

Curriculum Vitae

Name: Sausan Mousa Jaber Taha

Contact Information: University of Maryland School of Medicine
MSTF 5-18
Baltimore, MD 21201
smjaber@outlook.com

Degree and Date to be Conferred: Ph.D., Dec. 2018

Education:

2007 B.S., General Chemistry, Towson University (Cum Laude)
2007 B.S., Molecular Biology, Biochemistry & Bioinformatics, Towson University (Cum Laude)
2018 Ph.D., Biochemistry and Molecular Biology, University of Maryland School of Medicine Thesis Advisor – Brian Polster and Gary Fiskum
Title of Thesis – “Effects of Idebenone on the Mitochondrial Respiration of Neurons, Astrocytes, and Microglia”

Professional Society Memberships:

2014-Present General Member, Society for Neurochemistry
2017-present General Member, Society for Neuroscience
2017-present General Member, Society for Redox Biology and Medicine
2017-Present General Member, Women in Bio

Honors and Awards:

2011-2018 Meyerhoff Graduate Fellowship, University of Maryland, Baltimore and University of Maryland Baltimore County
2007 Undergraduate Research Grant, Towson University, awarded for distinguished clinical performance as an intern

Publications:

1. Kalakonda S, Nallar SC, **Jaber S**, Keay SK, Rorke E, Munivenkatappa R, Lindner DJ, Fiskum GM, & Kalvakolanu DV. (2013) Monoallelic loss of tumor suppressor GRIM-19 promotes tumorigenesis in mice. *Proceedings of the National Academy of Sciences*, 110 (45): E4213-E4222.
2. **Jaber S** & Polster BM (2015) Idebenone and neuroprotection: antioxidant, pro-oxidant or electron carrier? *Journal of Bioenergetics and Biomembranes* 47: 111-118.

3. **Jaber SM** & Polster BM. (2017) An in vitro model yields ‘importin’ new insights into chronic traumatic encephalopathy: damaged astrocytes stop ‘thrombospondin’ to the injury. *Journal of Neurochemistry*, 140: 531–535.
4. **Jaber SM**, Bordt EA, Bhatt NM, Lewis DM, Gerecht S, Fiskum G, & Polster BM (2018) Sex differences in the mitochondrial bioenergetics of astrocytes but not microglia at a physiologically relevant brain oxygen tension. *Neurochemistry International*, 117:82-90.
5. Bhalla K, **Jaber S**, Nahid M N, Underwood K, Beheshti A, Landon A, Bhandary B, Bastian P, Evens AM, Haley J, Polster B, Gartenhaus RB. (2018) Role of hypoxia in Diffuse Large B-cell Lymphoma: Metabolic repression and selective translation of HK2 facilitates development of DLBCL. *Scientific Reports*, 8:744.
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4. **Jaber SM**, Milstein JM, Hampton B, Barrett JP, Loane DJ, Polster BM. Upregulation of enzymatic idebenone reduction activity after proinflammatory stimulation of microglia. *Society for Redox Biology and Medicine*. Baltimore, MD. Poster Presentation, 2017
5. **Jaber SM**, Bordt EA, Clerc P, Ge SX, Fiskum G, Polster BM. The influence of oxygen tension on the mitochondrial respiratory inhibition of astrocytes by proinflammatory microglia. *American Society for Neurochemistry*. Riverside, CA. Poster Presentation, 2018

Abstract

Title of Dissertation: Effects of Idebenone on the Mitochondrial Respiration of Neurons, Astrocytes, and Microglia

Sausan Mousa Jaber Taha, Doctor of Philosophy, 2018

Dissertation Directed by: Brian M. Polster, Associate Professor, Department of Anesthesiology Research

Neuroinflammation and mitochondrial bioenergetic dysfunction are present in most neurodegenerative diseases. Microglia, the resident immune cells of the brain, release cytotoxic factors such as nitric oxide (NO), superoxide, and cytokines following proinflammatory activation. NO mediates mitochondrial respiratory inhibition in microglia and neighboring cells by competing with oxygen at complex IV of the electron transport chain. Additional modes of inhibition by NO include post-translational modifications to upstream complexes I and II. Idebenone is a clinically safe prodrug that, in its reduced form idebenol, can act in place of ubiquinol and donate electrons directly to complex III. This dissertation tested the overarching hypothesis that idebenone can support mitochondrial respiration in cells with sufficient quinone-reduction capacity to convert idebenone to idebenol, despite complex I and II impairment by NO. In astrocytes but not neurons, idebenone and two related quinones could rescue maximal oxygen consumption rate (OCR) when a complex I inhibitor was present. This difference between astrocytes and neurons was due to a disparity in cellular quinone-reduction capacity mediated by the expression of NADPH:quinone oxidoreductase 1 (NQO1). Astrocytes were sensitive to respiratory impairment by an NO donor or co-cultured proinflammatory microglia at a physiologically-relevant oxygen level and idebenone was able to partially reverse this impairment. Interestingly, microglia upregulated their

quinone-reduction capacity following proinflammatory stimulation and idebenone was also able to partially reverse respiratory impairment in microglia following activation. Surprisingly, in contrast to astrocytes, NQO1 was not responsible for idebenone reduction in activated microglia. Biochemical isolation of the responsible enzyme identified inducible nitric oxide synthase (iNOS) among the few candidates common to three distinct fractionation approaches. Assays performed with recombinant iNOS revealed a novel idebenone reduction activity with exciting implications for future studies. This dissertation's findings suggest that insufficient quinone-reduction capacity in diseased target cells may be a mechanistic reason for the failure of idebenone in clinical trials. These results support new strategic approaches for the use of idebenone and similar drugs to overcome mitochondrial bioenergetic dysfunction.

Effects of Idebenone on the Mitochondrial Respiration of
Neurons, Astrocytes, and Microglia

by
Sausan Mousa Jaber Taha

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List of Abbreviations

- AA, Antimycin A
- AD, Alzheimer's disease
- ADP, Adenosine diphosphate
- ANOVA, Analysis of variance
- ARE, Antioxidant response element
- ATP, Adenosine triphosphate
- BSA, Bovine serum albumin,
- CoQ, coenzyme Q₁₀
- CPTIO, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
- Cyt *c*, Cytochrome *c*
- DCPIP, Dichloroindophenol
- DETA-NO, Diethylenetriamine/nitric oxide adduct
- DMSO, Dimethylsulfoxide
- DNP, Dinitrophenol
- ECAR, Extracellular acidification rate
- ELISA, Enzyme linked immunosorbent assay
- ETC, Electron transport chain
- FBS, Fetal bovine serum
- FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
- FMN, Flavin mononucleotide
- GSH-EE, Glutathione-ethyl ester
- H₂O₂, Hydrogen peroxide
- HAPI, Highly aggressively proliferating immortalized
- HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IdBH₂, Idebenol
Ideb, Idebenone
IFN- γ , Interferon-gamma
IL-10, Interleukin-10
IL-1 β , Interleukin-1 beta
IL-6, Interleukin-6
iNOS, Inducible nitric oxide synthase
KEAP, Kelch-like ECH-associated protein 1
KO, Knockout
LPS, Lipopolysaccharide
MS, Multiple sclerosis
NO, Nitric oxide
NOX, NADPH oxidase
NQO1, NAD(P)H:quinone acceptor oxidoreductase 1
Nrf2, nuclear factor erythroid 2-related factor 2
O₂, Oxygen
OCR, Oxygen consumption rate
OLIGO, Oligomycin
ONOO⁻, Peroxynitrite
PD, Parkinson's disease
Pier A, Piericidin A
RNS, Reactive nitrogen species
ROS, Reactive oxygen species
Sap, Saponin
TBI, Traumatic brain injury

TCA, Tricarboxylic acid

TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine

TNF- α , Tumor necrosis factor-alpha

Chapter 1: Introduction¹

1.1 Introduction to Brain Metabolism

The brain is a highly complex organ that serves as the centralized control center for most of the body's functions. It is primarily composed of neurons and glial cells that include astrocytes, oligodendrocytes, and microglia. These cell types are further divided into several subtypes depending on their location and function. *In vivo*, these cells work in synergy with one another to facilitate the many functions of the brain that are critical to an organism's survival. However, our understanding of how the brain functions as a whole is limited by our need to study cells in a controlled environment. Mostly due to technical limitations, scientific studies of the brain are often conducted *in vitro* on isolated cells with minimal consideration paid to the drastic differences in extracellular environment.

Although the human brain makes up only 2% of the body's total mass, it disproportionately contributes to the body's energy requirements. The high-energy functions of the brain demand approximately 20% of the body's energy consumption (Magistretti & Allaman 2015). The majority of this energy was thought to be dedicated to neuronal functions. We have only recently begun to appreciate the importance of glial cell function (van Hall *et al.* 2009).

While mitochondrial respiratory impairment is associated with numerous pathologies, evidence for association comes primarily from studies using isolated brain

¹ Jaber, S. and Polster, B. M. (2015) Idebenone and neuroprotection: antioxidant, pro-oxidant, or electron carrier? *J. Bioenerg. Biomembr.* **47**, 111-118.

mitochondria, which are derived from both neurons and glia, or from primary neuronal cultures. Roles for mitochondrial respiratory function/dysfunction in neuroglia such as astrocytes and microglia are comparatively understudied, which is surprising since neuroglia are thought to comprise ~50% of the brain (Azevedo *et al.* 2009, Herculano-Houzel 2014).

1.1.2 Neurons

Neurons are the most studied brain cell type. They perform critical functions that facilitate cognition and behavior normally associated with the brain. These cells respond to extracellular stimuli to produce action potentials to receive, process, and transmit information. After an action potential, the recovery of the neuron involves neurotransmitter recycling and the repolarization of ion gradients across the plasma membrane, which are energetically expensive processes (Ames 2000). The majority of ATP is produced by oxidative phosphorylation, which can consequently generate high levels of reactive oxygen species (Hall *et al.* 2012). To supplement neuronal energy demands, neurons also work synergistically with astrocytes, which provide additional bioenergetic and antioxidant support through the transfer of metabolic intermediates (Magistretti & Allaman 2015).

1.1.3 Astrocytes

Astrocytes were initially regarded as “glue” or “housekeeping” cells of the brain. However, they are now recognized to have many pivotal functions such as regulation of ion homeostasis (Olsen *et al.* 2015, Magistretti & Allaman 2015), neurotransmitter recycling (Schousboe *et al.* 1993), and control of cerebral blood flow (Cabezas *et al.* 2014). Astrocytes also carry out important bioenergetic roles in the brain, including

regulation of brain glucose uptake, production and storage of brain glycogen, and provision of metabolic and antioxidant support for neurons (Belanger *et al.* 2011).

1.1.4 Microglia

Microglia are the resident immune cells of the brain that play diverse roles in physiology, including clearing cellular debris, synaptic pruning, and promotion of synaptogenesis (Tremblay *et al.* 2011, Wu *et al.* 2015). Microglia are activated to a proinflammatory state in many neurodegenerative diseases such as Alzheimer's, Parkinson's, multiple sclerosis and stroke (Dheen *et al.* 2007). Proinflammatory microglia release various inflammatory factors such as cytokines, chemokines, reactive oxygen and nitrogen species (Dheen *et al.* 2007). Initially, the microglial response to brain pathology may be neuroprotective; however, prolonged activation can be detrimental (Brown & Bal-Price 2003, Block *et al.* 2007b). Microglial shift their energy dependence from oxidative phosphorylation to glycolysis during activation (Voloboueva *et al.* 2013, Orihuela *et al.* 2015). Excessive or prolonged glial activation contributes to neurodegeneration at least in part by impairing mitochondrial bioenergetic function (Block *et al.* 2007a, Dheen *et al.* 2007, Brown & Bal-Price 2003). The mechanisms by which the respiratory complexes are affected are not fully understood.

1.2 Neuroinflammation

Neuroinflammation is a broad term used to describe an innate immune response in the central nervous system. The immune response within the brain is cell-type specific and very complex, with varying degrees of severity. Activation of the immune response in microglia can be initiated by interaction of either damage- or pathogen- associated molecular pattern (DAMPs and PAMPs respectively) with pattern recognition receptors

(PRRs) such as toll-like receptors TLR2 and TLR4. DAMPs such as HMGB1, s100, and heat shock proteins are released by dying neuronal and non-neuronal cells; while PAMPs such as lipopolysaccharides (LPS) are microbial molecules produced by pathogens. Co-treatment with LPS and the leukocyte secreted proinflammatory cytokine interferon- γ (IFN- γ) is commonly used to induce proinflammatory activation of microglia *in vitro*.

Activated microglia then release proinflammatory cytokines and reactive oxygen/nitrogen species such as nitric oxide into the extracellular space, further propagating the inflammatory response (Brown & Bal-Price 2003, Block et al. 2007). Prolonged and excessive exposure to these factors can lead to mitochondrial dysfunction, oxidative damage and death in neighboring cells (Boje & Arora 1992). In the case of traumatic brain injury, for example, glial cells become activated soon after the primary injury and release proinflammatory mediators. This initial response is observed within days of the primary injury and peaks at around 7 days (Loane & Byrnes 2010). Following the initial inflammatory response is a secondary response, wherein neuronal damage continues for months and even years afterwards (Loane *et al.* 2014). This secondary injury is attributed in part to chronic microglial activation (Loane et al. 2014). While treatment of the patient in time to prevent damage from the primary injury is challenging, we may be able to prevent damage from the secondary injury.

Nitric oxide (NO) is a short-lived diffusible free radical gas. Nitric oxide plays an important role as a vasodilator through activation of the enzyme guanylyl cyclase and formation of cyclic guanosine monophosphate (cGMP). Sources of NO can either be exogenous (food) or endogenous. NO is produced endogenously by nitric oxide synthases (NOS), of which there are three isoforms. In the brain, inducible NOS (iNOS) is

predominantly expressed in proinflammatory microglia while neuronal NOS (nNOS) is expressed in neurons. Endothelial NOS (eNOS) is expressed in non-neuronal cells such as the endothelial cells of the blood vessels and the meninges. NOS enzymes catalyze the oxidation of L-arginine to L-citrulline. This occurs in a two-stage reaction wherein NADPH and oxygen are consumed and nitric oxide is formed.

Nitric oxide released by activated microglia can inhibit respiration by competing with oxygen at complex IV of the electron transport chain. Additionally, nitric oxide can react with superoxide to form peroxynitrite. Nitric oxide and its derivatives can induce post-translational modifications such as thiol nitrosations in other respiratory enzymes (Brown 2001).

1.3 Mitochondria Introduction:

Mitochondria are cellular organelles that are most famously known for their role as the powerhouse of the cell. Not only are they the major source of cellular ATP production, but they also play additional important roles such as maintaining cellular ion homeostasis, producing cellular reactive oxygen species, and regulating cellular death mechanisms.

Mitochondria have two phospholipid bilayers referred to as the inner and outer mitochondrial membranes. The space between the inner membrane and outer membrane is called the intermembrane space. The outer mitochondrial membrane is readily permeable to ions and small molecules, while the inner membrane is impermeable to most molecules. The inner mitochondrial membrane is extensively folded into complex structures known as cristae, which provide a large surface area in which are embedded with the components of the electron transport chain. Encapsulated within the inner

membrane lies the matrix. The matrix contains several copies of mitochondrial DNA and a high concentration of proteins including pyruvate dehydrogenase and the enzymes of the citric acid cycle.

Mitochondria depictions are often oversimplified to resemble bacteria-like structures that are between 0.5-1.0 μM long; however, time-lapse imaging has revealed that they are in fact continuously undergoing fission and fusion with other mitochondria.

Bioenergetics is the study of energy transduction in living cells. Aerobic organisms produce energy in the form of cellular ATP. Energy sources such as carbohydrates, fats, and amino acids are broken down by several different catabolic pathways to provide the energy for ATP synthesis. Bioenergetic pathways in the cytosol such as glycolysis provide substrates for the dehydrogenases of the citric acid cycle within the matrix. The reducing equivalents produced by the citric acid cycle are then utilized by the electron transport chain.

The systematic flow of electrons through the electron transport chain leads to the final reduction of oxygen to water. The flow of electrons provides the energy required to pump protons across the inner mitochondrial membrane. This results in an electrochemical gradient between the intermembrane space and the matrix referred to as the proton-motive force. Protons are pumped into the intermembrane space, giving it higher positive charge than the matrix. The electrochemical potential of this gradient is what ultimately drives the phosphorylation of ADP to ATP.

1.3.1 Electron Transport Chain

The mitochondrial electron transport chain (ETC) consists of complexes I-IV, the lipophilic inner membrane electron carrier ubiquinone and the soluble intermembrane

space electron carrier cytochrome *c* (Nicholls and Ferguson 2013). The ATP synthase, which couples the electrochemical proton gradient established by the respiratory chain to phosphorylation of ADP to ATP, is often called complex V.

1.3.2 Complex I

Complex I, also known as NADH:ubiquinone oxidoreductase, of the electron transport chain is a large transmembrane protein assembly that is composed of 44 different subunits, a flavin mononucleotide (FMN)-containing flavoprotein, and eight iron–sulfur clusters. Seven of these subunits are encoded for in the mitochondrial genome while the remaining subunits are encoded for in the nuclear genome. The main function of complex I within oxidative phosphorylation is to transfer two electrons from NADH to ubiquinone to form ubiquinol; this reaction is coupled to the pumping of four protons from the matrix to the intermembrane space. The initial transfer of electrons from NADH to FMN within the flavin-binding site is a two-electron reduction while the subsequent transfers to the iron-sulfur clusters, and finally ubiquinone, are each a one-electron reduction. Consequently, the final reduction of ubiquinone to ubiquinol is a two-step reaction involving the formation of a semiquinone radical intermediate. A small percentage of semiquinones formed at complex I can react with oxygen to form superoxide, making complex I a significant source of cellular reactive oxygen species.

1.3.3 Complex II

Complex II, also known as succinate dehydrogenase, is composed of four subunits, two hydrophobic located within the membrane and two hydrophilic that extend into the matrix. It also contains three iron-sulfur clusters. Electrons are first transferred from succinate to an FAD⁺ prosthetic group, before being tunneled along the iron-sulfur

clusters to finally reduce ubiquinone. As with complex I, complex II is also a site of potential semiquinone formation and reactive oxygen species generation. Unlike complex I, this reaction is not coupled to the pumping of protons across the membrane.

1.3.4 Ubiquinone

Ubiquinone, also known as Coenzyme Q₁₀ (CoQ), is an essential non-vitamin cofactor and antioxidant. It is composed of a 1,4-benzoquinone ring and a tail comprised of ten isoprenyl chemical subunits. In high abundance, a ubiquinone pool is found in the mitochondrial inner membrane. It acts as an intermediate electron carrier from complexes I and II to complex III. Ubiquinone's quinone head group is believed to aggregate deep within the lipid bilayer and, upon reduction, gravitates toward the lipid-water interface. As described above, free radical quinones may react with water to form superoxide.

1.3.5 Complex III

Ubiquinone:cytochrome *c* oxidoreductase, referred to as complex III, is composed of 11 subunits, two iron-sulfur clusters and three heme prosthetic groups. The iron-sulfur clusters are located in a subunit known as the Rieske Iron Sulfur protein. Two of the heme groups (*bH* and *bL*) are located in the cytochrome B subunit and the third (*C1*) in the cytochrome C1 subunit. Complex III catalyzes the transfer of electrons from ubiquinol to cytochrome *c*. This reaction is coupled to the pumping of four protons per ubiquinol into the intermembrane space.

Ubiquinol is a two-electron carrier while cytochrome *c* is a one-electron carrier; complex III therefore oxidizes ubiquinol in a two-stage process called the Q cycle. During this process, two molecules of ubiquinol are oxidized and two molecules of cytochrome *c* are reduced, however one ubiquinol is regenerated; thus, a net of two

molecules of cytochrome *c* are reduced per ubiquinol. During the first stage, the first ubiquinol binds the quinone binding site (QP) located on the intermembrane space side of the inner membrane. This ubiquinol is fully oxidized to ubiquinone and its protons are released into the intermembrane space. One of its electrons is transferred to the iron-sulfur cluster and the other is transferred to the *bL* heme. The electron of the iron-sulfur group is then transferred to the C1 heme before it is finally transferred to cytochrome *c*. The other electron is transferred from the *bL* heme to the *bH* heme before it is finally transferred to a new molecule of ubiquinone bound at a different quinone binding site (QN) near the matrix side of the membrane. The one electron reduction of this quinone forms a semiquinone radical. During the second stage, the second ubiquinol is fully oxidized and two protons are again pumped into the intermembrane space as described above. As before, the next destination of one of the electrons is a new molecule of cytochrome *c*; however, the other electron is donated to the semiquinone generated in the previous stage to form a new molecule of ubiquinol.

1.3.6 Cytochrome c

Cytochrome *c* is a small water-soluble protein that contains a heme prosthetic group. It is loosely associated with the intermembrane side of the inner membrane and functions as an electron carrier by accepting electrons from complex III and donating them to complex IV. Cytochrome *c* also plays an important role in the initiation of apoptosis that is independent from its function within the electron transport chain.

1.3.7 Complex IV

Complex IV, also known as cytochrome *c* oxidase, transfers electrons from cytochrome *c* to reduce molecular oxygen to form water. This reaction is coupled to the

pumping of two protons from the matrix to the intermembrane space. Complex IV is composed of 13 subunits, two heme groups and three copper ion centers. Cytochrome *c* is a one-electron carrier and molecular oxygen (O₂) can accept four electrons to produce two molecules of water. One electron is transferred through the heme groups and copper ion center to oxygen.

1.3.8 Oxygen

Oxygen is the final acceptor of electrons in the electron transport chain. Oxygen also can react with semiquinone radicals produced by complexes I, II, and III to produce superoxide. Physiological brain oxygen levels range from 1-6% (5-45 mm Hg, 10-50 μM) *in vivo* while the oxygen level in the atmosphere is much higher at 21% (150 mm Hg, ~150-200 μM) oxygen (Murphy 2009). The rate of superoxide production by isolated mitochondria is estimated to be 0.12-2% of total oxygen consumed; this is estimated to be much lower *in vivo* (Jagannathan *et al.* 2016, Wagner *et al.* 2011, Murphy 2009).

1.3.9 The Electrochemical Gradient

During oxidative phosphorylation, protons are pumped from the matrix into the intermembrane space. This creates a pH and charge difference across the membrane referred to as the electrochemical gradient (also referred to as the chemiosmotic gradient or the proton-motive force). This gradient provides the energy needed for ATP synthase to produce ATP.

1.3.10 ATP synthase

The ATP synthase, sometimes referred to as complex V, is composed of nine subunits. ATP synthase passes protons from the intermembrane space to the matrix,

which is driven by the electrochemical gradient. As protons pass through the enzyme the subunits mechanically rotate. This reaction is coupled to the conversion of ADP and inorganic phosphate (Pi) into ATP. ATP synthase yields 2.5 molecules of ATP per NADH and 1.5 per succinate (FADH₂). In the absence of the electrochemical gradient, ATP synthase can function as an ATPase and hydrolyze ATP back to ADP. This reverse reaction is coupled to the pumping of electrons into the intermembrane space, thereby establishing an electrochemical gradient.

1.4 Seahorse Extracellular Flux Analyzer

Mitochondrial respiration measurements can be made from intact cells using the Seahorse Extracellular Flux Analyzer (“Seahorse,” Agilent Technologies). Using oxygen-sensitive probes, it is able to track changes in oxygen consumption rate. See Figure 1.1. Oxygen consumption rate (OCR) is an indicator of oxidative phosphorylation. OCR is expressed as picomoles O₂/min over time. The Seahorse can measure OCR in up to 24 wells at a time. Drug injection ports within the Seahorse cartridge allow for OCR measurements in response to up to four drug additions. Experimental design is highly centered on the consecutive injection of respiratory inhibitors and substrates. Basal respiration is the resting cell respiration observed prior to any drug additions. With the addition of the ATP synthase inhibitor, oligomycin, we can measure the cellular respiration that is dedicated to the production of ATP. The maximum rate at which cells can consume oxygen is an indicator of how well they may be able to respond to higher energy demands. Maximal respiration can be achieved by using an uncoupler, such as carbonyl cyanide-4-phenylhydrazone (FCCP) or dinitrophenol (DNP), to dissipate the electrochemical gradient and therefore dissociate the electron transport and

phosphorylation reactions. By adding the complex III inhibitor, antimycin a, we can measure the amount of oxygen consumption that is non-mitochondrial and may be due to other cellular processes that require oxygen.

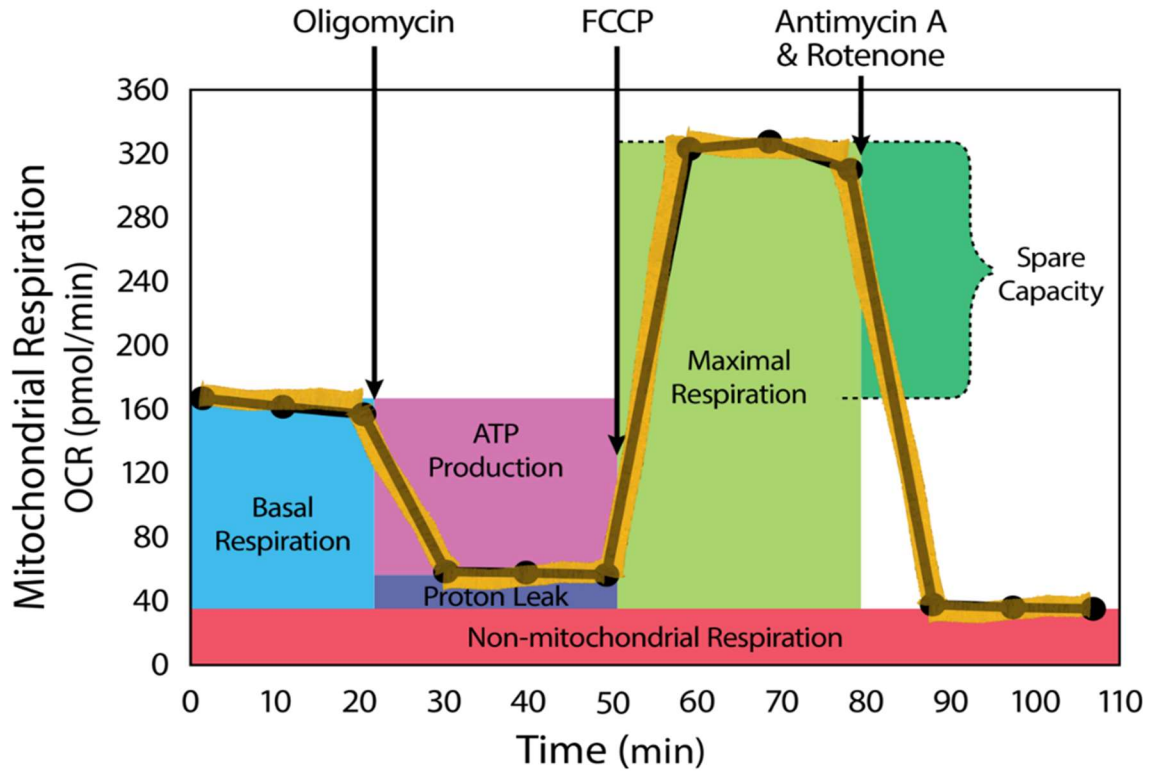


Figure 1.1 Schematic of respirometry measurements obtained with the Seahorse Extracellular Flux Analyzer. Parameters of mitochondrial function include: basal respiration, defined as resting rate respiration prior to drug addition; maximal respiration, defined as the respiration following the addition of an uncoupler; spare respiratory capacity, defined as the difference between maximal respiration and basal respiration; ATP linked respiration, defined as the basal respiration and the respiration after the addition of the ATP synthase inhibitor oligomycin; and finally non-mitochondrial respiration, defined as the respiration after the addition of a complex III inhibitor, such as antimycin A.

1.5 Electron Transport Bypass Therapeutic Strategy

Neuroinflammation and mitochondrial bioenergetic dysfunction are commonly associated with various neurodegenerative diseases (van Horssen *et al.* 2017, Witte *et al.* 2010). Proinflammatory microglia can disrupt mitochondrial respiration by releasing nitric oxide (van Horssen *et al.* 2017, Witte *et al.* 2010). Nitric oxide inhibits respiration by competing with oxygen at complex IV of the electron transport chain. Additional mechanisms of respiratory impairment include post-translational modifications to upstream complexes I and II which are more commonly associated with neurodegenerative diseases (Brown 2001). Strategies to overcome this impairment have included a mechanistic bypass of complexes I and II by direct electron donation to complex III. This can theoretically be achieved by providing the cell with reduced coenzyme Q.

Found in other cellular membranes in addition to mitochondria (Kalen *et al.* 1987), the reduced ubiquinol form of CoQ is a powerful antioxidant which can accept electrons from free radicals and inhibit lipid peroxidation (Geromel *et al.* 2002; Beyer *et al.* 1996). The antioxidant potential of CoQ and its role in cellular respiration make it a promising therapeutic agent for neurodegenerative diseases. However, due to its lipophilic nature and poor solubility, dietary supplementation with CoQ results in low bioavailability and little effect (Tomono *et al.* 1986). Largely for this radical scavenging property, CoQ supplements and skin cream are touted as “anti-aging” elixirs, though there is no hard scientific evidence that CoQ supplementation extends lifespan. Due to its essential role in electron transport, CoQ is sometimes loosely advertised as an “energy” supplement useful for treating a wide variety of pathological conditions. Nevertheless,

aside from rare disorders caused by CoQ deficiency, there is little evidence that CoQ therapy acts by mitigating electron transport chain deficiency (Geromel *et al.* 2002).

1.5.1 Idebenone Introduction

A series of short chain analogues of CoQ were synthesized with the aim of improving pharmacokinetics for patient treatment (Geromel *et al.* 2002, Erb *et al.* 2012, Suno & Nagaoka 1984b). Idebenone (2-(10-hydroxydecyl)-5,6-dimethoxy-3-methyl-cyclohexa-2,5-diene-1,4-dione) is one such quinone developed in Japan in the 1980s for the treatment of neurodegenerative conditions (Figure 1.2) (Suno & Nagaoka 1984b). When in its reduced form, idebenol, idebenone may act as an antioxidant or electron donor for cellular respiration and would therefore seem like a promising drug for the treatment of many disorders characterized by oxidative stress and mitochondrial dysfunction.

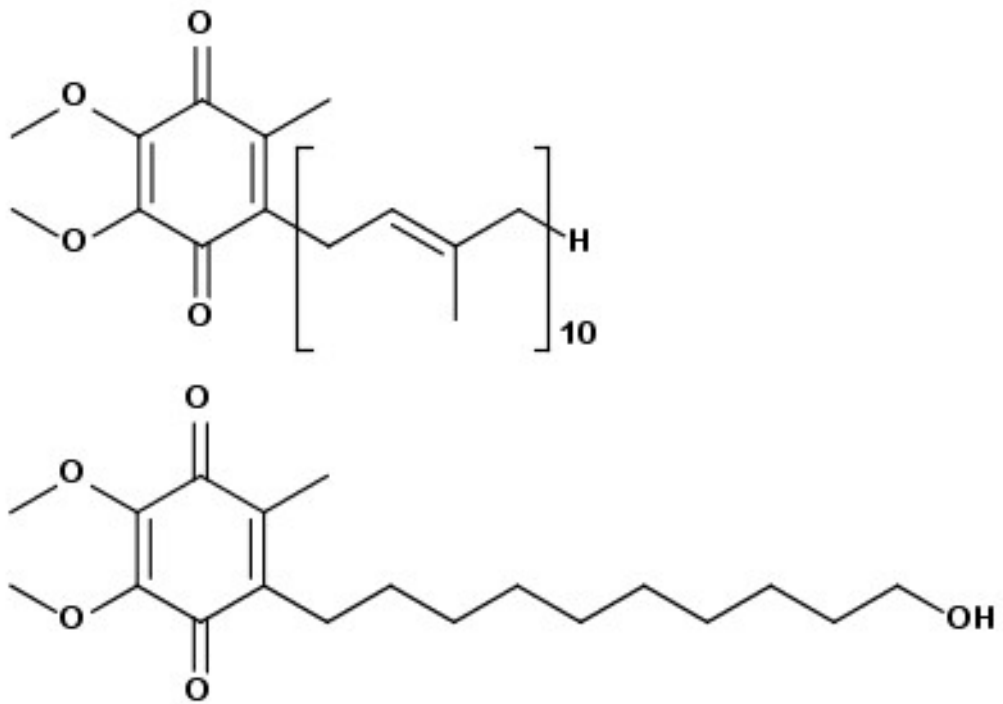


Figure 1.2 Chemical structures of coenzyme Q₁₀ (upper) and idebenone (lower)

Consistent with predicted antioxidant activity, idebenone inhibits lipid peroxidation in isolated brain mitochondria, synaptosomes, and cells (Suno & Nagaoka 1989, Suno & Nagaoka 1984a, Suno & Nagaoka 1984b, Erb et al. 2012, Yamada *et al.* 1999, Cardoso *et al.* 1998). In one study, idebenone was the most potent of 70 related quinones tested (Erb et al. 2012). Cell-free experiments demonstrated that idebenone *per se* lacks antioxidant activity, with activity conferred solely by the reduced hydroquinone form idebenol (Mordente *et al.* 1998). In addition to impairing lipid peroxidation, idebenol is capable of detoxifying a wide variety of free radicals, including peroxy and tyrosyl radicals and peroxynitrite (Mordente et al. 1998).

In vitro, idebenone attenuates oxidative injury to primary cortical neurons (Murphy *et al.* 1990, Ratan *et al.* 1994) and immortalized neural cells (Miyamoto *et al.* 1989, Pereira & Oliveira 2000) caused by depletion of the endogenous intracellular antioxidant glutathione. ¹⁴C drug radiolabelling experiments established that idebenone crosses the blood-brain barrier following oral administration (Nagai *et al.* 1989, Torii *et al.* 1985). Idebenone protects against neuronal death caused by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor stimulation *in vitro* (Bruno *et al.* 1994) and *in vivo* (Miyamoto & Coyle 1990). However, protection against N-methyl-D-aspartate (NMDA) receptor-induced death is observed only *in vitro* (Bruno et al. 1994, Miyamoto & Coyle 1990). Nevertheless, neurological improvement in response to idebenone treatment was reported in rodent models of stroke (Nagaoka *et al.* 1989) and Alzheimer's disease (Yamada et al. 1999). Idebenone is well-tolerated in humans even at daily doses of 1000-2000 mg and shows linear pharmacokinetics following single or repeated oral dosing (Kutz *et al.* 2009). Millions of humans have taken idebenone as part

of clinical trials or as approved treatment in various countries (Meier & Buyse 2009). Its excellent bioavailability and safety profile make idebenone an attractive neurotherapeutic drug candidate. But is it effective?

1.5.2 Idebenone in clinical trials—a short synopsis

Idebenone has been investigated most extensively for the treatment of Friedreich's ataxia, a rare autosomal recessive disease that typically affects children and young adults (Parkinson *et al.* 2013, Meier & Buyse 2009). Friedreich's ataxia is a progressive disorder characterized by degeneration of spinal cord nerve tissue (Parkinson *et al.* 2013). Adverse neurological symptoms include impaired movement and speech. Hypertrophic cardiomyopathy is a serious and prominent non-neurological feature (Parkinson *et al.* 2013, Meier & Buyse 2009).

The majority of Friedreich's ataxia patients are deficient in the mitochondrial protein frataxin, a protein involved in iron metabolism and redox homeostasis (Parkinson *et al.* 2013). Frataxin deficiency results in loss of essential iron-sulfur containing proteins found in respiratory chain complexes I, II, and III, leading to impaired ETC function and reduced ATP production (Rotig *et al.* 1997, Cooper & Schapira 2003). Dysfunctional mitochondria are further burdened by iron build-up, causing unmanaged oxidative stress (Wong *et al.* 1999). Treatment of Friedreich's ataxia patients with idebenone generally led to a reduction in oxidative stress markers, with many patients also showing improvement of non-neurological symptoms (Parkinson *et al.* 2013, Meier & Buyse 2009). However, although a six-month, randomized, double-blind, placebo-controlled trial for idebenone in Friedreich's ataxia patients found improvement in neurological function and activities associated with daily living (Di Prospero *et al.* 2007a), a

subsequent trial with a separate cohort of patients failed to recapitulate the neurological benefits (Lynch *et al.* 2010). Nevertheless, a twelve-month open label extension of the second study provided evidence for neurological improvement at the highest idebenone dose (Meier *et al.* 2012). Initially “authorized with conditions” for the treatment of Friedreich’s ataxia in Canada under the trade name CATENA[®], idebenone was voluntarily withdrawn from the Canadian market in 2013 by Santhera Pharmaceuticals without safety concerns, citing lack of efficacy.

Trials with idebenone for the most prevalent neurodegenerative disorder, Alzheimer’s disease, also generated mixed results. An early double-blind trial with only a moderate patient number (102) found statistically significant improvement in memory, attention and behavior (Senin *et al.* 1992). Several additional trials yielded favorable results (Bergamasco *et al.* 1994, Weyer *et al.* 1997, Gutzmann *et al.* 2002, Gutzmann & Hadler 1998). However, idebenone failed to slow Alzheimer’s-associated cognitive decline in a larger multicenter, double-blind, placebo-controlled trial (Thal *et al.* 2003). Approval for the use of idebenone to treat Alzheimer’s disease or related cognitive dementias in the U.S. has not been obtained. Thus, as in the case of Friedreich’s ataxia, the initial high hopes for idebenone in the treatment of Alzheimer’s disease remain unfulfilled.

A one-year, double-blind Huntington’s disease trial for idebenone disappointingly found no significant improvement in primary outcome measures related to disease progression (Ranen *et al.* 1996). Idebenone has also seen clinical trials for the mitochondrial disorders Mitochondrial Encephalopathy Lactic Acidosis and Stroke-like Episodes (MELAS) and Leber's Hereditary Optic Neuropathy (LHON), as well as for

Duchenne muscular dystrophy and multiple sclerosis (www.clinicaltrials.gov). Idebenone was recently approved in Europe for the treatment of Leber's Hereditary Optic Neuropathy. While some trials are still ongoing, idebenone has yet to be approved by the U.S. Food and Drug Administration for the treatment of any human disease.

1.5.3 Idebenone Structure, Function, Complex I Inhibition:

A greater understanding of how idebenone interacts with mitochondria in cells is needed to further clinical efforts with this drug. Because idebenone is described as a short chain CoQ analogue, a common misconception is that idebenone can substitute for the function of endogenous CoQ in the ETC. Although early work demonstrated that idebenone can restore complete succinate oxidation in CoQ-depleted brain mitochondria, NADH oxidation in the presence of idebenone was independent of downstream components of the ETC (Imada *et al.* 1989). These findings suggest that idebenone is effective at transferring electrons from complex II (succinate dehydrogenase) but not from complex I (NADH dehydrogenase) to complex III. Experiments using intact cells, which oxidize primarily NADH-linked substrates, confirmed that idebenone cannot substitute for the electron transfer function of CoQ in CoQ-deficient fibroblasts (Lopez *et al.* 2010). However, several studies support the ability of idebenone to act as an effective electron carrier from complex II or glycerol-3-phosphate dehydrogenase to complex III (Imada *et al.* 1989, Rauchova *et al.* 2008, Briere *et al.* 2004).

At apparent odds with its presumptive neuroprotective potential, multiple groups reported that idebenone can inhibit complex I and promote superoxide production (Genova *et al.* 2001, Genova *et al.* 2003, Esposti *et al.* 1996, Imada *et al.* 2008, Ohnishi *et al.* 2005, King *et al.* 2009, Fato *et al.* 2008, Briere *et al.* 2004, Fash *et al.* 2013). Most

of these studies were done with isolated complex I or submitochondrial particles—mitochondrial membrane fractions containing functional ETC complexes. However, it was also demonstrated that idebenone inhibits ADP-stimulated and uncoupled respiration by intact rat brain mitochondria in the presence of complex I-linked substrates (Imada et al. 1989). Detailed biochemical studies by King et al. elucidated likely mechanisms of complex I inhibition and superoxide production by idebenone (King et al. 2009). In the model of King et al., idebenone is reduced at the hydrophobic quinone-binding site within complex I like CoQ but exhibits very slow dissociation. Consequently it acts as a competitive substrate that impairs endogenous CoQ function without substituting for its electron transfer function to complex III (Figure 1.3) (King et al. 2009). Idebenone binds a second quinone-binding site within complex I that overlaps with the NADH binding site and flavin mononucleotide (FMN) moiety (Figure 1.3) (King et al. 2009). Due to its high lipophilicity, endogenous CoQ is incapable of binding this “non-physiological” hydrophilic quinone-binding site. However, idebenone, which is less lipophilic than CoQ, is reduced by the complex I flavin in the hydrophilic site to form an unstable semiquinone that generates superoxide (Figure 1.3) (King et al. 2009).

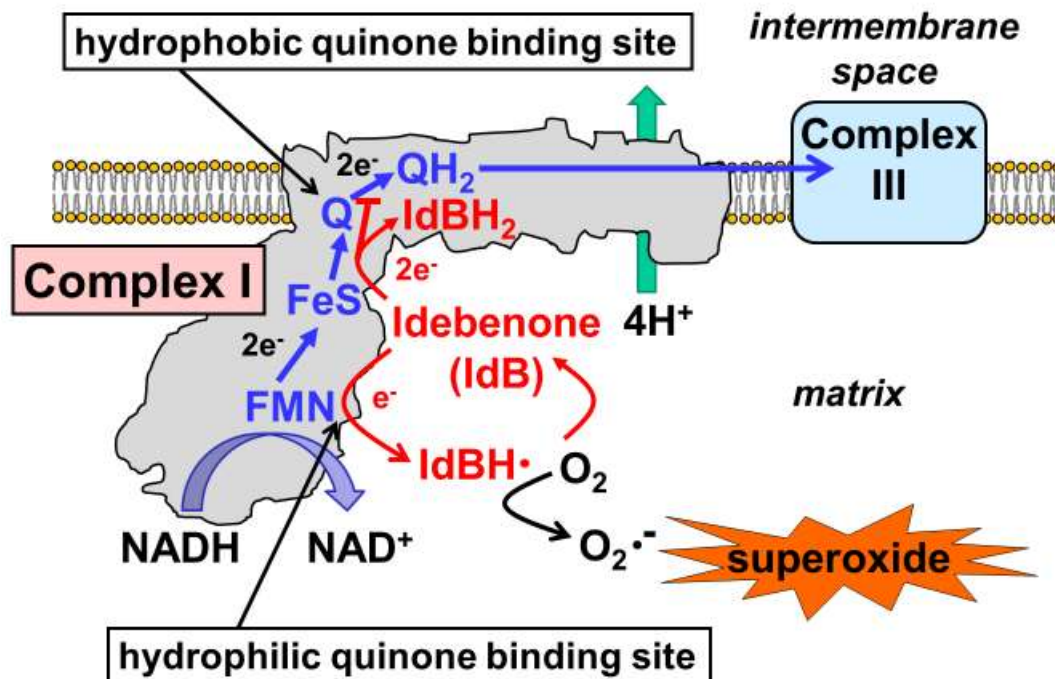


Figure 1.3 Model of how idebenone interacts with electron transport chain complex I. The oxidation of NADH by complex I results in the transfer of two electrons to complex III via ubiquinone/coenzyme Q₁₀ (Q), accompanied by the pumping of four protons (H⁺) from the mitochondrial matrix to the intermembrane space. Idebenone (IdB) competes with endogenous Q at the physiological hydrophobic quinone binding site for electrons from upstream iron-sulfur (FeS) clusters. The two-electron reduction of idebenone results in a product, idebenol (IdBH₂), with very slow dissociation kinetics from the quinone binding site. This slow dissociation not only makes idebenol ineffective at electron transfer to complex III but also allows it to competitively inhibit the electron transfer to complex III that normally occurs via endogenous ubiquinol (QH₂). A second interaction of idebenone with complex I occurs at a non-physiological hydrophilic quinone binding site that overlaps with the flavin mononucleotide (FMN) moiety. Here, the one electron reduction of idebenone yields an unstable semiquinone intermediate (IdBH·). This semiquinone causes the one-electron reduction of oxygen (O₂) to superoxide (O₂^{·-}), regenerating idebenone. The net results of idebenone-complex I interactions are impaired complex I functionality and superoxide generation.

Mitochondrial complex I inhibition combined with superoxide generation sounds like a nice recipe for neurotoxicity. Both events are thought to contribute to dopaminergic neurodegeneration in Parkinson's disease (Sherer *et al.* 2007). So why isn't idebenone toxic? Interestingly, idebenone at concentrations ≥ 25 μM is in fact toxic to the human dopaminergic neuroblastoma cell line SH-SY5Y (Tai *et al.* 2011). In addition, Giorgio *et al.* found that idebenone causes mitochondrial depolarization and NADH depletion within a fibroblast/osteosarcoma cybrid cell line (Giorgio *et al.* 2012). These events are inhibited by cyclosporine A, an inhibitor of the cyclophilin D-regulated permeability transition pore implicated in neurodegeneration (Schinzel *et al.* 2005). Since the permeability transition pore is a large conductance ion channel in the mitochondrial inner membrane that opens in response to oxidative stress and calcium overload (Kowaltowski *et al.* 2001, Petronilli *et al.* 1994), a likely explanation is that idebenone acts as a pro-oxidant in these cells by forming semiquinone radicals at complex I. Consistent with the possibility that idebenone promotes permeability transition pore opening via oxidative stress, N-ethylmaleimide, an agent that blocks permeability transition pore-regulating sulfhydryl groups (Petronilli *et al.* 1994), prevents idebenone from initiating pore-induced swelling of calcium-loaded mitochondria (Giorgio *et al.* 2012). Remarkably, supplementation of idebenone with the reducing agent dithiothreitol not only attenuates permeability transition pore opening but enables idebenone to restore oxygen consumption in cells with deficient or inhibited complex I (Giorgio *et al.* 2012). Respiration nevertheless remains sensitive to the complex III inhibitor antimycin A, indicating that distal components of the ETC are required for idebenone to rescue oxygen consumption. Idebenol but not idebenone maintains ATP synthesis in permeabilized cells incubated

with complex I and II inhibitors rotenone and malonate, respectively (Giorgio et al. 2012), suggesting that idebenone in its reduced form idebenol can bypass these complexes and transfer electrons to complex III. Although complex I proton pumping remains impaired, proton pumping at complex III and complex IV is recovered, allowing restoration of proton-motive force for ATP synthesis.

The human safety of idebenone is well established. Therefore it follows that most cells must have an efficient means of reducing idebenone to idebenol to avert the potentially disastrous consequences of oxidative stress-induced permeability transition pore opening. Idebenone is reduced by succinate dehydrogenase, and succinate greatly potentiates the ability of idebenone to impede lipid peroxidation in brain mitochondria (Suno & Nagaoka 1989). However, once idebenone reaches mitochondria, reduction at complex I is also likely to occur, leading to respiratory inhibition and superoxide production. Recent studies by Haefeli et al. identified NAD(P)H:quinone acceptor oxidoreductase (NQO) enzymes, also known as DT-diaphorases, as major enzymes catalyzing extramitochondrial idebenone reduction at the expense of cytoplasmic NAD(P)H (Haefeli *et al.* 2011). Idebenone is able to rescue ATP levels in a variety of cell types exposed to the complex I inhibitor rotenone and the extent of rescue correlates with NQO1 mRNA expression level (Haefeli et al. 2011). Idebenone also protects against the loss of retinal ganglion cells *in vivo* caused by intravitreal rotenone injection (Heitz *et al.* 2012). *In vitro* evidence supports an electron bypass model of cytoprotection whereby idebenone shuttles electrons from cytoplasmic NAD(P)H to complex III of the ETC in NQO1-expressing cells (Figure 1.4), restoring mitochondrial ATP synthesis following complex I inhibition (Erb et al. 2012, Haefeli et al. 2011, Heitz et al. 2012). A similar

NQO1-dependent complex I bypass mechanism was previously proposed for the related quinones CoQ₁ and menadione (Conover & Ernster 1962, Chan *et al.* 2002). Notably, virtually no NQO1 mRNA expression is observed in SH-SY5Y cells (Haefeli *et al.* 2011), perhaps explaining the reported toxicity of idebenone to this cell type (Tai *et al.* 2011). Regarding idebenone's neuroprotective potential, a critical question now emerges: where in the central nervous system is NQO1 expressed and can its expression be manipulated?

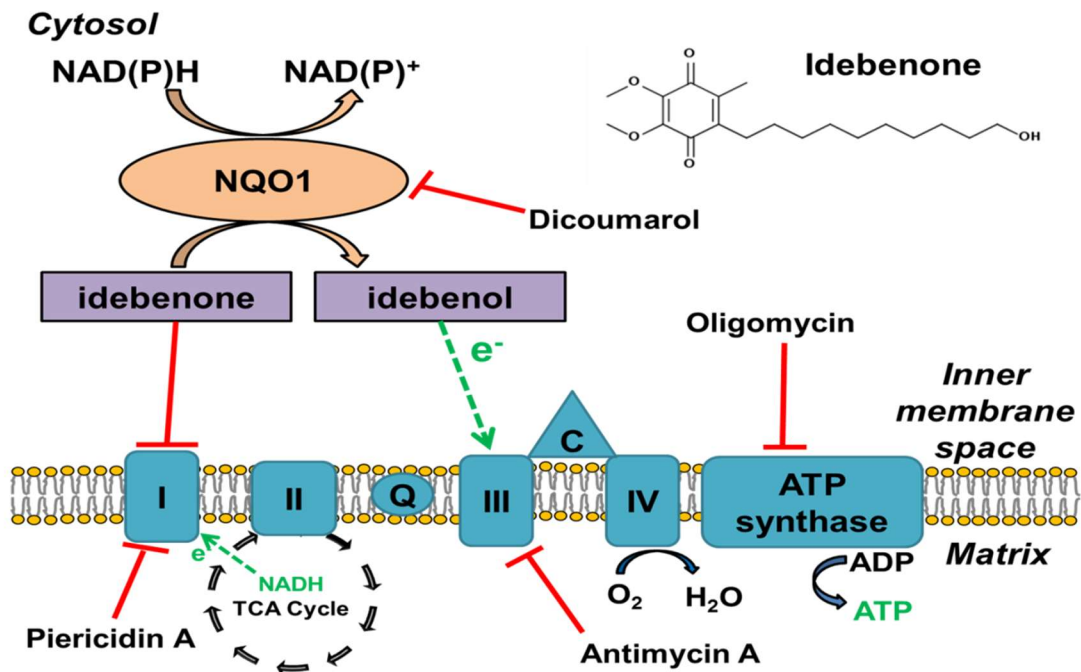


Figure 1.4 Model of NQO1-dependent shuttling of reducing equivalents from cytoplasmic NAD(P)H to mitochondrial electron transport chain complex III via idebenone. The two-electron reduction of idebenone to idebenol is catalyzed by NAD(P)H:quinone oxidoreductase 1 (NQO1) and occurs primarily in the cytoplasm. Idebenol is hydrophilic enough to traverse the cytoplasm but lipophilic enough to mediate electron transfer to complex III in the mitochondrial inner membrane. NQO1-catalyzed idebenone reduction may elude the deleterious consequences of idebenone-complex I interaction while idebenol may exert cytoprotection by bypassing disease-associated inhibition of complex I or upstream tricarboxylic acid cycle (TCA) enzymes. This bypass would restore oxygen (O₂) consumption at complex IV and ATP production by the ATP synthase. Stimuli which activate the nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor, including mild oxidative stress and the drugs sulforaphane and *tert*-butylhydroquinone, are predicted to act synergistically with idebenone by upregulating NQO1 expression

1.6 NQO1 Introduction

NQO1 is an antioxidant flavoprotein regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway (Ahlgren-Beckendorf *et al.* 1999, Dinkova-Kostova & Talalay 2010). While NQO1 is found mainly in the cytoplasm, small amounts localize to the mitochondria, endoplasmic reticulum and nucleus (Dong *et al.* 2013). NQO1 catalyzes the two-electron reduction of quinones to hydroquinones (Dinkova-Kostova & Talalay 2010, Lind *et al.* 1982). This reaction counteracts the one-electron reduction of quinones to free radical semiquinones catalyzed by NADPH:cytochrome P450 reductase and other enzymes (O'brien 1991, Joseph & Jaiswal 1994, Lind *et al.* 1982). Thus, the antioxidant activity of NQO1 limits superoxide formation by minimizing redox cycling of quinones between their semiquinone and quinone forms (O'brien 1991, Lind *et al.* 1982).

1.6.1 NQO1 Expression

NQO1 is expressed in many tissues throughout the body including the brain (Siegel & Ross 2000, Stringer *et al.* 2004, Schultzberg *et al.* 1988). In the healthy brain, NQO1 expression is predominately restricted to astrocytes and a subset of oligodendrocytes (Stringer *et al.* 2004), although expression in narrow neuronal subpopulations is also reported (Schultzberg *et al.* 1988).

1.6.2 NQO1 Regulation

Because the transcription factor Nrf2 is implicated in basal as well as inducible NQO1 expression (Bell *et al.* 2011), the relative lack of neuronal NQO1 expression may be due to the low basal level of Nrf2 in these cells (Shih *et al.* 2003). Given that NQO1 promotes the ability of idebenone to act as an effective antioxidant and electron carrier to

complex III (Haefeli et al. 2011), the low expression of NQO1 in neurons may mean that idebenone has limited potential to protect these cells by cell autonomous mechanisms.

Fortuitously, NQO1 is an inducible enzyme (Dinkova-Kostova & Talalay 2010). Relevant to neurodisease, a primary inducer is oxidative stress (Greco & Fiskum 2010). Oxidation of cysteine residues on Kelch-like ECH-associated protein 1 (KEAP1) releases the transcription factor Nrf2 from an inhibitory cytoplasmic interaction, enabling Nrf2 relocalization to the nucleus where it initiates transcription of NQO1 and other ARE-controlled genes (Figure 1.5) (Li & Kong 2009).

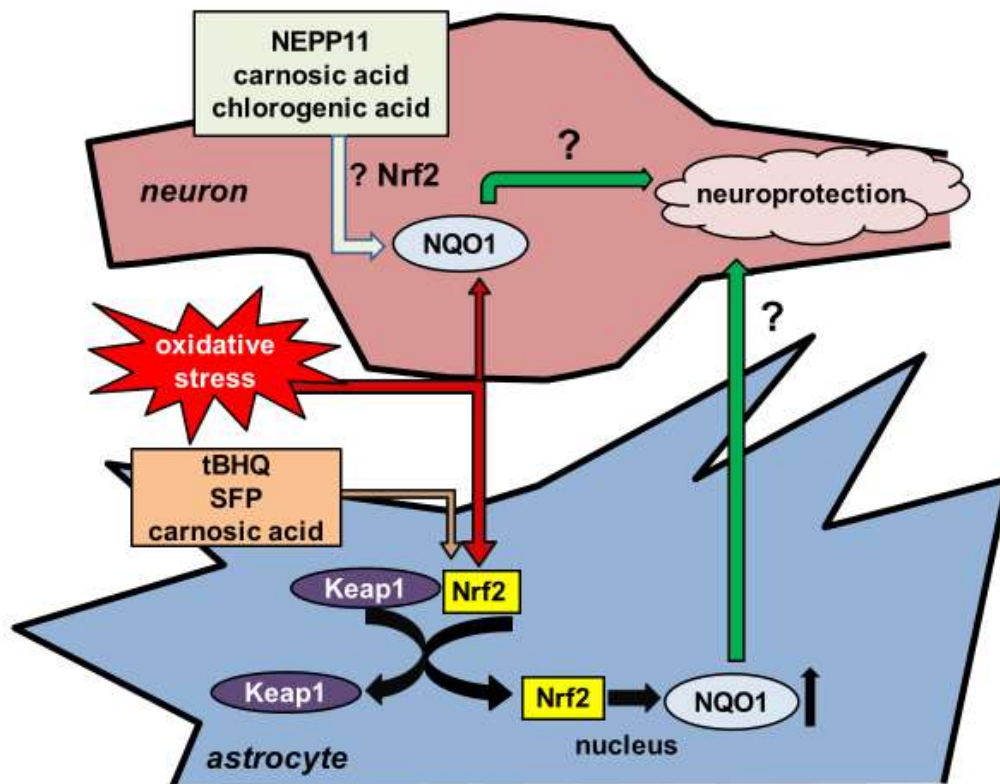


Figure 1.5 Oxidative stress leads to Keap1 dissociation and Nrf2 activation. Nrf2 is inactive when sequestered by Keap1. Nrf2-dependent NQO1 expression can be activated by small molecule electrophiles, including sulforaphane (SFP) or tert-butylhydroquinone (tBHQ) in astrocytes, by carnosic acid in both astrocytes and neurons, and by NEPP11 in neurons. Chlorogenic acid may induce neuronal NQO1 expression by Nrf2-independent mechanisms, although this has yet to be firmly established.

Interestingly, while NQO1 is expressed sparingly by neurons in the healthy brain, neuronal NQO1 is elevated in Alzheimer's disease (Raina *et al.* 1999, SantaCruz *et al.* 2004, Wang *et al.* 2000) and Parkinson's disease (van Muiswinkel *et al.* 2004), two neurodegenerative conditions with a major oxidative component (Lin & Beal 2006). In Alzheimer's disease, NQO1 expression and activity are observed in hippocampal neurons in close proximity to pathology, including in neurofibrillary tangles (Raina *et al.* 1999, SantaCruz *et al.* 2004, Wang *et al.* 2000). Therefore it is possible that oxidative stress associated with the disease process itself will enhance the antioxidant and electron transport functions of idebenone by upregulating NQO1. However, it is also possible that NQO1 is expressed primarily by dying neurons that are beyond rescue.

Chapter 2: Idebenone has distinct effects on mitochondrial respiration in astrocytes compared to neurons due to differential NQO1 activity

2.1 Introduction

Many disorders affecting the nervous system are characterized by oxidative stress and mitochondrial bioenergetic dysfunction, which contribute to pathology (Lin & Beal 2006). Complex I of the mitochondrial electron transport chain (ETC) is particularly susceptible to impairment by oxidative stress (Danielson *et al.* 2011, Srinivas Bharath 2017a) and is dysfunctional in various pathological conditions, including Parkinson's disease (Schapira *et al.* 1990, Keeney *et al.* 2006), Friedreich's ataxia (Heidari *et al.* 2009, Salehi *et al.* 2014), and Leber's Hereditary Optic Neuropathy (LHON) (Chinnery *et al.* 2001). Complex I shuttles electrons to ubiquinone, also known as Coenzyme Q₁₀ (CoQ), which subsequently carries electrons downstream to complex III (Nicholls & Ferguson 2013). Complex I also pumps protons out of the matrix to help generate the proton motive force required for ATP production. Complex I of the mitochondrial electron transport chain (ETC) is particularly susceptible to impairment by oxidative stress and is dysfunctional in various pathological conditions, including Parkinson's disease, Friedreich's ataxia, and Leber's Hereditary Optic Neuropathy (LHON). Complex I shuttles electrons to ubiquinone, also known as Coenzyme Q₁₀ (CoQ), which subsequently carries electrons downstream to complex III.

CoQ, in addition to its important role as an electron carrier, is an antioxidant abundantly found in cellular membranes (James *et al.* 2004). However, CoQ displays poor bioavailability (Tomono *et al.* 1986), hindering its therapeutic use. Idebenone contains the same quinone moiety as CoQ but has a shorter, less hydrophobic tail that

increases solubility and improves bioavailability (Gueven *et al.* 2015). Idebenone-mediated neuroprotection was observed in various *in vitro* and *in vivo* models of neuronal injury, including oxidative stress (Ratan *et al.* 1994), stroke (Nagaoka *et al.* 1989), Alzheimer's disease (Yamada *et al.* 1999), and LHON (Heitz *et al.* 2012).

Despite initially positive reports (Gutzmann & Hadler 1998, Gutzmann *et al.* 2002), idebenone ultimately failed in clinical trials for Alzheimer's disease (Thal *et al.* 2003). Clinical trials of idebenone in Friedreich's ataxia patients gave mixed results with regard to neurological symptoms (Di Prospero *et al.* 2007a, Lynch *et al.* 2010, Meier *et al.* 2012) and idebenone has yet to be approved in the U.S. for any disease treatment. However, it is still being tested in several active clinical trials, including for LHON and Duchenne muscular dystrophy. Importantly, idebenone was recently approved in Europe for the treatment of LHON and mitigates loss of visual acuity (Klopstock *et al.* 2011, Lyseng-Williamson 2016). A better understanding of how idebenone affects the bioenergetics of different types of brain cells may help improve upon its clinical efficacy for LHON treatment and promote success in ongoing disease trials. Therefore, our objective was to elucidate the mitochondrial bioenergetic effects of idebenone on neurons and astrocytes

NAD(P)H:quinone oxidoreductase 1 (NQO1) is an enzyme regulated by the nuclear erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) transcriptional antioxidant response pathway (Dinkova-Kostova & Talalay 2010, Li & Kong 2009) that is able to reduce idebenone to idebenol via cytoplasmic NAD(P)H oxidation (Haefeli *et al.* 2011). Idebenol can then transfer electrons to complex III like CoQ, bypassing a need for upstream electron transport chain components, including

complex I. The relative ability of idebenone to restore ATP levels in the presence of a complex I inhibitor across several cell types correlated well to the level of NQO1 mRNA expression (Haefeli et al. 2011). NQO1 immunoreactivity was detected in mouse retinal ganglion cells, the main degenerating cell type in LHON (Heitz et al. 2012), coinciding with the clinical efficacy of idebenone in that disease. However, in the brain, NQO1 is predominately expressed by astrocytes, with little to no expression in cortical neurons (Schultzberg et al. 1988, Stringer et al. 2004, Bell *et al.* 2015). We therefore tested the hypothesis that deficient NQO1 expression in cortical neurons relative to astrocytes is limiting for the ability of idebenone to stimulate complex I-independent respiration. Our results support this hypothesis and additionally show that idebenone impairs neuronal mitochondrial respiratory capacity by inhibiting complex I-dependent respiration. We predicted that pharmacological stimulation of NQO1 expression by activators of the Nrf2 pathway would overcome this idebenone-mediated respiratory inhibition. We provide proof-of-principle that induction of NQO1 in cells with poor NQO1 expression promotes the ability of idebenone to act as a direct respiratory chain electron donor.

2.2 Materials and Methods

2.2.1 Astrocyte culture

All animal procedures were approved by the University of Maryland Institutional Animal Care and Use Committee and were consistent with the NIH Guide for the Care and Use of Laboratory Animals. Cortical astrocytes were isolated from postnatal day 1 Sprague Dawley rat pups as described (Zielke *et al.* 1990, Danilov & Fiskum 2008). Following euthanasia, cortices were separated from midbrain and meninges and mechanically homogenized in Eagle's Minimal Essential Medium (EMEM, Quality

Biological, Gaithersburg, MD) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO), 1% non-essential amino acids (NEAA, Lonza, Walkersville, MD), and 100 µg/ml gentamicin (Quality Biological). The homogenate was vortexed for one minute and then filtered through a 70 µm mesh filter. Cells were plated in EMEM containing 10% FBS and 1% NEAA into 150 cm² vented flasks (Sigma-Aldrich) and maintained in a humidified incubator in 95% air/5% CO₂ at 37°C. Cultures were passaged after 18 days *in vitro* (DIV) using TrypLE Express (Thermo Fisher Scientific) and utilized for experiments between DIV 20-27. This method yields a >93% astrocyte-enriched culture, with <5% microglia and <2% oligodendrocytes or neurons (Danilov & Fiskum 2008). Astrocytes were seeded at least 2 days prior to measurement on Seahorse XF24 V7-PS Cell Culture Microplates (Agilent Technologies, Santa Clara, CA) at a density of 6 x 10⁴ cells per well. Cells derived from both males and females were used for experiments.

2.2.2 Neuron culture

Primary cortical neurons were prepared from E18 Sprague Dawley male and female pooled rat embryos (one litter per preparation, ~12 embryos) as previously described (Yakovlev *et al.* 2001, Stoica *et al.* 2005, Laird *et al.* 2013). Briefly, XF24 V7-PS cell plates were coated using poly-D-lysine hydrobromide (0.1 mg/ml, Sigma-Aldrich) at least 4 hours in advance of plating, incubated at 37°C, and washed once with sterile water. Isolated cortices were digested in trypsin and treated with DNase I (Sigma-Aldrich). Cell suspension was filtered through a 70 µm mesh filter and plated in Neurobasal media containing either B27 (Thermo Fisher Scientific, Waltham, MA) or Gem21 supplement (Gemini Bio-Products, Sacramento, CA), 10% FBS, and 100 IU/ml

penicillin with 100 mg/ml streptomycin (Gemini Bio-Products). Neurons were plated at 6×10^4 to 8×10^4 cells per well for respiration measurements. After 2 hours, medium was replaced with Neurobasal containing B27 or Gem21 without FBS. Cytosine arabinofuranoside (5 μ M, Sigma-Aldrich) was added on DIV 4 to inhibit glial proliferation. Additional FBS-free Neurobasal media equal to 50% of the existing total media volume was added on DIV 6. This method yields a >95% neuron-enriched culture (Laird et al. 2013). Neurons were utilized for experiments between DIV 10-14.

2.2.3 COS-7 cell culture.

Subjects included the following cell line: CLS Cat# 605470/p532_COS-7, RRID: CVCL_0224 (COS-7 cells). COS-7 cells were maintained in Dulbecco's Minimal Essential Medium (DMEM, Corning, Corning, NY) containing 10% FBS and 100 IU/ml penicillin with 100 mg/ml streptomycin. COS-7 cells were passaged using TrypLE Express and were plated at 2×10^4 cells per well for respiration studies. Cells were utilized after 72 hours of carnosic acid (3 μ M) or vehicle treatment.

2.2.4 Cellular respirometry

Cellular respiration was measured using a Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA) as described (Wu *et al.* 2007, Clerc & Polster 2012). Assay medium was artificial cerebrospinal fluid (aCSF) consisting of 120 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 1 mM MgCl₂, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 mg/ml fatty-acid free bovine serum albumin (BSA), and 15 mM glucose (pH 7.4) at 37°C. When employed, saponin and sodium pyruvate were always prepared fresh from powder. Prior to assays, cell culture medium was removed, cells were washed once in 1 ml aCSF, and then cells were

incubated in 675 μ l of aCSF in a CO₂-free incubator (37°C) for 45 minutes. Drug treatments were diluted in 75 μ l aCSF and added to the XF24 Seahorse assay cartridge (Agilent Technologies) at 10X, 11X, 12X, or 13X the final working concentration for ports A-D, respectively. Three measurements of oxygen consumption rate (OCR) were made prior to any drug additions to establish a baseline.

Enzymatically active recombinant human NQO1 (Sigma-Aldrich Cat# D1315) and NADPH substrate (Sigma-Aldrich) were delivered to neurons via saponin permeabilization. In permeabilized cell experiments, saponin was used at low enough concentrations to permeabilize cholesterol-containing membranes such as the plasma membrane while leaving the cholesterol-poor mitochondrial inner and outer membranes intact (Clerc & Polster 2012). Saponin (25 μ g/ml) was added with the complex I-linked substrates pyruvate and malate (5 mM each), the ATP synthase substrates ADP (1 mM) and phosphate (3.6 mM KH₂PO₄) to stimulate respiration, and the calcium chelator EGTA (5 mM) to reduce calcium to a normal cytoplasmic level (~100 nM). Subsequent injection of succinate (5 mM) was used to support complex II-dependent respiration in permeabilized cells.

2.2.5 Antibodies

The following antibodies were used: primary rabbit polyclonal anti-NQO1 (Sigma-Aldrich Cat# N5288, RRID:AB_1841045), primary rabbit monoclonal anti- β -glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cell Signaling Technology, Danvers, MA, Cat# 2118, RRID:AB_561053), and secondary goat anti-rabbit IgG (H+L) HRP antibody (Thermo Fisher Scientific Cat# 65-6120, RRID: AB_2533967).

2.2.6 *Western blot*

Cells were hypotonically lysed in 25 mM Tris-HCl and 1:200 protease inhibitor cocktail (EMD Millipore, Billerica, MA). Novex Tris-Glycine SDS Sample Buffer and Novex NuPAGE Sample Reducing Agent (Thermo Fisher Scientific) were added and the samples were heated for 10 minutes at 95°C. Samples were then centrifuged for 10 minutes at 14,000g and protein concentrations were measured using a NanoDrop ND-1000 (Thermo Fisher Scientific). Novex WedgeWell Tris-Glycine gels (Thermo Fisher Scientific) were loaded with 10 µg of protein per well. Transfer to a polyvinylidene difluoride (PVDF) membrane was performed using the Bio-Rad Trans-Blot Transfer System (Bio-Rad Laboratories, Hercules, CA). Membranes were probed overnight at 4°C using the NQO1 and GAPDH antibodies described above at 1:10,000 dilution. Blots were then washed and treated with horseradish peroxidase-conjugated secondary anti-rabbit antibody (Thermo Fisher Scientific). Bands were visualized using SuperSignal West Pico ECL (Thermo Fisher Scientific) and imaged using the Bio-Rad ChemiDoc XRS. Densitometric quantification of band intensities was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

2.2.7 *Experimental Design and Statistical Analysis*

NQO1 protein levels were analyzed by Student's *t* test. OCR data from the experiment evaluating the effects of idebenone on neurons compared to astrocytes were analyzed using a two-way analysis of variance (ANOVA), with cell type and idebenone treatment as factors. OCR data from the experiment evaluating the effect of idebenone treatment on astrocytes plus and minus concomitant dicoumarol treatment were analyzed using two-way ANOVA, with idebenone and dicoumarol treatment as factors. OCR data

from the experiment evaluating the effect of recombinant NQO1 treatment on astrocytes plus and minus idebenone were analyzed using two-way ANOVA, with idebenone treatment and NQO1±NADPH co-treatment as factors. OCR data from the experiment evaluating the effect of idebenone on carnosic acid pre-treated vs. vehicle pre-treated cells were analyzed using a two-way ANOVA, with the pre-treatment and the subsequent treatment as factors. All other OCR data were analyzed by one-way ANOVA. Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Significance level was set at $p < 0.05$. When a significant overall difference was detected, Tukey's post-hoc analysis was used to compare individual groups and p-values for comparisons of interest are reported. Data from 2-3 wells (technical replicates) per experiment were averaged to obtain one biological replicate from an independent cell culture preparation. Biological replicates are reported as mean \pm standard error (SE) in all figures.

2.3 Results

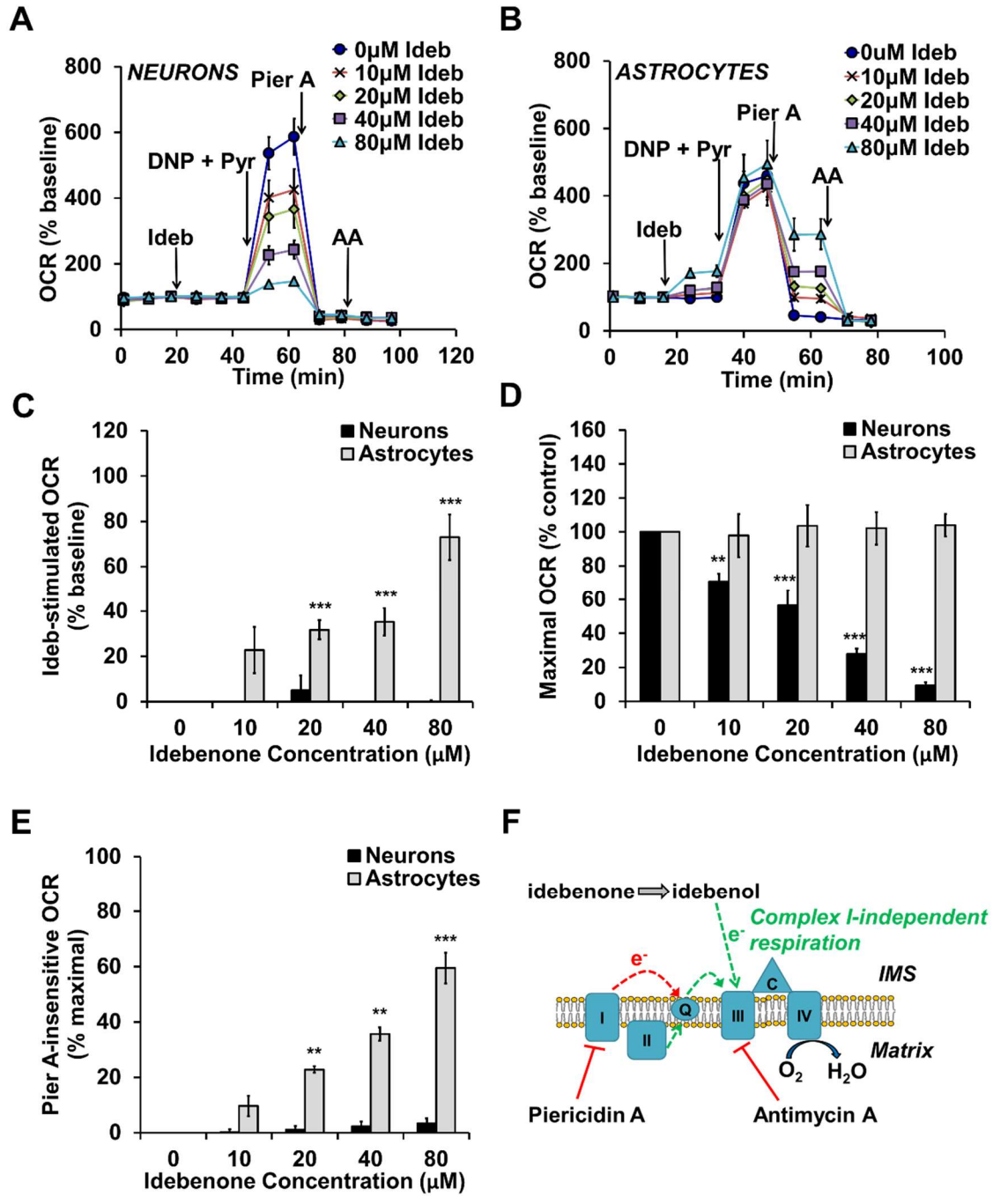
2.3.1 Idebenone differentially affects oxygen consumption by neurons compared to astrocytes

The effects of idebenone on cellular respiration of primary rat cortical neurons and astrocytes were investigated using a Seahorse XF24 Extracellular Flux analyzer. We found a significant interaction between idebenone concentration and cell type on basal and maximal oxygen consumption rate ($F_{(4,37)}=11.74$, $p < 0.001$ and $F_{(4,33)}=24.28$, $p < 0.001$, respectively). Oxygen consumption rate (OCR) by cortical neurons was not altered in response to idebenone injection (all *post-hoc* comparisons $p \geq 0.998$, Figure 2.2.1A,C), but maximal respiration, measured after addition of the uncoupler 2,4-dinitrophenol (DNP)

and excess mitochondrial substrate, was inhibited in a dose-dependent manner ($p=0.0359$ for 10 μM idebenone compared to control (0 μM idebenone), $p<0.001$ for all other *post-hoc* idebenone to control comparisons, Figure 2.1A,D). Idebenone can donate electrons to complex III of the mitochondrial electron transport chain in some cell types, bypassing complex I (Haefeli et al. 2011, Giorgio et al. 2012). There was a significant interaction between idebenone concentration and cell type (neurons vs. astrocytes) on the measured OCR subsequent to addition of the complex I inhibitor piericidin A ($F_{(4,33)}=22.28$, $p<0.001$). However, piericidin A addition to neurons after idebenone and DNP were added impaired respiration to the same extent as in cells that received no idebenone (all *post-hoc* comparisons $p>0.999$, Figure 2.1A,E), suggesting that idebenone does not support electron flux directly to complex III in neurons.

In contrast to neurons, primary rat cortical astrocytes treated with idebenone demonstrated a rapid, dose-dependent stimulation of basal respiration compared to control (p -values were 0.288, 0.0036, 0.0015, and <0.001 for 10, 20, 40, and 80 μM idebenone, respectively, Figure 2.1B,C) and no effect on maximal O_2 consumption rate (all *post-hoc* comparisons $p>0.848$, Figure 2.1B,D). Additionally, a dose-dependent rescue of piericidin A-impaired respiration was observed with increasing concentrations of idebenone ($p<0.001$ for 20, 40, or 80 μM idebenone compared to control, and for 40 μM compared to 80 μM idebenone, Figure 2.1B,E,F), suggesting bypass of inhibited complex I. Oxygen consumption in the presence of piericidin A and idebenone was blocked by injection of the complex III inhibitor antimycin A (Figure 2.1B,F), indicating that complex III and downstream components of the ETC are required for the idebenone rescue of respiration in astrocytes.

Figure 2.1 Idebenone stimulates complex I-independent respiration in astrocytes but inhibits maximal respiration in neurons. (A), O₂ consumption rate (OCR) measurements from primary rat cortical neurons. Idebenone (Ideb) was added at 0 (ethanol vehicle) to 80 μM, as indicated. The uncoupler 2,4-dinitrophenol (DNP, 200 μM) was then added in the presence of pyruvate (Pyr, 10 mM) to induce maximal OCR. Piericidin A (Pier A, 100 nM) and Antimycin A (AA, 1 μM) were added to inhibit complex I and complex III, respectively. (B), The same experiment described in (A) but using primary rat cortical astrocytes in place of neurons and 150 μM DNP rather than 200 μM. Data in (A) are mean ± SE from 3-5 independent experiments and data in (B) are mean ± SE from 3-7 independent experiments. (C-E), Bar graph representations of data from (A) and (B). C, OCR after idebenone injection relative to basal OCR. Some values were slightly negative compared to baseline but are depicted as zero since OCR stimulation is plotted. (D), OCR after injection of DNP plus pyruvate (“Maximal OCR”) with increasing idebenone concentration relative to vehicle (0 idebenone). (E), OCR after injection of piericidin A with increasing idebenone concentration, relative to maximal OCR of control (0 idebenone). (F), Schematic diagram illustrating the ability of idebenone to support complex I-independent respiration. The complex I inhibitor piericidin A suppresses the flow of electrons (e⁻, shown in red) from complex I to CoQ (Q). Idebenone, when reduced to idebenol, transfers e⁻ to complex III, bypassing the block at complex I. Electron entry through complex II (green arrows) can also bypass complex I inhibition (see Figure 4). IMS, intermembrane space. **p<0.01 relative to vehicle control; ***<0.001 relative to control, two-way ANOVA followed by Tukey’s *post hoc* test.



2.3.2 Idebenone-stimulated respiration in astrocytes is coupled to ATP synthesis

To test whether uncoupling contributes to idebenone-stimulated OCR in astrocytes, cells were treated with the ATP synthase inhibitor oligomycin after idebenone addition (Figure 2.2A). Oligomycin suppresses oxygen consumption dedicated to ATP synthesis (Brand & Nicholls 2011). Residual OCR is primarily due to proton leak across the mitochondrial inner membrane. The oligomycin sensitivity of respiration can therefore be used to estimate the amount by which electron transport is coupled to ATP synthesis compared to that which is uncoupled due to proton leak (Brand & Nicholls 2011). Oligomycin suppressed OCR to the same extent in the absence and presence of idebenone (one-way ANOVA, $F_{(4,14)}=0.429$, $p=0.786$, Figure 2.2B). Because the enhanced OCR upon idebenone addition was abolished by oligomycin, idebenone-stimulated OCR is therefore likely coupled to ATP synthesis. Residual OCR is primarily due to proton leak across the mitochondrial inner membrane. The oligomycin sensitivity of respiration can therefore be used to estimate the amount by which electron transport is coupled to ATP synthesis compared to that which is uncoupled due to proton leak.

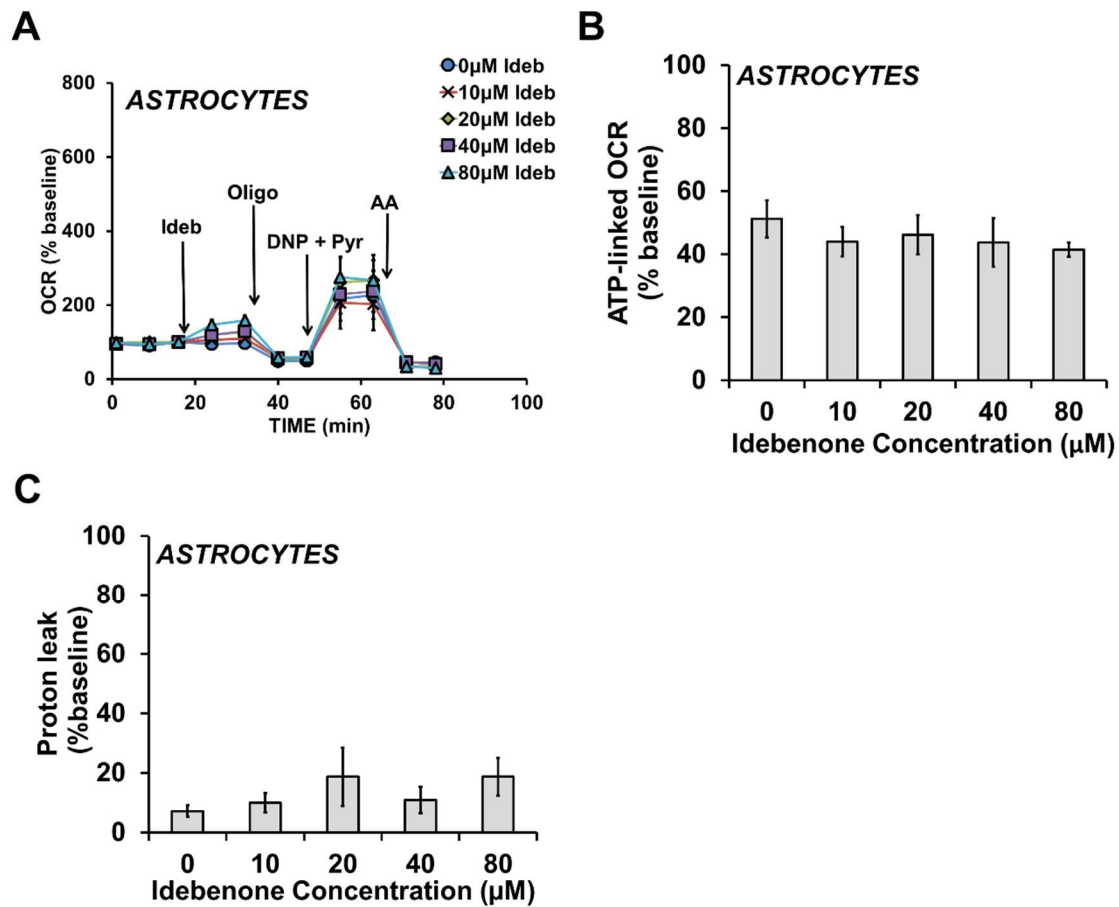


Figure 2.2 Idebenone-stimulated astrocytic respiration is coupled to ATP synthesis. (A), OCR measurements from primary rat cortical astrocytes. Idebenone (Ideb) was added at 0 (vehicle) to 80 µM, as indicated, followed by oligomycin (Oligo, 0.3 µg/ml) and finally Antimycin A (AA, 1µM). Data are mean ± SE from 3 independent experiments. (B and C), Bar graph representation of the data in (A). (B), ATP-linked respiration and (C), proton leak are relative to basal respiration. There were no significant differences (one-way ANOVA).

2.3.3 Inhibition of NQO1 in astrocytes reverses the ability of idebenone to promote Complex I-independent respiration

The enzyme NQO1 reduces idebenone to idebenol in the presence of NAD(P)H, inducing bioactivity (Haefeli et al. 2011). We quantified NQO1 protein expression levels in whole cell lysates of primary rat cortical astrocytes and neurons. As predicted by the reported expression pattern of NQO1 in the brain (Schultzberg et al. 1988, Stringer et al. 2004, Bell et al. 2015), astrocytes displayed abundant expression of NQO1 while NQO1 immunoreactivity was essentially absent in neurons (Figure 2.3A, $t_{(4)}=22.96$, $p<0.001$). To test whether NQO1 activity is required for the direct electron transfer activity of idebenone in astrocytes, idebenone was added in the presence or absence of the NQO1 inhibitor dicoumarol (Figure 2.3B). Dicoumarol was used at concentrations (20-80 μM) that did not induce uncoupling or respiratory inhibition (Figure 2.3B and data not shown). There was a significant interaction between idebenone and dicoumarol treatment on maximal OCR ($F_{(3, 27)}=4.399$, $p=0.0121$). In the presence of dicoumarol (40 or 80 μM), 80 μM idebenone significantly impaired maximal respiration compared to control ($p=0.0144$ and $p=0.0100$ for 40 and 80 μM dicoumarol, respectively, Figure 2.3C) and piericidin A-insensitive respiration was reduced ($p<0.001$ for both 40 and 80 μM dicoumarol added with 80 μM idebenone compared to 80 μM idebenone alone, Figure 2.3D). Thus, addition of NQO1 inhibitor to astrocytes recapitulates the bioenergetic effects of idebenone on neurons that lack NQO1.

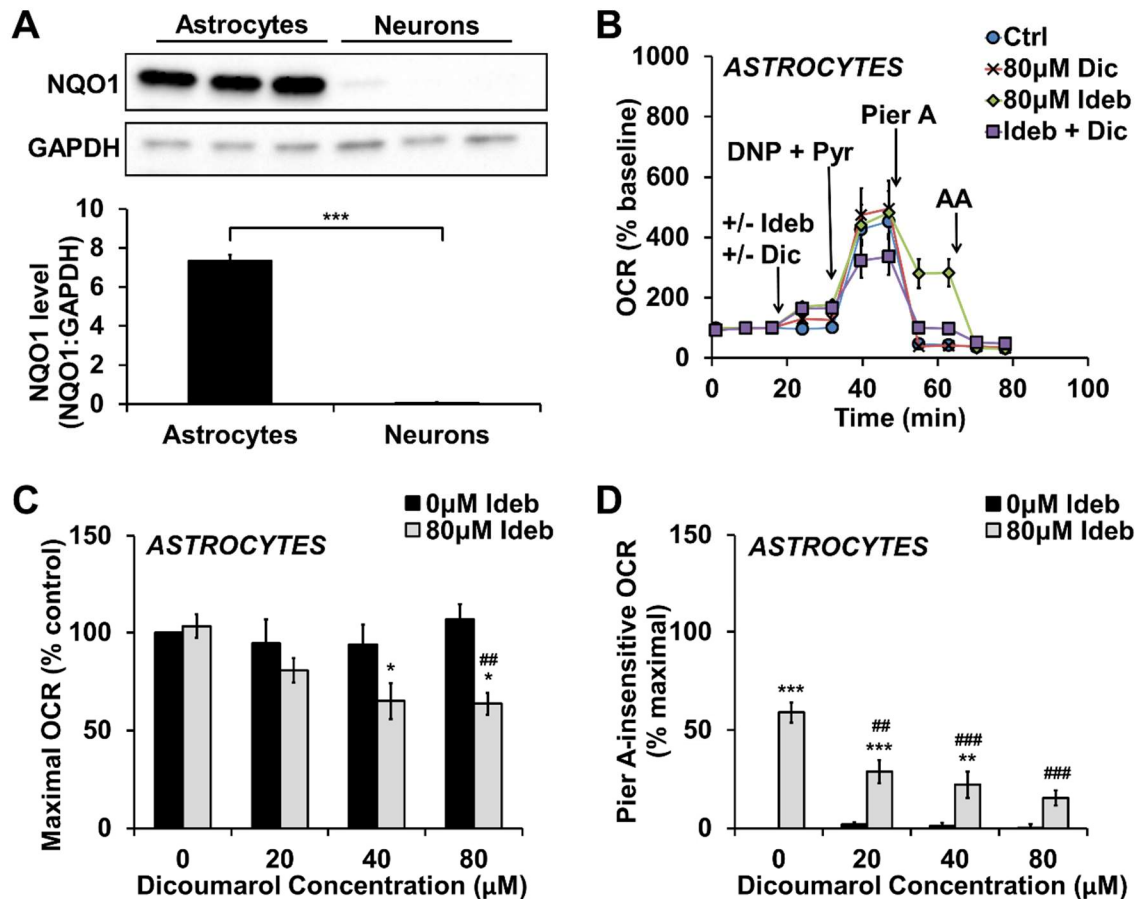


Figure 2.3 The ability of idebenone to support complex I-independent respiration in astrocytes is reversed by NQO1 inhibitor. (A), Immunoblot for NQO1 in primary rat cortical astrocytes and neurons, along with immunodetection of GAPDH as a loading control. NQO1 levels are expressed as a ratio to GAPDH and are mean \pm SE from 3 independent experiments. *** p <0.001, Student's t test. (B), OCR measurements from primary rat cortical astrocytes. The initial injection contained vehicle, dicoumarol (Dic, 80 μ M), idebenone (Ideb, 80 μ M), or the two drugs combined. DNP+Pyr was added as in Figure 1, followed by piericidin A (Pier A, 500 nM), and then antimycin A (AA, 1 μ M). Data are mean \pm SE from 3-7 independent experiments. (C, D), Bar graph representations of data in (B) and including additional dicoumarol treatments not depicted in (B) for clarity. (C), Maximal OCR with and without idebenone and increasing concentrations of dicoumarol, expressed as a percentage of control (no dicoumarol or idebenone). (D), OCR after injection of piericidin A with and without idebenone and increasing concentrations of dicoumarol, relative to maximal OCR of control (no dicoumarol or idebenone). * p <0.05 relative to vehicle control; ** p <0.01 relative to control; *** p <0.001 relative to control; ## p <0.01 relative to 80 μ M idebenone alone; ### p <0.001 relative to 80 μ M idebenone alone, two-way ANOVA followed by Tukey's *post hoc* test.

2.3.4 Idebenone inhibits complex I-dependent neuronal respiration

Next, we sought to determine the site of respiratory inhibition by idebenone in neurons (see Figure 2.1A,D), with the goal of reversing inhibition. Neurons were permeabilized by saponin to deliver substrates for specific ETC complexes. Saponin, when titrated carefully, permeabilizes the plasma membrane without affecting the integrity of mitochondrial membranes (Safiulina *et al.* 2004, Clerc & Polster 2012). When idebenone was added to permeabilized neurons exposed to complex I-linked substrates, we found that idebenone impaired ADP-stimulated oxygen consumption in a dose-dependent manner (Figure 2.4A,B, $F_{(4,10)}=11.87$, $p<0.001$). The complex II substrate succinate rescued the majority of the respiratory impairment by idebenone, suggesting that idebenone inhibits the electron transport chain at or upstream of complex I (Figure 2.4A,C). Following succinate treatment, we still detected a minor respiratory impairment across all concentrations of idebenone ($F_{(4,10)}=3.495$, $p=0.0494$), however none of the *post-hoc* comparisons were significant.

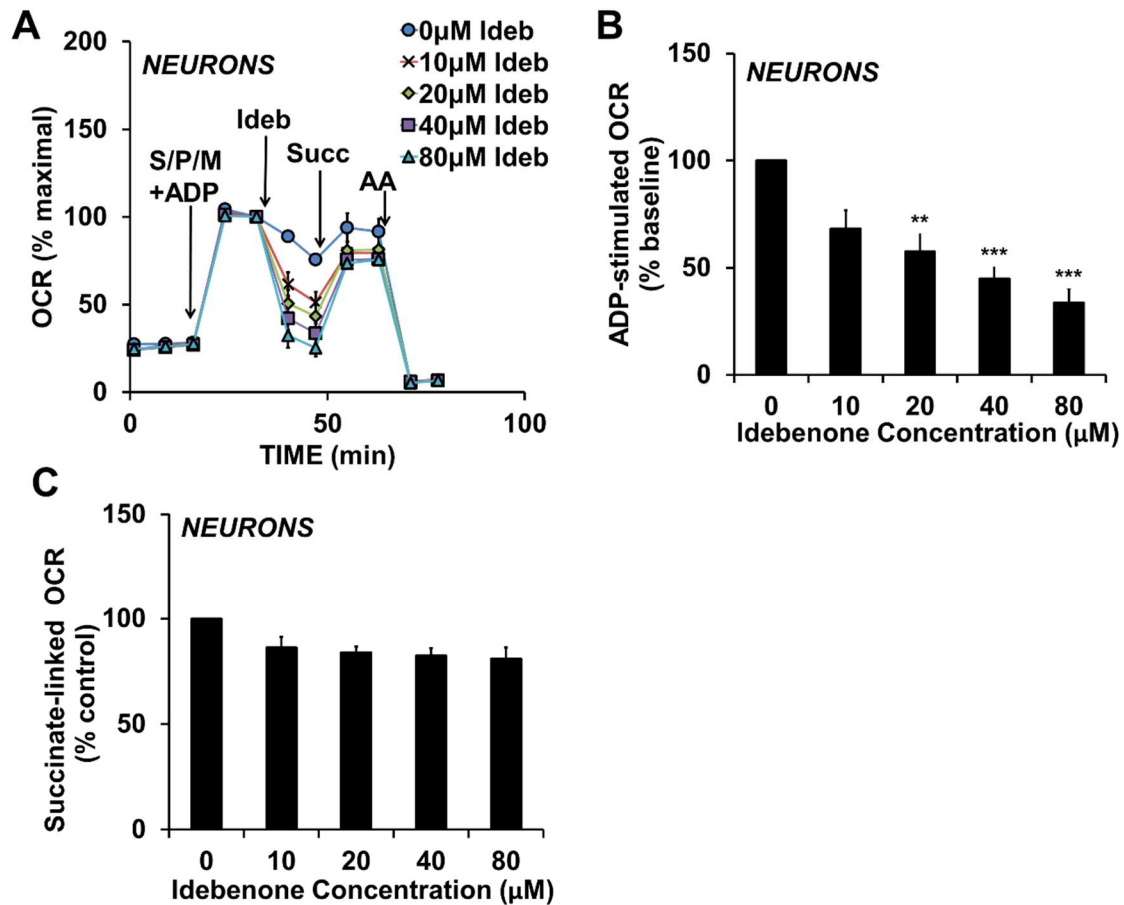


Figure 2.4 Idebenone inhibits complex I-dependent respiration, but not the downstream electron transport chain. (A), OCR measurements from cortical neurons. Neurons were first permeabilized by the injection of saponin in the presence of the complex I-linked substrates pyruvate and malate (collectively abbreviated as S/P/M), as described under Materials and Methods, with ADP (1 mM) added to stimulate respiration. Idebenone (Ideb) was then added at a concentration of 0 (vehicle) to 80 μM , as indicated. Subsequent additions were succinate (Succ, 5 mM) followed by antimycin A (AA, 1 μM). OCR measurements are normalized to the point just prior to idebenone/vehicle addition (ADP-stimulated baseline). Data are mean \pm SE from 3-4 independent experiments. (B), (C), Bar graph representations of data in (A). (B), OCR after idebenone addition with ADP and complex I substrates present (pyruvate and malate). (C), OCR after idebenone addition once the complex II substrate succinate was added. ** $p < 0.01$ relative to vehicle control; *** $p < 0.001$ relative to control, one-way ANOVA followed by Tukey's *post hoc* test.

2.3.5 Recombinant NQO1 and NADPH reverse respiratory inhibition by idebenone

If the absence of NQO1 expression in neurons accounts for the distinct effects of idebenone on respiration relative to astrocytes, then supplementation of neurons with exogenous NQO1 and its substrate NADPH should prevent respiratory inhibition by idebenone and induce an astrocyte-like phenotype. Primary cortical neurons were permeabilized and provided with complex I substrates in the presence or absence of recombinant human NQO1 and NADPH (Figure 2.5A). We detected a significant interaction between idebenone treatment and NQO1/NADPH treatment on both ADP-stimulated OCR and piericidin A-insensitive OCR ($F_{(3,30)}=4.213$, $p=0.0134$ and $F_{(3,22)}=3.985$, $p=0.0208$, respectively). As shown earlier, ADP-stimulated oxygen consumption was inhibited after addition of idebenone alone ($p<0.001$). Respiratory inhibition was reversed when idebenone was added in the presence of both exogenous NQO1 and its substrate, NADPH ($p=0.0014$), but not when NQO1 alone ($p=0.975$) or NADPH alone ($p>0.999$) was added (Figure 2.5A,B). The joint addition of idebenone, NQO1, and NADPH also induced a significant increase in piericidin A-insensitive oxygen consumption ($p<0.001$, Figure 2.5A,C) that was inhibited by antimycin A (Figure 2.5A).

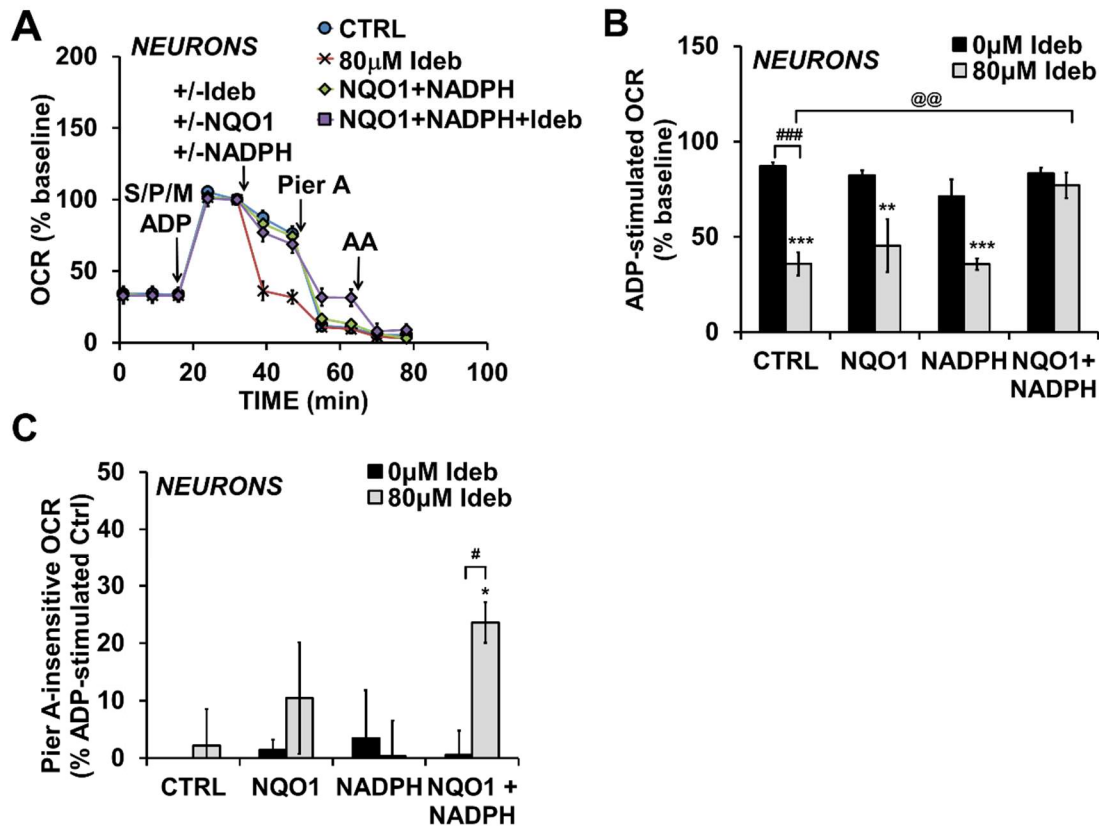


Figure 2.5 Delivery of recombinant NQO1 and NADPH reverses neuronal respiratory inhibition by idebenone. (A), OCR measurements from cortical neurons. Cells were first permeabilized with complex I substrates present (S/P/M), as in Figure 2.4 (A). Next, vehicle, idebenone (Ideb, 80 μ M), NADPH (0.5 mM), and recombinant human NQO1 (15-25 U/ml) were injected either individually or in the combinations indicated. Piericidin A (Pier A, 500 nM) was then added to inhibit complex I, followed by antimycin A (AA, 1 μ M). OCR measurements are normalized to the point just prior to idebenone/vehicle addition (ADP-stimulated baseline). Data are mean \pm SE from 4-6 independent experiments. (B), (C), Bar graph representations of data in (A) and including additional controls not depicted in (A) for clarity. (B), OCR after addition of idebenone/vehicle plus and minus the indicated treatments, expressed as a percentage of the ADP-stimulated baseline OCR. (C), OCR after injection of piericidin A with and without idebenone and the indicated treatments, relative to the ADP-stimulated control (Ctrl) group (point just prior to piericidin A addition). ** $p < 0.01$ relative to vehicle control; *** $p < 0.001$ relative to control. ### $p < 0.01$ between idebenone-treated and vehicle-treated groups; @@ $p < 0.01$ relative to 80 μ M idebenone alone, two-way ANOVA followed by Tukey's *post hoc* test.

2.3.6 Pharmacological NQO1 induction abolishes the inhibitory effect of idebenone on cellular respiration

NQO1 expression is regulated by the Nrf2-ARE antioxidant response pathway and is induced in many cell types by activators of the transcription factor Nrf2 (Dinkova-Kostova & Talalay 2010, Li & Kong 2009). We hypothesized that Nrf2 activators would reverse respiratory inhibition by idebenone in neurons and allow for bypass of complex I-impaired respiration by enhancing NQO1 activity. We failed to increase Nrf2 or NQO1 in primary cortical neurons using several different Nrf2 activators, including sulforaphane, *tert*-butylhydroquinone, and carnosic acid (data not shown). However, carnosic acid treatment for 72 hours led to a robust elevation of NQO1 in COS-7 cells, indicating that the drug was active ($t_{(4)}=19.33$, $p<0.001$, Figure 2.6A). Because basal NQO1 expression in COS-7 cells was low, we tested whether COS-7 cells could act as a surrogate for neurons to show proof-of-principle. We detected a significant interaction between carnosic acid pre-treatment and idebenone treatment on the basal OCR, maximal OCR, and piericidin A-insensitive OCR of COS-7 cells ($F_{(3,32)}=4.828$, $p=0.0070$, $F_{(3,32)}=7.018$, $p<0.001$, and $F_{(3,32)}=12.44$, $p<0.001$, respectively). As in neurons, idebenone inhibited maximal respiration in COS-7 cells that were not pre-treated with carnosic acid ($p<0.001$, Figure 6B,C). Although idebenone supported significant piericidin A-insensitive respiration in untreated COS-7 cells ($p=0.0070$, Figure 2.6B,D), the extent of this respiration was limited relative to that observed in astrocytes (19.5% compared to 59.5% of maximal OCR, Figure 2.6D compared to Figure 2.1E). In carnosic acid-treated COS-7 cells, idebenone stimulated basal respiration ($p=0.0440$ compared to control, Figure 2.6B, first arrow), did not impair maximal respiration ($p=0.2617$, Figure 2.6B,C), and greatly

augmented piericidin A-insensitive oxygen consumption (to 52.2% of maximal OCR, $p < 0.001$, Figure 2.6B,D). Thus, the bioenergetic responses of astrocytes to idebenone were recapitulated in COS-7 cells following carnosic acid treatment. Addition of the NQO1 inhibitor dicoumarol significantly reversed these effects ($p < 0.001$ for the effect on basal OCR, Figure 2.6B, first arrow, $p = 0.0053$ for maximal OCR, Figure 2.6B,C, and $p < 0.001$ for piericidin A-insensitive OCR, Figure 2.6B,D), suggesting that carnosic acid-induced NQO1 activity is causally involved in the transformed response of COS-7 cells to idebenone.

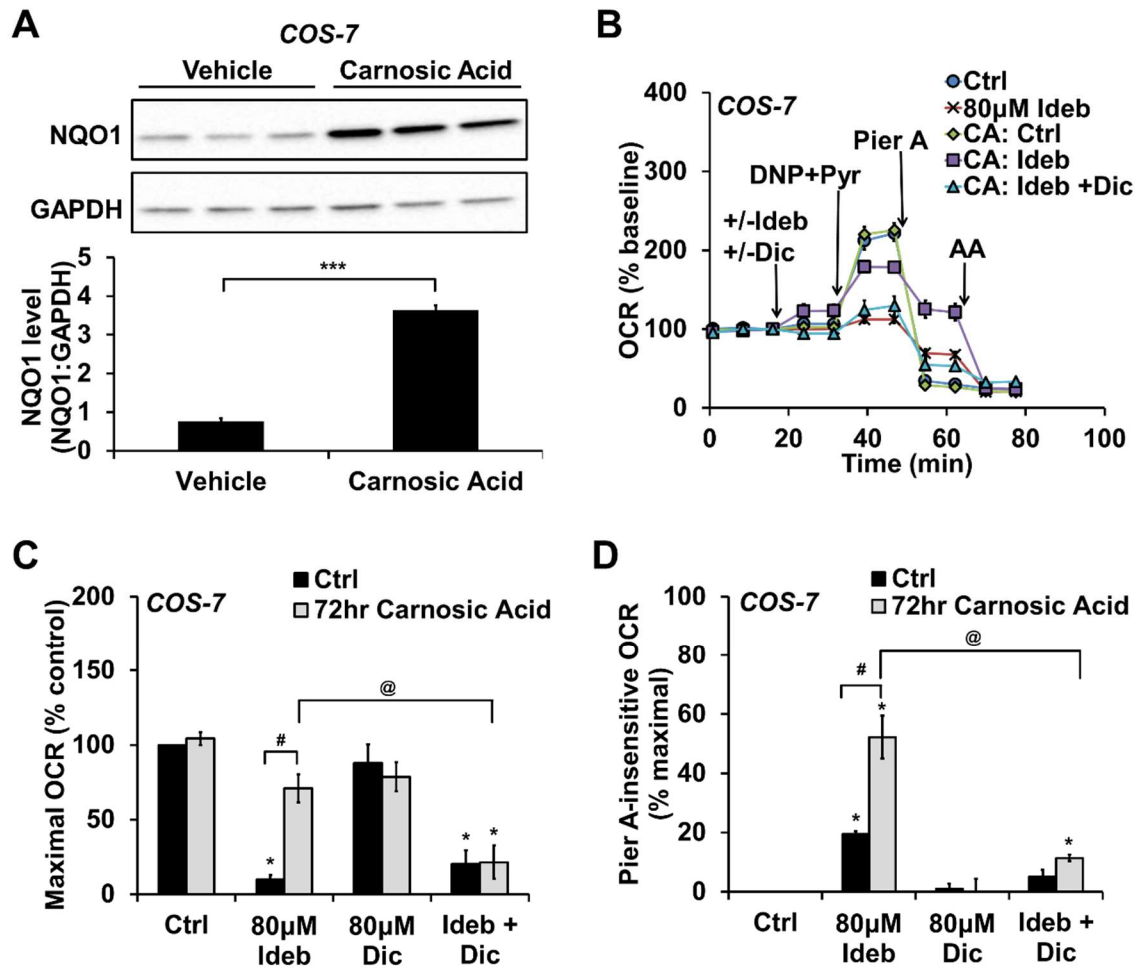


Figure 2.6 Upregulation of NQO1 by the Nrf2 activator carnosic acid mitigates the inhibitory effect of idebenone on cellular respiration. (A), Immunoblots for NQO1 and GAPDH in COS-7 cells after a 72 hour treatment with carnosic acid (CA, 3 μ M) or vehicle. NQO1 levels are expressed as a ratio to GAPDH and are mean \pm SE from 3 independent experiments. *** p <0.001, Student's t test. (B), OCR measurements from COS-7 cells after the 72 hour CA or vehicle treatment described in (A). Idebenone (Ideb, 80 μ M), dicoumarol (Dic, 80 μ M), DNP (150 μ M) + pyruvate (Pyr, 10 mM), piericidin A (Pier A, 500 nM) and antimycin A (AA, 1 μ M) were added as indicated. Data are mean \pm SE from 3-7 independent experiments. (C), (D), Bar graph representations of the data in (B) and including additional controls not depicted in (B) for clarity. (C), Maximal OCR with and without idebenone and/or dicoumarol, expressed as a percentage of control (no dicoumarol or idebenone). (D), OCR after injection of piericidin A with and without idebenone and/or dicoumarol, relative to maximal OCR of control (no dicoumarol or idebenone). ** p <0.01 relative to vehicle control; *** p <0.001 relative to control; ### p <0.001 between carnosic acid-treated and vehicle-treated groups; @@ p <0.01 for carnosic acid plus idebenone-treated groups with and without dicoumarol treatment, @@@ p <0.001 for carnosic acid plus idebenone-treated groups with and without dicoumarol treatment, two-way ANOVA followed by Tukey's *post hoc* test.

2.3.7 Analysis of other quinones with the potential for respiratory bypass in neurons and astrocytes

Other quinones with different efficacies, bioavailability and off-target effects may also help provide insights into the therapeutic value of this strategy. Here we evaluate the effects of some of these compounds on astrocyte and neuronal bioenergetics. Primary cortical neurons and astrocytes were treated with an initial injection of 0-80 μ M of the indicated quinone, followed by an injection of DNP and pyruvate, then piericidin A, and finally antimycin A, as described in Figure 2.1.

Like idebenone, the vitamin E derivative α -tocopherolquinone (α -TPQ) has been tested in clinical trials for the treatment of mitochondrial diseases such as Friedreich ataxia (Lynch et al. 2012). However, α -TPQ showed no significant effect on the basal, maximal or piericidin A-insensitive respiration of either neurons (one-way ANOVA, $F_{(4,10)}=0.3518$, $p=0.8371$, $F_{(4,10)}=0.3694$, $p=0.8252$, and $F_{(4,10)}=1.263$, $p=0.347$ respectively; Figure 2.7A) or astrocytes (one-way ANOVA, $F_{(4,15)}=0.1654$, $p=0.9527$, $F_{(4,15)}=0.6166$, $p=0.6574$, and $F_{(4,15)}=1.598$, $p=0.2263$ respectively; Figure 2.7B).

In a recent study, the Mootha group screened ~40,000 natural products and demonstrated the complex I bypass potential of six novel compounds, including potentiachimaphilin, 3-chloro-chimaphilin, dehydro- α -lapachone and dehydroiso- α -lapachone, 2-methoxy-1,4-naphthoquinone, and 2-methoxy-3-methyl-1,4-naphthoquinone (Vafai et al. 2016). Here we demonstrate the bioenergetic effects of two of these compounds, 2-methoxy-1,4-naphthoquinone (2MNQ) and 2-methoxy-3-methyl-1,4-naphthoquinone (2M3MNQ), on neurons and astrocytes.

2MNQ, also known as Lawsone methyl ether, is a naturally occurring compound found in the plant *Impatiens glandulifera* and studied for its antimicrobial and anticarcinogenic properties (Ding et al. 2008). 2MNQ dose dependently increased basal respiration (one-way ANOVA, $F_{(4,17)}=7.167$, $p=0.0014$; Figure 2.7C), and inhibited maximal respiration in neurons (one-way ANOVA, $F_{(4,17)}=7.116$, $p=0.0015$; Figure 2.7C). 2MNQ induced a relatively small increase in piericidin A-insensitive respiration in neurons (one-way ANOVA, $F_{(4,17)}=3.206$, $p=0.03292$; Figure 2.7C). In astrocytes 2MNQ dose dependently increased basal and piericidin A-insensitive respiration (one-way ANOVA, $F_{(4,21)}=4.599$, and $p=0.0080$, $F_{(4,21)}=5.907$, $p=0.0024$ respectively; Figure 2.7D), with no effect on maximal respiration (one-way ANOVA, $F_{(4,21)}=0.283$, $p=0.8857$; Figure 2.7D). However, the piericidin A-insensitive OCR was also not sensitive to the complex III inhibitor antimycin A, indicating a significant increase in non-mitochondrial respiration in response to 2MNQ (one-way ANOVA, $F_{(4,21)}=5.059$, $p=0.0052$; Figure 2.7D).

2M3MNQ is also a naturally occurring compound and is one of the many active compounds found in henna (*Lawsonia inermis*) (Viani et al. 1992, Mikhaeil et al. 2004). 2M3MNQ dose dependently increased basal respiration and inhibited maximal neuronal respiration (one-way ANOVA, $F_{(4,15)}=5.878$, $p=0.0047$ and $F_{(4,15)}=5.112$, $p=0.0084$; Figure 2.7E). In astrocytes, 2M3MNQ increased basal and piericidin A-insensitive respiration in a dose dependent manner (one-way ANOVA, $F_{(4,26)}=7.518$, $p=0.0004$, and $F_{(4,26)}=4.000$, $p=0.0117$ respectively; Figure 2.7F) and had no effect on maximal respiration (one-way ANOVA, $F_{(4,26)}=1.409$, $p=0.2588$; Figure 2.7F). However,

2M3MNQ also induced a significant increase in non-mitochondrial oxygen consumption (one-way ANOVA, $F_{(4,26)}=5.331$, $p=0.0028$; Figure 2.7F).

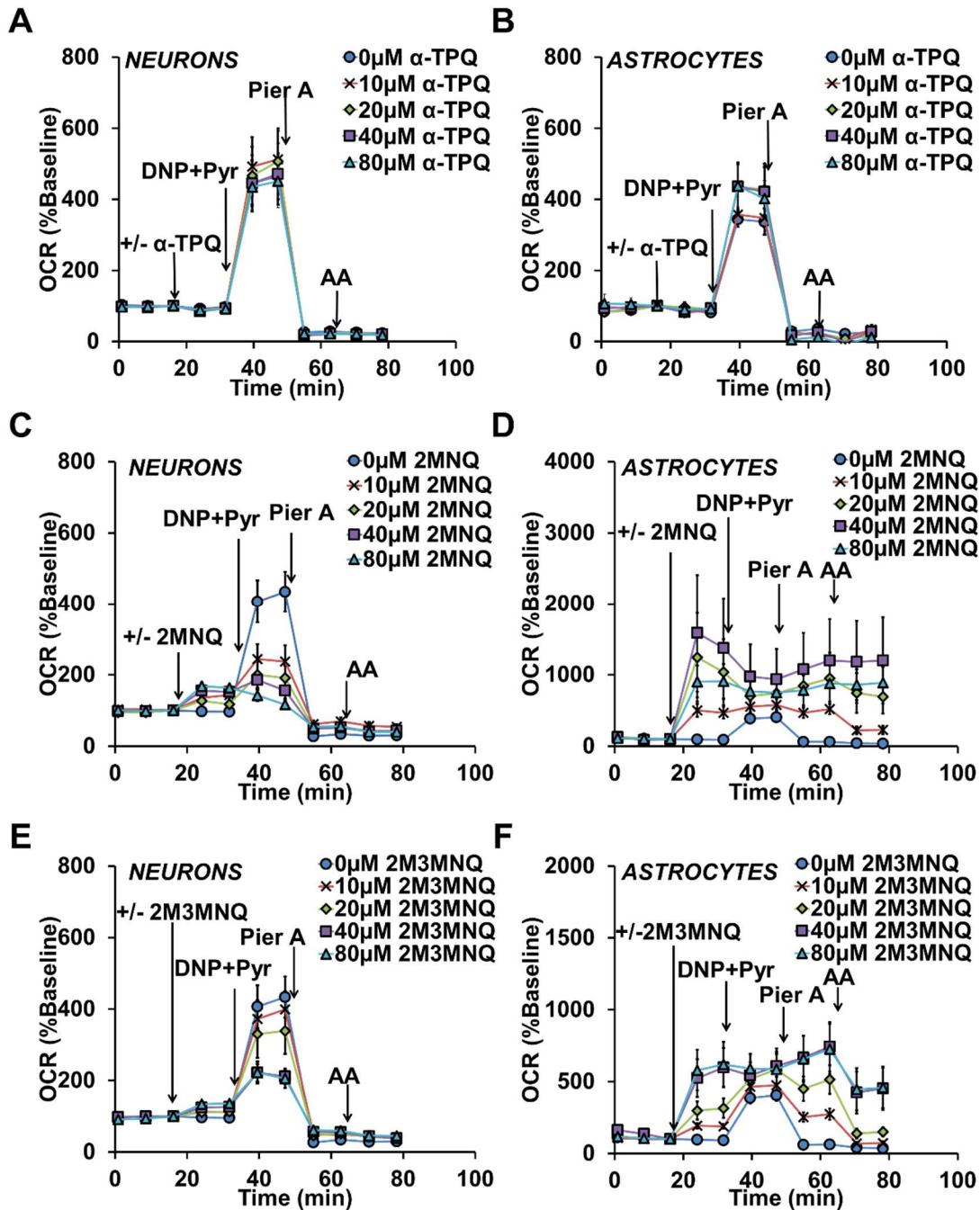


Figure 2.7 The effects of other quinones on the respiration of primary rat cortical neurons and astrocytes. (A-F), OCR measurements of neurons and astrocytes following the initial injection of 0-80 μM of the indicated quinone. Analogous to the experiments described in Figures 2.1A and 2.1B, subsequent injections of DNP (150 μM) + pyruvate (Pyr, 10 mM), followed by piericidin A (Pier A, 500 nM) and finally antimycin A (AA, 1 μM) were then added. (A), (B), α -Tocopherolquinone (α -TPQ). Data are mean \pm SE from 3-4 independent experiments. (C), (D), 2-Methoxy-1,4-naphthoquinone (2MNQ). Data are mean \pm SE from 3-8 independent experiments. (E), (F), 2-Methoxy-3-methyl-1,4-naphthoquinone (2M3MNQ). Data are mean \pm SE from 3-8 independent experiments.

2.3.8 Quinones can be carefully titrated to maximize complex I bypass and minimize non-mitochondrial respiration

Non-mitochondrial respiration can be attributed to several cellular processes. For example, quinones that undergo a one-electron reduction to semiquinone radicals can react with oxygen to form superoxide. We therefore further titrated the respiratory responses of 2MNQ and 2M3MNQ in astrocytes to demonstrate whether optimal concentrations could be achieved to produce a complex I bypass without inducing non-mitochondrial respiration (Figure 2.8A, B). In astrocytes, 2MNQ dose dependently increases piericidin A-insensitivity (one-way ANOVA $F_{(3,19)}=7.893$, $p=0.0013$; Figure 2.8C), and is statistically significant at $10\mu\text{M}$ relative to control ($p=0.0009$; Figure 2.8C). However, increasing concentrations of 2MNQ also trends towards an increase in non-mitochondrial respiration (one-way ANOVA $F_{(3,19)}=6.212$, $p=0.0040$) where $10\mu\text{M}$ induced a significant amount of non-mitochondrial respiration ($p=0.0043$; Figure 2.8E). This concentration also caused a reduction of maximal respiration in neurons ($p=0.0014$; Figure 2.7C).

2M3MNQ also induces a dose dependent increase in piericidin A-insensitivity (one-way ANOVA $F_{(3,19)}=11.31$, $p=0.0002$), which is significant at $10\mu\text{M}$ relative to control ($p<0.0001$; Figure 2.8D). At the tested concentrations, 2M3MNQ does induce non-mitochondrial respiration (one-way ANOVA $F_{(3,19)}=2.802$, $p=0.0678$; Figure 2.8F) and does not inhibit maximal respiration in neurons ($p=0.9813$; Figure 2.7E).

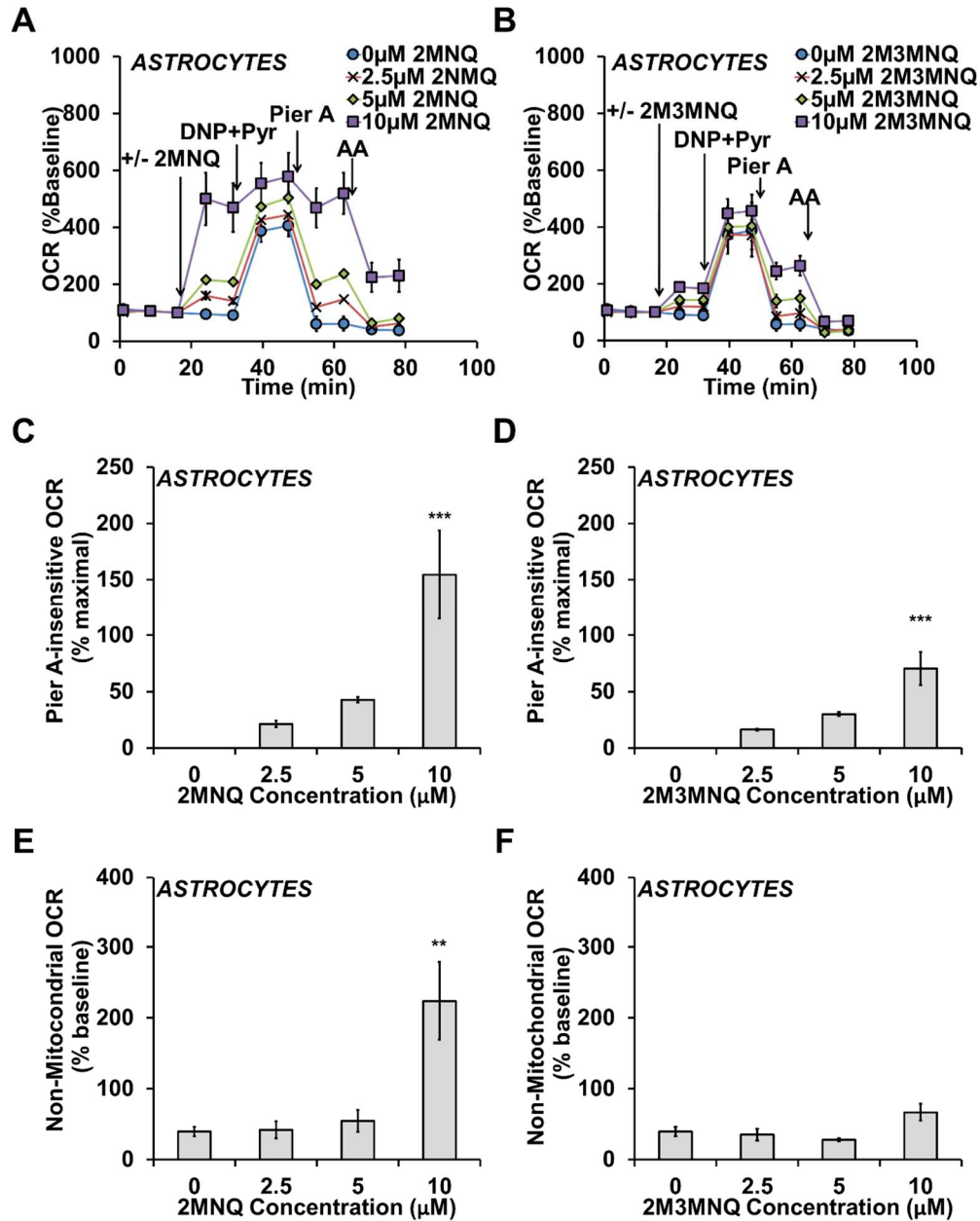


Figure 2.8 Quinone concentrations are titrated to demonstrate complex I bypass without excess non-mitochondrial respiration in primary cortical astrocytes. (A) (B), OCR measurements from astrocytes following treatment with 0-10 μ M of the indicated quinone, followed by DNP (150 μ M) + pyruvate (Pyr, 10 mM), piericidin A (Pier A, 500 nM) and antimycin A (AA, 1 μ M). (A) 2-Methoxy-1,4-naphthoquinone (2MNQ). Data are mean \pm SE from 3-8 independent experiments. (B), 2-Methoxy-3-methyl-1,4-naphthoquinone (2M3MNQ). (C), (D), Bar graph representations of OCR data from (A) (B), after the addition of piericidin A. (E), (F), Bar graph representation of the OCR from (A) (B), after the addition of antimycin A. * p <0.05 relative to vehicle control; ** p <0.01

relative to control; *** $p < 0.001$ relative to control. One-way ANOVA followed by Tukey's *post hoc* test.

2.4 Discussion

Clinical trials for idebenone in neurodegenerative disorders such as Alzheimer's disease (Gutzmann & Hadler 1998, Thal et al. 2003) and Friedreich's ataxia (Di Prospero et al. 2007a, Lynch et al. 2010, Meier et al. 2012) have not yielded convincing positive results. Previous studies indicate that idebenone acts as a prodrug that may influence cellular and mitochondrial function in different ways depending on its oxidation/reduction state; acting as an antioxidant, pro-oxidant, or electron carrier (Haefeli et al. 2011, Giorgio et al. 2012). In this study, we identified a fundamental difference in the way idebenone influences mitochondrial function in neurons compared to astrocytes. Idebenone was able to act as a direct electron donor to complex III only in astrocytes, whereas it inhibited complex I-dependent respiration in neurons. The difference between astrocytes and neurons was attributable to a large disparity in expression of the idebenone-reducing enzyme NQO1. Importantly, we showed proof-of-principle that pharmacological induction of NQO1 through the Nrf2-ARE transcriptional pathway can reverse the negative bioenergetic effects of idebenone while promoting the potentially cytoprotective complex I bypass mechanism.

Several enzymes are predicted to have idebenone-reducing capability, including succinate dehydrogenase (complex II), glycerol-3-phosphate dehydrogenase, NQO1, and NQO2 (Haefeli et al. 2011, Rauchova *et al.* 2012). However, our data suggest that idebenone is reduced primarily by NQO1 in astrocytes. Additionally, cortical neurons, which lack NQO1, are unable to compensate for the absence of NQO1 by reducing idebenone via other enzymes. The Hardingham group recently reported low Nrf2

expression in mature neurons stemming from epigenetic repression (Bell et al. 2015). Given the tight association between NQO1 levels and Nrf2 expression in other cell types (Stringer et al. 2004, Dinkova-Kostova & Talalay 2010, Bell et al. 2015), this observation likely explains neuronal NQO1 deficiency.

Our results showed that exogenous NQO1, when added with its substrate NADPH, was able to reverse the respiratory impairment by idebenone in neurons and induce complex I-independent respiration. Furthermore, an inhibitory effect of idebenone on maximal respiration appeared in astrocytes following addition of an NQO1 inhibitor. Prior evidence suggests that idebenone impairs complex I activity only when in the oxidized quinone form by competitively occupying the CoQ binding site (King et al. 2009)). Thus, idebenone reduction by NQO1 appears to not only allow idebenone to act as a complex III electron donor, but also to eliminate complex I inhibition by removing the oxidized form of the quinone. These findings led us to predict that pharmacological induction of NQO1 in neurons by activation of the Nrf2-ARE pathway would abolish inhibition of complex I-dependent respiration by idebenone and enable direct electron transfer to complex III.

There is evidence for drug-induced Nrf2-ARE pathway activation in primary neuronal cells, typically from reporter constructs or examination of Nrf2 targets at the mRNA level (Satoh *et al.* 2006, Satoh *et al.* 2008, Soane *et al.* 2010, Skibinski *et al.* 2017). We were unable to upregulate NQO1 protein in cortical neurons using several known Nrf2-ARE activators, including carnosic acid. Carnosic acid is a natural compound found in rosemary leaves (Aruoma *et al.* 1992) and was found to activate Nrf2 in both primary rat cortical neurons and COS-7 cells (Satoh et al. 2008), among several

cell types. To ensure the compound was active and establish proof-of-principle, we tested for synergistic bioenergetic effects of carnosic acid with idebenone in COS-7 cells, which display relatively low basal NQO1 expression. The effect of idebenone on mitochondrial respiration in untreated COS-7 cells was phenotypically similar to neurons, namely, inhibition of respiratory capacity. In contrast, cells treated with carnosic acid were phenotypically comparable to astrocytes, exhibiting preserved respiratory capacity and robust complex I-inhibitor insensitive respiration. As predicted, carnosic acid treatment led to strong upregulation of NQO1 enzyme. NQO1 inhibition reversed the effect of carnosic acid treatment on the bioenergetic response to idebenone, shifting the astrocyte-like phenotype back to one resembling neurons. In sum, these results suggest that pharmacological NQO1 upregulation mitigates the inhibitory action of idebenone on respiratory capacity and induces the complex I bypass mechanism mediated by the reduced form, idebenol.

Whether idebenone is the ideal compound for therapeutic complex I bypass is not yet known; we therefore emphasize the importance of characterizing target cell quinone reduction capacity prior to further investigating drug efficacy. We tested a few other quinones with the potential for complex I bypass on neurons and astrocytes. Results ranged from no effect at all on respiration (α -TPQ) to an undesirable increase in non-mitochondrial respiration that was dose dependent (2MNQ and 2M3MNQ). While idebenone required an ~8-fold higher concentration to produce a similar measure of complex I bypass to 2MNQ and 2M3MNQ, it did not increase non-mitochondrial respiration. However, we demonstrate how careful dosing of 2MNQ and 2M3MNQ may still make them promising candidates for future studies, particularly if they can be

optimized through structure-activity relationship studies. 2M3MNQ, when added at 10 μ M, induced a level of complex I bypass comparable to 80 μ M idebenone without inducing non-mitochondrial respiration and, in contrast to idebenone, without impairing neuronal respiratory.

Despite our finding that idebenone can impair complex I-dependent respiration in neurons and previous studies suggesting a direct inhibitory effect of idebenone on complex I (Esposti et al. 1996, King et al. 2009), idebenone doses of up to 75 mg/kg were found to be safe in humans, with few side effects (Di Prospero *et al.* 2007b). In clinical trials, plasma idebenone levels peaked at 55 mg/kg idebenone administration, yielding a plasma concentration of \sim 27 μ M in the adult cohort (Di Prospero et al. 2007b). Rodent experiments using 14 C idebenone showed brain penetration following oral administration (Nagai et al. 1989). However, extrapolating from the rodent data, the concentration of idebenone in human cortical brain tissue is likely on the order of \sim 0.5 μ M. Being hydrophobic, idebenone binds to proteins and also concentrates in membranes. We included bovine serum albumin in our assays at a concentration (4 mg/ml) designed to model extracellular protein in cerebrospinal fluid (Jekabsons & Nicholls 2004) and also to inhibit the binding of hydrophobic drugs to the polystyrene assay plates (Bordt *et al.* 2017). Nevertheless, the idebenone concentrations added in our *in vitro* assays may result in intracellular idebenone concentrations that are greater than those achieved in neurons and astrocytes of the human cortex. Encouragingly, structural modifications of idebenone have already generated compounds with a more potent ability to rescue mitochondrial function and cellular viability following oxidative stress (Duveau *et al.* 2010, Fash et al.

2013). Hence, it is plausible that the direct electron transfer function of idebenone can be harnessed *in vivo* without neurotoxicity.

The beneficial effect of idebenone to Leber's Hereditary Optic Neuropathy patients (Klopstock et al. 2011, Lyseng-Williamson 2016) and the detection of NQO1 immunoreactivity in mouse retinal ganglion cells (Heitz et al. 2012, Smith *et al.* 2016) suggests that some neurons may be able to reduce idebenone to idebenol effectively. Interestingly, neuronal NQO1 expression was observed in the brains of Alzheimer's disease (Raina et al. 1999, SantaCruz et al. 2004, Wang et al. 2000) and Parkinson's disease patients (van Muiswinkel et al. 2004), including in hippocampal pyramidal neurons and dopaminergic neurons of the substantia nigra, respectively. Thus, although little to no NQO1 is expressed by most neurons of the healthy brain, disease-associated changes may overcome the epigenetic repression of Nrf2, enabling activation of the antioxidant response pathway in stressed cells. Importantly, such cells may regain the ability to respond to Nrf2-ARE activators, enabling further elevation of NQO1 to levels where idebenone or analogues become effective. Multiple studies have demonstrated a protective effect of Nrf2 overexpression in neurons (Xiong *et al.* 2015, Skibinski et al. 2017), indicating that although Nrf2 expression is normally repressed in mature neurons, its induction is tolerated and can be beneficial.

In this study, we demonstrated synergism at the mitochondrial functional level between idebenone and carnosic acid. Like idebenone, carnosic acid crosses the blood-brain barrier and is non-toxic to healthy animals (Romo *et al.* 2013, Miller *et al.* 2015). Treatment with carnosic acid alone shortly after controlled cortical impact traumatic brain injury to mice improved the function of non-synaptic brain mitochondria assessed

ex vivo (Miller et al. 2015). Combining idebenone with carnosic acid treatment may have further protective potential by promoting NQO1-dependent mitochondrial bioenergetic rescue in cells competent to activate the Nrf2-ARE pathway. As cytoplasmic NAD(P)H is necessary for idebenone reduction by NQO1 (Haefeli et al. 2011), interventions that promote replenishment of NQO1 substrate supply, e.g. stimulation of the pentose phosphate pathway, may also exhibit synergistic neuroprotection with idebenone.

Finally, while mitochondrial bioenergetic rescue in neurons is an important goal, the potential for targeting astrocytes in neurodegenerative diseases should not be overlooked. Recently, an aberrant “A1” astrocyte phenotype was described in multiple neurodegenerative disease states, including amyotrophic lateral sclerosis (ALS) (Liddelow *et al.* 2017). Extensive evidence indicates that astrocytes from both familial and spontaneous ALS brain acquire a neurotoxic phenotype (Nagai *et al.* 2007, Haidet-Phillips *et al.* 2011, Re *et al.* 2014). Our finding that idebenone promotes mitochondrial bioenergetic function in cultured cortical astrocytes but not neurons suggests that idebenone may be more effective in diseases in which astrocytes contribute significantly to pathology.

Although much work is still necessary, our findings hint at why idebenone has not lived up to its initial promise as a neurotherapeutic agent—insufficient neuronal idebenone-reducing activity. Only in its reduced form, idebenol, can idebenone act as an antioxidant and an electron donor (Mordente et al. 1998, Haefeli et al. 2011, Giorgio et al. 2012, Erb et al. 2012). Overall, our results suggest that combination therapy with NQO1-inducing drugs like carnosic acid may be necessary to maximize the therapeutic potential of idebenone. In the long run, discovery of drugs displaying protective

synergism with idebenone *in vivo* could have a positive impact on how idebenone is used to treat LHON patients, as well as in the design of clinical trials for other diseases.

Chapter 3: Sex differences in the mitochondrial bioenergetics of astrocytes but not microglia at a physiologically relevant brain oxygen tension¹

3.1 Introduction

Mitochondria are organelles responsible for ATP production, consuming oxygen in the process. Mitochondrial ATP production is especially important in the brain, as the central nervous system (CNS) consumes ~20% of the total oxygen inspired, while only accounting for ~2% of the total body weight (Silver & Erecinska 1998). Mitochondrial dysfunction is a nearly ubiquitous occurrence in neurodegenerative diseases (Lin & Beal 2006, Fiskum *et al.* 1999) and acute CNS injuries (Fiskum 2000, Demarest & McCarthy 2015, Demarest *et al.* 2016, Robertson *et al.* 2006). While mitochondrial respiratory impairment is associated with numerous pathologies, evidence for association comes primarily from studies using isolated brain mitochondria, which are derived from both neurons and glia, or from primary neuronal cultures. Roles for mitochondrial respiratory function/dysfunction in neuroglia such as astrocytes and microglia are comparatively understudied, which is surprising since neuroglia are thought to comprise ~50% of the brain, with variation by species (Azevedo *et al.* 2009, Herculano-Houzel 2014).

Astrocytes were initially regarded as “glue” or “housekeeping” cells of the brain. However, they are now recognized to have many pivotal functions such as regulation of ion homeostasis (Olsen *et al.* 2015), neurotransmitter recycling (Schousboe *et al.* 1993), and control of cerebral blood flow (Cabezas *et al.* 2014). Astrocytes also carry out

¹ Jaber, S. M., Bordt, E. A., Bhatt, N. M., Lewis, D. M., Gerecht, S., Fiskum, G. and Polster, B. M. (2018) Sex differences in the mitochondrial bioenergetics of astrocytes but not microglia at a physiologically relevant brain oxygen tension. *Neurochem. Int.* **117**, 82-90.

important bioenergetic roles in the brain, including regulation of brain glucose uptake, production and storage of brain glycogen, and provision of metabolic and antioxidant support for neurons (Belanger et al. 2011).

Microglia are the resident immune cells of the brain (Kreutzberg 1996) that play diverse roles in physiology, including surveying the surrounding environment to clear cellular debris, synaptic pruning, and promotion of synaptogenesis (Tremblay et al. 2011, Wu et al. 2015). In response to brain pathology, microglia become “activated” and secrete cytokines, an initially protective process that becomes maladaptive when failing to resolve in a timely fashion (Brown & Bal-Price 2003, Block et al. 2007b). A shift in cellular bioenergetics from oxidative phosphorylation to glycolysis occurs during microglial activation (Voloboueva et al. 2013, Orihuela et al. 2015).

There is increasing evidence of sex differences in mitochondrial function in both health and disease. A recent study found that isolated brain mitochondria from female mice have higher complex I-linked respiration than male mitochondria at 3 months, a difference that no longer exists by 20 months or following ovariectomy (Gaignard *et al.* 2015). Multiple studies demonstrated that the glutathione antioxidant defense system, which protects mitochondrial bioenergetic function, is elevated in females compared to males (Gaignard et al. 2015, Demarest et al. 2016). In adults, this effect is abolished by ovariectomy (Gaignard et al. 2015). While these studies suggest that sex hormones may directly regulate mitochondrial function, fewer studies have examined whether there are intrinsic sex differences in mitochondrial function at the cellular level. A sex difference in respiration was not apparent in brain mitochondria isolated from postnatal day 7 rats

(Demarest et al. 2016). However, isolated forebrain mitochondria are derived from multiple cell types which may mask cell type-specific sex differences.

Sexual dimorphism of astrocyte morphology is already evident by the day of birth (Mong & McCarthy 2002), suggesting that there can be early sex differences in glial cell properties. Dimorphism is thought to occur via an organizational effect of gonadal steroids in males (Mong & McCarthy 2002). Thus, neonatally prepared glial cells from males will have had hormone exposure *in utero*, allowing for organizational effects (Mong *et al.* 1996, Mong & McCarthy 2002), while being devoid of continued gonadal hormone exposure during *in vitro* development. In addition to neonatal astrocytes, sex is also thought to influence neonatal microglial cell properties, including proinflammatory gene expression (Loram *et al.* 2012). The precedence for intrinsic sex differences in XX vs. XY glial cells makes neonatal primary cultures an excellent system to investigate fundamental mitochondrial sex differences in defined cell populations.

The primary goal of this study was to test the hypothesis that there are sex differences in the mitochondrial respiratory properties of relatively pure populations of rat cortical astrocytes or microglia. An additional goal was to determine whether experimental oxygen tension influences the astrocytic or microglial bioenergetic function of either sex. The vast majority of *in vitro* work on mitochondrial function has been performed at atmospheric oxygen (160 mm Hg, 21% O₂). However, atmospheric O₂ tension is far higher than the pO₂ that reaches cells within the brain (typically 5-45 mm Hg or ~1-6% O₂) (Grote *et al.* 1996). Oxygen tension regulates many biochemical processes with the potential to impact mitochondrial respiration, including superoxide production (Hoffman *et al.* 2007), nitric oxide formation (Rengasamy & Johns 1996) and

the stability of oxygen-sensitive transcription factors such as HIF-1 α (Semenza 2012). Therefore, we hypothesized that conducting experiments at a physiologically relevant O₂ level may reveal sex differences in mitochondrial bioenergetics that would not otherwise be observed. The effects of 3% O₂, specifically, were investigated because early studies suggested a reduction in oxidative stress-induced changes in cultured cells at this O₂ level compared to supraphysiological O₂ (Parrinello *et al.* 2003, Busuttill *et al.* 2003). We refer to 3% O₂ as low physiological O₂ because several studies have considered *in vitro* 5% O₂ as brain physiological O₂ (Tiede *et al.* 2011, Zhu *et al.* 2012, Sun *et al.* 2015, Dussmann *et al.* 2017).

We found that male astrocytes exhibit a higher respiratory capacity than female astrocytes when cultured and assayed at 3% O₂ (dissolved O₂ of ~1.2-3.0%), but not when experiments are conducted at atmospheric 21% O₂. However, we did not find sex differences in bioenergetic parameters or release of two key proinflammatory cytokines in non-stimulated or activated microglia, irrespective of experimental O₂.

3.2 Materials and Methods

3.2.1. Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

3.2.2. Preparation of primary rat cortical astrocytes

All procedures were approved by the University of Maryland Institutional Animal Care and Use Committee and were compliant with the NIH Guide for the Care and Use of Laboratory Animals. Primary rat cortical astrocytes were prepared from postnatal day

1 Sprague Dawley rat pups as described (McKenna 2012). Briefly, rats were sexed visually by comparing the distance between the genitals and anus. Only rats that were easily identifiable as male or female by at least two parties were used in this study. Rat pups were euthanized by decapitation. Cortices were then removed, homogenized by trituration, and vortexed for one minute. Brain homogenate from two pups of the same sex were then passed through a 70 μm filter before plating into two separate tissue culture-treated flasks in Eagle's Minimal Essential Medium (EMEM, Quality Biological, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA, Lonza, Walkerville, MD) and 50 $\mu\text{g}/\text{mL}$ gentamicin. One flask was placed in a standard 37°C incubator at 95% air/5% CO₂ (20% O₂). The other flask was placed in a 37°C 92% N₂/5% CO₂/3% O₂ incubator (referred to as 3% O₂). After two days, cell culture medium was changed to gentamycin-free medium and cells were thereafter maintained on medium without gentamicin. At 18 days *in vitro*, cells were trypsinized using TrypLE Express (Thermo Fisher, Waltham, MA), and sub-cultured for at least 24 hours prior to assays. Data represents preparations across 3-4 litters.

3.2.3. Preparation of primary rat cortical microglia

Primary rat cortical microglia were prepared from cortices of one day old Sprague-Dawley rats as previously described (Wu *et al.* 2010). Each primary culture preparation combined pups from 2 separate litters, with 3-4 separate preparations being utilized for each study. Briefly, cerebral cortices were dissected, homogenized by serial trituration with progressively narrower serological pipets, and plated in poly-D-lysine-coated culture flasks. Cells were maintained at 20% or 3% O₂ as described above in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM)/F12

supplemented with penicillin (100 IU/mL) plus streptomycin (100 μ g/mL) and 10% FBS. Seven days after preparation, flasks were shaken for one hour at 100 rpm using an Orbi-Shaker™ (Benchmark Scientific, Edison, NJ), after which medium was collected and centrifuged at 1,000g for 10 min to isolate microglia. To induce proinflammatory microglial activation, cells were treated with a combination of 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, cat# L2654) and 10 ng/mL recombinant interferon- γ (IFN- γ ; R&D Systems, Minneapolis, MN, cat# 485-MI-100) for 18 hours prior to bioenergetic measurements or cytokine quantification.

3.2.4. Dissolved Oxygen Measurements

Dissolved oxygen at the cell monolayer surface was measured using a commercially available sensor dish reader (SDR; PreSens, Regensburg, Germany) in specialized 24-well O₂-sensing plates (Oxo-Dish OD-24; PreSens). These plates contain an immobilized O₂-sensitive fluorescent patch within the plating surface of each well for measuring dissolved oxygen and come sterilized and calibrated by the manufacturer. Measurements were made at 15 second intervals over a 16 hour period in either a conventional 37°C, 95% air/5% CO₂ humidified incubator or in the 37°C 3% O₂ humidified incubator described above. To account for differences in proliferation rates at the different O₂ levels, astrocytes were plated at 3.6×10^5 cells/well for measurements in the 20% O₂ incubator and at 2.4×10^5 cells/well for measurements at 3% O₂. A plating volume of 1.2 mL was used for both O₂ levels. Data were acquired using SDR v4.0.0 software (PreSens), and then exported into Microsoft Excel for analysis. Dissolved O₂ at the cell monolayer of microglial cultures was calculated based on the relative O₂ consumption rate of the microglial cultures using Fick's first law:

$$J = D\nabla C - R$$

where J is the flux of O₂ into the system; D is the diffusion coefficient of O₂ into the media; ∇C is the change in O₂ concentration in the x, y, and z directions in the culture media; and R is the cellular O₂ consumption rate (Lewis *et al.* 2017).

3.2.5. XF24 microplate-based respirometry

Oxygen consumption rate (OCR) measurements from primary rat cortical astrocytes or microglia were performed using a Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA) as previously described (Gerencser *et al.* 2009, Clerc & Polster 2012). Astrocytes were plated in XF24 V7 plates (Agilent Technologies) at 0.6×10^5 cells per well at 20% O₂ and at 0.4×10^5 cells per well at 3% O₂. Microglia were plated in V7 plates at 1.0×10^5 cells per well. Cells were allowed to attach and culture overnight. Microglia were either untreated or activated for 18 hours prior to assay, as described above. Prior to OCR assays, cells in three of the 24 wells were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, pH 8.0). Lysates from these wells were used for protein quantification by the Pierce Micro bicinchoninic acid (BCA) Assay (Thermo Fisher), allowing subsequent normalization of O₂ consumption rates to average protein per well. Artificial cerebrospinal fluid (aCSF) assay medium for respiration measurements consisted of 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 1 mM MgCl₂, 120 mM NaCl, 3.5 mM KCl, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 mM glucose, and 4 mg/mL fatty acid-free bovine serum albumin (BSA), pH 7.4. Cells were incubated in a CO₂-free incubator at 37°C for 45 minutes prior to assay to allow temperature and pH calibration. XF24 assays consisted of cycles of 3 min mix, 2 min

wait, and 2 min measure for astrocytes, and 2 min mix, 1 min wait, and 2 min measure for microglia.

For experiments performed at 3% O₂, the Seahorse XF24 instrument was placed into the workspace of an Xvivo System environmental chamber (Biospherix, Ltd., Parish, NY) that also contained four cell culture incubators with independent gas control. The Xvivo workspace was at room temperature (~25°C) and regulated to 3% O₂ (with no CO₂). A minimum of 4 hours prior to assays, aCSF assay medium and a calibration cartridge containing XF calibrant (Agilent Technologies) were placed into a partitioned 37°C, 3% O₂/97% N₂/0% CO₂ incubator to equilibrate to temperature and O₂. Cells to be assayed at 3% O₂ were cultured within another partitioned incubator set to 3% O₂/92% N₂/5% CO₂ within the Xvivo System. Forty-five minutes prior to assays, cell culture medium was exchanged for 3% O₂-equilibrated aCSF assay medium within the 3% O₂-regulated Xvivo workspace and cells were then transferred to the CO₂-free 3% O₂ partitioned incubator. Injection port drug loading of the cartridge was also conducted at 3% O₂ within the environmental chamber. XF24 assays consisted of the same mix-wait-measurement cycles described above. Importantly, three empty wells of each assay plate (A6, B6, and C6) received four successive injections of 1.0 M sodium sulfite (1:10, 1:11, 1:12, and 1:13 dilutions in the assay wells, respectively) to chemically scavenge oxygen and provide a zero O₂ reference. Sodium sulfite stock (1.0 M) was made fresh immediately prior to each assay by dissolving powder in aCSF assay medium in a glass vial with a tight-fitting lid. For XF assays conducted at 3% O₂, OCRs generated by the XF software were recalculated using the XF Hypoxia Rate Calculator Program (Agilent

Technologies). The sodium sulfite zero oxygen reference specifically in wells A6, B6, and C6 is essential for the Hypoxia Rate software to calculate OCR at 3% O₂.

3.2.6. *ELISA analysis of TNF- α and IL-1 β*

Rat interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) were quantified by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols (R&D Systems).

3.2.7. *Statistical analysis*

Statistical analyses were performed using SigmaPlot 12.0 (Systat Software Inc, San Jose, CA). Two-way analysis of variance (ANOVA) was employed to evaluate the statistical significance of oxygen consumption measurements, while three-way ANOVA was used to evaluate the statistical significance of TNF- α and IL-1 β measurements. A p value < 0.05 was considered significant. Tukey's post-hoc analysis was used to compare individual groups.

3.3 Results

3.3.1 *Estimation of dissolved oxygen at the cell monolayer surface*

Cells that were assayed at 3% O₂ were also cultured at 3% O₂ and were not exposed to room air prior to measurements. Cells that were analyzed at 21% O₂ were cultured at 20% O₂ (95% of atmospheric 21% O₂) in an incubator containing 95% air and 5% CO₂. Due to cellular O₂ consumption and limitations in cell culture medium O₂ diffusion, dissolved O₂ at the cell monolayer surface is less than the regulated O₂ in air (Abaci *et al.* 2010). To get a better estimate of the O₂ level at the cell surface for our two O₂ setpoints, we used specialized Oxo-Dish plates containing O₂ sensor patches

directly embedded into the plates. For astrocytes cultured in 1.2 mL of media, a volume that produces a similar height of media to that used in Seahorse XF24 assay plates, O₂ at the cell surface during culture was $1.17 \pm 0.18\%$ at a set point of 3% O₂ and was $19.71 \pm 0.13\%$ O₂ at a set point of 20% O₂ (mean \pm SD, n=3).

Next, we used the relative O₂ consumption rates (OCR) of astrocytes and microglia to calculate the cell surface O₂ level for primary microglia cultured at 3% O₂. Because there were no sex differences in basal OCR (see below), cultures from males and females were combined for each cell type. The absolute (non-protein normalized) OCR of astrocytes was 69 ± 23 pmol O₂/min (mean \pm SD, n=11) while the absolute OCR of microglia was 49 ± 23 pmol O₂/min (mean \pm SD, n=6). Based on the slightly lower O₂ consumption rate of microglia, the dissolved O₂ at the microglial monolayer surface was calculated to be 1.69% during 3% O₂ culture.

During the acquisition of O₂ consumption rates by Seahorse respirometry, cells consume O₂ and frequent media mixing is required. Therefore, during assays, astrocytes likely experience a dynamic O₂ range of ~ 1.2 -3.0% O₂ when measured at 3% O₂ while microglia likely experience a range of ~ 1.7 -3.0% O₂ under the same conditions. Cells measured at 21% O₂ transiently drop below 21% O₂ during 2 min measurements but O₂ level quickly recovers after mixing.

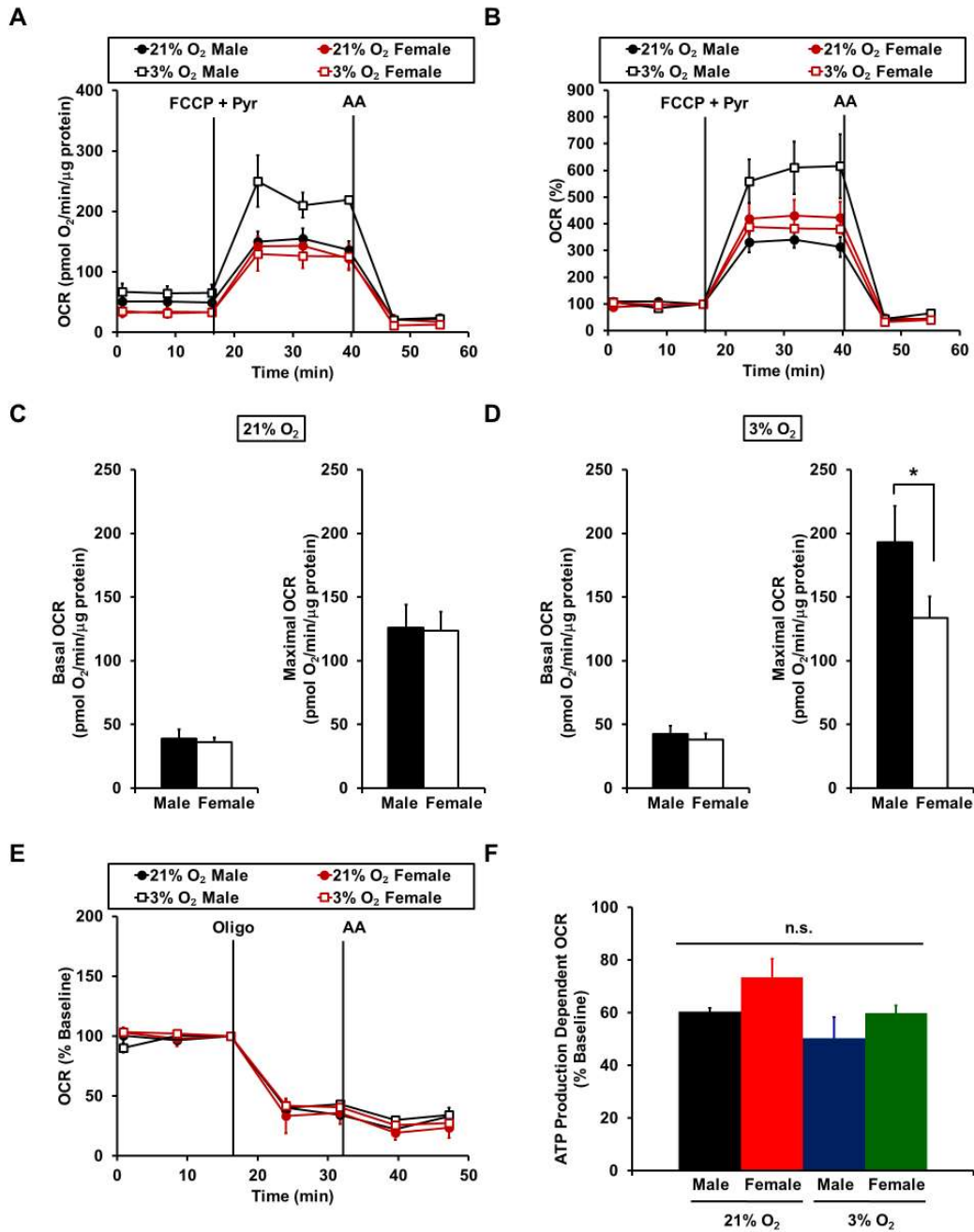
3.3.2 *Astrocyte bioenergetics*

The basal OCR of male and female primary rat glial cells was measured at 3% or 21% O₂. Following acquisition of basal OCR, maximal respiration was induced by the addition of the uncoupler FCCP and the cell permeable mitochondrial complex I substrate pyruvate. Provision of excess substrate was done to preclude any differences due to

insufficient substrate supply, enabling us to examine differences in OCR primarily due to differences in electron transport chain function. The complex III inhibitor antimycin A was added last and any remaining oxygen consumption was regarded as non-mitochondrial respiration.

Primary rat cortical astrocytes showed no significant differences in basal OCR among male and female astrocytes at either 21% or 3% oxygen (Fig. 1A and C-D). There were also no differences among these groups in the amount of basal O₂ consumption used for mitochondrial ATP synthesis, as estimated by the fraction of the basal respiration rate reduced by the ATP synthase inhibitor oligomycin (Fig. 1E and F). However, at 3% O₂ male astrocytes demonstrated a significantly higher maximal respiration rate than female astrocytes (Fig. 1A-D). Male astrocytes at 3% O₂ also had a higher maximal respiration rate than either male or female astrocytes at 21% O₂. The difference in maximal respiration rate remained significant even after respiration was normalized to basal respiration (Fig. 1B), indicating that male astrocytes have greater spare respiratory capacity than female astrocytes at 3% O₂. Spare respiratory capacity, the difference between basal and maximal respiration, is thought to reflect the capability of cells to respond to increased energy demand with an elevation in oxidative phosphorylation (Nicholls 2009).

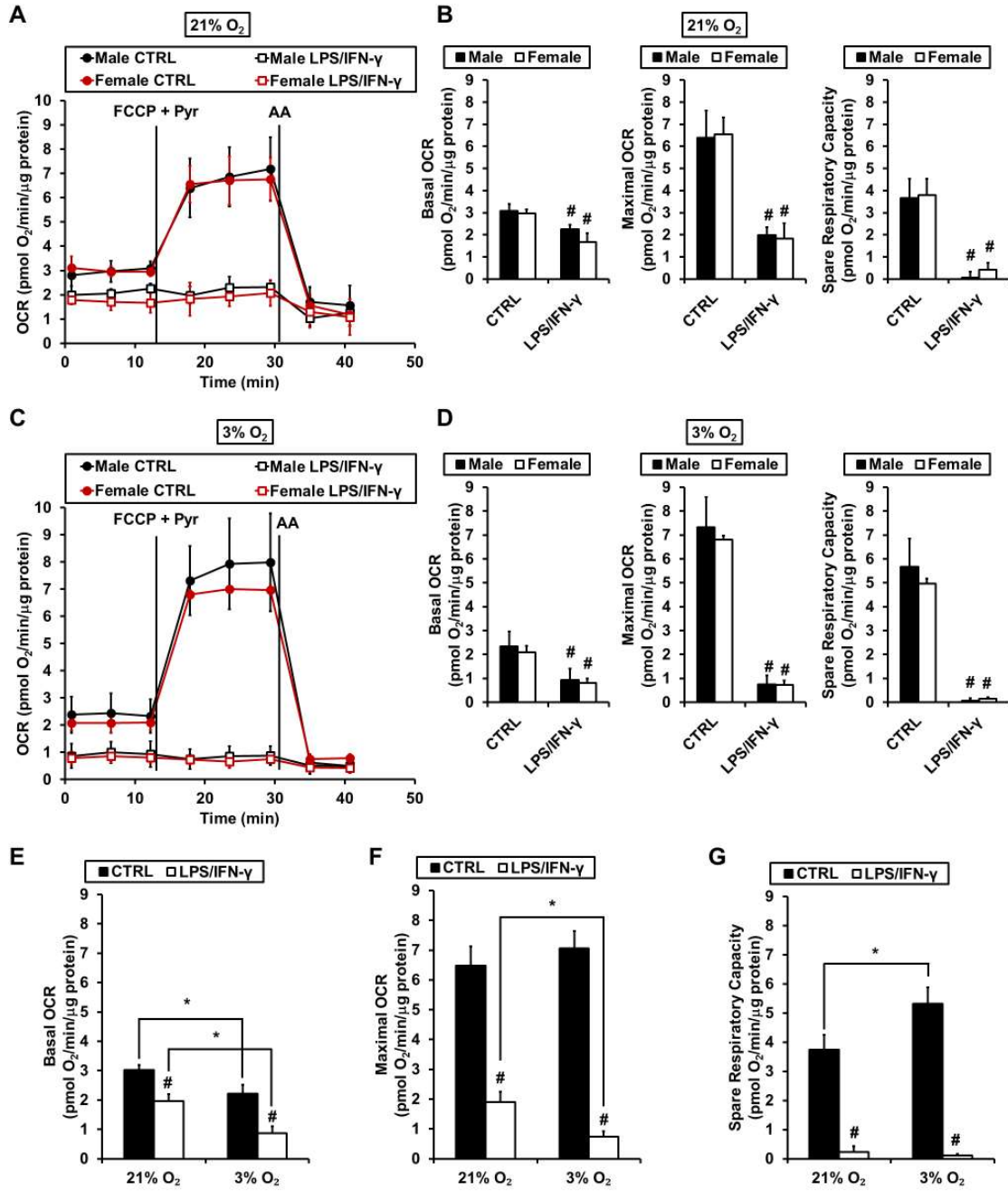
Figure 3.1 A sex difference in astrocyte respiration is observed at 3% oxygen but not at 21% oxygen. (A) Representative traces of oxygen consumption rate (OCR) measurements from male and female primary rat cortical astrocytes cultured and tested at 21% atmospheric or 3% physiological oxygen. FCCCP (6 μ M), pyruvate (Pyr, 10 mM) and antimycin A (AA, 1 μ M) were added when indicated. Traces are mean \pm standard deviation of three wells of cells from the same animal that were cultured and assayed at the different O₂ values (i.e. one male and one female). (B) Representative traces of the data described in A after normalization to the third basal respiration measurement prior to FCCCP + Pyr addition. (C-D) Bar graph representation of basal and maximal OCR at 21% O₂ (C) and at 3% O₂ (D). (E) Representative traces of normalized OCR measurements in male and female rat cortical astrocytes at 21% and 3% oxygen. Oligomycin (oligo, 0.3 μ g/mL), and antimycin A (AA, 1 μ M) were added when indicated. (F) Bar graph representation of ATP production-dependent OCR at 21% vs. 3% O₂. Data are presented as mean \pm standard error, n=5-8 astrocyte preparations across 3-4 separate litters derived from different dams. *p<0.05.



3.3.3 Microglial bioenergetics—the experiments in this section are accredited to the work of Evan Bordt

Primary rat cortical microglia were either untreated (CTRL) or stimulated with LPS plus IFN- γ (LPS/IFN- γ) for 18 hours prior to oxygen consumption measurements. There was no difference between male and female cells in basal OCR, maximal OCR, or spare respiratory capacity at either 3% or 21% O₂ (Fig. 2A-D). In both male and female controls, basal OCR was lower at 3% O₂ compared to 21% O₂, an effect that remained consistent following LPS/IFN- γ stimulation (Fig. 2E). There was no oxygen tension-dependent difference in maximal OCR (Fig. 2F), yet spare respiratory capacity of untreated microglia (CTRL) was significantly higher at 3% O₂ compared to 21% O₂ (Fig. 2G). At both 21% O₂ (Fig. 2A and B) and 3% O₂ (Fig. 2C and D), basal and maximal OCR were significantly suppressed by LPS/IFN- γ treatment. There were no sex differences in the extent of inhibition at either oxygen tension. However, both basal and maximal OCR were impaired to a greater degree at 3% O₂ compared to 21% O₂ following LPS/IFN- γ treatment (Fig. 2E and F).

Figure 3.2 LPS/IFN- γ induces a greater respiratory impairment in microglia at 3% O₂ compared to 21% O₂, regardless of sex. (A) Representative traces of oxygen consumption rate (OCR) measurements from primary rat cortical microglia at 21% O₂ following 18 hours of LPS (100 ng/mL) plus IFN- γ (10 ng/mL) (LPS/IFN- γ) stimulation or control (CTRL) treatment. FCCP (4 μ M), pyruvate (Pyr, 10 mM) and antimycin A (AA, 1 μ M) were added when indicated. (B) Quantification of basal OCR, maximal OCR, and spare respiratory capacity from the experiments described in (A). (C) Representative traces of OCR measurements from cortical microglia at 3% O₂ following 18 hours of LPS/IFN- γ stimulation or CTRL treatment. Drug additions were as in (A). (D) Quantification of basal OCR, maximal OCR, and spare respiratory capacity from the experiments described in (C). (E-G) Comparison of basal OCR (E), maximal OCR (F), and spare respiratory capacity (G) from microglia (combined data of male and female) at 21% vs. 3% O₂. Traces in (A) and (C) are mean \pm standard deviation of three wells of cells from the same microglial preparation and are representative of 3-4 independent experiments using different preparations. Data in (B) and (D) are mean \pm standard deviation, n = 3-4 microglial preparations derived from different animals. Data in (E-G) are mean \pm standard deviation, n=6-8 microglial preparations derived from different animals. Each individual preparation utilized litters stemming from separate dams. #p <0.05 compared to CTRL of the same sex. *p<0.05 for 21% vs. 3% O₂



3.3.4 Microglial release of proinflammatory TNF- α and IL-1 β in response to LPS/IFN- γ —the experiments in this section are accredited to the work of Evan Bordt

A previous study found that neonatal male cortical microglia expressed more IL-1 β mRNA than female cells upon 4 hours of LPS stimulation (Loram et al. 2012). Therefore, despite finding no sex differences in the microglial mitochondrial response to LPS/IFN- γ , we decided to test whether sex influences microglial secretion of proinflammatory cytokines. We also evaluated whether physiological oxygen tension, compared to atmospheric O₂, affects cytokine secretion. There were no significant sex or oxygen tension-dependent differences in the levels of TNF- α (Fig. 3A) or IL-1 β (Fig. 3B) released by microglia stimulated with LPS/IFN- γ for 18 hours. There were also no differences in basal cytokine release from untreated cells (CTRL, Fig. 3A and B).

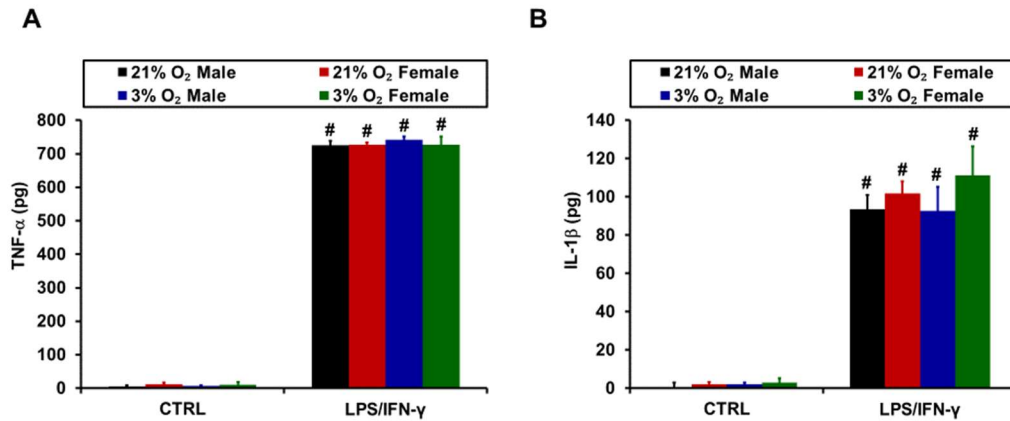


Figure 3.3 Lack of effect of sex or oxygen tension on microglial release of key proinflammatory cytokines. Primary rat cortical microglia were untreated (CTRL) or treated with 100 ng/mL LPS plus 10 ng/mL IFN- γ (LPS/IFN- γ) for 18 hours, and the release of (A) TNF- α and (B) IL-1 β were quantified by ELISA. Data are presented as mean \pm standard deviation, $n = 3-4$ microglial preparations derived from different animals, with each preparation utilizing litters stemming from separate dams. # $p < 0.05$ compared to CTRL.

3.4. Discussion

This study demonstrates an intrinsic sex difference in the mitochondrial bioenergetics of rat cortical astrocytes at low physiological 1.2-3.0% O₂ that was not observed at atmospheric O₂. Male astrocytes exhibited a higher maximal respiration rate than female astrocytes, but only when oxygen consumption rate was measured at 3% O₂ following 3% O₂ culture. In contrast to astrocytes, there were no sex differences in the respiratory characteristics of rat cortical microglia at either 3% or 21% O₂, regardless of whether they were non-stimulated or activated by LPS/IFN- γ . Nevertheless, there were some oxygen tension-dependent differences in both non-stimulated and LPS/IFN- γ - stimulated microglia.

Non-stimulated microglia displayed slightly lower basal respiration at 3% O₂ compared to atmospheric O₂. Although maximal OCR was not different, the difference in basal respiration resulted in greater spare respiratory capacity when microglia were at 3% O₂. As expected, LPS/IFN- γ stimulation curbed respiration at both oxygen levels, consistent with the reported metabolic shift from oxidative phosphorylation towards glycolysis upon activation (Voloboueva et al. 2013, Orihuela et al. 2015). However, LPS/IFN- γ induced a slightly greater suppression of both basal and maximal OCR at 3% O₂ compared to 21% O₂. Activated microglia produce nitric oxide (NO), which contributes to respiratory inhibition (Moss & Bates 2001). One mechanism by which NO may do so is by competing with molecular oxygen at complex IV of the electron transport chain (ETC) (Brown & Cooper 1994). A greater inhibitory effect of NO on the ETC at 1.2-3.0% O₂ compared to 21% O₂ is predicted based on a competition

mechanism, potentially accounting for the greater suppression of OCR that was observed when microglia were activated and assayed at 3% O₂.

The Organizational/Activational Hypothesis of hormone action states that gonadally-derived steroid hormones create lasting sex differences in brain circuitry that are then activated by sex-specific hormones in adulthood (McCarthy *et al.* 2012). Importantly, activation of the testes during a critical window during the late stages of embryonic development leads to a surge in production of the hormone testosterone in males (Gillies & McArthur 2010, Kight & McCarthy 2014). This testosterone is aromatized to estradiol. In rodents, it is estradiol that plays a significant role in the masculinization or de-feminization of neural circuitry (referred to as organization) by influencing processes such as synaptogenesis and neurite outgrowth (Wilson & Davies 2007). Because cultured cells are not exposed to the same levels of sex hormones that circulate in the brain, any sex differences observed *in vitro* would likely be caused by organizational modifications occurring *in utero* or intrinsic differences in gene expression between XX and XY chromosome-containing cells.

One concern involving *in utero* hormone exposure involves the role of alpha-fetoprotein, a plasma glycoprotein produced during fetal life (Andrews *et al.* 1982). Alpha-fetoprotein is thought to protect the developing female brain against masculinization through the binding of circulating estradiol (Bakker *et al.* 2006). As the level of alpha-fetoprotein may vary from mother to mother, we used litters from multiple dams to minimize differences due to variability in *in utero* hormone exposure. However, to determine the robustness and generalizability of the astrocyte sex difference identified

in Sprague Dawley cortical astrocytes, a future goal is to test whether our findings extend to astrocytes derived from other rat strains, or from other species.

The sex difference in mitochondrial respiration found in astrocytes but not microglia at 3% O₂ is consistent with the possibility of a lasting organizational modification occurring *in utero* specifically in astrocytes. Lasting *in vitro* sex differences have also been found in neurons, for example, in gamma-aminobutyric acid (GABA) responses in hippocampal neuronal cultures (Nunez & McCarthy 2009). Intrinsic neuronal sex differences in mitochondrial properties have also been reported, including XX-XY differences in mitochondrial biogenesis (Sharma *et al.* 2014), morphology (Sharma *et al.* 2014), and recruitment of cell death signaling (Du *et al.* 2004, Sharma *et al.* 2014). The observation of mitochondrial sex differences *in vitro* in neurons and astrocytes but not microglia may be explained by the developmental origin of the brain cells. Both neurons and astrocytes are thought to originate from radial glial cells (Malatesta *et al.* 2003, Malatesta *et al.* 2000), whereas microglia are thought to arise from precursor cells originating in the yolk sac (Alliot *et al.* 1999). It may be that lasting sex differences observed in neurons and astrocytes develop from alterations occurring in precursor radial glial cells, adaptations that would not be found in yolk sac-derived microglia. Nevertheless, literature supports sex differences in microglial cytokine production during early development (Loram *et al.* 2012, Crain *et al.* 2013) or following injury (Mirza *et al.* 2015), indicating that the topic requires further study.

The mechanism underlying the higher respiratory capacity observed in male astrocytes compared to female astrocytes at 3% O₂ remains to be elucidated. It may be that male astrocytes cultured and assayed at 3% O₂ express rate-limiting enzymes

involved in aerobic energy metabolism at higher levels than female cells or male cells at 21% O₂. A transcriptome study identified increased expression of the gene encoding complex I subunit Ndufa5 in astrocytes cultured at 4% O₂ relative to 20% O₂ (Chadwick *et al.* 2011). However, cells were pooled from both sexes, making the contribution of male astrocytes to this increase unclear. It is also possible that protein degradation/turnover of electron transfer chain subunits is differentially regulated, for example, by damage due to oxidative stress. Such alterations would not necessarily lead to a sex difference in basal respiration, which is regulated by energy demand. However, a relative increase in the levels of ETC proteins is predicted to provide males with a greater respiratory capacity, as indicated by maximal respiration measured in the presence of an uncoupler. Respiration in the presence of uncoupler is independent of energy demand because the protonmotive force required for ATP synthesis is dissipated. Therefore, uncoupled OCR measured with excess substrate is a good measure of ETC capacity.

Perhaps the best-characterized protein that is a candidate for mediating differences in mitochondrial protein expression is hypoxia-inducible factor-1 α (HIF-1 α), the oxygen-sensitive subunit of the transcription factor HIF-1 α (Semenza 2012). HIF-1 α is constitutively degraded at atmospheric O₂, with the prolyl hydroxylase enzymes necessary for this degradation using oxygen as a substrate (Epstein *et al.* 2001). Because prolyl hydroxylase activity is O₂-dependent, degradation is limited under low-oxygen conditions, including within the physiological oxygen range (Epstein *et al.* 2001). Stabilization of HIF-1 α is necessary for a wide variety of transcriptional processes, including regulation of cellular metabolism (Semenza 2012) and, specifically, ETC complex IV subunit composition (Fukuda *et al.* 2007). Interestingly, sex effects on

complex IV subunit transcription have also been reported (Roemgens *et al.* 2011). Alternatively, other factors could be responsible for modifying the function of the electron transport chain. For instance, if there is greater basal production of nitric oxide in female astrocytes compared to male cells, the higher NO concentration may result in inhibition of maximal respiration in female cells at 3% O₂. This would lead to a higher respiratory capacity in male cells. The absence of a sex difference at 21% O₂ might then be explained by an inability of NO to effectively compete with O₂ when oxygen is more abundant. Additionally, it is possible that the ETC is modified post-translationally in a sex-dependent manner, such as by S-nitrosylation, phosphorylation, or acetylation. Proteomic studies are needed to help resolve whether there are male-female differences in the composition or post-translational modification of the ETC in cultured astrocytes at 3% O₂.

A limitation of our study is that although the brain experiences a range of oxygen tensions, our experiments were performed at a single physiological oxygen tension. Notably, we were able to estimate O₂ level at the cell monolayer surface at the 3% O₂ set point, showing that dissolved O₂ is actually ~1.2% during culture. This O₂ level is closer to the bottom of the physiological range rather than the 3.5% midpoint. It is possible that the sex difference in astrocyte mitochondrial bioenergetics observed at 1.2-3.0% O₂ may decrease, increase, or disappear at the upper or lower O₂ limits of the physiological range. Similarly, it is possible that a sex difference in microglial bioenergetics would be revealed elsewhere within the physiological O₂ range.

The most significant contribution of our study is demonstration of the importance of controlling oxygen tension when studying *in vitro* sex differences of glial cells.

Additional studies are required to determine the mechanisms behind the dichotomous bioenergetic profiles of male and female astrocytes at low physiological 3% oxygen. It also remains to be seen whether the slight quantitative but not qualitative difference in microglial respiratory impairment at 3% O₂ compared to atmospheric O₂ during proinflammatory activation is functionally significant.

Chapter 4: The effects of idebenone on astrocytes and microglia in a proinflammatory environment

4.1 Introduction

Neuroinflammation is a component of most acute and chronic neurodegenerative disorders. Despite interest in the field, there are few FDA approved neuroprotective agents for the prevention or treatment of neuroinflammation. During an inflammatory response, activated microglia release cytokines and reactive nitrogen and oxygen species (Brown & Bal-Price 2003, Block et al. 2007). The effects of inflammation on the mitochondrial bioenergetics of the brain are not well understood. Many factors may contribute to mitochondrial dysfunction, including increased levels of oxidative and nitrosative stress. Activated microglia can have neuroprotective roles including the destruction of pathogens and phagocytosis of dead cell debris; however, prolonged microglia activation can persist for days or even years, resulting in catastrophic effects on the brain (Block et al. 2007a, Dheen et al. 2007, Brown & Bal-Price 2003)

Mitochondrial respiration is impaired in proinflammatory microglia, rendering them dependent on glycolysis to meet their energy demands (Voloboueva et al. 2013, Orihuela et al. 2015). The presence of high levels of nitric oxide produced by increased expression of inducible nitric oxide synthase (iNOS) is considered the primary contributor to impaired respiration (Figure 3.2). A growing body of evidence suggests that mitochondrial dysfunction plays a role in propagating microglial activation (Ye *et al.* 2016, Li & Stary 2016). The rescue of microglial respiration may attenuate the proinflammatory phenotype and is therefore an attractive topic for future studies.

Nitric oxide is thought to reversibly impair mitochondrial respiration by competing with oxygen at complex IV; however, this dogma fails to consider the influence of exposure to reactive oxygen species. Interestingly, it is complexes I and II that are primarily associated with the mitochondrial respiratory deficiency of most degenerative disorders and not complex IV.

Proinflammatory microglia can also produce superoxide by enzymes such as NADPH oxidase (NOX) and iNOS (Xia *et al.* 1998). Oxidation of nitric oxide by superoxide forms peroxynitrite, which can irreversibly impair the activity of multiple respiratory complexes. Additionally, reactive nitrogen species can induce post-translational modifications that inhibit complexes I and II, such as thiol-nitrosations by nitric oxide and tyrosine-nitration by peroxynitrite. The various inhibitory mechanisms introduce a complexity to the system that challenges the prospect of a simple explanation for nitric oxide induce respiratory inhibition.

Idebenone is a clinically safe Coenzyme Q analog mostly known for its powerful antioxidant properties. In Chapter 2, we demonstrated that the reduced form of idebenone, idebenol, can be utilized as a complex III substrate by directly providing electrons for oxidative phosphorylation and bypassing any upstream impairment. The reactive nitrogen and oxygen species produced by activated microglia impairs their mitochondrial respiration (Orihuela *et al.* 2016, Papageorgiou *et al.* 2016). Furthermore, the released cytotoxic factors of microglia are sufficient to induce mitochondrial dysfunction and death in nearby cells (Orihuela *et al.* 2016, Papageorgiou *et al.* 2016). Here we test the hypothesis that idebenone will ameliorate the mitochondrial respiratory impairment of proinflammatory microglia. Our current understanding of the brain's

inflammatory response is highly centered on the role of microglia and the effects of microglial activation on neurons. Due to the misconception that astrocytes are almost exclusively glycolytic cells, the effects of neuroinflammatory insult on astrocyte bioenergetics remain to be explored (Olsen et al. 2015, Magistretti & Allaman 2015). We previously demonstrated that astrocytes, and not neurons, expressed sufficient levels of NADPH:oxidoreductase 1 (NQO1) for idebenone to support respiration in the presence of a complex I inhibitor. Idebenone may therefore support astrocyte respiration in the presence of proinflammatory activated microglia.

In vitro models used to study the role of neuroinflammation in disease often do not account for the influence of oxygen concentrations on these studies. Most *in vitro* studies are performed at atmospheric oxygen levels (~160 mm Hg, 21% O₂) that exceed physiological brain O₂ levels by 4-7 fold (5-45 mm Hg, 1-6% O₂) (Grote et al. 1996). Oxygen tension is known to influence ROS generation and peroxynitrite formation. While oxygen does not affect the viability of cell cultures, cells cultured at atmospheric oxygen produce more mitochondrial reactive oxygen species than cells cultured at 2% O₂ (Ferguson *et al.* 2018). Cells cultured at supraphysiological oxygen had higher antioxidant capacity and were more resistant to oxidative stress (Ferguson et al. 2018). Therefore, oxygen levels may affect the severity and mechanism of mitochondrial respiratory dysfunction induced by proinflammatory factors released by activated microglia.

4.2 Materials and methods

4.2.1 Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

4.2.2 Preparation of primary rat cortical astrocytes

Primary rat cortical astrocytes were prepared as described in 3.2.2

4.2.3 Preparation of primary rat cortical microglia

Primary rat cortical microglia were prepared as described in 3.2.3

4.2.4 HAPI microglia cell culture

The HAPI rat microglial cell line was used for preliminary studies due to the low abundance of primary microglia. HAPI cells are cultured in DMEM containing 10% FBS and 1% Pen/Strep. A proinflammatory response was stimulated by treating cells with 100 ng/mL lipopolysaccharide plus 10 ng/mL interferon- γ (LPS+IFN- γ) for 18 hours prior to assay.

4.2.5 XF24 microplate-based respirometry

Respirometry assays were performed as described in 3.2.5

4.2.6 Microplate-based Idebenone Assay

Idebenone-dependent NADPH oxidation was measured in fresh cell lysates of primary cell cultures or following addition of recombinant enzyme. Primary microglial cells were treated with 100 ng/mL LPS and 10 ng/mL IFN- γ for 18 hours. Cells were washed twice with PBS and then scraped into a 25mM Tris-HCl pH 7.4 hypotonic lysis

solution containing 1:200 protease inhibitor cocktail (Sigma-Aldrich). Lysates were sonicated twice for 15 seconds on ice, then centrifuged at 17,000g for 20 minutes at 4°C. Supernatants were concentrated by centrifuging again at 14,000g for 20 minutes at 4°C using 50 kDa MWCO Amicon Ultra 0.5mL Spin columns. The above 50 kDa sample was collected and protein was estimated using a Nanodrop 1000 (Thermo Fisher Scientific). Idebenone-dependent oxidation of NADPH was detected by measuring NADPH absorbance at 340 nm over 10-20 minutes in the presence or absence of 0.2 µg/µL sample protein, 200 µM NADPH, and 200 µM idebenone and the indicated inhibitor. Assays were run in a 96-well plate format (BMG Labtech).

4.2.7 In-gel activity assays for detection and isolation of idebenone-reducing enzymes.

Cell lysates of HAPI microglia cells were collected as described above after treatment with 100 ng/mL LPS and 10 ng/mL IFN- γ for 18 hours. Precast Tris-Glycine WedgeWell gels (Invitrogen) were used for this assay. Similar to native polyacrylamide gel electrophoresis (PAGE), the anode buffer was composed of a 1x solution of Novex Tris-Glycine Native Running Buffer. The cathode buffer was made from running buffer with the addition of 100 µM of the redox dye 2,6-dichloroindophenol (DCPIP). A 2x loading buffer was made using 1 mM DCPIP and 10% Glycerol in running buffer. Wells were loaded to maximum capacity with ~300 µg protein in 1x loading buffer. Gels were run at 100V for 150 minutes at 4°C. Gels were then removed from their cassette and washed briefly in 25 mM Tris-HCl. Gels were then incubated in 15 mL of 25 mM Tris-HCl, 200 µM NADPH, and 100 µM DCPIP for 5-10 minutes. Pictures of gels were taken using an iPhone 6 (Apple). Images were converted to grayscale for clarity. Enzyme activity is indicated by the absence of color after enzymatic reduction of the dye.

4.2.8 Griess Assay

Nitric oxide concentration was estimated indirectly by detecting the more stable nitric oxide derivative nitrite using a Griess assay (ThermoFisher). After Seahorse respirometry assays, aCSF was collected from each well and the Griess assay was performed according to the manufacturer's protocol. Absorbance was measured at 560 nm and a standard curve of sodium nitrite was used to calculate concentrations.

4.2.9 Statistical analysis

Statistical analyses were performed using SigmaPlot 12.0 (Systat Software Inc, San Jose, CA). Two-way analysis of variance (ANOVA) was employed to evaluate the statistical significance of oxygen consumption measurements. A p value < 0.05 was considered significant. Tukey's post-hoc analysis was used to compare individual groups.

4.3 Results

4.3.1 Idebenone can support respiration in microglia after proinflammatory activation

The mechanism by which the mitochondrial respiration of proinflammatory microglia is impaired is still a controversial topic. The reversible inhibition by nitric oxide may not be the sole mechanism by which the electron transport chain may be impaired. Complex I and II impairment are implicated in most cases of neurodegenerative mitochondrial dysfunction. We therefore tested the effect of idebenone on non-activated and activated microglia. The rat HAPI microglial cell line was used in lieu of primary microglia for preliminary results. Prior to experiments, HAPI cells were activated using the bacterial endotoxin lipopolysaccharide (LPS) and the proinflammatory cytokine interferon- γ (IFN- γ).

Respiration was measured using the Seahorse XF24 Extracellular Flux Analyzer (Figure 4.1). When treated with idebenone, non-activated microglia did not incur a change in basal respiration after addition of idebenone and no change in maximal respiration was observed following the injection of DNP and excess substrate. Non-activated microglia demonstrated some insensitivity to the complex I inhibitor piericidin A in the presence of idebenone. This response was not fully blocked by the complex III inhibitor antimycin A. As expected, the proinflammatory microglia had impaired maximal respiration and no insensitivity to the complex I inhibitor. Interestingly, acute treatment with idebenone induced a small increase in basal respiration as well as a partial rescue of maximal respiration. This effect was mostly insensitive to piericidin A and predominantly inhibited by antimycin A, indicating that complex III and downstream components of the electron transport chain (ETC) are required for the idebenone rescue of respiration. As observed in non-activated microglia, respiration following complex III inhibitor addition was not fully reduced to control levels, suggesting that idebenone-induced oxygen consumption is not exclusively mitochondrial. These results were confirmed in primary microglial cultures in our lab by Evan Bordt (data not shown).

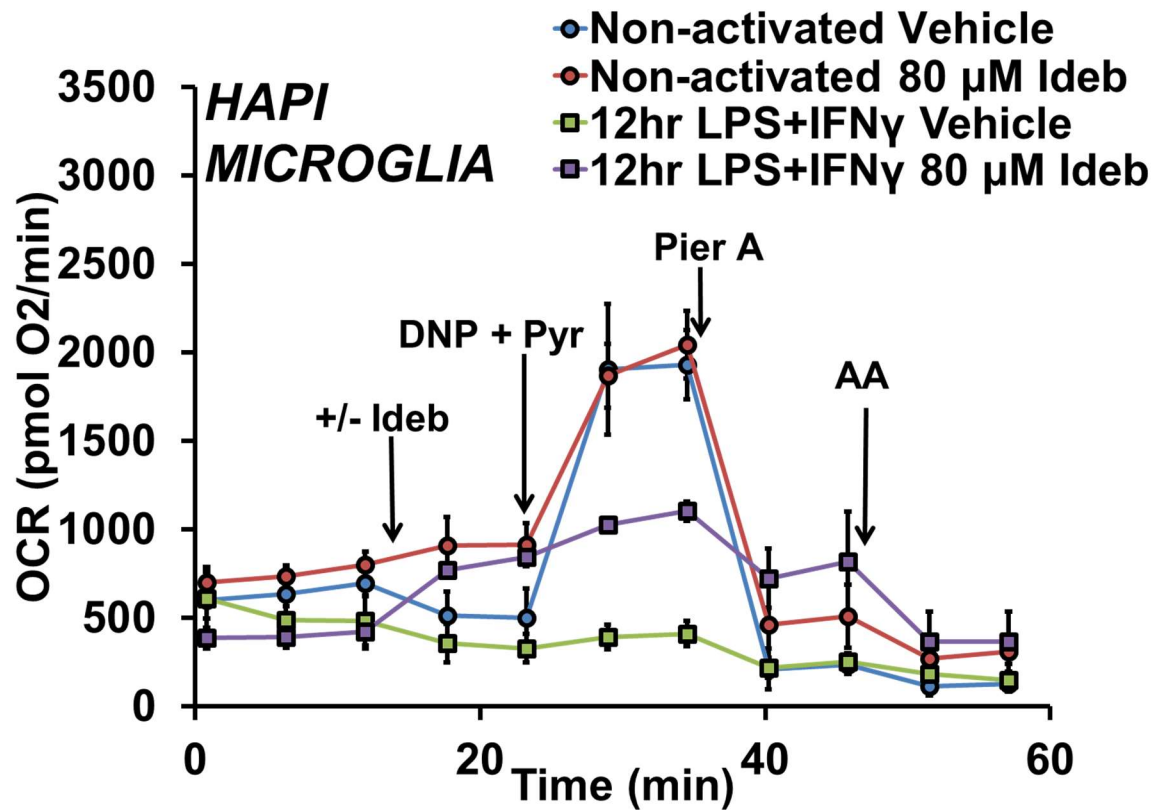


Figure 4.1 Idebenone partially rescues the respiration of proinflammatory microglia
 Oxygen consumption rate (OCR) measurements from non-activated HAPI microglia or HAPI microglia after 12 hours of proinflammatory activation by lipopolysaccharide (LPS) and interferon- γ (IFN- γ). The uncoupler 2,4-dinitrophenol (DNP, 200 μM) was then added in the presence of pyruvate (Pyr, 10 mM) to induce maximal OCR. Piericidin A (Pier A, 100 nM) was added to inhibit complex I and Antimycin A (AA, 1 μM) was added to inhibit complex III. n=1

4.3.2 NQO1 is not responsible for the microglial quinone reduction capacity

In Chapter 2, we showed that idebenone requires extra-mitochondrial enzymatic reduction by NQO1 to restore complex I-inhibited respiration in astrocytes. To compare the quinone reduction capacity among cell types, we measured the rate at which cell lysates could oxidize NADPH in an idebenone-dependent manner. Rat cortical astrocytes had the highest capacity for idebenone reduction among tested cell types (Figure 4.2). This activity was inhibited by the NQO1 inhibitor dicoumarol. Little to no activity was observed in rat cortical neurons or non-activated microglia (Figure 4.2). Proinflammatory microglia showed a significant increase in idebenone-dependent NADPH oxidation, measured after 18 hours of LPS+IFN- γ treatment. Surprisingly, this activity was not sensitive to dicoumarol, suggesting an enzyme other than NQO1 is responsible for this activity.

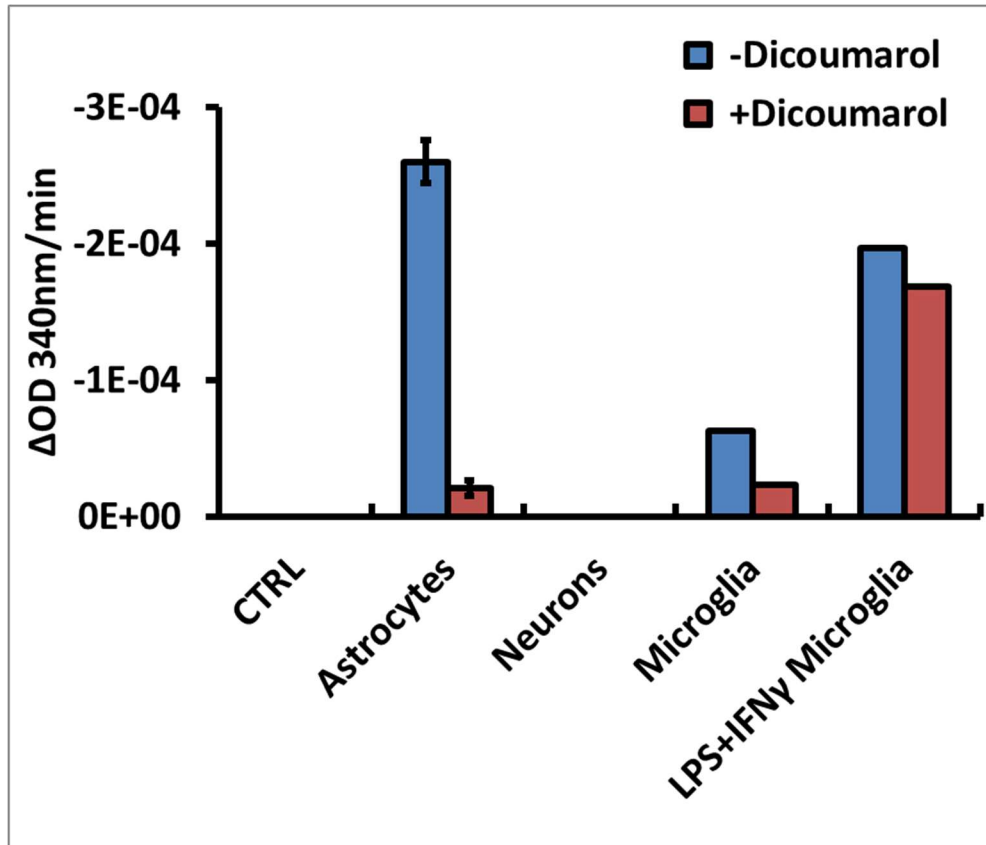


Figure 4.2 Idebenone-dependent NADPH oxidation occurs primarily in astrocytes and proinflammatory microglia Idebenone-dependent NADPH oxidation in primary rat neurons, astrocytes, and microglia was measured using a microplate-based assay. Oxidation of NADPH was detected by measuring absorbance at 340 nm over 10-20 minutes in the presence of 200 μ M NADPH and 200 μ M idebenone, with or without the co-presence of the NQO1 inhibitor dicoumarol (20 μ M). Control (CTRL) indicates measurements performed with 200 μ M NADPH and 200 μ M idebenone but no cell lysate present. n=2-3

4.3.3 Inducible nitric oxide synthase may be responsible for idebenone reduction by proinflammatory microglia.

Identification of the enzyme responsible for this novel idebenone-reducing activity in microglia may lead to improved therapeutic strategies using idebenone, as well as suggest other diseases wherein idebenone may have a beneficial effect. To identify the enzyme or enzymes responsible, cell lysate from non-activated or activated HAPI microglia were loaded onto non-denaturing gels in the presence of the redox dye DCPIP and electrophoresis was performed. Gels were then treated with NADPH and additional DCPIP. The location of in-gel enzyme activity was indicated by the absence of color due to enzymatic dye reduction (Figure 4.3A, yellow arrow). These clear bands were excised and submitted to the Mass Spectrometry Center Core Facility at the University Of Maryland School Of Pharmacy for protein identification. In parallel, we also utilized three distinct approaches to sample fractionation, including cell fractionation by ultracentrifugation, size exclusion chromatography, and cibacron blue NADPH affinity chromatography (data not shown). Among the proteins identified by mass spectrometry following the described techniques above, inducible nitric oxide synthase (iNOS) was one of four NADPH-dependent enzymes present following all approaches to enrich for idebenone-reducing activity. We therefore tested murine recombinant-iNOS (r-iNOS, Sigma-Aldrich) for idebenone-dependent NADPH oxidation in the absence of cell lysate, using the assay described above. r-iNOS was able to oxidize NADPH in the presence but not the absence of idebenone (Figure 4.3B). This indicates that idebenone may be acting as the NADPH electron acceptor. In addition, this finding is consistent with the possibility that iNOS expression induced by LPS+IFN- γ is responsible for idebenone

reduction in proinflammatory microglia. Whether idebenone is reduced to a semi-quinone radical or fully reduced to idebenol by iNOS remains to be seen and is important to explore in future studies.

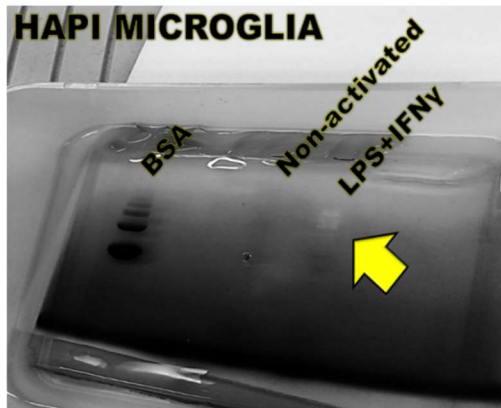
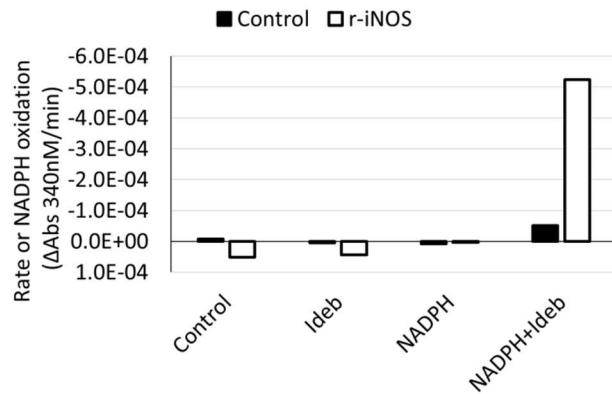
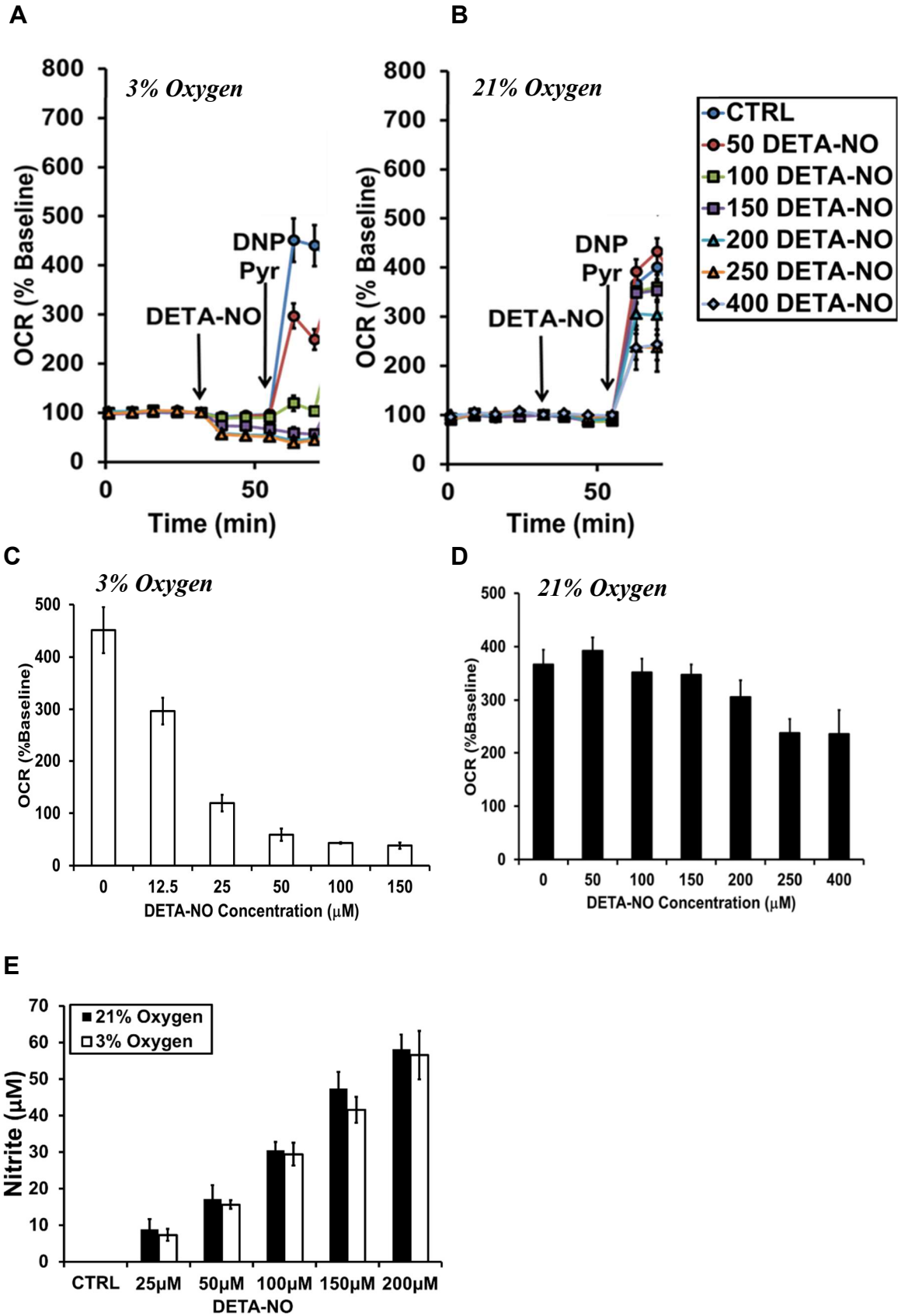
A**B**

Figure 4.3 The idebenone-dependent oxidation of NADPH by recombinant iNOS (A), In-gel activity assay of activated and non-activated HAPI cell microglia lysate. Lysates were run in the presence of DCPIP and treated with 200 μ M NADPH. Bands in which DCPIP was reduced are colorless and are indicated by the yellow arrow. The image is depicted in black and white for clarity. (B), Microplate-based assay of idebenone-dependent NADPH oxidation by recombinant inducible nitric oxide synthase (r-iNOS). Absorbance measurements were collected at 340 nm over 5-10 minutes in 25 mM Tris-HCl, pH 7.4 assay buffer in the presence or absence of 200 μ M NADPH, 200 μ M idebenone, and 1 U/mL of r-iNOS. n=2-3

4.3.4 Primary rat cortical astrocytes cultured at 3% oxygen are more susceptible to respiratory impairment by nitric oxide than cells cultured at 21% oxygen

The effect of idebenone on inflammation during inflammatory processes may depend on the redox state of the cell. The oxygen levels in which cells are cultured and experiments are performed can affect many cellular functions, including respiration and antioxidant defenses. Oxygen levels are known to influence the production of reactive oxygen and nitrogen species produced by cells. Proinflammatory microglia release nitric oxide, which is diffusible and may inhibit the respiration of surrounding astrocytes. We first tested the effects of acute treatment with the nitric oxide donor DETA-NO on the respiration of primary rat cortical astrocytes cultured and measured at 3% oxygen were compared to that of astrocytes cultured and measured at 21% oxygen (Figure 4.4 A and B). Maximal respiration was inhibited by DETA-NO at both 3% and 21% oxygen. However, the respiration of astrocytes at 3% oxygen was impaired by as little as 12.5 μM DETA-NO and fully inhibited by 50 μM DETA-NO, whereas respiratory impairment at atmospheric oxygen required 400 μM DETA-NO (Figures 4.4 C and D). This indicates that astrocytes at a physiologically relevant oxygen level are more susceptible to respiratory impairment by nitric oxide than those at atmospheric oxygen. To ensure that this result was not due to a difference in the behavior of the nitric oxide donor at the different oxygen levels, we measured the levels of nitrite in the media following the assay and found no oxygen-dependent differences(Figure 4.4E).

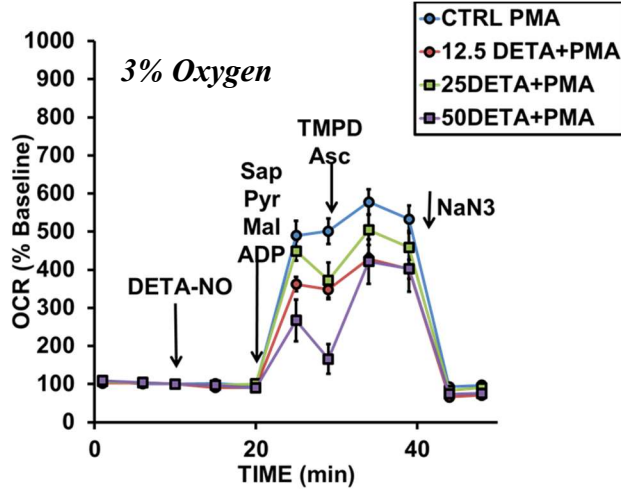
Figure 4.4 Effects of NO donor DETA-NO on maximal astrocyte oxygen consumption rate at 3% vs 21% O₂. (A) and (B), Representative Seahorse OCR traces of primary rat cortical astrocytes OCR measurements performed in triplicate at either 3% or 21% O₂ (A and B respectively). Astrocytes were treated with an acute injection of 0-400 μM of the nitric oxide donor, DETA-NO. The uncoupler DNP (150 μM) plus pyruvate (10 mM) was then added to induce maximal mitochondrial respiration. (C and D) Bar graph representations of maximal respiration from (A and B). (E) Nitrite concentration measured in assay media after Seahorse respirometry assays. n = 5-10 *P < 0.05



4.3.5 DETA-NO impairs astrocyte utilization of complex I and II-linked substrates, but not a complex IV substrate, at physiological oxygen

The respiration of astrocytes at 3% O₂ showed a greater sensitivity to nitric oxide compared to that of astrocytes at 21% O₂. In order to determine if the impairment of oxygen consumption is upstream of or at complex IV, we tested the astrocytes' ability to use different substrates for respiration in the presence of DETA-NO. Astrocytes were treated with DETA-NO and then permeabilized and provided with substrates for complex I-linked respiration (NADH is indirectly provided by pyruvate and malate metabolism) or complex II (succinate). ADP-stimulated OCR in the presence of substrates for complexes I or II-linked respiration was dose dependently inhibited by DETA-NO (Figures 4.5A and B). Respiration was fully reversed following an injection of TMPD/ascorbate, which donates electrons to cytochrome *c* and subsequently complex IV. A final injection of the complex IV inhibitor sodium azide fully reversed this rescue, demonstrating that the respiration was indeed complex IV-dependent.

A



B

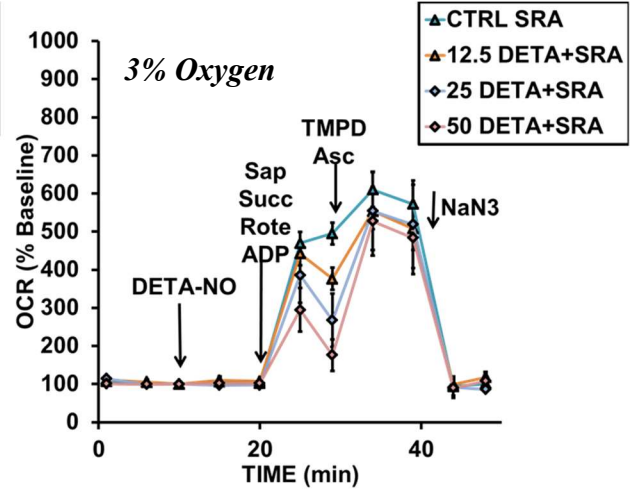


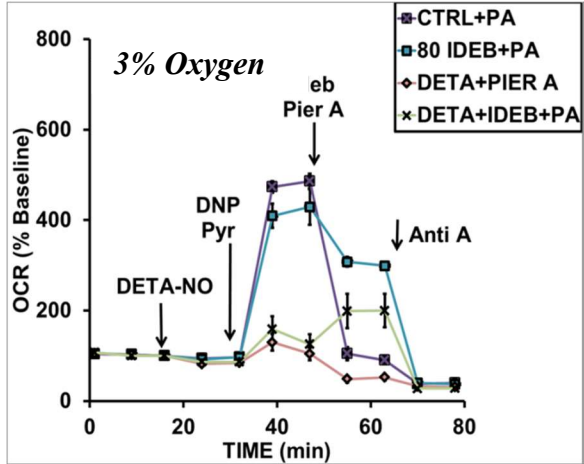
Figure 4.5 Seahorse OCR measurements of astrocytes at 3% O₂ in the presence of substrates for specific electron transport chain complexes OCR measurements from cortical astrocytes at 3% oxygen. Astrocytes were first permeabilized by an injection of saponin (Sap, 2 μ g/mL) in the presence of ADP (1 mM) to stimulate respiration and either substrates for complex I or II. (A) Complex I-linked substrates pyruvate (Pyr, 5mM) and malate (Mal, 5mM); collectively referred to as PMA. (B) the complex II substrate succinate (Succ, 5mM) and the complex I inhibitor rotenone (Rote, 0.5 μ M); collectively referred to as SRA. Injections of the complex IV-linked substrate TMPD (0.4 mM) plus ascorbate (0.4 mM) and finally the complex IV inhibitor sodium azide (NaN₃, 5mM) then followed.

4.3.6 Respiratory impairment by DETA-NO can be rescued by idebenone

Nitric oxide is thought to inhibit at complex IV; however, we found that in astrocytes treated with DETA-NO at 3% O₂, complex IV-linked respiration remained intact. Idebenone's electron carrier function enables it to directly donate electrons to complex III, thereby bypassing upstream impairments at either complex I or complex II.

To determine if idebenone could support respiration in the presence of nitric oxide, we tested the effect of 80 μM idebenone on astrocytes at 3% O₂ following DETA-NO addition. Idebenone was able to partially rescue maximal respiration following DETA-NO even in the presence of the complex I inhibitor piericidin A, in a manner that was reversed by antimycin A, (Figure 4.6A). Reduction of idebenone can also be achieved non-enzymatically by added a reducing agent such as ascorbate. To determine if the concentration required for idebenone to induce a respiratory rescue could be lowered, we titrated the concentration of idebenone in the presence of 2 mM ascorbate (data not shown). We found that in the presence of ascorbate, as little as 10 μM idebenone was sufficient to induce a respiratory rescue (Figure 4.6B). This data indicates that complex III remains functional following DETA-NO addition and provides further evidence that the respiratory impairment caused by NO is indeed upstream of complex IV, as the previous experiments with TMPD/ascorbate suggested.

A



B

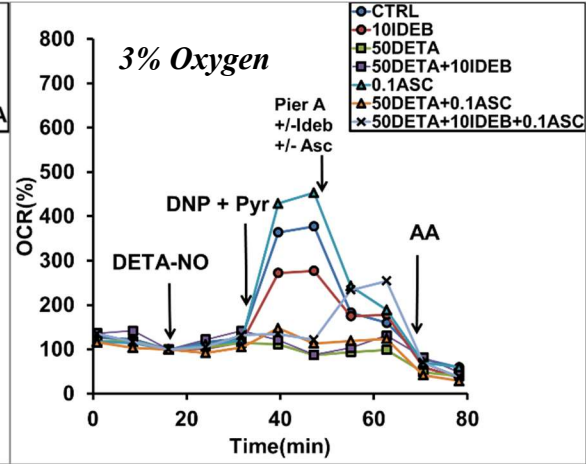


Figure 4.6 Respiratory inhibition by DETA-NO can be partially rescued by idebenone in a complex I-independent manner. Representative traces of Seahorse respiratory experiments using astrocytes at 3% O₂. (A) An initial injection of 50 μ M DETA-NO was followed by DNP+Pyruvate as described above. Idebenone (Ideb, 80 μ M) was added in the presence of the complex I inhibitor piericidin A (100 nm Pier A) followed by a final injection of the complex III inhibitor antimycin a (AA, 1 μ M). (B) Experiment is the same as in (A) except that the third injection is of Pier A plus 10 μ M idebenone in the presence and absence of ascorbate (Asc, 0.1mM).

4.3.7 DETA-NO-impaired astrocyte respiration is rescued at 21% and 3% O₂ by glutathione-ethyl ester or dithiothreitol

The previous experiments demonstrated that complexes I and II activities may be impaired in the presence of nitric oxide. Aside from direct inhibitory action at complex IV, nitric oxide and its derivatives can lead to post-translational modifications, including reversible S-nitrosation of cysteine residues on electron transport chain proteins that potentially inhibit enzyme activity. Reversing S-nitrosation is predicted to rescue nitric oxide-mediated respiratory inhibition. Glutathione is an important endogenous antioxidant regulated by the Nrf2/ARE transcriptional pathway that is essential for cellular defense against oxidative damage (Schulz *et al.* 2000). Additionally, glutathione can protect against nitric oxide mediated S-nitrosations of cysteine residues (Borutaite *et al.* 2000, Clementi *et al.* 1998). Glutathione-ethyl ester (GSH-EE) is a cell permeable glutathione derivative. Impressively, treatment with 1 mM GSH-EE reversed the respiratory impairment of astrocytes caused by DETA-NO concentrations up to 50 μ M (Figure 4.7A). The thiol-reducing agent dithiothreitol (DTT) also rescued respiration (Figure 4.7B). Taken together, these results suggest that a reversible thiol protein modification may, in part, be responsible for nitric oxide-mediated respiratory inhibition.

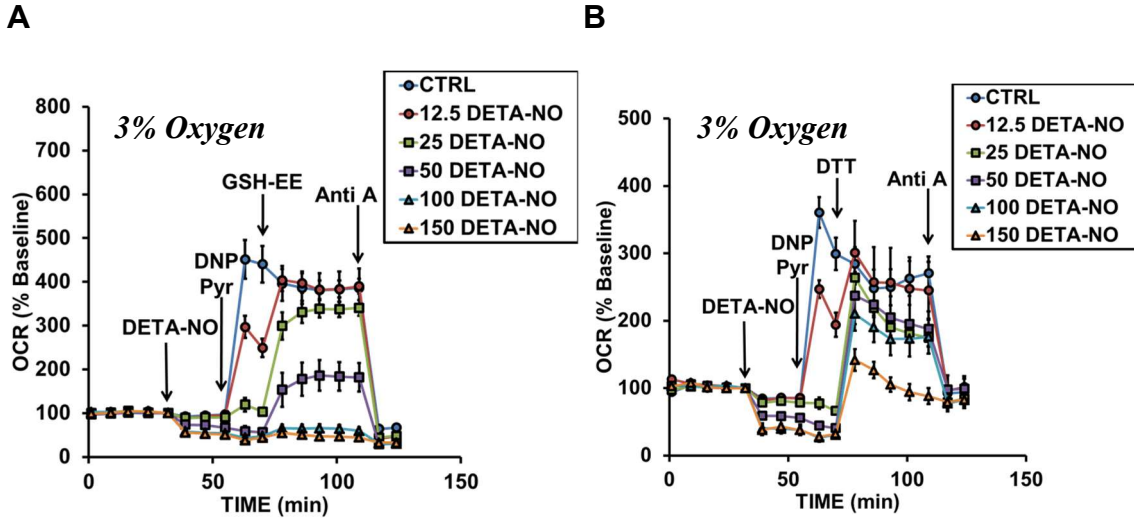


Figure 4.7 Respiration of astrocytes treated with DETA-NO at 3% O₂ is rescued by thiol reducing agents. Astrocytes cultured and tested at 3% O₂ were treated with, (A) glutathione-ethyl ester (1 mM, GSH-EE) or (B) the thiol reducing-agent dithiothreitol (1 mM, DTT) following the sequential administration of DETA-NO and DNP+pyruvate.

4.3.8 Astrocyte respiration in the presence of co-cultured microglia is oxygen dependent

Experiments with the nitric oxide donor, DETA-NO, suggested that NO impairs astrocyte respiration upstream of complex IV. Activated microglia produce multiple forms of reactive oxygen/nitrogen species, potentially modifying the mechanism of astrocyte respiratory impairment compared to exposure to NO alone. We therefore examined the respiration of astrocytes in the presence of proinflammatory activated microglia. HAPI microglia cultured at 3% or 21% oxygen were treated with LPS/IFN- γ for 16 hours. Activated or non-activated HAPI cells were then trypsinized and re-plated on 3% or 21% astrocytes for 1 hour. The respiration of astrocytes was not affected by non-activated HAPI cells; however, respiration was strongly inhibited in the presence of activated microglia at 3% oxygen compared to 21% oxygen (Figure 4.8). Interestingly, the addition of the complex IV linked-substrate TMPD/ascorbate only partially rescued the suppression of maximal respiration of astrocytes co-cultured with activated microglia. However, following addition of the nitric oxide scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), respiration was fully rescued. The addition of sodium azide completely reversed the rescue by CPTIO, indicating that oxygen consumption was dependent on complex IV activity. Taken together, results suggest that inhibition of maximal respiration in astrocytes by proinflammatory microglia may only partially mediated by inhibition of complex IV.

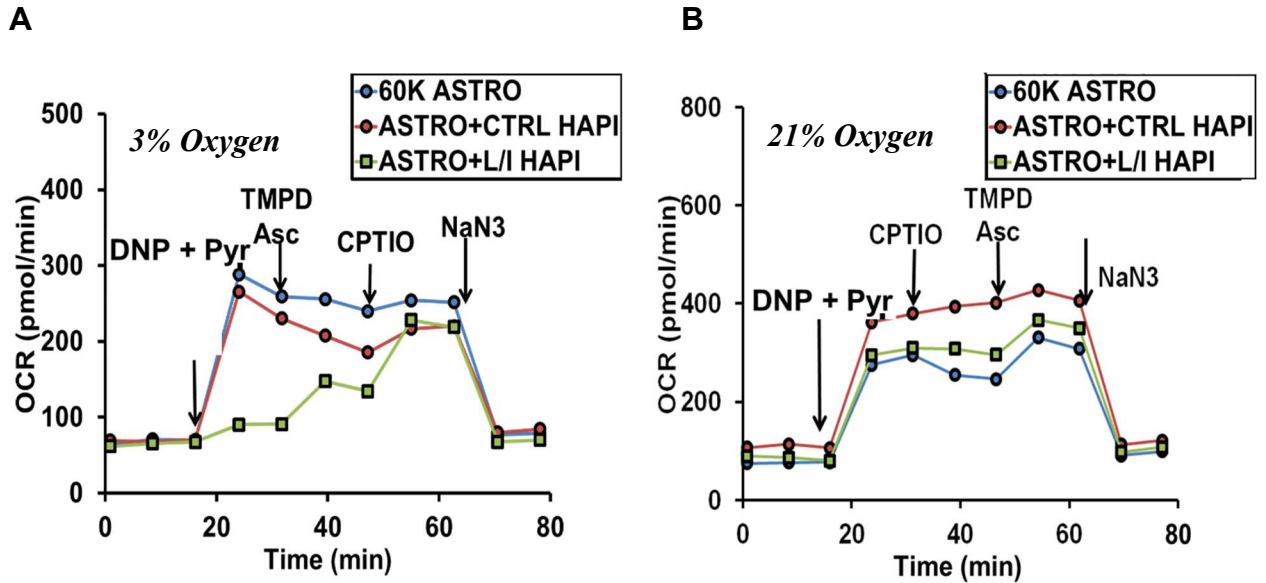


Figure 4.8 Astrocytes are more sensitive to mitochondrial respiratory impairment induced by co-culture with proinflammatory microglia at 3% oxygen compared to at 21% oxygen. Representative Seahorse OCR traces of primary rat cortical astrocytes co-cultured for one hour with 15k HAPI microglial cells. Proinflammatory HAPI microglia were activated for 16 hours with LPS/IFN- γ before re-plating onto astrocytes. OCR measurements at either 3% or 21% O₂ (A and B respectively). The uncoupler DNP (150 μ M) and pyruvate (10 mM) were added to induce maximal mitochondrial respiration. Subsequent injections of either 200 μ M CPTIO or TMPD (0.4 mM) plus ascorbate (0.4 mM) then followed. Finally, sodium azide (NaN₃, 5 mM) was added.

4.4 Discussion

Mitochondrial respiratory dysfunction in the context of neurodegeneration is an attractive target for the development of neuroprotective drugs. Idebenone is a synthetic partial analogue of ubiquinone that, upon reduction to idebenol, can act as an electron carrier from cytosolic NADPH to complex III of the mitochondrial electron transport chain (Haefeli et al. 2011). In Chapter 2 we demonstrated the therapeutic potential of idebenone in astrocytes and suggested a strategy for use in neurons. We also demonstrated the requirement for idebenone reduction by the cytosolic enzyme NADPH:quinone oxidoreductase. In this chapter, we explored the effects of idebenone on microglial bioenergetics and its potential use as an astroprotective agent in the context of neuroinflammation. Upon proinflammatory stimulation, microglia release several cytotoxic agents that may affect mitochondrial respiration. Nitric oxide produced by proinflammatory microglia is believed to be inhibit respiration by competing with oxygen at complex IV; however, microglia also produce other factors that may complicate the inhibitory mechanism (Brown & Cooper 1994, Bolanos & Heales 2010).

Seahorse respirometer measurements of rat cortical primary microglia were taken following proinflammatory stimulation with LPS/IFN- γ for 18 hours. Idebenone was able to partially support respiration of microglia following proinflammatory stimuli indicating that idebenone may have a therapeutic potential for the treatment of neuroinflammation (Ye et al. 2016, Li & Stary 2016). Originally, this finding led us to believe that NQO1 may be upregulated upon proinflammatory stimuli. We therefore tested for idebenone-dependent NADPH oxidizing activity in the lysates of primary rat cortical neurons, astrocytes and non-activated and activated microglia. We found that, as expected, activity

was observed in astrocytes and proinflammatory microglia but not neurons or non-activated microglia. Furthermore, as predicted, the activity was inhibited in astrocyte lysate by the NQO1 inhibitor dicoumarol. To our surprise however, dicoumarol did not similarly inhibit idebenone-dependent NADPH oxidation in microglia, indicating that another enzyme unique to activated microglia may be responsible for the observed activity.

Upon further investigation, inducible nitric oxide synthase (iNOS) emerged as a potential candidate for future studies. To determine the quinone reduction capability of iNOS, recombinant iNOS (r-iNOS) was assayed and was indeed found to have idebenone-dependent NADPH oxidation activity. While iNOS does seem to have the capacity to reduce idebenone, more experimentation is required before more concluding that iNOS is the enzyme responsible for idebenone reduction in proinflammatory microglia.

In Chapter 3 we demonstrated the effect that atmospheric 21% and physiologically relevant 3% oxygen environments can have on primary rat cortical astrocyte and microglial respiration. Primary microglia cultured and assayed at 3% oxygen had a greater overall respiratory impairment following proinflammatory stimulation than those at 21% oxygen.

Previous studies have demonstrated that oxygen can regulate cytokine-induced iNOS expression (Berge *et al.* 2001). Prior to further testing, we first considered the effects of nitric oxide on astrocytes at 3% vs 21% oxygen. Astrocyte oxygen consumption was measured after an acute treatment with a nitric oxide donor, DETA-NO. Similar to our earlier findings in microglia, astrocyte respiration was more sensitive

to respiratory inhibition at 3% than 21% oxygen. DETA-NO impaired oxygen consumption in a dose dependent manner at concentrations 4-7 fold less at 3% oxygen than was required at 21% oxygen. These results indicate that oxygen is an important variable to control for in studies of mitochondrial respiration and neuroinflammation.

The mechanism by which nitric oxide inhibits respiration in astrocytes is not clear. We therefore tested the ability of complexes I, II and IV-linked substrates to support respiration in the presence of DETA-NO. We found that complexes I and II had impaired substrate utilization while complex IV activity remained intact. Idebenone can donate electrons to complex III. We therefore tested whether idebenone could support respiration in the presence of DETA-NO at 3% oxygen. We found that idebenone was able to rescue much of the respiration and that co-treatment with the reducing agent ascorbate could augment the effect. Taken together, these data indicate a mechanism of respiratory impairment upstream of complex IV and give rise to some hope that idebenone will prove to be of therapeutic use in diseases involving neuroinflammation.

One mechanism of action by which nitric oxide may inhibit respiration is through the S-nitrosation of protein cysteine residues. To provide evidence that this is a potential mechanism of respiratory inhibition by nitric oxide, we demonstrated a rescue of DETA-NO-impaired astrocyte respiration at 3% oxygen by the glutathione derivative glutathione-ethyl ester (GSH-EE) and by the thiol-reducing agent dithiothreitol (DTT). Both GSH-EE and DTT can reverse thiol modifications, further suggesting that NO-mediated competition with oxygen at complex IV is insufficient to explain respiratory inhibition upon DETA-NO treatment.

The use of chemical nitric oxide donors such as DETA-NO is useful for preliminary understanding of inflammatory bioenergetic impairment mechanisms in the brain. However, microglia release several cytotoxic factors that can affect the respiration of surrounding cells. We therefore measured the respiration of primary astrocytes in the presence of proinflammatory HAPI microglia cells at 3% oxygen. HAPI cells were activated 16 hours prior to transferring onto primary astrocytes 1-2 hours before the start of the assay. Corroborating the data with DETA-NO in earlier studies, astrocytes co-cultured with proinflammatory HAPI cells were more sensitive to respiratory impairment at 3% than 21% oxygen. However, in contrast to the experiments conducted with DETA-NO, respiration was not fully rescued upon addition of complex-IV linked substrate. Respiration could, however, be rescued by the nitric oxide scavenger CPTIO. Taken together, this suggests that astrocyte complex IV is partially impaired by nitric oxide in the presence of proinflammatory microglia. This work demonstrates a clear need for controlling for oxygen availability in *in vitro* studies of neuroinflammation. New literature has emerged indicating that the level of oxygen in the human brain is closer to 5% than 3% O₂. Future directions for this study include co-culturing primary microglia on primary astrocytes at 5% oxygen and further exploring the therapeutic use of idebenone in mitochondrial mechanisms of neuroinflammation.

Chapter 5: Discussion

Neuroinflammation and mitochondrial bioenergetic dysfunction are associated with many neurodegenerative diseases (van Horssen et al. 2017, Witte et al. 2010). Microglia are the resident immune cells of the brain and, when activated to a proinflammatory state, they release cytotoxic factors, such as nitric oxide (NO) that can disrupt mitochondrial respiration (van Horssen et al. 2017, Witte et al. 2010). Nitric oxide can inhibit respiration by competing with oxygen at complex IV of the electron transport chain; however, the literature suggests other modes of inhibition through post-translational modifications to upstream complexes I and II (Brown 2001). Impairment of complexes I and II activity is more commonly associated with neurodegenerative disease. Strategies to overcome this impairment have included a mechanistic bypass of complexes I and II by direct electron donation to complex III. This can theoretically be achieved by providing the cell with reducing equivalents in the form of ubiquinol analogs.

In Chapter 1, we provided an overview of energy metabolism in the brain and a review of the mitochondrial electron transport system. Ubiquinone, commonly referred to coenzyme Q10 (CoQ), is a lipophilic electron carrier and endogenous antioxidant found in all cellular membranes. In the mitochondrial inner membrane, CoQ transfers electrons from complexes I or II to complex III. We then introduced the synthetic prodrug idebenone and discussed how it can influence mitochondrial function within the context of cytoprotection. Idebenone was developed to be a short chain CoQ partial analogue that can achieve a higher bioavailability and antioxidant capacity than that of CoQ supplementation. Additionally, we reviewed the performance of idebenone in various clinical trials and suggested a mechanistic reason for its disappointing results. Idebenone

was recently approved in Europe for the treatment of Leber's Hereditary Optic Neuropathy, a mitochondrial disease caused by deficient complex I activity (Klopstock et al. 2011, Lyseng-Williamson 2016). However, despite a strong premise, it has been less successful in clinical trials for other neurodegenerative diseases involving mitochondrial dysfunction such as Alzheimer's disease. Although championed as an antioxidant, idebenone can also act as a pro-oxidant by forming an unstable semiquinone at complex I (King et al. 2009). Furthermore, idebenone has been shown to act as an inhibitor of complex I in its oxidized form, causing it to fall short as a full CoQ analogue (Shivaram *et al.* 1998).

We then discussed a potential role for cytoplasmic NADPH:quinone oxidoreductase 1 (NQO1) in idebenone-mediated cytoprotection. NQO1 was identified as a major enzyme catalyzing idebenone reduction to idebenol (Haefeli et al. 2011). While reduction allows idebenone to act as an antioxidant, evidence also suggests that NQO1 enables idebenone to shuttle reducing equivalents from cytoplasmic NADPH to mitochondrial complex III, bypassing any upstream damage to the electron transport chain (Haefeli et al. 2011). Importantly, we pointed out that in the brain, NQO1 is primarily expressed by astrocytes rather than neurons. The differential expression of NQO1 may imply that idebenone could be more effective at supporting the mitochondrial respiration of astrocytes than neurons.

In Chapter 2, we tested the hypothesis that cortical astrocytes, and not neurons, have sufficient intracellular NQO1 activity to use idebenone as an electron donor to support mitochondrial respiration. We demonstrated that in astrocytes, idebenone stimulated basal respiration and rescued maximal respiration in the presence of a complex

I inhibitor. However, idebenone was not able to support respiration in neurons and instead inhibited respiration at complex I. Delivery of active recombinant NQO1 and NADPH rescued impaired neuronal respiration. Pharmacological Nrf2 activators failed to induce NQO1 expression in neurons but increased NQO1 in COS-7 cells that also displayed a low endogenous level of NQO1. These cells effectively used idebenone as a complex III donor only when NQO1 was both elevated and active. Other quinones evaluated were either ineffective or demonstrated a significant potential for non-mitochondrial off-target effects. Overall, results indicate that idebenone inhibits mitochondrial respiration in the absence of sufficient intracellular quinone reduction activity, including in cortical neurons, but stimulates respiration through complex III when NQO1 is present or transcriptionally induced by Nrf2. Optimizing NQO1 expression in appropriate cell types within a specific disease context may be key to delivering on idebenone's therapeutic potential.

To determine the bypass potential of idebenone on proinflammatory nitric oxide-mediated respiratory inhibition, we focused on astrocytes for their endogenous ability to utilize idebenone for respiration. Prior to beginning these studies, we first characterized the bioenergetic profile of astrocytes and microglia at a physiologically relevant oxygen concentration. Oxygen tension is an often-overlooked variable in many cell culture studies. The excess levels of oxygen in the atmosphere have been shown to regulate nitric oxide and induce oxidative stress in cultured cells (Boveris *et al.* 2000, Witte *et al.* 2010). Reactive oxygen species such as superoxide can react with nitric oxide and its derivatives to form other harmful reactive radicals (Adams *et al.* 2015). In Chapter 3, we demonstrated that controlling for oxygen availability is an important consideration for

studies on mitochondrial respiration that are relevant to neuroinflammation. This may be of particular significance in cellular respiration studies where mitochondria are considered to be the main producers of reactive oxygen species. Prior to investigating the effects of nitric oxide, we first characterized the bioenergetic profiles of male and female rat cortical astrocytes and microglia at different oxygen tensions.

Biological sex is thought to influence mitochondrial bioenergetic function. This study tested the hypothesis that sex and/or brain physiological oxygen tension influence the mitochondrial bioenergetic properties of primary rat cortical astrocytes and microglia. Previous respiration measurements examining brain mitochondrial sex differences were made at atmospheric oxygen using isolated brain mitochondria. Oxygen is 160 mm Hg (21%) in the atmosphere, while the oxygen tension in the brain generally ranges from ~5-45 mm Hg (~1-6% O₂) (Grote et al. 1996). Oxygen consumption was measured with a Seahorse XF24 cell respirometer in an oxygen-controlled environmental chamber. Strikingly, male astrocytes had a higher maximal respiration than female astrocytes when cultured and assayed at 3% O₂. Three percent O₂ yielded a low physiological dissolved O₂ level of ~1.2% (9.1 mm Hg) at the cell monolayer during culture and 1.2-3.0% O₂ during assays. No differences in bioenergetic parameters were observed between male and female astrocytes at 21% O₂ (dissolved O₂ of ~19.7%, 150 mm Hg during culture) or between either of these cell populations and female astrocytes at 3% O₂. In contrast to astrocytes, microglia showed no sex differences in mitochondrial bioenergetic parameters at either oxygen level, regardless of whether they were non-stimulated or activated to a proinflammatory state by LPS/IFN- γ . However, both basal and maximal OCR were impaired to a greater degree at 3% O₂ compared to 21% O₂ following LPS/IFN- γ

treatment. There were also no O₂- or sex-dependent differences in proinflammatory TNF- α or IL-1 β cytokine secretion measured at 18 hours activation. Overall, results revealed an intriguing sex variance in astrocytic maximal respiration that requires additional investigation. Findings also demonstrated that sex differences could be masked by conducting experiments at non-physiological O₂. The impaired oxygen consumption of microglia following proinflammatory activation is thought to be mediated by nitric oxide. Greater impairment of proinflammatory microglial maximal and basal OCR at 3% oxygen compared to atmospheric oxygen provides a strong rationale for considering oxygen as a variable in the study of nitric oxide-mediated respiratory impairment. We would therefore recommend adjusting for oxygen during *in vitro* experiments by both culturing and performing experiments at oxygen levels that are specific to the tissue of interest.

In Chapter 4, we explored the potential for therapeutic use for idebenone as a mitoprotective agent in the context of neuroinflammation. Neuroinflammation occurs in most acute and chronic neurodegenerative disorders. Due to the misconception that astrocytes are almost exclusively glycolytic cells, the effects of neuroinflammatory insult on astrocyte bioenergetics remain relatively unexplored (Olsen et al. 2015, Magistretti & Allaman 2015). Prior to using astrocytes, we first tested the effect of idebenone on proinflammatory HAPI microglia. Idebenone was able to partially support respiration of HAPI microglia after proinflammatory activation with LPS/IFN- γ . To compare the cellular capacity for reducing idebenone in astrocytes, neurons, and activated and non-activated primary rat cortical microglia, we estimated the capacity for idebenone reduction indirectly by measuring idebenone-dependent NADPH oxidation in cell lysates.

NADPH quinone oxidoreductases and other enzymes capable of quinone reduction together form a cell's quinone reduction capacity. Rat cortical astrocytes had the highest capacity for idebenone reduction among tested cell types. This activity was inhibited by the NQO1 inhibitor dicoumarol. Little to no activity was observed in rat cortical neurons or non-activated microglia. Proinflammatory microglia had a significant increase in idebenone reduction potential. This activity was not sensitive to dicoumarol, suggesting NQO1 was not responsible for this activity. To isolate the responsible enzyme, we used an in-gel activity assay followed by mass spectrometry analysis of the enzymatically active bands. Inducible nitric oxide synthase emerged as a potential candidate for quinone reduction activity. Upon testing recombinant iNOS for idebenone-dependent NADPH oxidation, we found that iNOS was able to reduce idebenone. More studies are required to determine whether iNOS is the enzyme responsible for the idebenone-mediated rescue of proinflammatory microglial respiration. A recent computational analysis study found a molecular docking site for quinones on iNOS and suggested quinones as potential inhibitors of iNOS-mediated nitric oxide production (Narayanaswamy et al. 2017). The rescue of mitochondrial respiration in proinflammatory microglia by idebenone along with a potential inhibitory function on nitric oxide production are promising avenues to pursue for future therapeutic studies. On the other hand, iNOS may also catalyze the one electron reduction of idebenone to a semiquinone and contribute to the formation of reactive oxygen species.

During an inflammatory response, activated microglial release cytokines, nitric oxide (NO), and superoxide which cause mitochondrial dysfunction in neighboring cells (Block et al. 2007a, Dheen et al. 2007, Brown & Bal-Price 2003). Nitric oxide in

particular is thought to directly impair mitochondrial respiration by competing with oxygen at complex IV (Brown & Cooper 1994). However, most studies pertaining to this dogma were performed in isolated mitochondria at super-physiological atmospheric oxygen. We attempted to account for this by culturing and assaying under a more physiologically relevant oxygen as described above. Primary rat cortical astrocytes demonstrated a far greater (>25-fold) sensitivity to mitochondrial respiratory impairment by acute treatment with the NO donor DETA-NO at 3% oxygen compared to 21% oxygen. The glutathione analogue glutathione-ethyl ester (GSH-EE)—a potent antioxidant—reversed this effect. Respiration was also rescued by the NO scavenger CPTIO. The respiratory impairment caused by nitric oxide was originally thought to occur at complex IV. However, we found that the utilization of substrates for complex I and II was impaired by DETA-NO, while complex IV substrate rescued respiration. Idebenone was also able to partially rescue respiration, implying that the respiratory impairment caused by NO was upstream of both complexes III and IV. Consistent with these results, co-culture of astrocytes with proinflammatory HAPI microglia demonstrated a respiratory impairment of astrocytes at 3% oxygen while astrocyte respiration at 21% oxygen remained minimally affected. Respiration was also fully rescued by the nitric oxide scavenger CPTIO. However, unlike DETA-NO, respiration in astrocytes co-cultured with proinflammatory microglia was only partially rescued by complex IV substrate. This may suggest a potentially different mechanism of action that requires further investigation.

Reactive oxygen and nitrogen species act as signaling molecules in the healthy brain (Ghasemi *et al.* 2018). In excess, nitric oxide and its derivative peroxynitrite can

induce reversible and irreversible post-translational modifications of cysteine and tyrosine residues of mitochondrial respiratory complexes, respectively, which can contribute to the pathogenesis of neurodegenerative diseases (Srinivas Bharath 2017b). The mechanism by which thiol nitrosations occur is still under study; some proposed mechanisms may involve reaction of nitric oxide with reactive oxygen species or transition metals such as iron.

The effect of oxygen on superoxide production is an important factor when considering the regulation of iron-sulfur cluster-containing enzyme activity. Iron sulfur clusters are critical to the function of several metabolic enzymes, including electron transport chain complexes I, II, and III, as well as aconitase (Ghasemi et al. 2018). Superoxide can oxidize and destroy iron-sulfur clusters, resulting in the release of iron and an increase in cellular free iron levels. Superoxide can also be reduced to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide can then react with iron to form hydroxyl radicals, which can damage cells by oxidizing lipids, nucleic acids, and proteins (Liochev & Fridovich 1999).

While oxygen concentration may have direct effects on nitric oxide metabolism and action, the strong association of oxygen, nitric oxide and cellular iron homeostasis is greatly underappreciated in the brain. Iron dysregulation has been reported in many neurodegenerative diseases (Carocci *et al.* 2018). Bosworth et al. found that nitrosothiol formation is independent of nitric oxide concentration and only observed at very low oxygen (Bosworth *et al.* 2009). This group later proposed that nitrosations occur by rapid reaction of NO with free iron to form dinitrosyliron complexes (DNIC) followed by a slower transnitrosation to thiol groups (Bosworth et al. 2009). Importantly, microglia

increase their iron uptake upon proinflammatory stimulation and the effect of this on mitochondrial dysfunction has not been studied (McCarthy *et al.* 2018, Riederer *et al.* 1989). The association of iron, nitric oxide and oxygen is a complex and exciting topic for future studies in neurodegeneration. A better understanding of these mechanisms can contribute to the approach of designing combined treatment strategies for mitochondria-targeted therapeutics such as the use of iron chelators to modulate intracellular iron levels, activators of the Nrf2/ARE pathway and antioxidants to reduce oxidative damage, and idebenone to support mitochondrial function.

Idebenone has been investigated in human disease as a single-agent therapy. What if we could upregulate NQO1 in the degenerating brain in a cell-specific and controlled fashion? Given the underwhelming success of idebenone in clinical trials, targeted induction of NQO1 in concert with idebenone treatment would seem to be the wave of the future. In addition to evidence that the Nrf2 antioxidant response pathway *per se* is neuroprotective, NQO1 is predicted to greatly increase the neuroprotective potential of idebenone by lessening unstable semiquinone formation while at the same time enhancing antioxidant and electron transfer activity. Therefore, idebenone and an Nrf2-inducing agent may be a strongly synergistic drug combination. Several small molecule electrophiles, including sulforaphane and *tert*-butylhydroquinone, initiate Nrf2-dependent gene expression (Kraft *et al.* 2004, Li & Kong 2009). However, in most cases induction of Nrf2-responsive genes by these agents is selective for astrocytes over neurons both *in vitro* and *in vivo* (Kraft *et al.* 2004, Habas *et al.* 2013, Murphy *et al.* 2001, Ahlgren-Beckendorf *et al.* 1999, Shih *et al.* 2003). Neuroprotective changes in neuronal gene expression seem to depend on altered neuron-glia interactions initiated by Nrf2 activity

in astrocytes (Kraft et al. 2004, Habas et al. 2013). While enhancing idebenone metabolism in astrocytes is indeed desirable, as this may increase the neuroprotective potential of astrocytes, improving NQO1-catalyzed idebenone reduction in both neurons and astrocytes may ultimately yield the greatest therapeutic benefit.

Evidence that the Nrf2 pathway can also be recruited by small molecules in neurons as well as astrocytes is gradually accumulating. Uptake of a class of electrophilic neurite outgrowth-promoting prostaglandin compounds known as NEPPs is reportedly selective for neurons compared to astrocytes (Sato et al. 2006). NEPP11 crosses the blood-brain barrier, activates the Nrf2 pathway in neurons *in vitro* and *in vivo*, and exhibits neuroprotection in an animal stroke model (Sato et al. 2006). Carnosic acid was described by the same group to activate the Nrf2 pathway in both neurons and astrocytes and exhibit protection against focal ischemia/reperfusion brain injury (Sato et al. 2008). Interestingly, chlorogenic acid, a compound found in both caffeinated and decaffeinated coffee, increases NQO1 expression in primary embryonic neurons without induction of other prototypical Nrf2-responsive genes such as heme oxygenase-1 (Kim *et al.* 2012).

The idea of combination therapy is not without caveats. One important caveat is that NQO1-dependent shuttling of electrons to complex III by idebenone has not been demonstrated to occur in neurons, astrocytes, or other glia *in vivo*; cell-specific metabolic differences may preclude this mechanism of action. Another caveat is that QS-10, a primary metabolite of idebenone (Torii et al. 1985), is not capable of transferring electrons to complex III though it is reduced by NQO1 (Haefeli et al. 2011). Thus, idebenone metabolism upstream of NQO1 may limit the efficacy of the proposed electron bypass mechanism. A third caveat is that while the human safety of idebenone is well

established, combination therapy may cause unexpected, dose-limiting toxicity that precludes usefulness. For example, a high level of cytoplasmic NADPH oxidation by NQO1 may alter the redox state of cells if NADPH-regenerating capacity is low or impaired (Kim *et al.* 2013, Dragan *et al.* 2006).

Finally, cells may have enzymes in addition to NQO1 that can reduce idebenone effectively without semiquinone radical formation. As we learn more about the cellular metabolism of this intriguing molecule, new avenues for its therapeutic use may emerge. Efforts are underway to synthesize idebenone analogues with preserved electron transfer function but reduced ability to impair complex I activity (Fash *et al.* 2013). It remains to be seen whether the ability of idebenone to inhibit complex I activity and stimulate mitochondrial superoxide production, phenomena so far observed only *in vitro*, impacted the success of idebenone in clinical trials.

The results in this dissertation demonstrate the importance of controlling for oxygen level in studies of bioenergetics and neuroinflammation. Our understanding of how and why mitochondria are affected in neurodegenerative diseases is hindered by our limited ability to study mitochondrial function *in vivo*. Ideally, *in vitro* studies should provide us with an efficient and reproducible model system that reliably mimics events occurring *in vivo*. Unfortunately, the targeted approach of *in vitro* models often fails to accurately recreate the unforeseen physiological events that occur *in vivo*. When culturing cells in isolation, we blind ourselves to innovative discoveries that are truly translatable, which in turn, compromises the credibility of *in vitro* studies. Ironically, the over-generalized approach to clinical methods and unforeseen variabilities can pose the same risk of inconclusive and lackluster results.

One of the highlights of this dissertation is to demonstrate the importance of a more physiologically inclusive approach to scientific methodology *in vitro*. Failing to control for the contributions of environmental variables and cell-type specificity may be a disadvantage to our understanding of diseases and therapeutics. Using a more accurate simulation of physiological conditions by accounting for the contributions of neighboring cell types and extracellular oxygen levels *in vitro* may potentially lead to experimental results that are more predictive and correlative to what is observed *in vivo*.

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