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HIF-1 Regulates Cytochrome Oxidase Subunits to Optimize Efficiency of Respiration in Hypoxic Cells

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SUMMARY

O_2 is the ultimate electron acceptor for mitochondrial respiration, a process catalyzed by cytochrome *c* oxidase (COX). In yeast, COX subunit composition is regulated by *COX5a* and *COX5b* gene transcription in response to high and low O_2, respectively. Here we demonstrate that in mammalian cells, expression of the COX4-1 and COX4-2 isoforms is O_2 regulated. Under conditions of reduced O_2 availability, hypoxia-inducible factor 1 (HIF-1) reciprocally regulates COX4 subunit expression by activating transcription of the genes encoding COX4-2 and LON, a mitochondrial protease that is required for COX4-1 degradation. The effects of manipulating COX4 subunit expression on COX activity, ATP production, O_2 consumption, and reactive oxygen species generation indicate that the COX4 subunit switch is a homeostatic response that optimizes the efficiency of respiration at different O_2 concentrations. Thus, mammalian cells respond to hypoxia by altering COX subunit composition, as previously observed in yeast, but by a completely different molecular mechanism.

INTRODUCTION

The goal of oxidative phosphorylation is the transfer of electrons through a series of acceptor cytochromes in order to generate a proton gradient within the inner mitochondrial membrane. The potential energy of this gradient is used to synthesize ATP. O_2 is the ultimate electron acceptor, resulting in the production of H_2O in a process that is catalyzed by cytochrome *c* oxidase (COX; complex IV). This process is not completely efficient; electron transfer to O_2 may occur at complex I or III, resulting in generation of reactive oxygen species (ROS) that oxidize cellular proteins, lipids, and nucleic acids and, by doing so, cause cell dysfunction or death.

COX, which is located in the inner mitochondrial membrane, is a dimer in which each monomer consists of 13 subunits (Tsukihara et al., 1996). Subunits I, II, and III are encoded by the mitochondrial genome, constitute the catalytic core of the enzyme, and are highly conserved in eukaryotes. The crystal structure of bovine COX revealed that subunit IV (COX4) interacts with both COX1 and COX2 (Tsukihara et al., 1996). In mammalian cells, the first step of COX assembly is the association of COX1 with COX4 (Nijtmans et al., 1998). Within the complex, COX4 binds ATP, leading to allosteric inhibition of COX activity at high ATP/ADP ratios and demonstrating a regulatory role for COX4 (Napiwotzki and Kadenbach, 1998).

Molecular modeling indicates that the spatial relationships of yeast homologs of COX1, COX2, and COX4 (which in yeast is designated COX5) are similar to the mammalian complex (Burke and Poyton, 1998). The yeast genome contains two genes encoding COX5 proteins, which show reciprocal patterns of expression: at high O_2 concentrations, *COX5a* transcription is activated and *COX5b* transcription is repressed, whereas at low O_2 concentrations, *COX5a* transcription is inactivated and *COX5b* transcription is derepressed (Lowry and Zitomer, 1984; Forsburg and Guarente, 1989; Kwast et al., 1998). Substitution of COX5b for COX5a increases the turnover number (TN_{max}) of COX by increasing the rate of electron transfer from heme *a* to the binuclear reaction center within COX1 (Waterland et al., 1991; Allen et al., 1995). Mammals possess paralagous genes encoding alternative isoforms of COX6, COX7a, COX8, and COX4 (Hutteman et al., 2001). Although tissue-specific expression of these genes has been demonstrated, neither molecular nor physiological regulatory mechanisms have been elucidated.

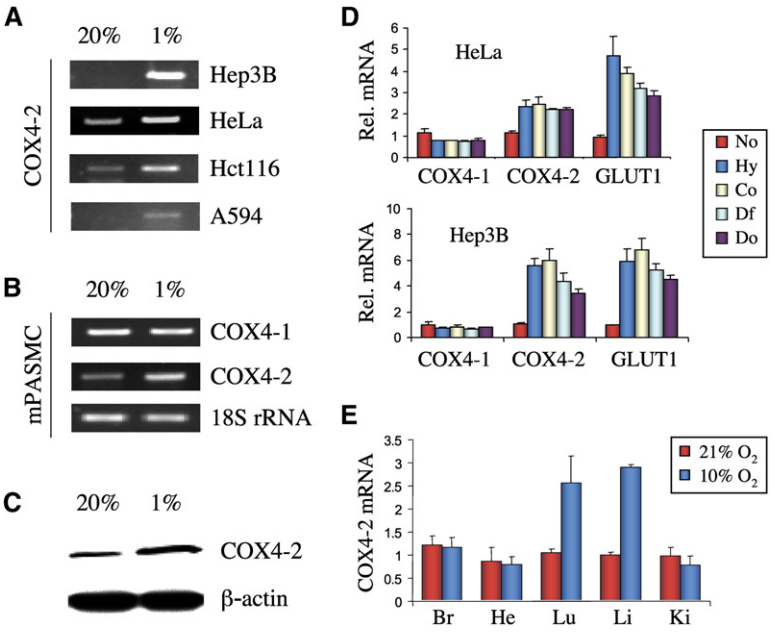

Figure 1. Expression of COX 4-1 and COX 4-2 mRNA

(A) COX4-2 mRNA expression was analyzed by RT-PCR in Hep3B, HeLa, Hct116, and A594 cells that were cultured under nonhypoxic (20% O_2) or hypoxic (1% O_2) conditions for 24 hr.

(B) COX4-1 and COX4-2 mRNA expression was analyzed in primary cultures of mouse pulmonary artery smooth muscle cells (mPASMC).

(C) Immunoblot assay of COX4-2 and β-actin in HeLa cells exposed to 20% or 1% O_2 for 24 hr is shown.

(D) Quantitative real-time (qrt) RT-PCR analysis of COX4-1, COX4-2, and GLUT1 mRNA expression (mean ± SEM) in Hep3B and HeLa cells exposed to: 20% O_2 with no treatment (No); 1% O_2 (Hy); or 20% O_2 in the presence of $CoCl_2$ (Co), desferrioxamine (Df), or dimethyloxalylglycine (Do).

(E) qrtPCR was performed to analyze expression of COX4-2 mRNA (mean ± SEM) in brain (Br), heart (He), lung (Lu), liver (Li), and kidney (Ki) tissue from mice (n = 3 each) exposed to 21% or 10% O_2 for 3 weeks.

Hypoxia-inducible factor 1 (HIF-1) is a transcriptional activator that functions as a master regulator of oxygen homeostasis in all metazoan species. Previous studies have demonstrated that HIF-1 transactivates genes encoding glucose transporters and glycolytic enzymes in response to reduced O_2 availability (Semenza et al., 1996; Iyer et al., 1998; Seagroves et al., 2001). HIF-1 is a heterodimeric protein composed of a constitutively expressed HIF-1β subunit and an O_2-regulated HIF-1α or HIF-2α subunit (Wang et al., 1995; Jiang et al., 1996; Tian et al., 1997; Wiesener et al., 1998). Under aerobic conditions, HIF-1α and HIF-2α are subjected to prolyl hydroxylation by enzymes that utilize O_2 as a substrate (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). Hydroxylation is required for binding of the von Hippel-Lindau (VHL) protein (Maxwell et al., 1999), which targets HIF-1α and HIF-2α for ubiquitination and proteasomal degradation (Salceda and Caro, 1997). The interaction of HIF-1α with the coactivators CBP and p300 is blocked by O_2-dependent asparaginyl hydroxylation mediated by FIH-1 (Lando et al., 2002). Under hypoxic conditions, the rate of hydroxylation declines, leading to HIF-1 activation and thus providing a mechanism by which changes in oxygenation are transduced to the nucleus as changes in gene expression. In this study, we tested the hypothesis that in mammalian cells the expression of COX4-1 and COX4-2 is O_2 regulated through the activity of HIF-1.

RESULTS

COX4-2 mRNA Expression Is Induced by Hypoxia

To investigate whether COX4-2 mRNA expression is O_2 regulated in human cells, Hep3B (liver), HeLa (uterus), Hct116 (colon), and A594 (lung) cells were incubated at 20% or 1% O_2 for 24 hr. COX4-2 mRNA expression was induced under hypoxic conditions in all four cell lines as determined by reverse transcriptase (RT)-PCR (Figure 1A). Hypoxia-induced expression of COX4-2 mRNA was also observed in primary cultures of mouse pulmonary artery smooth muscle cells (mPASMC; Figure 1B). COX4-2 protein expression was induced in hypoxic HeLa cells as determined by immunoblot assay (Figure 1C) using an antibody that specifically recognizes COX4-2 (Figure S1).

COX4-1 mRNA expression was not O_2 regulated in mPASMC, HeLa, or Hep3B cells as determined by both conventional RT-PCR (Figures 1B and S2) and quantitative real-time RT-PCR (qrtPCR; Figure 1D). COX4-2, but not COX4-1, mRNA expression was induced in HeLa and Hep3B cells exposed to $CoCl_2$, desferrioxamine (DFX), or dimethyloxalylglycine (DMOG), which are inducers of HIF-1α expression that inhibit hydroxylase activity (Epstein et al., 2001; Jaakkola et al., 2001) and thereby increase the expression of mRNAs encoded by HIF-1 target genes such as *GLUT1* (Figures 1D and S2). COX4-2 mRNA expression was increased in liver and lung tissue from mice exposed to 10% O_2 for 3 weeks relative to control mice exposed to room air (21% O_2), indicating that COX4-2 induction is a physiological response to hypoxia (Figure 1E). Taken together the data shown in Figure 1 demonstrate that expression of the *COX4I2* gene, which encodes COX4-2, is induced in response to hypoxia and suggest that this response may be regulated by HIF-1.

Identification of Hypoxia-Response Elements in the Human *COX4I2* gene

We searched the human *COX4I2* gene for matches to a consensus sequence, which was generated from hypoxia-response elements (HREs) in the *EPO, ALDA, Vegf,*

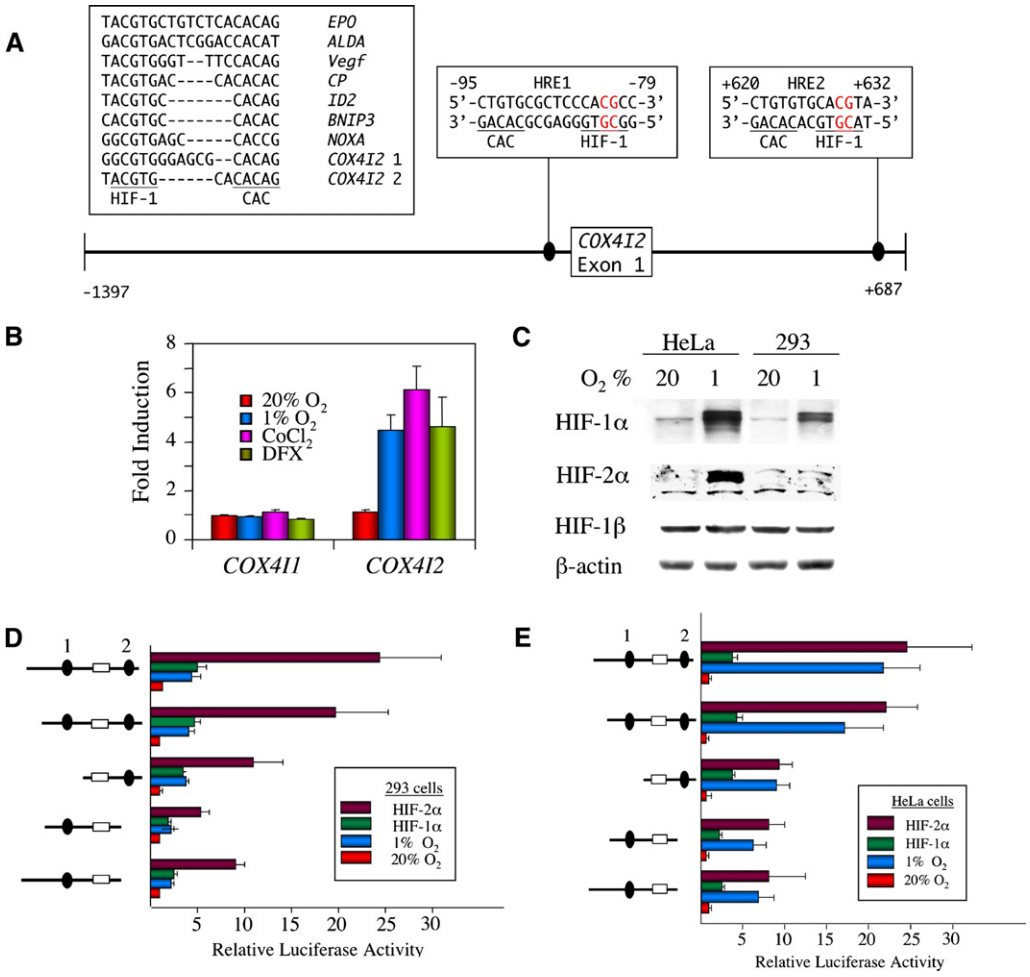

Figure 2. Identification of Hypoxia-Response Elements in the *COX4I2* Gene

(A) Nucleotide sequences matching the consensus hypoxia-response element (HRE) from seven mammalian genes [5′-RCGTG(N)$_{1-8}$CACAG-3′] are present in the 5′-flanking region (HRE1) and intron 1 (HRE2) within a 2.1 kb DNA fragment of the human *COX4I2* gene. The nucleotide sequences are numbered in relation to the transcription initiation site, which is designated "+1." The nucleotides that were mutated to eliminate HIF-1 binding are shown in red.

(B) Luciferase reporter genes containing the 2.1 kb *COX4I2* gene fragment or an analogous 2.7 kb *COX4I1* gene fragment were transfected into 293 cells and cultured under the following conditions for 24 hr: 20% O_2; 1% O_2; or 20% O_2 in the presence of 100 μM $CoCl_2$ or desferrioxamine (DFX). For each reporter, mean luciferase activity under each experimental condition is shown (± SEM, n = 3) relative to the activity in control cells (20% O_2).

(C) Immunoblot assay of HIF-1α, HIF-2α, HIF-1β, and β-actin protein in HeLa and 293 cells cultured under nonhypoxic (20% O_2) or hypoxic (1% O_2) conditions.

(D and E) Luciferase reporter genes containing full-length 2.1 kb *COX4I2* gene fragment with both HREs (black ovals) spanning exon 1 (open rectangle) or deletion constructs with only a single HRE were cotransfected into HeLa (D) or 293 (E) cells with expression vector encoding HIF-1α or HIF-2α, then incubated at 20% O_2 or cotransfected with empty vector and incubated at 20% or 1% O_2 for 24 hr. For each experimental condition, mean luciferase activity is shown (± SEM, n = 3) relative to activity in cells transfected with empty vector and incubated at 20% O_2.

CP, ID2, BNIP3, and *NOXA* genes (Semenza et al., 1996; Shima et al., 1996; Bruick, 2000; Mukhopadhyay et al., 2000; Kim et al., 2004; Lofstedt et al., 2004) and consisted of the core HIF-1-binding site 5′-RCGTG-3′ followed, after 1–8 nucleotides, by a CAC sequence (consensus, 5′-CA CAG-3′). Candidate HREs were identified in the 5′-flanking region (HRE1) and intron 1 (HRE2) of *COX4I2* (Figure 2A). A 2.1 kb DNA fragment spanning these HREs was inserted into the promoterless luciferase reporter plasmid pGL2-Basic. A second plasmid containing 2.7 kb spanning the analogous region of the human *COX4I1* gene, which encodes COX4-1, was also constructed. The reporter plasmids were transfected into human embryonic kidney 293 cells, which were incubated under four conditions: control nonhypoxic conditions (20% O_2); hypoxia (1% O_2); and nonhypoxic conditions in the presence of 100 μM $CoCl_2$ or DFX. Analysis of cell lysates 24 hr later revealed that *COX4I2* promoter activity was induced by hypoxia, $CoCl_2$, or DFX, whereas *COX4I1* promoter activity was unaffected (Figure 2B). These results are consistent with the

analyses of COX4-1 and COX4-2 mRNA expression (Figure 1) and indicate that hypoxia selectively induces *COX4I2* gene transcription.

Immunoblot assays revealed that HIF-1α protein expression was induced by hypoxia in HeLa and 293 cells, whereas HIF-2α expression was poorly induced in hypoxic 293 cells (Figure 2C). *COX4I2* promoter activity in HeLa cells was dramatically increased by hypoxia or cotransfection of an expression vector encoding HIF-2α, whereas cotransfection of an expression vector encoding HIF-1α had a more modest effect (Figures 2D and S4) despite high levels of HIF-1α protein in transfected cells (Figure S3). In 293 cells, *COX4I2* promoter activity was greatly increased by cotransfection of a HIF-2α expression vector, whereas hypoxia or cotransfection of HIF-1α expression vector had a more modest effect (Figures 2E and S4). These data suggest that HIF-2α is a more potent transactivator of the *COX4I2* promoter than HIF-1α, and the immunoblot data (Figure 2C) suggest that the more modest effect of hypoxia on *COX4I2* promoter activity in 293 cells is due to the limited induction of HIF-2α expression. Analysis of deletion constructs containing only HRE1 or HRE2 suggested that both sequences are required for maximal induction of *COX4I2* promoter activity in response to hypoxia or overexpression of HIF-1α or HIF-2α (Figures 2D, 2E, and S4).

The data presented in Figure 2 suggest that both HIF-1α and HIF-2α can contribute to increased *COX4I2* promoter activity in hypoxic cells. To further test this hypothesis, HeLa and 293 cells were cotransfected with pCEP4-HIF-1αDN, which encodes a dominant-negative form of HIF-1α that competes with endogenous HIF-1α and HIF-2α for dimerization with HIF-1β but results in transcriptionally inactive heterodimers (Jiang et al., 1996). HIF-1αDN inhibited hypoxia-induced *COX4I2* promoter activity in a dose-dependent manner in both 293 and HeLa cells (Figure 3A), which is similar to its effect on reporter plasmid p2.1 (Figure S5), which contains a HIF-1-dependent HRE from the *ENO1* gene (Semenza et al., 1996).

To determine the effect of specifically interfering with HIF-1α or HIF-2α expression, HeLa and 293 cells were transfected with: empty vector (EV); expression vector encoding short hairpin RNA (shRNA) directed against HIF-1α (Shr-HIF-1α) or HIF-2α (Shr-HIF-2α); or vector encoding a scrambled negative control sequence (Scr). Immunoblot assays revealed that target protein expression was reduced in Shr-transfected cells (Figure S6). The cells were cotransfected with reporter plasmid p2.1 or the *COX4I2* promoter reporter. Shr-HIF-1α significantly inhibited hypoxia-induced p2.1 in both HeLa and 293 cells, whereas Shr-HIF-2α had no significant effect on hypoxia-induced p2.1 activity in HeLa or 293 cells (Figure S7). In contrast, Shr-HIF-2α significantly inhibited hypoxia-induced *COX4I2* promoter activity in HeLa cells but not in 293 cells (Figure 3B), which have low HIF-2α expression (Figure 2C). These data indicate that: different gene promoters (*ENO1* versus *COX4I2*) are differentially regulated by HIF-1α versus HIF-2α and that regulation of the same gene (*COX4I2*) in different cell types (293 versus HeLa) may vary depending upon the extent to which HIF-2α is induced by hypoxia.

To demonstrate that the HIF-1-binding sites in HRE1 and HRE2 were required for hypoxia-induced *COX4I2* promoter activity, CG→AA mutations that destroy the HIF-1-binding sites were introduced into the HREs (Figure 2A). Mutation of HRE2 resulted in a partial loss of activity, whereas mutation of both HREs resulted in a complete loss of hypoxia-induced *COX4I2* promoter activity in both HeLa and 293 cells (compare Figure 3C with Figures 2D and 2E).

To demonstrate that HIF-1 binds to these sequences within living cells, chromatin immunoprecipitation (ChIP) assays were performed using DFX-treated HeLa and 293 cells. Binding of HIF-2α to HRE1 and HRE2 in HeLa cells and binding of HIF-1α to HRE2 in 293 cells was demonstrated when PCR was performed using a single set of flanking primers (Figures 3D and 3E). A more sensitive but nonquantitative nested PCR was required to demonstrate the binding of HIF-1α to HRE2 in HeLa cells. No PCR product was detected when nonspecific IgG was used instead of specific antibody against HIF-1α or HIF-2α for ChIP assay of DFX-treated cells or when chromatin from untreated cells was used for ChIP with antibodies against HIF-1α or HIF-2α (data not shown). The data presented in Figures 1–3 show that *COX4I2* expression is induced by HIF-1 and that HIF-2α-containing heterodimers preferentially, but not exclusively, bind to two HREs within the 5′-flanking region and intron 1 to activate gene transcription.

Regulation of COX4-1 Protein Levels by HIF-1

We next investigated whether HIF-1 regulates COX4-1 protein levels using a subunit-specific antibody (Figure S1). HeLa and Hct116 cells were cultured for 24 or 48 hr in the presence or absence of $CoCl_2$. Exposure of the cells to $CoCl_2$ induced increased HIF-1α expression as expected but also resulted in a dramatic reduction in COX4-1 protein levels, whereas β-actin levels were unaffected (Figure 4A). A time course analysis of $CoCl_2$-treated HeLa cells revealed a gradual reduction in COX4-1 protein levels over 24 hr, whereas β-actin protein levels were unchanged (Figure 4B). In addition to $CoCl_2$, the HIF-1 hydroxylase inhibitors DMOG and dihydroxybenzoate (DHB) increased HIF-1α and decreased COX4-1 protein levels (Figure 4C).

To investigate whether the effect of hydroxylase inhibitors on COX4-1 was due to their induction of HIF-1 activity, we utilized two other HIF-1 gain-of-function (GOF) models. First, we analyzed RCC4 human renal carcinoma cells in which VHL loss-of-function (LOF) results in constitutive high-level expression of HIF-1α and HIF-2α (Hu et al., 2003). In these cells, COX4-1 protein levels were low even in the absence of $CoCl_2$ treatment, whereas in a subclone stably transfected with a VHL expression vector to inhibit HIF-1α and HIF-2α expression under nonhypoxic conditions, COX4-1 protein levels were increased in the

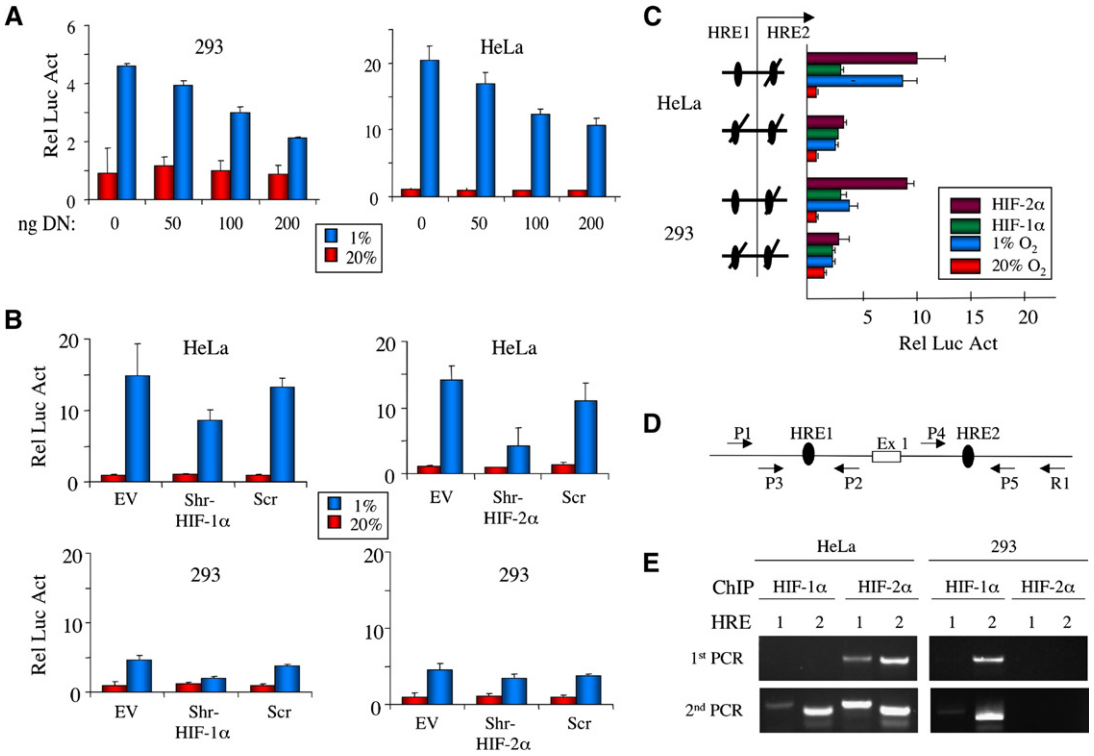

Figure 3. Involvement of HIF-1α and HIF-2α in Transactivation of the *COX4I2* Promoter

(A) Cells were transfected with the *COX4I2*-luciferase reporter and increasing amounts (in ng) of expression vector pCEP4-HIF-1αDN, which encodes a dominant-negative form of HIF-1α, and were incubated at 20% or 1% O_2 for 24 hr. Mean luciferase activity is shown (± SEM, n = 3) relative to the activity in cells transfected with no pCEP4-HIF-1αDN (200 ng of empty vector pCEP4) and incubated at 20% O_2.

(B) HeLa and 293 cells were cotransfected with *COX4I2*-luciferase reporter and empty vector (EV) or vector encoding a short-hairpin RNA that blocks the expression of HIF-1α (Shr-HIF-1α) or HIF-2α (Shr-HIF-2α) then incubated for 24 hr at 20% O_2 (red bars) or 1% O_2 (blue bars). Mean luciferase activity is shown (± SEM, n = 3) relative to cells transfected with EV and incubated at 20% O_2.

(C) HeLa and 293 cells were cotransfected with reporter plasmids containing a CG→AA mutation in the putative HIF-1-binding site of HRE1 or HRE2 (slashed oval) in the presence of: 20% O_2; 1% O_2; or 20% O_2 with cotransfected HIF-1α or HIF-2α expression vector. Mean luciferase activity (± SEM, n = 3) was determined.

(D and E) Chromatin immunoprecipitation (ChIP) assays were performed using the indicated primers (D) and antibodies (E) to demonstrate binding of HIF-1α or HIF-2α to HRE1 and HRE2 in desferrioxamine-treated HeLa or 293 cells. The first PCR was performed using P1-P2 and P4-R1 primer pairs for HRE1 and HRE2, respectively. The second PCR was performed using P3-P2 and P4-P5 primer pairs.

absence but not in the presence of $CoCl_2$ (Figure 4D). In a second HIF-1 GOF model, we analyzed COX4-1 levels in HeLa cells that were infected with an adenovirus encoding either β-galactosidase (AdLacZ) or a constitutively active form of HIF-1α (AdCA5) that activates target gene expression under nonhypoxic conditions (Kelly et al., 2003). COX4-1 levels were dramatically reduced by infection with AdCA5 as compared to AdLacZ (Figure 4E). Thus, HIF-1 GOF leads to loss of COX4-1 protein expression.

We also investigated the effect of HIF-1 LOF using two independent experimental approaches. In wild-type mouse embryo fibroblasts, which express HIF-1α but not HIF-2α, COX4-1 protein levels were reduced in response to hypoxia, whereas in HIF-1α null cells, no induction of HIF-1α or loss of COX4-1 protein occurred (Figure 4F). In the second LOF approach, expression of Shr-HIF-1α in HeLa cells partially inhibited the induction of HIF-1α in response to $CoCl_2$ and partially inhibited the loss of COX4-1 protein in response to $CoCl_2$, whereas β-actin protein levels were unaffected by $CoCl_2$ or Shr-HIF-1α (Figure 4G).

To investigate the mechanism by which COX4-1 was degraded in response to hypoxia, cells were treated with the proteasome inhibitor MG132, which blocks HIF-1α degradation (Figure 4H). However, $CoCl_2$-induced degradation of COX4-1 was not blocked by MG132. In pulse-chase assays, degradation of COX4-1 was dramatically increased by $CoCl_2$ (Figure 4I). The results presented in Figure 4 demonstrate that COX4-1 degradation is induced in cells exposed to hypoxia or $CoCl_2$ by a HIF-1-dependent, proteasome-independent mechanism.

HIF-1 Regulates a Mitochondrial Protease that Is Required for COX4-1 Degradation

The activity of the mitochondrial protease LON is induced by hypoxia, ischemia, or endoplasmic reticulum stress

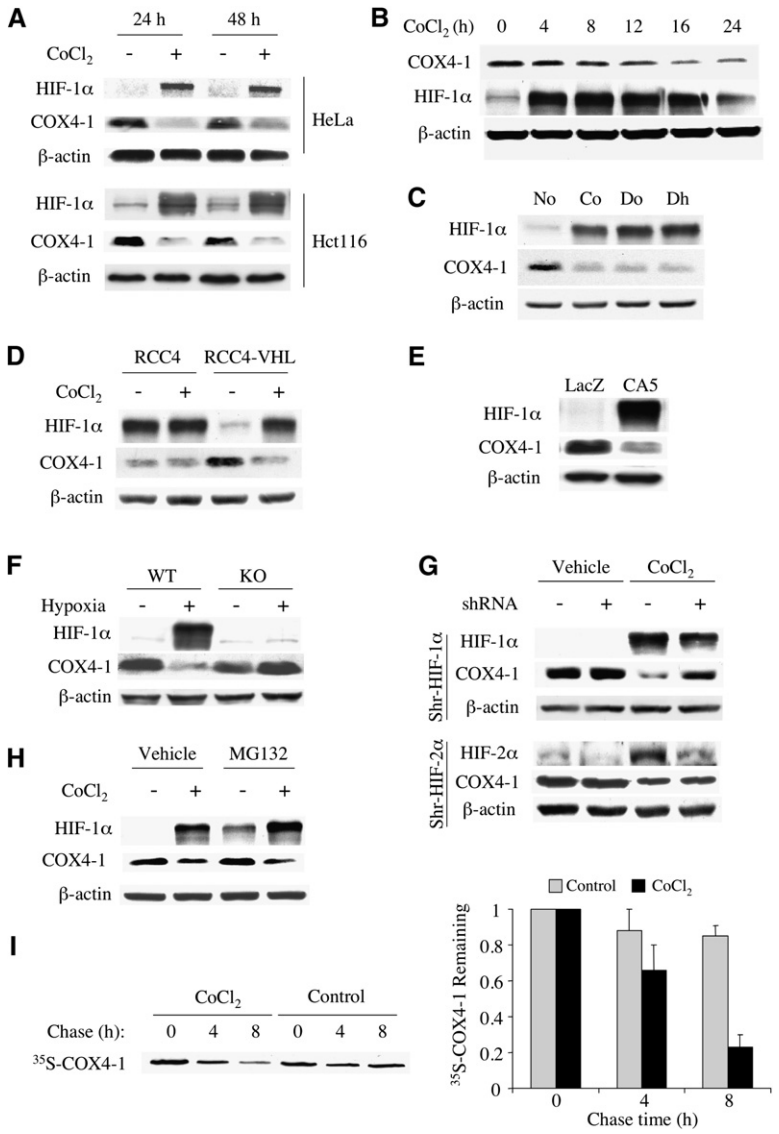

Figure 4. O_2-Dependent Regulation of COX4-1 Protein Levels

(A) Immunoblot assay of HIF-1α, COX4-1, and β-actin protein in HeLa and Hct116 cells that were treated with vehicle (–) or 100 μM $CoCl_2$ (+) for 24 or 48 hr.

(B) HeLa cells were exposed to $CoCl_2$ as indicated, and lysates were analyzed for expression of COX4-1, HIF-1α, and β-actin protein.

(C) Cells were untreated (No) or treated with $CoCl_2$ (Co), dimethyloxalylglycine (Do), or dihydroxybenzoate (Dh). Whole-cell lysates were prepared after 4 hr for HIF-1α or after 24 hr for COX4-1 and β-actin immunoblot assays.

(D) RCC4 cells, which lack VHL expression, and RCC4-VHL cells, which are stably transfected with a VHL expression vector, were untreated (–) or treated with $CoCl_2$ for 24 hr (+), and lysates were subjected to HIF-1α, COX4-1, and β-actin immunoblot assays.

(E) HeLa cells were infected with 50 plaque-forming units per cell of AdLacZ or AdCA5, and lysates were analyzed 24 hr after infection.

(F) Mouse embryo fibroblasts that were wild-type (WT) or homozygous for a knockout (KO) allele at the locus encoding HIF-1α were incubated at 20% or 1% O_2. Whole-cell lysates were prepared after 4 hr for HIF-1α or after 24 hr for COX4-1 and β-actin immunoblot assays.

(G) HeLa cells were transfected with vector (+) encoding short-hairpin RNA against HIF-1α (Shr-HIF-1α), HIF-2α (Shr-HIF-2α), or empty vector (–) and exposed to vehicle or $CoCl_2$ for 24 hr prior to immunoblot assays.

(H) Cells were untreated (–) or $CoCl_2$-treated (+) for 24 hr with 10 μM MG132 or vehicle added for the last 4 hr.

(I) Cells were pulsed with [^{35}S]-methionine and chased with unlabeled methionine for 0, 4, or 8 hr in the absence (control) or presence of $CoCl_2$ followed by immunoprecipitation of COX4-1, PAGE, autoradiography (left), and quantification (right; mean ± SEM, n = 3).

(Hori et al., 2002). We hypothesized that LON is responsible for hypoxia-induced degradation of COX4-1. LON mRNA expression was induced in: HeLa cells exposed to 1% O_2 for 24 or 48 hr (Figures 5A and S8); heart, lung, and liver of hypoxic mice (Figure 5B); and AdCA5-infected cells (Figure 5C). LON mRNA expression was induced by hypoxia in RCC4-VHL cells but was constitutively expressed in the VHL-deficient parental RCC4 cells (Figure 5D, left panels). RCC4 cells stably transfected with expression vector encoding Shr-HIF-1α expressed lower levels of LON mRNA than did RCC4 cells transfected with empty vector (Figure 5D, right panels). Compared to scrambled control shRNA, shRNA against LON mRNA blocked degradation of COX4-1 protein that was induced by hypoxia or $CoCl_2$ (Figure 5E).

Analysis of the human *LON* gene promoter revealed the presence of five potential HIF-1-binding sites within 0.6 kb 5′ to the transcription start site (Figure S9). Activity of the full-length promoter was markedly induced by hypoxia, and deletion analysis suggested that the middle and proximal, but not distal, candidate HIF-1 sites contributed to the response in HeLa (Figure 5F) and 293 (Figure S10) cells. These results were corroborated by ChIP assays, which demonstrated the binding of protein complexes containing HIF-1α and HIF-2α to the proximal and middle, but not distal, HIF-1 sites in DFX-treated, but not in untreated, HeLa cells (Figure 5G). The data presented in Figure 5 indicate that HIF-1 induces *LON* expression and that LON is required for the degradation of COX4-1 protein in hypoxic cells.

Functional Consequences of Modulating COX4 Subunit Expression

Little is known regarding the functional properties of the human COX4-1 and COX4-2 proteins. We analyzed the effect of increasing (Figure S11) or decreasing (Figures S12

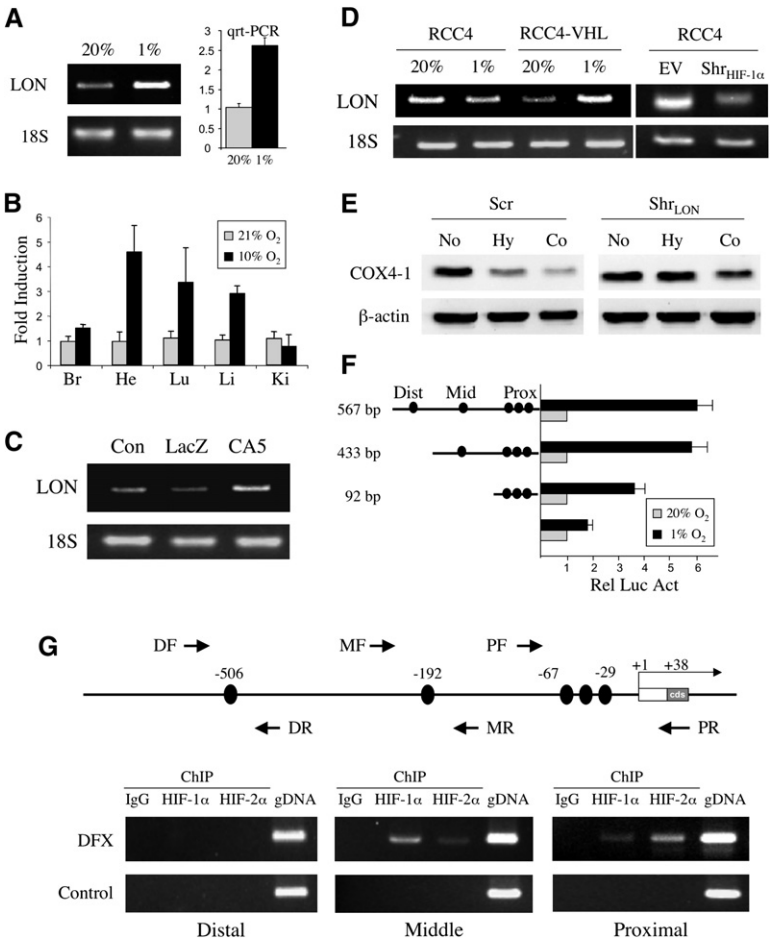

Figure 5. Regulation of LON Mitochondrial Protease Expression

(A and B) LON mRNA expression relative to 18S rRNA was determined by RT-PCR and qrtPCR (mean ± SEM, n = 3) in: (A) HeLa cells incubated for 24 hr at 20% or 1% O_2 and (B) tissues from mice exposed to 10% or 21% O_2 for 3 weeks.

(C) LON mRNA expression in control HeLa cells (Con) and cells infected with AdLacZ or AdCA5.

(D) LON mRNA expression was analyzed in RCC4 and RCC4-VHL cells incubated at 1% or 20% O_2 and RCC4 cells stably transfected with empty vector (EV) or expression vector encoding short-hairpin RNA (Shr) against HIF-1α ($Shr_{HIF\text{-}1\alpha}$).

(E) Effect of LON loss-of-function (LOF) on the expression of COX4-1 is shown. Cells were transfected with an expression vector encoding Shr-targeting LON mRNA (Shr_{LON}) or a scrambled control sequence (Scr) and were exposed to no treatment (No), $CoCl_2$ (Co), or hypoxia (Hy) for 24 hr. COX4-1 and β-actin protein levels in cell lysates were determined by immunoblot assays.

(F) HeLa cells were transfected with luciferase reporter plasmids with *LON* promoter sequences containing candidate HIF-1-binding sites (ovals) and incubated for 24 hr at 20% or 1% O_2.

(G) HIF-1 binding to *LON* gene sequences was analyzed by ChIP. Chromatin from cells that were exposed to vehicle (Control) or to desferrioxamine (DFX) was immunoprecipitated (ChIP) with IgG, anti-HIF-1α, or anti-HIF-2α antibody and analyzed by PCR using primers spanning candidate HIF-1-binding sites. gDNA is a positive control in which PCR was performed using total human genomic DNA rather than immunoprecipitated chromatin.

and S13) the levels of these proteins in 293T cells cultured under nonhypoxic and hypoxic conditions. 293T cells were transiently transfected with expression vectors encoding either FLAG- or V5-epitope-tagged COX4-1 or COX4-2 protein. Immunoblot assays revealed that all four proteins were expressed at high levels and localized to the mitochondrial fraction of cell lysates (Figure S14), and all subsequent experiments were performed with FLAG-tagged proteins.

Compared to cells transfected with EV, nonhypoxic cells transfected with vector encoding COX4-1 manifested modest, but significant, increases in COX activity and O_2 consumption (COX and JO_2, respectively, in Figure 6A, left panels), which is consistent with the already high level of COX4-1 in these cells (Figure S12). COX4-2 GOF also increased COX and JO_2 in nonhypoxic cells. COX4-2, but not COX4-1, increased COX and JO_2 in hypoxic cells. Immunoblot assay revealed that overexpression of FLAG-COX4-1 successfully overwhelmed the proteolytic machinery in hypoxic cells (Figure S11), thus ruling out the possibility that the ineffectiveness of FLAG-COX4-1 to increase COX and JO_2 in hypoxic cells was due to its degradation. ATP levels were increased in nonhypoxic cells with either COX4-1 or COX4-2 GOF, but only COX4-2 GOF increased ATP levels in hypoxic cells. COX4-1 LOF led to a reduction in COX, JO_2, and ATP in nonhypoxic cells but had no effect on these indices in hypoxic cells (Figure 6A, right panels). COX4-2 LOF had no effect in nonhypoxic cells but led to reduced COX, JO_2, and ATP in hypoxic cells.

We hypothesized that COX4-1 and COX4-2 may be necessary to optimize electron transfer chain activity under nonhypoxic and hypoxic conditions, respectively. Nonoptimal efficiency may result in decreased ATP production and/or increased production of H_2O_2 due to reaction of electrons with O_2 prior to complex IV. H_2O_2 levels increased in response to hypoxia, an effect that was increased by COX4-1 GOF and decreased by COX4-2 GOF (Figure 6A, left panels). COX4-1 and COX4-2 LOF selectively increased H_2O_2 levels in nonhypoxic and hypoxic cells, respectively. Activation of caspase 3 was increased in hypoxic cells, an effect that was amplified by COX4-1 GOF, whereas COX4-2 GOF increased caspase activation in nonhypoxic cells. COX4-1 and COX4-2 LOF selectively

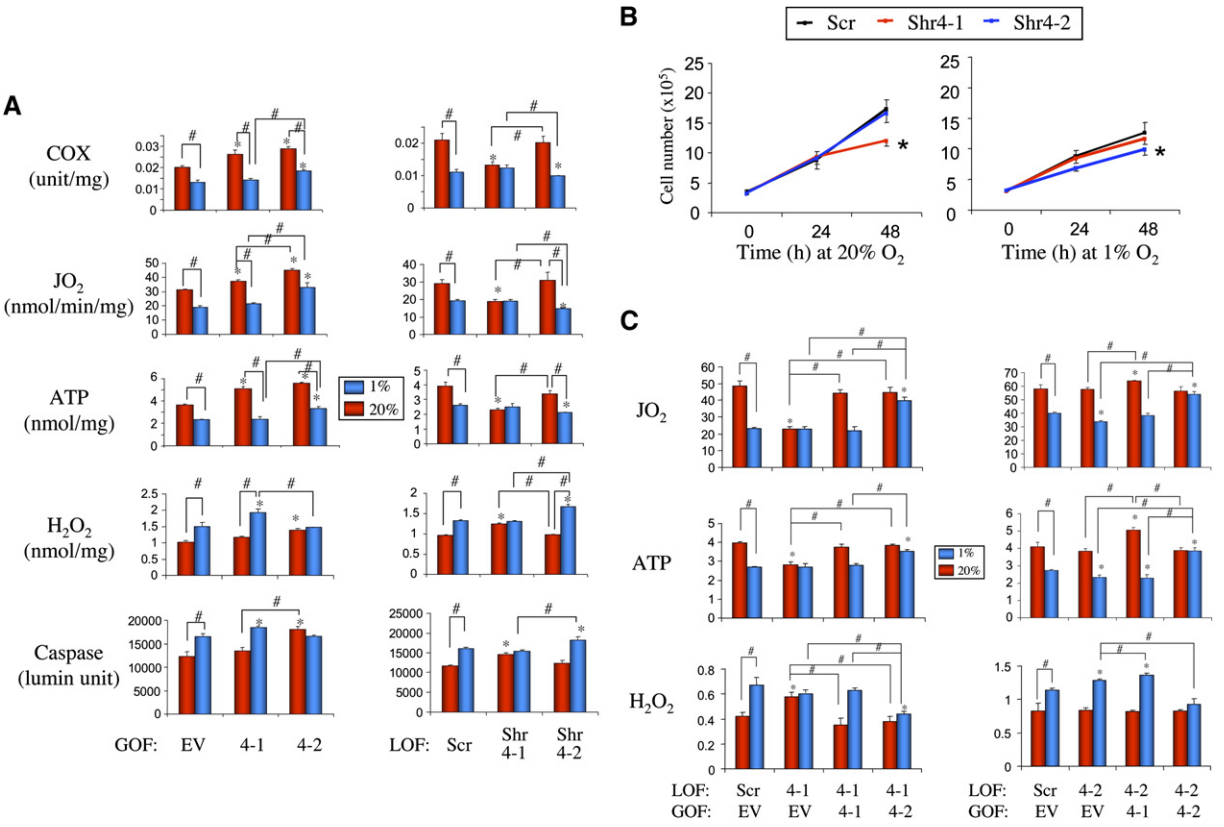

Figure 6. Effects of Altered COX4 Subunit Expression on Cellular Homeostasis

(A) 293T cells were transiently transfected with empty vector (EV), FLAG-COX4-1, or FLAG-COX4-2 (left panels) or with vector encoding short-hairpin RNA (Shr) with no target (Scr) or targeting COX4-1 (Shr4-1) or COX4-2 (Shr4-2) mRNA (right panels) and incubated under nonhypoxic (red bars) or hypoxic (blue bars) conditions for 24 hr, followed by determination of mean (± SEM) cytochrome *c* oxidase activity (COX), O_2 consumption (JO_2), ATP, H_2O_2, and activated caspase 3 activity. *, $p < 0.05$ compared to Scr/EV; #, $p < 0.05$ for indicated comparison.

(B) 293T cells were transiently transfected with expression vectors encoding scrambled negative control (Scr), COX4-1 (Shr4-1), or COX4-2 (Shr4-2) shRNA and incubated at 20% (left) or 1% (right) O_2. Cells were harvested 24 or 48 hr later, and the number of viable cells was determined by trypan blue exclusion. Mean values are shown (± SEM, n = 3); *, $p < 0.05$ by Student's t test compared to Scr.

(C) Left panels: 293T cells were transiently transfected with: vector encoding Shr with no target (Scr) or Shr resulting in COX4-1 (Shr4-1) LOF and EV or vector for COX4-1 (4-1) or COX4-2 (4-2) gain-of-function (GOF). Right panels: 293T cells were transfected with: vector encoding Shr with no target (Scr) or Shr resulting in COX4-2 (Shr4-2) LOF and EV or vector for COX4-1 (4-1) or COX4-2 (4-2) GOF. Cells were incubated under nonhypoxic (red bars) or hypoxic (blue bars) conditions for 24 hr, followed by determination of mean (± SEM) JO_2, ATP, and H_2O_2. *, $p < 0.05$ compared to Scr/EV; #, $p < 0.05$ for indicated comparison (Student's t test). For all panels, each graph represents an independent experiment, and mean ± SEM (n = 3) are shown.

increased caspase activation in nonhypoxic and hypoxic cells, respectively (Figure 6A, right panels). Trypan blue staining revealed that COX4-1 LOF led to a selective reduction in the number of viable cells under nonhypoxic conditions, whereas COX4-2 LOF led to selective reduction in the number of viable cells under hypoxic conditions (Figure 6B). All of these experiments were also performed in HeLa cells with similar results (Figures S15 and S16).

We next performed subunit rescue assays. The effects of COX4-1 LOF on JO_2, ATP, and H_2O_2 levels could be rescued by COX4-1 expression vector (Figure 6C, left panels) even though the increase in COX4-1 protein levels was modest (Figure S17). Overexpression of COX4-2 also rescued COX4-1 LOF. In contrast, the effects of COX4-2 LOF on JO_2, ATP, and H_2O_2 levels could be rescued by COX4-2, but not by COX4-1, GOF (Figure 6C, right panels). All of these experiments were also performed in HeLa cells with similar results (Figure S18). The data presented in Figure 6 demonstrate that in hypoxic cells COX4-2 plays a unique and essential role. In nonhypoxic cells, COX4-1 GOF increased COX, JO_2, and ATP without increasing levels of H_2O_2 and activated caspase 3, whereas COX4-2 increased COX, JO_2, and ATP as well as H_2O_2 and activated caspase. Taken together, the comprehensive analyses we have performed indicate that the COX4 subunit switch constitutes a critical adaptive response to hypoxia.

DISCUSSION

In this study we demonstrate that COX structure and function are regulated in response to changes in the

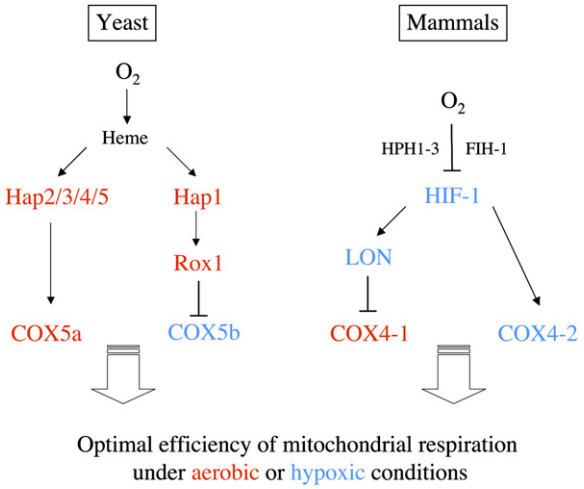

Figure 7. Mechanisms and Consequences of O_2-Regulated COX Subunit Switching

In yeast (left) growing under aerobic conditions that are permissive for heme synthesis, Hap2/3/4/5 transactivates the *COX5a* gene and Hap1 transactivates the *Rox1* gene, the protein product of which represses *COX5b* transcription. Under hypoxic conditions, inhibition of heme synthesis results in loss of Hap2/3/4/5 and Hap1 activity, loss of *COX5a* transcription, and derepression of *COX5b* transcription. In mammals (right), HIF-1 activity is inhibited by prolyl (HPH1-3) and asparaginyl (FIH-1) hydroxylases, which utilize O_2 as a substrate to hydroxylate HIF-1α and HIF-2α. In hypoxic cells, hydroxylation is inhibited, leading to HIF-1-mediated transactivation of the *COX4I2* gene, which encodes COX4-2, and the *LON* gene, which encodes a protease that is required for the degradation of COX4-1. In yeast and mammals, COX subunit switching represents a homeostatic mechanism to maintain optimal efficiency of mitochondrial respiration in response to changes in cellular oxygenation.

oxygenation of mammalian cells. In a striking example of convergent evolution, mammalian COX4 and yeast COX5 subunit expression are both regulated in an O_2-dependent manner. However, the molecular mechanisms underlying this common strategy for regulating COX activity differ (Figure 7). In *Saccharomyces cerevisiae*, *COX5a* transcription is activated by the Hap2/3/4/5 protein and *COX5b* transcription is repressed by the Rox1 protein under aerobic conditions, whereas under hypoxic conditions, *COX5a* transcription is no longer activated and *COX5b* is no longer repressed (Burke and Poyton, 1998). The involvement of O_2 in this pathway is indirect: Hap2/3/4/5 and Hap1 (the transactivator of *Rox1*) are activated by binding heme, which is only synthesized under aerobic conditions.

In hypoxic mammalian cells, HIF-1, which is negatively regulated by O_2-dependent hydroxylation, activates transcription of the *COX4I2* and *LON* genes, which leads to increased COX4-2 mRNA and protein synthesis and increased COX4-1 proteolysis. Both HIF-1α and HIF-2α contribute to the regulation of *LON* gene expression (Figure 5), whereas knockdown of HIF-1α but not HIF-2α blocks COX4-1 degradation (Figure 4G). Further studies are necessary to determine whether other mitochondrial proteases that are regulated exclusively by HIF-1α may participate in the degradation of COX4-1. While this work was in progress, reduced levels of COX4-1 in $CoCl_2$-treated cells were reported by investigators who concluded that inhibition of precursor processing led to degradation of the protein (Hervouet et al., 2006), but they did not identify the protease nor observe the induction of COX4-2 expression that is critical for the subunit switching phenomenon we have demonstrated.

Mammalian COX4 and yeast COX5 have remarkably similar structures and functions. The crystal structure determined for bovine COX4-1 (Tsukihara et al., 1996) and the predicted three-dimensional structure of yeast COX5a are superimposable, thus allowing a similar predicted spatial relationship between these proteins and the catalytic COX1 and COX2 subunits (Burke and Poyton, 1998). Both mammalian COX4 and yeast COX5 are required for proper assembly of COX1, COX2, and COX3 (McEwen et al., 1986; Nijtmans et al., 1998). Most importantly, complexes containing the hypoxic COX5b subunit have higher TN_{max} values at low O_2 concentrations than do complexes containing the aerobic COX5a subunit (Waterland et al., 1991; Allen et al., 1995).

Our data indicate that the switch from COX4-1 to COX4-2 in mammalian cells represents a similar adaptive response that optimizes COX activity under hypoxic conditions. COX4-2 and LON mRNA expression are induced by hypoxia in the liver and lungs of mice exposed to 10% O_2, indicating that the pathway we have delineated represents a physiological response to hypoxia. In considering the factors that determine how respiratory chain function is optimized at any given O_2 concentration, we hypothesize that a balance is maintained between transfer of electrons to O_2 at complex IV to form water as opposed to premature electron transfer at complex I or complex III to form superoxide radical, which is converted to H_2O_2 by superoxide dismutase. Our demonstration that the effect of manipulating COX4-1 and COX4-2 expression on H_2O_2 levels is O_2 dependent provides strong support for this model.

Recent studies have demonstrated that respiration is also O_2-regulated at an earlier step, the conversion of pyruvate to acetyl CoA by pyruvate dehydrogenase, which is inactivated when phosphorylated by pyruvate dehydrogenase kinase 1 (PDK1), the product of a HIF-1 target gene (Kim et al., 2006; Papandreou et al., 2006). Hypoxic HIF-1α null mouse embryo fibroblasts undergo apoptosis caused by increased ROS production and are rescued by forced expression of PDK1 (Kim et al., 2006). Whereas PDK1 reduces tricarboxylic acid cycle activity and flux through the respiratory chain, COX4 subunit switching provides a mechanism to maximize the efficiency of respiration under conditions of reduced O_2 availability. The extent to which PDK1 expression or COX4 subunit switching is utilized as a strategy for maintaining oxygen homeostasis may be tissue specific, as evidenced by the induction of COX4-2 and LON mRNA expression in several, but not all, tissues of chronically hypoxic mice (Figures 1E and

5B). These results are consistent with previous studies demonstrating that the battery of HIF-1 target genes expressed in response to hypoxia differ from one cell type to another (Kelly et al., 2003). Further studies are required to investigate the adaptive significance of tissue-specific metabolic responses to hypoxia. HIF-1 also plays a key role in regulating glycolysis, and analysis of cells cultured in the presence of galactose and glutamine rather than glucose (in order to eliminate glycolytic metabolism) may provide additional mechanistic insight.

While these strategies may be focused on preventing the generation of toxic levels of ROS, physiological levels of ROS generated by complex I (Bonnet et al., 2006) or complex III (Brunelle et al., 2005; Guzy et al., 2005; Mansfield et al., 2005) may play an important signaling role in the inhibition of hydroxylase activity under hypoxic conditions that leads to HIF-1 activation. The complexity of mitochondrial function reflects the critical role of this organelle in generating ATP and ROS and, when intracellular adaptation fails to balance these factors, in triggering programmed cell death. The remarkable similarities in the regulation of COX activity in yeast and human cells indicate that the selection for O_2-dependent homeostatic regulation of mitochondrial respiration is ancient and likely to be shared by all eukaryotic organisms.

EXPERIMENTAL PROCEDURES

Tissue Culture

Cells were cultured in DMEM (293, 293T, HeLa, Hep3B, A495, and RCC4) or McCoy's 5A medium (HCT116) with 10% FBS (Gibco). Mouse embryo fibroblasts were cultured in DMEM with 15% FBS. Mouse pulmonary artery smooth muscle cells were cultured as described (Wang et al., 2006). Cells were maintained in 5% CO_2/95% air at 37°C. For hypoxic exposure, cells were placed in a modular incubator chamber (Billups-Rothenberg) that was flushed with 1% O_2/5% CO_2/balance N_2 and incubated at 37°C. Other reagents were from Sigma ($CoCl_2$ and DFX), Frontier Scientific (DMOG), Calbiochem (MG132), and Aldrich (DHB).

RT-PCR and qrtPCR

Total RNA was extracted from cells and tissue using Trizol (Invitrogen) and treated with DNaseI (Ambion). One microgram of total RNA was used for first-strand DNA synthesis using iScript cDNA Synthesis system (BioRad), and PCR was performed using primers shown in Table S1. qrtPCR was performed as described (Kelly et al., 2003). The hypoxia-induced change in target mRNA expression was calculated based on the threshold cycle (Ct) as $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{target} - Ct_{18S}$ and $\Delta(\Delta Ct) = \Delta Ct_{1\%} - \Delta Ct_{20\%}$.

Luciferase Reporter Assays

COX4I1 and *COX4I2* sequences were amplified from human genomic DNA by PCR using primers with MluI and BglII restriction sites (Table S1), digested, and ligated into pGL2-Basic (Promega), which encodes firefly luciferase. *LON* sequences were amplified, digested, and ligated into pGL2-Promoter. Mutations were introduced into both HREs in *COX4I2* by using mutated PCR primers (Table S1). 4×10^4 cells were transfected with firefly luciferase reporter plasmid (200 ng) and control reporter plasmid pSV-Renilla (40 ng), which encodes *Renilla* luciferase, using Fugene-6 (Roche). In GOF/LOF studies, cells were transfected with pSV-Renilla, luciferase reporter (100 ng), and 100 ng of pSUPER vector encoding shRNA, pcDNA3.1 vector encoding HIF-1α or HIF-2α, or pCEP4 expression vector encoding HIF-1αDN plasmid. The ratio of firefly to *Renilla* luciferase activity was determined using the Dual-Luciferase Assay System (Promega).

Immunoblot Assays

Whole-cell lysates were prepared in RIPA buffer and fractionated by SDS-PAGE. Primary antibodies against the following proteins were used: HIF-1α, HIF-2α, and HIF-1β (Novus Biologicals); β-actin (Santa Cruz); FLAG (Sigma); and COX4 (Abcam). A polyclonal antibody against COX4-2 was raised in a rabbit using a peptide corresponding to human COX4-2 amino acids 133–145. HRP-conjugated anti-rabbit (Roche), -mouse, and -goat (Santa Cruz) secondary antibodies were used. Signal was detected using ECL Plus (GE Healthcore).

ShRNA and COX Expression Vectors

The shRNA targeting sequences are shown in Table S1. shRNA oligonucleotides were annealed and ligated into BglII/HindIII-digested pSUPER-retro-puro (OligoEngine). A 19 nt scrambled negative control vector was also prepared (Okuyama et al., 2006). COX4-1- and COX4-2-coding sequences were reverse transcribed from 293 and Hep3B mRNA, amplified from cDNA using *Pfu* polymerase (Stratagene), and ligated into pCR-BluntII-TOPO (Invitrogen). Coding sequences were amplified with primers containing HindIII (5′-end) and BglII (3′-end) restriction sites, digested, and ligated into p3xFLAG-CMV-7 (Sigma).

ChIP Assays

Cells were treated with vehicle or 100 μM DFX for 4 hr, 1% formaldehyde for 10 min, and 0.125 M glycine for 5 min and analyzed using the Chromatin Immunoprecipitation Assay (Upstate) with anti-HIF-1α and HIF-2α antibodies (Novus) and rabbit IgG (Santa Cruz). PCR was performed using primers (Table S1) to amplify DNA sequences from the *COX4I2* or *LON* gene.

Adenoviral and Retroviral Transduction

HeLa cells were infected with 50 plaque-forming units per cell of adenovirus AdCA5 or AdLacZ and analyzed 24 hr later (Kelly et al., 2003). RCC4 cells were stably transduced with retrovirus $rvShRNA_{HIF\text{-}1\alpha}$ (Krishnamachary et al., 2006).

Pulse-Chase Labeling of COX4-1

0.5×10^6 HeLa cells were plated in 6 cm dishes, exposed to 100 μM $CoCl_2$ for 16 hr, and labeled with [^{35}S]methionine (200 μCi/ml; Amersham Pharmacia Biotech) for 40 min, which was followed by incubation for 4–8 hr in standard culture medium. Whole-cell lysates were prepared using 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 1% deoxycholate, and 1 mM PMSF; immunoprecipitated with anti-COX4 antibody (Abcam); and subjected to SDS-PAGE followed by autoradiography.

COX, ATP, O_2-Consumption, H_2O_2, and Caspase Assays

Mitochondrial fractions isolated from HeLa and 293T cells were analyzed by Cytochrome *c* Oxidase Assay (Sigma). ATP levels were measured by Somatic Cell ATP Assay (Sigma). To measure O_2 consumption, cells were transfected and after 24 hr incubated in 20% or 1% O_2 for 24 hr, removed from the chamber, and resuspended in DMEM at 3×10^6 cells/ml; then 350 μl was analyzed using an Oxygraph (Hansatec Instruments). To measure H_2O_2, cells were transfected and incubated for 24 hr at 20% or 1% O_2. Lysates were prepared in M-PRE (Pierce), and 50 μl were analyzed by Amplex Red Hydrogen Peroxide Assay (Invitrogen). Caspase activities were measured using Caspase-Glo 3/7 Assay kit (Promega). Cells were plated in white-wall 96-well plate and transfected either with FLAG-tagged COX4 expression plasmids or COX4-shRNA plasmids. After 24 hr, cells were exposed to 1% O_2 for 24 hr. Lysis of hypoxic cells and H_2O_2 and caspase analysis were performed inside a hypoxic chamber to avoid reoxygenation.

Statistical Analysis

Data are presented as mean ± SEM of at least three independent experiments. Statistical significance ($p < 0.05$) was assessed by t test.

Supplemental Data

Supplemental Data include eighteen figures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/129/1/111/DC1/.

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