (CoCl2), or DFO using 2',7'-dichlorofluorescein diacetate (DCFH) (27). This probe enters the cells and can be oxidized in the presence of ROS, generating the fluorescent compound, DCF. Previous studies have indicated that DCFH oxidation can be mediated by H2O2 but not by superoxide (28). Hep3B cells incubated with DCFH for 6 h during hypoxia (1.5% O2) or with CoCl2 (100 μM) during normoxia demonstrated an increase in DCF fluorescence (Fig. 1, A and B). By contrast, DFO (100 μM) did not elicit an increase in DCF fluorescence over the same period (Fig. 1C). The increase in DCF fluorescence induced by hypoxia or CoCl2 was abolished by the thiol reductant pyridoline dithiocarbamate (PDTC, 20 μM) (Fig. 1, A and B). PDTC can enhance the scavenging of H2O2 by maintaining cellular stores of reduced glutathione (GSH). ROS can be generated at a number of intracellular sites, including NADPH oxidases and at mitochondrial Complex III. DPI inhibits electron transport at the flavin-containing systems including NADPH oxidase and mitochondrial Complex I. DPI (10 μM) attenuated the increase in DCF fluorescence in response to hypoxia but not to CoCl2 (Fig. 1, A and B). Mitochondrial ROS generation at Complex III can be attenuated by inhibiting electron flux at more upstream sites. Rotenone (1 μg/ml), an inhibitor of electron transport at
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Complex I, abolished the increase in DCF fluorescence during hypoxia but not during CoCl2 incubation (Fig. 1, A and B). By contrast, antimycin A (1 μg/ml), an inhibitor of the downstream end of Complex III, did not alter the increase in DCF fluorescence. Superoxide generated within mitochondria would require anion channels to enter the cytosol, where it would be converted to H2O2 by cytosolic superoxide dismutase (29). The inhibitor of mitochondrial anion channels 4,4′-dithioothiocyanato-2,2′-disulfonate (DIDS) should therefore suppress the egress of superoxide from the mitochondria (30). Indeed, DIDS (150 μM) abolished the increase in DCF fluorescence during hypoxia (Fig. 1A). By contrast, DIDS failed to attenuate the CoCl2-stimulated increase in the DCF signal (Fig. 1B). These results support the conclusion that hypoxia stimulates mitochondrial ROS generation, that CoCl2 stimulates ROS generation by a non-mitochondrial mechanism, and that desferoxamine does not affect ROS production.

Stabilization of HIF-1α during Hypoxia Requires an Increase in Mitochondrial ROS—Hep3B cells were exposed to hypoxia, CoCl2, or DFO in the presence of PTDC to examine whether increases in ROS are required for the HIF-1α stabilization. PTDC abolished the hypoxia- and CoCl2-induced stabilization of HIF-1α but had no effect on DFO-induced stabilization of HIF-1α protein (Fig. 2A). Furthermore, human epithelial kidney 293 cells transiently co-transfected with a luciferase reporter construct driven by the HRE and a construct containing catalase (31) were exposed to hypoxia, CoCl2, or DFO. Catalase expression significantly attenuated the expression of luciferase in response to hypoxia or CoCl2, whereas the response to DFO was unaffected (Fig. 2B). HIF-1α protein accumulation and HRE-luciferase expression were examined in Δb-He3B cells and Δb-293 cells, respectively, to determine whether mitochondrial function as the source of ROS required for activation of HIF-1 during hypoxia. The Δb-He3B cells failed to accumulate HIF-1α protein during hypoxia (Fig. 2C). By contrast, CoCl2 and DFO still stabilized HIF-1α protein in Δb-293 cells (Fig. 2C). Hypoxia-induced HRE-luciferase expression was not detected in Δb-293 cells, but these cells still demonstrated HRE-luciferase expression in response to CoCl2 or DFO (Fig. 2D). The CoCl2-induced increase in HRE-luciferase expression in Δb-293 cells was abolished in cells co-transfected to overexpress catalase (Fig. 2D). To clarify further the role of mitochondria in the hypoxic response, wild-type Hep3B cells were exposed to hypoxia, CoCl2, or DFO in the presence of rotenone (1 μM/ml), DPI (10 μM), DIDS (150 μM), or antimycin A (1 μg/ml). Rotenone, DPI, and DIDS all abolished the accumulation of HIF-1α during hypoxia but not during CoCl2 or DFO treatment (Fig. 3, A–C). Antimycin A augments ROS generation at Complex III (32, 33), which should mimic the response to hypoxia and lead to stabilization of HIF-1α. Yet antimycin A failed to stabilize HIF-1α during normoxia and did not abrogate the induction of HIF-1α during hypoxia, CoCl2, or DFO (Fig. 3D). We had previously reported that the ROS response to antimycin A during normoxia was smaller than the response to hypoxia in Hep3B cells (21), which suggests that ROS signaling by antimycin A may have been inadequate. We therefore tested whether stabilization of HIF-1α during normoxic antimycin A treatment might be amplified in cells whose ability to degrade H2O2 is impaired. Hep3B cells were treated for 16 h with buthionine-SR-sulfoximine (100 μM) to deplete glutathione stores in an attempt to amplify the response to an oxidizing stimulus evoked by antimycin A (34). Glutathione-depleted Hep3B cells demonstrated HIF-1α accumulation under normoxia in the presence of antimycin A (Fig. 3E). Rotenone, DPI, and DIDS all abolished this response, confirming the specificity of mitochondrial involvement (Fig. 3E). These results support the hypothesis that mitochondrial ROS are required and sufficient for stabilization of HIF-1α during hypoxia and that non-mitochondrial ROS generation is involved in the response to CoCl2. DFO responses require neither the mitochondria nor ROS, suggesting that this compound acts at a more distal step in the signaling cascade.

Exogenous Peroxide Stabilizes HIF-1α Protein during Normoxia—The observation that catalase abolishes the hypoxia- and CoCl2-induced stabilization of HIF-1α suggests that H2O2 acts as a signaling element in this response. If so, then exogenous administration of peroxide should stabilize HIF-1α during normoxia. Accordingly, boluses of H2O2 or t-buty1 hydroperoxide, a more stable analogue of H2O2, were added to culture media of normoxic Hep3B cells. Serum-free media was used because catalase in serum would otherwise rapidly degrade any added peroxide. In the absence of serum, a bolus of H2O2 added to achieve an initial concentration of 25 or 40 μM was progressively degraded over 30 min (Fig. 4A). t-Buty1 hydroperoxide (25 or 40 μM) produced a more sustained elevation, but it, too, was largely degraded within 1 h (Fig. 4B). To produce a more sustained elevation in H2O2 levels, Hep3B cells were treated with repeated boluses of H2O2 or t-buty1 hydroperoxide (25 μM or 40 μM) every 15 min for 2 h. This regimen was sufficient to trigger the stabilization of HIF-1α protein levels (Fig. 4C). Hydrogen peroxide failed to stabilize HIF-1α when bolus concentrations were lowered to 10 μM (data not shown). In 293 cells transfected with the HRE-luciferase expression

**Fig. 1.** DCF fluorescence as a measure of ROS generation Hep3B cells. Cells were incubated with DCFH-DA (10 μM) for 6 h in the presence of 1.5% O2 (A), CoCl2 (100 μM) (B), or DFO (100 μM) (C). Results are expressed relative to normoxic controls (21% O2). n = 3; mean ± S.D. ROT, rotenone.
vector, bolus additions of H$_2$O$_2$ (40 mM H$_2$O$_2$ every 15 min for 10 h) caused expression of luciferase compared with normoxic controls (Fig. 4D). By contrast, 293 cells co-transfected with the HRE-luciferase and with catalase demonstrated an attenuated expression in response to a similar regimen of H$_2$O$_2$ administration (Fig. 4D). To determine whether H$_2$O$_2$ acts upstream or downstream of mitochondria, HRE-luciferase expression was examined in p$^o$-293 cells exposed to a similar regimen of H$_2$O$_2$ (40 mM). These p$^o$-293 cells increased HRE-luciferase expression, indicating that peroxide must act downstream from the mitochondria (Fig. 4D).

**Kinase and Phosphatases Are Activated Downstream from the H$_2$O$_2$ Step**—Protein phosphorylation participates in the signaling cascade mediating the accumulation of HIF-1α as well as in the transactivation by the HIF-1 complex in response to hypoxia (35–38). The phosphatase inhibitor calyculin A (5 nM) abolished HIF-1α stabilization in response to hypoxia, CoCl$_2$, or DFO in Hep3B cells (Fig. 5A). Wortmannin (0.1–1.0 μM), an inhibitor of PI-3 kinase, attenuated HIF-1α accumulation in response to hypoxia, CoCl$_2$, or DFO (Fig. 5B). Wortmannin (500 nM) also attenuated the expression of HRE-luciferase in 293 cells (Fig. 5C). In the presence of calyculin A or wortmannin, the protease inhibitor N-carbobenzyloxy-l-leucinyl-l-leucinyl-l-norvalinal (10 μM) was still able to stabilize HIF-1α (Fig. 5D). These observations reveal the following: (a) the inhibited stabilization of HIF-1α in the presence of these compounds did not reflect a nonspecific toxic effect, and (b) phosphatases and PI-3 kinases must act upstream of the proteolytic degradation machinery. To demonstrate that phosphatases and PI-3 kinases act downstream of H$_2$O$_2$, Hep3B cells were treated with boluses of H$_2$O$_2$ (40 μM every 15 min for 2 h) in the presence of calyculin A or wortmannin. Calyculin A and wortmannin independently abolished the stabilization of HIF-1α protein effected by H$_2$O$_2$ (Fig. 5E). Thus, phosphatases and PI-3 kinases are required for the stabilization of HIF-1α and act at steps downstream of ROS. To determine whether the inhibition of the HIF-1α protein response to hypoxia, CoCl$_2$, or DFO by wortmannin was specific to that compound, additional studies were carried out using the PI-3 kinase inhibitor LY 294002. At a concentration of 20 μM, this compound also inhibited the stabilization of HIF-1α in response to hypoxia, CoCl$_2$,  

FIG. 2. A, HIF-1α protein levels in Hep3B cells exposed to 21% O$_2$, 1.5% O$_2$, CoCl$_2$ (100 μM), or DFO (100 μM) for 4 h with or without PDTC (20 μM). B, luciferase expression in 293 cells transfected with HRE-luciferase and in cells co-transfected with catalase and exposed to 1.5% O$_2$, CoCl$_2$ (100 μM), or DFO (100 μM) for 16 h. n = 4; mean ± S.D. C, HIF-1α protein levels in wild-type 293 cells and in p$^o$-Hep3B cells exposed to 21% O$_2$, 1.5% O$_2$, CoCl$_2$ (100 μM), or DFO (100 μM) for 4 h. D, luciferase expression in p$^o$-293 cells transfected with HRE-luciferase and co-transfected with catalase and exposed to 1.5% O$_2$, CoCl$_2$ (100 μM), or DFO (100 μM) for 16 h. n = 4; mean ± S.D.
or DFO (Fig. 5F). These results support the conclusion that PI-3 kinase participates in the signaling regulating HIF-1α stabilization by acting downstream from the activation by hypoxia, CoCl2, and DFO.

Complex III Is the O2 Sensor for Stabilization of HIF-1α during Hypoxia—We previously found that cytochrome c oxidase undergoes a decrease in Vmax during hypoxia (39). Cytochrome oxidase in rat hepatocytes requires 90–120 min under hypoxia to effect this change (40). Such a decrease could augment the reduction state at more proximal locations in the electron transport chain, conceivably explaining the increase in superoxide generation during hypoxia (41). To evaluate the temporal relationship between the changes in cytochrome oxidase Vmax and the stabilization of HIF-1α, isolated rat hepatocytes were studied under hypoxia. Surprisingly, HIF-1α protein stabilization was detected within 30 min at 1.5% O2 (Fig. 6A). Rotenone and PDTC abolished this response, whereas antimycin A maintained the hypoxia-induced accumulation of HIF-1α (Fig. 6A). These results are incompatible with the hypothesis that changes in cytochrome c oxidase Vmax function as the O2 sensor mediating stabilization of HIF-1α during hypoxia. Rather, the data suggest that Complex III exhibits intrinsic sensitivity to [O2] allowing it to serve as an O2 sensor by increasing its generation of superoxide at low O2 levels.

ROS generation at Complex III increases when antimycin A inhibits electron flux at its downstream end while succinate is used to supply electrons into it (32, 33). However, the effects of low [O2] on this process have not been explored. Hence, we assessed the influence of [O2] on ROS generation by Complex III during graded hypoxia by incubating isolated rat liver mitochondria for 30 min with succinate (5 mM, an electron source) in the presence of rotenone (1 μg/ml, to block electron transport to/from Complex I) and antimycin A (1 μg/ml, to fully reduce Complex III). DCFH (10 μM) was used to assess ROS generation at [O2] ranging from 1 to 5%. Under these conditions, graded increases in DCFH oxidation were seen at decreasing O2 tensions, which were inhibited by further addition of myxothiazol (100 ng/ml) (Fig. 6B). Myxothiazol inhibits Complex III upstream of the site of ROS generation, thereby confirming the role of Complex III in this response (Fig. 6B).

ROS production by Complex I has also been reported (33). To determine if [O2]-dependent ROS production by Complex I also occurs, mitochondria were incubated under 1% O2 for 30 min with DCFH, using malate (5 mM) and glutamate (5 mM) to supply electrons to Complex I and rotenone (1 μg/ml) to block electron transfer to Complex III. DCFH oxidation by Complex I did not increase during hypoxia (data not shown), indicating that the [O2]-dependent increase in ROS generation during hypoxia is restricted to Complex III.

Many elements of the mitochondrial electron transport system are highly conserved through evolution, including Complex III. The Gram-negative bacterium P. denitrificans contains a respiratory chain in the cell membrane that is similar to eukaryotic mitochondria (42). To determine whether P. denitrificans demonstrate graded increases in ROS generation during hypoxia, bacterial suspensions were incubated under 1% O2 with 25 mM DCFH, using malate (5 mM) and glutamate (5 mM) to supply electrons to Complex I and rotenone (1 μg/ml) to block electron transfer to Complex III. DCFH oxidation by Complex I did not increase during hypoxia (data not shown), indicating that the [O2]-dependent increase in ROS generation during hypoxia is restricted to Complex III.

Additional studies were therefore carried out using suspensions of E. coli, to determine whether an organism that lacks a semiquinone step in its respiratory chain would fail to generate ROS during hypoxia. During 1% O2 incubation, E. coli demonstrated consistent decreases in oxidation of DCFH, compared
with control experiments incubated under 21% O₂ (data not shown). These findings provide further support for the conclusion that electrons released from the ubisemiquinone site of Complex III are required for the generation of ROS during hypoxia.

**DISCUSSION**

ROS have been proposed to participate in the signal transduction process mediating the stabilization of the transcription factor HIF-1α during hypoxia. However, controversy exists regarding whether ROS levels increase or decrease under hypoxia. The present study extends our previous work by demonstrating that HIF-1α stabilization by hypoxia or CoCl₂ requires an increase in ROS. Hypoxia and CoCl₂ both elicited an increase in DCF fluorescence, revealing that oxidation of the probe increases prior to the stabilization of HIF-1α. The antioxidant PDTC abolished the stabilization of HIF-1α and oxidation of DCFH in response to hypoxia or CoCl₂, further suggesting the involvement of ROS.

Recent studies using exogenous administration of H₂O₂ (0.1–1 mM) did not detect stabilization of HIF-1 under normoxia. In fact, H₂O₂ bolus treatment (1 mM) abolished HIF-1-mediated gene transcription during subsequent hypoxia (1% O₂ for 8 h) (19). Our measurements revealed that a bolus of peroxide is rapidly degraded by cells even in serum-free medium, suggesting that administration of repeated boluses might be required to sustain active signaling. Indeed, Hep3B cells responded to H₂O₂ boluses (every 15 min for 2 h) by demonstrating accumulation of HIF-1α, whereas 293 cells given H₂O₂ (40 μM every 15 min for 10 h) induced the expression of HRE-luciferase. These observations indicate that low levels of an oxidizing stimulus are sufficient to stabilize HIF-1α.

The iron chelator DFO also induces HIF-1α stabilization and HIF-1-dependent gene activation, but it appears to act at a more downstream site in the signaling cascade. Stabilization of HIF-1α by DFO did not appear to involve ROS, as DFO neither increased nor decreased DCF fluorescence during normoxia. The response to DFO was not abolished by either PDTC or catalase, both of which enhanced the scavenging of H₂O₂. The flavin inhibitor DPI and the Complex I inhibitor rotenone also

**Fig. 5.** A, HIF-1α protein levels in Hep3B cells exposed to 21% O₂, 1.5% O₂, CoCl₂ (100 μM), or DFO (100 μM) for 4 h in the presence of calyculin A (Caly.A) (5 nM). B, HIF-1α protein levels in Hep3B cells exposed to 21% O₂, 1.5% O₂, CoCl₂ (100 μM), or DFO (100 μM) for 4 h in the presence of wortmannin (0–1.0 μM). Cells were pretreated with wortmannin for 90 min. C, luciferase expression in 293 cells transfected with HRE-luciferase and catalase and exposed to 1.5% O₂, cobalt chloride (100 μM), or DFO (100 μM) in the presence of wortmannin (500 nM) for 16 h, n = 4; mean ± S.D. D, HIF-1α protein in Hep3B cells exposed to 21% O₂ and 10 μM N-carbenzoxyl-l-leucinyl-l-leucinyl-l-norvalinal in the presence calyculin A (5 nM) or wortmannin (Wort., 1.0 μM). E, HIF-1α protein in Hep3B cells exposed to boluses of H₂O₂ (40 μM) every 15 min for 2 h in the presence of calyculin A (5 nM) or wortmannin (1 μM). F, HIF-1α protein in Hep3B cells exposed to 21% O₂, 1.5% O₂, CoCl₂ (100 μM), or DFO (100 μM) for 4 h in the presence of LY 294002 (LY, 20 μM). Cells were pretreated with LY 294002 for 2 h prior to study.
failed to abolish HIF-1α stabilization during DFO, and ρ0 Hep3B cells were still able to respond to DFO. However, wortmannin and calyculin A both abolished the HIF-1α stabilization during DFO. These findings indicate that DFO does not require mitochondrial electron transport or ROS and appears to be acting at a site downstream from the oxidant-mediated step but upstream from the ubiquitin-proteasome degradation step.

What is the source of ROS that trigger stabilization of HIF-1α during hypoxia? Mitochondrial Complex III can generate superoxide anions during normoxia (32, 33) (Fig. 7). Our data reveal that hypoxia increases the generation of ROS at Complex III to an extent sufficient to stabilize HIF-1α protein. In support of this, respiration-incompetent ρ0 cells failed to stabilize HIF-1α, were unable to express HRE-luciferase, and failed to increase oxidation of DCFH during hypoxia. However, ρ0 cells were able to respond to CoCl2 and to DFO, indicating that they retained the ability to respond. Moreover, ρ0 293 cells responded to exogenous H2O2 by inducing expression of HRE-luciferase. Collectively, these data indicate that DFO does not require mitochondrial electron transport or ROS and appears to be acting at a site downstream from the oxidant-proteasome degradation step.

FIG. 6. A, HIF-1α protein in rat hepatocytes exposed to 1.5% O2 in the presence of rotenone (1 μg/ml), PDTC (20 μM), or antimycin A (1 μg/ml) for 30 min. B, DCF fluorescence in media containing suspended isolated rat liver mitochondria incubated for 30 min under 1–5% O2 with DCFH-DA (10 μM), rotenone (1 μg/ml), succinate (5 mM), and antimycin (1 μg/ml). Myxothiazol (myxo., 100 ng/ml) was added to one group to confirm involvement of Complex III. C, typical pattern of DCFH oxidation by P. denitrificans as a function of [O2]. P. denitrificans were incubated for 60 min under 1–5% O2 with DCFH-DA (10 μM). D, typical pattern of DCFH oxidation by P. denitrificans during incubation for 60 min at 1 or 21% O2. Rotenone (Rot., 5 μg/ml) or myxothiazol (myxo., 200 ng/ml) attenuated DCFH oxidation under 1% O2.

ROS Are Required and Sufficient for HIF-1α Stabilization

FIG. 7. Mitochondrial model for O2 sensing. Hypoxia stimulates superoxide generation at Complex III. Superoxide enters the cytosol via anion channels and is converted to H2O2 by superoxide dismutase. H2O2 activates PI-3 kinases and phosphatases, triggering HIF-1α stabilization. CoCl2 generates H2O2 by a non-mitochondrial mechanism, thereby stimulating the effects of hypoxia on HIF-1α stabilization. DFO does not require mitochondria or H2O2 but does require PI-3 kinases and phosphatases to trigger HIF-1α stabilization.
signaling in a cell reflects a balance between the rate of oxidant generation and the efficacy of antioxidant systems. In Hep3B cells depleted of glutathione stores, the ability to metabolize H$_2$O$_2$ would be compromised, which should amplify the effects of a smaller oxidant source. Indeed, HIF-1α protein was stabilized in response to antimycin A during normoxia, whereas rotenone, DPI, and PDTC blocked that response in glutathione-depleted Hep3B cells. These observations provide further support for the conclusion that mitochondrial ROS generation at Complex III is required and sufficient for the stabilization of HIF-1α during hypoxia.

How do ROS generated in mitochondria escape to the cytosol? Superoxide generated in the mitochondrial matrix can be dismutated to H$_2$O$_2$ by Mn-superoxide dismutase. The H$_2$O$_2$ so produced could diffuse into the cytosol or could be degraded by the mitochondrial antioxidant system. Superoxide generated at Complex III could alternatively enter the cytosol via anion channels in the mitochondrial membranes (29). Our data show that anion channel inhibition by DIDS abolished the DCFH oxidation observed during hypoxia or with antimycin A during normoxia. By suppressing the egress of mitochondrial superoxide, anion channel inhibition should attenuate the cytosolic oxidant signals that are required for HIF-1α stabilization in hypoxia but have no effect on the response to CoCl$_2$ or to DFO because these responses do not require mitochondria. Indeed, the data show that HIF-1α accumulation was abolished by DIDS during hypoxia in Hep3B cells and during antimycin A treatment in glutathione-depleted cells studied during normoxia. However, DIDS did not block the response to CoCl$_2$ or to DFO. These observations further support the conclusion that an oxidizing stimulus localized to the cytosol is required for the stabilization of HIF-1α.

Stabilization of HIF-1α protein by CoCl$_2$ or DFO does not require a functional mitochondrial electron transport system, as both were able to stabilize HIF-1α protein and induce expression of HRE-luciferase in ρ0 cells. However, CoCl$_2$, unlike DFO, increased the oxidation of DCFH dye, and the CoCl$_2$-induced expression HRE-luciferase was attenuated in ρ0 cells overexpressing catalase. Stabilization of HIF-1α by CoCl$_2$ was not inhibited by DPI, suggesting that cobalt stimulates ROS generation by a non-enzymatic, non-mitochondrial mechanism.

The observation that DFO does not require ROS or mitochondria led us to examine whether phosphorylation might serve as a common signaling event linking hypoxia, CoCl$_2$, and DFO-induced stabilization of HIF-1α. Insulin and insulin-like growth factors have been shown to stabilize HIF-1α protein and HIF-1-dependent gene expression during normoxia (46). Two major signaling components in the insulin-activated pathway are phosphatases and PI-3 kinase (47, 48). Our data indicate that both PI-3 kinases and phosphatases are required for the HIF-1α stabilization effected by hypoxia, CoCl$_2$, and by DFO. We conclude that PI-3 kinases and phosphatases must be acting upstream of the ubiquitin/proteasome degradation step in the O$_2$-sensing pathway, because H$_2$O$_2$-induced HIF-1α stabilization was abolished by wortmannin as well as by calyculin A. Protease and PI-3 kinase inhibitors are toxic to cells at higher concentrations, and toxicity could conceivably inhibit HIF-1α stabilization nonspecifically. However, accumulation of HIF-1α still occurred during normoxia in response to the proteasome inhibitor N-carbobenzyoxyl-L-leucinyl-L-leucinyl-L-nor- valinal in the presence of wortmannin or calyculin A, indicating that the cells retained the ability to respond in the presence of these inhibitors. To confirm further that wortmannin was not inhibiting HIF-1α stabilization by a nonspecific mechanism, the PI-3 kinase inhibitor LY 294002 was also tested. Like wortmannin, this compound inhibited the response to hypoxia, CoCl$_2$, and DFO when used at a concentration of 20 μM, thereby supporting the conclusion that PI-3 kinase participates in the signaling pathway required for HIF-1α stabilization.

Does cytochrome oxidase act as the O$_2$ sensor during hypoxia? We previously demonstrated that hypoxia causes a decrease in cytochrome c oxidase V$_{max}$, which led us to hypothesize this as a mechanism affecting the redox state of cytochromes at more proximal locations. We then postulated that such redox changes might explain the stimulation of superoxide production at low [O$_2$]. In support of this, we noted a similar temporal relationship between the changes in cytochrome c oxidase V$_{max}$ and the ROS generation in cardiomyocytes (41). However, our current observations appear to be at odds with that hypothesis. Cytochrome c oxidase in rat hepatocytes required 90–120 min under hypoxia to undergo a decrease in V$_{max}$ (40), yet these cells displayed HIF-1α protein accumulation within 30 min in the present study. Based on the discrepancy between the duration of hypoxia required to effect a change in V$_{max}$ and the duration required to stabilize HIF-1α, it is unlikely that cytochrome c oxidase could serve as the primary O$_2$ sensor in hypoxia. Rather, it appears that Complex III must possess inherent sensitivity to [O$_2$], allowing it to adjust its generation of ROS inversely with the O$_2$ tension. Indeed, our previous observation that ROS generation increased at lower [O$_2$] in Hep3B cells treated with antimycin A (21) is consistent with this conclusion, because electron transport inhibition by antimycin A would fully reduce Complex III, rendering its redox state insensitive to the redox state of cytochrome c oxidase. In the present study, increased oxidant generation by Complex III under hypoxia was confirmed in isolated mitochondria. When electrons were supplied exclusively to Complex III, ROS generation increased during hypoxia confirming the results in intact cells. By contrast, ROS generation tended to decrease during hypoxia when electrons were supplied only to Complex I, demonstrating the specificity of this response for Complex III. We therefore conclude that Complex III functions as the O$_2$ sensor during hypoxia by regulating ROS generation inversely with [O$_2$].

In a broader context, it is interesting to note that mitochondria are ubiquitous in eukaryotic cells and could provide a mechanism of O$_2$ sensing in other physiological responses. Our observation of an increased DCFH oxidation by P. denitrificans, a bacterium that demonstrates important similarities to eukaryotic mitochondria, supports this notion by showing that [O$_2$]-dependent ROS generation by Complex III is not unique to higher organisms. By contrast, E. coli oxidation of DCFH decreased at low O$_2$ levels, indicating that not all organisms generate an increased oxidant signal under hypoxia. The failure to increase ROS generation by E. coli may relate to its lack of a bc$_1$ complex, allowing electron transfer to occur without the formation of a ubiquinone intermediate (43). This further supports the conclusion that univalent electron transfer from a semiquinone to O$_2$ acts as the source of superoxide in cells that contain a functioning bc$_1$ complex. Future studies will need to address the possibility that mitochondria function as an O$_2$ sensors and ROS as signaling molecules in other systems.

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