The Role of Mitochondria in the Regulation of Hypoxia-inducible Factor 1 Expression During Hypoxia

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Running title: Mitochondria and regulation of HIF-1

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SUMMARY

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that regulates transcriptional activation of several genes responsive to the lack of oxygen, including erythropoietin, vascular endothelial growth factor, glycolytic enzymes and glucose transporters. Because the involvement of mitochondria in the regulation of HIF-1 has been postulated, we tested the effects of mitochondrial electron transport chain deficiency on HIF-1 protein expression and DNA-binding in hypoxic cells. The neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydroperidine (MPTP) inhibits electron transport chain at the level of complex I. MPTP is first converted to a pharmacologically active metabolite 1-methyl-4-phenylpiridinium (MPP+). MPP+ effectively inhibited both complex I activity and hypoxic accumulation of HIF-1α protein in dopaminergic cell lines PC12 and CATH.a. In C57BL/6 mice, a single dose of MPTP (15 mg/kg, i. p.) inhibited complex I activity and HIF-1α protein accumulation in the striatum in response to a subsequent hypoxic challenge (8% O2, 4 h). In a genetic model system, 40% complex I-inhibited human-ape xenomitochondrial cybrids, hypoxic induction of HIF-1α was severely reduced and HIF-1 DNA binding diminished. However, succinate, the mitochondrial complex II substrate, restored the hypoxic response in cybrid cells, suggesting that electron transport chain activity is required for activation of HIF-1. A partial complex I deficiency and a mild reduction in intact cell oxygen consumption effectively prevented hypoxic induction of HIF-1α protein.
INTRODUCTION

Mammalian cells are able to sense decreased oxygen availability and activate adaptational responses including transcriptional activation of several hypoxia-inducible genes: erythropoietin, vascular endothelial growth factor, glycolytic enzymes and glucose transporters. This allows increased O₂ delivery through enhanced erythropoiesis and angiogenesis, and metabolic adaptations that facilitate glycolytic ATP production (reviewed in ref. 1). Transcriptional activation of the erythropoietin gene is controlled via an enhancer element located in the 3'-flanking region of the gene, and requires binding of a specific transcription factor termed hypoxia-inducible factor 1 (HIF-1)* (2). Identification and cloning of HIF-1 revealed a heterodimeric protein consisting of two subunits, HIF-1α and HIF-1β (3, 4). Both subunits belong to a family of basic helix-loop-helix transcription factors containing a PAS (PER-ARNT-SIM) domain (3, 4). HIF-1β, previously identified as aryl hydrocarbon receptor nuclear translocator (ARNT), is a common binding partner for other members of the family (5), while HIF-1α is the subunit regulated by cellular O₂ tension (3, 6). Both subunits are required for DNA binding and transactivation of HIF-1 target genes (3). The consensus DNA binding sequence for HIF-1 is 5'-RCGTG-3' (7).

Under normoxic conditions HIF-1α protein undergoes rapid degradation by a proteasome.

*Abbreviations: HIF-1, hypoxia-inducible factor 1; PAS, Per-Arnt-Sim homology; ARNT, aryl hydrocarbon nuclear receptor translocator; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP+, 1-methyl-4-phenylpiridinium; VHL, von Hippel-Lindau; ROS, reactive oxygen species.
Hypoxia increases HIF-1α protein stability by inhibiting its degradation (8, 9). It has been reported that von Hippel-Lindau (VHL) protein binds to HIF-1α and targets it for proteasome degradation (10). In hypoxia, degradation is blocked perhaps because a targeting modification of HIF-1α requires oxygen (10). It was suggested that hypoxia-mimicking agents like cobalt chloride and desferrioxamine induce HIF-1α by disrupting its association with VHL and impairing HIF-1 degradation by the proteosome (10).

The mechanisms of sensing low oxygen and transducing this signal to activate HIF-1 are not well understood. A role for a putative oxygen sensor molecule has been suggested (11). Another model proposes that the process of sensing decreased oxygen concentration involves NADPH oxidase activity (12). A decrease in available oxygen would result in decreased reactive oxygen species (ROS) formation by NADPH oxidase, and this in turn, would activate pathway/s leading to HIF-1 induction (12). Recently, a different model based on the role of the mitochondrial electron transport chain in hypoxia sensing was suggested (13). Inhibition of complex I and complex III of mitochondrial respiratory chain blocked HIF-1 DNA-binding activity and expression of HIF-1 target genes in Hep3B cells under hypoxic conditions (13, 14). In addition to hypoxia, several growth factors can activate HIF-1 and its target genes via different signaling pathways (15, 16).

The potential involvement of the mitochondrial electron transport chain in the physiologic regulation of the hypoxic response could be important because respiratory chain abnormalities underlie a number of human clinical disorders (reviewed in ref. 17). Specific respiratory chain defects are associated with encephalopathies, myopathies, and cardiomyopathies. Among the neurodegenerative diseases, the presence of the defect in the
Mitochondria and regulation of HIF-1 α complex I activity is clearly established in Parkinson’s disease (17). Parkinson’s disease can be induced in primates and mice by MPTP, which is first converted by monoamine oxidase B in astrocytes to an active toxin 1-methyl-4-phenylpyridinium (MPP+), followed by selective accumulation in the nigrostriatum (18). MPP+ is taken up by dopaminergic neurons via an active transport by dopamine transporters (reviewed in ref. 19). Accumulation of MPP+ in the cell is thought to inhibit oxidative phosphorylation via inhibition of complex I which results in energy failure and death of nigrostriatal neurons (18, 20, 21).

This study was undertaken to determine the consequences of electron transport chain deficiency on hypoxic induction of HIF-1α protein in cultured cells and in an animal model. We used the mouse model of Parkinson’s disease based on administration of MPTP to responsive C57BL/6 mice. To further ascertain the consequences of partial complex I inhibition on HIF-1α expression during hypoxia, we made use of human xenomitochondrial cybrids harboring a partial 40% complex I deficiency (22, 23). The cybrid lines were derived from fusion of the human cells depleted of mitochondria (ρ0) with mitochondrial DNA from primates (22, 23). The decreased complex I activity in these cell lines is related to a number of differences in mitochondrial DNA-encoded subunits between humans and apes (22, 23).
EXPERIMENTAL PROCEDURES

**Cell culture and reagents.** A human osteosarcoma cell line 143B (ATCC CRL 8303) was maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. PC12 cells (ATCC CRL 1721) were grown in RPMI 1640 medium, supplemented with 10% FBS and 5% horse serum. Mouse neuroblastoma CATH.a cells (ATCC CRL 11179) were grown in RPMI 1640 medium, supplemented with 8% horse serum and 4% FBS. All media and antibiotics were purchased from Life Technologies Inc. All reagents were purchased from Sigma (St. Louis, MO). Cells were pretreated with or without rotenone and MPP+ for 1 hour and subsequently exposed to hypoxia (1% O₂, 5% CO₂, 94% N₂) for 4 hours in plexiglass modular chambers (Billups, Rothenburg).

Human xenomitothochondrial cybrid cell lines (a generous gift from Carlos T. Moraes, University of Miami, Miami, Florida) were derived following fusion of human ρ₀ cells (143B cells selected with ethidium bromide to destroy mitochondrial DNA) with primate mitochondria as previously described (22, 23). The cybrid cell lines used in this study were: HCA1-2 (human-chimpanzee), Hugr (human-gorilla), HCB1-2 (human-chimpanzee) and Hupig (human-pigmy chimpanzee). All four cybrid cell lines show an approximate 40% decrease in complex I activity (22, 23). Cells were maintained in DMEM medium supplemented with 10% FBS and pyruvate (100 µg/ml). Hypoxic induction of HIF-1α was further tested in one of cybrid cell lines subjected to hypoxia (1% O₂, 5% CO₂ and 94% N₂, 4 h) using the complex II substrate-succinate. In this experiment HCB1-2 cells were permeabilized with digitonin (40 μg/ml) and medium was supplemented with succinate (4 mM).

**Animals.** C57BL/6 mice were obtained from Harlan Laboratories. Mice were treated with MPTP (15mg/kg weight, intraperitoneally) or vehicle (phosphate buffered saline, PBS). 24 hours
after MPTP or PBS administration, mice were subjected to hypoxia in a plexiglass chamber (8% O₂, balance N₂, 4 h) or kept in room air. A group of mice was kept in room air 4 days after MPTP administration and then subjected to hypoxia (8%O₂, balance N₂; 4 h) to test the delayed effects of MPTP on HIF-1α hypoxic induction.

**Western blot analysis.** Mice were anesthetized, their brains were removed and immediately frozen at -80°C. The striatum and brain cortex were dissected and homogenized in ice-cold buffer (20 mM HEPES, pH 7.5; 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1 M NaCl) freshly supplemented with 0.2 mM dithiothreitol, 0.5 mM sodium vanadate and protease inhibitors (0.4 mM phenylmethyl-sulfonyl fluoride and 2 µg/ml each of leupeptin, pepstatin and aprotinin). Subsequently NaCl was added to a final concentration of 0.45 M and the homogenate was centrifuged at 10,000 g for 30 min and the supernatant collected. Samples (total of 200 µg proteins) were separated in SDS-7% polyacrylamide gel and transferred to nitrocellulose membranes using standard procedures. To test for HIF-1α protein expression in cultured cells, nuclear extracts were prepared and 20 µg of protein was subjected to electrophoresis. Membranes were blocked with 5% non-fat dry milk powder, followed by incubation with a monoclonal anti-HIF-1α antibody (Novus Biologicals), and secondary antibody detection by enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ). Protein concentrations were determined by the Bradford protein assay (Bio-Rad Inc., CA). Densitometric analysis was performed in autoradiograms of samples from striatum and cortex. Ratios of optical densities (O. D.) from MPTP or hypoxia-treated vs. PBS-treated (control) mice were calculated and analysed statistically as described below.

**Nuclear extracts and DNA electrophoretic mobility shift assay.** Cells were harvested in cold PBS, washed in 5 volumes of packed cell volume of buffer containing: 10 mMTris-HCl
(pH 7.5), 1.5 mM MgCl₂, 10 mM KCl; incubated on ice 10 min and homogenized in a glass Dounce homogenizer. Nuclei were pelleted and resuspended in buffer (0.42 M KCl, 20mM Tris-HCl, 20% v/v glycerol, 1.5 mM MgCl₂), rotated 30 min at 4°C followed by centrifugation. Supernatant nuclear proteins were used for an electrophoretic mobility shift assay (2), using oligonucleotide probe from the erythropoietin enhancer region containing HIF-1 binding site (5'-GCCCTACGTGCTGTCTCA-3').

**Measurement of complex I activity.** Mitochondria from cultured cells were prepared by discontinuous Percoll gradient centrifugation (24). In the case of brain samples, the crude mitochondrial pellet was used for enzymatic activity. Brains were rapidly transferred to ice-cold isolation buffer containing 0.32 M sucrose, 1 mM EDTA (K+ salt) and 10 mM Tris-HCl (pH 7.4). The striatum was dissected and homogenized by hand in a glass Dounce homogenizer. The homogenate was centrifuged at 1,330 g for 30 min and supernatant centrifuged at 21,200 g for 10 min. The resulting crude mitochondrial pellet was resuspended in a minimum volume of buffer and stored at -80°C until used. In all the cases, mitochondrial samples were freeze-thawed several times before enzymatic activity analysis. Complex I activity was measured as described previously (22), by measuring the oxidation of NADH at 340 nm using decylubiquinone as the electron acceptor. A total of 20-40 µg of mitochondrial protein were used and assays were performed at 37°C in a Beckman DU 640 spectrophotometer.

**Statistical Analysis**

Data were reported as mean ± standard deviation. Comparisons between multiple groups were performed by one-way ANOVA test with Tukey correction (program version SPSS PC V8.0). For comparison between two groups the unpaired Student’s t-test was used. In all cases, p< 0.05 was considered significant.
RESULTS

The effect of MPP+ on HIF-1 DNA-binding, hypoxic induction of HIF-1α and complex I activity. The CATH.a cell line was established from a brain tumor of a transgenic mouse. The PC12 cell line used in this study derives from rat adrenomedullary tumor and has many properties of catecholamine neurons including neurotransmitter synthesis, storage and selective uptake (25). PC12 cells have saturable membrane binding sites for MPP+, and have the ability to accumulate MPP+ (25). MPP+ (50 and 100 µM) inhibited HIF-1α DNA binding in nuclear extracts from hypoxic CATH.a and PC12 cells (Fig. 1A). A similar effect was observed using another complex I inhibitor-rotenone (1µM). To determine whether MPP+ and rotenone also affect HIF-1α protein induction, an immunoblot assay was performed. Both agents effectively prevented HIF-1α protein induction in hypoxic CATH.a (Fig. 1B) and PC12 cells (Fig. 1C). Complex I activity decreased by 56% in PC12 cells after 5 h of treatment with 100 µM MPP+ (Fig. 1D), and correlated with the lack of HIF-1α hypoxic induction.

HIF-1 DNA binding and HIF-1α protein are almost completely absent in hypoxic cybrid cell lines. We next investigated the effect of partial complex I inhibition on HIF-1α protein induction and HIF-1 DNA binding. For this purpose we used four xenomitochondrial cybrid cell lines. These cell lines harbor approximately 40% complex I deficiency (22, 23). Previous characterization of cybrid cell lines indicated an isolated defect in complex I of the respiratory chain (22). HIF-1α protein accumulation in hypoxic cybrid cell lines was drastically reduced (Fig. 2). Accordingly, HIF-1 DNA-binding activity was diminished in all four cybrid lines after exposure to hypoxia for 4 h (Fig. 2). Therefore, a partial complex I deficiency dramatically affected HIF-1 activation in hypoxic cells.
HIF-1 DNA binding and HIF-1α protein accumulation is restored in cybrid cell lines using complex II substrate-succinate. The lack of HIF-1 activity in cybrid cell lines could potentially result from an unsuspected defect (other than the defect in complex I) in the signal transduction pathway leading to HIF-1 activation. Thus, we investigated whether the cybrid cell lines have retained the ability to activate HIF-1 in response to hypoxia by using complex II as an indirect route of electron flow to complex III. In this experiment succinate was used as a complex II substrate. Succinate restores oxygen consumption in cybrid cell lines (22). Parental cell line 143B and cybrid line-HCB1-2 were exposed to normoxia or hypoxia, in the presence or absence of digitonin (40µg/ml) or succinate (4 mM) and assayed for HIF-1 DNA binding and HIF-1α protein levels (Fig. 3A and B). HIF-1 DNA binding was present in nuclear extracts from 143 B cells exposed to hypoxia regardless of other treatments (Fig. 3A, lanes 2, 3, 4, 5). In contrast, no HIF-1 DNA binding could be detected in HCB1-2 hypoxic cells, unless both digitonin (permeabilizing agent) and succinate (the complex II substrate) were present in the medium (Fig. 3A, lane 10). In agreement, HIF-1α protein was detectable in nuclear extracts of hypoxic 143B cells regardless of treatment (Fig. 3B, lanes 2 and 3), while hypoxic HCB1-2 cells in the presence of digitonin and succinate regained the ability to induce HIF-1α (Fig. 3B, lane 6). These results suggest that cybrid cell lines are able to induce HIF-1α protein during hypoxia, however electron transport chain activity is necessary for HIF-1α induction.

Inhibition of complex I activity blocks HIF-1α hypoxic induction in vivo. Inhibition or damage to complex I or other complexes of electron transport chain is encountered in human mitochondrial disorders including some neurodegenerative diseases, and this might inhibit induction of HIF-1 and its target genes. Therefore, we extended our study in an animal model of complex I deficiency caused by MPTP administration. MPTP replicates biochemical and
pathological features of Parkinson’s disease. Typically, administration of 4 doses of MPTP (20-30 mg/kg each) is reported to cause nigrostriatal neuronal loss in C57Bl/6 mice (26). To avoid the effect of significant cell death, the administered dose was reduced in our study and mice received only a single dose of 15 mg/kg MPTP. Maximum reduction of complex I activity in the mouse striatum occurs approximately 16-24 h after MPTP administration (26), thus we chose to challenge the mice with hypoxia (8% O₂, 4 h) 24 h after MPTP. MPTP treatment prevented hypoxic HIF-1α protein induction in the striatum (Fig. 4A and 4C). Correspondingly, complex I activity in the striatum decreased by 38% compared to mice that received PBS only (Fig. 4D). MPP+ accumulation in the neurons requires a specific dopamine transporter and it appears that neurons that express higher levels of dopamine transporters are more susceptible to the neurotoxic effects of MPP+ (thus, the striatum is expected to be more susceptible to MPP+ than, for example, the brain cortex) (19). Indeed, our results show that HIF-1α hypoxic induction (Fig. 4A and C) and complex I activity (Fig. 4D) in the brain cortex were not affected significantly by MPTP treatment.

Since MPP+ accumulation is associated with neuronal cell toxicity (27), potentially irreversible cell damage could have rendered cells unresponsive to hypoxia. To test this possibility, in a group of mice the hypoxic challenge was delayed for 4 days after MPTP administration. The striatum recovered normal response to hypoxia at this point, and HIF-1α hypoxic induction could be demonstrated (Fig. 4B and Fig. 4C). In agreement, the complex I activity in the striatum decreased by 38 % after 24 hours, but returned to baseline level after 4 days, paralleling the hypoxic induction of HIF-1 in the tissue (Fig. 4D). Therefore, the inhibition of HIF-1 hypoxic induction observed 24 hours after MPTP cannot be explained by the possible cell damage/death due to MPP+ toxicity.
DISCUSSION

The molecular events preceding HIF-1 protein stabilization are of central importance for understanding the mechanisms of cellular oxygen sensing. It was reported that inhibition of complex I and III, but not of complex IV of mitochondrial respiratory chain blocked expression of HIF-1 target genes in Hep3B cells during hypoxia (13, 14). Thus, we investigated in several different ways whether complex I inhibition affects HIF-1 protein stabilization in hypoxic cells and in an animal model. We used human xenomitochondrial cybrids as a cellular model of partial complex I inhibition. Mammalian complex I has at least 43 subunits, seven of which are encoded by mitochondrial DNA. In addition to complex I, respiratory complex III, complex IV and complex V also contain mitochondrial DNA-coded subunits, however their activities in cybrid cell lines are not affected (22). In the four cybrid cell lines complex I activity is decreased by approximately 40% and the intact cell oxygen consumption is decreased by only 20% compared to the human parental cell line (22). Therefore a partial deficit in complex I activity and a mild reduction of oxygen consumption blocked almost completely HIF-1α hypoxic induction. Importantly, in the cybrid cell line the hypoxic induction of HIF-1α could be restored by complex II substrate succinate which maintains electron flow from complex II to complex III, confirming that electron transport chain activity is necessary for HIF-1α hypoxic induction. Consistent with this, mitochondrial complex I inhibitor MPP+ blocked the HIF-1α induction in hypoxic PC12 and CATH.a cells (Fig. 1). Rotenone (22) and MPP+ (21) have other complex I-independent toxic effects. These confounding factors were avoided by including cybrid cell lines in the present study. These cybrid lines have an additional advantage over ρ0 cells because they harbor an isolated complex I defect, which allowed us to test the importance of maintaining electron flow from complex II to complex III, while bypassing complex I. These results further establish
the crucial role of mitochondria in the complex process of oxygen sensing in mammalian cells. Our data are in agreement with previous reports that electron flux into complex III is necessary for HIF-1 activity and up-regulation of HIF-1 target genes in hypoxic cells (29, 13). The mechanisms that underlie involvement of the electron transport chain, and complex III in particular, in oxygen sensing remain to be investigated. Reactive oxygen species generated at complex III are proposed as a possible candidate that mediates HIF-1 activation in hypoxia (29, 13).

In a mouse model of Parkinson’s disease, administration of MPTP replicates biochemical (complex I inhibition) and clinical features of the disease. MPP+ is considered to inhibit complex I activity through direct binding to the site in the complex I that is either identical to or adjacent to the rotenone-binding site (28). A single, low dose of MPTP was sufficient to cause a partial inhibition of complex I activity in the striatum and complete inhibition of HIF-1α induction after hypoxic challenge (Fig. 4). Many human mitochondrial diseases result from complex I deficiency, including Leber’s hereditary optic neuropathy, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), several neuromuscular disorders, severe infantile lactic acidosis, Parkinson’s disease, etc. (30). Because of the crucial role of oxidative energy metabolism, respiratory chain deficiency can have severe consequences for cell homeostasis. Organs with high oxidative energy requirements, such as brain or muscle, are particularly vulnerable and rely on glycolysis as a means of energy production. Interestingly, some of these diseases, for example MELAS, are associated with episodes of stroke, which could be due to small vessel angiopathy (30). These small vessel abnormalities could potentially lead to local hypoperfusion and brain hypoxia. Similarly, some neurodegenerative disorders characterized by a respiratory chain deficiency are also associated with reduced cerebral blood
flow (31). Because HIF-1 typically stimulates glucose transport into cells and increases glycolytic capacity through up-regulation of genes encoding glucose transporters and glycolytic enzymes during hypoxia, HIF-1 may be an important factor in promoting neuronal metabolic adaptations under these conditions. However, cells with electron transport chain deficiency may not be able to respond by activating HIF-1, which could result in a general perturbation of neuronal metabolic adaptations. Thus, HIF-1 might have neuroprotective roles in these disorders. Another possible way HIF-1 could be protective in neurodegenerative diseases, some of which are associated with electron transport chain deficiency, was recently suggested (32). In this study, structurally different pharmacologic agents (cobalt chloride and iron chelator-desferrioxamine) that induce HIF-1 and mRNAs regulated by HIF-1, prevented cell death in neuronal cultures subjected to oxidative stress (32). This was explained by their ability to stimulate aerobic glycolysis, which results in decreased ROS formation due to two cooperative effects, increase in antioxidant pyruvate and prevention of ROS production by reducing the rate of glucose oxidation (32).

In summary, results of this study establish the importance of a functional electron transport chain in regulating HIF-1α protein stabilization during hypoxia. Importantly, a partial complex I deficiency and a mild reduction of intact cell oxygen consumption effectively blocked HIF-1 activity in xenomitochondrial cybrid cells. In agreement, a partial decrease in complex I activity caused by MPP+ blocked HIF-1α hypoxic induction in cultured cells and in an animal model. Partial complex I deficit of a similar degree is encountered in various human mitochondriopathies suggesting that HIF-1-mediated hypoxic responses might be impaired in these disorders.
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FIGURE LEGENDS

Figure 1. The effects of MPP+ on HIF-1 DNA binding, HIF-1α protein level and complex I activity. Cells were left untreated or were exposed to hypoxia (H), or treated with 1 μM rotenone (R), 50 μM MPP+ (M*) or 100 μM MPP+ (M). (A) HIF-1 DNA-binding diminished in nuclear extracts from hypoxic CATH.a and PC12 cells treated with rotenone or MPP+ (C, constitutive factor). (B) and (C), rotenone and MPP+ prevented hypoxic induction of HIF-1 protein in CATH.a and PC12 cells, respectively. (D) Complex I activity in PC12 cells decreased from 120 ± 7 to 53 ± 11 nmol/min/mg protein (56% decrease) after 5 h of treatment with 100 μM MPP+ (n=3 in each treatment group; *p<0.05, unpaired t-test).

Figure 2. The effect of hypoxia on HIF-1 DNA-binding and HIF-1α protein induction in cybrid cell lines. Nuclear extracts are from parental 143B cells (lanes 1 and 2), HCA1-2 (lanes 3 and 4), Hugr (lanes 5 and 6), HCB1-2 (lanes 7 and 8) and Hupig (lanes 9 and 10). HIF-1α protein induction is severely reduced in hypoxic cybrid cell lines (lanes 4, 6, 8 and 10) compared to 143B cells (lane 2). HIF-1 DNA binding is detectable only in parental 143B cells exposed to hypoxia (lane 2).

Figure 3. The effect of succinate on HIF-1 DNA binding and HIF-1α induction in hypoxic HCB1-2 cells. Parental cell line 143B (lanes 1-5) and HCB1-2 cell line (lanes 5-10) were maintained in medium supplemented with sodium pyruvate (100 μg/ml). Cells were exposed to hypoxia (H) in the presence or absence of 40μg/ml digitonin (D) or 4 mM succinate (S). (A) HIF-1 DNA binding is present in 143B hypoxic cells regardless of other treatments (lanes 2-5), and is restored in HCB1-2 hypoxic cells only when both digitonin and succinate are present in the medium (lane 10). (B) Nuclear extracts from 143B (lanes 1-3) and HCB1-2 cells (lanes 4-6).

During this experiment, all the cells were maintained in the medium supplemented with...
digitonin. HIF-1α protein induction is restored in hypoxic HCB1-2 cybrid cells in the presence of digitonin and succinate (lane 6).

**Figure 4. The effect of MPTP on hypoxic induction of HIF-1α and complex I activity in the mouse striatum and brain cortex.** (A) 24 hours after MPTP or PBS administration mice were exposed to hypoxia (8% O₂, 4 h) or kept in room air, and brain lysates prepared for immunoblot assay. In the striatum, HIF-1α protein levels were undetectable in mice kept in room air (lane 3) and were induced strongly after hypoxia (lane 4). Similarly, HIF-1α levels were undetectable in the brain cortex of mice in room air (lane 7) and were induced after hypoxia (lane 8). MPTP had no effect on baseline HIF-1α expression in the striatum and brain cortex (lanes 5 and 9, respectively), blocked hypoxic induction of HIF-1α in the striatum (lane 6) but not in the cortex (lane 10). Lanes 1 and 2, nuclear extracts from non-hypoxic and hypoxic Hep3B cells, respectively. (B) 1 day after PBS (lanes 1 and 2) or MPTP administration (lane 3) and 4 days after MPTP administration (lane 4), mice were exposed to hypoxia (8% O₂, 4 h) or kept in room air. Delaying hypoxic challenge for 4 d after MPTP administration restored HIF-1α induction in the striatum (lane 4). Lanes 5 and 6, nuclear extracts from non-hypoxic and hypoxic Hep3B cells, respectively. (C) Densitometric analysis of the ratios of HIF-1α signal relative to control (PBS-treated mice) in the striatum and brain cortex after exposure to hypoxia, delayed hypoxia (HD), or MPTP. The ratios of optical densities are presented as mean ± SD (number of treated mice shown in parenthesis). MPTP significantly inhibited hypoxic induction of HIF-1α in the striatum (* p< 0.05, one-way ANOVA), but had no significant effect in the cortex. HIF-1α hypoxic induction was restored in the striatum when hypoxic exposure was delayed for 4 days after MPTP administration. (D) Complex I activity in the isolated mitochondria from the striatum and brain cortex after MPTP treatment (n=3 for each treatment group; mean ± SD). Mice received a
single dose of PBS (control) or MPTP (15 mg/kg weight) and complex I activity was measured in the isolated mitochondria from striatum (1 and 4 days after MPTP) and brain cortex (1 day after MPTP). In the striatum, complex I activity (nmol/min/mg protein) decreased by 38% (103 $\pm$ 14 vs. 64 $\pm$ 6, *p*<0.05, one-way ANOVA test) 1 day after MPTP, but returned to control levels 4 days after MPTP (101 $\pm$ 12). No significant difference in complex I activity was observed in the mitochondrial preparations from brain cortex before and after MPTP.
Mitochondria and regulation of HIF-1

| Hypoxia | - | + | - | + | - | + | - | + | - | + |
|---------|---|---|---|---|---|---|---|---|---|---|
|         | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10|

HIF-1α

HIF-1 C
A

D - - + - + - - + - +
S - - - + + - - - + +
Hypoxia - + + + + - + + + +

B

S - - + - - +
Hypoxia - + + - + +

HIF-1α
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