Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing

Robert D. Guzy,[1] Beatrice Hoyos,[2] Emmanuel Robin,[1] Hong Chen,[1] Liping Liu,[3,4] Kyle D. Mansfield,[4] M. Celeste Simon,[3,4] Ulrich Hammerling,[2] and Paul T. Schumacker[1,*]

[1]Department of Medicine, The University of Chicago, Chicago, Illinois 60637
[2]Immunology Program, Memorial Sloan Kettering Cancer Center, New York, New York 10021
[3]Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104
[4]The Abramson Family Cancer Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104
*Correspondence: p-schumacker@northwestern.edu

Summary

Multicellular organisms initiate adaptive responses when oxygen (O_2) availability decreases, but the underlying mechanism of O_2 sensing remains elusive. We find that functionality of complex III of the mitochondrial electron transport chain (ETC) is required for the hypoxic stabilization of HIF-1α and HIF-2α and that an increase in reactive oxygen species (ROS) links this complex to HIF-α stabilization. Using RNAi to suppress expression of the Rieske iron-sulfur protein of complex III, hypoxia-induced HIF-1α stabilization is attenuated, and ROS production, measured using a novel ROS-sensitive FRET probe, is decreased. These results demonstrate that mitochondria function as O_2 sensors and signal hypoxic HIF-1α and HIF-2α stabilization by releasing ROS to the cytosol.

Introduction

Multicellular organisms require oxygen (O_2) for respiration and respond to decreases in O_2 availability (Bunn and Poyton, 1996) by activating transcriptional responses that preserve tissue O_2 supply and increase glycolytic capacity. Hypoxia-inducible factors HIF-1 and -2 are the principal factors mediating these changes. HIFs are heterodimers consisting of O_2-regulated (HIF-α) and constitutively expressed (HIF-β/ARNT) subunits (Kallio et al., 1997; Wang and Semenza, 1995). HIF-α is degraded under normoxia following proline hydroxylation by prolyl hydroxylases (PHDs; Jaakkola et al., 2001; Ivan et al., 2001). This facilitates its recognition by VHL protein, an E3 ubiquitin ligase (Maxwell et al., 1999; Huang et al., 1998). Hypoxia inhibits HIF-α hydroxylation, thereby allowing it to accumulate, interact with ARNT, and initiate transcription (Berra et al., 2003).

The O_2 sensor responsible for inhibiting HIF-α degradation during hypoxia is not established. PHDs require O_2 as a substrate and exhibit a relatively high Km for O_2 in vitro (Hirsilae et al., 2003), so it has been proposed that PHDs function as O_2 sensors because the decreasing availability of O_2 during hypoxia could limit their ability to hydroxylate HIF (Hagen et al., 2003; Epstein et al., 2001).

We propose that mitochondria function as O_2 sensors and signal hypoxia by releasing ROS to the cytosol (Chandel et al., 1998; Chandel et al., 2000). The resulting oxidant signal then mediates an inhibition of PHDs, leading to HIF-α stabilization. This mitochondrial model has been controversial because previous studies relied on pharmacological tools, because conflicting reports have appeared (Vaux et al., 2001; Srinivas et al., 2001) and because technical concerns have arisen regarding the measurement of ROS production in living cells. The present study therefore utilizes RNA interference to disrupt mitochondrial function, overexpression of cellular antioxidant enzymes to modify intracellular oxidant signals, and a novel redox-sensitive FRET protein sensor to assess cytosolic oxidant signaling. We show that a functional mitochondrial ETC is required for hypoxia-induced ROS production and that this ROS production is specifically required for hypoxia-induced HIF-α stabilization.

Results

To determine whether the ETC is required for hypoxic stabilization of HIF-1α, human 143B osteosarcoma cells were depleted of mitochondrial DNA (King and Attardi, 1989). The resulting ρ^0 genotype is characterized by a lack of mitochondrial DNA encoding a variety of genes located in diverse regions of the mitochondrial genome (Figure 1B). Further characterization of ρ^0 cells is described in the accompanying Supplemental Data available with this article online. The 143B ρ^0 cells fail to stabilize HIF-1α during hypoxia yet still respond to anoxia, which blocks HIF-1α hydroxylation by depriving PHDs of substrate (Schroedl et al., 2002) in a mitochondria-independent manner (Figure 1A). Stabilization of HIF-1α in wild-type 143B cells is inhibited by the complex I inhibitor rotenone. However, anoxic activation of HIF-1α is insensitive to rotenone in both wild-type and 143B ρ^0 cells. Thus, a functional ETC is required for cellular sensing of hypoxia but is not required for HIF-1α stabilization response to anoxic inhibition of PHDs.

To assess cytosolic ROS signaling, we introduce a novel fluorescence resonance energy transfer (FRET) sensor consisting of cyan and yellow fluorescent proteins (CFP/YFP) linked by a 69 amino acid cysteine-containing regulatory domain from the redox-regulated heat-shock protein HSP-33 (Jakob et al., 1999). The resulting protein (HSP-FRET) was transiently ex-

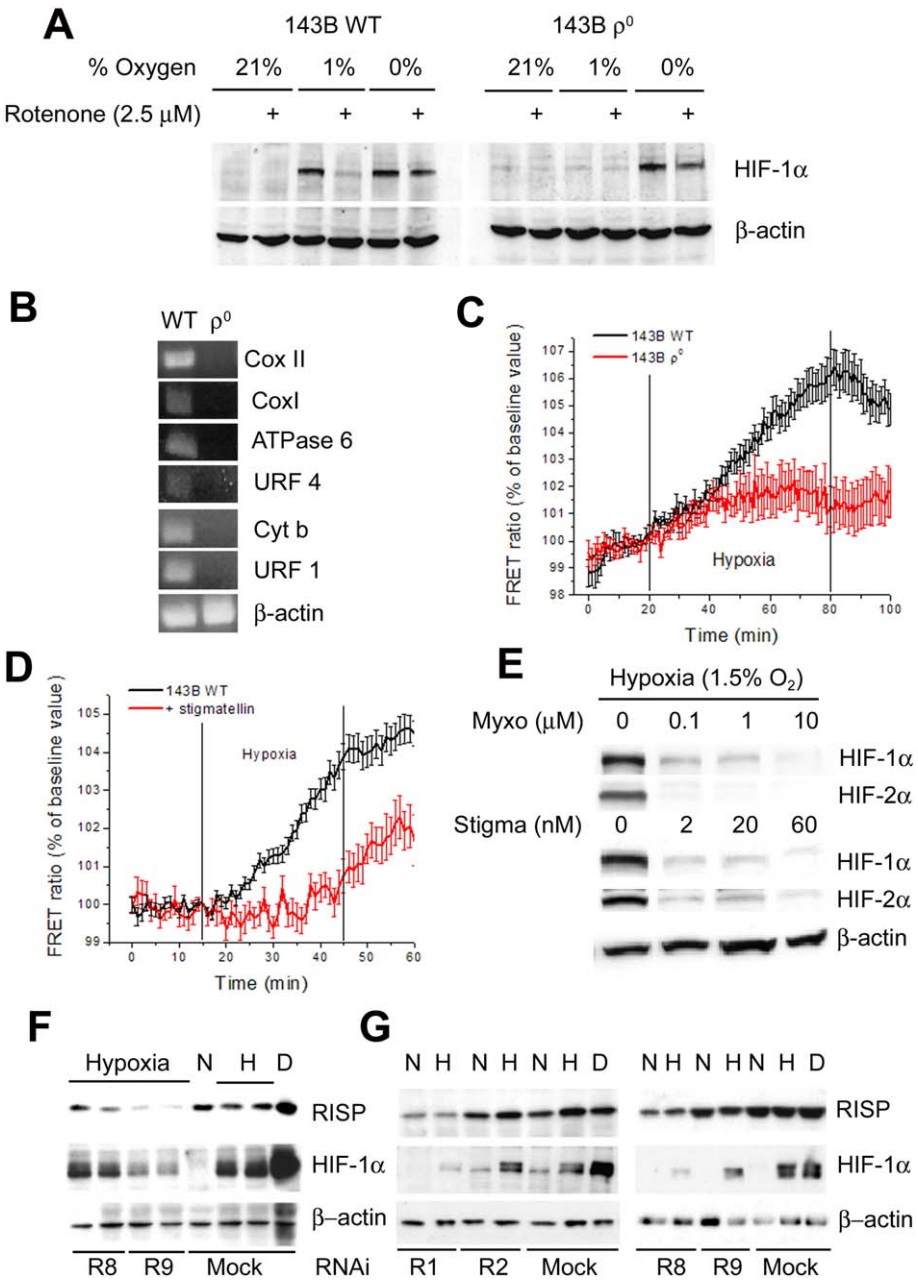

Figure 1. Mitochondrial complex III is required for hypoxic stabilization of HIF-1α

A) 143B ρ^0 cells were generated by long-term exposure to EtBr (50 ng/ml). The 143B wild-type (wt) and ρ^0 cells were exposed to 21%, 1%, or 0% O_2 for 3 hr with or without rotenone (2.5 μM). HIF-1α and β-actin levels were analyzed from whole-cell extracts by immunoblotting.

B) Loss of mitochondrial DNA was confirmed in the 143B ρ^0 cells by semiquantitative RT-PCR analysis of mitochondrial genes in comparison to β-actin.

C) 143B wt and ρ^0 cells were transfected with a plasmid expressing the HSP-FRET probe and exposed to hypoxia (1% O_2). (Means ± SEM.)

D) 143B wt cells transfected with HSP-FRET were incubated with stigmatellin (100 nM) and exposed to 30 min of hypoxia (1% O_2). (Means ± SEM.)

E) Hep3B cells exposed to hypoxia for 3 hr in the presence of myxothiazol or stigmatellin. HIF-1α, HIF-2α, and β-actin levels were analyzed from whole-cell extracts by immunoblotting.

F) HEK293 and (**G**) Hep3B cells were transfected with RNA hairpins complementary to RISP mRNA (R1, R2, R8, R9) or random sequences (Mock). Twenty-four hours after transfection, cells were selected with puromycin (0.5 g/ml) for 7 days. Cells were then exposed to 21% O_2 (N), 1.5% O_2 (H) or 100 μM DFO (D) for 4 hr. Whole-cell extracts were analyzed for levels of RISP, HIF-1α, and β-actin via immunoblotting. Constructs producing significant knockdown of RISP (R1, R8, and R9) significantly attenuate HIF-1α responses to hypoxia.

pressed in cells, permitting optical interrogation of cytosolic thiol redox in live cells. Oxidation of cysteine thiols in the linker domain causes separation of the CFP and YFP fluorophores, increasing the CFP/YFP HSP-FRET ratio. Oxidation of HSP-FRET occurs in response to exogenous H_2O_2 (Figure 4A) but not during exposure to nitric oxide (500 nM; Figure S3A). However, hypoxia increases cytosolic oxidant signaling, as demonstrated by a change in HSP-FRET ratio in 143B cells (Figure 1C). Administration of the thiol reductants 2 mercaptopropriony glycine or pyrrolidine dithiocarbamate abrogate the HSP-FRET response to hypoxia by maintaining thiols in a reduced state (Figure S3B). Control studies using an empty FRET construct in which the redox-sensitive domain is replaced by a control peptide lacking cysteine residues fails to respond to hypoxia. Using HSP-FRET, hypoxic responses in ρ^0 cells are significantly attenuated, indicating that oxidant signals during hypoxia originate from the ETC (Figure 1C).

The majority of H_2O_2 detected during hypoxia by HSP-FRET appears to originate from complex III of the ETC, since it is attenuated by stigmatellin (Figure 1D), which inhibits electron flux into complex III at the same site as myxothiazol. Myxothiazol and stigmatellin inhibit hypoxic stabilization of HIF-1α and -2α in Hep3B cells (Figure 1E) and 143B wild-type cells (data not shown) dose-dependently, but fail to inhibit HIF-α stabilization in response to direct PHD inhibition with dimethyloxallyl glycine (DMOG; data not shown). Therefore, a functional com-

plex III of the ETC is required for O_2 sensing and HIF-α stabilization through its involvement in ROS production.

While ρ^0 cells provide insight into the role of mitochondria in O_2 sensing, other investigators report conflicting results (Vaux et al., 2001). We therefore employed RNA interference to inhibit expression of the Rieske iron-sulfur protein (RISP), which normally transfers an electron from ubiquinol to cytochrome c1 in complex III (Link and Iwata, 1996). This oxidation produces a ubisemiquinone radical, which may generate superoxide (Figure 4F). Genetic attenuation of RISP should therefore inhibit ROS production at complex III.

Transient knockdown of RISP was achieved by transfection with RNA hairpins complementary to RISP mRNA (R1, R8, R9) or scrambled sequences (Mock) in HEK293 (Figure 1F) and Hep3B cells (Figure 1G). After selection in puromycin for 7 days, heterogeneous populations of cells with knockdown of RISP show an attenuated hypoxic stabilization of HIF-1α compared with Mock cells, further supporting the proposed role of complex III in the hypoxic response.

Subsequently, 143B cells were stably transfected with other short-hairpin RNA (shRNA) oligonucleotides targeted to six different regions of human RISP mRNA (Figure S2A; Paddison et al., 2002). We then isolated stable clones expressing shRNA from those constructs. Significant heterogeneity in the extent of RISP knockdown was observed among clonal populations (Figure S2B). RISP shRNA clones demonstrated a highly specific knockdown of RISP compared to other proteins (Figure 2A) and significant reduction in O_2 consumption (Figure S1A). Further characterization of those cells is depicted in the accompanying Supplemental Data.

143B cells with stable knockdown of RISP achieved with two different targeting constructs exhibit decreased ROS production during hypoxia (Figure 2B) and decreased hypoxic stabilization of HIF-1α (Figures 2C and S4B), compared with wild-type controls. However, they retain responsiveness to the hypoxic mimetics deferoxamine (DFO), cobalt chloride ($CoCl_2$), and DMOG (Figures 2C and 2D). While hypoxic HIF-1α stabilization in wild-type 143B cells is inhibited by myxothiazol, the small increase in HIF-1α retained during hypoxia in RISP shRNA clones is insensitive to myxothiazol and appears to mimic the wild-type response in the presence of myxothiazol (Figure 2D). RISP shRNA clones also exhibit normal HIF-1α stabilization during anoxia (Figure 2E). Overall, RISP shRNA clones show a 2.5- to 3-fold decrease in hypoxic HIF-1α stabilization but maintain normal responses to DFO, $CoCl_2$, DMOG, and anoxia (Figure 2F), which appear to directly interfere with PHD activity. Thus, loss of RISP and complex III activity selectively undermines hypoxic sensitivity without abrogating the capacity for HIF-1α stabilization in response to PHD inhibition, which is proposed to act downstream from the O_2 sensor. Furthermore, RISP knockdown decreases hypoxia-induced ROS production, strongly suggesting a link between ROS production from complex III and HIF-1α stabilization.

To determine whether ROS is necessary for the response, we tested whether antioxidants block hypoxic HIF-α stabilization. The glutathione mimetic ebselen (Schewe, 1995) decreases hypoxia-induced HSP-FRET oxidation (Figure 3A) and attenuates hypoxic HIF-1α and HIF-2α stabilization (Figure 3B). Furthermore, overexpression of catalase, which degrades H_2O_2, was achieved using a recombinant adenovirus. Catalase overexpression inhibits hypoxia-induced oxidation of HSP-FRET (Figures 3C and S3B), indicating that HSP-FRET is sensitive to changes in cytosolic thiol redox balance induced by H_2O_2. However, overexpression of Cu,Zn-superoxide dismutase (SOD-I) or Mn-superoxide dismutase (SOD-II), which convert superoxide to H_2O_2, has no significant effect on hypoxia-induced HSP-FRET oxidation (Figure 3D), suggesting that HSP-FRET is oxidized by H_2O_2 rather than by superoxide. Catalase overexpression reduces hypoxic stabilization of HIF-1α (Figure 3E) and HIF-2α (data not shown) but does not affect mitochondria-independent stabilization by DFO. Expression of similar levels of catalase targeted to the mitochondrial matrix has a only a small effect on hypoxic HIF-1α stabilization, suggesting that H_2O_2 release to the cytosol is more important for HIF-1 activation than is H_2O_2 release to the matrix. Overexpression of SOD-I or SOD-II failed to inhibit hypoxic stabilization of HIF-1α (Figure 3E) or HIF-2α (data not shown). Therefore, mitochondrial release of H_2O_2, but not superoxide, is required for hypoxic stabilization of HIF-α, but not for stabilization caused by direct PHD inhibition.

To determine whether exogenous H_2O_2 is sufficient to oxidize HSP-FRET and to trigger HIF-α stabilization during normoxia, glucose oxidase was administered to Hep3B cells in order to generate H_2O_2 (Figure 4A). Either glucose oxidase (10 μg/ml) or exogenous H_2O_2 (10–100 μM; data not shown) can oxidize HSP-FRET to the same degree as hypoxia. Doses of H_2O_2 that oxidize HSP-FRET administered to Hep3B (Figure 4B) and HEK293 (data not shown) cells in serum-free media cause normoxic stabilization of HIF-1α and HIF-2α. Moreover, exogenous tert-butyl hydroperoxide administered to 143B ρ^0 cells also elicits stabilization of HIF-1α during normoxia (Figure S4A).

Since exogenous H_2O_2 can stabilize HIF-1α independently of mitochondria (Chandel et al., 2000), we tested whether RISP shRNA clones exhibiting deficient hypoxic HIF-1α stabilization retain the ability to stabilize HIF-1α in response to exogenous H_2O_2. H_2O_2 (50 μM; data not shown), tert-butyl hydroperoxide (TBP; 10-100 μM; Figure 4C), or glucose oxidase (10 μg/ml; Figure 4D), stabilize HIF-1α in normoxic wild-type and RISP shRNA clones. During hypoxia, glucose oxidase produces only a slight increase in HIF-1α stabilization in wild-type 143B cells. However, in hypoxic RISP shRNA clones, glucose oxidase restores HIF-1α stabilization to the level induced by hypoxia in wild-type cells (Figure 4D). While hypoxic stabilization of HIF-1α is not affected by addition of catalase to the cell-culture medium, the effects of glucose oxidase can be inhibited with extracellular catalase (200 U/ml; data not shown). These data indicate that RISP is crucial for hypoxic stabilization of HIF-1α due to its involvement in the generation of superoxide (Figure 4F), which is rapidly dismuted to the signaling molecule H_2O_2.

To test the effect of exogenous oxidants on hydroxylation and subsequent ubiquitination of HIF-1α, we administered tert-butyl hydroperoxide to Hep3B cells in the presence of the proteasome inhibitor MG-132. Tert-butyl hydroperoxide stabilized HIF-1α during normoxia, but it failed to abolish the accumulation of ubiquitinated HIF-1α (Figure 4E). Similarly, treatment of Hep3B cells with hypoxia or DMOG resulted in HIF-1α stabilization without preventing accumulation of ubiquitinated HIF (Figure 4E). By contrast, anoxia stabilized HIF-1α while it decreased ubiquitinated HIF levels significantly. Thus, inhibition of PHD activity by 1% O_2 or by administration of ROS during

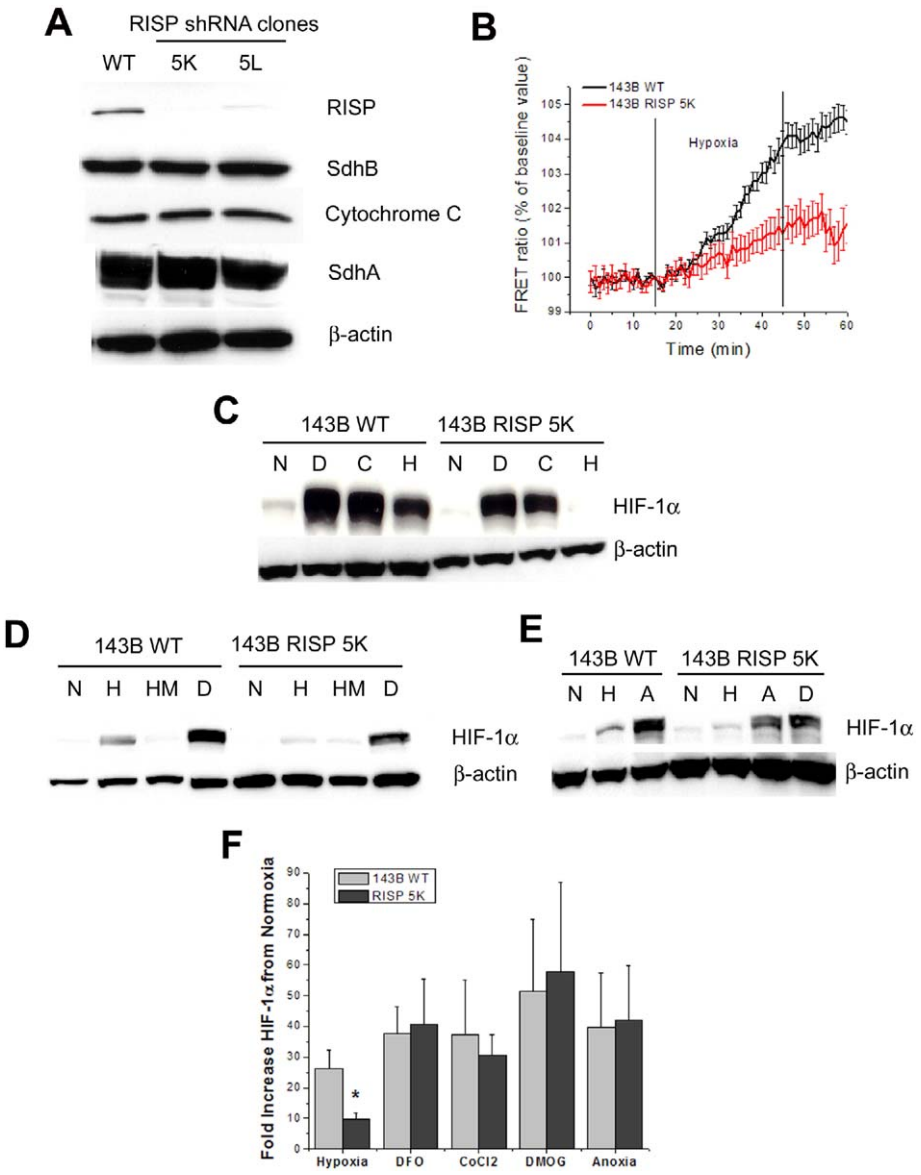

Figure 2. Complex III RISP knockdown attenuates hypoxic ROS production and stabilization of HIF-1α

A) 143B cells were transfected with a plasmid expressing RISP shRNA sequence 5. After 24 hr, cells were selected with G418 (0.5 mg/ml) until stable clones could be isolated. Whole-cell extracts from 143B RISP shRNA clones 5K and 5L were analyzed for expression of β-actin, RISP, succinate dehydrogenase subunit B (SdhB), cytochrome c, and SdhA by immunoblotting.

B) 143B wt or RISP shRNA 5K cells transfected with the HSP-FRET probe were exposed to hypoxia (1% O_2). (Means ± SEM.)

C) 143B wt and RISP shRNA 5K cells grown to 80% confluence were exposed to normoxia (N), DFO (D; 100 μM), $CoCl_2$ (C; 100 μM), or hypoxia (H; 1.5% O_2) for 3 hr.

D) 143B wt and RISP shRNA 5K cells were grown as in (**C**) and exposed to hypoxia (H) in the presence or absence of myxothiazol (M; 0.1 μg/ml) for 2.5 hr. DFO (D) was added as a positive control.

E) Immunoblots showing 143B wt and RISP shRNA 5K cells exposed to normoxia (N), hypoxia (H), anoxia (A), or DFO (D) for 3 hr.

F) HIF-1α immunoblots from numerous experiments where wt and RISP shRNA 5K cells were exposed to hypoxia (1.5% O_2; n = 13), DFO (n = 7), $CoCl_2$ (n = 3), DMOG (n = 3), and anoxia (n = 4) were quantified via densitometric analysis and normalized to β-actin. Data is presented as fold increase from normoxia; *indicates a significant difference from wt values (p = 0.01). (Means ± SEM.)

normoxia is incomplete, such that accumulation of nonubiquitinated HIF-1α can still continue, albeit to a lesser extent than in normoxia. Inhibition of PHD activity by anoxia, however, is sufficient to abolish the accumulation of ubiquitinated HIF due to the inability to hydroxylate HIF-1α.

Discussion

Whether ROS production increases or decreases during hypoxia has been a focus of debate, based on the behavior of existing oxidant-sensitive probes (Chandel et al., 2000; Srinivas et al., 2001; Gorlach et al., 2003). Using a ratiometric sensor, we find compelling evidence for increased H_2O_2 production from mitochondria during hypoxia. This increase requires electron transport in complex III, and it triggers HIF-α stabilization during hypoxia. By contrast, HIF-α stabilization during anoxia or in response to DMOG, DFO, or $CoCl_2$ does not require mitochondria because these interventions directly inhibit PHDs, thereby preventing recognition by VHL protein. Hence, mitochondria act as O_2 sensors during hypoxia, whereas PHDs act as O_2 sensors during anoxia. These characteristics assure continued activation of HIF-mediated adaptive responses when tissue hypoxia progresses to anoxia, when ROS production presumably would halt.

Superoxide can be generated at the Qi or Qo sites in complex III, resulting in ROS release to the matrix and/or the intermembrane space/cytosol (Figure 4F). Superoxide released to the matrix from complex I or III is unlikely to escape to the cytosol (Muller et al., 2004), although H_2O_2 formed in the matrix could conceivably escape. Cytosolic catalase attenuates HSP-FRET oxidation and abrogates HIF-α stabilization in hypoxia, whereas HIF responses are preserved in cells expressing catalase targeted to the matrix. This indicates that cytosolic, rather than matrix, oxidant signals are required for hypoxic activation

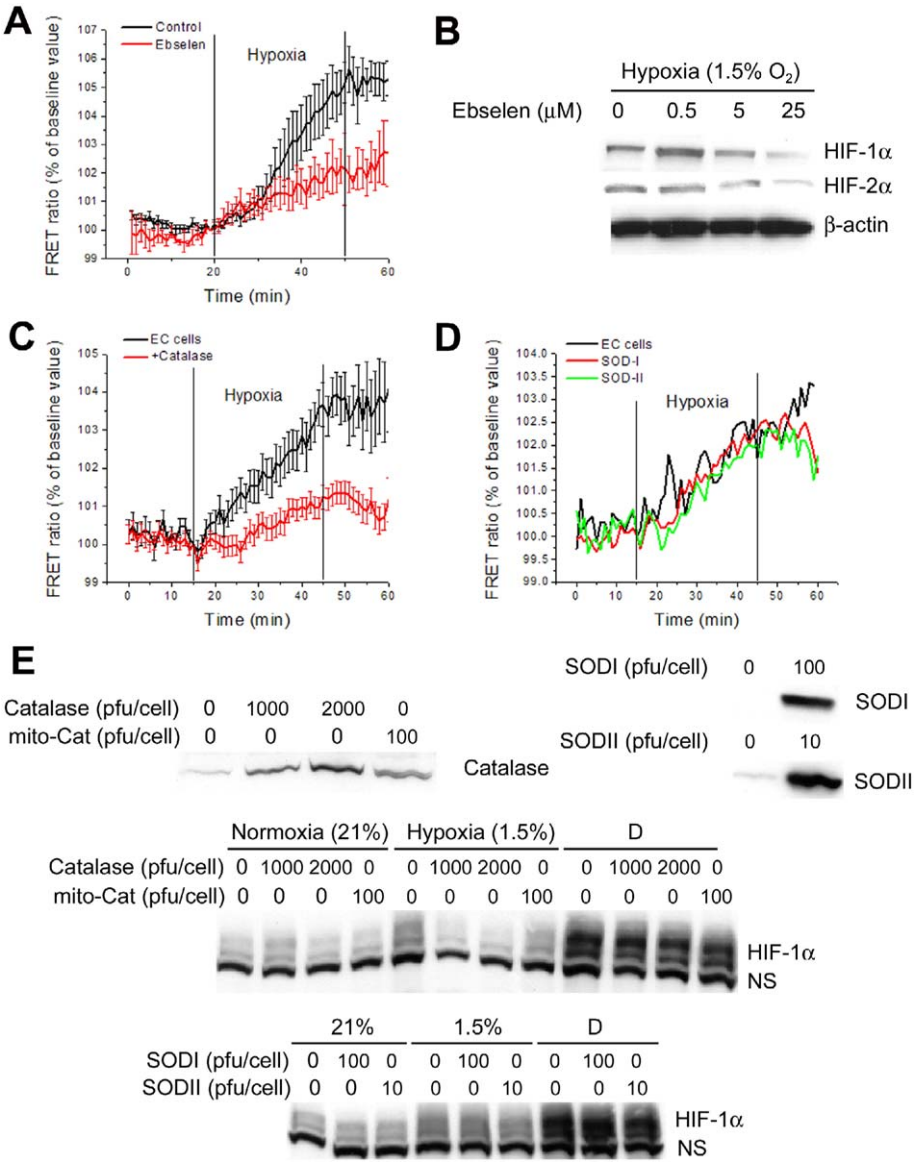

Figure 3. Requirement of cytosolic H_2O_2 for hypoxic stabilization of HIF-1α

A) Embryonic chick cardiomyocytes expressing HSP-FRET were exposed to hypoxia (1% O_2) with or without ebselen (25 μM). (Means ± SEM.)

B) Hep3B cells at 80% confluence were exposed to hypoxia in the presence of ebselen. Whole-cell extracts were analyzed for HIF-1α and HIF-2α by immunoblotting.

C and D) Mouse embryonic culture EC cells were transfected with HSP-FRET. Cells were infected with the recombinant adenoviruses expressing catalase (1000 pfu/cell; [**C**]) and SOD-I (100 pfu/cell) or SOD-II (10 pfu/cell; [**D**]) 24 hr prior to the experiment. (Means ± SEM.)

E) Mouse EC cells were infected with adenoviruses expressing catalase, mitochondria-targeted catalase (mito-Cat), SODI, or SOD-II for 24 hr. Then, the media were changed and cells were exposed to normoxia, hypoxia, or normoxia + DFO (D; 100 μM) for 2.5 hr. Overexpression of catalase, SOD-I, and SOD-II were verified, and stabilization of HIF-1α was analyzed via immunoblotting. NS: nonspecific signal.

of HIF-α. However, loss of complex III activity also disrupts electron flux in complex I and II, so the possible involvement of these systems cannot be excluded. Inhibition of complex III would be expected to augment superoxide formation at I and/or II, yet we observe a decrease in hypoxia-induced ROS formation in response to myxothiazol, stigmatellin, and by genetic depletion of RISP. These interventions block the oxidation of ubiquinol to ubisemiquinone, prevent subsequent superoxide production, and inhibit HIF-α stabilization in hypoxia. Our findings implicate superoxide release from the Qo site of complex III to the intermembrane space and subsequent H_2O_2 release to the cytosol. The mechanism by which hypoxia increases ROS generation at complex III is unknown, but it could involve O_2-dependent structural changes that prolong the lifetime of ubisemiquinone, that increase accessibility of O_2 to a site where single electrons can be captured, or that alter the directional escape of superoxide to the intermembrane space versus matrix compartments (Muller et al., 2004).

Neither SOD I nor II overexpression abolishes HIF-α responses to hypoxia, indicating that superoxide is not directly required, although undoubtedly it is the source of H_2O_2 during hypoxia. Exogenous oxidants stabilize HIF-α during normoxia, indicating a requirement for peroxide as a signaling agent. Exogenous oxidants also stabilize HIF in RISP knockdown cells and in ρ^0 cells, indicating that the ETC is not required for the response to H_2O_2.

PHDs require O_2 as a substrate, allowing the enzyme to function as an O_2 sensor in anoxia. We previously reported that Hep3B ρ^0 cells fail to stabilize HIF-1α in 1.5% O_2, but this was challenged when others reported that ρ^0 cells retain HIF responses to anoxic conditions (<0.1% O_2; Vaux et al., 2001). We now clarify this issue by showing that, similar to data presented by Schroedl et al. (2002), ρ^0 cells retain HIF responses to anoxia while they lose the ability to increase ROS and stabilize HIF-1α during hypoxia because they lack a functional ETC. Furthermore, other genetic models in addition to the present

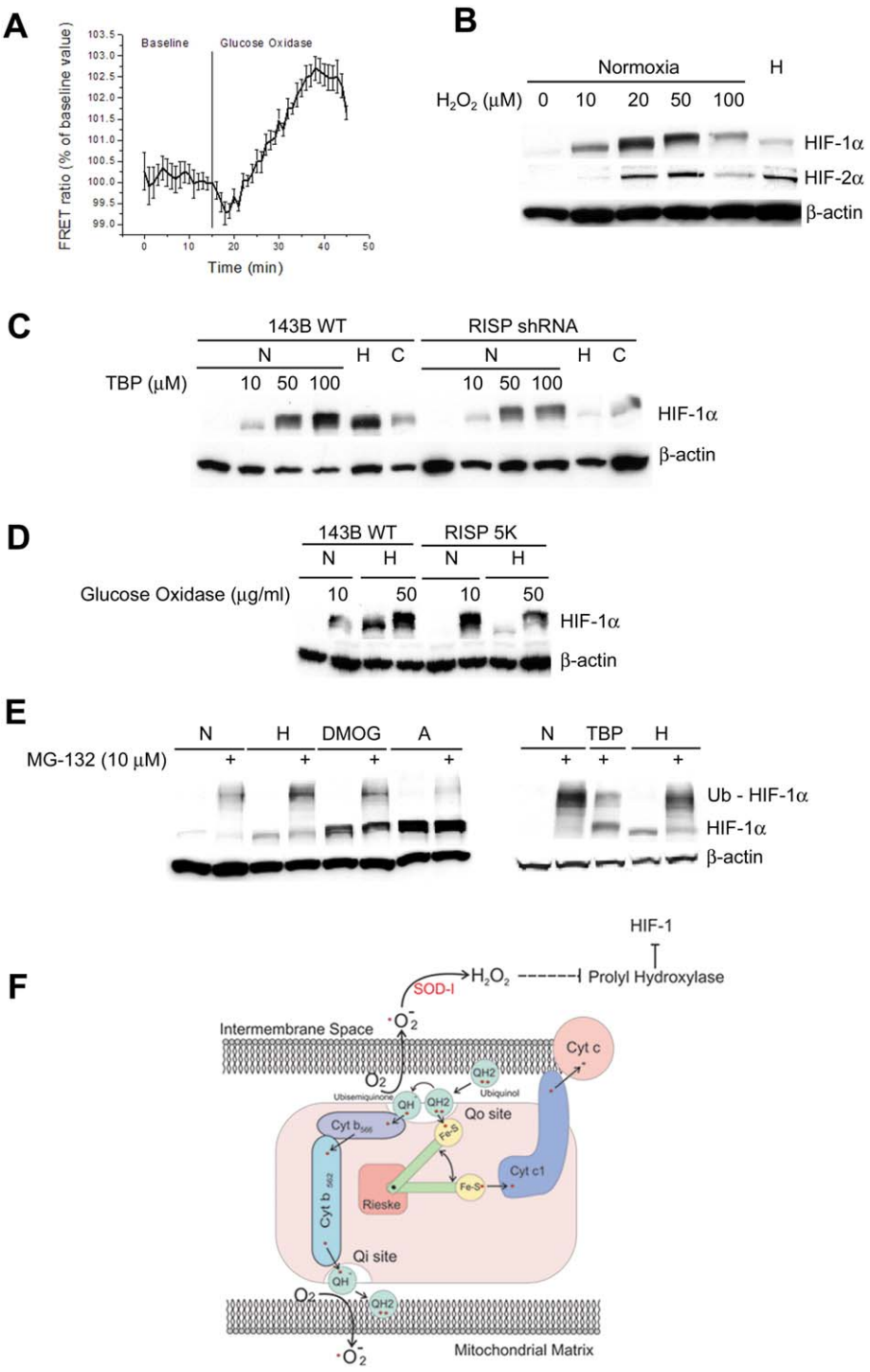

Figure 4. Exogenous H_2O_2 is sufficient to oxidize HSP-FRET and stabilize HIF-1α during normoxia

A) Hep3B cells expressing HSP-FRET. Glucose oxidase (10 μg/ml) was added to the perfusate to induce H_2O_2 production. (Means ± SEM.)

B) Normoxic Hep3B cells were administered boluses of H_2O_2 every 20 min or exposed to hypoxia (H) for 2 hr, and HIF-1α, HIF-2α, and β-actin levels were assessed via immunoblotting.

C) 143B wt and RISP shRNA 5K cells were incubated in normoxia (N), hypoxia (H), or with $CoCl_2$ (C) for 2 hr. Exogenous tert-butyl hydroperoxide was applied as indicated every 20 min.

D) 143B wt and RISP shRNA 5K cells were incubated with glucose oxidase for 2 hr in normoxia (N) or hypoxia (H).

E) Hep3B cells were exposed to normoxia (N), hypoxia (H), DMOG (DG), or anoxia (A) for 4 hr, and TBP for 2 hr. MG-132 (10 μM) was added to cells for the last 1 hr.

F) Schematic diagram of mitochondrial complex III illustrating sites of superoxide production believed to be augmented during hypoxia.

study independently demonstrate the significance of complex III for hypoxic HIF-α stabilization (Mansfield et al., 2005 [this issue of Cell Metabolism]; Brunelle et al., 2005 [this issue of Cell Metabolism]).

We show that cells continue to hydroxylate and ubiquitinate HIF-1α during hypoxia, during pharmacologic inhibition of PHD, and in response to exogenous oxidants. The similar patterns suggest that these interventions all cause HIF-α stabilization by partially inhibiting PHD activity. By contrast, anoxia abrogates the accumulation of ubiquitin-labeled HIF-1α, indicating a complete inhibition of PHDs. These findings reveal that mitochondrial oxidants regulate HIF-α responses during physiological hypoxia. The regulation of PHD by oxidant signals is poorly understood, and further studies are required to understand the details of the signal transduction pathway linking hypoxia-induced mitochondrial ROS to inhibition of PHDs.

Experimental procedures

Cell culture and reagents

Hep3B, HEK293, and 143B cells were obtained from ATCC and maintained in a humidified, 37°C, 5% CO_2 incubator. Media were formulated according to manufacturer's recommendations. 143B ρ^0 cells were generated by incubating cells in media containing ethidium bromide (50 ng/ml) and supplemented with uridine (500 ug/ml) and pyruvate (2.5 mM). Cells were determined to be ρ^0 based upon respiration phenotype and loss of a variety of mitochondrial genes after a number of weeks. Rotenone, myxothiazol, stigmatellin, ebselen, hydrogen peroxide, tert-butyl hydroperoxide, glucose oxidase, DFO, and $CoCl_2$ were purchased from Sigma Chemicals. MG-132 was obtained from EMD Biosciences. Recombinant adenoviruses expressing catalase and mitochondria-targeted catalase were kind gifts from Drs. Cederbaum and Bai (Bai et al., 1999), and adenoviruses expressing SOD-I and SOD-II were obtained from J.F. Engelhardt (Zwacka et al., 1998a; Zwacka et al., 1998b).

RNA interference

RNAi for RISP used in HEK293T cells and Hep3B cells were generated by selecting nine sequences (21–29 base pair-long, named R1–R9) with a G at 5′ end and TT at 3′ end that are unique for RISP. A PCR-based strategy previously described (Fox et al., 2003) was used to generate U6-promoter shRNA cassettes as well as a control Mock vector. Human 143B osteosarcoma cells were electroporated with a pPNT or pPNT-shRNA vector (as detailed in the accompanying Supplemental Data), and after 24 hr, cells were selected with G418 (0.5 mg/ml). For short-term knockdown, cells were selected for 7 days and collected to assess RISP knockdown via immunoblotting. Stable G418-resistant clones were maintained in media containing G418, uridine, and pyruvate until clonal colonies emerged, at which time clones were collected. Clones generated from each of the six shRNA vectors or the empty pPNT vector (wild-type) were screened via immunoblotting for expression of RISP, and clones demonstrating significant knockdown of RISP expression were further analyzed.

Hypoxia, anoxia, and exogenous oxidants

Hypoxic experiments were carried out in a Coy Laboratories hypoxic glove box equilibrated to 1.5% O_2, 5% CO_2, and 93.5% N_2. Experiments were 2–3 hr in length unless otherwise noted. Anoxic experiments were carried out in an anoxic glove box equilibrated to 5% CO_2, 3%–4% H_2, balance N_2, where anoxia was maintained with the use of a palladium catalyst. Experiments were 3 hr in length unless otherwise noted. Cells exposed to exogenous H_2O_2 or tert-butyl hydroperoxide were incubated in normoxic serum-free media, and boluses of oxidants were applied every 20 min for 2 hr. Cells exposed to glucose oxidase were incubated in serum-free media throughout the experiment. All hypoxic and anoxic whole-cell extracts were collected within the experimental workstations.

Western blotting

Whole-cell lysates were generated and analyzed via Western blot analysis as described in the accompanying Supplemental Data.

Measurement of ROS

The HSP-FRET probe was created as described in the accompanying Supplemental Data. Cells were plated on glass coverslips and transfected (Geneporter, Gene Therapy Systems) with a plasmid encoding the HSP-FRET probe expressed under the CMV promoter. The cells were then placed in a flow-through chamber and mounted on an inverted epifluorescence microscope. Individual cells were monitored over time for their YFP and CFP fluorescence, and the HSP-FRET ratio is defined as the YFP/CFP fluorescence ratio in individual cells. Ratios were normalized to normoxic baseline after 20 min of equilibration. Cells were perfused with a basic salt solution bubbled with a 21% O_2, 5% CO_2, 74% N_2 gas mixture. Hypoxia was induced by bubbling with 0%–1% O_2, 5% CO_2, 96% N_2 gas.

Supplemental data

Supplemental Data include four figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/1/6/401/DC1/.

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