Oxygen Tension Regulates Mitochondrial DNA-encoded Complex I Gene Expression*

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Oxygen is a major regulator of nuclear gene expression. However, although mitochondria consume almost all of the O_2 available to the cells, little is known about how O_2 tension influences the expression of the mitochondrial genome. We show in O_2-sensitive excitatory rat PC12 cells that, among the mtDNA-encoded genes, hypoxia produced a specific down-regulation of the transcripts encoding mitochondrial complex I NADH dehydrogenase (ND) subunits, particularly ND4 and ND5 mRNAs and a stable mRNA precursor containing the ND5 and cytochrome b genes. This unprecedented effect of hypoxia was fast (developed in <30 min) and fairly reversible and occurred at moderate levels of hypoxia (O_2 tensions in the range of 20–70 mm Hg). Hypoxic down-regulation of the mitochondrial complex I genes was paralleled by the reduction of complex I activity and was retarded by iron chelation, suggesting that an iron-dependent post-transcriptional mechanism could regulate mitochondrial mRNA stability. It is known that cell respiration is under tight control by the amount of proteins in mitochondrial complexes of the electron transport chain. Therefore, regulation of the expression of the mitochondrial (mtDNA)-encoded complex I subunits could be part of an adaptive mechanism to adjust respiration rate to the availability of O_2 and to induce fast adaptive changes in hypoxic cells.

Oxygen (O_2) is absolutely required for cell survival due to its fundamental role in mitochondrial oxidative phosphorylation. Decreases in O_2 tension (PO_2) trigger acute and chronic adaptive cellular responses to increase O_2 delivery to the tissues and to minimize the deleterious effects of hypoxia. In mammals, acute hypoxia (occurring over a time scale of seconds to minutes) regulates the activity of ion channels existing in specific O_2-sensitive cells, which mediate fast respiratory and cardiovascular counterregulatory adjustments (for review, see Refs. 1 and 2 and references therein). Protracted (chronic) hypoxia (lasting hours or days) activates hypoxia-inducible transcription factors (hypoxia-inducible factor-1α (HIF-1α) and isofoms) that regulate the expression of a broad and growing set of nuclear genes involved in anaerobic glucose metabolism, angiogenesis, or oxygen transport as well as in development, tissue remodeling, and tumor transformation (3–5).

In recent years, it has been shown that HIF activity depends on prolyl and asparaginyl hydroxylases, which utilize ambient O_2 as substrate. Under normoxic conditions, hydroxylation of proline and asparagine accelerates HIF-1α degradation by the proteasome (6, 7) and prevents its transcriptional activity (8, 9). Inhibition of HIF-1α hydroxylation during hypoxia leads to its stabilization and nuclear translocation and to the subsequent activation of HIF-dependent genes (10–12). Stabilization of HIF-1α by hypoxia seems to require the release of reactive oxygen species (ROS) from mitochondria to the cytosol (13–16).

Despite the progress in the understanding of the signaling pathway responsible for the regulation of nuclear gene expression by O_2, little is known about how the mitochondrial genome responds to hypoxia and in which way the O_2-sensitive mitochondrial and extramitochondrial processes interact. This is rather surprising if one considers that mitochondria consume almost all of the O_2 available to the cells and that they have been classically postulated as O_2 sensors. Mitochondrial respiration is known to be depressed even during moderate hypoxia (17–19), but the underlying molecular mechanisms accounting for this regulation have not yet been studied in detail. Among the little information available, there is a study reporting that extreme hypoxia (<1 mm Hg) produces transcriptional down-regulation of some nuclear and mitochondrial genes encoding complex IV proteins (20).

This study was designed to perform a systematic analysis of the effect of hypoxia on the expression of the mitochondrial genome. Mammalian mitochondrial (mtDNA) is a circular molecule of ~16.6 kb, present in several copies per mitochondrion. The mtDNA contains genes for two rRNAs (12 S and 16 S), 22 tRNAs, and 13 mRNAs encoding proteins in mitochondrial complexes I and III–V (21). In most of the experiments, we used the oxygen-sensitive rat pheochromocytoma-derived PC12 cell line (22–25) as a model system. In addition, confirmatory experiments were done on non-excitable Buffalo rat liver (BRL) cells. Among all of the mtDNA-encoded genes, hypoxia induced a specific down-regulation of the mRNAs encoding complex I proteins. This regulation was fast and reversible and occurred at physiologic O_2 tensions. The hypoxia-induced decrease in these transcripts was paralleled by a reduction of the NADH dehydrogenase activity of complex I. In contrast, hypoxia did not alter the transcripts of the genes encoding the complex III–V subunits. Our data suggest that regulation of the mtDNA-encoded complex I subunits participates in mitochondrial O_2 sensing. This could be part of an adaptive mechanism evolved to tune respiration rate during hypoxia and to optimize mitochondrial oxygen consumption and energy production by the cells.

MATERIALS AND METHODS

Cell Cultures and Hypoxic Treatments—Rat pheochromocytoma PC12 and BRL cells were cultured in Dulbecco’s modified Eagle’s...
medium (Invitrogen) containing 4.5 g/liter D-glucose, l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. PC12 cultures were supplemented with 5% (v/v) fetal bovine serum and 10% (v/v) horse serum and maintained under "normoxic" conditions at 37 °C in a humidified atmosphere of 10% CO₂ and 21% O₂. BRL cultures were supplemented with 10% (v/v) fetal bovine serum and grown at 37 °C in a humidified atmosphere of 5% CO₂ and 21% O₂ for normoxic conditions. For hypoxic treatments, cells were grown at 1, 3, 6, or 10% O₂ (7, 21, 42, and 70 mm Hg, respectively) in a hypoxic incubator (ThermoForma). Chemical reagents were added to the culture medium at the following final concentrations: 100 µM Deferoxamine mesylate (DFX; Sigma), 100 µM cobalt chloride, 1 mM dimethylglyoxaline (DMOG), and 2 mM glutathione ethyl ester (reduced form; Sigma). To test the effect of iron chelation during hypoxia, DFX was added to the culture medium 18 h before incubation under hypoxic conditions. To test the effect of glutathione ethyl ester during hypoxia, the reducing agent was added 30 min before incubation under hypoxic conditions. To study the effect of hypoxia in the absence of extracellular calcium, cells were incubated in calcium-free Dulbecco's modified Eagle's medium (Invitrogen catalog no. 21068) supplemented with 4 mM l-glutamine, 1 mM EGTA, 1 mM sodium pyruvate, and 100 units/ml penicillin/100 µg/ml streptomycin without serum added. For these experiments, cells were first preincubated under normoxic conditions for 30 min and then transferred to hypoxic conditions. For all experiments, exponentially growing cells under normoxic conditions (60–80% confluence) cultured in 60-mm Petri dishes were transferred to an incubator set for hypoxic conditions. After different time intervals (typically 0.5, 2, 6, and 24 h), dishes were withdrawn for total RNA preparation. As a control (0 h of hypoxia), cultures kept in normoxia were used.

**RNA Analysis**—Total RNA was prepared from cells using TRIzol® reagent (Invitrogen) according to the recommended protocol. For Northern blotting, 10–15 µg of RNA/sample was loaded onto formaldehyde-agarose gel. Electrophoresis and transfer to Hybond-N nylon membranes were performed following standard procedures. RNA/DNA hybridization was performed at 42 °C in ULTRAhyb hybridization buffer (Ambion, Inc.). Gel-purified DNA probes were radiolabeled with [α-32P]dCTP using the Rediprime kit (Amersham Biosciences). Riboprobes were labeled with [α-32P]dUTP by in vivo transcription of the corresponding genes following standard procedures. After each hybridization experiment, probes were stripped off in boiling 0.5% SDS solution before hybridization with the cyclophilin probe (Ppia gene) for a loading control. Northern RNA band intensity was quantified with the Fujifilm FLA-3000 system.

**Probes**—Probes hybridizing with mitochondrial mRNAs (see Fig. 1A) were synthesized by PCR as follows: probe 1 (to hybridize with the ND1, ND2, COI, and COII genes; nucleotides 2773–7605 of the rat mitochondrial chromosome, GenBankTM accession number AJ428514), primers Rmit1 (5’TGGCATGGCCTTACTCA-3’) and Rmit2 (5’GCCGCAAATTCAGAGC-3’); probe 2 (to hybridize with the ATP6 and ATP8 genes; nucleotides 7755–8562), primers Rmit5 (5’-ATGCCA-CACTGACACATC-3’) and Rmit6 (5’-AGGGTGAAATACATAGGGCTTG-3’); probe 3 (to hybridize with the COII, ND4, ND4/4L, ND5, and ND6 genes; nucleotides 8600–14160), primers Rmit3 (5’-CCCCACCAAAAACCATGCA-3’) and Rmit4 (5’-GGGGGTGAGATTTCCGG-3’); probe 4 (to hybridize with the cytochrome b (Cyt-b) gene; nucleotides 14145–15227), primers Rmit7 (5’-CGAAATATCTACCCCTAT-T-3’) and Rmit8 (5’-GTGTTCCTACGGTTGGCCT-3’); probe COIII (nucleotides 8600–9219), primers Rmit3 and Rmit5 (5’-TACGTGGAGCCCATGA-3’); probe ND4 (to hybridize with the ND4/4L gene; nucleotides 10335–10725), primers Rmit16 (5’-CATTATCACCAGGGGTGAGATTTCCGG-3’) and Rmit17 (5’-CATGTTGGAGGTAGGGG-3’); probe ND5-5p (to hybridize with the 5’-region of ND5; nucleotides 11784–12263), primers Rmit11 (5’-CCAATCTCATTTCCCAT-3’) and Rmit12 (5’-GACTCGGTGTGAGAGGATT-3’); probe ND6-5p (to hybridize with the 3’-region of ND5; nucleotides 12883–13560), primers Rmit13 (5’-TCTTCACAGGATTCTACC-3’) and Rmit14 (5’-TTGGAATCATGCGGATTA-3’); and probe ND6 (nucleotides 13540–14029), primers Rmit9 (5’-TAAATCCCGAGGTATTCTT-3’) and Rmit10 (5’-ACTGTGTTGTTGGTTG-3’). For riboprobes rND5 and rND6, the PCR-generated ND5-5p and ND6 probes were cloned in the pGEM-T Easy® vector (Promega Corp.). The rat cyclophilin cDNA probe (Ppia) was obtained as a 743-bp EcoRI-HindIII fragment from plasmid pBl15 as described (26).

**Mitochondrial isolation**—PC12 cells were grown on four to six 15-mm Petri dishes per sample and incubated either under normoxic or hypoxic conditions as described above. Mitochondrial isolation was carried out at 4 °C with samples kept on ice. About 10⁶ cells were harvested in ice-cold phosphate-buffered saline, spun for 5 min at 1000 rpm, washed once with ice-cold buffer A (250 mM sucrose, 2 mM Heps, and 0.1 mM EGTA), and resuspended in 1.5 ml of buffer A. Cells were broken by 10–15 strokes in a Dounce homogenizer with a motor-driven pestle. The homogenate was centrifuged at 4000 rpm for 6 min in microcentrifuge, and the supernatant containing mitochondria was collected. Mitochondria were spun down by centrifugation for 10 min at 13,000 rpm, washed twice with buffer A, and resuspended in a final volume of ~250 µl of buffer A. The mitochondrial suspension was aliquoted (40 µl), flash-frozen in liquid nitrogen, and kept at ~80 °C until used. The protein concentration was determined by the Bio-Rad colorimetric assay, diluting samples in 0.05% SDS.

**Measurement of Activities of Mitochondrial Complexes**—Mitochondrial complex activities were determined in a Beckman DU-640 spectrophotometer according to Birch-Machin and Turnbull (27) with slight modifications. All reagents were purchased from Sigma unless indicated otherwise. For mitochondrial complex I and II activities, 30–50 µg of protein were assayed at 30 °C. Samples were diluted 1:4 in assay reaction buffer (25 mM KH₂PO₄ (pH 7.2), 5 mM MgCl₂, 3 mM potassium cyanide, and 2.5 mg/ml bovine serum albumin) and liquid nitrogen frozen/thawed three times before the assay. For mitochondrial complex I activity, rotenone-sensitive ND activity was measured as the decrease in absorbance at 340 nm (referred to 425 nm) due to oxidation of 130 µM NADH (Roche Applied Science) in the presence of 3.6 µM antimycin and 130 µM ubiquinone-1. Absorbance was measured for 2 min before and after addition of 5 µM rotenone to the reaction. Differences between rates were considered for determination of activity due to mitochondrial complex I. To evaluate mitochondrial complex II activity, succinate dehydrogenase activity was measured for a period of 2 min as the decrease in absorbance at 600 nm due to the reduction of 50 µM 2,6-dichlorophenolindophenol coupled with reduction of 130 µM ubiquinone-1. The reaction was carried out in the presence of 3.6 µM antimycin, 5 µM rotenone, and 10 mM succinate. The activities of complex I and II were normalized to the mitochondrial matrix citrate synthase activity of the same samples. For citrate synthase activity, the formation of 5,5′-dithiobis(2-nitrobenzoic acid) anion coupled with oxidation of oxalacetate was measured as the increase in absorbance at 412 nm after addition of acetyl-CoA for 1 min.

**Statistical Analysis**—Data are expressed as means ± S.E., and statistical significance was estimated with a parametric Student’s t test. Statistical significance is given in the figure legends.
RESULTS

Down-regulation of Complex I Mitochondrial mRNA by Hypoxia—A transcriptional analysis of the mitochondrial genome was performed using several probes that hybridize with all of the genes. The rat mitochondrial genome and the probes used in our study are schematically depicted in Fig. 1A. Transcription of mtDNA is initiated mainly at the D-loop region by the mitochondrial transcription factor A (mETFA) and occurs bidirectionally from two divergent promoters (P_H and P_L) that regulate the heavy (H-strand) and light (L-strand) strands, respectively. The H-strand encodes the two ribosomal RNAs, most of the mRNAs and 15 tRNAs; the L-strand encodes the ND6 gene and the remaining seven tRNAs. Thus, the H- and L-strands are synthesized as polycistronic RNA molecules that must be subsequently processed (21). The tRNAs scattered through the circular mtDNA act as a punctuation system for cleavage of precursors. Stabilization of mature RNA species and some precursors is achieved mainly through polyadenylation of their 3' ends (28). Rat PC12 cells were incubated either under normoxic (21% O_2; P_O_2 = 147 mm Hg) or hypoxic (3% O_2; P_O_2 = 21 mm Hg) conditions for different time periods, after which total RNA was prepared and subjected to Northern blot analysis. Among the six complex I subunits encoded by the H-strand, we analyzed the mRNAs of the ND1, ND2, ND5, ND4, and ND4/4L genes, with the last two appearing on the same transcript (29). The transcript encoding the ND3 subunit was not studied because its steady-state expression was below the level of reproducible detection of the Northern blot technique. We also analyzed the mRNAs of the Cyt-b (complex III); COI, COII, and COIII (complex IV); and ATP6 and ATP8 (complex V) genes. Hypoxia induced a decrease in mRNAs corresponding to ND1 and ND2 (which were detected at the same location in the blot), ND4/4L, and ND5 (Fig. 1B). The ND6 mRNA transcript was not detected with these probes, as the steady-state level of the L-strand is much lower than that of the H-strand (30). The ND6 mRNA transcript was not regulated by hypoxia (Fig. 1B). For quantification purposes, the mRNA levels of the ND subunits were normalized to the mRNA levels of either COIII (detected with probe 3) (Fig. 1C, upper panel) or COII (detected with probe 1) (lower panel). This eliminates the variability in different cultures due to unnoticed changes in the amount of mtDNA relative to

FIGURE 1. Effect of hypoxia on mtDNA expression in PC12 cells. A, scheme of the mitochondrial genome. Genes are indicated separated by short segments representing tRNAs. P_H and P_L are the divergent promoters from which transcription (indicated by arrows) starts. The heavy (H) transcript encodes all of the proteins except ND6, which is encoded by the light (L) transcript. The probes (probes 1–4) used for Northern analysis as well as the complementary regions of the chromosome are indicated. B, Northern analysis of PC12 cell cultures incubated under hypoxic conditions (3% O_2; P_O_2 = 21 mm Hg) for 2, 6, and 24 h using probes 1–4. A DNA probe hybridizing with the nuclear Ppia gene, which encodes the cyclophilin A gene, was used as a loading control. C6/8, ATP6/ATP8. C, quantification of the RNA levels of genes encoding mitochondrial complex I (MCI) proteins. Values were normalized to COIII for ND4/4L, ND5, and the ND5 + Cyt-b precursor and to COII for ND1 + ND2. These two transcripts were quantified together because of their same length. Values are relative to the levels under normoxic conditions (0 h). The data points and error bars represent means ± S.E. of at least six experiments. Statistical significance (p < 0.01) with respect to the values under normoxic conditions is indicated by asterisks. D, quantification of the RNA levels of genes encoding proteins of mitochondrial complexes III–V (MCIII, MCIV, and MCV, respectively). Values were normalized to the Ppia transcript. Statistical significance was as described for C. COX, cytochrome c oxidase.
nuclear DNA. The decrease in the mRNA of the complex I ND subunits occurred within the first 2 h of hypoxic exposure, reaching a maximum plateau level after 6–24 h of culture (Fig. 1C). Fig. 1D illustrates quantitatively that the mRNA levels of Cyt-b (complex III); COI, COII, and COIII (complex IV); and ATP6 and ATP8 (complex V) normalized to those of the nuclear encoded cyclophilin transcript (Ppia gene) were not significantly altered by hypoxia. In addition to ND4 and ND5 mRNAs, we also detected with probe 3 a 3.6-kb precursor molecule containing the ND5 and Cyt-b genes (30–32). This precursor (ND5 + Cyt-b), processed more slowly probably due to the lack of tRNA between the various genes, becomes polyadenylated and remains as a stable RNA species (30, 32). Interestingly, the ND5 + Cyt-b mRNA was the one most affected by hypoxia (Fig. 1, B and C). The ratio of ND5 + Cyt-b mRNA to the COIII transcript reached a minimum of ~25% of the normoxic value after 24 h of hypoxia (3% O2; PO2 = 21 mm Hg). It is noteworthy that, despite the effect on the precursor, hypoxia did not alter mature Cyt-b, which strengthens the view that PO2 regulates specifically the H-strand-encoded ND transcripts. Other precursor mRNA species of larger molecular weight appearing at more proximal parts of the blots were not significantly modified by hypoxia. In addition to PC12 cells, which belong to the class of acutely responding neurosecretory cells that release transmitters upon exposure to hypoxia (1), we also examined the mitochondrial response to hypoxia in non-excitable BRL cells. Qualitatively, the effect of hypoxia (3% O2) on mtDNA expression in BRL cells was identical to that reported in PC12 cells. After 24 h of hypoxia, the ND4, ND5, and ND5 + Cyt-b transcripts were down-regulated to 54, 90, and 32%, respectively, of their values under normoxic conditions. This suggests that down-regulation of the steady-state levels of mRNAs encoding the ND proteins is a general phenomenon that probably occurs in all eukaryotic cells.

Identification of the mRNAs from the ND5/ND6/Cyt-b Region—Because the transcripts more sensitive to hypoxia (ND5 + Cyt-b, ND4, and ND5) were those detected with probe 3, we confirmed their identity by performing Northern hybridizations using individual DNA probes for each of the genes as well as riboprobes for the two overlapping ND5 and ND6 genes (Fig. 2A). As shown in Fig. 1B, after hybridization with DNA probe 3, the ND5 + Cyt-b precursor appeared in the upper part of the blot. This precursor, although clearly visible, was less abundant than the mature transcripts detected with the same probe (with the exception of ND3) (Fig. 2B, lane 1) (30, 32). The precursor was also detected in hybridizations with DNA probes for ND5, both for the 5’-region (ND5-5p DNA probe) (Fig. 2B, lane 5) and the 3’-region (ND5-3p DNA probe) (lane 6), as well as with DNA probes for ND6 (lanes 7 and Cyt-b (DNA probe 4) (lane 4). As expected, the ND5-5p, ND5-3p, and ND6 DNA probes also detected the mature ND5 mRNA (Fig. 2A). The fact that a DNA probe for ND6 detected mature ND5 (Fig. 2B, lane 7) is not surprising because it is known that the ND5 transcript has a trailer of ~600 bp corresponding to antisense ND6. Hybridization with a riboprobe for ND5 (rND5) showed both the ND5 + Cyt-b precursor and the mature ND5 transcript (Fig. 2B, lane 8), whereas a riboprobe for ND6 (rND6) detected only the mature ND6 transcript (lane 9). The mature ND6 transcript was also detected with the ND5-3p and ND6 DNA probes (Fig. 2B, lanes 6 and 7, respectively), but not with the ND5-5p DNA probe (lane 5). This was expected because the mature ND6 transcript has a region of ~600 bp of antisense ND5 as a trailer that does not cover the region recognized by the ND5-5p DNA probe (Fig. 2A) (30). In Fig. 2B (lanes 6 and 7), the ND6 transcript appeared with low intensity because the processed transcripts from the L-strand are intrinsically less stable than those from the H-strand (30, 33). Finally, the transcript corresponding to the ND5 + Cyt-b precursor was not present in hybridizations with DNA probes specific for ND4/4L (Fig. 2B, lane 3) and COIII (lane 2). Taken together, these experiments demonstrate that, in our preparations, the 3.6-kb precursor contains only the ND5 (with its antisense ND6 trailer of ~600 bp) - and Cyt-b-encoding regions; the other more intense bands detected with DNA probe 3 correspond to the ND5, ND4, and COIII genes. Therefore, all subsequent studies on the hypoxic regulation of complex I mRNAs were done using probe 3.

Hypoxic Regulation of mtDNA Expression Is Rapid and Reversible and Occurs at Physiologic O2 Levels—We studied in detail the time course and reversibility as well as the dose dependence of the hypoxic down-
regulation of ND5 + Cyt-b, ND5, and ND4/4L mRNAs. The levels of these mRNA species markedly decreased during the first 6 h of exposure to hypoxia (3% \(O_2; \text{PO}_2 = 21 \text{ mm Hg}\)) (Fig. 3A). A significant decrease in ND5 + Cyt-b mRNA (to \(\sim 75\%\) of the control value) was already observed after 30 min of incubation. At the end of the 6-h incubation under hypoxic conditions, the levels of ND5 + Cyt-b, ND5, and ND4/4L mRNAs were 35, 85, and 65% of their respective control values (Fig. 3C). Upon re-exposure of the cells to normoxia (21% \(O_2; \text{PO}_2 = 147 \text{ mm Hg}\)), recovery of the mRNAs was almost complete in only 30 min. Full recovery (100%) was observed within the first 2 h of normoxia (Fig. 3, B and C). Therefore, the effect of hypoxia on the ND mRNA levels is fast and fully reversible, suggesting a rapid turnover of the transcripts.

Regulation of ND mRNAs by hypoxia is a graded phenomenon. ND5 + Cyt-b precursor mRNA levels relative to COIII mRNA levels decreased gradually with the intensity of hypoxia. With 6 h of incubation, the decrease in ND5 + Cyt-b mRNA levels was appreciable with exposures to \(O_2\) tensions \(\leq 70 \text{ mm Hg}\) (Fig. 4A). Qualitatively, a similar dose dependence was observed for the hypoxic down-regulation of ND5 and ND4/4L mRNAs (Fig. 4, B and C). These data demonstrate that regulation of mitochondrial complex I gene expression by \(O_2\) is concentration-dependent and occurs at relatively moderate \(O_2\) values.

**Hypoxia Decreases Mitochondrial Complex I Activity**—The seven complex I proteins encoded by mtDNA are conserved in all organisms from prokaryotes to the most complex eukaryotes. They are part of the “core” of the complex and are therefore necessary for its function (34). In fact, frameshift or nonsense mutations of mtDNA-encoded complex I subunits result in marked decreases in malate/glutamate-dependent respiration and NADH:quinone oxidoreductase activity (35). To test whether low \(O_2\) has a functional impact on mitochondrial complex I, we measured rotenone-sensitive ND activity in isolated mitochondria from PC12 cell cultures incubated for 24 h under hypoxic conditions. Fig. 5 shows that, upon incubation under hypoxic conditions (3% \(O_2; \text{PO}_2 = 21 \text{ mm Hg}\)), complex I activity decreased on average to \(\sim 75\%\) of the activity measured under normoxic conditions. For comparison, we measured in the same mitochondrial preparation the activity of complex II, a multisubunit structure composed of only nuclear DNA-encoded proteins. Complex II function (measured as succinate dehydrogenase activity) was unaltered or even slightly increased after incubation of the cells under hypoxic conditions (Fig. 5). Therefore, although ND mRNA levels are probably higher than those required for normal respiration rate under normoxic conditions (35), destabilization of these transcripts under hypoxic conditions could contribute to produce inhibition of ND activity. Obviously, we did not rule out additional regulatory steps participating in the decay of complex I activity (see “Discussion”).

**Hypoxic Regulation of the Mitochondrial Complex I Transcripts Is Delayed by Iron Chelation**—The activities of \(O_2\)-dependent prolyl and asparaginyl hydroxylases that regulate HIF also depend on the availability of Fe\(^{2+}\) and oxoglutarate. Both DFX, an iron chelator, and DMOG, a membrane-permeant analog of oxoglutarate that competitively inhibits prolyl hydroxylases, block HIF hydroxylation and retard its degradation (10). Cobalt is another HIF stabilizer that prevents the binding of the von Hippel-Lindau protein required for HIF ubiquitination and proteasome degradation (36). Therefore, DFX, DMOG and cobalt have the same effect as hypoxia on the expression of HIF-dependent nuclear genes (4, 5, 25, 37, 38). To test whether HIF stabilization is involved in...
the hypoxic regulation of mtDNA expression, we assayed the effect of these agents on the levels of mtRNA. Fig. 6A illustrates that, under normoxic conditions, the expression of transcripts detected with DNA probe 3 was not affected by 6 h of incubation with DFX, cobalt, or DMOG. The absence of effect was maintained even after 24 h of incubation with these agents (data not shown), thus suggesting that HIF does not play a role in the hypoxic regulation of mtDNA gene expression. As a control, up-regulation of the classical HIF-1α-dependent phosphoglycerate kinase-1 (PGK1) gene in PC12 cells (39) was observed for all the three agents (Fig. 6A).

Iron is involved in the regulation of gene expression, particularly in cases in which it occurs at the post-transcriptional level. A paradigmatic example is the regulation of mRNA stabilization and translational activity of nuclear genes involved in iron metabolism or in the Krebs cycle and heme synthesis as well (40, 41). These genes contain iron regulatory elements in their untranslated sequences that allow the corresponding mRNA to be regulated by binding of iron regulatory proteins (IRPs), a phenomenon favored by the absence of iron (40, 42). Hypoxia decreases the binding of IRP1 to RNA, and this effect is prevented by iron chelation with DFX (43, 44). Therefore, we investigated whether the stability of mtRNA under hypoxic conditions is also regulated by DFX. Fig. 6 (B and C) shows that the hypoxia-induced decrease in the ND mRNAs (particularly ND5 + Cyt-b mRNA) was markedly retarded in PC12 cells incubated in medium containing 100 µM DFX. In control cultures without DFX, 2 h of hypoxia induced the precursor RNA level to decrease to ~45% of the value measured under normoxic conditions (Fig. 1C). However, this inhibitory effect of hypoxia fully disappeared in the presence of DFX (Fig. 6C). At 6 h of incubation under hypoxic conditions, the decrease had already reached a plateau of ~40% of the value under normoxic conditions, but in the presence of DFX, this value was still ~80% of the control value. Only in cells incubated for 24 h under hypoxic conditions did the decrease persist even in the presence of DFX.

As in previous experiments, the changes in ND4/4L and ND5 mRNA levels followed that in the ND5 + Cyt-b precursor mRNA levels (Fig. 6B). To test a possible role of iron in mitochondrial mRNA synthesis, we incubated normoxic cells in the presence of either 100 µM DFX (Fig. 6A) or 50 µg/ml ferric ammonium citrate (data not shown). Neither of these treatments altered the amount of the mRNAs, indicating that the effect of iron chelation is specific on the mechanism of mRNA regulation by hypoxia.

Because DFX can partially chelate other external cations (such as Ca<sup>2+</sup>) known to regulate gene expression in PC12 cells (45), we assayed the effect of external Ca<sup>2+</sup> removal on hypoxia-induced down-regulation of mitochondrial gene expression. Fig. 7 (A and C) shows that the effect of hypoxia on the complex I subunit mRNAs and on the precursor was preserved in cells incubated in Ca<sup>2+</sup>-free medium and with 1 mM EGTA added. We also tested the effect of ROS production on the hypoxic regulation of mtRNA because DFX is known to be a potent inhibitor of iron-dependent ROS formation (46). Fig. 7 (B and C) shows that the decrease in the RNA levels induced by hypoxia was unaltered in cells incubated with 2 mM glutathione ethyl ester, a membrane-permeant glutathione donor used to inhibit oxidative stress (39). Similar effects were observed with dithiothreitol (5 mM), another reducing agent. Altogether, these results strongly suggest that hypoxic down-regulation of the ND subunits is independent of extracellular Ca<sup>2+</sup> or ROS production and that an iron-dependent protein could be involved in the regulation of mitochondrial mRNA species stability.

**DISCUSSION**

In this work, we have show that, among the various mtDNA-encoded genes, hypoxia produces a selective down-regulation of the expression of complex I subunits encoded by the H-strand, particularly of ND4 and a 3.6-kb RNA precursor containing the Cyt-b and ND5 genes. The effect of lowering PO<sub>2</sub> on ND gene expression was relatively fast and reversible and occurred at moderate levels of hypoxia. This previously unknown...
Down-regulation of Complex I mtDNA Expression by Hypoxia

The effect of hypoxia on mitochondrial gene expression was paralleled by the decline of complex I activity and appears to depend on post-transcriptional mechanisms that regulate mRNA stability. There is evidence that regulation of mtDNA expression may be a mechanism for fine-tuning the amount of respiratory chain components in response to chronic energy demands (47). Moreover, it has been proposed that the level of mitochondrial mRNA changes with a rapid turnover in response to modifications in intramitochondrial ATP concentration (47). Down-regulation of the mitochondrial expression of complex I genes by hypoxia described in this study represents an example of how environmental factors can influence the mitochondrial genome. The information available on the regulation by O2 tension of the mitochondrial genome or even the nuclear encoded mitochondrial proteins is very scant. A coordinated decrease in all of the mitochondrial encoded mRNAs has been observed after 48 h of hypoxia (Po2 < 40 mm Hg) (48); however, we interpret this response as nonspecific and obviously different from the fast and selective effect described here. Recently, it has been reported in several mammalian cell lines (including PC12) that extreme hypoxia (Po2 < 1 mm Hg) decreases the cytochrome c oxidase mRNA content and decelerates the rate of mitochondrial transcription and catalytic activity of the enzyme (20). This reduction of cytochrome c oxidase mRNA levels is observed in both the mtDNA-encoded (COI and COII) and nuclear genome-encoded (COIV and COVb) subunits (20). The regulation of complex I mRNA by

FIGURE 6. Effect of hypoxia-mimicking agents on mitochondrial transcription. A, effect of 100 μM DFX, 100 μM CoCl₂, and 1 mM DMOG on the mRNA levels of transcripts analyzed with DNA probe 3. The mRNA levels of the nuclear encoded phosphoglycerate kinase-1 gene (PGK1) are also shown as a control. Before mRNA analysis, PC12 cells were incubated for 6 h (Po2 = 147 mm Hg) in the presence of each of the indicated compounds. B, delay of hypoxia-induced down-regulation of the ND5 + Cyt-b precursor and ND mRNAs by DFX. PC12 cell cultures were incubated under hypoxic conditions (Po2 = 21 mm Hg) for 2, 6, and 24 h in the presence of DFX (100 μM). C, time course of ND5 + Cyt-b RNA inhibition by hypoxia (Po2 = 21 mm Hg) in the absence (■) or presence (○) of DFX. Data points and error bars indicate means ± S.E. of four experiments. *, statistical significance (p < 0.001) with respect to the corresponding values under normoxic conditions; #, significantly different (p < 0.001) with respect to the corresponding values obtained in the absence of DFX.

FIGURE 7. Hypoxic inhibition of mtRNA expression is independent of extracellular calcium or the production of reactive oxygen species. A, down-regulation of mtRNA assayed with DNA probe 3 in PC12 cells incubated under hypoxic conditions (Po2 = 21 mm Hg) for 2, 6, and 24 h in medium lacking calcium and containing 1 mM EGTA; B, down-regulation of mtRNA assayed with DNA probe 3 in PC12 cells incubated under hypoxic conditions (Po2 = 21 mm Hg) for 2, 6, and 24 h in medium containing 2 mM glutathione ethyl ester (G-SH); C, quantification of the ND5 + Cyt-b RNA level relative to the COIII mRNA level. The data values and error bars represent means ± S.E. of four experiments.
hypoxia reported here affects mainly genes (or precursors) encoding ND subunits that are strictly necessary for complex I activity (35, 49, 50). The observation that hypoxia decreases the 3.6-kb ND5 + Cyt-b precursor without an effect on the mature Cyt-b mRNA reinforces the specificity of the regulation by oxygen of complex I genes. Interestingly, alterations in the levels of the same precursor along with minor changes in the level of mature Cyt-b mRNA have been reported in a rat model of kidney ischemia-reperfusion (32).

Although changes in mtDNA replication and transcription occur in numerous circumstances (51–53), the fact that most of the mitochondrial genes are transcribed into a single mRNA molecule suggests that the effect of hypoxia on the H-strand-encoded ND genes is post-transcriptional. Additionally, we have observed that hypoxia does not alter mtDNA replication (data not shown). Oxygen regulation of mtDNA expression was also independent of HIF-1α, the major PO2-dependent transcriptional regulator of nuclear encoded genes (4, 5), or of extracellular Ca2+ influx and ROS production, which have also been associated with regulation of the expression of some O2-dependent nuclear genes in PC12 cells (45, 54). It has been demonstrated in plants and Protozoa, and recently in Metazoa, that polyadenylation by mitochondrial poly(A) transferase plays a role in the stabilization of processed mitochondrial mRNAs (28, 55, 56). Whether this is the case for the observed hypoxia-dependent post-transcriptional regulation of mitochondrial mRNAs remains to be investigated. It is noteworthy that the 3.6-kb ND5 + Cyt-b precursor was the mitochondrial mRNA most affected by hypoxia. Stable polyadenylated mitochondrial mRNA molecules that are precursors of the mature species have been found before (57, 58). Our results thus strengthen the concept that stepwise processing of the polycistrionic transcripts is an additional regulatory element in the expression of the mitochondrial genome.

Regarding the possible mechanism of O2-dependent mitochondrial mRNA stabilization, it is particularly interesting that incubation of PC12 cells with the iron chelator DFX prevented or at least retarded the complex I mRNA down-regulation by hypoxia. This was quite specific, as EGTA, which chelates other divalent cations, did not have a similar effect. Iron is involved in the post-transcriptional regulation of genes containing iron regulatory elements in their 3′- or 5′-untranslated sequences that allow the corresponding mRNA stability or translational activity to be regulated by binding of IRPs (42, 59). There are reports indicating that hypoxia decreases the binding of IRP1 to RNA and that this effect is prevented by iron chelation with DFX (43, 44, 60). Although we did not find typical iron regulatory element consensus sequences in the rat mitochondrial genome, these observations raise the possibility that a specific IRP/iron regulatory element-like system imported by the mitochondria regulates the organelle mRNA stability/amount in response to hypoxia. The observation that, under normoxic conditions, neither the presence of DFX nor saturating amounts of free iron alter mitochondrial mRNA levels suggests that a specific mechanism for hypoxic regulation that depends on iron exists. This is an attractive hypothesis that should be explored in future experimental work.

The most obvious functional role of mitochondrial complex I gene regulation by PO2 is the adjustment of respiration rate to the availability of O2. Although cell O2 uptake and oxidative phosphorylation during brief exposures to hypoxia remain independent of PO2 as long as ambient O2 tension exceeds ~7 mm Hg (61, 62), when this situation is prolonged, cells undergo a significant suppression of respiration (63). This mitochondrial adaptation to hypoxia has been suggested to result from changes in the kinetic properties of cytochrome c oxidase (13, 18). Another proposed mechanism involves NO because it is produced in hypoxic cells due to induction of NO synthase and can bind to the heme group of cytochrome c oxidase to reduce the affinity of the enzyme for O2 (see Ref. 64 and references therein). As discussed above, the PO2 values required to evoke changes in the expression of complex IV subunits (20) are clearly lower than those utilized in our work. In addition, hypoxic induction of ND synthase requires HIF-dependent activation of nuclear genes (1, 4, 5). In contrast, mitochondrial down-regulation of ND mRNAs is observed at moderate levels of hypoxia and, in <30 min, can either develop or fully reverse after hypoxia or re-oxygenation, respectively. Thus, a plausible hypothesis is that, in the short term, down-regulation of these genes and concomitant inhibition of complex I activity contribute to mitochondrial depression during hypoxia. However, the direct mechanistic link between the mRNA down-regulation reported here and the decay in mitochondrial complex I function remains unproven due to technical limitations. To date, the antibodies available and those generated in our laboratory against the ND proteins are quite nonspecific. In addition, a possible effect of hypoxia on mitochondrial protein synthesis and stability was investigated. Preliminary studies of methionine-labeled incorporation into mtDNA-encoded proteins were inconclusive and did not reveal major effects of hypoxia on protein turnover (data not shown).

Down-regulation of ND mRNAs by hypoxia could have pathophysiological implications, as mitochondrial dysfunction and particularly defects in mitochondrial complex I are involved in several human diseases (65). A variety of mutations affecting ND subunits result in complex I deficiency in individuals affected by Leber hereditary optic neuropathy and distonia (66), and ND5 is a hot spot for atypical mtDNA deletions observed in mitochondrial neurogastrointestinal encephalomyopathy (67). On another hand, defects in complex I activity are well established in Parkinson disease (68, 69). Given the high energy requirement of neurons and the exquisite relationship between PO2, mitochondrial complex I protein expression, and cell respiration, it could be that repeated episodes of hypoxia (causing transient ND down-regulation) contribute to the development of neurodegenerative diseases.

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