The $Q_0$ site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production

Eric L. Bell, Tatjana A. Klimova, James Eisenbart, Carlos T. Moraes, Michael P. Murphy, G.R. Scott Budinger, and Navdeep S. Chandel

Mammalian cells increase transcription of genes for adaptation to hypoxia through the stabilization of hypoxia-inducible factor $\alpha$ (HIF-1$\alpha$) protein. How cells transduce hypoxic signals to stabilize the HIF-1$\alpha$ protein remains unresolved. We demonstrate that cells deficient in the complex III subunit cytochrome b, which are respiratory incompetent, increase ROS levels and stabilize the HIF-1$\alpha$ protein during hypoxia. RNA interference of the complex III subunit Rieske iron sulfur protein in the cytochrome $b$-null cells and treatment of wild-type cells with stigmatellin abolished reactive oxygen species (ROS) generation at the $Q_0$ site of complex III. These interventions maintained hydroxylation of HIF-1$\alpha$ protein and prevented stabilization of HIF-1$\alpha$ protein during hypoxia. Antioxidants maintained hydroxylation of HIF-1$\alpha$ protein and prevented stabilization of HIF-1$\alpha$ protein during hypoxia. Exogenous hydrogen peroxide under normoxia prevented hydroxylation of HIF-1$\alpha$ protein and stabilized HIF-1$\alpha$ protein. These results provide genetic and pharmacologic evidence that the $Q_0$ site of complex III is required for the transduction of hypoxic signal by releasing ROS to stabilize the HIF-1$\alpha$ protein.

Introduction

Oxygen homeostasis is important for normal cellular function (Semenza, 2000). As oxygen levels decrease in the surrounding environment (hypoxia), cells respond by activating hypoxia-inducible factor (HIF) dependent gene transcription to facilitate cellular adaptation to hypoxia. HIF is a heterodimer of two basic helix-loop-helix/Per/Arnt/Sim domain proteins, HIF-\(\alpha\) and the aryl hydrocarbon nuclear translocator (ARNT or HIF-\(\beta\); Wang et al., 1995). Under normal oxygen conditions, ARNT is constitutively stable, whereas the $\alpha$ subunit is labile. In normal oxygen conditions, the $\alpha$ subunit is hydroxylated at proline residues by a family of prolyl hydroxylase enzymes (PHDs). Proline hydroxylation targets the protein for ubiquitination by the von Hippel-Lindau protein (pVHL)/E3 ubiquitin ligase and for subsequent proteasomal degradation (Maxwell et al., 1999; Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). The $\alpha$ subunit is also hydroxylated at an asparagine residue by the enzyme factor inhibiting HIF-1 (FIH-1) under normal oxygen conditions (Mahon et al., 2001; Lando et al., 2002a,b). Asparagine hydroxylation blocks the binding of the transcriptional coactivators p300 and CREB binding protein (CBP) to HIF-1 (Dames et al., 2002; Freedman et al., 2002). Under hypoxic conditions, the $\alpha$ subunit is not hydroxylated by the PHDs or FIH, resulting in the stabilization of the HIF-$\alpha$ protein, dimerization with ARNT, and association with p300/CBP to initiate gene transcription.

The mechanism by which cells transduce the hypoxic signal to activate HIF is a subject of ongoing research. Previous studies indicate that mitochondria are involved in the transduction of hypoxic signals; however, the mechanism is not fully understood. One model proposes that mitochondria regulate the ability of the PHDs to hydroxylate HIF-1$\alpha$ protein because of their capacity to consume oxygen (Hagen et al., 2003; Doerge et al., 2005). Mitochondrial oxygen consumption would generate a gradient of oxygen within the cytosol of the cell, thereby limiting the availability of oxygen, a necessary cosubstrate for PHD activity. Another model proposes that mitochondria increase the levels of cytosolic reactive oxygen species (ROS) during hypoxia to activate HIF (Bell et al., 2005). Initial evidence to support this model...

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Abbreviations used in this paper: DMOG, dimethyloxalylglycine; HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase enzyme; ROS, reactive oxygen species; siRNA, short hairpin RNA; TATM, mitochondrial translation factor A; TMPD, $N,N,N,N'$-tetramethyl-p-phenylenediamine; WT, wild-type.

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Figure 1. Cells that contain a cytochrome b-deficient bc1 complex are respiratory incompetent but still generate ROS and stabilize HIF-1α in hypoxia. (A) Mitochondrial complex III generates ROS through the ubiquinone (Q) cycle. The Q cycle generates ROS at the Qo and Qi sites. The Rieske Fe-S protein is required for ROS production at the Qo site. The loss of complex III subunit cytochrome b abolishes the Qo site. (B) Oxygen consumption of 143B, 143Bρ0, WT cybrids, and ΔCyt b cybrids subjected to 21% O2 (N), 1.5% O2 (H), or 1 mM DMOG (D) for 4 h. Representative blot from four independent experiments. (D) Intracellular H2O2 levels were measured by Amplex red in 143B cells, 143Bρ0 cells, WT cybrids, and ΔCyt b cybrids exposed to 1.5 or 21% O2 for 4 h. n = 4 (mean ± SEM); *, P < 0.05 (all groups were compared with the normoxia sample of WT cells).

Results

Hypoxic stabilization of HIF-1α protein is independent of respiration and cytochrome b

Mitochondrial complex III consists of 11 subunits, three of which have known electron transport activity (the Rieske Fe-S protein, cytochrome b, and cytochrome c1). The electron flux from ubiquinol (QH2) to cytochrome c occurs through the ubiquinone (Q) cycle within complex III (Hunte et al., 2003). The first electron from ubiquinol is transferred to the Rieske Fe-S/cytocrome c1/cytochrome c axis transiently, making the radical ubisemiquinone. The second electron from ubisemiquinone is transferred to cytochrome b. However, ubisemiquinone does have the capability of transferring an electron to oxygen to generate superoxide. This allows for the generation of ROS at the Qo site of complex III through the interaction between ubisemiquinone (Q) and molecular oxygen within the bc1 complex (Fig. 1 A). To explore the role of complex III in the stabilization of the HIF-1α protein, we used cells that are deficient in cytochrome b. These cells are cybrids that were generated by repopulating 143Bρ0 cells with mitochondria that contain either a wild-type (WT) mitochondria DNA or a 4-base pair deletion of the cytochrome b gene (Rana et al., 2000). The cytochrome b–deficient cells (∆Cyt b) do not consume oxygen, similar to ρ0 cells (Fig. 1 B). However, the ΔCyt b cybrid cells retain the ability to stabilize HIF-1α protein under hypoxia (Fig. 1 C). These data indicate that the ability of cells to consume oxygen is not related to their ability to stabilize HIF-1α protein.

Moreover, under hypoxia, the ΔCyt b cybrids increase H2O2 levels measured in the cytosol using Amplex red (Fig. 1 D). ρ0 cells did not display an increase in ROS in the cytosol during hypoxia, indicating that mitochondria are the major source of ROS production during hypoxia. These data indicate that the ability of mitochondria to increase cytosolic ROS and stabilize HIF protein in hypoxia is independent of both cytochrome b and oxygen consumption. Additionally, the levels of cytosolic antioxidant proteins Cu/Zn superoxide dismutase and catalase did not change drastically in hypoxic conditions.
Mitochondrial-targeted antioxidant MitoQ prevents hypoxic stabilization of HIF-1α protein. (A) Intracellular H₂O₂ levels were measured in ΔCyt b cybrids using Amplex red in the presence of 1 μM MitoQ or 1 μM of the control compound TPMP for 4 h. n = 4 [mean ± SEM]; *, P < 0.05 [TPMP hypoxia samples compared with MitoQ hypoxia samples]. (B and C) HIF-1α protein levels of whole cell lysates from ΔCyt b cybrids (B) and WT cybrids (C) preincubated with 1 μM MitoQ or 1 μM of the control compound TPMP for 4 h and then subjected to 21% O₂ (N), 1.5% O₂ (H), or 1 mM DMOG (D) for 4 h. Representative blot from three independent experiments.

Figure 2.

Mitochondrial DNA is required for hypoxic stabilization of HIF-1α protein. To ensure that our results were not due to any adaptation to the loss of cytochrome b protein or shRNA against Rieske Fe-S protein, we corroborated our genetic findings in WT cells using well-established pharmacological inhibitors of complex III. Incubating WT cells with the complex III inhibitor antimycin A, which preserves the ROS generation at the Qo site is dispensable for hypoxic increase in cytosolic ROS (Fig. 5 B). However, activity of the Q₁ site is abolished in cells deficient in the cytochrome b protein (Fig. 1). Therefore, the data indicate that the Q₁ site is dispensable for hypoxic increase in cytosolic ROS and stabilization of HIF-1α. Stably expressing a shRNA against the Rieske Fe-S protein in the ΔCyt b cybrid cells decreases expression of the Rieske Fe-S protein in the ΔCyt b cybrid cells decreases expression of the Rieske Fe-S protein (Fig. 5 A). These cells do not stabilize the HIF-1α protein when exposed to hypoxia but retain HIF-1α protein stabilization in the presence of DMOG (Fig. 5 B). As expected, neither the TFAM shRNA cells nor Rieske Fe-S shRNA cells were able to increase cytosolic ROS under hypoxic conditions (Fig. 5 C).

Figure 3.

Cytosolic antioxidant EUK-143 prevents hypoxic stabilization of HIF-1α protein. HIF-1α protein levels of whole cell lysates from WT cybrids (A) and ΔCyt b cybrids (B) preincubated with 10 μM EUK-143 for 2 h and then subjected to 21% O₂ (N), 1.5% O₂ (H), or 1 mM DMOG (D) for 4 h. Representative blot from three independent experiments.

Hypoxic stabilization of HIF-1α protein requires generation of ROS from the Q₁ site of the mitochondrial complex III

To further validate that the mitochondrial electron transport chain is required for hypoxic stabilization of the HIF-1α protein in the ΔCyt b cybrids, we used short hairpin RNA (shRNA) against the mitochondrial transcription factor A (TFAM). TFAM is required for the proper transcription and replication of mitochondrial DNA (Ekstrand et al., 2004). In the absence of TFAM, cells become depleted of their mitochondrial DNA (ρ₀ cells; Larsson et al., 1998). Expression of TFAM shRNA in the ΔCyt b cybrids lowered TFAM mRNA expression and mitochondrial copy number by 75% compared with cells expressing the control shRNA against Drosophila melanogaster HIF (dHIF; Fig. 4, A and B). The cell containing TFAM shRNA diminished their ability to stabilize HIF-1α protein under hypoxia (Fig. 4 C). These data indicate that mitochondrial electron transport has an important role in hypoxic stabilization of HIF-1α protein.

To determine whether ROS generation from the Q₁ site is responsible for the increase in cytosolic ROS and stabilization of the HIF-1α protein, the ΔCyt b cybrid cells were stably infected with retrovirus containing shRNA against the Rieske Fe-S protein. In the absence of the Rieske Fe-S protein, the Q cycle is not initiated and ROS are not generated at the Q₁ site. It is theoretically possible that the Q₁ site might generate ROS. However, activity of the Q₁ site is abolished in cells deficient in the cytochrome b protein (Fig. 1). Therefore, the data indicate that the Q₁ site is dispensable for hypoxic increase in cytosolic ROS and stabilization of HIF-1α. Stably expressing a shRNA against the Rieske Fe-S protein in the ΔCyt b cybrid cells decreases expression of the Rieske Fe-S protein (Fig. 5 A). These cells do not stabilize the HIF-1α protein when exposed to hypoxia but retain HIF-1α protein stabilization in the presence of DMOG (Fig. 5 B). As expected, neither the TFAM shRNA cells nor Rieske Fe-S shRNA cells were able to increase cytosolic ROS under hypoxic conditions (Fig. 5 C).

To ensure that our results were not due to any adaptation to the loss of cytochrome b protein or shRNA against Rieske Fe-S protein, we corroborated our genetic findings in WT cells using well-established pharmacological inhibitors of complex III. Incubating WT cells with the complex III inhibitor antimycin A, which preserves the ROS generation at the Q₁ site of complex III, did not decrease hypoxic stabilization of the HIF-1α protein (Fig. 6 A). In contrast, the complex III inhibitor antimycin A, which preserves the ROS generation at the Q₁ site of complex III, did not decrease hypoxic stabilization of the HIF-1α protein (Fig. 6 A).
ROS regulate hydroxylation of HIF-1α protein

Under normal oxygen conditions, HIF-1α protein is hydroxylated by the PHDs, thereby facilitating ubiquitination and subsequent proteasomal degradation. Exogenous ROS are sufficient to stabilize HIF-1α protein under normal oxygen conditions (Chandel et al., 2000). Using an antibody that specifically recognizes HIF-1α protein hydroxylated on proline 564, we demonstrate that ROS inhibit the ability of the PHDs to hydroxylate HIF-1α protein. Quenching the increase in cytosolic ROS under hypoxia with MitoQ recovers hydroxylation of HIF-1α protein, indicating that the ability of the PHDs to hydroxylate HIF-1α protein is indeed regulated by ROS.

Cytochrome c reduction is not sufficient to stabilize HIF under hypoxia

Previous findings indicate that loss of cytochrome c prevents the hypoxic stabilization of the HIF-1α protein (Mansfield et al., 2005). To determine whether cytochrome c-generated ROS are required for the hypoxic stabilization of the HIF-1α protein, we exposed p66Shc cells to normoxia or hypoxia in the absence of functional complex III and IV, resulting in a loss of electron flux through cytochrome c. TMPD donates electrons to cytochrome c, thereby fully reducing cytochrome c. The electrons from cytochrome c could then be donated to p66Shc in the absence of a functional complex IV, resulting in ROS generation and HIF stabilization. WT 143B or A549 cells stabilize the HIF-1α protein during hypoxia (1.5% O2) or in the presence of DMOG (Fig. 9 A). In contrast, the 143Bp66 or A549p66 cells do not stabilize HIF-1α protein during hypoxia.
Our data indicate that the Qo site of complex III is part of the hypoxic signal transduction machinery. Cells deficient in cytochrome b protein are able to generate ROS at the Qo site of complex III. During hypoxia, these cells stabilize the HIF-1α protein. Preventing ROS generation at the Qo site in the cytochrome b–deficient cells with MitoQ or by shRNA against the Rieske Fe-S protein prevents the increase in cytosolic ROS and stabilization of the HIF-1α protein during hypoxia. The present data also demonstrate that ROS regulate the ability of the PHDs to hydroxylate HIF in both normoxic and hypoxic conditions. Quenching ROS with MitoQ in hypoxic conditions allows for continued hydroxylation of HIF-1α protein, whereas addition of exogenous ROS in normal oxygen conditions inhibits the ability of the PHDs to hydroxylate HIF-1α protein. These data suggest that the Qo site of the bc1 complex participates in hypoxic signal transduction via ROS generation to initiate HIF-mediated transcriptional responses that facilitate cellular adaptation to low oxygen.

The present data are in contrast with other groups that have proposed that the ability of mitochondria to consume oxygen is the major requirement for stabilization of the HIF-1α protein in hypoxic conditions. Their model proposes that respiring mitochondria generate an oxygen gradient, preventing hydroxylation, and thereby increasing stabilization of the HIF-1α protein (Hagen et al., 2003; Doee et al., 2005). According to this model, in the absence of a functioning respiratory chain, the oxygen gradient would be reduced, resulting in hydroxylation and degradation of the HIF-1α protein. However, the cytochrome b–null cells are respiratory incompetent and therefore unable to generate an oxygen gradient. Contrary to this model, these cells still retain the ability to stabilize the HIF-1α protein during hypoxia. Furthermore, the mitochondrial-targeted antioxidant MitoQ or the cytosolic antioxidant EUK-134 prevents stabilization of the HIF-1α protein in the cytochrome b–deficient cells, indicating ROS involvement in HIF stabilization. MitoQ has been shown to prevent hypoxic stabilization of the HIF-1α protein in

### Discussion

Mammalian cells transduce signals that couple decreases in oxygen levels to initiate HIF-dependent gene expression. The mechanism of how cells transduce hypoxic signals is not fully understood. Mitochondrial electron transport chain has been proposed as part of the hypoxic signal transduction machinery. Indeed, previous genetic evidence indicates that loss of cytochrome c or the Rieske Fe-S protein prevents the hypoxic stabilization of HIF-1α, indicating that these proteins are involved in the increase in cytosolic ROS during hypoxia (Brunelle et al., 2005; Guzy et al., 2005; Mansfield et al., 2005). RNAi of the Rieske Fe-S protein or loss of cytochrome c prevents the formation of ubisemiquinone, thus preventing ROS generation at the Qo site of complex III. The loss of cytochrome c or RNAi of Rieske Fe-S protein would also not cause a reduction in cytochrome c, thereby preventing cytochrome c–dependent ROS generation. In the present study, we demonstrate that cytochrome c is not the primary site of ROS generation in hypoxia. We demonstrate that fully reducing cytochrome c levels is not sufficient to stabilize the HIF-1α protein during hypoxia or normoxia. Therefore, cytochrome c does not contribute to hypoxic signal transduction through its ability to generate ROS via p66Shc.

### Figure 6

**Hypoxic HIF-1α protein stability is attenuated by pharmacological inhibitors of the Qo, but not Qr, site in WT cells.** (A) HIF-1α protein levels of whole cell lysates from WT cybrids incubated with 1 μM stigmatellin and subjected to 21% O2 (N), 1.5% O2 (H), or 1 mM DMOG (D) for 4 h. Representative blot from three independent experiments. (B) HIF-1α protein levels of whole cell lysates from WT cybrids incubated with 1 μM antimycin A and subjected to 21% O2 (N), 1.5% O2 (H), or 1 mM DMOG (D) for 4 h. Representative blot from three independent experiments.

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### Figure 7

**Hypoxic increase in cytosolic ROS generated from the Qo site of complex III inhibits hydroxylation of HIF-1α protein.** Immunoblot analysis of whole cell lysates for hydroxylated HIF-1α protein from WT cybrids [A] and ΔCyt b cybrids [B] treated with 20 μM MG132 to stabilize HIF-1α protein preincubated with 1 μM MitoQ or 1 μM control compound TPMP for 4 h and then subjected to 21% O2 (N), 1.5% O2 (H), or 1 mM DMOG (D) for 4 h. Representative blot from three independent experiments.

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MT 1.43Bp⁰ or A549p⁰ cells also did not stabilize the HIF-1α protein under normoxia (21% O2) or hypoxia (1.5% O2; Fig. 9 B). This was not due to reduced levels of p66Shc or cytochrome c in the p⁰ cells (Fig. 9 C). TMPD did reduce cytochrome c, and under these conditions it did not generate ROS (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200609074/DC1). Previous results indicate that ROS due to electron transfer between cytochrome c and p66shc is observed only during DNA damage and may not be the normal physiological response in healthy cells (Giorgio et al., 2005). These results indicate that reduction of cytochrome c is not sufficient for hypoxic stabilization of HIF-1α protein.
respiratory-competent cells, demonstrating the importance of ROS in HIF-1α protein stabilization (Sanjuan-Pla et al., 2005). However, there are instances when an oxygen gradient created by the mitochondria during normoxia can create a hypoxic environment within cells, causing HIF-1α protein accumulation (Doeg et al., 2005). For example, if metabolically active cells are cultured at high confluence, their demand for oxygen exceeds the supply of oxygen, resulting in a local hypoxia. Under these conditions, respiratory inhibition would result in restoration of the oxygen levels to normoxia within the cells, resulting in the degradation of the HIF-1α protein. Cells that are cultured at a high confluence under hypoxia (1–2% O2) would experience anoxia (0% O2). Under these conditions, respiratory inhibition would result in restoration of oxygen levels only to the hypoxic levels. If respiratory inhibition does not result in attenuating ROS generation, such as in the cytochrome b–deficient cells, then cells would still be able to stabilize the HIF-1α protein in conditions of high confluence under hypoxia. Collectively, our data indicate that the ability of mitochondria to generate ROS and not an oxygen gradient is required for the stabilization of the HIF-1α protein during hypoxia.

In summary, we demonstrate that the Qo site of complex III is necessary to increase cytosolic ROS in hypoxic conditions, which results in the inhibition of the ability of the PHDs to initiate degradation of HIF-1α protein (Fig. 10). We also conclusively demonstrate that the ability to consume oxygen by mitochondria is not required for hypoxic stabilization of the HIF-1α protein. The link between ROS and the PHDs is currently unknown. As oxygen levels fall, the enzymatic activity of PHDs decreases (Schofield and Ratcliffe, 2004). It is possible that the link could be an oxidant-dependent signaling pathway in which a posttranslational modification of the PHDs, such as phosphorylation, turns off the catalytic activity. In fact previous studies have implicated multiple signaling molecules that are required for hypoxic activation of HIF-1 (Aragones et al., 2001; Hirota and Semenza, 2001; Turcotte et al., 2003; Emerling et al., 2005; Hui et al., 2006). Alternatively, the link between ROS and the PHDs could be due to changes in the cytosolic redox state. The ROS may induce a shift in iron redox state from Fe2+ to Fe3+ as a result of the Fenton reaction, thereby limiting an essential cofactor of the PHDs, resulting in an inhibition of hydroxylating HIF protein (Gerald et al., 2004). It could also be that the low oxygen levels decrease PHD activity and the ROS produced during hypoxia further decrease PHD activity to prevent hydroxylation of HIF-α protein. Furthermore, multiple factors affecting cellular redox state and metabolism are likely to affect hydroxylation of the HIF-1α protein (Pan et al., 2006). Our study also suggests that the targeting of mitochondrial ROS could serve as a therapeutic target for many HIF-dependent pathological processes, including cancer. It will be of interest in future studies to examine whether the Qo site of complex III serves as part of a signal transduction machinery for other hypoxia-initiated cellular events, such as calcium signaling.
Hydroxylated
HIF

Fe
Fe

PHDs (inactive)

Degradation

Fe

H2O2

Cytochrome c

Mitochondrial Matrix

Intermembrane Space

Hyoxia

Intracellular Space

O2

ROS

Figure 10. Schematic model of mitochondrial-generated ROS stabilization of HIF-1α in hypoxic conditions. Hypoxia increases generation of ROS from the Q2 site of the bc1 complex. These ROS are released into the intermembrane space and enter the cytosol to decrease PHD activity, thus preventing hydroxylation of the HIF-1α protein. We speculate that ROS decrease the PHD activity from a combination of a posttranslational modification of the PHDs, such as phosphorylation or decreasing the availability of Fe(II), which is required for hydroxylation to occur.

Materials and methods

Cell culture

WT A549 and 143B cells were cultured in DME, whereas the p0 derivatives were cultured in DME supplemented with 100 μg/ml uridine. The p0 derivatives were made as previously described (Brunelle et al., 2005). The WT and ΔCyt b 143B cybrid cells (provided by I.F.M. de Coo, University Medical Center, Rotterdam, Rotterdam, Netherlands) were previously described by Naka et al. (2000) and were cultured in DME supplemented with 100 μg/ml uridine. Cells were cultured at 37°C in 5% CO2 humidified incubators for normoxic conditions. Hypoxic conditions (1.5% O2) were achieved in the humidified variable aerobic workstation InVivo2 (Biotrace).

ROS measurement

Intracellular ROS was measured using Amplex red (Invitrogen) according to manufacturer’s protocol. In brief, cells were lysed in 100 μM Amplex red solution supplemented with 2 U/ml HRP and 200 μM superoxide dismutase (OXS International) and incubated in the dark for 30 min. Fluorescence was measured in a plate reader (SpectraMax Gemini; Molecular Devices) with excitation of 540 nm and emission of 590 nm. ROS were measured using 10 μM of the oxidant-sensitive fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA; Invitrogen). Cells were plated at equal density probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Invitrogen). Cells were plated at equal density and exposed to different experimental conditions.

Immunoblotting

Protein levels were analyzed in whole cell lysates using lysis buffer (Cell Signaling), and 50 μg of samples were resolved on a 5% polyacrylamide gel. Gels were analyzed by immunoblotting with antibodies for HIF-1α, cytochrome c, p65NFKB (BD Biosciences), Rieske Iron Sulfur protein (Invitrogen), and hydroxylated HIF-1α (a gift from P. Ratcliffe, University of Oxford, Oxford, UK), and α-tubulin (Sigma-Aldrich) was used as a loading control.

Oxygen consumption

Cellular O2 consumption rates were measured in aliquots of 1–3 × 106 subconfluent cells removed from flasks and studied in a magnetically stirred, water-jacketed (37°C) anaerobic respirometer fitted with a polarographic O2 electrode (Oxygraph system; Hansatech Instruments). Oxygraph Plus software was used to determine oxygen consumption rate.

Mitochondrial copy number assay

The number of mitochondria was determined by analyzing the abundance of the mitochondrial DNA encoded gene cytochrome c oxidase subunit 1 (COX1) relative to the nuclear gene 18S. Total DNA was isolated using the DNeasy Tissue kit (Qiagen). 10 ng of total DNA was subjected to quantitative Real-Time PCR using Sybr Green Chemistry. Primers were used as follows: 18S forward, 5′-GTCAGTGACAGATTGATGC-3′; 18S reverse, 5′-CAAATGTCCACACCAATAAGA-3′; COX1 forward, 5′-CCACCAGGCCTCAAAAGATT-3′; and COX1 reverse, 5′-TTTGCTAATA-CAATGGCAGTCGG-3′.

Statistical analysis

The data presented are means ± SEM. Data were analyzed by two-way analysis of variance using Graph Pad Prism 4. When the analysis of variance indicated a significant difference, individual differences were explored with paired t-test. Statistical significance was determined at the 0.05 level.

Online supplemental material

Fig. S1 shows antioxidant protein profile of whole cell lysates from WT cybrids and ΔCyt b cybrids subjected to either 21% O2 (N2) or 1.5% O2 (H2) for 4 h. Fig. S2 shows HIF-1α protein levels in WT and ΔCyt b cybrids subjected to 21% O2 (N2), 1.5% O2 (H2), or 1 mM DMOG (D) for 4 h at various concentrations of MitoQ. Fig. S3 shows p53 cells treated with TMPD/ascorbate for 15 min and then subjected to either 21 or 1.5% O2; cytochrome reduct state in isolated mitochondria or ROS measurement via DCH oxidase was then assessed in whole cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200609074/DC1.

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