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Quantitative proteomics reveals oxygen-dependent changes in neuronal mitochondria affecting function and sensitivity to rotenone

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Abstract

Mitochondria are implicated in a variety of degenerative disorders and aging. Mitochondria are responsive to the oxygen in their environment yet tissue culture is performed at atmospheric (21%) oxygen and not at physiological (1-11%) oxygen levels found in tissues. We employed imaging of mitochondrial probes, mass spectrometry, western blots and ATP assays of the human neuroblastoma cell-line SH-SY5Y; and imaging of mitochondrial probes in human primary neurons in standard non-physiological oxygen conditions (atmospheric) and in physiological oxygen levels in the nervous system to assess the impact of oxygen on mitochondrial function. SH-SY5Y cells cultured in physiological 5% oxygen exhibited the lowest reactive oxygen species (ROS) production, indicating that culture at 5% oxygen is favored; these results were mimicked in primary human cells. Mass spectrometric analysis revealed extensive mitochondrial proteomic alterations in SH-SY5Y cells based upon oxygen culture condition. Among these the rotenone-sensitive subunit of complex I NDUFV3 was increased in cells cultured at 5% oxygen. Rotenone is a Parkinson's disease-linked toxin, and correspondingly SH-SY5Y cells cultured at 5% oxygen also exhibited over 10-fold greater sensitivity to rotenone than those cultured in atmospheric, 21%, oxygen. Our results indicate that neuronal mitochondria are responsive to oxygen levels and produce differential responses under different oxygen levels.

Keywords

Mitochondria; Neurodegeneration; Oxidative stress; Bioenergetics/electron transfer complex; Hypoxia; Hyperoxia

INTRODUCTION

Oxygen is essential to the function of complex life forms. Oxygen is reduced to water during oxidative phosphorylation, greatly increasing the cellular energy production from a single molecule of glucose. Adequate oxygen is essential to mitochondrial and, ultimately, cellular health. Elevated oxygen levels result in increased amounts of reactive oxygen species

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Author Contributions

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Supporting Information

A complete list of identified proteins is listed in the supplementary. This material is available free of charge via the Internet at <http://pubs.acs.org>.

(ROS). Although ROS are a critical part of communicating the redox state of a cell, overproduction of these same ROS can lead to increased protein and lipid oxidation, DNA damage and ultimately cell death.

Alterations in proteins known to bind to the mitochondria and modify mitochondrial morphology and function occur in a variety of neurodegenerative conditions including aging, Parkinson's disease (PD), Huntington's disease (HD), and Alzheimer's disease (AD). For example, parkin (PARK2), PTEN-induced putative kinase 1 (PINK1) and other PD proteins are known to play roles in mitochondrial protection and energy production.^{1, 2} For HD, axonal transport of mitochondria is believed to be impaired even before symptoms of the disease emerge.³ In AD there seems to be an intricate interplay between ROS, AD proteins and mitochondria (for review see ⁴) with two AD-linked proteins, A β and tau, capable of interaction with the mitochondria. Mitochondrial DNA deletions have been noted to play a potential role in multiple sclerosis.⁵ Numerous other links between mitochondria and neurodegeneration exist, including dysregulation of the fission and fusion processes^{6, 7} and mitochondrial autophagy or mitophagy.⁸ These are only some of the examples of how mitochondrial health and function are tied to neurodegeneration.

While *in vitro* studies have contributed greatly to our knowledge of mitochondrial function and mechanisms of dysfunction, most tissue culture is currently conducted in incubators at atmospheric oxygen levels (21%), which is much greater than the oxygen levels experienced *in vivo* (1-11%) (for review see ⁹). Culturing immune cells at physiological oxygen as opposed to atmospheric oxygen impacts functional responses to stimuli.¹⁰ In our own work, the response of the mitochondria of rat striatal neurons to HIV viroproteins Tat and Nef was significantly altered by oxygen condition.¹¹ Additionally, experiments on neuronal cells and their precursors performed at these lower, physiological oxygen conditions (2-5%) have documented increased survival, proliferation and dopaminergic differentiation of cultured neurons.¹²⁻¹⁵

In the following study, we show that different tissue culture oxygen conditions alter the levels of proteins associated with oxidative phosphorylation and ROS scavenging. In turn, changes in protein levels are associated with functional differences including changes in mitochondrial ROS production levels, altered electron transport chain complex compositions, and modified sensitivity to a mitochondrial toxin implicated in PD. Functional tests were also performed in human fetal neurons and produced similar results as SH-SY5Y cells demonstrating that the results from SH-SY5Y cells translate to a primary culture system. Together these results indicate that altering culture oxygen may be a useful tool in the modeling of metabolic disorders and neurodegeneration with a variety of different mitochondrial conditions.

EXPERIMENTAL PROCEDURES

Tissue Culture

SH-SY5Y cells were counted and plated in 35-mm, poly-D-Lysine coated dishes (MatTek; Ashland, MA) with No. 1.5 coverslip bottoms at 5×10^5 cells per dish for imaging or in T-75 flasks for western blots. Cells were cultured in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum and 1,000 units/ml penicillin, and 1,000 μ g/ml streptomycin. Cells were cultured in a standard tissue culture incubator containing 5% carbon dioxide. Media was changed every 2-3 days. To culture cells in 5% or 2% oxygen, cells were cultured using identical pre-oxygen-equilibrated media and kept in a tri-gas incubator (Heracell 150i; Thermo Scientific, Waltham, MA) adjusted to the appropriate oxygen, 5% carbon dioxide, and the balance nitrogen. Low oxygen (5% and 2% O₂) cultures were established three separate times with atmospheric cultures run in parallel for the 8-12 passages corresponding

to several weeks to allow for adequate mitochondrial protein turnover. For cell culture maintenance, media was switched once every 3 days. Cells were rinsed with PBS and trypsinized at the corresponding oxygen condition. Media equilibrated to the corresponding oxygen condition was added to the proper cells.

Human fetal neurons were cultured from aborted fetuses obtained from University of Washington, handled in full compliance with the University of Nebraska Medical Center (UNMC) and NIH ethical guidelines under institutional review board (IRB) approval. The cerebral cortical tissue was digested with (0.25% trypsin) and dissociated into a single cell suspension, followed by selective neuronal culture in Neurobasal MEM supplemented with 2% B-27-AO and 0.5 mM L-glutamine. Dishes and plates were kept in chambers with 2% or 5% oxygen, and 5% carbon dioxide. Media was half-exchanged after 5 days and then every 3–4 days thereafter until the neurons had been in culture for 14 days. Other care is as noted for the SH-SY5Y cells above. Three biological replicates were obtained from separate donors ranging in age from 94 to 113 days.

For quantitative proteomics, SH-SY5Y cells were equilibrated to either 21% or 5% oxygen as described above. Cells from the 21% and 5% oxygen condition were cultured in DMEM-containing ^{13}C - and ^{15}N -labeled (“heavy media”; Invitrogen, Grand Island, NY) or unlabeled arginine and lysine (“light media”; Invitrogen, Grand Island, NY) respectively for seven generations. Cells from both oxygen conditions were differentiated into neurons using 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich, St. Louis, MO) and retinoic acid (Sigma-Aldrich, St. Louis, MO) as previously described.¹⁶

Immunoblotting

Cells were lifted with an enzyme-free PBS-based cell dissociation buffer (Versine, Gibco, CA). Mitochondria were then isolated as before. A 12% tris-glycine gel (Invitrogen, Carlsbad, CA) was loaded with 20 μg of protein per lane. The following antibodies were used for antigen detection by western blot: the oxidative phosphorylation (OXPHOS) panel (1:5,000) (MS604; Mitosciences, Eugene, OR), SOD2 (1:10,000) (ab16956; Abcam, Cambridge, MA), VDAC (1:10,000) (D73D12; Cell Signaling, Cambridge, MA), GAPDH (1:10,000) (437000; Invitrogen, Grand Island, NY), NDUFS3 (1:10,000) (MS110; Mitosciences, Eugene, OR), NDUFV3 (1:2,000) (13430-1-AP; Proteintech, Chicago, IL), UQCRCB (1:2000) (10756-1-AP; Proteintech, Chicago, IL), and ATP5H (1:10,000) (MS504; Mitosciences, Eugene, OR). The OXPHOS antibody panel is a mix of antibodies that include: NDUFB8, SDHB, UQCRC2, COXI, and ATP5A. Bands were visualized with an Image Station 4000MM Pro and densitometry was conducted with Carestream Molecular Imaging software (Carestream, Rochester, NY). For all blots, results were normalized to ATP5A, ATP5H or VDAC1.

Imaging

Cells were incubated in the appropriate dye and imaged on a Nikon swept-field confocal microscope using a 60x oil-immersion (1.45 NA) objective. A Live Cell imaging chamber (Pathology Devices, Inc.) was used to maintain appropriate oxygen and carbon dioxide conditions during experiments. To examine the rate of ROS production in the mitochondria, neurons were incubated in 100 μM dihydrorhodamine-123 (DHR123) (Invitrogen, Carlsbad, CA) for 20 minutes before imaging. The excitation wavelength used was 488 nm with a 536/40 nm fluorescence emission filter. Images were taken for 10 minutes at a rate of 1/min. After 10 minutes, a new imaging region was selected. In ROS images, measurements were conducted in au which is a measure of image intensity. For live/dead assays, dishes were stained with 5- μM calcein (Invitrogen, Carlsbad, CA) and 5- $\mu\text{g}/\text{ml}$ propidium iodide (PI) (Invitrogen, Carlsbad, CA) media for 30 minutes. Cells were imaged on a Zeiss Cell

Observer microscope with the appropriate green and red filters to determine which dye(s) were present in each individual cell for counting purposes.

Image Analysis

To calculate the rate of ROS production in neurons cultured under various conditions, DHR123 image stacks were analyzed in Image J. ROIs were selected and propagated through the stack using the multimeasure tool.¹¹ Average fluorescence values were normalized to the first image ROI. All normalized values were averaged at each time point. These average values were plotted and then analyzed with a linear fit to determine the slope. The slope was proportional to the rate of ROS production while the uncertainty in the slope represented the standard error for the measurement.

For live/dead assays using calcein/PI staining, images were taken, and the number of green and red cells were counted and totaled over all images taken for each experimental condition. The percent of dead cells was determined using the formula: $(N_{red}/N_{green}) * 100$.

Mass Spectrometry Preparation

Cells were washed two times with PBS and harvested from dishes. Cells from the 5% and 21% condition were mixed in equal proportions. Mitochondria were isolated by sequential differential centrifugation (Mitosciences, Eugene, OR) and an immunomagnetic based affinity isolation (Miltenyi Biotech, Auburn, CA). Protein amount was quantitated using a Pierce 660 assay (Thermo Fisher, Rockford, IL). Fifty- μ g of protein was trypsinized (Promega, Madison, WI) using the filter aided proteome preparation technique with a 20- μ m filter¹⁷ (Pall Corporation, Ann Arbor, MI). The resultant peptides were cleaned with an Oasis mixed-mode weak cation exchange cartridge (Waters, Milford, MA). Isoelectric focusing was performed using an Agilent 3100 Offgel Fractionator pH 3-10 low resolution kit (Agilent Technologies, Santa Clara CA). Fractions were prepared for mass spectrometry with Pierce C-18 PepClean Spin Columns (Thermo Fisher, Rockford, IL) in accord with the manufacturers' instructions. Samples were dehydrated with a Savant ISS 110 SpeedVac Concentrator (Thermo Fisher, Rockford, IL) and resuspended in 6 μ L of 0.1% formic acid for LC-MS/MS analysis. The experiment was performed using 3 technical replicates.

Mass Spectrometry

Mass spectrometry was conducted using a LTQ Orbitrap XL nano-LC system featuring two alternating peptide traps and a PicoFrit C18 column emitter (New Objective, Woburn, MA). Samples were resuspended in 1% formic acid in water mixed with 1% formic acid in acetonitrile in a 98:2 ratio. Peptides were injected with an autosampler and eluted with a linear gradient of acetonitrile from 0–60% over the course of 60 mins. The machine was calibrated before samples were analyzed using the manufacturers' standards. Peptides were identified in a data-dependent acquisition mode. One precursor scan in the Orbitrap identified the 5 most abundant peptide peaks for fragmentation and detection in the LTQ. System variables were set to values as previously described.¹⁸ Briefly, precursor peaks were scanned from 300 to 2000 m/z with a resolution of 60,000 and dynamically excluded after two selections for 60 s. Previously detected background peaks were included in a mass rejection list. Collision energy was set to 35 using an isolation width of 2 and an activation Q of 0.250

Data obtained from the LTQ-Orbitrap was analyzed with MaxQuant (version 1.2.2.2) to generate a peak list. Using the Andromeda algorithm, the peak lists were compared against the Uniprot human database. Ratios of the amount of heavy-to-light peptide were generated. The search parameters were set as a maximum of two missed cleavages, carbamidomethyl (C) as fixed modification, N-acetyl (protein) and oxidation (M) as variable modifications,

top 6 MS/MS peaks per 100 Da; and MS/MS mass tolerance of 0.5 Da. Exclusion criteria to remove proteins from analysis were as follows: FDR of 0.1 both peptides and proteins, peptides must contain at least 6 amino acids, contaminants as identified through the database search and proteins identified as being in the reverse database. All data analysis was performed on data normalized through MaxQuant.

The heat maps of protein expression levels indicate the change in mass spectrometry intensity-measurements based upon oxygen condition. All values represent the changes in protein expression levels in cells cultured at 5% oxygen relative to 21% (atmospheric) oxygen. The protein expression changes were imported into Multiple Experiment Viewer (Dana-Faber Cancer Institute, Boston, MA). The heat maps were generated by this program and the color scale was set to encompass all values.

ATP Assay

An ATP determination kit (A22066; Invitrogen, Carlsbad, CA) was used to assess ATP levels in untreated cells or cells treated with 0.2 μ M rotenone for 20 minutes. Cells were scraped and then lysed using a flash freeze fracture method. Thawed cell lysate was immediately used to perform the luciferase assay according to the manufacturer's recommendations. Luminescence was read on a Victor3 1420 Multilabel Plate Counter (Perkin Elmer, Waltham, MA). ATP levels were normalized by cell count.

Statistical Analysis

Statistical analysis of imaging and western blot data were performed using one-or two-way ANOVA with Bonferroni post-hoc tests to compare three samples, or t-test in the cases where only two conditions were studied. All statistical analysis and linear fits were completed using Prism software (GraphPad Software Inc., La Jolla, CA).

RESULTS

Reactive Oxygen Species

Since overproduction of ROS is linked to harmful effects on neurons and other cells, we first assessed the effects of alternative oxygen levels on mitochondrial ROS production using the fluorescent probe DHR123. DHR123 localizes to the mitochondria and fluoresces when oxidized. Evaluation of the ROS levels in SH-SY5Y cells grown at 2%, 5% and atmospheric (21%) oxygen levels revealed inherent ROS productions differences (Figure 1A). ROS production was depressed in both the 2% and 5% oxygen condition ($p < 0.0001$). The lowest levels occurred in the 5% oxygen (0.23 ± 0.02 adu/min) with the 2% (0.39 ± 0.08 adu/min) being median and the highest rate belonging to the cells cultured in atmospheric conditions (0.62 ± 0.2 adu/min). Interestingly similar differences in ROS levels were found in human neurons, with the lowest level of ROS production found in those cultured at 5% oxygen (Figure 1B) suggesting that these differences are translatable to a primary culture system.

While production of ROS occurs in all cells, mechanisms exist for protecting the cells from elevated levels of ROS. In the mitochondria, the keystone protein in ROS scavenging is superoxide dismutase 2 (SOD2). Examination of SOD2 protein levels by western blotting (Figure 1C and D) demonstrate that SOD2 protein levels are increased significantly ($p < 0.01$) for SH-SY5Y cells cultured in 5% oxygen and 2% oxygen compared to those cultured in atmospheric oxygen. Our results suggest cells cultured at physiologically relevant oxygen levels have a greater capacity for reducing ROS which, in turn, may be beneficial to overall cell health and longevity. Our results on ROS production and SOD2 levels suggest that cells cultured in 5% oxygen are in a more optimal cellular condition than the cells cultured in the other conditions we examined.

Proteomic Analysis by Mass Spectrometry

While we found an alteration in levels of SOD2, we hypothesized the alterations in the proteome may be more widespread. In order to identify alterations within the mitochondrial proteome, a quantitative mass spectrometry experiment was performed using stable isotope labeling of amino acids in cell culture (SILAC).¹⁹ Cells cultured at 5% oxygen and atmospheric oxygen were labeled with amino acids containing either light or heavy isotopes, respectively. These two conditions were chosen because cell culture normally occurs at atmospheric oxygen levels and both SH-SY5Y cells and human embryonic neurons grown at 5% oxygen exhibited decreased ROS production (Figure 1A and 1B). To better mimic neurons, metabolically labeled SH-SY5Y cells were differentiated into neurons following subsequent treatments of retinoic acid and TPA.¹⁶ Mitochondria were isolated and the proteomic data was obtained using a high mass accuracy LTQ Orbitrap mass spectrometer.

In total, 714 proteins were identified. The complete output from the MaxQuant software analysis is provided in the supplementary. Analysis by the Database for Annotation, Visualization, and Integrated Discovery (DAVID) demonstrated enrichment in mitochondrial proteins (Table 1). As expected, DAVID analysis indicated through gene ontology (GO) that the samples were enriched for mitochondrial proteins. A large number of the proteins identified were integral to bioenergetic processes. Examination of the proteins identified in the electron transport chain (ETC) revealed elevation of ETC complex subunit expression levels between cells cultured at 5% as opposed to atmospheric oxygen (Figure 2). Similarly elevation of mitochondrial protein import/export proteins were also identified in cells cultured at 5% oxygen (Figure 2).

Orthogonal Confirmation of MS Expression Data

To verify the protein expression data generated by mass spectrometry, we first determined a set of proteins whose expression levels were unchanged to be utilized as controls. This set of proteins for our experiments included VDAC, GAPDH, ATP5A, and ATP5H (Figure 3A).

To assess the validity of the expression ratios, immunoblots were performed on a number of proteins with different levels of change: NDUFS3, NDUFV3, SDHB and UQCRCB (Figure 3B and 3C). The data obtained from immunoblots were similar to the data obtained from mass spectrometry for each protein examined. Based upon western blots and mass spectrometry, differences in expression level were evident between cells cultured at 5% and atmospheric oxygen, demonstrating that proteomic alterations are indeed present in the ETC when cells are cultured under different oxygen conditions.

Oxygen-mediated Rotenone Responsiveness

It was intriguing that NDUFV3, the target for rotenone inhibition of complex I, was found to be elevated in cells cultured in the physiological oxygen range (5% oxygen). Rotenone is a natural compound used as a pesticide and insecticide that, due to its lipophilic nature, crosses cellular membranes as well as the blood brain barrier. Rotenone inhibits mitochondrial complex I, and rotenone exposure has been linked to PD in humans.²⁰ To assess whether the differences resulting from tissue culture oxygen conditions affect the rotenone/PD system, we measured the toxicity of rotenone on SH-SY5Y cells cultured under the two oxygen conditions. The rotenone LD₅₀ varied significantly between SH-SY5Y cells cultured at the physiological (5%) and atmospheric (21%) oxygen conditions ($p < 0.05$). Cells cultured at atmospheric oxygen had a relatively high resistance to rotenone, with an LD₅₀ of $49.8 \pm 0.03 \mu\text{M}$, whereas cells cultured at 5% oxygen were more sensitive to rotenone, with an LD₅₀ of $1.98 \pm 0.04 \mu\text{M}$ (Figure 4A). This correlates with our finding of an increase in the incorporation of the rotenone-sensitive subunit (NDUFV3) in complex I at 5% oxygen (Figure 3B and 3C). As rotenone is commonly used to model PD *in vivo* and *in vitro*²¹ our

results indicate that tissue culture oxygen levels may be an important consideration for *in vitro* neurotoxicological studies of PD and, possibly, other neurodegenerative disorders.

Rotenone Inhibition Assay

In addition to affecting cell survival, the rotenone inhibition of the ETC should impact ATP production. To examine this we performed a luciferase-based ATP determination assay (Figure 4B) to assess ATP levels in cells culture under atmospheric and 5% oxygen in the presence and absence of rotenone, using a dose that does not produce cellular toxicity, 0.2 μ M. In the absence of rotenone, cells cultured in 5% oxygen produced more ATP than those in atmospheric oxygen, again confirming the physiological oxygen levels as been optimal. Treatment with rotenone lowered ATP production in cells cultured at 5% oxygen. These results indicate a heightened sensitivity of SH-SY5Y cells cultured at 5% oxygen to rotenone and understandable is congruent with the increased expression of the rotenone target, NDUFV3, in this condition.

DISCUSSION

Our results indicate that cells are sensitive to environmental oxygen and respond by altering mitochondrial protein levels in an adaptive manner. This adaptive response results in altered mitochondrial function, increasing ETC protein subunits in order to optimize for energy. Many studies have shown that neuronal cell survival improves at lower, physiological oxygen levels, and that cellular responses to stimuli are altered at these lowered oxygen levels as well.^{10-12, 22, 23} Similarly, our finding that mitochondrial ETC subunits are elevated in 5% oxygen suggest that cells cultured at 5% oxygen rely more heavily on oxidative phosphorylation for energy production. Still, the vast majority of tissue culture is done in atmospheric oxygen.

Cell culture at atmospheric oxygen remains valuable tool for modeling some disorders. The possible down regulation of oxidative phosphorylation (implied from the lower ATP levels shown in Figure 4B) in cells cultured at atmospheric oxygen may be very similar to the Warburg effect observed in cancer cells. The Warburg effect occurs in both primary and transformed cancer cells and may provide a protective mechanism that allows for survival and proliferation of cancer cells in abnormal oxygen conditions.

In systems where oxidative phosphorylation is essential, like healthy neurons, a physiological oxygen level results in a model system that more closely resembles the *in vivo* condition. Maintenance of oxygen levels is particularly relevant as many studies on the pathogenesis of neurodegenerative disorders rely on the disturbances of mitochondrial functions, by, for example, the use of rotenone to study PD. Our results show a dramatic increase in rotenone sensitivity for cells cultured in 5% oxygen likely due to an increased in the expression of the rotenone-sensitive subunit in NADH-ubiquinone oxidoreductase. Understanding the intricate interplay between oxygen level and subunit expression will allow for modeling of complex I inhibition with rotenone at lower concentrations. Using lower rotenone concentrations may both better mimic the true effects of this PD-linked toxin in experimental conditions and avoid spurious results from the secondary off-targets effects of high concentrations of rotenone. Although our data only explains how oxygen tension modifies rotenone sensitivity, it is likely that many mitochondrial responses can be altered by oxygen tension.

The observed ETC complex subunit alterations are interesting as changes in subunits have been primarily noted to differ among tissue or cell types rather than under experimental conditions.^{24, 25} We show that changes in different ETC complex subunit expression are a mechanism of environmental adaptation. Given the resulting changes in rotenone toxicity,

similar subunit switching may be responsible for differences in cellular susceptibility to a wide variety of conditions involving mitochondrial proteins and mitochondrial dysfunction, including but not limited to PD, HD, and other neurodegenerative disorders. While we have demonstrated a considerable differential expression of subunits, further work should be performed to identify how these alterations affect cellular homeostasis.

In our examination of primary neurons, we have found that neurons from different animal models and different brain locales (human cortical, this study, versus rat striatal¹¹) were in optimal conditions at different oxygen levels. While at first this difference may be puzzling, it is logical that regions of the brain exposed to different oxygen levels would have neurons optimized accordingly. Oxygen levels in different regions of the brain have been assessed in the rat, and range from 2.53–5.33% in cortical grey matter, 1.47–2.13% in the hypothalamus, and 0.55–1.07% in the midbrain.²⁶ It is intriguing that the midbrain, where degeneration of the dopaminergic neurons in the substantia nigra results in PD, has a quite low oxygen level, and whether alterations in the mitochondrial proteome contribute to the susceptibility of these cells to the environmental, toxic, genetic, and other potential contributors to PD is unknown but is a tenable hypothesis.

Our findings demonstrate the effects of oxygen tension on mitochondrial responses, however, the mechanism behind intracellular oxygen-sensing remains elusive. Currently, there are multiple hypotheses on the mechanism behind oxygen sensation including the hypoxia-induced factor-1 (HIF-1) pathway (reviewed in ²⁷) and the peroxisome proliferator activated receptor co-activator, PGC, pathway (reviewed in ²⁸). Most likely, oxygen sensing is a complex relationship between both these mechanisms. The ubiquinol-cytochrome c reductase binding protein (UQCRB) mechanism of the HIF-1 pathway has the most relevance to our studies.^{29, 30} UQCRB, being a subunit of complex III of the electron transport chain, means subunit expression differences would directly alter the complex III function and, possibly, the oxygen-sensing properties of this protein. Further research should be performed to identify the oxygen-sensing pathway pertinent to our system.

As yet, much remains to be done to determine the cellular response mechanism for altered oxygen levels. While many have studied the effects of short-term hypoxia and occasionally hyperoxia (however not, as we show here, accounting for the fact that culture in atmospheric oxygen is in fact physiological hyperoxia), far fewer studies examine the long-term effects of varying the availability of oxygen to cultured cells. Still there may be elements in common to both responses such as the so far elusive oxygen sensor. Understanding long-term mechanisms of oxygen-sensation is likely to be the key to understanding differential expression of mitochondrial proteins during homeostasis, aging, and neurodegeneration.

CONCLUSION

Tissue culture oxygen is an important factor that results in alterations in protein levels and mitochondrial function in living cells. For translation of *in vitro* studies on neurons, the use of physiological oxygen levels is essential, as cells are able to sense the available oxygen and adjust to maximize survival, adding a spurious factor to experimentation and limiting applicability to the *in vivo* situation. In addition, for studies of mitochondrial function, alterations of oxygen tension can be used to replicate different conditions that might occur in the body, allowing for modeling of a variety of conditions with the alteration of mitochondrial oxygen. Specifically, we show this is relevant for a commonly used PD model. Through better understanding of the mechanism that allows cells to adapt to different oxygen environments we can better understand neurodegenerative conditions and other brain diseases such as stroke as well as non-nervous system disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ROS	reactive oxygen species
PD	Parkinson's disease
HD	Huntington's disease
AD	Alzheimer's disease
PARK2	parkin
PINK1	PTEN-induced putative kinase 1
ETC	electron transport chain
SOD2	superoxide dismutase 2
DAVID	Database for Annotation, Visualization, and Integrated Discovery

References

1. Xie W, Wan OW, Chung KK. New insights into the role of mitochondrial dysfunction and protein aggregation in Parkinson's disease. *Biochim Biophys Acta*. 2010; 1802(11):935–41. [PubMed: 20674742]
2. Navarro A, Boveris A. Brain mitochondrial dysfunction and oxidative damage in Parkinson's disease. *J Bioenerg Biomembr*. 2009; 41(6):517–21. [PubMed: 19915964]
3. Bilsland LG, Sahai E, Kelly G, Golding M, Greensmith L, Schiavo G. Deficits in axonal transport precede ALS symptoms in vivo. *Proc Natl Acad Sci U S A*. 2010; 107(47):20523–8. [PubMed: 21059924]
4. Bobba A, Petragallo VA, Marra E, Atlante A. Alzheimer's proteins, oxidative stress, and mitochondrial dysfunction interplay in a neuronal model of Alzheimer's disease. *Int J Alzheimers Dis*. 2010
5. Campbell GR, Ziabreva I, Reeve AK, Krishnan KJ, Reynolds R, Howell O, Lassmann H, Turnbull DM, Mahad DJ. Mitochondrial DNA deletions and neurodegeneration in multiple sclerosis. *Ann Neurol*. 2011; 69(3):481–92. [PubMed: 21446022]
6. Cho DH, Nakamura T, Lipton SA. Mitochondrial dynamics in cell death and neurodegeneration. *Cell Mol Life Sci*. 2010; 67(20):3435–47. [PubMed: 20577776]
7. Frank S. Dysregulation of mitochondrial fusion and fission: an emerging concept in neurodegeneration. *Acta Neuropathol*. 2006; 111(2):93–100. [PubMed: 16468021]
8. Batlevi Y, La Spada AR. Mitochondrial autophagy in neural function, neurodegenerative disease, neuron cell death, and aging. *Neurobiol Dis*. 2011; 43(1):46–51. [PubMed: 20887789]

9. Ndubuizu O, LaManna JC. Brain tissue oxygen concentration measurements. *Antioxid Redox Signal*. 2007; 9(8):1207–19. [PubMed: 17536959]
10. Atkuri KR, Herzenberg LA. Culturing at atmospheric oxygen levels impacts lymphocyte function. *Proc Natl Acad Sci U S A*. 2005; 102(10):3756–9. [PubMed: 15738407]
11. Tiede LM, Cook EA, Morsey B, Fox HS. Oxygen matters: tissue culture oxygen levels affect mitochondrial function and structure as well as responses to HIV viroproteins. *Cell Death Dis*. 2011; 2:e246. [PubMed: 22190005]
12. Kaplan FS, Brighton CT, Boytim MJ, Selzer ME, Lee V, Spindler K, Silberberg D, Black J. Enhanced survival of rat neonatal cerebral cortical neurons at subatmospheric oxygen tensions in vitro. *Brain Res*. 1986; 384(1):199–203. [PubMed: 3098354]
13. Brewer GJ, Cotman CW. Survival and growth of hippocampal neurons in defined mediums at low density: advantages of a sandwich culture technique or low oxygen. *BRES*. 1989; 494:65–74.
14. Stacpoole SR, Bilican B, Webber DJ, Luzhynskaya A, He XL, Compston A, Karadottir R, Franklin RJ, Chandran S. Efficient derivation of NPCs, spinal motor neurons and midbrain dopaminergic neurons from hESCs at 3% oxygen. *Nat Protoc*. 2011; 6(8):1229–40. [PubMed: 21799491]
15. Stacpoole SR, Bilican B, Webber DJ, Luzhynskaya A, He XL, Compston A, Karadottir R, Franklin RJ, Chandran S. Derivation of neural precursor cells from human ES cells at 3% O₂ is efficient, enhances survival and presents no barrier to regional specification and functional differentiation. *Cell Death Differ*. 2011; 18(6):1016–23. [PubMed: 21274009]
16. Presgraves SP, Ahmed T, Borwege S, Joyce JN. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. *Neurotox Res*. 2004; 5(8):579–98. [PubMed: 15111235]
17. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods*. 2009; 6(5):359–62. [PubMed: 19377485]
18. Kraft-Terry SD, Engebretsen IL, Bastola DK, Fox HS, Ciborowski P, Gendelman HE. Pulsed Stable Isotope Labeling of Amino Acids in Cell Culture Uncovers the Dynamic Interactions between HIV-1 and the Monocyte-Derived Macrophage. *Journal of Proteome Research*. 2011; 10:2852–2862. [PubMed: 21500866]
19. Ong SE, Blagoev B, Karatchmarova I, Kristensen DB, Steen H, Pandley A, Mann M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics*. 2002; 1(5):376–86. [PubMed: 12118079]
20. Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, Marras C, Bhudhikanok GS, Kasten M, Chade AR, Comyns K, Richards MB, Meng C, Priestley B, Fernandez HH, Cambi F, Umbach DM, Blair A, Sandler DP, Langston JW. Rotenone, paraquat, and Parkinson's disease. *Environ Health Perspect*. 2011; 119(6):866–72. [PubMed: 21269927]
21. Greenamyre JT, Cannon JR, Drolet R, Mastroberardino PG. Lessons from the rotenone model of Parkinson's disease. *Trends Pharmacol Sci*. 2010; 31(4):141–2. author reply 142–3. [PubMed: 20096940]
22. Sahaf B, Atkuri K, Heydari K, Malipatlolla M, Rappaport J, Regulier E, Herzenberg LA, Herzenberg LA. Culturing of human peripheral blood cells reveals unsuspected lymphocyte responses relevant to HIV disease. *Proc Natl Acad Sci U S A*. 2008; 105(13):5111–6. [PubMed: 18364393]
23. Sahaf B, Heydari K, Herzenberg LA. The extracellular microenvironment plays a key role in regulating the redox status of cell surface proteins in HIV-infected subjects. *Arch Biochem Biophys*. 2005; 434(1):26–32. [PubMed: 15629105]
24. Schlerf A, Droste M, Winter M, Kadenbach B. Characterization of two different genes (cDNA) for cytochrome c oxidase subunit VIa from heart and liver of the rat. *EMBO J*. 1988; 7(8):2387–91. [PubMed: 2461293]
25. Schillace R, Preiss T, Lightowers RN, Capaldi RA. Developmental regulation of tissue-specific isoforms of subunit VIa of beef cytochrome c oxidase. *Biochim Biophys Acta*. 1994; 1188(3):391–7. [PubMed: 7803453]
26. Erecska M. Tissue oxygen tension and brain sensitivity to hypoxia. *Respir Physiol*. 2001; 128(3):263–76. [PubMed: 11718758]

27. Semenza G. Oxygen Sensing, Homeostasis, and Disease. *The New England Journal of Medicine*. 2011; 365(6):537–547. [PubMed: 21830968]
28. Wenz T. Regulation of mitochondrial biogenesis and PGC-1 α under cellular stress. *Mitochondrion*. 2013; 13:134–142. [PubMed: 23347985]
29. Crivellone M, Wu M, Tzagoloff A. Assembly of the mitochondrial membrane system. Analysis of structural mutant of the yeast coenzyme QH₂-cytochrome c reductase complex. *J Biol Chem*. 1988; 263:14323–14333. [PubMed: 2844766]
30. Jung HJ, Shim JS, Lee J, Song YM, Park KC, Choi SH, Kim ND, Yoon JH, Mungai PT, Schumacker PT, Kwon HJ. Terpestacin inhibits tumor angiogenesis by targeting UQCRB of mitochondrial complex III and suppressing hypoxia-induced reactive oxygen species production and cellular oxygen sensing. *J Biol Chem*. 2010; 285:11584–11595. [PubMed: 20145250]

FIGURE 1. Mitochondrial ROS production rates for SH-SY5Y cells (A) and human cortical neurons (B). Western blots indicating changes in (C) SOD2 levels of mitochondria cultured under differing oxygen levels with quantification (D) of n=3 independent biological replicates normalized against ATP5H. All measurements are in adu/min. Adu is a measure of image intensity. ATM represents atmospheric (21%) oxygen. * indicates $p < 0.05$

FIGURE 2.

Heat map displaying the change in protein expression of mitochondrial electron transport chain subunits and import/export proteins in SH-SY5Y cells cultured in 5% oxygen from those cultured in atmospheric oxygen, based on proteins quantitated in the mass spectrometry experiment. The values are displayed on a log₂ scale.

FIGURE 3.

Representative western blots of mitochondrial lysates from SH-SY5Y cells, cultured at the indicate oxygen conditions, for (A) unchanged mitochondrial proteins used as controls, (B) components of the oxidative phosphorylation complexes, and (C) quantitation of the western blots from n=3 independent biological replicates. * indicates $p < 0.05$. For the other proteins the change did not reach statistical significance.

FIGURE 4.

Cell death curve displaying rotenone toxicity (A) for calculating LD₅₀s of n=3 independent biological replicates. Changes in SH-SY5Y cell ATP production (B) normalized to cell count for rotenone based upon oxygen condition for n=3 independent biological replicates. All comparisons are against the corresponding controls. * indicates p<0.05

TABLE 1

Gene ontology (GO) annotation for biological process, cellular component and molecular function obtained from the Database for Annotation, Visualization, and Integrated Discovery (DAVID).

Gene Ontology Cellular Components		Fold Enrichment	P-Value
1	Mitochondrial part	6.29	3.49E-119
2	Mitochondrion	4.48	5.98E-117
3	Organelle envelope	5.19	1.09E-84
4	Envelope	5.17	2.00E-84
5	Mitochondrial envelope	6.29	1.50E-81
6	Mitochondrial membrane	6.38	1.89E-78
7	Organelle inner membrane	6.57	1.09E-68
8	Mitochondrial inner membrane	6.67	2.19E-65
9	Organelle membrane	3.22	5.92E-55
10	Mitochondrial lumen	7.13	1.97E-54
Gene Ontology Biological Processes			
1	Generation of precursor metabolites and energy	5.75	1.41E-43
2	Chromatin assembly or disassembly	8.65	6.23E-37
3	Chromatin assembly	10.34	2.36E-34
4	Nucleosome organization	9.88	5.18E-34
5	Oxidation reduction	3.56	8.83E-34
6	Nucleosome assembly	10.23	1.43E-32
7	Protein-DNA complex assembly	9.66	5.41E-32
8	DNA packaging	8.20	6.52E-31
9	Cellular macromolecular complex assembly	4.78	8.58E-31
10	Cellular respiration	9.06	1.55E-30
Gene Ontology Molecular Function			
1	Structural constituent of ribosome	6.19	2.68E-27
2	NADH dehydrogenase (ubiquinone) activity	10.49	9.23E-18
3	NADH dehydrogenase (quinone) activity	10.49	9.23E-18
4	NADH dehydrogenase activity	10.49	9.23E-18
5	Oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	9.61	1.96E-17
6	Oxidoreductase activity, acting on NADH or NADPH	7.09	2.25E-17
7	Structural molecule activity	2.57	2.68E-15
8	Cofactor binding	3.78	3.31E-15
9	Coenzyme binding	4.33	8.81E-15
10	Monovalent inorganic cation transmembrane transporter activity	5.66	2.18E-14