Role of Astrocytes in Ischemic Preconditioning-Induced Neuroprotection

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ROLE OF ASTROCYTES IN ISCHEMIC PRECONDITIONING-INDUCED NEUROPROTECTION

By

Srinivasan Narayanan

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

August 2015
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

ROLE OF ASTROCYTES IN ISCHEMIC PRECONDITIONING-INDUCED
NEUROPROTECTION

Srinivasan Narayanan

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Cerebral ischemia, most notably in the form of stroke, is a leading cause of morbidity and mortality resulting in long-term disability in the United States (U.S.). Investigating therapies that could be preemptively administered to individuals with a risk factor for stroke could improve prognosis following this devastating condition. One such prophylactic therapy could be represented by ischemic preconditioning (IPC). IPC is the intrinsic neuroprotective response from a brief sub-lethal ischemic event which increases the tolerance towards a future lethal ischemic event. However, previous preconditioning studies in the brain have been studied mainly from the perspective of neurons; as a result, the role of astrocytes in mediating IPC-induced neuroprotection is poorly understood.

Two functions ascribed to astrocytes are antioxidant production and lactate production. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor involved in upregulating endogenous antioxidant production, and may help ameliorate oxidative stress following cerebral ischemia. In addition, astrocyte-derived lactate has been implicated in maintaining neuronal metabolism, particularly under ischemic conditions. As oxidative stress and bioenergetic failure act cooperatively to contribute to the pathogenesis of stroke, the central hypothesis of my thesis work was that IPC
treatment modulates astrocyte function, leading to neuroprotection in the context of cerebral ischemia.

My findings suggest that Nrf2 is significantly enriched in rodent astrocytes compared to neurons. Subsequent work demonstrates that IPC and resveratrol preconditioning (RPC), an IPC-mimetic treatment, had reduced neuroprotective effects in Nrf2−/− mice compared to WT mice. These studies suggest that Nrf2 contributes to preconditioned-induced protection of astrocytes and neurons.

To investigate the effects of IPC-treatment of astrocytes on neuroprotection, we developed a modified astrocyte-neuronal co-culture system that allows us to precondition astrocytes independent of neurons. IPC-treatment of astrocytes alone induced protection of neurons against lethal oxygen-glucose deprivation (OGD), an *in vitro* model of cerebral ischemia. In addition, we found that astrocytes increased lactate production following IPC treatment. Finally, exogenous lactate administration to neurons for 48 hours induces neuroprotection against lethal OGD. These findings suggest that IPC-treatment of astrocytes can induce ischemic tolerance to neurons. In addition, exogenous administration of lactate may represent a neuroprotective agent to be used in the context of cerebral ischemia.

From my current thesis work, our results suggest that the astrocyte-enriched transcription factor Nrf2 contributes to IPC and RPC-induced neuroprotection. Furthermore, IPC-treatment of astrocytes alone transferred ischemic tolerance to neurons.
through soluble mediators. One of these mediators may be lactate, as exogenous lactate administration induced neuroprotection to neurons *in vitro*. As oxidative stress and bioenergetic failure are hallmarks of stroke, the ability of astrocytes to produce antioxidants and lactate in the context of cerebral ischemia could represent viable targets for future stroke therapy.
To my father Shri, my mother Usha, my sister Sheela, and my fiancé Pinal for their continued love and support.
ACKNOWLEDGMENTS

This project would not have been possible without the help of many people. My sincerest thanks to my mentor, Dr. Miguel Perez-Pinzon, for his excellent guidance and help with my thesis work, dissertation revisions, and development as a scientist. Also, thanks to my committee members, Dr. Antonio Barrientos, Dr. Grace Zhai, and Dr. Carlos Moraes, who offered guidance and support throughout my doctoral tenure. I would also like to extend my deepest appreciation to my colleagues, past and present: Dr. Kahlilia Morris-Blanco, Dr. Hung Wen Lin, Dr. Jacob Neumann, Dr. Kunjan Dave, Dr. Ami Raval, Charles Cohan, Holly Stradecki, Kevin Koronowski, and Daniel Perez for their technical help and guidance throughout my thesis. I would like to thank the University of Miami M.D./Ph.D. program and the Neuroscience Graduate Program for giving me the opportunity to complete my dissertation. And finally, I want to thank to my fiancé, parents, and numerous friends and family who endured this long and arduous process with me, always offering their love and support.
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Publication of Chapters and Role of Author

**Chapter 1:** This was modified from previously published review papers and book chapters:

*co-first authors


*co-first authors

I prepared and co-wrote these reviews with my co-authors and mentor Dr. Miguel Perez-Pinzon. The illustrations were designed by me or Dr. John Thompson (Figure 1.6) with Dr. Perez-Pinzon.

**Chapter 2:** I designed the research, analyzed data, and prepared and wrote the chapter. I performed all of the research. Dr. Kunjan Dave gave technical assistance and scientific development of the mitochondrial experiments in Figure 2.5 and 2.7 under my and Dr. Perez-Pinzon’s direction.

**Chapter 3:** This chapter was modified from a previously published peer-reviewed original basic science manuscript:

Narayanan SV, Dave KR, Saul, I., Perez-Pinzon MA. Stroke. 2015. Resveratrol preconditioning protects against cerebral ischemic injury via Nrf2. IN PRESS. PMID: 25908459

I performed all of the research. Dr. Kunjan Dave gave technical assistance and scientific development of the mitochondrial experiments in Figure 3.4 and 3.5 under my and Dr. Perez-Pinzon’s direction.

**Chapter 4:** I performed all of the research, with assistance and development of the chapter under the guidance of Dr. Perez-Pinzon.

**Disclosures:**

Dr. Perez-Pinzon was supported in part by the University of Miami Miller School of Medicine. All authors were supported by grants from the National Institutes of Health,
National Institute of Neurological Disease and Stroke (NINDS) NS45676, NS054147, NS34773, F31NS080344.

**Sources of Funding**
This work was supported by grants from the National Institutes of Health, National Institute of Neurological Disease and Stroke (NINDS) NS45676, NS054147, NS34773, F31NS080344, and the Lois Pope Life Science Research Award.
Chapter 1: Introduction

1.1 Background: Cerebral ischemia and pathogenesis of stroke

Cerebrovascular disease, primarily “stroke,” is the fourth leading cause of death in the United States. Approximately 800,000 strokes occur each year in the U.S. and 87% of all strokes in the world are caused by embolism, thrombosis, or systemic hemorrhage/hypoperfusion, all of which are a form of cerebral ischemia. Thus, development of novel prophylactic therapies that target individuals at high-risk of developing stroke (i.e. patients with hypertension, diabetics, or history of smoking) could lead to improvements in survival and neurological outcomes following such a devastating condition. Focal cerebral ischemia is caused by a lack of oxygen due to little or no blood flow in the cerebral circulation, which affects the entire tissue distribution of the hypoperfused vessel (as reviewed in Sierra et. al). The resultant ischemic damage can cause neuronal cell death and irreversible tissue damage to vital motor and sensory centers of the region supplied by the hypoperfused vessels. During stroke, decreased or absent blood flow is prevalent leading to revival of cerebral circulation through recanalization of the clot or diversion of blood flow to collateral circulation. Upon reperfusion, enhanced superoxide generation is observed leading to tissue damage of different areas of the brain as well as decreased blood flow. This may result in yet another possible ischemic/hypoxic condition.

One main consequence of cerebral ischemia is the loss of cellular bioenergetics, which is the conversion of oxygen and glucose to adenosine triphosphate (ATP) through oxidative respiration by mitochondria. Mitochondria are not the only source of ATP; however, it is the cellular organelle that produces ATP at the most efficient rate or quantity. The
principal and initial detriment during cerebral ischemia is that ATP production becomes a limiting factor to maintain normal cellular processes. Neurons in the brain are dependent on proper mitochondrial function due to the high metabolic energy demand and decreased ability to anaerobically respire. Neurons require high levels of ATP to maintain axonal/dendritic outgrowth, ionic gradients and produce/release/reuptake neurotransmitters. During cerebral ischemia, the availability for oxygen and glucose become limited to the neuron, leading to diminished ATP production. Oxygen is depleted within 10 seconds, glucose is depleted in 2 to 4 minutes while ATP levels are completely exhausted within 5 minutes of cerebral ischemia.

1.2 Role of mitochondrial dysfunction in cerebral ischemia

In addition to cellular bioenergetics, mitochondria also play an intricate role in the initiation and propagation of the apoptotic intrinsic pathway. Apoptosis (programmed cell death) is propagated through cytosolic Ca$^{2+}$ influx by a variety of mechanisms including: increased protease activation, reactive oxygen species (ROS) production, depolarization of the mitochondrial membrane potential and opening of the mitochondrial permeability transition pore (MPTP). Maintaining normal mitochondrial function could prove to be of great therapeutic value following many pathologic processes (i.e. cerebral ischemia).

Mitochondria and synaptic dysfunction

Electrical information is transmitted from one neuron to another via the synapse. Under normal oxygen levels (normoxia), an action potential from the neuron is propagated from the axon to the dendrites; subsequently, neurotransmitters such as glutamate,
acetylcholine or γ-aminobutyric acid (GABA) are released from presynaptic terminals to adjacent postsynaptic neurons or targets (non-neuronal), activating respective receptors. The basis of neurotransmission involves ion gradients established by various ion pumps and transporters on the cell membrane, which either exchange ions or use ATP as an energy source to extrude ions against their own gradient. In the brain, neurons are surrounded by glial cells, which provide substrates to neurons and aid in the removal of glutamate from the synapse through the use of the glutamate-aspartate transporter. This transporter shuttles one glutamate and two Na⁺ ions into the glial cell, as one K⁺ ion is extruded. The importance for the regulation of these ion gradients is observed during periods of extreme cellular stress (i.e. cerebral ischemia), whereby a decrease in mitochondrial respiration and increase in the cytoplasmic levels of Ca²⁺ can be detrimental to neurons.

Cerebral ischemia triggers severe stress in neurons, as cerebral blood flow is interrupted to a focal or global region of the brain decreasing oxygen/glucose levels. Oxygen/glucose deprivation (OGD) leads to decreased mitochondrial ATP production and potentially resulting in apoptosis (see mitochondria and apoptosis section). Before ATP levels are significantly decreased in ischemia, a small transient depolarization and enhanced synaptic excitability increases the release of neurotransmitters. In addition, decreased ATP production and increased presynaptic depolarizations lead to decreased cytoplasmic ATP caused by the consumption of ATP by various ATPases in the extracellular membrane, mitochondria and endoplasmic reticulum (ER). Consequently, during severe and prolonged ischemic conditions, the Na⁺/K⁺ ATPase function is
attenuated. The Na\(^+\)/K\(^+\) ATPase normally counteracts cytoplasmic Na\(^+\) accumulation from the glutamate transporter, where glutamate and two Na\(^+\) ions in the synapse are exchanged for one K\(^+\) ion 17. The inability for glutamate reuptake results in enhanced glutamate in the synapse, increasing the activity of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors 18. NMDA receptors are widely accepted as the excitotoxic response to excessive amounts of glutamate, through the massive influx of Ca\(^{2+}\) 19, 20. The increase in cytoplasmic Ca\(^{2+}\) leads to amplified amount of Ca\(^{2+}\) sequestered by the mitochondria and eventually causes the induction of apoptosis 21, 22.

As neurons gain access to the necessary oxygen/glucose during and after reperfusion, recovery from ischemia is dependent on the ability of the mitochondria to resume ATP production with the replenishment of oxygen/glucose. ATP production after reperfusion allows ATPases to extrude cations to the cytosol and re-establish proper pH and ion gradients 23, 24. As ion gradients are re-established after ischemia and subsequent reperfusion, the glutamate-aspartate transporter sequesters extracellular glutamate into glia and neurons decreasing NMDA receptor activation and influx of Ca\(^{2+}\) ending Ca\(^{2+}\)-induced-NMDA-mediated excitotoxicity (Figure 1).

**Cerebral Ischemia and Apoptosis**

Glutamate excitotoxicity and Ca\(^{2+}\) overload generated during ischemic injury facilitate opening of the MPTP 19, 25, an irreversible step in the apoptotic cascade that leads to whole-cell metabolic derangement 26. The MPTP complex consists of regulatory protein
cyclophilin D, voltage dependent anion channel (VDAC), and adenine nucleotide translocase (ANT). VDAC may be affected by glutamate excitotoxicity and Ca\(^{2+}\) overload, as Ca\(^{2+}\) binds to VDAC sites and facilitate opening of the MPTP \(^{27}\). The opening of the MPTP results in uncoupling of oxidative phosphorylation, mitochondrial swelling, and subsequent release of proapoptotic molecules such as Bak and Bax to the cytosol \(^{28}\).

Neuroprotection can be achieved through IPC-mediated mitochondria protection through the ATP sensitive K\(^+\) (mK\(^+_\text{ATP}\)) channel in mitochondria, which is analogous to the cell surface ATP-linked potassium channels found in cardiac myocytes \(^{29,30}\). These channels allow K\(^+\) entry into the mitochondrial matrix leading to mitochondrial uncoupling and decreased membrane potential. The induction of IPC activates mK\(^+_\text{ATP}\) by upregulation of the enzyme that phosphorylates mK\(^+_\text{ATP}\) [protein kinase C epsilon (PKCe)] \(^{31}\). Opening of mK\(^+_\text{ATP}\) by IPC results in cytoprotection in multiple tissue types such as the gut, heart, liver, brain, and kidney \(^{30,32-35}\). Numerous studies have suggested that activation of mK\(^+_\text{ATP}\) channels have antiapoptotic properties \(^{34,36-38}\), whereby opening of mK\(^+_\text{ATP}\) channels decrease Ca\(^{2+}\) accumulation in mitochondria inhibiting the opening of MPTP following ischemia \(^{37}\). Activation of mK\(^+_\text{ATP}\) channels also suppresses apoptotic signaling by hindering translocation of Bax and cytochrome c release \(^{34}\) and induces preconditioning through mitochondria swelling and ROS generation \(^{39}\). Mitochondrial K\(^+\)\text{ATP}-specific activators reduce ischemic injury in the brain by increasing regional cerebral blood flow, attenuating ROS, and maintaining the mitochondrial membrane potential \(^{40}\). Together, these results suggest that mK\(^+_\text{ATP}\) channels may play a major role
in IPC-mediated protection and could be the subject of future therapeutic interventions to ameliorate damage resulting from cerebral ischemia (Figure 2).

**Mitochondria and Calcium Buffering**

During normoxia, the levels of Ca\(^{2+}\) in the extracellular space (1 mM) are similar to those inside the lumen of the ER; in contrast to low levels of cytosolic Ca\(^{2+}\) (100 nM). Rises in cytosolic Ca\(^{2+}\) are buffered by voltage-gated uniporters located in the inner mitochondrial membrane and sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) in the ER membrane \(^{41-43}\). During ischemia, depressed mitochondrial respiration reduces the amount of available ATP, as a consequence, SERCA is unable to pump Ca\(^{2+}\) into the ER. However, mitochondria continue to buffer this rise in cytosolic Ca\(^{2+}\) by increasing mitochondrial Ca\(^{2+}\) uptake via voltage-gated uniporters \(^{44,45}\). Following cerebral ischemia, excess Ca\(^{2+}\) continues to flood the post-synaptic terminal via glutamate-NMDA receptor activation \(^{46}\), where the Ca\(^{2+}\) uniporter continues to uptake Ca\(^{2+}\) into the mitochondria until a Ca\(^{2+}\) threshold is reached, resulting in collapse of the inner membrane collapses and opening of the MPTP \(^{47,48}\).

The detrimental consequence of MPTP opening is a result of the massive mitochondrial influx of Ca\(^{2+}\), which opens the large conductance Ca\(^{2+}\)-activated potassium channel (K\(_{Ca}\)) in the inner mitochondrial membrane to influx K\(^+\) into the mitochondria \(^{49,50}\). During cerebral ischemia, this K\(_{Ca}\)-mediated-K\(^+\)-influx depolarizes the mitochondrial membrane potential (MMP) and decreases Ca\(^{2+}\) sequestration \(^{51}\); however, IPC activation induces hyperpolarization of MMP and increases the Ca\(^{2+}\) buffering capacity in
hippocampal neurons of the mitochondria for future neuroprotection. Moreover, in isolated rat hearts, the induction of IPC conferred cardioprotection following ischemia through the activation of $K_{Ca}$. In addition, $K_{Ca}$ activation decreased MPTP opening in the mitochondria, improving cardiac function following ischemia. These cardiac studies may be extended to the brain suggesting that IPC may ameliorate toxic cytosolic loading of $Ca^{2+}$ following ischemia.

The damaging effects of $Ca^{2+}$ on the mitochondria occur from the inability of the ER to continue to sequester $Ca^{2+}$ during periods of reduced ATP; however, the ER is also the location where new proteins are synthesized/folded. Under normoxia, high ER $Ca^{2+}$ levels allow for proper protein synthesis and folding. Under physiological $Ca^{2+}$ levels, an ER chaperone glucose-regulated protein (GRP) binds and inactivates stress proteins: PKR-like ER kinase, inositol-requiring enzyme and activating transcription factor 6.

### 1.3 Neuroprotective adaptations to ischemic injury: IPC

Cerebral ischemia resulting from cardiac arrest and stroke is one of the leading causes of mortality and morbidity in the world. During decades of research, a large number of neuroprotective agents have shown efficacy in animal models but have failed in clinical trials. This failure may have been due to the use of wrong animal models, poor design of clinical trials, and the use of dosages that are much lower than those utilized in animal studies, among many others. As a result, investigators have been pushed to rethink
strategies to develop new therapies for cerebral ischemia. One new strategy that has gained attention is ischemic preconditioning (IPC).

The phenomenon of IPC was discovered when studies in rabbit heart demonstrated that induction of mild ischemia followed by a period of reperfusion made the heart more resistant to a subsequent, ordinarily lethal ischemic insult. IPC has since proven to be a powerful strategy to induce tolerance to ischemic insults in most organs studied, and has been replicated in many laboratories around the world.

IPC in brain consists of an early and a late phase, during which different neuroprotective responses are elicited in specific time windows (time interval between the first sublethal insult and the second, ordinarily lethal insult). There is a rapid phase for which the combination of released factors is maximally effective if the window between initial and final insult is approximately 1 hr in duration. The combination of factors released in the second phase, defined as delayed preconditioning, elicits maximal efficacy if the window is extended to several days after the preconditioning insult, and has been shown to provide more robust and longer-lasting neuroprotection than the first phase. It is now understood that IPC is comprised of several key steps, during which: 1) triggering factors are released in response to the short-duration sublethal ischemic insult; 2) signaling pathways are activated by receptors of the triggering factors; and 3) gene expression is orchestrated by the delayed preconditioning-activated signaling pathways. Activation of these pathways results in brain cells having a phenotype that is highly resistant to ischemic insults.
Although many triggering factors are activated by IPC, our lab has shown two such factors to be critical, and they appear to have opposite effects. We and others showed that adenosine is released after IPC and initiates a signaling pathway that promotes ischemic tolerance in brain via activation of A1 receptors (A1AR)\(^{62,68,69}\). Adenosine is a neuro- and vaso-modulator that is normally released when ATP levels decline\(^{70}\). Inhibition of synaptic activity is observed when adenosine binds to the A1 receptor\(^{62}\), which is believed to be the key receptor in the induction of ischemic tolerance.

In contrast to the inhibitory pathway activated by adenosine, we and others have shown that activation of excitatory post-synaptic NMDA receptors is also required for IPC-induced ischemic tolerance in brain\(^{71-74}\). It is now known that these two receptors (NMDA and A1AR), via different mechanisms, activate a novel type of protein kinase C (εPKC)\(^{71,75}\) which plays a key role in the induction of ischemic tolerance. Although there are multiple key signaling pathways that mediate preconditioning, it is plausible that significant cross talk occurs among pro-survival kinases following IPC. Since this area of research is beyond the scope of this review, the reader is directed to previously written reviews that provide an overview of these signaling pathways and their effect on IPC\(^{66,67,76-78}\).

Cerebral ischemia targets many sensitive cellular sites that seldom recover thereafter. Owing to the excitotoxicity that ensues following cerebral ischemia, synaptic plasticity and functional recovery are significantly impaired. In addition, mitochondria are
particularly sensitive to both the ischemic insult as well as the reperfusion phase. IPC, via activation of key survival signaling pathways, promotes synaptic and mitochondrial alterations that make neurons more resistant to ischemia. In the following sections we will describe some of these effects mediated by IPC.

**Synaptic targets of IPC and functional recovery**

Excitotoxicity was identified as one of the first steps in the pathology of cerebral ischemia. IPC was found to ameliorate excitotoxicity by inhibiting glutamate release, increasing inhibitory neurotransmitter GABA release, and enhancing GABA pre- and postsynaptic activities, thus making neurons more resistant to the excitotoxic insult. These findings suggest that IPC promotes synaptic modifications that may preserve synaptic function and functional recovery following cerebral ischemia. This hypothesis is further supported by findings that show IPC improves spatial memory, a well-established memory system in humans and in animal models. In animals, spatial memory allows them to acquire and retain information about a surrounding environment. This type of memory resides in the hippocampus and entorhinal cortex. In rats, spatial memory, evaluated by the Morris water maze, was shown to be impaired following asphyxial cardiac arrest. However, IPC ameliorated spatial memory deficits after global cerebral ischemia, suggesting that IPC targets synaptic terminals in order to improve functional recovery.
Mitochondrial targets of IPC

As producers of ROS, mitochondria are key mediators of cell death following cerebral ischemia as well as in many neurological disorders, and therefore have become important targets for potential neuroprotective therapies \(^{88}\). In fact, mitochondria have been shown to play an important role in IPC-induced neuroprotection. IPC mitigates cerebral ischemia-induced neuronal death by several mechanisms, including prevention of decreased oxidative phosphorylation (OXPHOS) capacity, mitochondria-dependent cell death pathways, and reactive oxygen species (ROS) generation from the mitochondrial electron transport chain (ETC) \(^{34, 89-95}\). Because of their crucial role in IPC-induced neuroprotection, Dirnagl and Meisel described mitochondria as “gatekeepers of preconditioning” \(^{89}\).

In addition, IPC appears to activate several pathways, which in turn modify mitochondrial proteins. In a previous study we observed that within 1 hr after IPC, the mitochondrial ATP-sensitive K\(^+\) (mitoK\(^+_\text{ATP}\)) channel was phosphorylated/activated by \(\varepsilon\)PKC. This pathway led to the induction of ischemic-tolerance pathways, potentially by sub-lethal mitochondrial release of ROS \(^{31}\). Our group observed delayed IPC-mediated increases in synaptosomal \(\varepsilon\)PKC levels, which contributed to improved mitochondrial OXPHOS capacity following an ordinarily lethal ischemic insult. The increased OXPHOS capacity was attributed to \(\varepsilon\)PKC-induced post-translational modifications of mitochondrial ETC components \(^{96}\).
IPC-induced improvement in brain mitochondrial OXPHOS capacity may also be due to IPC-mediated changes in OXPHOS gene expression. Several transcription factors have been shown to translocate to the nucleus following IPC, such as nuclear factor-kappa B (NF-κB) and cyclic-AMP response element-binding protein (CREB) pathways, gene products of which can mediate IPC-induced neuroprotection; Liu and colleagues identified 19 differentially expressed microRNAs (miRNAs) in brains of hypoxia-preconditioned mice following middle cerebral artery occlusion. Bioinformatics analysis of the miRNA target genes of two conventional PKC (βIIPKC and γPKC)- and one novel PKC (εPKC)-interacting proteins predicted involvement of major energy-generating pathways (glycolysis/gluconeogenesis, citrate cycle and oxidative phosphorylation) in hypoxia preconditioning-induced neuroprotection. However, two similar studies suggested that IPC-induced alterations in miRNA expression pattern had no effect on mitochondrial OXPHOS. Gene ontology analysis in another study identified mitochondria as one of the cellular organelles affected by IPC. These studies suggest that IPC and hypoxic preconditioning differentially affect miRNA expression in relation to mitochondrial oxidative phosphorylation pathways. It appears that IPC positively regulates mitochondrial functions by directly affecting gene expression of specific mitochondrial proteins rather than by altering miRNA expression.

Following cerebral ischemia-induced excitotoxicity, altered neuronal calcium (Ca^{2+}) buffering capacity plays a key role in potentiating neuronal cell death. In vitro models of excitotoxicity demonstrated that a tolerance-inducing stimulus protected neurons from lethal excitotoxicity by increasing mitochondrial Ca^{2+} buffering capacity and by
decreasing mitochondrial Ca\(^{2+}\) uptake \(^{103}\). Iijima \textit{et al.} reported that brief oxygen-glucose deprivation increased mitochondrial calcium loading capacity \(^{104}\). These studies highlighted another important role for mitochondria in protecting cells against lethal ischemia-induced excitotoxicity.

1.4 \textbf{Astrocytes}

\textbf{Astrocytes: function and physiology}

While neuronal cell death can significantly impact the outcome of a patient following stroke, it is important to note that a cerebral infarction encompasses many cell types other than neurons. These include cells of the vasculature and astrocytes. While traditionally viewed as housekeeping cells of the CNS, astrocytes have a tremendous diversity in roles that support neuronal functioning.

Astrocytes are important in the regulation of blood flow \(^{105,106}\). The current neurovascular unit model stipulates that astrocytes are able to induce vasodilation of blood vessels at their end-feet through the uptake of glutamate from the synaptic cleft following neuronal activity. Internalization of glutamate leads to increases in intracellular calcium, subsequent activation of arachidonic acid and cyclooxygenase, and the production of vasodilatory prostaglandins. As a result, astrocytes are critical in coupling neuronal activity to blood flow. The consequence of vasodilatation is increased glucose delivery to and uptake by astrocyte end-feet processes; this glucose can either be stored as glycogen in astrocytes, or utilized glycolytically to form lactate. It is this substrate that can be shuttled to neurons as a bioenergetic fuel, as discussed further below and in Chapter 4.
Finally, astrocytes are an important source of glutathione in the brain, and can shuttle glutathione and its precursors, such as cysteine and glycine, to neurons\textsuperscript{107}. Studies have previously shown that there are 10 times lower levels of glutathione in neurons compared to astrocytes\textsuperscript{108}, further validating the high antioxidant potential of astrocytes.

**Role of astrocyte in cerebral ischemia**

While astrocytes serve many important neuronal functions, they can also be a contributory factor to the pathogenesis of cerebral ischemia\textsuperscript{109}. The primary deficit of ischemia is the lack of oxygen and glucose delivery to tissues, resulting in decreased ATP, metabolic failure, and ultimately cell death. As described earlier, excitotoxicity prevails as a principal mediator of cerebral ischemia-induced damage. Following loss of ATP due to limited glucose and oxygen supply, Na\textsuperscript{+}/K\textsuperscript{+} ATPases fail and ionic homeostasis is unable to be maintained. The resulting depolarization of neurons results in massive calcium influx and depolarization of synaptic terminals. This event results in unregulated release of neurotransmitters, which in the case of glutamate, can induce activation of NMDA receptors and potentiate Ca\textsuperscript{2+} influx into cells. Calcium subsequently activates several proteases and induces mitochondrial apoptotic pathways, resulting in cell death.

Fortunately, a key role of astrocytes is in the uptake of glutamate; astrocytes are essential in the recycling of glutamate to neurons in the form of glutamine, a function achieved by the astrocyte specific expression of glutamine synthetase\textsuperscript{110}. Similarly, astrocytes express GABA transporters which can take up GABA and metabolize this neurotransmitter back
into glutamine for subsequent export to neurons\textsuperscript{111, 112}. Recent evidence has suggested that astrocytes can also impact glutamate signaling in the brain by releasing glutamate. Astrocytic release of glutamate, along with ATP\textsuperscript{113}, can sustain increases in extracellular glutamate at the synaptic cleft and aid in prolonged neuronal depolarization through NMDA receptor activation. In addition, the ATP that is released from astrocytes can activate the family of purinergic receptors (P2Y)\textsuperscript{114}, which also aid in transporting glutamate into the synaptic cleft\textsuperscript{115}. Finally, a cofactor required for proper modulation of the NMDA receptor is D-serine; D-serine is an outer transmitter that can be released from astrocytes and thus has been thought to contribute to further neuronal damage during ischemia\textsuperscript{116}. Thus, while astrocytes are instrumental in glutamate buffering, astrocyte dysfunction can contribute to excite-toxic injury of neurons following cerebral ischemia.

**Astrocytes: sources of glycogen and lactate in the CNS**

A distinguishing feature between astrocytes and neurons is that astrocytes are able to harbor glycogen in their cells. Glycogen stores are critical, as they can be utilized for energy through glycogenolysis and subsequent lactate production, which can be transferred to neurons for metabolic incorporation. Neurons, due to their increased density of ionic transporters and ATPases, require large amounts of ATP to maintain basal metabolic function. As a result, they are more susceptible to a drop in ATP production as a result of ischemia, and lactate may enable maintenance of ATP levels during this time.
Astrocytes are in exclusive possession of the enzymatic machinery to produce and breakdown glycogen. Rather than produce glucose for subsequent influx into the oxidative phosphorylation pathway, previous studies suggest that astrocyte glycogenolysis eventually results in production of lactate. It is this lactate that is a basis of a well-documented, but still controversial, theory in regards to cerebral metabolism known as the astrocyte-neuronal lactate shuttle. The theory posits that glucose taken up by astrocyte end-feet from cerebral circulation and astrocyte glycogenolysis yield lactate, which can be shuttled to neurons as a preferential oxidative fuel. Reviews for the lactate shuttle hypothesis and the controversial debate are listed here, but further discussion is beyond the scope of this introduction.

What could be the possible benefits of lactate? As lactate is deemed to be metabolized by oxidative phosphorylation in neurons, it stands to reason that under severe ischemia, as with what occurs in the core of an ischemic insult, lactate would not be able to be utilized by neurons. However, in less-severe ischemic areas of the brain, particularly in the penumbra surrounding the core infarcted region, oxygen tension could remain high enough to allow oxidative utilization of lactate by neurons. Indeed, studies have documented that under glucose-deprivation or hypoxia, neurons can readily utilize lactate derived from astrocytes to maintain ATP levels. As discussed in Chapter 4, there are many metabolism-independent effects of lactate on neurons. These include alterations to synaptic transmission, long-term memory in the hippocampus, and modulation of AMPA and NMDA receptor activity. Thus, astrocyte derived lactate can have diverse protective functions on neurons in the context of cerebral ischemia.
1.5 Role of oxidative stress in cerebral ischemia

Following ischemia, mitochondria are important organelles for maintaining ATP levels and contributing to free radical production. Mitochondrial dysfunction is a consequence of ischemia and has received considerable attention as a major contributor to both ischemia and reperfusion mediated injury\textsuperscript{124}. Numerous studies have demonstrated that mitochondrial respiration is severely affected by ischemia and by reperfusion\textsuperscript{125, 63}. Hyperoxidation of respiratory chain proteins is characteristic of the mitochondria following ischemia and may stem from substrate unavailability, reaction with reactive oxygen species (ROS), or through the release of cytochrome c and NADH following mitochondrial permeability transition pore opening (MPTP)\textsuperscript{126}. Mitochondrial dysfunction following ischemia is the primary site of reactive species production and free radical mediated damage in the reperfused brain following ischemia\textsuperscript{127, 128, 129}. Previous studies have extensively demonstrated that tissue reperfusion following an ischemic episode contributes significantly to the pathophysiology of ischemic injury\textsuperscript{130, 131, 132}. The formation and subsequent reactions of reactive species are critical in understanding the pathophysiology of ischemic injury, and may help identify pathways that can be targeted to ameliorate ischemic damage.

Reactive oxygen species

The generation of ROS occurs under normal physiological conditions and is normally balanced by the cellular antioxidant defense system. However, ROS formation may be increased during periods of ischemia and reperfusion. Previous studies have suggested
that the increased rate of ROS production during reperfusion is a major cause of the pathophysiology of ischemia/reperfusion injury in both the heart and brain.\textsuperscript{133, 134}

Multiple sites of ROS production have been suggested, with the mitochondria accounting for the majority of the ROS generated inside cells. ROS are produced primarily by complex I and III of the mitochondrial electron transport chain.\textsuperscript{135} In organotypic hippocampal slice cultures, ischemic/reperfusion-mediated free radical formation was reduced by the complex I inhibitor rotenone.\textsuperscript{136} During conditions of hypoxia, the electron transport chain stalls, allowing donated electrons from NADH\textsuperscript{+} and FADH\textsubscript{2} sufficient time to interact with oxygen in the mitochondria. The main types of ROS produced by the mitochondria are superoxide and hydrogen peroxide. Hydrogen peroxide, if not degraded by antioxidants, will form highly reactive hydroxyl radicals, which increase during the early reperfusion phase following brief periods of global cerebral ischemia.\textsuperscript{137} Increases in oxidative stress, occurring either by increased ROS formation or decreased antioxidant defenses, result in lipid peroxidation, protein and DNA damage. Attenuation of ROS is therefore critical to cell survival during ischemic conditions, and is mediated through hypoxic adaptation through gene transcription, and amelioration of oxidative stress by antioxidants.

**Endogenous antioxidants**

Although neurons are highly sensitive to oxidative stress due to their high-energy consumption,\textsuperscript{138} there are certain antioxidant enzymes that aid to quell a substantial rise in ROS following exposure to ischemia (Figure 1). The superoxide ion, or \( \text{O}_2^- \), is formed...
under normal physiologic conditions upon the reaction of molecular oxygen ($O_2$) and an electron. The electron may be donated from complex I or complex III of the mitochondrial ETC, or from other metabolic reactions in the cell. These reactions include xanthine oxidase, alpha ketoglutarate, and the cyclooxygenase and lipoxygenase pathways\textsuperscript{139}. In the cytoplasm of the cell, copper/zinc (Cu/Zn) superoxide dismutase (SOD1) converts superoxide into hydrogen peroxide ($H_2O_2$). Similarly, manganese (Mn) superoxide dismutase in the mitochondria converts superoxide produced in the mitochondrial matrix into hydrogen peroxide as well\textsuperscript{140}.

Further removal of hydrogen peroxide is performed through the actions of the glutathione and thioredoxin reductase systems, both of which decompose hydrogen peroxide into molecular oxygen and water\textsuperscript{141}. Catalase is another enzyme that can reduce hydrogen peroxide, and is found in peroxisomes\textsuperscript{142}. Lastly, NQO1 (NAD(P)H quinone oxidoreductase 1) may scavenge superoxide\textsuperscript{143}, as well as reducing endogenous quinones such as vitamin E quinine and coenzyme Q10 in the mitochondria ETC\textsuperscript{144}. The reduction of these molecules produces more stable quinones, which increase their antioxidant ability. Coenzyme Q10 may react with vitamin E to scavenge free radicals that accumulate in the mitochondria, and increased accumulation of coenzyme Q10 has been shown to be neuroprotective in various animal models of neurodegeneration\textsuperscript{145, 146}.

1.6 **Nuclear factor erythroid 2-related factor 2**

**Nrf2: Master regulator of endogenous antioxidant production**

The regulation of most of these antioxidant enzymes is through the transcription factor nuclear factor erythroid-2 related factor (Nrf2)\textsuperscript{147}. Nrf2 dissociates from its cytosolic
repressor protein (Figure 4) following electrophilic and oxidative stress, and will translocate to the nucleus to transcribe endogenous antioxidant genes\textsuperscript{148}. Thus, activation of Nrf2 is an important pathway that can upregulate endogenous antioxidant production, and prevent the pathogenesis

Nrf2 is involved in protecting the cell from the damaging effects of oxidative stress by binding to antioxidant response elements (ARE), located in the regulatory domains of its target genes\textsuperscript{149}. Under basal conditions of oxygen tension, there exists a cytosolic protein, known as Keap1 (Kelch-like ECH-associated protein 1), that functions to down regulate the activity of Nrf2\textsuperscript{150}. Keap1 complexes with Nrf2 and activates Nrf2 ubiquitination, eventually leading to its degradation under normal conditions. However, under certain conditions, Keap1 or Nrf2 may be chemically modified through phosphorylation\textsuperscript{148, 151}, deacetylation\textsuperscript{152}, and S-nitrosylation\textsuperscript{153}. These chemical modifications enhance Nrf2 disassociation from Keap-1, thus facilitating Nrf2 nuclear translocation and subsequent Nrf2-dependent gene expression. Upon nuclear translocation, Nrf2 binds to ARE in the regulatory domain of its target genes; examples of antioxidant genes under Nrf2 transcriptional control include glutathione synthase\textsuperscript{154}, heme oxygenase-1\textsuperscript{155}, and catalase\textsuperscript{156, 157}.

**ROS in Nrf2 signaling**

ROS has been suggested to regulate activation of Nrf2 following ischemia through kinase activation. Subsequent phosphorylation of Nrf2 enhances Nrf2 dissociation from Keap1 and allows Nrf2 to express antioxidant enzymes and other proteins to better adapt the cell
to oxidative stress\textsuperscript{158}. Nrf2 has a ubiquitous expression, as Nrf2 has been shown to induce antioxidant gene transcription in rat liver\textsuperscript{159}, lung\textsuperscript{160}, brain\textsuperscript{161} and heart tissue\textsuperscript{156}. Nrf2 was shown to be up-regulated following a 50\% reduction in cerebral blood flow in mice. Cerebral oligemia in mice led to increased oxidative stress and subsequent activation of Nrf2 in neurons predominantly in cerebellar Purkinje cells and cingulate cortex\textsuperscript{162}.

In addition to ROS, NO has been shown to activate Nrf2 through S-nitrosylation of cysteine residues residing on Keap1 in cultured rat pheochromocytoma cells\textsuperscript{153}. This chemical modification allowed Keap1 to dissociate from Nrf2, allowing Nrf2 to translocate from the cytosol to the nucleus. In addition to S-nitrosylation, this study suggested that nitric oxide could activate PKC-dependent phosphorylation and subsequent dissociation of Nrf2 from Keap1\textsuperscript{153}. A recent study has also suggested that SIRT1 was inhibitory on Nrf2’s transcriptional activity\textsuperscript{152}. More importantly, this study also suggested a novel regulation of Nrf2 after separation from Keap1, such that acetylation of the dissociated and nuclear-translocated Nrf2 enhanced its binding to ARE. However, conflicting results have been reported with the use of resveratrol, a polyphenolic antioxidant known to activate SIRT1. Resveratrol was demonstrated to stabilize and restore levels of Nrf2 in the cerebellum in a rodent model of fetal alcohol syndrome\textsuperscript{163}. Yet another study suggested that the use of histone deacetylase inhibitors increased Nrf2 activation following focal cerebral ischemia in mice, and resulted in decreased infarct volumes when administered shortly after induction of focal cerebral ischemia\textsuperscript{164}. This last study suggests that inhibition of Sirt1 and other sirtuin enzymes may activate Nrf2, conferring neuroprotection to ischemia.
There has been extensive debate as to whether transient hypoxic stress activates Nrf2 protective pathways. A previous study demonstrated upregulation of Nrf2-targeted gene transcription following ischemic preconditioning in human and rat astrocytes; more importantly, the observed decrease in cell death due to induction from IPC was abrogated in homozygous Nrf2 knockout rats, suggesting that Nrf2 could mediate an important role in IPC mediated neuroprotection\textsuperscript{165}. The group of *Bell et. al* demonstrated that pure mice neuronal cultures were unable to upregulate antioxidant genes under Nrf2-transcriptional control, suggesting that astrocytes are the primary source of Nrf2 production and activation. Finally, homozygous Nrf2 knockout mice were not protected from induction of IPC or exposure hydrogen peroxide following OGD, supporting the contributive role of Nrf2 to mediating IPC-induced neuroprotection\textsuperscript{166}. Activation of Nrf2 following IPC treatment could present an important pathway that results in cytoprotection following ischemia, and future studies will need to investigate how low levels of oxidative stress regulate induction of Nrf2.

**Interaction of Nrf2 with mitochondria**

A recent group has described novel mitochondrial targets of Nrf2\textsuperscript{167}. These targets include nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM\textsuperscript{16}), and peroxisome-activated receptor gamma coactivator 1 alpha (PGC-1\alpha), all of which regulate mitochondrial biogenesis. Nrf2 has been shown to induce transcription of nuclear respiratory factor 1 (NRF1) following exposure of mice cardiomyocytes to a cardiotoxic agent\textsuperscript{168}. In addition, previous studies suggested that PGC-1\alpha may be under
transcriptional control of Nrf2 as well \(^{169}\), suggesting that Nrf2 may activate mitochondrial biogenesis through NRF1 and PGC-1\(\alpha\). In addition, p62 (a factor involved in mitophagy \(^{170}\) is also under Nrf2 transcriptional control \(^{171}\) and was shown to localize to mitochondria following IPC \(^{170}\). In Nrf2 deficient mice, lack of Nrf2 was associated with age-related developments of retinopathy; however, an interesting observation of this study was that mice deficient in Nrf2 were observed to have dysregulated lysosome-dependent degradation. Through electron microscopy, swollen mitochondria were observed to be in close proximity to phagosomes in intermediate stages. These results were suggestive of accumulation of intermediate structures that were improperly removed through the lysosomal pathway. This study suggests that Nrf2 plays a role in regulating autophagy, which is a critical role of the retinal pigment epithelium for proper eye functioning.

In addition, these results suggest that Nrf2 deficiency is associated with abnormal phagocytosis in central nervous system structures \(^{172}\). Furthermore, the role of IPC-treatment in activating mitophagy was recently examined \(^{170}\). In Langendorff-perfused rat hearts, IPC treatment increased Parkin translocation to mitochondria. Parkin has been associated with binding to PINK1, a kinase on the outer mitochondrial membrane, which signals mitochondria for selective degradation. In addition, p62, a protein that binds to mitochondria and mediates fusion with the phagosome, was also shown to translocate to the mitochondria following induction of IPC. However, this effect was absent when Parkin was absent, suggesting that Parkin activates p62 mitochondrial translocation. In addition, depletion of p62 attenuated mitophagy, and was associated with increased cell
death following lethal ischemia in perfused rat hearts. Most importantly, loss of Parkin decreased p62 translocation and abrogated cardioprotection following induction of IPC. Finally, previous studies have suggested that Nrf2 can be tethered to mitochondria through an outer mitochondrial membrane protein PGAM 5, a member of the phosphoglycerate mutase family \(^\text{173}\). The consequences of this observation are not known, but were suggested in this study that Nrf2 may serve as a signaling factor to alert the nucleus of increased mitochondrial ROS production. While the role of Nrf2 in mitochondria is relatively unknown, these aforementioned studies suggest an important role of Nrf2 and mitochondrial quality control and mitochondrial gene expression, perhaps in an effort to suppress ROS production from the causative organelle in the cell.

### 1.7 Clinical scenarios for IPC

There exist many clinical scenarios for when IPC treatment could be beneficial, most of which involve patients undergoing invasive or long surgeries and are subjected to procedures that could result in a relative state of ischemia. As a result, patient outcome following surgery might be improved through IPC treatment. Additionally, chronic IPC treatments may afford ischemic and anti-inflammatory protection in patients who suffer from metabolic syndrome, cardiovascular and cerebrovascular disease or in patients at risk for recurrent ischemic attacks.

#### Models of IPC in clinic

Most *in vivo* models of IPC performed in rodent animals are usually invasive and thus impractical to translate into a clinical setting. However, a modified form of IPC known as remote ischemic preconditioning (RIPC) could prove to have high translational value.
RIPC involves cycles of temporary occlusion/restoration of blood flow in a forelimb far removed from the desired sight of cytoprotection. Repeated cycles of temporary ischemia in this area can trigger the release of soluble protective factors into the blood, which can be delivered to the target organ and confer protection. For example, clinical trials have already utilized blood pressure cuffs to induce temporary occlusion and restoration of blood flow in an arm or thigh of patients, thus constituting one model of RIPC. A previous study reported that RIPC-treated patients showed improved ejection fraction, graft patency, and electrocardiogram parameters following coronary artery bypass surgery. In stable angina pectoris patients, three 2-minute coronary artery balloon inflation/deflation cycles demonstrated improved cardiac contractility and decreased chest pain. In patients undergoing coronary angioplasty or coronary artery bypass procedures, the induction of carotid artery balloon inflations/deflations just prior to the main surgical procedure could mimic “early-window” IPC-induced cytoprotection. Lastly, RIPC has been shown to be safe and well tolerated in critically ill patients with subarachnoid hemorrhage, suggesting that RIPC may represent a feasible, prophylactic therapy.

**Resveratrol: an IPC-mimetic**

Perhaps more clinically applicable is the use of a pharmacologic agent that can activate pathways critical for IPC-induced neuroprotection. Our lab has previously shown the contribution of Sirtuin 1, (SIRT1, a class III NAD^+ - dependent histone deacetylase), to mediate delayed IPC-induced neuroprotection. Therefore, a potent activator of SIRT1 such as the polyphenol resveratrol could represent a potential therapy for cerebral
ischemia. Although the safety of resveratrol in humans has already been profiled \(^{179}\), further understanding of its mechanisms is imperative before utilizing this compound for clinical IPC treatment.

### 1.8 Concluding remarks and hypotheses

Cerebral ischemia induces numerous processes that attempt to protect the neuron from innate cellular damage. OGD leads to mitochondria dysfunction and inadequate ATP levels as various ATPases attempt to remove/pump ions and maintain ion gradients. The influx of Ca\(^{2+}\), via NMDA receptors, persists as glutamate increases in the synapse, further depolarizing the plasma membrane to activate VGCC. This, in turn, deposits additional Ca\(^{2+}\) in the cytosol, which is sequestered by the mitochondria and incites an array of detrimental processes ultimately leading to cell death. There is a complex interaction between many pathways and signaling cascades that regulate mitochondrial adaptation and hence cellular adaptation to hypoxia. The exploitation of these mechanisms through IPC could provide a therapy that increases brain tissue viability following cerebral ischemia, while decreasing the long-term neurological impairment typically associated with stroke and other cerebral vascular-related diseases.

In addition, the therapeutic potential of IPC has increased interest in understanding the ROS-activated neuroprotective signaling pathways. As discussed in this introduction, the level of ROS are thought to be a pivotal trigger in activating either survival or death pathways of the cell following ischemia. Although many proteins and molecular pathways have been implicated in IPC-mediated neuroprotection and oxidative stress
signaling, there is still much to be learned about the cytoprotective effects of ROS against ischemic injury. The role of Nrf2, a transcription factor involved in upregulating the endogenous antioxidant response in cells, appears to be a promising pathway to explore for neuroprotection. The goal of this dissertation work was to investigate if Nrf2 is involved in mediating IPC-induced protection, and through what mechanisms Nrf2 could confer neuroprotection. Our central hypothesis for this work was that Nrf2 contributes to IPC-induced neuroprotection through modulation of mitochondrial function and antioxidant expression.
1.9 Figures

**Figure 1.1: Pathogenesis of cerebral ischemia leading to apoptosis.**

Cerebral ischemia leads to widespread loss of ATP production, and thus failure to maintain ion homeostasis between extracellular and intracellular compartments. This leads to uncontrolled Ca\(^{2+}\) influx and neurotransmitter vesicular fusion with the membrane of the presynaptic terminal. If excitatory glutamate is released, NMDA receptors become activated resulting in Ca\(^{2+}\) influx to the postsynaptic nerve terminal. Cytosolic Ca\(^{2+}\) activates a host of deleterious molecules, leading to protease activation, DNA degradation, and mitochondrial Ca\(^{2+}\) overload. This mitochondrial Ca\(^{2+}\) leads to MPTP opening, the irreversible step towards apoptosis. Opening of mK\(^{+}\)\(_{ATP}\) and K\(_{Ca}\) channels dissipate mitochondrial membrane potential, which increases the Ca\(^{2+}\) buffering capacity of mitochondria. Together, these mechanisms allow the cell to stave off apoptotic signaling during an ischemic challenge. (ATP: adenine triphosphate; Ca\(^{2+}\): calcium; NMDA: N-methyl-D-aspartate; MPTP: mitochondrial permeability transition pore; mK\(^{+}\)\(_{ATP}\): mitochondrial ATP-linked potassium channel; K\(_{Ca}\): mitochondrial calcium linked potassium channels, Cyt c: cytochrome c)
Figure 1.2: Summary of pathways involved in mediating IPC- and RIPC induced neuroprotection.

IPC can increase recruitment of DNA repair enzymes and transcription factor Nrf2 involved in upregulating antioxidant enzymes. In addition, IPC can modulate the mitochondrial K$_{\text{ATP}}$ channel and further suppress mitochondrial ROS production. Lastly, IPC can activate TLR to induce a mild inflammatory response, eventually triggering anti-inflammatory cytokines to suppress ischemia-induced recruitment of immune cells and thus inflammation. Alternatively, RIPC produces neuroprotection by increasing soluble cytokines in a vascular bed that is far removed from the desired location of cytoprotection. IPC: Ischemic Preconditioning; RIPC: Remote Ischemic Preconditioning; Nrf2: Nuclear factor erythroid 2 related factor 2; TLR: Toll-like Receptor; mitoK$_{\text{ATP}}$: mitochondrial ATP-sensitive potassium channels; GPCR: G-protein coupled Receptor; PKC: Protein Kinase C; NO: Nitric Oxide; ROS: Reactive oxygen species; OXPHOS: Oxidative Phosphorylation; cytC: cytochrome C; $\Delta\psi$m: mitochondrial membrane potential.
In the presence of ROS generated during ischemia, PKC will become activated to phosphorylate Nrf2 at the Serine 40 residue. This particular residue is the critical site of PKC control, although other kinases can phosphorylate additional sites on Nrf2. Nrf2 will then dissociate from its cytosolic repressor, Keap1, and translocate into the nucleus. Upon binding to ARE, Nrf2 transcribes several genes including those for energy regulation, mitochondria biogenesis, and antioxidant expression. Under normal or high oxygen tension, Nrf2 remains bound to Keap1, and is eventually targeted for ubiquitination and proteasomal degradation. The pathway on the right describes activation of HIF1 in the presence of ROS. The inducible subunit of HIF1, HIF1α, is regulated by PHD. Under normal oxygen levels, PHD will hydroxylate HIF1α. This allows VHL to ubiquitinate HIF1α and target it for proteasomal degradation. However, in the presence of oxidative stress, PHD is inactivated and HIF1α can translocate to the nucleus. In the nucleus, HIF1α binds to the constitutively expressed HIF1β subunit, and together these subunits form the functional HIF1 factor. This factor then binds to HRE on the genome and transcribes several genes involved in hypoxic adaptation, including genes for energy metabolism, angiogenesis, and red blood cell production. Nrf2: Nuclear factor (erythroid-derived 2)-like 2; Keap1: Kelch-like ECH-associated protein 1; PHD: Prolyl hydroxylase; HIF: Hypoxic Inducible Factor; Ser: Serine; OH: Hydroxyl; VHL: Von Hippel-Lindau; VEGF: Vascular endothelial growth factor; EPO: Erythropoietin; HO-1: Heme Oxygenase 1; iNOS: inducible nitric oxide synthase; eNOS: endothelial-derived nitric oxide synthase; GS: Gluthathione Synthase; GR: Glutathione Reductase; NQO-1: NAD(P)H dehydrogenase [quinone] 1; SOD1: Super Oxide Dismutase 1; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha.; ARE: Antioxidant Response Element; HRE: Hypoxic Response Element; ROS: Reactive Oxygen Species; PKC: Protein Kinase C
During ischemia, ROS is produced from complexes I and III of the ETC in the mitochondria. ROS can activate several pathways leading to cell damage and cell death. \( \text{O}_2^- \cdot \) can oxidize and fragment both mtDNA and nuclear DNA of a cell. mtDNA damage results in decreased synthesis of ETC proteins, resulting in decreased ATP production. In addition, DNA damage can activate DNA repair enzymes, and if damage exceeds the capacity of these repair enzymes, the cell will be signaled for apoptotic cell death. ROS produced in the mitochondria can also oxidize mitochondrial membranes, eventually leading to leakage of \( \text{cytC} \), a pro-apoptotic signaling molecule. Similarly, ROS can stimulate opening of the MPTP, leading to loss of mitochondrial membrane potential and leakage of \( \text{cytC} \) from the intermembrane space, both of which contribute to activation of apoptosis. If \( \text{O}_2^- \cdot \) were to react with NO residing in close proximity, ONOO\(^-\) is formed. ONOO\(^-\) has a multitude of effects, sharing several downstream pathways with ROS. ONOO\(^-\) can nitrate, and thus inactivate, many proteins. Some targets include SOD, DNA repair enzymes, and myelin. The result is increased burden of oxidative stress and inability to cope with ROS, especially in the nervous system. In addition, ONOO\(^-\) can oxidize proteins, such as LDL, in vascular tissue. This process stimulates potent inflammation and compromises vascular integrity. Similar to ROS, ONOO\(^-\) can oxidize membrane lipids and fragment DNA, all of which contribute to apoptosis. However, low levels of ROS which do not exceed the coping ability of the cell may be potentially beneficial, as these radical species may activate several cytoprotective and adaptive pathways to ameliorate oxidative stress. ONOO\(^-\) : Peroxynitrate; \( \text{O}_2^- \cdot \) : Superoxide ion; mtDNA: mitochondrial DNA; \( \text{cytC} \) : cytochrome C; MPTP: Mitochondrial Permeability Transition Pore; \( \text{O}_2 \) : molecular oxygen; \( e^- \) : electron; ETC: Electron transport chain; IMS: Intermembrane space. Gluthathione reductase; GS: Glutathione synthase; TR: thioredoxin; \( \text{H}_2\text{O} \) : water; \( \text{O}_2 \) : molecular oxygen.

**Figure 1.4: Summary diagram of cytotoxic effects resulting from ROS and RNS generation.**

![Image of diagram showing the effects of ROS and RNS on mitochondria](image-url)
ROS is endogenously produced primarily from complex I and III of the ETC in the mitochondria. The production of ROS would quickly buildup if there weren’t mechanisms in places to quickly convert ROS into inert byproducts. In the matrix of the mitochondria, O$_2^•$ is formed between molecular oxygen reacting with electrons from the ETC. In the case of ischemia, oxygen is limiting and thus transport of electrons through the ETC stalls; stalling of the ETC allows more time for electrons to react with remaining intramitochondrial oxygen and form the potentially devastating O$_2^•$ ion. However, the matrix of the mitochondria contains MnSOD, which converts O$_2^•$ into H$_2$O$_2$. The hydrogen peroxide can be further reduced to water and oxygen through catalase or glutathione/thioredoxin reduction pathways, both of which are conveniently located in the mitochondrial matrix. ROS can also be generated in the intermembrane space of mitochondria from complex III. In the intermembrane space, O$_2^•$ is acted upon by either ZnSOD or CuSOD, and reduced to H$_2$O$_2$. However, if O$_2^•$ exceeds the capacity of these enzymes, O$_2^•$ could activate opening of the MPTP, releasing cytC and stimulating apoptotic pathways. To prevent this occurrence, ROS plays a role in cell signaling, and stimulates transcription of cytoprotective genes involved in adaptation to oxidative stress and antioxidant expression. Together, the induction of these cytoprotective genes will eventually reduce ROS formation; this negative feedback provides the continual generation of enzymes designed to quell a significant rise in ROS production before irreversible cell damage and cell death occur. MnSOD: Manganese Super Oxide Dismutase; CuSOD: Copper Super Oxide Dismutase; ZnSOD: Zinc Super Oxide Dismutase; O$_2^•$: Superoxide ion; mtDNA: mitochondrial DNA; cytC: cytochrome C; MPTP: Mitochondrial Permeability Transition Pore; O$_2$: molecular oxygen; e$^-$: electron; ETC: Electron transport chain; I-V: Denotes complex number of ETC; ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; IMM: Inner mitochondrial membrane; OMM: Outer mitochondrial membrane; NADH: Nicotinamide adenine dinucleotide; GR:
Gluthathione reductase; GS: Glutathione synthase; TR: Thioreduxin; H₂O: water; O₂: molecular oxygen.
Low levels of ROS formation following IPC exposure has been implicated in activating numerous signaling pathways involved in IPC neuroprotection. Of the proteins targeted by ROS, PKC\(\varepsilon\) is central to IPC mediated neuroprotection. Once activated, PKC\(\varepsilon\) phosphorylates and thereby opens the mK\(^{+}\)\text{ATP} channel leading to further ROS formation by the mitochondria and inhibiting the opening of the mitochondrial permeability transition pore (MPTP). Low levels of ROS are also known to activate HIF-1, SIRT1, and Nrf2. ROS may also regulate neuroprotective signaling pathways indirectly through signaling pathway cross-talk. ROS: Reactive oxygen species; IPC: Ischemic Preconditioning; PKC\(\varepsilon\): Protein Kinase C Epsilon; mK\(^{+}\)\text{ATP}: mitochondrial ATP sensitive potassium channel; MPTP: Mitochondrial Permeability Transition Pore; HIF-1: Hypoxic Inducible Factor 1; SIRT1: Sirutin 1; Nrf2: Nuclear factor (erythroid-derived 2)-like 2;
Chapter 2: IPC treatment of astrocytes promotes tolerance against oxygen glucose deprivation via the Nrf2 pathway

2.1 Summary

Aims: Ischemic preconditioning (IPC) represents a potential therapy against cerebral ischemia by activation of adaptive pathways and modulation of mitochondria to induce ischemic tolerance to various cells and tissues. Mitochondrial dysfunction has been ascribed to contribute to numerous neurodegenerative conditions and cerebral ischemia. Nuclear erythroid 2-related factor 2 (Nrf2) is a transcription factor that has traditionally been involved in upregulating cellular antioxidant systems to combat oxidative stress in the brain; however, the association of Nrf2 with mitochondria in the brain remains unclear. In the present study, we investigated the effects of Nrf2 on (i) IPC-induced protection of astrocytes; (ii) OXPHOS protein expression and (iii) mitochondrial supercomplex formation.

Methods: Oxygen-glucose deprivation (OGD) was used as an in vitro model of cerebral ischemia and IPC in cultured rodent astrocytes. OXPHOS proteins were probed via Western blotting and supercomplexes were determined by Blue Native Gel Electrophoresis (BNGE).

Results: IPC induced cytoprotection in WT, but not Nrf2-/-, mouse astrocyte cultures following a lethal duration of OGD. In addition, our results suggest that Nrf2 localizes to the outer membrane in non-synaptic brain mitochondria, and that a lack of Nrf2 in vivo produces altered supercomplex formation in mitochondria.

Conclusions: Our findings support a role of Nrf2 in mediating IPC-induced protection in astrocytes, which can profoundly impact the ischemic tolerance of neurons. In addition,
we provide novel evidence for the association of Nrf2 to brain mitochondria and supercomplex formation. These studies offer new targets and pathways of Nrf2, which may be heavily implicated following cerebral ischemia.

2.2 Background

A potential therapy that could mitigate the morbidity and mortality of cerebral ischemic injury may be represented as ischemic preconditioning (IPC); previous studies from our group have shown that IPC treatment induced neuroprotection in rodent models of global cerebral ischemia\textsuperscript{97, 180, 181}. However, preconditioning treatment has historically focused mainly on neuronal physiology and amelioration of neuronal cell death following \textit{in vitro} and \textit{in vivo} models of ischemia. As a result, the role of astrocytes in mediating IPC-induced cytoprotection has not been thoroughly characterized, despite the well-known functions of astrocytes in mediating several neuroprotective mechanisms\textsuperscript{182}.

In addition to astrocytes, mitochondria have gained much recognition as a potential contributor to neurodegenerative diseases. In many of these diseases, disorders in mitochondrial quality control, bioenergetics, and reactive oxygen species production have been ascribed as the causative dysfunction in neurodegenerative conditions, including cerebral ischemia (reviewed in\textsuperscript{183}). As a result, a better understanding of the role of mitochondria in neurodegenerative diseases may help to identify potential therapeutic targets to intervene and restore normal functioning in the brain.

The antioxidant transcription factor, nuclear erythroid 2-related factor 2 (Nrf2), has been extensively studied in the context of ischemia\textsuperscript{184, 185}. Nrf2 has been suggested to be highly
expressed in astrocytes as opposed to neurons\textsuperscript{165}. As an antioxidant transcription factor, Nrf2 has been shown to translocate to the nucleus in various cell types after exposure to oxidative stress. Upon nuclear translocation, Nrf2 increases gene transcription of some common antioxidants, such as catalase, superoxide dismutase, and glutathione\textsuperscript{186}. As mitochondria are organelles that contribute to the majority of ROS production in the cell, an interaction of Nrf2 with mitochondria may serve to keep the nucleus apprised of mitochondrial functioning. However, the relationship of Nrf2 to mitochondria in the brain has not been extensively investigated.

As Nrf2 has a well-documented role in mediating nuclear gene transcription, its involvement with mitochondria may suggest that Nrf2 could facilitate mitochondrial-nuclear communication. Mitochondrial-nuclear communication and coordination, particularly in oxidative phosphorylation (OXPHOS) regulation, are critical to maintaining normal bioenergetics within a cell. In addition, key functions like mitochondrial biogenesis and mitophagy also rely on the coordination of nuclear transcription factors and mitochondrial-targeted downstream pathways\textsuperscript{187}. Therefore, Nrf2 may represent a novel transcription factor that can coordinate nuclear-mitochondrial communication and OXPHOS regulation.

Therefore, the focus of the present study was to determine if IPC treatment could induce cytoprotection in astrocytes through Nrf2. We found that Nrf2 was enriched in astrocytes compared to neurons, and that IPC preconditioning was able to activate Nrf2-dependent gene transcription in rat astrocytes. Absence of functional Nrf2 protein reduced IPC-
induced cytoprotection in astrocyte cultures following an *in vitro* model of cerebral ischemia. Finally, we provide novel evidence which suggests that Nrf2 associates with the outer mitochondrial membrane in non-synaptic mitochondria, and that Nrf2 plays a role in OXPHOS supercomplex association. These studies highlight the contribution of Nrf2 to IPC-induced protection of astrocytes and novel interactions of Nrf2 with mitochondria.

### 2.3 Methods

**Materials**

Minimum Essential Medium (MEM), Hanks Balanced Salt Solution (HBSS) and Fetal Bovine Serum (FBS) were purchased from Gibco/Life Technologies (Grand Island, NY). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

**Animal Use**

All animal protocols were approved by the Animal Care and Use Committee of the University of Miami. Experiments were conducted in accordance to ARRIVE guidelines. 16-17 day-pregnant Sprague-Dawley rats were purchased from Charles Rivers Laboratories and housed in a temperature controlled environment with 12 hr light -12 hr dark cycle and *ad libitum* food and water. 5 week old male and female homozygous knockout mice (Nrf2−/−, Jackson Laboratories) were bred to establish homozygous Nrf2−/− colonies.
Preparation of embryonic post-natal astrocyte cultures

Astrocyte and neuronal cultures were prepared as previously described\textsuperscript{188} with slight modifications. For astrocyte cultures, cortices from postnatal Sprague Dawley rat pups (P2-P4) were harvested, followed by digestion with 0.25% trypsin and DNase. Following trituration and filtration through a 70 µm filter, the resulting cell suspension was centrifuged at 200 x g for 5 minutes and plated in Minimal Essentials Media (MEM) supplemented with 20 mmol/L glucose, 1% GlutaMAX, 1% Penicillin/Streptomycin, and 10% FBS before plating. Complete media changes were performed every 2-3 days until cultures reached 70% confluency 7 days following the initial plating. Astrocyte cultures were then passaged and plated in appropriate culture vessels at a density of 50,000 cells/cm\textsuperscript{2}, and allowed to reach full confluency and maintained for an additional 6-7 days before experimental use.

Oxygen Glucose Deprivation

To mimic IPC \textit{in vitro}, astrocyte cultures were exposed to oxygen and glucose deprivation (OGD) as previously described\textsuperscript{189} for 1 hr. Through empirical testing, 1 hr was determined to be a sublethal duration of OGD that induced the highest degree of protection to astrocytes following a lethal OGD insult (6 hrs). The 6 hr time point was chosen because greater than 50% cell death occurred along with minimal cell detachment from the tissue culture dishes, allowing for more accurate lactate dehydrogenase release assays. To induce OGD, cells were washed two times with glucose-free HBSS (in mmol/L) (CaCl\textsubscript{2} 1.26, KCl 5.37, KH\textsubscript{2}PO\textsubscript{4} 0.44, MgCl\textsubscript{2} 0.49, MgSO\textsubscript{4} 0.41, NaCl 136., NaHCO\textsubscript{3} 4.17, Na\textsubscript{2}HPO\textsubscript{4} 0.34, sucrose 20, pH 7.4) and exposed to an oxygen-free
environment (90% nitrogen, 5% hydrogen, and 5% CO₂, 37°C) using a COY anaerobic chamber (COY Laboratory Products Inc, Lake Charter Township, MI). OGD was terminated by placing the cells back into glucose-containing maintenance media and returning cultures to a 5% CO₂, 37°C incubator. Sham IPC was performed using similar number of washes and glucose-Free HBSS, except glucose (20 mmol/L) was substituted for sucrose and cells were placed back into normoxic conditions.

**Cell Death Assay**

To assay cell death, astrocyte culture media was assayed for lactate dehydrogenase using a commercially available kit (Roche Inc.). Cell death assays were performed according to manufacturer’s instructions. Briefly, cell culture media was collected from astrocyte cultures prior to IPC or Sham treatment, prior to lethal OGD treatment, 48 hours-post lethal OGD treatment, and finally following complete lysis of cells with 2% triton in PBS solution from the same well. LDH release into cell culture media was measured colorimetrically at an absorbance of 490 nm. LDH release results were normalized to the total amount of LDH present in each corresponding well, thereby normalizing for slight differences in cell density across each individual well of the cell culture plate. Values are represented as a percentage of maximum LDH release.

**Isolation of non-synaptic mitochondria**

Non-synaptic mitochondria were isolated from WT or Nrf2-/- mice. Mouse cortex were homogenized in isolation medium (250 mM sucrose, 1 mg/ml bovine serum albumin (fraction V essentially fatty acid free, BSA), 1.0 mM ethylenediaminetetra-acetic acid
(EDTA), and 0.25 mM dithiothreitol, pH 7.4) Tissue was minced with a pair of scissors and rinsed thoroughly with the isolation medium. The minced tissue was homogenized in a hand-operated Teflon glass homogenizer by 7-8 strokes. The homogenate was diluted to yield 10% (w/v) homogenate and centrifuged at 720 × g for 5 min using a Sorvall (Newton, CT) RC5 centrifuge. The supernatant was collected in another tube and centrifuged again at the same speed to reduce nuclear contamination of the eventual mitochondria sample. To isolate glial and neuronal cell body mitochondria, non-synaptic mitochondria was collected by layering the supernatant obtained from the final slow-speed centrifuge on a 24% (v/v) percoll gradient (percoll diluted in isolation media with BSA). The gradients were centrifuged at 32,500 × g for 5 min. The resulting pellet was washed once with isolation media and centrifuged at 15,000 × g for 10 min. The pellet was again washed with 0.25 M sucrose by centrifugation at 15,000 × g for 10 min. The resulting pellet was resuspended in 0.25 M sucrose, and protein content was determined by bicinchoninic acid (BCA) assay. All mitochondrial isolation procedures were performed at 4°C.

**Western Blot**

Cells were lysed in RIPA Buffer (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na3VO4 and 1 mmol/L PMSF). Protein concentration was determined by BCA protein assay and 30 µg of protein was loaded onto a 12% SDS-polyacrylamide gel and electroblotted to nitrocellulose. Membranes were blocked in 5% dry milk/TBST and hybridized with primary antibodies overnight at 4°C. Blots were
probed with rabbit anti-Nrf2 (1:500, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-UCP2 (1:500, Calbiochem Inc.), rabbit anti-GAPDH (1:10,000, Cell Signaling Technology, Danvers, MA), mouse anti-β-actin (1:10,000, Sigma), rabbit anti-MnSOD (1:2,000, Cell Signaling Technology) or goat anti-Lamin-B (1:1,000, Cell Signaling Technology). Membranes were washed with TBST followed by incubation with anti-mouse, anti-goat, or anti-rabbit HRP-conjugated secondary antibodies (1:5,000, Pierce, Thermo Scientific; Rockford, IL) for 1 hr at room temperature. Proteins were detected using enhanced chemiluminescence (ECL) system (Pierce, Thermo Scientific). Western blot densitometry was analyzed using ImageJ (NIH).

**Submitochondrial localization experiments**

For the proteinase K treatment, the mitochondria were prepared without protease inhibitor and were incubated with 0, 25, 50, 100, and 150 μg of proteinase K at 37°C for 30 minutes. After this, mitochondrial samples were centrifuged at 13000g for 10 min. The supernatant was discarded, and proteinase k–treated mitochondria were subjected to SDS-PAGE and immunoblotting.

**Blue Native Gel Electrophoresis**

Separation of electron transport supercomplexes was performed using BN-PAGE as previously described. Mitochondrial membranes were solubilized with digitonin in a buffer composed of 750 mM 6-aminohexanoic acid, 50 mM Bis-Tris and 0.5 mM EDTA, pH 7.0 at 4°C to a digitonin; protein ratio of 8:1 (w/w). This concentration of digitonin was empirically determined to solubize mitochondrial membranes while still maintaining
oligomeric association of respiratory supercomplexes. Following treatment with digitonin, samples were centrifuged for 10 min at 13000 × g at 4°C. Serva G Blue was added to the resulting supernatants using a detergent-to-dye ratio of 8:1 (w/w). Proteins (100 μg aliquots per lane) were separated on a Native PAGE Novex 3-12% Bis-Tris gradient gel (Invitrogen, Carsbad, CA, USA). Following electrophoresis, protein bands were visualized using Bio-Safe Coomassie-G250.

Separation of individual electron transport complexes by 2D SDS Page
Following Blue native PAGE, supercomplexes were further resolved in the second dimension under reducing conditions to determine their composition, as previously described\textsuperscript{191}. In brief, a lane from the Blue Native gel was excised and placed in a solution of SDS sample buffer. These lanes were microwaved briefly for 30 seconds until the SDS buffer began to boil. After a subsequent 10 minute incubation in the sample buffer, the lane was placed atop a 12% SDS-PAGE gel and ran under normal SDS page conditions. Following previously described immunoblotting methods, the composition of the supercomplexes were analyzed using an OXPHOS western blotting cocktail of antibodies which probed complexes I-V. The bands were imaged with Kodak Film and were analyzed with ImageJ software.

Statistical Analysis
All data are expressed as mean±STDEV. Statistical analysis between two groups was performed using the unpaired Student’s \textit{t}-test. Statistical analysis between more than two groups was performed using a one-way ANOVA with Bonferroni’s multiple comparison
post-hoc test unless otherwise specified. \( p \leq 0.05 \) was considered statistically significant (GraphPad Prism v. 5.00 for Windows, GraphPad Software, San Diego California USA).

2.4 Results

2.4.1 Nrf2 is predominately located in rat cortical astrocytes

Previous studies have shown that astrocytes contain a higher antioxidant potential than neurons. Therefore, we first determined if Nrf2 levels were increased in astrocytes compared to neurons. Whole cell lysates prepared from rat astrocyte and neuronal cultures were immunoblotted for Nrf2. Whole-cell astrocyte lysates contained higher levels of Nrf2 protein as compared to neuronal cultures under naïve conditions (Figure 2.1A). The results show that neurons had approximately 92% less basal Nrf2 levels compared to astrocytes (Figure 2.1B, \( p < 0.005 \), n=3). These results indicate that Nrf2 is predominantly found in cortical astrocytes.

2.4.2 IPC protects astrocytes against lethal OGD

IPC has been described in many different cells and tissues, but has not been well-defined in rodent astrocytes. Therefore, we investigated if IPC could induce cytoprotection in post-natal astrocytes against lethal ischemia using an in vitro model of ischemia (oxygen glucose deprivation, OGD). Following lethal OGD, astrocytes that were treated with IPC had 44 ± 5% cell death compared to 78 ± 7% cell death in Sham-treated cultures (Figure 2.2) as determined by LDH release assay. Our results suggest that IPC treatment was able to induce tolerance in rat astrocyte cultures against lethal ischemic injury.
2.4.3: IPC treatment activates Nrf2 in rat astrocyte culture

After establishing a preconditioning paradigm in astrocyte cultures, we next investigated whether IPC treatment could activate Nrf2 in astrocytes. To determine if downstream targets of Nrf2 were increased following preconditioning, whole-cell astrocyte lysates were probed for NQO-1, a gene under strict Nrf2 transcriptional control. 48 hrs following IPC treatment, NQO-1 protein levels were significantly increased in astrocyte cultures compared to sham treatments, respectively (Figure 2.3B). The results indicate that IPC activate Nrf2 by increasing Nrf2-dependent gene transcription.

2.4.4: IPC-induced protection is reduced in Nrf2\textsuperscript{-/-} astrocyte cultures following lethal OGD

To investigate the role of Nrf2 in IPC-induced cytoprotection, we subjected cortical astrocytes cultured from WT and Nrf2\textsuperscript{-/-} mice to IPC treatment 48 hrs prior to lethal OGD exposure. Based on results obtained from the LDH release assay, 1 hr and 2 hr durations of OGD protected WT astrocytes against lethal (5 hr OGD) ischemia as compared to sham preconditioning (1 hr: 14 ± 11%; 2 hr: 6 ± 0.3%; Sham: 32 ± 3.3%, p≤0.005, n=4-6) but no cytoprotection was observed in Nrf2\textsuperscript{-/-} cultures following IPC (Figure 2.4). In addition, Nrf2\textsuperscript{-/-} sham-treated cultures exhibited increased cell death compared to WT sham-treated astrocyte cultures following lethal OGD (52 ± 1.2% vs. 32 ± 3.3%, respectively, p≤0.05). These results suggest that Nrf2\textsuperscript{-/-} astrocytes are more susceptible to OGD injury, and that loss of Nrf2 protein decreased IPC-induced astrocyte protection.
2.4.5: Nrf2 colocalizes to non-synaptic mitochondria in WT mice

Previous studies from our group have suggested that Nrf2 plays a role in mediating mitochondrial bioenergetics and mitochondrial antioxidant protein levels in non-synaptic mitochondria\textsuperscript{193}. A previous study has suggested that Nrf2 can associate with mitochondria in a human cell line following overexpression of an outer mitochondrial membrane adapter protein, \textit{phosphoglycerate mutase} family member 5\textsuperscript{173}. Therefore, we investigated whether Nrf2 is integrally associated with non-synaptic cortical mitochondria \textit{in vivo}. Non-synaptic mitochondria were isolated from WT mice and purified on a percoll gradient. We subjected 100 µg aliquots of WT non-synaptic mitochondria to increasing concentrations of proteinase K treatment, which degrades exposed outer mitochondrial membrane proteins\textsuperscript{194}. In Figure 2.5, we found that proteinase K was able to degrade Nrf2 in unpermeabilized mitochondria. As a positive control, Tom20, an integral outer mitochondrial protein, showed a similar pattern of degradation with increasing proteinase K treatment. Finally, a matrix protein, mtHSP70, did not show signs of degradation following proteinase K, indicating that proteinase K was limited to degradation of outer mitochondrial membrane proteins. As a further control, the control sample displayed in Figure 2.5 shows the immunoblotting of proteins on unpermeabilized mitochondria. When mitochondria were lysed with 2% solution of triton and subjected to proteinase K, we observed almost complete degradation of protein by proteinase K as expected, since the enzyme now had access to proteins contained in all the compartments of mitochondria. These results suggest that Nrf2 localizes to non-synaptic mitochondria, and that it is located on the outer mitochondrial membrane.
2.4.6: Nrf2 knockout mice do not have altered OXPHOS protein expression compared to WT mice.

In mitochondria, OXPHOS protein expression is contributed by both mitochondrial and nuclear genomes. From Figure 2.5, given that Nrf2 appears to associate with WT mitochondria we surmised that Nrf2 may coordinate nuclear and mitochondrial OXPHOS gene expression. To determine this, we measured the protein level ratio of a nuclear-encoded OXPHOS subunit, SDHA, and of a mitochondrial-encoded OXPHOS subunit, MCTO1 (COX-1) under basal conditions from isolated WT or Nrf2−/− non-synaptic mitochondria. The ratio of a nuclear-encoded OXPHOS protein subunit to a mitochondrial-encoded OXPHOS subunit has been suggested to be an indicator of mitochondria-nuclear OXPHOS balance and mitochondrial biogenesis. From our results, there was a non-significant decrease in SDHA/MCTO1 ratio in Nrf2−/− non-synaptic mitochondria compared to WT non-synaptic mitochondria. These results suggest that Nrf2−/− mitochondria may have altered mitochondria-nuclear OXPHOS balance (Figure 2.6A, 2.6B, p=0.07). Once again, however, this decrease was statistically non-significant.

In addition, we investigated whether the protein levels of the electron transport chain complexes were altered by Nrf2. We therefore isolated non-synaptic mitochondria from naïve WT or Nrf2−/− and measured protein levels of individual electron transport chain complexes. From Figure 2.6C, we did not observe any significant difference between the levels of Complexes I-V between WT and Nrf2−/− mice. From these results, there appears to be no alterations in OXPHOS protein expression or mitochondrial-nuclear OXPHOS balance between WT and Nrf2−/− mice.
2.4.7: Nrf2 knockout mice have altered expression of electron chain supercomplexes compared to WT mice.

A previous study suggested that electron transport chain complexes can aggregate to form “supercomplexes”, and that this structure confers a bioenergetic advantage and a reduction in ROS production in mitochondria\textsuperscript{196}. Given that we did not observe any gross alterations in the expression of specific complexes in mitochondria from WT or Nrf2\textsuperscript{-/-} mice, we investigated whether Nrf2 could play a role in regulating mitochondrial supercomplex formation. Non-reducing electrophoresis conditions and 2-d separation of supercomplexes obtained from WT non-synaptic mitochondria resulted in the protein pattern observed in Figure 7A. Based on Western blotting of the OXPHOS complex subunits, we detected supercomplexes containing complexes I/III/IV/V, I/III/V, I/III/IV, I/V, III/IV, and IV/V. Complex II, as previously described, does not typically associate with other complexes to form a supercomplex.

The profile of supercomplexes in Nrf2\textsuperscript{-/-} exhibited marked differences (Figure 2.7B). There was a decrease of supercomplex assemblies, as Nrf2\textsuperscript{-/-} only contained supercomplexes composed of complexes I/III/V, I/V, and III/IV. Once again, complex II was isolated from interacting with other complexes. The results from Figure 2.7 suggest that under naïve conditions, Nrf2 increased supercomplex formation. Loss of Nrf2, as observed in Figure 2.7B, is associated with a reduction in total OXPHOS supercomplex assemblies.
2.5 **Discussion**

Our investigation sought to determine if Nrf2 plays a role in IPC-induced protection in astrocytes and if Nrf2 plays a role in mitochondrial OXPHOS expression. From the current studies, we developed a model of IPC that induces protection in rat astrocytes against lethal durations of oxygen-glucose deprivation (Figure 2). In addition, this duration of IPC can activate Nrf2 as determined by measuring NQO-1, a downstream gene product under strict transcriptional control of Nrf2 (Figure 2.3). Our focus on astrocytes was due to our results in Figure 2.1 that highlighted the enriched presence of Nrf2 in astrocytes compared to neurons. Therefore, our results suggested that Nrf2 due to its previously reported regulatory roles, could be a key player in IPC-induced cytoprotection in astrocytes. This conjecture was supported since IPC-induced protection was significantly reduced in mouse astrocytes cultured from Nrf2\(^{-/-}\) mice, suggesting that Nrf2 contributes to the protective effects of IPC treatment (Figure 2.4). Next, we showed that Nrf2 can localize to the outer mitochondrial membrane of non-synaptic mitochondria from WT mice (Figure 2.5). While we did not observe any differences in OXPHOS expression between WT and Nrf2\(^{-/-}\) mice (Figure 2.6), Nrf2\(^{-/-}\) mice had fewer non-synaptic mitochondrial supercomplexes compared to WT mice (Figure 2.7). These studies provide novel evidence that Nrf2 not only colocalizes with mitochondria in the brain, but may play an important role in mediating supercomplex formation.

Mitochondrial dysfunction, particularly high levels of mitochondrial ROS production, has been shown to be a precipitating factor in the pathogenesis of cerebral ischemia.
However, sub-injurious levels of ROS serve as important signaling molecules that can be exploited to produce long-term adaptation in cells against a second lethal ischemic insult. Studies have shown that treatment of cells with antioxidants prior to induction of IPC decreases the induction of ischemic tolerance and subsequent protection against lethal ischemic injury. As a result, for our studies we focused on the Nrf2 pathway as this transcription factor is involved in cellular adaptation to oxidative stress through the upregulation of endogenous antioxidants.

Because mitochondrial function is critical to cellular adaptation and cellular dysfunction during cerebral ischemia, we sought to determine if there was a relationship between Nrf2 and brain mitochondria. Previous studies have implicated the role of Phosphoglycerate Mutase Family Member 5 (PGAM5) as functioning as a tether to which Nrf2 and its cytosolic repressor, Keap1, are retained on the outer mitochondrial membrane in HeLa cells. Therefore, we first determined if Nrf2 could associate and co-localize with non-synaptic mitochondria from the mouse cortex. We utilized non-synaptic mitochondria because this population contained more glial and astrocytic mitochondria, as compared to the synaptic fraction which is essentially synaptosomes from neurons. However, non-synaptic mitochondria also have cell body neuronal mitochondria. But, since we found that Nrf2 levels in mitochondria are several fold greater in astrocytes than in neurons (Figure 2.1), we surmised that in Nrf2<sup>-/-</sup> mice, absence of Nrf2 in this mitochondrial fraction could shed light into Nrf2’s mitochondrial function. A previous study by Lo et al provided evidence that after overexpression of PGAM5, Nrf2 was able to associate with this outer mitochondrial membrane. Studies
with proteinase K suggest that Nrf2 can co-localize to the outer mitochondrial membrane in non-synaptic mitochondria. To our knowledge, this is the first study to provide evidence of the localization of Nrf2 to mitochondria in the brain.

As Nrf2 is a nuclear transcription factor, our results with Nrf2’s localization to non-synaptic mitochondria may suggest a novel role of Nrf2 in coordinating mitochondria and nuclear OXPHOS expression. Therefore, we investigated the protein expression of a nuclear-encoded OXPHOS subunit (SDHA from Complex II) and a mitochondrial-encoded OXPHOS subunit (MCTO-1 from Complex I) in non-synaptic mitochondria. From Figure 2.6, we found a non-significant trend suggestive of decreased SDHA/MCTO-1 ratio in Nrf2−/− mitochondria compared to WT mice. In addition, we found no significant differences between these two strains of mice in regards to protein expression of individual electron transport chain complexes.

In regards to the electron transport chain (ETC), previous work has suggested that the complexes can exist in oligomeric forms called supercomplexes. These supercomplexes are assemblies of 2 or more of the ETC complexes, and have been suggested to provide more efficient bioenergetics and produce less ROS198. Therefore, we then investigated whether Nrf2 influenced the presence of supercomplexes in non-synaptic mitochondria. From Figure 2.7, blue native gel electrophoresis and 2-d separation suggest that Nrf2−/− mice have decreased supercomplex formation compared to WT mice. While the mechanism of supercomplex formation is still largely unknown, Nrf2 may facilitate supercomplex formation in an effort to suppress ROS production from mitochondria. In addition to these findings, previous studies from our lab have shown that Nrf2−/− mice
have a lower respiratory control ratio compared to WT mice, suggestive of mitochondrial uncoupling\textsuperscript{193}; therefore, the activation of Nrf2 following IPC could therefore facilitate more efficient mitochondrial bioenergetics and decreased ROS production, perhaps through mitochondrial supercomplex formation, which could help sustain cellular function in the face of lethal cerebral ischemia.

The association of Nrf2 to mitochondria may have several additional implications. Previous studies have shown that Nrf2, following nuclear translocation, can bind to p62\textsuperscript{171, 199}; p62 has been implicated in stimulation of mitochondrial autophagy (mitophagy)\textsuperscript{200}. In addition, Nrf2 has been implicated in regulating mitochondrial biogenesis\textsuperscript{168}; Nrf2 can also bind to nuclear respiratory factor 1 (NRF1), a prominent gene that can subsequently bind to a promoter found on the transcription factor of mitochondria (TFAM)\textsuperscript{167}. TFAM translocates to mitochondria, where it initiates mitochondrial gene transcription. Therefore, Nrf2 may associate with mitochondria to serve as a messenger to relay the function of mitochondria, represented in its production of ROS, to the nucleus. Once in the nucleus, Nrf2 can promote increase in antioxidant enzymes to suppress ROS production, but may induce more long-term adaptation to oxidative stress by modulating mitochondrial gene transcription and biogenesis. While our studies only looked at one measurement of mitochondrial-nuclear OXPHOS balance (SDHA/MCTO1 ratio), future studies will further investigate this relationship. Investigating other markers of nuclear-mitochondrial balance, such as mitochondrial quality control (mitophagy and biogenesis) as well as apoptosis would further highlight
the role of Nrf2 as a novel communicator between the mitochondrial and nuclear compartments.

In conclusion, our studies implicate the astrocyte-enriched transcription factor Nrf2 as being required, as least partially, for IPC-induced protection. In addition, loss of Nrf2 results in abrogation of IPC-mediated protection in astrocytes, which could have importance consequences to the ischemic tolerance of neurons. Finally, our studies provide novel evidence for the association of Nrf2 with brain mitochondria, and that Nrf2 plays a role in supercomplex formation. As a result, Nrf2 may represent an important pathway in nuclear-mitochondrial communication, whose activation could be therapeutic in the context of ischemic preconditioning and cerebral ischemia.

Our next study investigated if resveratrol, an IPC-mimetic, could activate Nrf2 similarly to IPC treatment in astrocytes. Furthermore, we extend this study to an in vivo model of focal cerebral ischemia, the middle cerebral artery occlusion (MCAO) utilizing WT and Nrf2−/− mice. Through these studies, our goal was to extend our in vitro studies regarding IPC and Nrf2 to in vivo studies with resveratrol preconditioning. As discussed in chapter 1, clinical implementation of ischemia can be beneficial. However, a more attractive feature of IPC is the replication of neuroprotection through more compliant pharmacologic treatments, such as resveratrol.
2.6 Figures

A) Western blot analysis of whole cell lysates from astrocyte and neuronal cultures derived from postnatal and embryonic rat pups, respectively. Samples were immunoblotted with Nrf2 and Actin (whole-cell loading control). B) Quantification of western blots from A). Nrf2 protein levels were normalized to Actin. Data represented as a percentage of astrocyte Nrf2/Actin ratios. **p ≤ 0.05. n = 3.

Figure 2.1: The antioxidant transcription factor, Nrf2, is enriched in astrocytes compared to neurons.
Figure 2.2: IPC treatment protects astrocytes against lethal oxygen glucose deprivation injury.

LDH release assay results of rat astrocyte cultures following lethal OGD previously treated with Sham, or IPC treatment. n = 6 *p < 0.05.
Figure 2.3: IPC or RPC treatment activate Nrf2 in astrocyte cultures.

A) Whole cell astrocyte lysates were probed for NQO-1 expression following IPC or Sham treatment. B) Quantification of western blot results. NQO-1 protein was normalized to actin (whole cell loading control). n = 4, *p ≤ 0.05.
Figure 2.4: IPC treatment of WT astrocyte cultures induces neuroprotection against lethal OGD, which is abrogated in Nrf2-/- astrocyte cultures.

A) Cell death assessment of WT and Nrf2-/- astrocyte cultures following lethal OGD. Cultures were treated with 1 hr and 2 hr durations of OGD as preconditioning treatments, along with sham treatments. Cell death was determined using LDH release assay, and data is represented as a percent of the maximal LDH release n = 4-6, * p ≤ 0.05.
Figure 2.5: Nrf2 localizes to mitochondrial fraction of rat astrocyte cultures and rat cortex.

Mitochondria was isolated from WT C57 black mice and purified on a percoll gradient. Mitochondria from WT C57Bl/6J mice were treated with proteinase K to determine submitochondrial localization of Nrf2. mtHsp70 (matrix protein) and Tom20 (outer mitochondrial membrane protein) were used as controls for proteinase K treatment. Ctrl: unpermeabilized non-synaptic mitochondria. Triton: treatment of mitochondria with 2% triton solution. n=3.
Figure 2.6: Nrf2 knockout mice do not have altered mitochondrial-nuclear ratio of OXPHOS protein expression compared to WT mice.

100 µg of crude mitochondria from WT or Nrf2-/- mice were isolated and subjected to SDS-page electrophoresis. A subunit of COX-1 (MCTO1), a subunit of complex II (SDHA), and a cocktail of antibodies directed against the ETC complexes (I-V) were probed via Western blotting. A) Representative image is presented. B) Quantification of SDHA/MCTO1 ratio, a measurement of mitochondrial biogenesis and mitochondrial-nuclear OXPHOS balance. N.S.: not significant. C) Quantification of each of the 5 ETC complexes (I-V), normalized to VDAC (mitochondrial loading control). n = 4-6.
Figure 2.7: Nrf2 knockout mice have altered expression of electron chain supercomplexes compared to WT mice.

100 µg of crude mitochondria from WT or Nrf2 KO mice were isolated and subjected to BNGE and 2-d SDS-page electrophoresis. For 2-dimensional electrophoresis, bovine heart mitochondrial lysate was also included in the electrophoresis as a positive control to indicate the molecular weight of the respiratory complexes. The complexes and supercomplexes of the original 1-d BNGE lane are indicated at the top based on the resolved complexes in the 2nd dimension. Representative image, n=3.
**Figure 2.8: Genotyping of Nrf2-/- and WT C57Bl/6J mice.**

LAC Z denotes the portion of the Nrf2 locus that has been replaced with a LAC Z and Neomycin resistance gene cassette. Presence of LAC Z indicates animals that have this gene in place of exon 4 and exon 5 of the Nrf2 gene, and denotes a knockout animal. Presence of WT allele denotes presence of Nrf2 gene. Presence of both LAC Z and WT gene indicate a heterozygous knockout animal. Genotyping was performed by Transnetyx Inc.
Chapter 3: Resveratrol preconditioning protects against cerebral ischemic injury via Nrf2

3.1 **Summary**

**Background and Purpose:** Nuclear erythroid 2 related factor 2 (Nrf2) is an astrocyte-enriched transcription factor that has previously been shown to upregulate cellular antioxidant systems in response to ischemia. While resveratrol preconditioning (RPC) has emerged as a potential neuroprotective therapy, the involvement of Nrf2 in RPC-induced neuroprotection and mitochondrial reactive oxygen species (ROS) production following cerebral ischemia remains unclear. The goal of our study was to study the contribution of Nrf2 to RPC and its effects on mitochondrial function.

**Methods:** We used rodent astrocyte cultures and an *in vivo* stroke model with RPC. An Nrf2 DNA-binding ELISA and protein analysis via Western blotting of downstream Nrf2 targets were performed to determine RPC-induced activation of Nrf2 in rat and mouse astrocytes. Following RPC, mitochondrial function was determined by measuring ROS production and mitochondrial respiration in both wild-type (WT) and Nrf2−/− mice. Infarct volume was measured to determine neuroprotection, while protein levels were measured by immunoblotting.

**Results:** We report that Nrf2 is activated by RPC in rodent astrocyte cultures, and that loss of Nrf2 reduced RPC-mediated neuroprotection in a mouse model of focal cerebral ischemia. In addition, we observed that wild-type and Nrf2−/− cortical mitochondria exhibited increased uncoupling and ROS production following RPC treatments. Finally, Nrf2−/− astrocytes exhibited decreased mitochondrial antioxidant expression and were unable to upregulate cellular antioxidants following RPC treatment.
Conclusion: Nrf2 contributes to RPC-induced neuroprotection through maintaining mitochondrial coupling and antioxidant protein expression.

3.2 Background

In the United States, 1 in 20 deaths can be attributed to stroke, while more than 80% of the 800,000 people who suffer from stroke each year survive and require long-term rehabilitation\textsuperscript{201}. Ischemic preconditioning has emerged as a potential therapy that could mitigate the morbidity of cerebral ischemic injury; previous studies from our group have shown that ischemic preconditioning treatment induced neuroprotection in rodent models of global cerebral ischemia\textsuperscript{80, 181}. This protection has been recapitulated using the polyphenolic compound resveratrol as a pharmacologic preconditioning agent\textsuperscript{202}. The use of resveratrol as a preconditioning agent has been substantiated by numerous studies in a diverse range of in vitro and in vivo models\textsuperscript{203, 204}.

However, previous preconditioning studies have focused mainly on neuronal physiology and amelioration of neuronal cell death following cerebral ischemia. As a result, the role of astrocytes in mediating ischemic preconditioning is often neglected, despite the well-known functions of astrocytes in mediating several neuroprotective mechanisms\textsuperscript{182}. Astrocytes have been suggested to have increased resistance to ischemic injury when compared to neurons\textsuperscript{205}; however, astrocyte dysfunction has been shown to exacerbate various neurodegenerative conditions\textsuperscript{206-208} and increase susceptibility of neurons to ischemia\textsuperscript{209}. Rodents have both a lower astrocyte:neuron ratio and fewer astrocytic processes compared to humans\textsuperscript{210}. Indeed, the relative differences in cytoarchitecture
between rodents and humans may have contributed to the relative plateau of clinically translatable neuroprotective agents.

One of the many neuroprotective functions of astrocytes includes supplying neurons with antioxidants, the production of which is primarily controlled by the transcription factor Nuclear erythroid 2 related factor 2 (Nrf2). Nrf2 has been previously suggested to be highly expressed in astrocytes as opposed to neurons, and has been shown to increase the antioxidant proteins thioredoxin and NAD(P)H-quinone oxidoreductase 1 (NQO-1)\textsuperscript{165}. Since oxidative stress is a major consequence of cerebral ischemia, the function of Nrf2 to mitigate this stress makes Nrf2 and related downstream pathways attractive targets to combat cerebral ischemic injury.

In light of the aforementioned studies, the focus of our investigation was to determine if RPC treatment could induce neuroprotection through Nrf2 activation. We found that absence of functional Nrf2 protein reduced RPC-induced neuroprotection in a mouse model of focal cerebral ischemia. In addition, RPC treatment failed to increase mitochondrial and cellular antioxidants in cultured astrocytes when Nrf2 was absent. These studies highlight the contribution of astrocyte-derived Nrf2 to RPC-induced protection and a novel role of Nrf2 in maintaining mitochondrial function.

### 3.3 Methods

Additional detailed methods are described in the supplemental section. All animal protocols were approved by the Animal Care and Use Committee of the University of Miami. Minimum Essential Medium (MEM), Hank’s Balanced Salt Solution (HBSS) and
Fetal Bovine Serum (FBS) were purchased from Life Technologies (Grand Island, NY). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

**Preparation of primary cultures and in vitro preconditioning studies**

Astrocyte cultures were prepared as previously described. Cortical tissue was harvested from P2-P4 Sprague Dawley rats, C57Bl/6J wild-type (WT) mice, or Nrf2−/− mice. Following treatment with 0.25% trypsin and 0.1% DNase, single cell suspensions were plated onto cell-culture dishes and maintained for 10-14 days prior to experimental use. After reaching approximately 80% confluency, cultures were trypsinized and passaged. Passages 1-3 were used for experiments. For RPC treatment, astrocyte cultures were exposed to 2 hr of resveratrol (25 µmol/L) or DMSO (Vehicle) 48 hrs prior to harvesting cell lysates or nuclear fractions for downstream analysis.

**ELISA and Western blotting**

Nuclear and cytoplasmic fractionation was prepared according to manufacturer’s protocol by using a nuclear extract kit (Active Motif Cat. #: 40010). Nuclear and cytoplasmic extracts were probed with both Lamin B and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to establish purity of the nuclear and cytoplasmic fractions, respectively. 10 µg of nuclear samples were used for the TransAM Nrf2 enzyme-linked immunosorbent assay (ELISA) kit (Active Motif) to measure DNA-binding of activated Nrf2 nuclear protein, as determined by absorbance measurements at 450nm. For immunoblotting, cells were lysed in Radioimmunoprecipitation assay (RIPA) buffer and
immunoblotted for Nrf2, uncoupler protein 2 (UCP2), GAPDH, β-actin, manganese superoxide dismutase (MnSOD), or Lamin B. Proteins were detected using enhanced chemiluminescence system (Pierce, ThermoScientific) and densitometry was performed using ImageJ (National Institute of Health)\textsuperscript{190}.

**Animal model of Focal Cerebral Ischemia**

For our focal cerebral ischemia model, the left middle cerebral artery (MCA) was occluded for 1 hr using an intraluminal filament model as previously described\textsuperscript{211}. C57Bl/6J WT or Nrf2\textsuperscript{-/-} male mice between 7-11 weeks were randomly assigned to 2 treatment groups: resveratrol (10 mg/kg i.p.) or DMSO (Vehicle). The investigator was blinded to the administration of these agents 48 hrs prior to MCA occlusion. Twenty-four hours following reperfusion, infarct volume was assessed with 2,3,5-triphenyltetrazolium chloride (TTC) and quantified using ImageJ software. Exclusion criteria for these studies included (1) greater than 30% of baseline MCA blood flow persisting through the occlusion phase of the MCA occlusion (MCAO) injury as measured by laser Doppler flowmetry; and (2) lack of detectable infarct following TTC staining.

**Polarography**

Mitochondrial respiration studies were conducted as previously described\textsuperscript{96}. In brief, non-synaptic mitochondria were isolated from WT or Nrf2\textsuperscript{-/-} mice 48 hrs following resveratrol or vehicle treatment. The ratio of State III/State IV respiration was measured using a Clark-type oxygen electrode. This ratio represented the respiratory control index (RCI), an established measure of mitochondrial coupling\textsuperscript{212}. 

Measurement of mitochondria reactive oxygen species production

Mitochondrial reactive oxygen species (ROS) production was determined using a spectrophotometer following a previously established protocol\(^ {213} \). Isolated non-synaptic mitochondria from Nrf2\(^{-/-}\) or WT animals were added to a microplate containing horseradish peroxidase, Amplex Red Ultra, and superoxide dismutase. H\(_2\)O\(_2\) emission was measured spectrophotometrically at 555nm excitation/590nm emission wavelengths. After establishing baseline measurements, respiratory substrates were added in a similar manner to the polarographic studies. Rates of H\(_2\)O\(_2\) emission were recorded for each complex-specific substrate/inhibitor pair, and normalized to baseline.

Statistical Analysis

All data are expressed as mean±STDEV. Statistical analysis between two groups was performed using the unpaired Student’s t-test. Statistical analysis between more than two groups was performed using a one-way ANOVA with Bonferroni’s multiple comparison post-hoc test unless otherwise specified. \( p \leq 0.05 \) was considered statistically significant (GraphPad Prism v5.00 for Windows, GraphPad Software, San Diego, California USA).

3.4 Results

3.4.1 Loss of Nrf2 decreases RPC-induced neuroprotection following focal cerebral ischemia

Previous studies from our laboratory have shown that RPC can induce neuroprotection against cerebral ischemia \textit{in vivo}\(^{202} \). Therefore, we wanted to determine if loss of
functional Nrf2 decreased RPC-induced neuroprotection in a mouse model of focal cerebral ischemia. Nrf2\textsuperscript{−/−} mice were verified by standard genotyping and immunoblotting for NQO-1 (supplemental figure 3.1). WT and Nrf2\textsuperscript{−/−} mice were subjected to MCAO injury 48 hrs following either RPC or vehicle treatment. Quantification of TTC-stained brain slices from each treatment group indicated that RPC treatment of WT mice significantly reduced infarct volume compared to vehicle treatment (44.43±13.90%, n=9 vs. 28.31±9.93%, n=6; p<0.05) (Figure 3.1A and 3.1B). However, no significant difference was observed in Nrf2\textsuperscript{−/−} mice between RPC and vehicle-treated groups (40.86±17.10%, n=10 vs. 34.47±14.25%, n=9; p=0.39). In addition, there was no significant difference between WT and Nrf2\textsuperscript{−/−} vehicle-treated groups. Therefore, the results from Figure 3.1 indicate that RPC-induced neuroprotection was decreased in the absence of functional Nrf2.

3.4.2 Loss of RPC-induced neuroprotection in Nrf2\textsuperscript{−/−} mice is not due to altered cerebral blood flow

A previous group has suggested that the cerebral blow flow is altered in Nrf2\textsuperscript{−/−} mice, which led to their use of a modified \textit{in vivo} model to achieve adequate middle cerebral artery occlusion\textsuperscript{214}. To determine if the observed infarct volumes from Figure 3.1 were attributable to strain differences in cerebral blood flow, we analyzed blood flow throughout the MCAO injury via laser Doppler flowmetry for each treatment group of mice. The average blood flow for each group during the phases of the MCAO injury (baseline, occlusion, and reperfusion) were compared and expressed as a percentage of baseline laser Doppler Flowmetry measurements (Figure 3.2A); our results show that
there was no statistically significant difference between blood flow of any of the treatment groups for each phase of the MCAO injury. In Figure 3.2B, the average age and weight for each treatment group were analyzed, with no statistically significant differences observed. Therefore, the loss of RPC-induced neuroprotection in Nrf2−/− mice is not attributable to altered cerebral blood flow.

3.4.3 RPC treatments activate Nrf2 in rat astrocytes

Given that the reduction in RPC-induced neuroprotection in Nrf2−/− mice was not due to altered cerebral blood flow (Figure 3.2), the results from Figure 3.1 suggest that RPC-induced neuroprotection partially requires Nrf2. As there is greater Nrf2 protein abundance in astrocytes165, we next sought to determine if RPC can activate Nrf2 in vitro by utilizing astrocyte cultures. Using the TransAM Nrf2 ELISA kit, RPC treatment increased the amount of activated Nrf2 in nuclear astrocyte fractions at 48 hrs compared to vehicle-treated cultures as determined by absorbance measured at 450 nm (0.983±0.458 vs 0.625±0.352, p<0.05 n=3) (Figure 3.3A), which were not observed at earlier time points (1 or 24 hrs) following RPC treatment. To determine if downstream pathways of Nrf2 were increased following RPC, whole-cell astrocyte lysates were probed for NQO-1, an Nrf2-dependent gene target. 48 hrs following RPC treatments, NQO-1 protein levels were significantly increased in astrocyte cultures by ≈2.1 fold compared to vehicle treatments (Figure 3.3B). The results indicate that RPC activates astrocyte Nrf2 by increasing Nrf2 DNA binding and Nrf2-dependent gene transcription.

3.4.2 RPC-induced alterations to mitochondrial function in WT and Nrf2−/− cortical mitochondria
3.4.4 RPC induces uncoupling in WT and Nrf2\(^{-/-}\) mitochondria

Resveratrol has previously been implicated in modifying cerebral mitochondrial function. Therefore, we investigated mitochondrial coupling (represented as respiratory coupling index or RCI) in isolated non-synaptic mitochondria from WT and Nrf2\(^{-/-}\) mouse cortex following RPC treatment \textit{in vivo}. We utilized non-synaptic mitochondria because this fraction contains more astrocyte-derived mitochondria than the synaptic fraction, and therefore would better represent functional changes with the loss of Nrf2. RPC-induced treatment induced a mild uncoupling in both WT and Nrf2\(^{-/-}\) mitochondria compared to vehicle treatments of each respective mouse strain (WT: Vehicle 5.84±0.55 vs RPC 3.93±0.42, p<0.05; Nrf2\(^{-/-}\): Vehicle 3.272±0.67 vs RPC 2.27±0.15, p<0.05) (Figure 3.4A). In addition, we observed a significant difference between Nrf2\(^{-/-}\) and WT mice RCI values between vehicle groups, suggesting that Nrf2\(^{-/-}\) mitochondria have an innate respiratory dysfunction. Therefore, RPC treatment had similar effects on the RCI in both mouse strains, with RPC treatment further reducing the already decreased RCI in Nrf2\(^{-/-}\) mice.

3.4.5 RPC-induces increased UCP2 expression in Nrf2\(^{-/-}\) astrocytes

Previous studies from our lab have suggested an interaction between resveratrol and UCP2, an uncoupler protein which if altered by RPC could explain the results observed in Figure 3.4A. As isolation of astrocyte mitochondria from the brain was not feasible, we took advantage of highly-enriched astrocyte cultures to look at the effects of Nrf2 on UCP2 expression \textit{in vitro}. Therefore, 48 hrs following RPC treatment, whole cell mouse
WT and Nrf2−/− astrocyte lysates were prepared and probed for UCP2. Compared to vehicle treatments, UCP2 was significantly increased 48 hrs following RPC treatment in WT and Nrf2−/− astrocyte cultures (4.05±1.01 fold and 1.89±0.26 fold, respectively, p<0.05) (Figure 3.4B and 3.4C). Therefore, RPC treatment increased UCP2 protein expression in WT and Nrf2−/− mouse astrocyte cultures.

3.4.6 RPC treatment increases ROS production in WT and Nrf2−/− mitochondria

As Nrf2−/− mice are expected to have decreased antioxidant capacity, we also measured mitochondrial H2O2 production as a measure of ROS generation in non-synaptic mitochondria isolated from WT and Nrf2−/− mouse cortex following RPC treatment in vivo. RPC treatment significantly increased H2O2 production several-fold compared to baseline production following rotenone-induced complex I inhibition (WT: Vehicle 3.66±1.19 vs RPC 8.29±1.56 fold of baseline p<0.05, Nrf2−/−: Vehicle 3.90±2.82 vs. RPC 8.35±2.65 p<0.05) and antimycin-induced complex III inhibition (WT: Vehicle 7.54±2.43 vs RPC 31.06±10.17 p<0.005, Nrf2−/−: Vehicle 9.59±6.32 vs. RPC 23.25±10.05, p<0.05) for both strains of mice (Figure 3.5A). However, there were no significant differences between WT and Nrf2−/− mice for the same treatment group. These findings suggest a role of RPC in increasing ROS production at complex I and III in WT and Nrf2−/− cortical mitochondria.

3.4.7 RPC-induced antioxidant enzyme expression in WT and Nrf2−/− astrocytes

While RPC-induced increase in mitochondrial ROS production occurred in both WT and Nrf2−/− mice, we hypothesized that this phenomenon was detrimental in Nrf2−/− mice and
could explain loss of RPC-induced neuroprotection in this population. Therefore, we immunobinned WT and Nrf2⁻/⁻ astrocyte lysates for the antioxidants MnSOD and NQO-1 48 hrs following RPC treatment. (Figure 3.5B). RPC treatment significantly increased NQO-1 protein expression (normalized to actin) in WT astrocytes compared to vehicle treatment (1.91±0.35 fold increase, n=6, p<0.05). As expected, we were unable to observe or measure the Nrf2-dependent protein NQO-1 in Nrf2⁻/⁻ astrocyte lysates (Figure 3.5B and 3.5C). Next, we measured the mitochondrial antioxidant MnSOD proteins levels in WT and Nrf2⁻/⁻ astrocyte cultures via Western blotting. We found no statistically significant difference in MnSOD protein levels between vehicle and RPC-treated groups of either strain of mice (Figure 3.5D). However, Vehicle and RPC-treated Nrf2⁻/⁻ astrocytes had significantly less MnSOD protein compared to WT Vehicle MnSOD levels (40.3% and 37.2% less MnSOD protein, respectively, p≤0.005). These results suggest that Nrf2⁻/⁻ astrocytes have depressed mitochondrial and cellular antioxidants, and are unable to upregulate these proteins in response to RPC-induced ROS production observed in Figure 3.5A.

3.5 Discussion

In the present study, we investigated the contribution of Nrf2 to RPC-induced neuroprotection and the effect of Nrf2 on cortical mitochondrial function. We investigated the role of Nrf2 in RPC-induced neuroprotection in a rodent model of focal cerebral ischemia. Our MCAO studies suggest that in the absence of Nrf2 protein, RPC-induce neuroprotection is not effective in significantly ameliorating cerebral infarction in mice (Figure 3.1). Given that RPC treatment increased ROS production in both WT and
Nrf2−/− cortical mitochondria (Figure 3.4B), and that RPC was unable to induce antioxidant expression in Nrf2−/− mice (Figure 3.5), we believe this evidence suggests that RPC induced neuroprotection can be attributed to ROS-mediated signaling pathways, which ultimately activates astrocytic Nrf2 and confers cerebral ischemic tolerance.

The role of astrocyte pathways have not been fully elucidated in preconditioning research, but their importance to neuronal disease has been extensively studied. Previous studies by Belle et al.166 highlighted that astrocytic Nrf2 was necessary for ischemic tolerance in murine neuronal cultures. In direct contrast, Layon-Haskew et al determined that physiologic levels of H2O2 could induce ischemic tolerance in neurons independent of Nrf2215. Although our study is a different preconditioning paradigm (i.e., RPC), our current studies indicate that RPC is indeed able to increase Nrf2 DNA-binding and downstream expression of NQO-1, an Nrf2-dependent gene.

Similar to a previous study214, we did not observe any difference in infarct volume between vehicle-treated WT and Nrf2−/− mice at 24 hrs. While we observed significant neuroprotection with RPC treatment in WT mice following MCAO, there was no significant reduction of infarct volume in RPC-treated Nrf2−/− mice. While resveratrol has been shown to activate a multitude of pathways, resveratrol was still unable to significantly ameliorate infarct injury in Nrf2−/− mice following MCAO. This suggests that Nrf2 activation is a key pathway for RPC-induced neuroprotection. Our current investigation has also shown that resveratrol induced an increase in ROS production in WT and Nrf2−/− mitochondria, which can be detected 48 hr post-treatment.
This increase in ROS following resveratrol treatment has been seen previously in yeast cells\textsuperscript{216}, human adipocytes\textsuperscript{216}, and cancer cell-lines\textsuperscript{217}. In addition, the increase in ROS following preconditioning treatments is a well observed phenomenon, and inhibition of ROS production has been shown to ameliorate preconditioning-induced neuroprotective effects\textsuperscript{218}.

We also present findings that RPC treatment increased UCP2 expression in astrocyte cultures, and that this increase was several fold more in WT vs. Nrf2\textsuperscript{-/-} astrocytes (Figure 3.4). While our previous findings described an RPC-induced decrease in UCP2 protein expression in adult rat hippocampal mitochondria, our current studies investigated UCP2 in cortical post-natal mouse astrocyte cultures. This discrepancy could be due to the use of different models and cell types, and future studies may serve to elucidate the dependence of cell-type and brain region on the regulation of UCP2 by RPC treatment. Taken together, we propose that ROS production from RPC treatment induces UPC2 expression, leading to mild uncoupling and subsequent protection against oxidative stress. Furthermore, RPC-induced increase in UCP2 (Figure 3.4), basal MnSOD, and NQO-1 (Figure 3.5) protein levels were decreased in Nrf2\textsuperscript{-/-} mice compared to WT mice; we believe this implicates Nrf2 as a critical pathway in which RPC-mediated mitochondrial ROS production activates Nrf2, thus promoting the induction of antioxidant pathways and subsequent neuroprotection against focal cerebral ischemia.

Following RPC treatment, WT and Nrf2\textsuperscript{-/-} exhibited reductions in their RCI, suggestive of a resveratrol-induced uncoupling effect. By further decreasing the coupling of Nrf2\textsuperscript{-/-}
mitochondria, resveratrol may exacerbate already dysfunctional cortical mitochondria in Nrf2⁻/⁻ mice. Thus, decreased mitochondrial coupling, increased ROS production, and decreased antioxidant defenses could be plausible explanations as to explain why RPC was less effective in Nrf2⁻/⁻ mice than WT mice. Our findings that Nrf2⁻/⁻ non-synaptic mitochondria exhibited decreased coupling were in line with previous studies which suggested that Nrf2⁻/⁻ brain, heart and liver mitochondria exhibit decreased RCI²¹⁹. Interestingly, studies by Fiskum et al. did not describe any changes to brain non-synaptic mitochondrial respiration following activation of Nrf2 with sulforaphane. Future studies may serve to understand the relationship of Nrf2 to cortical non-synaptic and synaptic mitochondria.

In conclusion, our investigation provides new insight into the mechanism of RPC-induced protection, and implicates Nrf2 as an important pathway to induce RPC’s neuroprotective effects in the context of stroke.

As the previous studies have focused on the antioxidant function of astrocytes and implications Nrf2 in mediating preconditioning-induced protection, the next chapter discusses ongoing work regarding the role of astrocytes in mediating transference of ischemic tolerance to neurons through soluble mediators. Through the development of a modified co-culture system, IPC-treatment of astrocytes alone was found to induce neuroprotection in neurons. The next chapter elaborates on this technique, and the implications of astrocyte-derived lactate in mediating this transference of neuroprotection.
3.6 Figures

A) Representative image of TTC staining of brain slices to assess infarct volume of mice subjected to MCAO injury. Slices are distributed rostrocaudally. VEH: Vehicle. B) Quantification of infarct volume. Areas of cortical infarction at seven coronal levels were cumulated and normalized for edema. Number of subjects represented in inset of bar graph. Data are presented as mean ± STDEV; *p ≤ 0.05, (multiple comparison 1-way ANOVA followed by Bonferroni test).

Figure 3.1: RPC-induced neuroprotection is decreased in Nrf2−/− mice following MCAO injury.

A) Representative image of TTC staining of brain slices to assess infarct volume of mice subjected to MCAO injury. Slices are distributed rostrocaudally. VEH: Vehicle. B) Quantification of infarct volume. Areas of cortical infarction at seven coronal levels were cumulated and normalized for edema. Number of subjects represented in inset of bar graph. Data are presented as mean ± STDEV; *p ≤ 0.05, (multiple comparison 1-way ANOVA followed by Bonferroni test).
Figure 3.2: Reduced RPC-induced neuroprotection in Nrf2<sup>−/−</sup> mice is not due to altered cerebral blood flow during ischemia.

A) Blood flow measurements during the baseline, ischemic/occlusion, and reperfusion phases of the MCAO injury from the same treatment subjects as in Figure 1. Data represented as a percentage of baseline blood flow, as measured by laser Doppler flowmetry. B) Physiologic variables of WT and Nrf2<sup>−/−</sup> mice used for MCAO experiments.
Figure 3.3: RPC treatment activates Nrf2 in astrocyte cultures.

A) ELISA assay was used to measure Nrf2 DNA binding in rat astrocyte nuclear fractions harvested at 1, 24, or 48 hrs after RPC treatment. B) whole-cell astrocyte lysates were probed for NQO-1 expression following RPC treatment. n=4, *p≤0.05, **p≤0.01.
Figure 3.4: RPC treatment increases mitochondrial uncoupling in Nrf2\(^{-/-}\) and WT mice.

A) RCI of WT and Nrf2\(^{-/-}\) non-synaptic mitochondria following RPC and vehicle treatment. \(n=4\), \(*p \leq 0.05\) (WT vehicle vs. WT RPC); \(^{#}p \leq 0.05\) (WT vehicle vs. Nrf2\(^{-/-}\) vehicle); \(^{\wedge}p \leq 0.05\) (Nrf2\(^{-/-}\) vehicle vs. Nrf2\(^{-/-}\) RPC. B) UCP2 and Actin protein levels from WT and Nrf2\(^{-/-}\) astrocyte cultures treated with RPC and Vehicle. (+) positive control: mouse cortex tissue lysate. C) Quantification of UCP2 proteins levels normalized to Actin. \(n=3\), \(*p \leq 0.05\)
Figure 3.5: RPC increases non-synaptic mitochondrial ROS production and only increases antioxidant expression in WT astrocytes.

A) Complex I and Complex III \( \text{H}_2\text{O}_2 \) production rate in WT or Nrf2\(^{-/-}\) non-synaptic mitochondria. \( n=4-6, \ *p \leq 0.05 \) (WT vehicle vs. WT RPC); \(#p \leq 0.05 \) (Nrf2 vehicle vs. Nrf2\(^{-/-}\) RPC). B) Western blots of astrocyte cultures for MnSOD, NQO-1, and actin (loading control) 48 hrs after RPC or Vehicle treatment. C) Quantification of WT astrocyte NQO-1 levels and D) WT and Nrf2\(^{-/-}\) astrocyte MnSOD protein levels, normalized to Actin protein levels. \( n=3-6, \ *p<0.05, \ **p<0.005. \)
Chapter 4: Ischemic preconditioning treatment of astrocytes transfers ischemic tolerance to neurons

4.1 Summary

Aims: Ischemic preconditioning (IPC) represents a potential therapy against cerebral ischemia. While our group has previously shown IPC to induce neuroprotection through various pathways, the role of astrocytes in supporting IPC-induced neuroprotection has not been extensively studied. Astrocyte-derived lactate has gained attention as a potential soluble mediator through which astrocytes could impart ischemic tolerance to neurons. Therefore, the goal of this study was to determine if i) IPC-treatment of astrocytes alone could transfer ischemic tolerance to neurons; ii) if IPC-treatment of astrocytes increases lactate production; and if iii) exogenous lactate administration to neurons could induce neuroprotection against lethal ischemia in vitro.

Methods: A co-culture system was used and modified from a previous method. This system allows astrocytes and neurons to be separated by a physical barrier, while allowing secreted substances from either cell type to interact with each other. Oxygen-glucose deprivation was used as a model of cerebral ischemia and IPC in cultured rodent astrocytes and neurons. Lactate concentration from astrocyte media was measured using a commercially available calorimetric assay.

Results: Neurons incubated with IPC-treated astrocytes were significantly protected against lethal ischemic injury compared to neurons incubated with sham-treated astrocytes. In addition, IPC-treatment of astrocytes significantly increased lactate
secretion into the extracellular media. Finally, exogenous lactate administration can significantly attenuate cell death in neuronal cultures following exposure to lethal OGD.

**Conclusion**: Our studies defined a modified astrocyte-neuronal co-culture system. Using this model, our results suggest that IPC-treatment of astrocytes alone can transfer ischemic tolerance to neurons. In addition, the ability of IPC to increase lactate production in astrocytes suggest that lactate could represent a neuroprotective agent to protect neurons against lethal ischemic injury.

### 4.2 Background
Cerebrovascular disease, primarily “stroke,” is the fourth leading cause of death in the United States. Many of the individuals who succumb to stroke survive, yet require long-term rehabilitation and often have a poor prognosis. Thus, the development of novel prophylactic therapies that target individuals at high-risk of developing stroke (i.e. patients with hypertension, diabetes, or history of smoking) could lead to improvements in survival and neurological outcomes following such a devastating condition.

One such therapy could be represented by ischemic preconditioning (IPC). IPC is an intrinsic neuroprotective mechanism whereby a brief, sublethal ischemic exposure protects against a subsequent lethal ischemic insult. Our group has previously shown IPC to induce cytoprotection in both astrocytes and neurons, and shown to provide neuroprotection in *in vivo* rodent models of global cerebral ischemia. However, the field of IPC has focused on neuroprotection from a predominantly “neuro-centric” point of view; as a result, the role of astrocytes in mediating neuronal ischemic tolerance has not been well understood.
Astrocytes have been shown to serve many neuroprotective functions. Astrocytes can supply neurons with antioxidants, recycle neurotransmitters, and modulate synaptic transmission\textsuperscript{224} (reviewed in\textsuperscript{225}). Rat retinal ganglion cells cultured in the presence of astrocytes or astrocyte-conditioned media have been shown to have increased neurite outgrowth and increased density of synaptic connections\textsuperscript{226}. These results suggest that astrocytes can profoundly impact neuronal functioning at the synaptic level. As glutamate-excitotoxicity mediates much of the pathogenesis in cerebral ischemia\textsuperscript{227}, astrocytes can perhaps induce neuroprotection by modulating synaptic activity in the face of excess glutamate release. Indeed, glutamate uptake by astrocytes has been suggested as a principle function of these cells, and under basal conditions is thought to be a key mechanism in coupling neuronal activity to astrocyte glucose uptake from the cerebral vasculature\textsuperscript{105}.

While many of these functions have ample support, a more controversial role of astrocytes is the supplying of neurons with bioenergetic substrates, particularly lactate. This theorized function has received considerable attention in recent years, attributed to the astrocyte-neuronal lactate shuttle (ANLS). While controversial, the ANLS theory has focused on the bioenergetic properties of lactate and the compartmentalization of glycolysis and oxidative phosphorylation in the brain\textsuperscript{228, 229}. Lactate has traditionally been relegated as a waste-product of glycolysis. However, lactate has since been shown to have a dynamic role between astrocytes and neurons. The current model of lactate shuttling consists of astrocyte production of lactate through glycolysis or glycogenolysis;
the lactate is then exported via the astrocyte-specific monocarboxylate transporter 1 (MCT1). In addition, astrocytes express an isoform of lactate dehydrogenase (LDH), LDH5, which preferentially converts glycolysis-derived pyruvate into lactate. Lactate can then be exported out of astrocytes and taken up by neurons, which occurs by a different neuronal monocarboxylate transporter, MCT2. Finally, neurons express a different isoform of LDH (LDH1) which facilitates conversion of lactate to pyruvate for subsequent entry into the Krebs cycle and oxidative phosphorylation (reviewed in120).

Independent of bioenergetics and metabolism, however, previous studies have shown that astrocyte-derived lactate can have a profound impact on neuronal synaptic transmission. Glycogenolysis-derived astrocyte lactate can modulate AMPA and NMDA receptor transmission in neurons230, 231. In addition, lactate has been shown to induce long-term memory in the hippocampus in mice through synaptic plasticity changes and induction of LTP15. In these studies, inhibition of lactate transport from astrocytes to neurons by blocking their respective MCT or LDH isoforms inhibited many of these plasticity effects of lactate, further lending credence to the directionality of lactate transport between astrocytes and neurons. While lactate generation can occur even during periods of low glucose and oxygen availability (ischemia), the neuroprotective role of lactate in the context of cerebral ischemia has not been well investigated.

Therefore, our hypothesis was that IPC-treatment of astrocytes can transfer protection to neurons, and that the soluble transport of lactate mediates this protection. We present here a modified astrocyte-neuronal co-culture system that allows us to precondition
astrocytes independent of neurons. IPC-treatment of astrocytes induced protection of neurons against lethal oxygen-glucose deprivation (OGD), an *in vitro* model of cerebral ischemia. In addition, we found that in the early time-points following IPC-treatment, astrocytes can increase lactate production and secretion into the extracellular media. Finally, exogenous lactate administration to neurons for 48 hours induces neuroprotection against lethal OGD. These findings suggest that IPC-treatment of astrocytes can induce ischemic tolerance to neurons through the transfer of soluble lactate. In addition, exogenous administration of lactate may represent a neuroprotective agent to be used in the context of cerebral ischemia.

4.3 **Methods**

**Animal Use**

All animal protocols were approved by the Animal Care and Use Committee of the University of Miami (assurance number: A-3224-01). All experiments were conducted in accordance to ARRIVE guidelines. 16-17 day-pregnant Sprague-Dawley rats were purchased from Charles Rivers Laboratories and housed in a temperature controlled environment with 12 hr light -12 h dark cycle and *ad libitum* food and water.

**Materials**

Minimum Essential Medium (MEM), Hanks Balanced Salt Solution (HBSS), Fetal Bovine Serum (FBS), Propidium iodide, and NucBlue Hoechst 33342 nuclear stain were purchased from Life Technologies (Grand Island, NY). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.
**Preparation of post-natal astrocyte cultures**

Cortex from postnatal Sprague Dawley rat pups (P2-P4) was harvested, followed by digestion with 0.25% trypsin and DNase. Following trituration and filtration through a 70 μM filter, the resulting filtrate was centrifuged at 200 g for 5 minutes to remove trypsin and DNase. The pelleted cells were then resuspended in Astrocyte Media, consisting of MEM base supplemented with 20 mM glucose, 1% GlutaMAX, 1% Penicillin/Streptomycin, and 10% FBS and plated in 100 mm cell culture dishes at a density of 2 hemispheres per plate. Complete media changes were performed every 2-3 days until cultures reached 70% confluency 5-7 days following the initial plating. Astrocyte cultures were then passaged and plating in appropriate culture vessels at a density of 50,000 cells/cm², and allowed to reach full confluency and maintained for an additional 6-7 days. Passages P0-P3 were used for experiments.

**Preparation of neuronal cultures**

Cortical tissue from embryonic pups was isolated and digested with 0.25 % Trypsin and 1% DNase, triturated, and filtered through a 70 μM mesh filter to remove any undigested tissue debris. Neurons were plated in neuronal plating media (MEM supplemented with 5% FBS, 1% Glutamax, and 20 mM Glucose) at a density of 2 hemispheres for each 24 well plate, with each well containing a 12 mm poly-d lysine coated coverslip. Cultures were maintained 10-14 days with neuronal maintenance media (MEM supplemented with 1% Glutamax and 20 mM Glucose) with ½ media exchanges occurring every 3-4 days.
Proliferation of non-neuronal cells was inhibited by the addition of cytosine arabinoside (5 uM final) 72 hours after the initial plating.

**Astrocyte-Neuronal co-culture model**

A modified co-culture system was adapted from a previously described protocol\textsuperscript{188}. Astrocytes were prepared in 100 mm dishes containing paraffin pedestals, and were cultured until fully confluent. In parallel, neuronal cultures were prepared by culturing neurons on poly-D-lysine-coated 12 mm coverslips. Following treatment of astrocyte cultures with a preconditioning paradigm, astrocyte cultures were then placed in neuronal maintenance media. Neuronal coverslips were placed on top of the astrocyte cultures, such that the paraffin dots suspended the neuronal coverslips above the astrocytes without direct cell-to-cell contact, while still allowing both cell types to be bathed in the media. The coverslips were then incubated with the astrocytes for 48 hours. Following this incubation, the neuronal coverslips were removed and placed in a new 24 well culture dish, and were subjected to lethal OGD (3 hrs). 48 hours following lethal OGD, the neuronal coverslips were then assayed for cell death through immunofluorescence with propidium iodide and NucBlue nuclear stain.

**Oxygen Glucose Deprivation**

To mimic sublethal cerebral ischemia, astrocyte cultures were exposed to oxygen and glucose deprivation (OGD) as previously described\textsuperscript{97} for 2 hr. Through empirical testing, 2 hr was determined to be a sublethal duration of OGD that induced the highest degree of protection to astrocytes following a lethal OGD insult (6 hours). 6 hour time point was
chosen as both a significant amount of cell death occurred along with minimal cell detachment. Similarly, for lethal OGD treatment of neurons for *in vitro* neuroprotection studies, neuronal cultures were exposed to 3 hours of lethal OGD. To induce OGD, cells were washed two times with glucose-free HBSS (CaCl$_2$. 2H$_2$O 1.26 mM, KCl 5.37 mM, KH$_2$PO$_4$ 0.44 mM, MgCl$_2$ 0.49 mM, MgSO$_4$·7H$_2$O 0.41 mM, NaCl 136.9 mM, NaHCO$_3$ 4.17 mM, Na$_2$HPO$_4$·7H$_2$O 0.34 mM, sucrose 15 mM, pH 7.4) and exposed to an oxygen-free environment (90% nitrogen, 5% hydrogen, and 5% CO$_2$, 37°C) using a COY anaerobic chamber (COY Laboratory Products Inc, Lake Charter Township, MI). OGD was terminated by placing the cells back into normal aerobic media and returning the cultures to a 5% CO$_2$, 37°C incubator.

**Cell Death Assay**

In addition, neuronal cell death was determined by propidium iodide staining. Following removal from the modified astrocyte co-culture system and 48 hours following exposure to lethal OGD, neuronal coverslips were incubated in culture medium supplemented with 2 μg/ml propidium iodide for 15 minutes before imaging. Micrographs of the neuronal culture propidium iodide staining were captured (1) before preconditioning or sham treatment; (2) 48 hours after lethal ischemia; and (3) 24 hours after *N*-methyl-β-aspartate (NMDA) treatment (500 μmol/l) to determine maximum neuronal cell death. Fluorescence images were obtained using a SPOT CCD camera and were digitized using SPOT advanced software (Sterling Heights, MI, USA). Cell death was quantified as fraction of propidium iodide positive cells (number of propidium iodide-positive cells/
Hoechst 3324-positive cells) for at least 3 fields per treatment group for each independent experiment.

Alternatively, cell death was assessed in neuronal cultures by measuring lactate dehydrogenase release after sham or lethal OGD using a commercially available kit (Roche, Nutley, NJ, USA). Maximum neuronal lactate dehydrogenase release was determined by exposing cultures to 500 μmol/l NMDA for 24 hours. Lactate dehydrogenase was measured at 340 nm absorbance using a microplate reader (Bio-Tek Instrument, Winooski, VT, USA). Values are expressed relative to maximal NDMA-induced neuronal lactate dehydrogenase release.

**Lactate assay**

Lactate was measured from the extracellular media of astrocyte cultures using a colorimetric assay according to manufacturer’s protocol (Eton Biosciences, Lactate Assay Kit I catalogue# 120001100A), which measures lactate concentration at an absorbance of 490 nm. Extracellular media from astrocyte cultures were collected 0, 1, 24, or 48 hours following termination of ischemic preconditioning, sham preconditioning, or 1 μM oligomycin treatment for lactate measurements. Media was collected and centrifuged through a 0.22 um filter to clear the media of cellular debris. Media was then diluted 1:5 and added to the manufacturer’s developing solution. 30 minutes following development, the assay reaction was stopped with 0.5 M acetic acid and lactate concentration was measured at 490 nm. Absorbances were correlated with lactate
concentration based on a standard curve (0.05 – 3.2 mM) that were performed concurrently with the samples to be measured.

**Polarography**
Mitochondrial respiration studies were conducted as previously described\(^96\). In brief, naïve rat astrocytes were trypsinized and resuspended to a concentration of 2\(x10^6\)/mL of complete astrocyte media. The ratio of State III/State IV respiration was measured using a Clark-type oxygen electrode. State III was induced by the addition of astrocyte cells to the polarographic chamber, and basal respiration was allowed to occur for at least 1 minute. To produce State IV respiration, oligomycin (1 uM final) was added to the chamber to inhibit coupled-respiration, as previously described. This ratio represented the respiratory control index (RCI), an established measure of mitochondrial coupling.

**Statistical Analysis**
All data are expressed as mean ± SEM. Statistical analysis between two groups was performed using the unpaired Student’s \(t\)-test. Statistical analysis between more than two groups was performed using a one-way ANOVA with Bonferroni multiple comparison post hoc test. \(P < 0.05\) was considered statistically significant.

4.4  **Results**

4.4.1  **IPC treatment of astrocytes transfers ischemic tolerance to neurons**

For our first experiment, we wanted to investigate the ability of astrocytes to transfer neuroprotection to neurons. For these experiments, we developed a modified co-culture
system (Figure 1) that allows neurons and astrocytes to be physically separated through the use of paraffin pedestals, thus suspending neuronal coverslips above a confluent layer of astrocytes.

From Figure 2, our results indicate that neurons that were incubated with astrocytes treated with IPC were significantly protected from lethal OGD compared to neurons incubated with sham-preconditioned astrocytes, as determined by the fraction of propidium iodide-positive cells. Treatment of neurons with NMDA was performed to ensure that the neurons were mature enough to express NMDA receptors and were susceptible to excitotoxic injury. The use of the co-culture model from Figure 1 and results from Figure 2 suggest that IPC-treatment of astrocytes alone can transfer protection to neurons against lethal ischemic injury through the exchange of soluble mediators.

4.4.2 Lactate production following IPC

While there is a plethora of soluble factors that astrocytes and neurons can freely exchange with each other, previous studies have suggested an important role of lactate in neuronal bioenergetics and neuronal synaptic transmission. Therefore, to determine if lactate could be one of the soluble factors responsible for the transference of protection observed in Figure 2, we next investigated whether IPC-treatment of astrocytes could increase astrocyte lactate production. We measured lactate concentrations in extracellular media 0, 1, 24, and 48 hours following IPC or Sham treatment. Lactate concentration was normalized to total protein content of each sampled well of astrocyte cultures. As a positive control, oligomycin (1 µM final) was added to astrocyte cultures to inhibit
oxidative phosphorylation and to ensure that the assay could detect increases in lactate production \textit{in vitro}.

From the results in Figure 3A, lactate production was significantly decreased in the IPC-treatment group immediately following termination of treatment compared to Sham and oligomycin-treated astrocyte cultures (Figure 3A; IPC: 0.17±0.03; Sham: 1.092±0.08; oligomycin: 2.011±0.1, n = 4, p<0.005). As expected, oligomycin significantly increased lactate production compared to both Sham and IPC-treated cultures (p<0.005). However, at 1 hr post-IPC treatment, IPC-treated astrocytes had a significant increase in lactate production compared with either sham or oligomycin-treated groups (Figure 3B; IPC: 0.98±0.14; Sham: 0.70±0.15; Oligomycin: 0.63±0.08, n = 4, p<0.01). Finally, lactate production in both IPC and Sham-treated astrocyte cultures were not significantly different at the 24 and 48 hour time points (Figure 3C and 3D). However, oligomycin-treated astrocyte cultures significantly increased lactate production at these time points relative to the preconditioned groups. The time course of lactate production is summarized in the Figure 3E. To ensure that the positive control of oligomycin was sufficient to inhibit oxidative phosphorylation in astrocyte cultures, we performed polarographic experiments to measure basal respiration of astrocyte cultures and respiration in the presence of 1 μM oligomycin. From Figure 3F, 1 μM oligomycin was sufficient to inhibit astrocyte respiration, therefore supporting its use as a positive control to inhibit oxidative phosphorylation and increase lactate production.
### 4.4.3 Exogenous administration of lactate to neurons

Based on the previous findings, IPC-treatment of astrocytes was able to transfer ischemic tolerance to neurons, and IPC-treatment of astrocytes increased lactate production. These results suggest that lactate could represent one soluble compound that could mediate astrocyte-transference of ischemic tolerance in the context of IPC. To determine if lactate could represent a neuroprotective compound, we treated neuronal cultures with 5 mM lactate for 48 hours prior to exposure to lethal OGD. 48 hours following termination of lethal OGD, neurons treated with lactate had a significant reduction in cell death compared to control neurons, as determined by LDH release assay (Figure 4). To determine if this effect is specifically due to lactate, we incubated neurons with lactate in the presence of a 100 µM Alpha-cyano-4-hydroxycinnamate (4-CN). Previous studies have indicated that this concentration of 4-CN can preferentially inhibit MCT-2, the monocarboxylate transporter located on neurons and responsible for lactate uptake. When 4-CN was administered with lactate, the protection of neurons against lethal OGD was mitigated and was no longer significantly different compared to untreated neurons. Finally, 4-CN alone had no significant effect on neuronal cell death following lethal OGD. These results suggest that exogenous lactate administration can induce ischemic tolerance to neurons *in vitro.*
4.5 Discussion

The role of astrocytes in mediating IPC-induced neuroprotection in neurons is not well understood, despite astrocytes serving many well-documented functions that are important for normal neuronal functioning. Our current study provides evidence that IPC-treatment of astrocytes can transfer ischemic tolerance to neurons through soluble mediators. In addition, IPC was found to increase lactate production in astrocytes, suggesting that lactate plays a role in mediating this protection. Finally, neuroprotection induced by exogenous lactate administration may indicate that lactate could serve as a neuroprotective agent. Mechanisms that could stimulate lactate production from astrocytes could also serve as means to induce ischemic tolerance in neurons.

Our current co-culture system includes a slight modification from a previous method (Figure 1). This model allows us to manipulate astrocytes independent of neurons, while still allowing the two cell types to freely exchange soluble mediators. Incubating neurons with IPC-treated astrocytes induced protection against a subsequent lethal ischemic insult when compared to neurons incubated with sham-treated astrocytes (Figure 2). Astrocytes have previously been documented to secrete several soluble factors that could modulate neuronal function. Perhaps most well documented are factors belonging to the family of proteins known as glypicans and thrombospondins that modulate synaptogenesis and synaptic transmission in neurons. Indeed, incubation of neurons with astrocytes or astrocyte conditioned media induced significant increases in synaptic connections and neurite outgrowth. As cerebral ischemia consists of an excitotoxicity injury mediated by deranged glutametergic synaptic transmission, modulation of neuronal synapses by
astrocyte function could represent an important therapeutic strategy to combat cerebral ischemic injury.

One possible consequence of exposure of astrocytes to brief periods of ischemia could be an increase in lactate production. In general, most cells adapt to ischemia by increasing glycolysis and lactate production in an effort to sustain metabolism. Astrocytes are more capable of this function than neurons due to their glycogen content and expression of certain isoforms of lactate dehydrogenase (LDH) that favor the conversion of pyruvate to lactate. In addition, astrocytes express MCT isoforms that favor the efflux of lactate, while neurons express the necessary receptors and enzymatic machinery to uptake and convert lactate back to pyruvate. Therefore, we measured the lactate concentration in the extracellular media in astrocyte cultures following IPC. We found that 1 hour following termination of IPC, astrocytes were able to significantly increase lactate production compared to sham or OXPHOS inhibition via oligomycin treatment (Figure 3B). Interestingly, immediately following IPC treatment, there was a significant decrease in lactate production compared to sham or oligomycin-treated groups (Figure 3A).

While we expected brief periods of OGD to increase lactate production based on previous studies, our results appear to contradict the ability of astrocytes to significantly upregulate glycolysis during periods of ischemia. Following replenishment of glucose in IPC-treated astrocyte cultures, astrocytes appeared to recover their ability to produce lactate, as determined by Figure 3B. It is possible that under periods of OGD in our astrocyte cultures, astrocytes are readily producing and consuming lactate, which we
detect as an observed decrease in extracellular media lactate when compared to Sham or oligomycin-treated groups. When glucose is replenished in the media following IPC treatment, astrocytes can perhaps utilize the glucose for metabolic needs, sparing the consumption of lactate for use by neurons and resulting in the observed increased concentration of lactate in the extracellular media after 1 hr of IPC treatment (Figure 3B). At 24 and 48 hours, we did not observe any increase in lactate production compared to sham or IPC, although both were significantly different compared to 0 and 1 hr time points for both treatment groups. This increase in lactate during the first hour following IPC-treatment in astrocytes suggests a temporal profile of how ischemic tolerance is transferred from astrocytes to neurons by lactate. Further investigation of the temporal release of lactate and other soluble mediators between astrocytes and neurons could provide a wealth of information into the mechanisms of IPC-induced transference of ischemic tolerance.

To corroborate these findings, we wished to determine if exogenous lactate administration could induce neuroprotection. Following 48 hours of lactate treatment, neurons were significantly protected against lethal ischemic injury (Figure 4). In addition, these effects appeared to be specific to lactate as this protection was inhibited by the MCT2 blocker 4-CN. These findings suggest that lactate may be an important neuroprotective agent, and could represent an important soluble factor that could be transferred between astrocytes and neurons to induce ischemic tolerance. While the role of lactate in neuronal metabolism and the astrocyte-neuronal lactate shuttle remains
controversial, it is possible that lactate may serve bioenergetic-independent roles to induce neuronal ischemic tolerance through maintenance of synaptic connections.

In conclusion, our current investigation serves to better understand the role of astrocytes in ischemic preconditioning-mediated neuroprotection. The results suggest that lactate may induce neuroprotection and could be a soluble factor that can be transferred between astrocytes and neurons to induce ischemic tolerance. Future therapies that could selectively stimulate lactate production from astrocytes by either OXPHOS inhibition (such as the use of oligomycin) could represent an important neuroprotective therapy.
4.6 Figures

Figure 4.1: Schematic of modified segregated astrocyte-neuronal co-culture system.
Confluent astrocytes were plated in a cell culture dish containing paraffin pedestals. Following exposure to IPC or Sham preconditioning, neurons (10-14 DIV) were placed on top of the pedestals. The paraffin pedestals allowed for physical separation of neurons and astrocytes but free exchange of soluble mediates. Neurons were incubated with IPC- or Sham-preconditioned astrocytes for 48 hours. After 48 hours, coverslips were removed and placed in a new cell culture dish, and were exposed to lethal OGD (3 hr). 48 hours following termination of lethal OGD, cell death was assayed in neuronal cultures with LDH release assay.
Figure 4.2: IPC-treatment of astrocytes can transfer ischemic tolerance to neurons.

Neurons were subjected to lethal OGD following incubation with either IPC- or Sham-treated astrocytes. Panels above are representative images of propidium iodide and Hoechst 3324 staining of neuronal coverslips 48 hours following lethal OGD exposure. Ctrl: naïve neurons. NMDA: 500 µM N-Methyl-D-aspartic acid treatment (maximal cell death). Bar graph indicates quantification of cell death of neuronal cultures based on fraction of propidium iodide-positive cells compared to all Hoechst 3324-labeled cells. Sham: neurons incubated with sham-preconditioned astrocytes. IPC: neurons incubated with IPC-treated astrocytes. PI: propidium iodide. n = 5, *p<0.05 (IPC vs. Sham).
Figure 4.3: IPC increases lactate production in astrocyte cultures.

Lactate concentrations were measured and normalized to protein content in each sampled well. Extracellular lactate concentrations were measured by harvesting astrocyte culture media immediately following termination of preconditioning treatment and 1, 24, 48 hours following treatment (Panels 3A-3D). Oligomycin was used as a positive control to inhibit OXPHOS and promote lactate production through glycolysis. E) Compiled time
course of lactate production from astrocyte cultures from each treatment group. F) Polarographic measurement of astrocyte respiration in the presence and absence of oligomycin (1 uM final). n = 5, ** p<0.005, *p<0.05.
**Figure 4.4: Exogenous lactate administration decreases neuronal cell death following lethal OGD.**

Neurons were treated with 5 mM lactate, the MCT inhibitor 4-CN (100 µM final), or a combination of lactate and 4-CN for 48 hours prior to lethal OGD. 48 hours following lethal OGD, cell death was measured with LDH-release assay and normalized to NMDA-induced LDH release. MCT: monocarboxylate transporter. 4-CN: Alpha-cyano-4-hydroxycinnamate. n = 4-5, *p<0.05.
Chapter 5: Future Directions

The current thesis work suggests a unique role of astrocytes in mediating the neuroprotective effects of ischemic preconditioning by augmenting antioxidant and lactate production. As previous studies have suggested, both of these astrocyte-enriched functions could help stave off cerebral damage following stroke injury.

From Ch. 1 and Ch. 2, the role of Nrf2 has expanded to include novel interactions with astrocyte and non-synaptic brain mitochondria. While the current studies suggest a role of Nrf2 interacting with mitochondria, it would be invaluable to demonstrate the dynamic role of Nrf2 and its ability to translocate between the mitochondrial and nuclear compartments. Mitochondria depend on communication with the nucleus for various mitochondrial processes, including bioenergetics, quality control, and involvement in biochemical reactions. The effect of Nrf2 on mitochondrial compartmentalization in a cell would be an intriguing question to pursue, as previous studies have suggested that mitochondrial transport to perinuclear regions may alter the redox potential of the nucleus to better sense changes in oxidative stress\textsuperscript{237}. In addition, previous studies have suggested that mitochondria can move to areas in astrocytes that contain glutamate transporters\textsuperscript{238, 239}. This mobility of mitochondria has been demonstrated during periods of neuronal activation. These mitochondria may provide a sink for glutamate to enter the TCA cycle, or to help with ATP production to balance out the sodium ion co-transport of these receptors. How Nrf2 effects mitochondrial subcellular localization due to cues from the nucleus could be important in the coordinated adaptation to cerebral ischemia.
In addition, Nrf2 increases glutathione synthesis and has previously been shown to effect excitatory amino acid transporter 3 (EAAT3) and Cxt transporters required for glutamate and cysteine uptake, respectively. Therefore, future work may serve to understand how Nrf2 may be beneficial in excitotoxicity by increasing conversion of glutamate into glutathione. In addition, Nrf2 may help to increase density of glutamate transporters in astrocytes as well.

While Nrf2 may promote antioxidant support to neurons via astrocytes, the role of lactate in mediating bioenergetic support for neurons is also a burgeoning field. While controversial as a source of oxidative substrate for energy production in neurons, it is clear that there are many signaling effects of lactate in the brain, most of which alter synaptic transmission. The ability of lactate to affect long term potentiation may be critical in restoring cognitive function in the brain following cerebral ischemia, and could be a role fulfilled by glyogenolysis in astrocytes. While our studies have suggested that exogenous administration of lactate is neuroprotective in neurons, it remains to be seen whether this is due to an effect on OXPHOS metabolism, or if there is a functional consequence at the level of the pre- or post-synaptic bouton.

The model of a separated co-culture system between astrocytes and neurons may be utilized in the future to determine additional molecules that could be transferred between neurons and astrocytes. An important understanding of astrocyte and neuronal communication is critical in discovering new therapeutic avenues in combating stroke. Furthermore, our system could also incorporate coverslips of other cell types, such as
microglia or oligodendrocytes, to re-create an extracellular milieu that approaches the \textit{in vivo} condition of the brain. Combined with approaches in bioinformatics and high throughout analysis, it is possible that there may be dozens of candidates that are important mediators of transferred-neuroprotection by astrocytes.

In conclusion, the current work sheds light on the transcription Nrf2 and its role in both IPC and RPC-induced neuroprotection. Nrf2 also has novel interaction with mitochondria, including mitochondrial antioxidant expression and supercomplex formation. It remains to be seen how Nrf2 may further affect mitochondria-nuclear communication and coordination of gene expression in response to ischemia. We believe that Nrf2 is an important pathway in astrocytes that holds tremendous clinical potential due to the high functioning capacity of astrocytes in the human CNS. Therapies targeted at astrocytes may induce a broader range of neuroprotection, and could provide several new therapeutic targets.
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