5-2009

Delayed Anesthetic Preconditioning and Metallothioneins I+II: Novel Mediators of Anesthetic-Induced Protection

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DELAYED ANESTHETIC PRECONDITIONING AND METALLOTHIONEINS I+II:

NOVEL MEDIATORS OF ANESTHETIC -INDUCED PROTECTION

A dissertation presented

by

SCOTT EDMANDS

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Molecular and Cellular Biology
DELAYED ANESTHETIC PRECONDITIONING AND METALLOTHIONEINS-I+II: NOVEL MEDIATORS OF ANESTHETIC -INDUCED PROTECTION

A dissertation presented
by
SCOTT EDMANDS

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DEDICATION

To my wife and best friend, Debra, my daughter Allison, my parents, Clay and Judy Edmands.
ACKNOWLEDGEMENTS

I want to thank my advisor and mentor, Adam Hall, for all his support and wisdom over the years, including turning a short meeting at a café in Northampton into quick start on a graduate career. I also want to thank my committee members Chris White-Ziegler, Sallie Smith Schneider and Juan Anguita for their support and experience to help me on my way.

My parents, Clay and Judy, have provided love and support all of my life, even when they didn’t understand what I was doing or why I was doing it. They are amazing in their persistence and in their commitment to all the people they meet. I couldn’t have gotten anywhere without you. Elise, my sister, has always shown me how great life can be. I am constantly amazed by her energy and commitment to the people and causes that are important to her.

Allison, my daughter and my sweetheart, has brought more joy into my life over the two years that she’s been alive than I can ever express. She makes every day brighter, fuller, and more fun.

Last, but not least, Debra my wife and best friend has been my support through this entire process. She’s listened patiently to untold hours of technical and theoretical musings, only occasionally glazing over. She is my partner in crime and the one who I turn to when I need perspective. I can’t wait to see where the journey takes us.
ABSTRACT

DELAYED ANESTHETIC PRECONDITIONING AND METALLOTHIONEINS-I + II:

NOVEL MEDIATORS OF ANESTHETIC -INDUCED PROTECTION.

MAY 2009

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Ischemic injury is a common and debilitating outcome of natural illness and as a complication of commonly performed medical procedures. Whereas naturally occurring ischemic insults are often the result of unpredictable events, such as in the case of stroke or heart attack, the risk of operative and perioperative ischemia is somewhat better characterized in the clinical setting. Given the prevalence and severity of outcomes in ischemic injury, there is significant interest in developing better pharmacological and procedural approaches to improve patient outcomes. One approach that has shown significant promise in the laboratory setting, particularly in the context of planned medical procedures, is the use of delayed anesthetic preconditioning. Delayed anesthetic preconditioning is a phenomenon whereby a prior exposure to clinical concentrations of commonly used inhaled anesthetics, including isoflurane, induces the production of
endogenous protective proteins that are able to provide robust protection against subsequent, potentially toxic, ischemic insults. Although many aspects of delayed anesthetic preconditioning have been previously described, a complete understanding of preconditioning mechanism has yet to emerge.

The studies described in this dissertation aim to further our understanding of molecular mechanisms involved in delayed anesthetic preconditioning. In the first project, I used DNA microarray to identify genes that were differentially expressed in adult rat liver, kidney and heart following a clinically relevant exposure to the inhaled anesthetic isoflurane. By selecting those genes that were differentially expressed in multiple tissues, I was able to identify a small group of interesting genes for further study. In my second study, I chose from our list two related genes, metallothioneins I + II, to analyze for a role in anesthetic-mediated protection. Using a combination of approaches, I was able to establish that metallothioneins I + II play an essential role in delayed anesthetic preconditioning. In the final study of this dissertation I explore a possible role for metallothioneins I + II as sensor molecules, involved in detecting cellular oxidative stress.

Taken together, these three studies represent an important contribution to our understanding of the mechanisms of delayed anesthetic preconditioning and how they might contribute to protecting against ischemic stroke.
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CHAPTER 1

INTRODUCTION

Ischemia Reperfusion injury (IRI) is a common complication in both surgical and trauma patients that leads to high degree of morbidity and mortality in a wide range of organ systems including lung, heart, brain, liver, kidney, and intestine. In neurological tissue, ischemia-producing complications from neurosurgical, vascular and cardiac procedures can induce stroke, seizures, and neuropsychological deficits.

Despite advances in our understanding of what causes it, ischemic stroke remains a serious complication of surgery. For example, following coronary artery bypass grafting, recent studies show an incidence of stroke ranging from 1.6-5.2%, while carotid endarterectomy (CEA) carries a risk of 0.25% to 7%. In the North American CEA trial, 35% of strokes occurred during the procedure while the remainder occurred after the patient left the operating room. Of these, just over half of the delayed events occurred within 24 hours of surgery. Given this substantial rate of surgical complication, there is significant clinical interest in developing both procedures and pharmacological agents that are capable of either protecting or preconditioning tissues against the risk of ischemia-reperfusion induced injury. One approach that has been gaining significant interest for its potential to protect against ischemic injury in a number of tissues is the phenomenon of delayed anesthetic preconditioning (APC). In delayed anesthetic preconditioning, a clinical dose of an inhaled anesthetic is sufficient to set in motion an array of endogenous cellular pathways that are able to defend tissues against potentially toxic ischemia for days following the initial preconditioning treatment. However,
before detailing anesthetic preconditioning and possible mechanisms of protection, it’s useful to have a basic overview of ischemic damage and the pathways that are involved in order to better understand preconditioning.

**Biology of Ischemia-Reperfusion injury**

Although ischemic injury can occur in any tissue, this introduction focuses on ischemic injury in the brain, or ischemic stroke. Ischemic stroke is the result of a temporary or permanent reduction in cerebral blood flow due to blockage of a major cerebral artery from thrombosis (originating locally) or embolism (originating elsewhere). Reduced blood flow and concomitant reduction in glucose and oxygen delivery sets in motion a complex cascade of molecular and cellular events, which, if untreated, ultimately leads to cellular death. In animal models of ischemic injury, such as middle cerebral artery occlusion (MCAO), and in human stroke, the ischemic lesion can typically be divided into two distinct regions (See Figure 1.1). The core of the ischemic lesion is made up of the tissue immediately served by the occluded artery and receives blood flow that is 25% or less of normal flow. Radiating out from the core is a region called the penumbra that, while not neurologically functional, maintains structure as it receives collateral perfusion (< 40% normal) from nearby blood vessels. If blood flow is not restored within a few hours, evidence indicates that tissue in the penumbral region will ultimately die and become part of the core. Injury and death pathways in the core and penumbra differ markedly. In ischemic the core, due to a near complete lack of available energy, cell death proceeds primarily by necrosis while in the penumbra, cells die by a mixture of necrosis and apoptosis with apoptosis becoming more common.
the greater the distance from the core\textsuperscript{10}. In the context of neuroprotection, there appears to be little that can be done to protect against the near complete failure of energy systems in the core, however, there is evidence that targeting the penumbra for protective treatments could have significant long-term benefits for improving patient outcomes\textsuperscript{10}.

Figure 1.2 shows a simplified overview of the molecular and cellular events involved in the ischemic cascade. It is important to note that while the figure depicts a stepwise progression of ischemic injury, in reality many of the depicted events will overlap one another both spatially and temporally\textsuperscript{10}. Lacking energy stores of its own, the brain depends on a steady supply of oxygen and glucose to undergo mitochondrial oxidative-phosphorylation for the production of ATP. Therefore, attenuation of blood flow leads to rapid depletion of energy substrates for mitochondrially produced ATP, particularly oxygen and glucose, triggering a conversion to anaerobic glycolysis. Anaerobic glycolysis, with a resultant increase in cellular lactate, promotes increased reactive oxygen species (ROS) production and inhibition of cellular protein synthesis that may contribute to ischemic injury\textsuperscript{10}. Despite the energetic switch to anaerobic metabolism, ATP levels fall rapidly in the core of the ischemic lesion\textsuperscript{11}. Decreased ATP levels cause ionic gradients normally maintained by Na\textsuperscript{+}/K\textsuperscript{+}- ATPases and Ca\textsuperscript{2+}/H\textsuperscript{+}-ATPase pumps to dissipate, leading to neuronal and glial depolarization\textsuperscript{12,13}. This situation is further compounded by the reversal of Na\textsuperscript{+}/Ca\textsuperscript{2+} transporters\textsuperscript{14}. Depolarization of neuronal and glial membranes triggers voltage dependent calcium channels to open, facilitating an influx of extracellular calcium which, in turn, leads to the release of excitatory neurotransmitters, including glutamate\textsuperscript{13}. Accumulation of extracellular glutamate causes the ionotropic receptors N-methyl-D-aspartic acid
(NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), to open channels for Na\(^+\)/Ca\(^{2+}\) and Na\(^+\) respectively, compounding calcium overload and promoting cellular edema respectively\(^{12}\). Furthermore, release of Ca\(^{2+}\) via an IP\(_3\) –dependent pathway through activation of metabotropic glutamate receptors from organelles including endoplasmic reticulum, mitochondria, synaptic vesicles and Ca\(^{2+}\) binding proteins may further increase intracellular Ca\(^{2+}\) levels\(^{10}\).

Increased intracellular Ca\(^{2+}\) plays an important role in cerebral ischemic injury by triggering the activation of an array of Ca\(^{2+}\) -dependent enzymes including protein kinase C, phospholipase A2, phospholipase C, cyclooxygenase, calcium-dependent nitric oxide synthase, calpain, proteases and endonucleases\(^{10}\). These events in turn result in the production of cytotoxic products including ROS, reactive nitrogen species (RNS) and leukotrienes which, as discussed below, play an important role in ischemic damage\(^{10}\).

ROS are generated by multiple pathways involved in the ischemic cascade and, in turn, contribute significantly to ischemic injury. ROS are produced by inhibition of the oxidative phosphorylation machinery due to ionic imbalances in the mitochondria, as part of enzymatic processes such as the cyclooxygenase-dependent conversion of arachidonic acid to prostanoids, by the degradation of hypoxanthine and by increased cellular lactate\(^{10}\). Furthermore, inflammation can significantly elevate local ROS and RNS levels\(^{15}\). ROS react with and oxidize cellular lipids, proteins, and DNA, leading to dysregulation of cellular processes,
perturbations to ion homeostasis and interruption of normal cell signaling\textsuperscript{12,15,16}. Ultimately, if ROS damage accumulates, it will eventually trigger cellular apoptosis\textsuperscript{17}. At the brain structure level, damage to cellular membranes by ROS contributes to the breakdown of the blood/brain barrier and promotes cerebral edema\textsuperscript{16}.

Rapid \(\text{Ca}^{2+}\) influx along with increased ROS levels rapidly induces the upregulation and activation of a number of pro-inflammatory transcription factors including NF-Kappa B, hypoxia inducible factor 1 (HIF-1) and STAT3\textsuperscript{13}. Activation of these factors, leads to increased production of inflammatory cytokines including TNF-alpha and IL-1beta, and inflammation related enzymes including iNOS and COX-2\textsuperscript{13}. Ischemia also leads to increased expression of vascular adhesion molecules such as ICAM-1 and selectins in local vascular tissue, thereby increasing numbers of neutrophils, macrophages, and monocytes at the site of injury within hours of the original insult. The pathogenic role of immune cells at the site of injury is a subject of debate\textsuperscript{10}. Macrophages and microglia may be beneficial through their roles in tissue remodeling. However, activated phagocytes produce large quantities of ROS and RNS that may further increase the oxidative stress of the tissue and contribute cellular damage\textsuperscript{12,13,16}. The rate and severity of cellular damage are dependent on the degree of blood flow attenuation. In the core, where blood flow in the lowest, these events occur rapidly, however in the penumbra the progression of ischemic injury will play out over the course of days\textsuperscript{13}. 
Depending on the severity of the initial ischemic insult, restoration of blood supply alone may not be sufficient to rescue affected tissues from injury\textsuperscript{18}. Indeed, disruption of normal energy pathways due to dysregulated pathways, ionic imbalances and cellular damage, the return of higher glucose and oxygen levels can lead to large increases in toxic ROS stemming both from inefficient mitochondrial oxidative phosphorylation\textsuperscript{19} and from the energetic switch to the ROS producing NADPH oxidase pathway\textsuperscript{18}. These increases in ROS upon reperfusion lead to further oxidation of cellular proteins, lipids and DNA that can, in turn, trigger cellular apoptotic and necrotic pathways\textsuperscript{13}. The complexity and number of pathways involved in the cellular response to ischemia makes treatment extremely challenging\textsuperscript{10}. In the context of neuroprotective treatments, such as delayed anesthetic preconditioning, the aim is to control the degree of these potentially toxic pathways such that cells are ultimately able to return to normal metabolism following the reinstatement of a normal blood supply.

**Treatment of ischemic stroke**

Experimental animal models of focal ischemia have been developed in recent decades and have provided the basis for most of our understanding of the mechanisms involved in cerebral ischemia. From these models, two main approaches have emerged for the treatment of ischemic stroke: recanalization and neuroprotection\textsuperscript{10}.

Recanalization involves the clearing of the arterial blockage by either surgical or pharmacologic means. Mechanical recanalization, using a mechanical embolectomy device to clear the blockage has shown positive early results, but is not yet widely used for treatment\textsuperscript{20}. Currently the recombinant tissue-type plasminogen activator (rt-PA) is
the only approved pharmacological treatment for acute ischemic stroke when administered within three hours after the onset of symptoms\textsuperscript{21}. While somewhat effective, only a small number (1-8.5\%) of hospitalized patients receive rt-PA treatment due to the short window for treatment, the necessity for computed tomography scan and other exclusions \textsuperscript{10,22}.

Neuroprotection, on the other hand, complements recanalization with the aim of protecting the penumbral tissue and preventing growth of the ischemic core until normal blood flow can be restored\textsuperscript{10}. This approach arises from evidence, noted above, that reperfusion alone is not sufficient to prevent the progression of the ischemic lesion\textsuperscript{10}. Numerous potentially neuroprotective agents that include glutamate release inhibitors, NMDA and AMPA antagonists, free radical scavengers, calcium channel blockers and chelators, and NO antagonists, have been directed at the various harmful steps of the ischemic cascade with the intent of preventing or delaying damage until homeostasis can be restored\textsuperscript{10}. However, though many substances have shown promise \textit{in vitro} studies or in animal models of experimental stroke, the majority of agents have failed in clinical models. Reasons for this are unclear and are likely due to multiple variables including experimental design, small sample sizes and heterogeneity of the clinical population\textsuperscript{10}. It may be too, that the treatment of any one pathway may be insufficient to for cellular protection, especially when so many pathways are affected through the ischemic cascade. In support of this, recent preclinical data suggest that approaches utilizing multi-drug combinations might have greater clinical effectiveness than those targeting individual agents \textsuperscript{23}. Given the overall lack of effective measures against ischemic damage, delayed preconditioning has arisen as a potentially useful therapeutic intervention. A central
benefit of the preconditioning approach is that preconditioning, by its nature, induces endogenous proteins that address multiple important points in the ischemic cascade (See mechanism section below). By improving our understanding of preconditioning mechanisms, there is promise that therapies that are able to harness the power of these pathways will lead to improved patient outcomes.

**Introduction to general anesthetic agents**

General anesthetics comprise a wide range of structurally diverse molecules that are broadly categorized as volatile anesthetics, anesthetic gases, alcohols and intravenous agents (see Figure 1.3 for structures of these agents). Most general anesthetics will effectively produce unconsciousness/hypnosis, immobility, and amnesia along with varying degrees of analgesia, muscle relaxation and depression of autonomic reflexes. Because general anesthetics vary in their ability to confer all of these functions, modern anesthesia typically supplements general anesthetics with analgesics and neuromuscular blockers. General anesthetic agents are thought to exert their anesthetic properties largely through positive or negative allosteric modulation of agonist activity at pre- and post-synaptic ligand-gated ion channels, though voltage-gated ion channels and g-protein coupled receptors may also play a role. In simpler form, anesthetics induce unconsciousness by blocking the propagation of neuronal action potentials either by inhibiting pre-synaptic excitatory transmitter release or by strengthening post-synaptic inhibitory response and thus blocking the excitation of the post-synaptic neuron. While all classes of general anesthetics are considered to be effective at inducing anesthesia, currently only volatile anesthetics, especially the halogenated ethers including isoflurane,
halothane, sevoflurane and desflurane, are thought to induce delayed anesthetic preconditioning. The mechanisms by which these anesthetics induce delayed preconditioning are likely distinct from those by which they induce unconsciousness and will be discussed below.

**Background of Preconditioning**

Preconditioning is an important biological phenomenon where exposure to a sub-lethal stressor including heat stress, exercise, global or focal ischemia, inflammation, or exposure to an inhaled general anesthetic, can induce endogenous pathways that are able to protect against a subsequent, more severe ischemic insult. Preconditioning typically manifests in at least two distinct temporal windows. Early protection, also called “classical preconditioning” begins almost immediately following the preconditioning stimulus and dissipates after 1-2 hours. In this type of preconditioning, protection is derived primarily from post-translational modifications to existing proteins. Many of the pathways in classical preconditioning appear to converge on mitochondrial targets and include activation of phosphoinositide-3-kinase, protein kinase C, endothelial nitric oxide synthase, glycogen synthase kinase 3beta, and p38 mitogen-activated protein kinases. Activation of these signaling molecules leads to changes to key mitochondrial proteins resulting in altered metabolism and reduction in cellular death. By contrast, delayed preconditioning appears to rely on *de novo* protein synthesis in order for protection to emerge. As a result, protection begins later, 12-24 hrs following the preconditioning stimulus, and can provide protection lasting several days. The remainder of this dissertation will concern itself with delayed preconditioning.
The idea that endogenous pathways could be harnessed in order to protect against hypoxia and ischemia dates back more than six decades. Noble, in 1943, first proposed that brief bouts of global ischemia might protect the entire organism and improve brain function during more extreme hypoxia. Janoff in 1964, examining changes to rabbit lysosomal proteins following shock, coined the term “preconditioning” to denote sublethal stimulation that leads to later protection. Two decades later, in the first reported clinical test of classical, or early ischemic preconditioning (IPC), Murry, in 1986, reported that brief, non-injuring bouts of ischemia protected canine heart against more protracted, potentially lethal ischemia. Finally, in the first report of delayed IPC, Kitagawa in 1990, demonstrated that short periods of global ischemia in gerbil were protective against more potent ischemia 1-2 days following the preconditioning regimen.

While these early reports of preconditioning all used ischemia as the preconditioning agent, Kersten et al., in 1997, demonstrated anesthetic-induced classical preconditioning against ischemia-reperfusion injury in canine heart using the inhaled anesthetic isoflurane. Only very recently, in 2004, did Tanaka demonstrate that an inhaled anesthetic (again isoflurane) could induce delayed protection against the acute effects of ischemia in rabbit heart in a manner similar to that reported in regards to delayed ischemic preconditioning. The work of Kersten and Tanaka has been supported and expanded upon by numerous studies in both animal models and through clinical studies. Although numerous studies showed anesthetic-induced protection against the acute effects of ischemia, ie. the tissue damage immediately following the ischemic insult, there remained questions as to the long-term benefits of delayed
preconditioning both in terms of tissue health and in terms of functional outcomes \(^6\). This question was recently addressed in a study where rats preconditioned with isoflurane and subjected to middle cerebral artery occlusion showed better histological and neurological outcomes at one month compared to non-preconditioned controls \(^39\), thereby confirming that delayed preconditioning can improve long-term as well as acute outcomes in an animal model.

Since Tanaka’s first report of isoflurane-induced delayed preconditioning in rabbit cardiac tissue, inhaled anesthetics have also been shown to induce endogenous protection in a range of other tissues including brain \(^40\), spinal cord \(^41\), liver \(^42\), skeletal muscle \(^43\), and immune cells \(^44\). In addition to supporting the hypothesis that inhaled anesthetics can induce protection, these studies support Noble’s 1943 hypothesis that anesthetic preconditioning (although he envisioned hypoxia as the preconditioning agent) might be able protect the whole of the organism against ischemic injury. Taking an even broader view, a very interesting characteristic of delayed anesthetic preconditioning is that it appears to be conserved in a wide range of organisms including rabbit \(^33\), rat \(^40\), mouse \(^45\), gerbil \(^36\), and human \(^44\). Isoflurane has even recently been reported to effectively precondition the invertebrate *Caenorhabditis elegans* (*C. elegans*) against hypoxic injury, suggesting a fundamentally conserved cellular response to certain types of stress— a property that we exploited to the inform data analysis in our first study (See Chapter 2; Methods) \(^46\).
Current understanding of mechanisms involved in delayed anesthetic preconditioning.

The mechanisms by which inhaled anesthetics induce anesthesia are likely distinct from those that induce preconditioning. Although the mechanisms of anesthetic induction of preconditioning are far from clear, they almost certainly rely on a transient increase in reactive oxygen and nitrogen species \(^{47}\). Recent studies suggest that inhaled anesthetics may initiate APC through ROS production by inhibiting the mitochondrial electron transport chain at complex I, III or \(V^{26,27,48}\), leading to an increase in cellular levels of ROS including the oxygen radical, superoxide \((\text{O}_2)\)^{26,49}. The RNS nitric oxide, produced by endothelial nitric oxide synthase and/or inducible nitric oxide synthase, is also widely reported to be involved in the induction of delayed anesthetic preconditioning \(^{47,50}\). Though the specifics are lacking, ROS and RNS are thought to act as signaling molecules that trigger the production of other proteins involved in cellular protection, eg., superoxide dismutase, catalase, and hexokinase-2, \(^{19,47}\).

As described above, ischemic injury proceeds via a complex pathway involving a number of molecular players and pathways. Interestingly, in addition to NO and \(\text{O}_2\) described above, many of these molecular players and pathways involved in the ischemic cascade are the same as those described in relation to delayed anesthetic preconditioning (Figure 1.4) \(^{19}\). These include: transcription factors (STAT3 \(^{51}\), NFkB \(^{50}\)), inflammatory mediators (COX-2 \(^{37}\), iNOS, \(^{50}\), eNOS \(^{52}\)), Antioxidants (Mn-SOD \(^{53}\)), ion channels, and other cellular enzymes (12-lypoxgenase \(^{54}\),) Kinases (PI3Kinase \(^{55}\), mediators of apoptosis (Apaf1 \(^{46}\), and mitochondrial K(ATP) channel proteins (Vdac \(^{56}\), and \(K^+\)-ATP channels \(^{57,58}\)). Although some of the details of delayed preconditioning have been
described, our understanding of how these molecules interact with one another to confer protection is as yet incomplete. A better understanding of the molecular players and the interactions that they have with one another will be essential for us to understand how anesthetic-induced protective measures work and how they might be better utilized for clinical protection against ischemia.

Finally IPC and APC appear to protect via similar mechanisms. Indeed a recent survey of mechanisms involved in delayed preconditioning suggest that there is significant similarity, if not identity in the pathways ascribed to preconditioning, regardless of the source of preconditioning stimulus. Though both APC and IPC have been shown to protect in the clinical setting, APC has been gaining interest due to the ease with which it can be administered and to the increased safety of APC over IPC. For this reason, we chose to focus our efforts on the mechanisms of delayed anesthetic preconditioning.

In the chapters that follow, we describe three studies aimed at furthering our understanding of the phenomena of delayed anesthetic preconditioning. In Chapter 1, we describe the use of DNA microarray to identify previously unidentified anesthetic-regulated genes with the potential for involvement in anesthetic preconditioning. In the second chapter, we selected metallothioneins I + II, two of the genes identified in Chapter 1, and assessed their involvement in delayed anesthetic preconditioning. Finally, in Chapter 3, we examine how metallothioneins I + II fit in to what is already known about the mechanism of delayed preconditioning. Specifically, we ask if metallothioneins might act as sensors/transducers of oxidative stress during preconditioning. Taken
together, these three studies represent an important contribution to our understanding of the mechanisms of delayed anesthetic preconditioning.
Figure 1.1. Cartoon of ischemic lesion. The core of the ischemic lesion (light grey) is made up of the area immediately served by the occluded artery and is comprised of dead or dying cells. Radiating out from the core is the penumbra which is served by collateral blood flow from nearby blood vessels. Penumbral tissue, because of it’s potential to survive if blood flow is restored, is the target of neuroprotective approaches to ischemic injury. Figure adapted from: Acute Ischemic Stroke: New Concepts of Care © 1998-1999 Genentech Inc.
Figure 1.2. The ischemic cascade. A.) Normal cell: With no blockages, energy substrates oxygen and glucose are delivered in the amounts needed for normal cellular function. This allows ATP requirements to be met and energy requiring processes such as maintenance of ion gradients continue normally. B.) Shortly following arterial occlusion, glucose and oxygen levels drop, leading to rapidly diminished ATP levels. Low ATP levels causes the failure of Na⁺/K⁺-ATPases and the dissipation of membrane potential. C.) Drop in membrane potential triggers the opening of voltage dependent Ca²⁺ channels leading to massive influx of extracellular Ca²⁺. This in turn causes glutamate to be exported where it accumulates in the extracellular space. There it interacts with NMDA and AMPA to promote the entry of even more Ca²⁺ and Na⁺ into the cell. Increased Ca²⁺ can inhibit mitochondrial respiration further, leading to increased ROS. Ca²⁺ will also activates a variety of cellular enzymes that can lead to cellular damage. Finally, Ca²⁺ along with increased ROS and RNS activate transcription factors that code for greater numbers of the enzymes already described as well as inflammatory cytokines and the upregulation of vascular adhesion molecules that will facilitate the arrival of activated phagocytes to the site of the injury. D.) Within hours of the initial injury, activated phagocytes and microglia arrive to the site of injury where they produce large quantities of reactive oxygen and nitrogen species. They may also play a protective role by assisting in tissue remodeling and repair.
Figure 1.3. Chemical structures of selected general anesthetics. In the context of delayed preconditioning, the halogenated ethers including halothane, enflurane, sevoflurane and isoflurane have thus far proven most effective. From Krasowski and Harrison 1999⁴."
Figure 1.4. Reported molecular players in delayed anesthetic preconditioning.
Previously reported molecular participants are overlayed on figure of the ischemic cascade. Red stars indicate molecules or pathways that have been reported to be involved both in ischemic injury and in preconditioning-mediated protection from ischemia. Anesthetic preconditioning has been reported to be mediated by: 1.) Modulation of mitochondrial $K^+\text{-ATP}$ channels\textsuperscript{58}. 2.) Scavenging of ROS\textsuperscript{47,61}. 3.) Regulation through STAT3\textsuperscript{51}. 4.) Induction of NF-kappa B\textsuperscript{50,62}. 5.) Induction of HIF-1\textsuperscript{63}. 6.) Increases in COX-2 activity\textsuperscript{37}. 7.) Modulation of phospholipases\textsuperscript{64}. 8.) Increases in production of nitric oxide by endothelial and/or inducible NOS\textsuperscript{50,52}. 9.) Modulation of TNF-alpha and IL-1beta levels\textsuperscript{65}. 10.) Modulation of ICAM-1 and selectins expression\textsuperscript{66}. 11.) Modulation of inflammation\textsuperscript{67}. 
CHAPTER 2
IDENTIFICATION OF GENES DIFFERENTIALLY REGULATED IN RAT LIVER KIDNEY AND HEART FOLLOWING ISOFLURANE EXPOSURE

Introduction

For our initial study, we employed DNA microarray to identify novel anesthetic-regulated genes following a clinical exposure to the inhalational anesthetic agent, isoflurane. As a focus for our search we considered recently reported observations of ischemic injury and of anesthetic preconditioning to inform our experimental design and our data analysis. Firstly, ischemic injury proceeds via a complex cascade of molecular events involving a number of ontological pathways such as the disruption of energy pathways, modulation of cell death pathways, dissipation of ion gradients and enhancement inflammatory processes\(^\text{11}\). Furthermore, studies have shown that genes involved in delayed APC target a number of different ischemia-related pathways (including iNOS\(^\text{40}\), MT I + II\(^\text{45}\), COX-2\(^\text{37}\), and mitochondrial/sarcolemmal K\(^+\)/ATP channels\(^\text{58}\)). Many studies have considered individual molecular and cellular pathways involved in protection against ischemia, however given the complexity of ischemic injury, we hypothesize that there are additional protective elements yet to be revealed.

Furthermore, we noted that the protection provided by anesthetic preconditioning is a widely reported phenomenon and has been described in multiple organisms (mouse\(^\text{45}\), rat\(^\text{40}\), rabbit\(^\text{37}\), and human\(^\text{38}\)) and tissues (heart\(^\text{37}\), brain\(^\text{40}\), skeletal muscle\(^\text{43}\), kidney\(^\text{37}\)).
spinal chord\textsuperscript{41}). The concept that preconditioning may be a basic response to environmental stress is supported by a recent study by Jia and Crowder, describing isoflurane and halothane preconditioning in \textit{C. elegans}\textsuperscript{2,46}. Thus, we hypothesized that by selecting genes differentially regulated in multiple tissues, we might identify genes involved in a general mechanism for preconditioning.

**Methods**

**Animal Care**

Male Sprague-Dawley rats were acquired from Jackson Laboratories (250 and 300 grams; Bar Harbor, ME) and allowed to acclimatize for two weeks prior to experiments. Animals were maintained under a standard 12/12 light/dark cycle at room temperature and given access to food and water \textit{ad libitum}. Animals were housed and all experiments carried out with protocols approved by Smith College Institutional Animal Care and Use Committee (Northampton, MA, USA) according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

**Arrays**

Rat specific DNA microarrays were obtained from the Rutgers Neuronal Gene expression Lab (NGEL; Piscataway, NJ). Briefly, each array was constructed from a collection of 4967 probes representing approximately 4900 genes printed onto a poly-L-lysine slide. Probes were 65-70 nucleotides in length and were designed to optimize melting temperature and to minimize homology between probes. Further information about these arrays can be found at the web site http://www.ngelab.org.
Isoflurane Exposure and Tissue Collection

Animals receiving isoflurane treatment were placed in an induction chamber and induced with 3% isoflurane/97% medical grade air (vol/vol) until loss of righting reflex (2-3 minutes). Anesthesia was then maintained at 2% isoflurane/98% air ~1.5 MAC (minimum alveolar concentration) for the remainder of the 90 minute exposure period. Body temperature was maintained at 37°C throughout the treatment using heated gel packs and oxygen levels were monitored using a model V3304 pulse oximeter (Surgivet; Waukesha, WI). Animals were observed to maintain > 95% oxygen saturation at all times. Upon completion of the anesthetic exposure, test animals were immediately sacrificed by decapitation. Samples from liver, kidney cortex and heart ventricle were collected and homogenized with a Polytron homogenizer (Brinkmann; Westbury, NY) in ice cold Trizol (Invitrogen; Carlsbad, CA). Because stress has been demonstrated to influence gene expression 69, control animals were maintained in their cages until the time of sacrifice. Each control animal was handled gently and then rapidly decapitated. Tissue was processed in the same manner as test animals. Tissue/Trizol homogenates were stored at –20°C until further processing. All animals were sacrificed during the same daily 5hr window in order to diminish confounding factors from diurnal variations in gene expression 70.

RNA Preparation

Total RNA was isolated from Trizol/tissue homogenate by adding chloroform and performing a phase extraction. The aqueous phase was mixed with ethanol 1:3 v/v and added to an Rneasy column (Qiagen: Valencia, CA). Manufacturer instructions were
followed for column washing and elution of RNA. Purified RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies; Rockland, DE). All samples yielded $A_{260}/A_{280}$ ratios between 1.9 and 2.1. Isolated RNA was stored at −80°C until further use. The same RNA preparations were used for both microarray experiments and subsequent QRT-PCR verification of selected genes.

**Experimental Design**

For each tissue, RNA from an isoflurane-treated animal was competitively hybridized against control RNA from a pool comprised of equal amounts of RNA from 8 control individuals. For each tissue, this experiment was performed twice using the same labeling schema, and a third time reversing the labeling schema as a dye-flip control to ensure no dye-specific artifacts. These three experiments were repeated for each of 3 experimental animals, for a total of 9 arrays per tissue.

**Target Labeling and Hybridization**

Microarray targets were prepared using the Genisphere 350 dendrimer labeling system according to manufacturer instructions (Genisphere, Inc.; Hatfield, PA). Briefly, 2.5µg of isoflurane-treated and pooled control RNA were reverse-transcribed to cDNA in separate tubes using Superscript II (Invitrogen), and oligo d(T)$_{18}$ primers containing unique, label-specific capture sequences. Samples were then alkaline hydrolyzed to destroy residual RNA. Control and test tagged cDNAs were mixed, applied to arrays and sealed in watertight chambers (GeneMachines; Ann Arbor, MI), and allowed to hybridize for 48-72 hrs in a waterbath at 55°C. Arrays were washed one time
for 10 min (2X SSC, 55°C), followed by two washes for 10 min (0.2X SSC, room temperature). Arrays were then spin dried prior to labeling. Hybridized arrays were labeled with fluorescent-labeled dendrimer and incubated 2 hrs at 61°C. Labeled arrays were washed and dried as described above and scanned using an Axon GenePix 4100 scanner and GenePix 4.0 software (Axon Instruments; Union City, CA).

**Data Analysis**

Scanned array images were processed using Genepix 4.0 (Axon Instruments). Spots were eliminated if the signal to noise ratio of the spot fell below 2.0. Statistical analysis of each tissue group was performed using G-Processor (version 3.1, Zhong Guan (http://bioinformatics.med.yale.edu). This package performs a Lowess normalization and yields a differential expression ratio along with a Student’s *t*-test *p*-value for each probe. For each tissue, the gene list was screened for statistical significance in Excel (version 11.5.3 for MacIntosh; Microsoft, Redmond, WA) using the following criteria: for each gene selected, *p*-value ≤ 0.05, spot data were valid for ≥ 7 of the 9 arrays and level of differential expression was ≥ 1.2 compared to control. The final list of “interesting genes” was compiled by choosing genes that were differentially expressed in more than one tissue and whose expression pattern (up or down) was consistent in the different tissues.
QRT-PCR

In order to verify results from our microarray, three of the highly regulated genes from the liver array were selected for verification by quantitative reverse-transcriptase polymerase chain reaction (QRT-PCR). See Table 1 for primer and probe sequences. All primer/probe sets were acquired from Integrated DNA technologies (IDT Inc., Coralville, IA). Each QRT-PCR reaction was carried out in a single tube on an Applied Biosystems 7700 “Taqman” instrument (Applied Biosystems; Foster City, CA). The QRT-PCR reaction mix was comprised of 1X PCR buffer (Invitrogen, Carlsbad, CA), 6mM MgCl₂, 0.67 mM each dinucleotide, 6.75 pmols each of forward and reverse primers, 100 nM FAM/TAMRA probe, 1.5 units Superase-in (Ambion; Austin, TX), 15 units Superscript II (Invitrogen), 0.75 units Platinumum Taq Polymerase (Invitrogen) to a final volume of 15µl. Each reaction was run one cycle of 30’ at 42˚, 1 cycle of 3’ at 95˚ and 40 cycles of 1’ at 60˚ and 20” at 95˚. Universal mouse RNA (Stratagene, La Jolla CA) was used to create a standard curve for each gene of interest and for the normalization gene. Gene reactions were run in triplicate, averaged and normalized to rat protein tyrosine phosphatase, receptor type C, a gene that was chosen because it showed no differential regulation on any of the tissue arrays.

Results

Based on our selection criteria, all of the tissues tested showed differential gene expression in response to a 90 min dose of 2.0% isoflurane. Liver showed the greatest number of differentially regulated genes in response to isoflurane treatment with 725 of 4900 (~15%) genes showing either up or down differential regulation ≥ than 1.2 fold as
compared to the pooled control. Kidney had fewer with 214 (~4%) differentially regulated genes and heart had the least with 137 (~3%). The full list of differentially regulated genes for each tissue is available in Supplementary Tables 1-3.

Of the genes described above, 34 of the 4937 genes on the arrays were differentially regulated in the same direction in at least 2 tissues (see Table 2 for gene descriptions). Of these, 19 were up-regulated and 15 were down-regulated. Figure 2.1 shows a Venn diagram of the distribution of the 34 co-regulated genes. From the genes described in Table 2, a number of broad ontologies were represented including: apoptosis, necrosis and survival signaling, regulation of energetic pathways, modulation of cellular redox, maintenance of cellular ion gradients, regulation of inflammatory response, and regulation of vesicular transport.

We selected 3 of the genes that were significantly regulated on the liver arrays and assessed their expression levels by QRT-PCR using the same RNA as in the array experiments. As shown in Figure 2.2, there was good agreement between microarray and QRT-PCR differential expression levels, suggesting that array values reflect a reasonable approximation of differential expression levels under the conditions we used.

**Discussion**

Ischemic injury plays a central role in the pathology of a range of human illnesses from stroke and cardiac arrest to tissue transplant or complications from childbirth. In the clinical setting the risk of perioperative stroke following surgical procedures present a small but significant risk. In situations where there is a known risk of ischemic damage, such as during and after certain surgical procedures or organ transplant, one
A treatment option that has shown promise for improved clinical outcomes is the use of delayed anesthetic preconditioning. While an intense focus on anesthetic preconditioning has illuminated a number of the mechanisms involved in providing protection, a complete picture has yet to develop. We therefore employed a unique approach to identify novel genes that may be involved in common pathways of anesthetic preconditioning. In the current study, we used a novel approach to analyze microarray data from three rat tissues that prioritized genes differentially regulated in more than a single tissue. While this approach might prove less effective for the identification of tissue or process-specific pathways, it enabled us to focus on genes potentially involved in common pathways. Using these parameters, we identified 34 genes that were differentially regulated in multiple tissues following a clinical exposure to the volatile anesthetic isoflurane. Literature investigations of the genes contained on our list revealed a number of potential targets for further investigation in the context of anesthetic preconditioning.

Seven of the genes we identified (Vdac2, BAD, Pld1, Slc25a3, Atp1a1, MT-I, MT-II) are broadly involved in apoptosis, necrosis and survival signaling. Voltage dependent anion channel-2 (Vdac2) is a mitochondrial outer membrane protein. It is a component of the mitochondrial permeability transition pore that participates in transportation of metabolites and helps regulate cellular progression towards apoptosis. It is involved in the global regulation of mitochondria under stress, and has been implicated in the regulation of both apoptotic and necrotic pathways, both of which are involved in ischemic injury. Vdac2 has also been suggested to participate in APC. Vdac2 activity is regulated, at least in part, by another in our gene list, bcl-2 associated death agonist (BAD). BAD has been found to be modulated during early ischemic and
anesthetic preconditioning, although to our knowledge, differential regulation of BAD has not been associated with anesthetic treatment (others have found BAD to be down-regulated in response to oxidative stress).

Phospholipase D1, is a pleotropic protein whose cleavage by caspases has been reported to promote apoptosis via a p53-dependent pathway. Its activity during ischemia-reperfusion has also been tied to signaling for necrotic death. Solute carrier family 25, member 3 (Slc25a3) is another pleotropic protein and has been reported to be a regulator of mitochondrial cytochrome-c release (a protein important for caspase-dependent apoptosis). ATPase, Na\(^+\)/K\(^+\) transporting alpha 1 polypeptide (Atpa1) is the catalytic subunit of a membrane bound Na\(^+\)/K\(^+\) transporter ATPase. The n-terminus of this subunit has recently been shown to interact with inositol 1, 4, 5 triphosphate receptor to trigger Ca\(^{2+}\)-mediated survival signaling. MT I + II have been shown to be protective against apoptotic and necrotic death in a number of tissues. This protection may arise from roles in reducing oxidative stress and by triggering survival pathways that can protect against apoptosis and necrosis.

Disruption of Ca\(^{2+}\), Na\(^+\), and K\(^+\) ion gradients is an important step in the progression of ischemia-reperfusion injury. Two of the genes described above (Pld1 and Atpa1) have described roles in the regulation of cellular Ca\(^{2+}\) and Na\(^+\)/K\(^+\), ion gradients respectively. Pld1 is a membrane protein that catalyzes the hydrolysis of phosphatidyl choline to for phosphatidic acid. It has been shown to interact with protein kinase C (PKC) and extracellular responsive kinase (ERK) and is involved in sarcolemmal Ca\(^{2+}\) control in heart and skeletal muscle. Atpa1 is an integral membrane protein that is involved in establishing and maintaining Na\(^+\) and K\(^+\) gradients across the
plasma membrane and therefore its regulation may play a role in maintaining ionic balance in ischemic tissue\textsuperscript{81}.

Inflammatory response plays a significant role in later stages of ischemic injury and can greatly influence survival of penumbral tissue. Four of the genes we identified, interferon regulatory factor 1 (Irf1), basigin (bsg) and metallothioneins I + II have been reported to participate in inflammatory response\textsuperscript{86,87}. Irf1 regulates the transcription of the inflammatory cytokines interferons alpha and beta and acts as a transcriptional regulator of genes controlled by these proteins\textsuperscript{13}. Irf1 is also a transcription factor for iNOS, which has been implicated in delayed APC\textsuperscript{40,88}. Knockout of Irf1 was recently shown to improve outcomes in a mouse model of ischemic stroke\textsuperscript{86,89}. Basigin, also known as extracellular matrix metalloproteinase inducer (EMMPRIN), neurothel in and cluster of differentiation 147 (CD147), is a membrane bound protein of the immunoglobulin superfamily. Through an interaction with cyclophilin A (CypA), basigin has been shown to be an important regulator of neutrophil infiltration in a mouse model of lung injury\textsuperscript{90}. Blocking the interaction of basigin and CypA lead to a 50% decreased neutrophil infiltration at the site of injury and reduced tissue pathology\textsuperscript{90}. Finally, MT-I + II have also been shown to modulate inflammatory response in rat brain. In the rodent model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), intra-peritoneal administration of MT-I + II was shown to reduce levels of the proinflammatory cytokines TNF-alpha and IL-6\textsuperscript{91} while metallothionein knockout was associated with increased levels of proinflammatory cytokines IL-1, IL-3, IL-6, IL-12, TNF-alpha, lymphotoxin-alpha, macrophage activating factor and ICAM-1\textsuperscript{85}.

In addition to its role in modulating inflammatory response, basigin has other
reported roles that could potentially contribute to either increased ischemic damage or to protection\textsuperscript{92}. In its role as an inducer of matrix metalloproteinases, it is thought to play a role in tissue remodeling following ischemic damage in rat heart\textsuperscript{93} and brain\textsuperscript{94} and has been associated with negative outcomes. Upregulation of basigin and the associated lactic acid transporters MCT-1 and MCT-4 have been reported to protect against lactic acid damage in neuronal and cardiac tissue during ischemia\textsuperscript{95,96}. Finally, in addition to regulation of neutrophil infiltration, basigin/CypA complexes have been reported to protect neurons from ischemic and oxidative stress\textsuperscript{97}. Interestingly, the basigin/CypA interaction has been found to activate ERK1/2, p38MAPK, SAPK, and AKT: proteins that are widely accepted to play a role in early preconditioning\textsuperscript{92,31}.

Disruption of energy pathways is a core part of the pathology of ischemic injury\textsuperscript{11}. Four of the co-regulated genes on our arrays (Vdac2, Prkab1, Slc25a3, and G6pc) have been shown to participate in the monitoring and regulation of energetic pathways. Vdac2, in addition to its role in apoptosis, helps to regulate the ingress and egress of small metabolites, including ATP and ADP from the mitochondrial space\textsuperscript{31}. Protein kinase, AMP-activated protein (Prkab1) has been shown to be involved in nutrient sensing in conjunction with Akt and mTOR and is regulated by p53 in a stress-dependent manner\textsuperscript{98}. Slc25a3 is a mitochondrial transport protein that is involved in the transport of inorganic phosphates into the mitochondrial space\textsuperscript{99} and is therefore directly involved in regulating mitochondrial ATP production. Glucose-6-phosphatase is an endoplasmic reticulum protein that is involved in conversion of D-glucose-6-phosphate to D-glucose and inorganic phosphate in liver and kidney cortex\textsuperscript{100}.
In conclusion we identified a number of genes that were differentially expressed in rat liver kidney and heart following a clinical dose of isoflurane. Consistent with our prediction, there were a number of genes that were differentially regulated in a number of tissues. In order to assess functional roles for these molecules in the context of ischemic injury, further functional assays will have to be performed. For our second study, we selected two of the genes from our list, metallothioneins I + II, for further investigation into their role in delayed anesthetic preconditioning.
Table 2.1. Primer and probe sets for QRT-PCR confirmation of selected DNA microarray data.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>GenBank ID</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>FAM/TAMRA Probe</th>
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</thead>
<tbody>
<tr>
<td>Ccnd1</td>
<td>CyclinD1</td>
<td>D14014</td>
<td>GGGTCTGCGAGCCATGCT</td>
<td>CCGCATGGATGGCACAAAT</td>
<td>AGACCTGCGGCCCTCCGTTT</td>
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<tr>
<td>Pdx5</td>
<td>Peroxiredoxin 5</td>
<td>AF110732</td>
<td>GCTCCGTCATCGCTACTCTTT</td>
<td>ACCCGGCGACTGCTGAA</td>
<td>AGCAAGGCCGAAAGGAGCAGTTG</td>
</tr>
<tr>
<td>Mt2</td>
<td>Metallothionein 2</td>
<td>NM_008630</td>
<td>GCAAATGCACCTCCTGCAA</td>
<td>ACAAGCCTGGGCACATT</td>
<td>AAAGCTGTGCTCCTGCTGCC</td>
</tr>
<tr>
<td>Ptprc</td>
<td>Protein tyrosine phosphatase, receptor type, C</td>
<td>M10072</td>
<td>ACCTACATTGGAATGGATGCCATGCT</td>
<td>CCTCCACCTGCACCACAGACA</td>
<td>GAAGCAAGGCGAAAGTGATGTCTATGG</td>
</tr>
</tbody>
</table>

All sequences shown are 5'3'. Probes sequences were modified with a 5' 6-carboxy fluorescein and at the 3' end with a 6-carboxy-tetramethyl rhodamine. Primers and probes were designed in Primer express 1.0 (Applied Biosystems Inc.,)
Table 2.2. Genes differentially regulated in rat liver, kidney and heart following a clinically relevant isoflurane exposure. Selected genes had to be present on at least 7 of 9 arrays, found to have statistically significant (P ≤ 0.05) differential regulation compared to a pooled control in at least two separate tissues, and be regulated at least 1.2 either up or down in each of those tissues.

<table>
<thead>
<tr>
<th>Ontology</th>
<th>Full Name</th>
<th>Code</th>
<th>GenBank accession</th>
<th>Potential roles in protection</th>
<th>Liver Level</th>
<th>Kidney Level</th>
<th>Heart Level</th>
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</thead>
<tbody>
<tr>
<td>Apoptosis and necrosis, energy voltage-dependent anion channel 2</td>
<td>Vdac2</td>
<td>48018663</td>
<td>regulation of ATP consumption and regulation of apoptosis</td>
<td>1.63 1.23</td>
<td>NSR</td>
<td></td>
<td></td>
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<tr>
<td>Unknown</td>
<td>multiple EGF-like-domains 8</td>
<td>Mep8</td>
<td>48011534</td>
<td>too little data</td>
<td>-1.25</td>
<td>NSR -1.36</td>
<td></td>
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<tr>
<td>Unknown</td>
<td>ninjurin 2</td>
<td>Nj2</td>
<td>5045018</td>
<td>tissue regeneration, neurite outgrowth</td>
<td>-1.61</td>
<td>-1.38</td>
<td>NSR</td>
</tr>
<tr>
<td>DNA repair, cell cycling</td>
<td>cyclin-dependent kinase 7</td>
<td>Cdk7</td>
<td>1397</td>
<td>inhibits mTOR, thiol carrier suggests role in oxidative stress</td>
<td>2.22 1.66</td>
<td>NSR</td>
<td></td>
</tr>
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<td>cellular remodelling</td>
<td>basigin (EMMPRIN/Ox47/CE-9)</td>
<td>Basg</td>
<td>NM_013783</td>
<td>May play a role in basement membrane remodelling</td>
<td>-1.22</td>
<td>1.66</td>
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<td>energy metabolism</td>
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<td>Sc4mol</td>
<td>595599</td>
<td>involved in energy-linked phosphate transport</td>
<td>1.46 1.26</td>
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<td></td>
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<td>Neuronal growth and guidance</td>
<td>VDAC1</td>
<td>139379</td>
<td>known</td>
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<td>-1.50</td>
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<td>1.43</td>
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Figure 2.1. Venn diagram of genes differentially expressed in rat liver kidney and heart. Represented genes were differentially expressed on at least 7 of 9 arrays for each tissue, were significant (p < 0.05), and were differentially regulated in more than one tissue.
Figure 2.2. Comparison of differential regulation of three genes by DNA microarray and by QRT-PCR. The trendline represents perfect agreement between DNA microarray and QRT-PCR results. The same sample of RNA was used for both microarray and QRT-PCR experiments. Microarray values shown are the average “median of ratios” value from 9 independent arrays ± SEM. QRT-PCR values are the average ± SEM of 3 separate trials for each gene after being normalized to our control gene,
CHAPTER 3

A ROLE FOR METALLOTHIONEINS-I + II IN ISOFLURANE PRECONDITIONING OF PRIMARY MURINE NEURONAL CULTURES

Introduction

Given the potential for improved clinical outcomes, mechanisms of delayed anesthetic preconditioning have been intensely studied. Since first being demonstrated in rabbit heart, preconditioning by volatile anesthetics has been described in both in vivo and in vitro models, and in other tissues including the brain. In these studies, inducible nitric oxide synthase, intracellular calcium, mitogen-activated protein kinase, and a variety of mitochondria related proteins have all been implicated in the cellular mechanisms of APC. Despite these findings, a complete understanding of the pathways involved in APC has yet to be elucidated.

Recent work from our lab identified metallothionein I + II genes (MT-I + II) as among those significantly regulated in rat liver, kidney and heart following a 90min 2% isoflurane exposure. Furthermore, Carmel et al., found MT-I + II mRNAs were strongly and rapidly upregulated in response to ischemic preconditioning of rat spinal cord. Metallothioneins-I + II are small (6000-7000 Daltons), cysteine-rich metal-binding proteins and have been shown to protect against a wide range of stresses including cardiac ischemia-reperfusion and focal cerebral ischemia. To date, the relationship between MT-I + II proteins and anesthetic preconditioning remains to be explored. In the current study, we examine protective characteristics of delayed isoflurane
preconditioning in dissociated neuronal culture and the role that MT-I + II play in conferring APC-mediated protection.

**Methods**

**Animal Care**

C57BL/6J (C57), 129S7/SvImJ (129S, control strain for MT-I + II knockout) mice and 129S7/SvEvBrd-Mt1<sub>tm1Bri</sub> Mt2<sub>tm1Bri</sub>/J (MTKO, metallothionein-I + II knockout strain) were purchased from Jackson Laboratories (Bar Harbor, ME) and breeding colonies established. Animals were kept under a standard 12/12 light/dark cycle at room temperature and given access to food and water *ad libitum*. Animals were housed and all experiments carried out with protocols approved by Smith College Institutional Animal Care and Use Committee (Northampton, MA, USA) according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

**Primary neuronal cultures**

Neonatal mouse pups at postnatal day 1-3 (P1-3) were sacrificed by decapitation. Cortices were dissected out, minced and placed in modified DMEM (80% Dulbecco’s modified eagle medium; 10% fetal bovine serum, 10% F12 medium and 10 µg/ml Pen/Strep). Tissue was digested in dilute Trypsin/Hanks Balanced Salt Solution (1:1) for 25 min, quenched with 50% horse serum prior to brief centrifugation to pellet tissue. Tissue was triturated with a fire-polished Pasteur pipette, filtered through a Steriflip100µM nylon filter (Millipore; Billerica, MA) and plated onto poly-lysine coated wells, or in the case of immunofluorescence microscopy, on poly-lysine coated...
glass coverslips. Cultures were maintained in an incubator (5% CO₂, 95% air) at 37˚ C for 10-12 days in vitro.

**Isoflurane Preconditioning**

Isoflurane preconditioning was accomplished by placing cultures (10-12 days in vitro) inside a humidified exposure chamber with inlet and exhaust ports for the anesthetic mix. The entire exposure chamber was housed within a 37˚ incubator. A mixture of 95% air/5% carbon dioxide was passed through an Isotec 3 isoflurane vaporizer (GE Healthcare; Waukesha, WA) to deliver 1.5% isoflurane/93.5% air/5%CO₂ to the chamber at a rate of 0.4 l/min for 3 hrs. Following APC, cells were washed 1X fresh media was added prior to replacing cells in a standard humidified carbon dioxide incubator. For controls, all media changes were performed in parallel with preconditioned cultures.

**Oxygen-Glucose Deprivation**

Oxygen glucose deprivation (OGD) exposures were carried out as previously described with some modifications. Cell cultures were washed 4 times with zero-glucose DMEM (Invitrogen, Carlsbad CA) and placed in a humidified anoxic chamber at 37˚C. The chamber, was evacuated and flushed 3 times with 90% N₂, 5% H₂, and 5% CO₂ in order to remove oxygen. To further reduce oxygen levels, a palladium catalyst was also maintained in the chamber to react any remaining oxygen with the hydrogen. Non-OGD samples were treated the same as OGD receiving samples, but were washed with standard glucose-containing culture media.
Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) of MT-I + II RNA levels

Cells were harvested using a cell scraper, pooled, pelleted by centrifugation, and then added to Trizol reagent (Invitrogen) and stored at –20°C for further processing. Total RNA for QRT-PCR analysis was isolated from Trizol/tissue homogenate using Qiagen Rneasy Mini kits (Qiagen: Valencia, CA). In brief, chloroform was added to the Trizol/tissue homogenate and the aqueous phase was removed, mixed with 70% ethanol and placed on a separation column. After repetitive washes and elution in RNAse–free water, RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies; Rockland, DE). All samples yielded 260/280 absorbance ratios between 1.9 and 2.1.

QRT-PCR of MT-I and MT-II messenger RNA (mRNA) was performed using a mouse-modified primer/probe set (FAM/TAMRA) described by Tomita et al. Primers and probes were synthesized by IDT inc (Coralville, IA): MTI forward 5’-CTGCTCCACCGGCGG-3’, MT1 Reverse 5’-GCCCTGGGCACATTTGG-3’; MTI taqman probe: 5’-CTCCTGCAAGAAGAGCTGCTGCTCCT-3’; MTII forward 5’-TCCTGTGCCTCCGATGGATC-3’; MTII reverse 5’-GTCGGAAGCCTCTTTGCAGA-3’; MTII taqman probe 5’-AAAGCTGCTGCTCCTGCTGCCC-3’; LCA1 (leukocyte common antigen 1) forward 5’-ACCTACATTGGAATTGATGCCATGCT-3’; LCA1 reverse 5’-CCTCCACCTTGACACCACATCAGACA-3’; LCA1 taqman probe 5’-GAAAGCAGAGGGCAAAATGAGATGTCTATGCAAGCTGCT-3’. All QRT-PCR reactions were carried out on an Applied Biosystems 7700 Taqman instrument (Applied Biosystems; Foster City, CA) in single tubes. The QRT-PCR reaction mix was comprised of 1X PCR buffer
(Invitrogen, Carlsbad, CA), 6mM MgCl₂, 0.67 mM each dinucleotide, 6.75 pmols each of forward and reverse primers, 1.5 units Superase-in (Ambion; Austin, TX), 15 units Superscript II (Invitrogen), 0.75 units PlatinumIn Taq Polymerase (Invitrogen) to a final volume of 15µl. Each reaction was run one cycle of 30’ at 42˚, 1 cycle of 3’ at 95˚ and 40 cycles of 1’ at 60˚ and 20” at 95˚. QRT-PCR for the normalization gene, mouse protein tyrosine phosphatase, receptor type C (PTPRC), was performed as above but using PTPRC primers and probe set. Universal mouse RNA (Stratagene, La Jolla CA) was used to create a standard curve for each gene of interest and for the normalization gene, PTPRC. Gene reactions were run in triplicate, averaged and normalized to PTPRC.

Lactate Dehydrogenase assay for determination of cellular toxicity

Global cellular toxicity was assessed using the CytoTox LDH assay (Promega, Madison WI). Briefly, following experimental treatment, 50µl media were removed from each culture well and placed in a separate well-plate. 50 µl of LDH assay solution was added to each well and allowed to incubate in the dark at room temperature for 30 min. At the end of the incubation period, 50µl stop solution was added and color change was measured at 490nm on a Bio-TEK μQuant UV/Vis plate reader (BioTEK Instruments; Winooski VT). Net color change was determined by subtracting the average value of media-only wells from experimental values.

Lactate dehydrogenase release data were first normalized using the equation 
\[((LDH \text{ value}_{\text{Individual well}}) / (\text{Control Average}_{\text{Experiment}}))\] in order to compare results between different experiments. Values for each condition were further transformed by calculating
average percent level of protection compared to control using the equation: (100- (100 * (Experimental/Control)).

**Microtubule associated protein-2 (MAP2)/Glia fibrillary associated protein (GFAP) colorimetric assays for determination of cellular toxicity**

MAP2 and GFAP colorimetric assays were performed following a modified protocol described by Carrier *et al.* 108. Briefly, treated and control cultures were washed 3 times in phosphate buffered saline (PBS) and then fixed at room temperature in 4% paraformaldehyde in 0.1% sodium phosphate (pH 7.4) for 30 min. Fixed cells were subsequently permeabilized with 0.4% Triton-X 100 in PBS for 10 min at room temperature. Cells were then washed 3 times in PBS, and blocked for 30 min in 10% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) at room temperature. Either rabbit anti-GFAP (sc-65343, Santa Cruz Biotechnology Inc, Santa Cruz, CA) or anti-MAP2 (sc-20172; Santa Cruz Biotechnology Inc) primary antibody in 3% bovine serum albumin/0.4% Triton-X100 in PBS (1:100) was added to fixed cultures and allowed to hybridize overnight at 4°C. Fixed cells were then washed 3 times in 0.5% Tween/PBS and hybridized for 2 hrs at room temperature with horse radish peroxidase- conjugated 2’ antibody at 1:2000 (sc-2004; Santa Cruz Biotechnology) in 1% bovine serum albumin/0.4% Triton-X 100 in PBS. Cell survival was monitored by measuring the horse radish peroxidase-catalyzed conversion of Amplex Red to resorufin (Invitrogen) with 1mM H₂O₂ (1mM; Sigma) in 50mM phosphate buffer. Samples from each well were transferred to a 96 well plate and absorbance (at 565 nm) measured on a Bio-TEK µQuant UV/Vis plate reader.
MAP2, GFAP, MT-I + II immunofluorescence microscopy for determination of MT localization.

Cells for localization studies were grown on poly-lysine coated glass coverslips. Preparation for labeling was performed as described for colorimetric assays until the secondary labeling stage. Cultures were labeled with primary antibodies for MAP2 (rabbit anti-MAP2 polyclonal; sc-20172; Santa Cruz Biotechnology), GFAP (rabbit anti-GFAP polyclonal; ab16997-1; AbCam) and MT-I + II (mouse anti-MT-I + II monoclonal; E-9; Zymed). The E9 clone has been demonstrated to be specific for MT-I + II and not to label other metallothioneins. Secondary labeling was performed using fluorescent dye-conjugated 2° antibodies (Alexa 488 goat anti-rabbit and Alexa 594 goat anti-mouse; Invitrogen; 1:4000) in 1% bovine serum albumin/0.4% Triton-X100 in PBS. Nuclei were counterstained for 3 min with 1x Hoechst (Invitrogen). Coverslips were mounted on slides and visualized using an Olympus BX51 epifluorescence microscope (Olympus America Inc, Center Valley, PA).

siRNA Knockdown of MT-I + II

A Dicer-substrate (DsiRNA) duplex that targets both MT-I and MT-II and sham duplex (designed not to target any genes) were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). RNA sequences for the metallothionein-I + II knockdown duplex strands were \textit{AGAACUCUUCAAACCGAUCUCGT} and \textit{ACGAGAGAUCGGUUUGAAGAGUUCUAG}. Sequences for the scrambled sham duplex were not available from Integrated DNA technologies. Cultures 10-12 days in vitro were transfected with siRNA constructs following instructions for use of TransPass R1.
transfection reagent (New England Biolabs, Ipswich MA). In brief, for each well, 400µl of high-glucose serum-free DMEM (Invitrogen) was mixed for 20 min with 1.0 µl transfection solution A, 2.0 µl transfection solution B, and 20 µM duplex RNA to bring the final siRNA concentration to 5-10 nM. Cells were then washed 4 times with serum-free high-glucose DMEM (Invitrogen). Transfection mixture was added and cultures were returned to the incubator. After 2 hrs, the transfection mixture was removed and replaced with standard media. Cells were allowed to recover for 72 hrs prior to further treatment. Transfection efficiency was assessed using immunofluorescence microscopy to visualize the transfection of a fluorescein-labeled siRNA control (15 nM, Fluorescein-siRNA Transfection Control; New England Biolabs). siRNA knockdown of MT-I + II was assessed by QRT-PCR under both basal and under MT-stimulating conditions (20 µM ZnCl₂, 12 hrs prior to harvest. Cells were treated with sham siRNA for all control experiments.

**MT-I + II protein transfection**

Cells were transfected with either exogenous MT-I + II purified from horse liver (Sigma) or with a similar sized control protein (insulin oxidized β-chain, Sigma) using ProteoJuice transfection reagent (Novagen, San Diego CA). Briefly transfection reagent and protein were mixed 1:2 (v:w) in 25 µl of serum-free DMEM and incubated at room temperature for 20 min. Meanwhile cells were washed 4 times with serum-free DMEM and left in serum-free DMEM. The transfection mixture was added to each well to achieve a final protein concentration of 1.6-3.2 µM and cells were returned to the incubator at 37°C for 2 hrs. Transfected cells were washed 4 times in no-glucose DMEM
and immediately placed in an anoxic chamber for OGD treatment. OGD toxicity was assessed by LDH.

Statistics

All statistics were performed in SPSS 9.0 (SPSS Inc, Chicago IL). ANOVA analyses were applied to data in Figures 3.1, 3.2, and 3.3 with Bonferroni post-hoc analyses where applicable (Figure 1). Figure 4 required no statistics. Figure 5 was analyzed by ANOVA. Two-tailed Student’s t-tests were applied to the data in Figures 3.1, 3.3, 3.5, 3.6 and 3.7. Significance level was set at p < 0.05 unless stated otherwise.

Results

Isoflurane induced delayed protection against subsequent OGD-mediated toxicity in mouse cortical cultures.

Isoflurane preconditioning has been shown to protect against ischemic injury in rat brain providing improved histologic and neurologic outcomes one month following preconditioning/ischemia \(^{39}\) and in neuron-enriched cortical cultures 24 hrs following isoflurane preconditioning \(^{40}\). In order to determine if isoflurane preconditioning protected over an extended period in culture conditions, we evaluated OGD toxicity in mixed cortical cultures at 24, 48, 72 and 96 hrs post-preconditioning (Figure 3.1). A 3hr pretreatment with 1.5% v/v isoflurane (a clinically relevant exposure) provided protection that was significant (two-tailed Student’s t-test; p < 0.01) compared to paired, non-preconditioned controls for all time points. In detail, at 24 hrs the protection amounted to 11.6 ± 7.9% (n=21), increasing to peak protection of 37.5 ± 2.5% at 72 hrs (n=21). The
effect persisted and was still robust at 96 hrs (33.4 ± 1.6%; n=32). These data are consistent with isoflurane conferring delayed preconditioning via a direct pharmacological mechanism in dissociated primary cultures (see also Kapinya et al. 40).

Isoflurane preconditioning protects neurons and glia against OGD toxicity

To determine which cell lineages were protected against OGD by isoflurane preconditioning, we used an Amplex red-based immunoassay (Figure 3.2) to assess neuronal and glial survival following 3hrs OGD. Although 3hrs OGD was toxic to both neuronal and glial populations, neurons were particularly sensitive to this treatment with survival rates of only 39.6 ± 8.6% vs 79.0 ± 4.1% (n=16) for glial cells. Neurons were also more responsive to isoflurane preconditioning showing recovery to a 84.9 ± 4.3% (n=16) survival rate i.e. ~127% increase in survival. To a lesser extent glial cells were also protected with a relative increase in survival rate of only ~22% (Figure 3.2). For neuronal populations, a 2x2 ANOVA analysis showed significant main effects of OGD and of isoflurane preconditioning (APC) with a significant interaction between these variables (p < 0.01). This interaction indicates the effectiveness of APC in counteracting OGD-mediated cell death and is supported by a Student’s t-test that confirmed a significant (p<0.01) difference between ‘OGD alone’ and ‘APC + OGD’ conditions (Figure 3.2). For glial cells, the 2x2 ANOVA analysis revealed significant main effects of OGD and of isoflurane preconditioning (APC) (p < 0.05) however there was no significant interaction between these variables (p=0.53). The lack of a significant interaction can be interpreted to mean that isoflurane preconditioning is not effective in protecting glia against OGD. However, a Student’s t-test comparing the ‘OGD alone’
and ‘APC + OGD’ conditions revealed a significant (p<0.01) difference between these groups suggesting that APC was effective but that the level of OGD toxicity was not as pronounced in this cell population.

**MT-I + II protein transfection provides protection against OGD-mediated toxicity**

Previous studies have implicated MT-I + II in neuronal protection against ischemia (see Carmel et al. 105, and Wakida et al. 106). Kennette et al. 112 determined that basal levels of metallothioneins were protective against certain toxins (e.g. tertbutyl peroxide) while for other toxins (e.g. cadmium and cisplatin) MT levels had to increase above basal levels for protection to occur. To discern in our system whether increased MT protein levels provided direct protection against OGD, we transfected cultures with exogenous MT-I + II protein prior to a 3 hr OGD challenge and then assessed toxicity by LDH (Figure 3.3). Compared to transfection with a similarly-sized control protein (oxidized insulin β-chain), MT protein transfection conferred protection against 3hr OGD, rising from 2.9 ± 2.0% protection at 1.6 µM MT (not significant; n=8 ) to significant protection of 33.2 ± 3.1% (p < 0.001; n= 8) with 3.2 µM MT . In summary, these data confirm previous reports that metallothioneins are protective against ischemic toxicity in neuronal tissue 113.

**MT-I and MT-II gene expression increased following isoflurane-mediated preconditioning.**

Given the precedent for regulation of MT gene expression following an ischemic episode 12 or an anesthetic exposure 104, we examined whether MT-I and MT-II were
induced in mouse mixed neuronal/glial following preconditioning with a 3hr, 1.5% isoflurane exposure. Both MT-I and MT-II were rapidly upregulated with increases in mRNA levels observed as early as 3 hrs (Figure 3.4). For both metallothioneins-I + II, mRNA levels peaked at ~12 hrs with levels that were 2.2 ± 0.2 and 2.8 ± 0.4 fold the non-preconditioned cultures for MT I and MT II, respectively. Compared to untreated paired controls, MTI mRNA fold changes were significant (two-tailed Student’s t-test; p < 0.05) at 6 and 12 hrs and MTII mRNA fold changes were significant at 3, 6 and 12 hrs. These data are consistent with those described by Carmel et al. for ischemic-preconditioning of rat spinal cord and indicates that metallothioneins are also regulated in response to isoflurane preconditioning. Although it would be preferable to establish the timecourse of the changes in protein levels post-APC due to the small size and nature of the metallothioneins (e.g. highly hydrophilic, number of cysteine residues) we were unable to resolve metallothionein protein levels at the sensitivity required (see also Lu et al.).

**siRNA knockdown of MT-I + II enhances OGD-mediated toxicity and attenuates isoflurane-induced protection.**

In order to assess the protective role of MT-I + II in isoflurane preconditioning, prior to repeating the preconditioning/OGD regimen, we transfected cultures with either a sham siRNA construct or an siRNA construct that targeted both MT-I and MT-II. We first determined the degree of MT-I and MT-II mRNA knockdown using QRT-PCR (Figure 3.5 A). MT-I + II mRNA levels were assessed under basal and under MT stimulating conditions (addition of 20µM ZnCl₂, see Kim et al.). Though we were able
achieve only partial knockdown of either gene (Figure 3.5 A), MT-I and MT-II mRNA levels were diminished in a dose-dependent manner by MT-siRNA transfection. Zinc stimulation and siRNA knockdown both showed greater effect on MT-II than on MT-I.

We assessed the effect of sham knockdown (ShamKD) or metallothionein knockdown on isoflurane-mediated protection against 3hr OGD using an LDH assay (Figure 3.5 B). Isoflurane -preconditioned ShamKD cultures demonstrated substantial protection (17.5 ± 5.2%, n=27), similar to our findings for WT C57 cultures. ANOVA analysis of the siRNA data revealed main effects of metallothionein knockdown (p < 0.05) and of APC (p < 0.01). However, there was no significant interaction found between these variables (p=0.518). This implies that the partial knockdown of the metallothioneins was not effective in reducing the protective influence of APC significantly. However, metallothionein-I + II knockdown conditions without preconditioning showed substantially increased OGD-mediated toxicity compared to ShamKD (Figure 3.5 B). This suggests that MT-I + II below typical basal levels renders cells particularly vulnerable to OGD insults. Due to the incomplete knockdown of MT-I + II in the siRNA experiments, we extended our study to perform APC/OGD experiments with MT-I + II knockout mouse-derived cultures.

MT-I + II knockout abolishes isoflurane-mediated protection against OGD toxicity.

As a more definitive method of addressing the importance of metallothioneins to isoflurane preconditioning, we compared treatments on cultures prepared from MTI + II knockout mice (see Methods) to cultures derived from the background (129) strain (Figure 3.6). Similar to our previous experiments with C57 mice (Figure 3.1),
preconditioned background (129)-derived cultures showed significant protection (two-tailed Student’s t-test; \( p < 0.001 \)) against OGD toxicity (26.2 ± 3.6%; \( n=16 \)) as compared to paired non-preconditioned samples. By contrast knockout-derived cultures (MTKO) showed no protection from isoflurane preconditioning (1.5% ± 3.7%; \( n=25 \), Figure 3.6 B). Both 129 WT and MTKO cultures were assessed by LDH for toxicity from isoflurane preconditioning alone. Preconditioned 129 WT cultures showed LDH levels of 97 ± 11% of untreated cultures and MTKO cultures showed LDH levels 124% ± 19% their untreated counterparts at 48 hrs post-isoflurane exposure (\( n=16 \)). There was no significant difference between treated and untreated cultures in either 129 WT or MTKO cultures (two-tailed Student’s t-test, \( p = 0.86 \) and \( p =0.49 \) respectively). These data provide compelling evidence of a critical role for metallothioneins in isoflurane-mediated preconditioning.

Metallothionein-I + II proteins are primarily localized to neuronal cytoplasm in mouse neonatal cortical cultures.

In light of our finding that APC provided more protection from OGD to neurons rather than glia, we determined the cellular localization of MT-I + II proteins (Figure 3.7). Previous studies in mouse brain have shown metallothioneins-I + II to be localized primarily in glial cells\(^{115,116,106}\). Here we used immunofluorescence microscopy to assess the colocalization of a monoclonal MTI + II antibody with either neuron-specific MAP2 antibodies or glial-specific GFAP antibodies. In C57- and 129-derived cells the metallothionein antibody colocalized strongly with MAP2 labeled cell bodies indicating strong neuronal localization (Figure 3.7 A). GFAP-labeled cells showed metallothionein
co-localization to a lesser extent (data not shown). Neither MT knockout-derived cultures nor 129 wild type (WT) cultures coincubated with 10 µM horse MT-I + II protein demonstrated measurable MT labeling, consistent with MT-I + II specific labeling (Figure 3.7 B and C).

**Discussion**

Delayed anesthetic preconditioning has been shown to be a powerful tool for improving both histological and clinical outcomes against ischemia-mediated toxicity in a number of tissues \(^{101, 117}\). Given the relative safety of preconditioning with anesthetics vs surgically-induced ischemia \(^{59}\), there is emerging interest in anesthetic preconditioning. In this study, we used a dissociated cortical neuronal culture model to investigate the direct pharmacological effects of isoflurane preconditioning and the role that MT-I + II play in mediating that protection. An *in vitro* dissociated tissue culture model of neuronal APC/OGD presents a number of advantages over *in vivo* models including the ability to measure direct pharmacological effects of the anesthetic agent while avoiding the confounding influences of hemodynamics, cardiac depression and other anesthesia mediated physiological factors. An *in vitro* model also provides more flexibility for manipulation and direct observation of outcomes in studying molecular mechanisms. Inevitably, however, a dissociated culture model has limitations. For instance, the *in vitro* OGD model cannot be used to assess system-wide effects of ischemic injury such as inflammatory and immune response to injury. Furthermore focal ischemia models enable observations of long-term outcomes *in vivo*. Despite these constraints, we have shown that dissociated culture with OGD is a useful model for studying APC and OGD, and that
the time course of protection mirrors that of in vivo models. In future, it will be necessary to follow up our experiments with studies on the role of metallothioneins during anesthetic preconditioning in vivo. Moreover, as MTs can be induced through various conditions, this work may have important implications for improved clinical outcomes.

The principal findings of our study are as follows: 1) Pretreatment with 1.5% isoflurane provided robust protection against OGD toxicity that peaked 72 hrs following isoflurane exposure. 2) OGD was more toxic to neurons than to glia although preconditioning with isoflurane significantly reduced toxicity in both populations. 3) Exogenous MT protein supplementation was directly protective against OGD toxicity. 4) MT-I + II mRNAs were rapidly and robustly induced by treatment with clinically-relevant doses of isoflurane. 5) siRNA knockdown of MT-I + II diminished isoflurane diminished protection against an OGD insult. 6) MT-I + II gene knockout abolished isoflurane mediated protection and rendered cultures more susceptible to OGD than WT cultures. 7) MT-I + II localized to neuronal cell bodies, although were also seen in glial cytoplasm. Combined these data argue for an important role of metallothioneins-I + II in isoflurane-mediated protection against OGD. To our knowledge, this is the first time metallothioneins-I + II have been revealed as important mediators of isoflurane preconditioning.

There are several reports supporting a protective role of MT-I + II in a number of tissues. In particular, MT-I + II have been implicated in protection against ischemic injury in brain, heart, and kidney. Campagne et al. found MT overexpressing mice not only had better histologic outcomes, but also significantly
improved motor performance 3 weeks following focal cerebral ischemia/reperfusion. Wakida et al. 106 found erythropoietin-induced MT-I + II reduced infarct area and volume at 24 hrs following permanent middle carotid artery occlusion (MCAO) damage in rat brain. Of direct relevance to our study, Carmel et al. 105 found MT-I + II mRNAs were rapidly induced in rat spinal cord following brief ischemic preconditioning. Together these data complement our findings that MT-I + II are rapidly induced following isoflurane preconditioning and that the increased MT levels provide protection against subsequent ischemic injury.

A surprising result was the localization of MT-I + II to neuronal cell bodies along with weaker glial staining (Figure 3.7). While this localization correlates well with the robust APC-mediated neuronal protection (Figure 3.2), it contrasts with previous reports that metallothioneins-I + II localize primarily within glia in the brain with little to no expression in neurons 115. These localization differences may be accounted for by a number of experimental variations including developmental stage of our cells, differences in structure of cells grown in culture vs in vivo, or differences in fixing procedures (see Hidalgo et al., 2001115). Neuronal localization may be accounted for by recent work by Chung et al. 121, who found MT-I + II can be exported by astrocytes and readily taken up and utilized by neurons.

While our study demonstrated that MT-I + II can be protective against OGD, the mechanism by which MT-I + II confer this protection remains to be determined. Metallothioneins are small (~6000-7000 Da) metal-binding (primarily Zn²⁺ and Cu²⁺) proteins that are expressed throughout the body. They exhibit a number of cellular activities that could potentially account for their protective role against ischemia (see
Penkowa,\textsuperscript{85}). These roles include modulation of cellular redox, immune defense response, mitochondrial respiration, angiogenesis, cell-cycle progression, cell survival and differentiation. An intriguing role for MTs in preconditioning is as a critical component of the nitric oxide signaling cascade\textsuperscript{122} that has been identified as essential for induction of delayed isoflurane-induced preconditioning in heart.\textsuperscript{117} Stitt et al.,\textsuperscript{123} studying nitric oxide signaling in mouse lung endothelial cells, showed that metallothionein was essential for nitric oxide-mediated translocation of the transcription factor MTF-1 (metal-responsive transcription factor 1) to the nucleus and for subsequent upregulation of metallothionein. These data support a role for metallothioneins in nitric oxide signaling during ischemic stress.

In addition to possibly participating in nitric oxide signaling, another potential role for MT’s in APC-mediated protection is as an end effector of cellular protection. This is supported by the findings, shown in Figure 3.3, in which transfection of exogenous MT-I + II protein provided immediate protection against OGD damage. As an end effector, MTs might act as antioxidants to detoxify reactive oxygen species produced by the energy pathway imbalances of ischemia (see Penkowa\textsuperscript{85}). In support of this, increased MT levels have been shown to decrease both reactive oxygen species levels and peroxidation of cellular proteins and lipids\textsuperscript{124}. Furthermore the antioxidant effects of MTs during mitochondria-specific oxidative stress have been shown to result in greater protection than provided by Cu/Zn-SOD, glutathione peroxidase, Mn-SOD or catalase\textsuperscript{53}. Alternatively, MT-mediated protection may depend on the central role metallothioneins play in zinc homeostasis\textsuperscript{85}. Approximately 3\% of the mammalian proteome requires zinc for some aspect of functionality with 40\% of zinc binding proteins acting as transcription
factors and the remaining 60% proteins involved in ion transport or as enzymes\textsuperscript{125}. Through their role in zinc homeostasis, MTs may have pleiotropic effects in ischemic tissue which affect a range of responses from transcription to ion homeostasis through modulation of zinc availability throughout the cell.

In conclusion, this study describes an important role for metallothioneins-I + II in isoflurane preconditioning against OGD. Based on the role that MT’s play in isoflurane-mediated delayed protection against OGD, our results implicate metallothioneins-I + II as possible molecular targets for improving clinical outcomes during ischemic injury. Further studies to clarify the mechanisms of metallothionein I + II action will be essential for understanding metallothionein-mediated protection and their potential for protection against ischemic injury. In the final study of the dissertation, we address a possible role for metallothioneins in delayed anesthetic preconditioning as sensors in an oxidative stress signaling pathway.
Figure 3.1. Isoflurane induces delayed protection against oxygen glucose deprivation in murine mixed neuronal and glial cultures. Lactate dehydrogenase release (LDH) was used to assess oxygen-glucose deprivation (OGD)-mediated toxicity in both untreated and isoflurane preconditioned (1.5% isoflurane; 3 hrs) cultures at 24, 48, 72 and 96 hours post preconditioning. Percent protection (mean ± SEM) was calculated using the equation: 100 - (100 x (Mean \text{Test LDH}/\text{Mean Control LDH})). Protection is reported as compared to days in vitro-matched controls. *Significance: (p<0.01, two-tailed Student’s t-test). Inset indicates timecourse for experimental procedure. **Significant difference between timepoints and ‘NS’ denotes No significant difference for an ANOVA with Bonferroni post-hoc analysis (p<0.05).
Figure 3.2. Neurons and glia are protected by isoflurane preconditioning. Mouse cortical cell culture subpopulations were assessed for oxygen-glucose deprivation (OGD)-induced toxicity and isoflurane-induced protection by immunolabeling for neurons with horse radish peroxidase-conjugated antibodies to microtubule associated protein -2 (MAP2) and for glia with glial fibrillary associated protein GFAP. *Significance: (p<0.01, n=16, two-tailed Student’s t-test). Inset indicates timecourse for experimental procedure.
Figure 3.3. Transfection with exogenous metallothionein-I + II protein protects primary cortical cultures against oxygen glucose deprivation -mediated toxicity. Primary cortical cultures were transfected with 1.6µM or 3.2 µM metallothionein-I + II (MT-I + II) protein or 3.2 µM oxidized insulin β-chain (control). Toxicity was assessed by lactate dehydrogenase release assay. *Significance: (p<0.001, two-tailed Student’s t-test). Inset indicates time course for experimental procedure.
Figure 3.4. Metallothionein I and II mRNA levels are increased following isoflurane preconditioning. Primary cortical cultures were preconditioned with 1.5% isoflurane for 3hrs. Cells were collected at 5 time points (T=0, 3, 6, 12, and 24 hrs) post-exposure, and metallothionein-I (MT1) and metallothionein-II (MTII) mRNA levels were assessed by quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR), normalized to protein tyrosine phosphatase, receptor type C (PTPRC) expression. *Significance: (p < 0.05; n=5; two-tailed Student’s t-test).
Figure 3.5. Metallothionein short interfering RNA reduces Metallothioneins I and II mRNA levels in a dose-dependent manner and increases vulnerability to oxygen glucose deprivation. (A) Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) was used to assess metallothionein I + II knockdown by a short interfering RNA (siRNA) against metallothioneins-I + II (MTKD). A sham siRNA (ShamKD) construct was used as the control. ZnCl$_2$ was used to simulate metallothionein-stimulating conditions. (B) OGD-mediated toxicity was assessed by lactate dehydrogenase release assay in mixed cortical cultures transfected with either ShamKD or the MTKD siRNA constructs. See Results for discussion of significance (n=27). Insets indicate time course for experimental procedure.
Figure 3.6. Knockout of metallothionein-I + II genes abolishes isoflurane-mediated protection against oxygen-glucose deprivation. Oxygen glucose deprivation (OGD)-mediated toxicity was evaluated following 3hr, 1.5% isoflurane preconditioning in mixed neuronal/glial cultures from A.) Background strain (129) and B.) Metallothionein-I + II (MT-I + II) knockout mice (MTKO). *Significance: p<0.001, two-tailed Student’s t-test. Inset indicates time course for experimental procedure.
Figure 3.7. Metallothioneins-I + II localize to neuronal cell bodies cortical neuronal cultures. Metallothionein-I + II (MT-I + II) localization was visualized by epifluorescence microscopy. Cultures, 10 days in vitro, were labeled using a monoclonal antibody against MT-I + II and a polyclonal antibody against microtubule associated protein-2 (MAP2). Secondary labeling was accomplished with alexafluor 488 or alexafluor 594 fluorescent conjugates. A.) 129 cells, nuclei were stained with Hoechst. B.) MT-I + II knockout cells. C.) 129 cultures, antibody pre-incubated with 10 μM horse MT-I + II protein to show MT antibody specificity.
CHAPTER 4

A ROLE FOR METALLOTHIONEINS I + II AS SENSORS OF OXIDATIVE STRESS IN ISOFLURANE-MEDIATED DELAYED ANESTHETIC PRECONDITIONING.

Introduction

Ischemic injury progresses via a complex cascade of events that includes diverse, but intertwined ontological pathways. Recent studies have identified a range of molecules that are involved in delayed anesthetic preconditioning including COX-2, NF-Kappa B, PI3 kinase, K(ATP) channels, Mn-SOD, and 12-lipoxygenase. In addition to the above molecules, the reactive oxygen species, superoxide and the reactive nitrogen species, nitric oxide have received particular attention for their essential role in inducing delayed anesthetic preconditioning. Despite significant evidence implicating ROS and RNS in the induction of delayed APC, how they exert a protective effect is not well understood.

Superoxide-mediated oxidation of cysteines on the zinc binding proteins metallothioneins I + II has been shown to result in the release of zinc to the cell cytoplasm. Recent studies in mouse lung endothelial cells have demonstrated that metallothioneins I + II are also important targets of nitric oxide, again resulting in release of bound zinc. Furthermore, it has been shown that pharmacological nitric oxide donors are able to induce sufficient zinc release from metallothioneins to activate metal-responsive transcription factor-1 (MTF-1), the primary transcription factor.
regulating metallothionein expression during oxidative and heavy metal stress\textsuperscript{131}. Activation of MTF-1 by increased cellular zinc causes it to translocate from the cytoplasm to the nucleus leading to increased metallothionein production\textsuperscript{123}. It has been hypothesized that MTF-1, in conjunction with oxidative release of zinc from metallothioneins, may participate in a novel signal transduction pathway that provides protection against oxidative stress\textsuperscript{131-134}. Given that ROS and RNS are widely reported to play instrumental roles in the induction of delayed preconditioning, combined with our recent finding that metallothioneins I + II play a central role in delayed preconditioning\textsuperscript{45}, we, in this final study, investigated a possible role for metallothioneins as sensors of oxidative stress following preconditioning with the inhaled anesthetic isoflurane, the reactive nitrogen species nitric oxide and the oxygen radical superoxide.

**METHODS**

**Animal Care**

129S7/SvEvBrd-\textit{Mt}^\textit{tm1Bri}\textit{Mt}^\textit{tm2Bri/}J (MTKO, metallothionein-I + II knockout) and 129S7/SvImj (129, control strain for MT-I + II knockout) mice were initially purchased from Jackson Laboratories (Bar Harbor, ME) and a breeding colony established. Animals were kept under a standard 12:12 light:dark cycle at room temperature and given access to food and water \textit{ad libitum}. Animals were housed and all experiments carried out with protocols approved by Smith College Institutional Animal Care and Use Committee (Northampton, MA, USA) according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).
**Primary neuronal cultures**

Neonatal mouse pups postnatal day 1-3 (P1-3) were sacrificed by decapitation. Cortices were dissected out, minced and placed in modified DMEM (80% DMEM, 10% FBS, 10% F12 medium and 10 µg/ml Pen/Strep). Tissue was digested in dilute Trypsin/HBSS (1:1, v/v) for 25 min, then quenched with 50% horse serum prior 3 min centrifugation to pellet tissue. The tissue was triturated then filtered through a Steriflip™ 100µM nylon filter (Millipore; Billerica, MA) and plated onto poly-lysine coated tissue culture plates. Cultures were maintained in a 5% CO₂ incubator at 37˚C for 10-12 days *in vitro* (DIV 10-12).

**Preconditioning Regimens**

Isoflurane preconditioning was accomplished using a humidified exposure chamber containing inlet and outlet ports housed inside a 37˚ incubator. Cultures (DIV 10-12) were placed in the exposure chamber and an air/CO₂ mix (95% air/5% CO₂) was passed through an Isotec 3™isoflurane vaporizer (GE Healthcare; Waukesha, WA) to deliver 2.5% isoflurane/92.5% air/5% CO₂ at a rate of 0.4 L/min for 2 hrs. In experiments blocking iNOS and superoxide, cells were incubated with the inducible nitric oxide synthase inhibitor, aminoguanidine (AG, 1.0 mM, Sigma) and the superoxide dismutase mimetic manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP, 1.0 M, Sigma), respectively, 2 hrs prior to the anesthetic preconditioning.

Freshly made S-Nitroso-N-acetylpenicillamine (SNAP, 200µM, Sigma), degraded SNAP (SNAP degraded at 37˚ overnight in culture media), and paraquat (5µM, Sigma) were used for nitric oxide and superoxide preconditioning respectively. Cultures were
returned to the incubator for the 2 hr preconditioning period. All preconditioning regimens were followed by 3 washes with fresh media. Cultures used for toxicity assays, were returned to the incubator for 72 hrs until OGD (see below) in order to achieve peak isoflurane mediated- protection. Cultures being used for MTF-1 translocation experiments were processed immediately for nuclear protein extraction. Each media change performed in preconditioned cultures was performed in parallel in control cultures.

**Oxygen-Glucose Deprivation**

Oxygen glucose deprivation (OGD) exposures were carried out as previously described with some modifications. Cell cultures were washed 4 times with zero-glucose DMEM (Gibco) and placed in a humidified anoxic chamber (model A-141, Sheldon Mfg., Cornelius, OR) at 37°C. The chamber was evacuated and flushed 3 times with 90% N₂, 5% H₂, and 5% CO₂. A palladium catalyst was maintained in the incubator to further diminish O₂ levels. Cultures were maintained in the anoxic chamber for 4 hrs prior to LDH assay. All control cultures not receiving OGD treatment were handled in parallel, but washes were performed with standard media rather than with OGD media.

**Lactate Dehydrogenase assay for determination of cellular toxicity**

Global cellular toxicity was assessed using a CytoTox™ LDH assay (Promega, Madison WI). Briefly, following experimental treatment, 50µl media were removed from each culture well and placed in a separate well-plate. 50 µl of LDH assay solution was added to each well and allowed to incubate in the dark at RT for 30 min. At the end of the incubation period, 50µl stop solution was added and color change was measured at
490nm on a Bio-TEK µQuant™ UV/Vis plate reader (BioTEK Instruments; Winooski VT). Net color change was determined by subtracting the average value of media-only wells from experimental values. In order to compare results between experiments, data were normalized to “% normalized protection” by dividing each individual experimental value by the average control value. Thus the equation for generating normalized data was as follows: 100-100*(Abs_{Experimental} - Abs_{Average-media}) / Abs_{Average(-control)} - Abs_{Average-Media})%.

**Isolation of nuclear proteins for determination of MTF-1 translocation**

Immediately following preconditioning regimen, plates were washed 3X with ice cold PBS. Cells were then scraped with 1mL PBS on ice, placed in a microcentrifuge tube and spun at 500 x g for 3 min to pellet the cells. The supernatant was then removed and replaced with the outer membrane lysis buffer (0.01M Hepes, 1.5mM MgCl$_2$, 0.01M KCl, 1.0mM, DTT) at ~10X packed cell volume and incubated on ice for 15 min. Igepal-CA630 non-ionic detergent (10% v/v; Sigma) was added to the cell suspension for a final concentration of 1%. Samples were immediately vortexed and centrifuged at 21000 x g for 5 min. Supernatant containing cytosolic proteins was removed and the pellet containing nuclei was washed 2x in 500µL Buffer I (without Igepal). The nuclear pellet was then resuspended in 200µl Buffer II (nuclear extraction buffer; 0.02M HEPES, 1.5mM MgCl$_2$, 0.42 M NaCl, 200µM EDTA, 25% glycerol, 1.0mM, DTT), pipette-mixed to break up pellet, and incubated on ice for 40 min. Samples were then vortexed for 30 secs and centrifuged at 21000 x g for 15 min. Supernatant containing the nuclear fraction was transferred to a fresh tube. Protein concentration was determined by BCA
assay (Pierce), and samples were stored at -20°C. The nuclear pellet was observed prior
to nuclear lysis by phase-contrast and epifluorescence microscopy (with Hoeschst 33342
nuclear stain; Invitrogen) and found to contain only nuclei with little to no cellular debris
(not shown).

**Western Determination of MTF-1 levels**

Nuclear proteins (20µg) were separated by electrophoresis on 4-12% Bis-Tris
polyacrylamide gels (Novex, Invitrogen) and transferred to 0.45µM nitrocellulose
membrane (Invitrogen). Membranes were probed with a polyclonal antibody against
MTF-1 (1:750; Santa Cruz Bio, SC-48775) and detected with an HRP-conjugated anti-
rabbit 2° antibody (1:100,000 Santa Cruz Bio, SC-2004). Bands were visualized with
Supersignal West Pico chemiluminescent Substrate (Pierce) and detected on film (Kodak
X-Omat LS, Sigma). Relative densitometry was performed in Photoshop 9.02 (Abobe
Inc., San Jose, CA) for quantitative analysis of bands from 3 replicates of each
condition\textsuperscript{135}.

**Statistics**

All statistics were performed in SPSS 9.0 (SPSS Inc., Chicago IL). Normalized
toxicity/protection data were analyzed by ANOVA (Figures 4.1 A and 4.2 A and 4.3 A).
Significance level was $p < 0.05$ unless otherwise stated.
Results

Nitric oxide and superoxide generators precondition wild type cultures and promote nuclear translocation of MTF-1.

Growing evidence supports the role of reactive oxygen and reactive nitrogen species as the primary initiators of preconditioning \(^{47}\). In this group of experiments, we attempted to use the nitric oxide donor SNAP, a degraded form of SNAP, and the superoxide generator paraquat as preconditioning agents. Similar to previously described preconditioning experiments using isoflurane as the preconditioning agent\(^{40,45}\), SNAP preconditioned cultures demonstrated significant protection against OGD (37.6 ± 3.4%; n=28) compared to non-preconditioned cells in WT cultures (p < 0.001; n=28) (Figure 4.1 A). Cells treated with the same concentration of SNAP that had been heat degraded (37°, 24hrs) were not significantly protected 8.3 ± 6.7% (p = 0.4; n=14) and were significantly less protected than the SNAP preconditioned cultures (p < 0.01; n=14). Paraquat preconditioned cultures showed significant protection (24.2 ± 3.6%; n=14; p < 0.01) compared to untreated controls. In order to ensure that outcomes were the result of preconditioning only, cultures were assessed for treatment-mediated toxicity by taking media just prior to OGD treatment. SNAP, decomposed SNAP and paraquat showed only small differences from basal control LDH levels (75.2 ± 2.6%, p= 0.18, n= 6), (65.5 ± 1.1%; p < 0.05; n=6) and (82 ± 3.1%; p= 0.26, n=6) of control levels respectively.; p = 0.18 and p < 0.05 respectively; n=6). Though the degraded SNAP value was statistically significant, it represented a small absolute difference from the control value and were not considered to affect the final outcome.
Reactive oxygen and nitrogen species have previously been shown to induce nuclear translocation of MTF-1. We were interested to determine if the preconditioning described above correlated with increased nuclear MTF-1 localization. Following 2hrs on from the initiation of preconditioning, SNAP and paraquat treatments resulted in increased nuclear MTF-1 levels (by $72 \pm 21\%$ and $231 \pm 103\%$ above untreated control levels respectively; n=3, Figures 4.1 B and C). Degraded SNAP showed minimal MTF-1 translocation compared to non-degraded SNAP ($17 \pm 4.9\%$ above untreated control; n=3, Figures 4.1 B and C). Together these results support a role for MTF-1 in NO and O$_2$–induced delayed protection.

**Isoflurane preconditions wild type cultures and promotes nuclear translocation of MTF-1**

Given the above finding that nitric oxide and superoxide generators were able to induce delayed preconditioning and that the protection corresponded with increased nuclear MTF-1 localization, we determined whether a clinically relevant dose of isoflurane would yield similar results. In agreement with earlier reports, preconditioning with a 2 hr, 2.5% isoflurane exposure provided significant levels of protection against 4hr oxygen-glucose deprivation ($29.8 \pm 2.2\%$; $p < 0.001$; n=15) compared to non-preconditioned cultures (Figure 4.2 A). Also in agreement with previous results, the inducible nitric oxide inhibitor, aminoguanidine and the superoxide dismutase mimetic, MnTBAP, diminished isoflurane-mediated protection to $5.8 \pm 4.9\%$ ($p = 0.47$; n=18) and $6.8 \pm 4.8\%$ ($p = 0.28$; n=14) of control levels. Similar to findings with pharmacologic nitric oxide and superoxide donors, Western blot analysis
showed isoflurane treatment corresponded with increased nuclear localization of MTF-1 (55 ± 2.4% above untreated controls; n=3). Pretreatment with aminoguanidine and MnTBAP reduced these nuclear MTF-1 levels to -6.0 ± 7.3% (n=3) and 20 ± 2.9% (n=3) above control levels respectively (Figures 4.2 B and C). These data support a role for MTF-1 in NO and \( O_2^- \)-induced delayed protection as a result of isoflurane exposure.

**Knockout of MT-I + II diminishes isoflurane, nitric oxide, and superoxide-mediated preconditioning and diminishes nuclear MTF-1 levels.**

Metallothioneins I + II have been shown to play a central role in RNS/ROS mediated translocation of MTF-1\(^{123}\) and we recently showed that they are important for the induction of isoflurane-mediated delayed preconditioning \(^{45}\). In these experiments, we wanted to determine if the loss of MT-I + II genes would affect preconditioning by SNAP and paraquat and if this would correlate with nuclear MTF-1 translocation. In agreement with previous isoflurane preconditioning data \(^{45}\), isoflurane, nitric oxide and paraquat provided no significant protection against 4hr OGD compared to untreated controls in metallothionein I + II knockout-derived cultures (0.92 ± 1.8%; n= 22; p = 0.81), (-1.58 ± 2.7%; n=24, p = 0.76), and (-5.6 ± 4.1%; n=14, p = 0.24) respectively (Figure 4.3). Toxicity of the treatments alone in knockout-derived cultures was assessed by LDH following the 72 hr preconditioning window there was no significant toxicity from any of the treatments alone (paraquat 8 ± 4.2% above control levels ; n=8; p=0.21), anesthetic preconditioning (12 ± 8.5% above control levels; n=8; p=0.27) and SNAP (2.0 ± 3.7% below control levels; n=8; p= 0.57; n=8)
Western analysis of nuclear MTF-1 levels in metallothionein knockout cultures showed that nuclear MTF-1 levels were increased 3.0 ± 9.7%, 18 ± 3.0%, and 31 ± 2.7% respectively, above controls (n=3 for each). Though preconditioning appeared to increase nuclear MTF-1 localization, levels were sharply attenuated compared to wild type cultures (Figure 4.3 B and C). Taken together, these data support an important role for metallothioneins I + II both in propagating NO signaling, but also in NO and O$_2$ mediated protective signaling.

**Discussion**

Protection through delayed preconditioning has been demonstrated in variety of tissues$^{40-43,45,136}$ and animal models$^{38,40,45,46,136}$ resulting in improved histological and functional outcomes. Furthermore, the relative safety of anesthetic preconditioning compared to ischemic preconditioning$^6$,$^59$, and the global nature of its protection has contributed to growing interest in the mechanisms of APC. While many aspects of the delayed preconditioning mechanism have been described, there are still gaps in our understanding. Reactive nitrogen species, primarily in the form of nitric oxide$^{40,42,47,50,117}$ and reactive oxygen species such as superoxide$^{49,137}$ have been shown to be important mediators of delayed preconditioning. However, the mechanism by which these molecules are able to confer protection is not well understood. We recently reported that the metal-binding proteins metallothioneins I and II are important for isoflurane-mediated delayed preconditioning$^{45}$. In the current study, we used a dissociated neuronal culture model to examine the hypothesis that metallothioneins I + II play a role in RNS and ROS signaling and protection during delayed anesthetic preconditioning. Using an *in
vitro model allowed us to isolate direct pharmacologic effects of the preconditioning agents while avoiding confounding variables such as changes in hemodynamics, cardiac depression, and other anesthetic related physiological effects. Nevertheless, an in vitro model has limitations. For example, it is not well suited to address system effects such as inflammation, immune response, and drug clearance that may play an important role in long-term outcomes of ischemic injury. Furthermore, in vivo models are better able to evaluate long-term cognitive and neurological outcomes of protection and ischemia. Despite these constraints, in vitro models have been shown by us and others to be useful models for studying the acute effects of anesthetic preconditioning, with similar time courses and levels of protection to in vivo models.

In this study, we determined the following: 1.) As previously reported, isoflurane preconditioning provided significant cellular protection against 4hr OGD while the nitric oxide synthase inhibitor aminoguanidine and the superoxide scavenger MnTBAP diminished the protective effects of isoflurane preconditioning. 2.) Isoflurane treatment corresponded to increased nuclear localization of metal-responsive transcription factor-1 in wild type cultures. Pretreatment of cells with aminoguanidine or MnTBAP diminished the effects of isoflurane preconditioning on MTF-1 nuclear localization. 3.) Preconditioning with the nitric oxide generator, SNAP, (but not the oxidized version of the molecule) or the superoxide generator, paraquat, was able to protect WT-derived cultures against 4hr OGD with time courses and magnitudes of protection similar to isoflurane preconditioning. 4.) As in isoflurane preconditioning, treatment of wild type cultures with SNAP or paraquat induced nuclear translocation of MTF-1. 5.) Knockout of metallothionein I + II genes abrogated the protective effects of all of the
preconditioning agents. 5.) Isoflurane, SNAP and paraquat treated knockout cultures showed increased MTF-1 levels, though to a lesser extent than those seen in wild type cultures. These data support an essential role for metallothioneins I + II both transducing ROS and RNS signaling and protection. While we expected MTF-1 localization to drop to control levels or below in MT knockout experiments, the discrepancy may be due to the presence of another isoform of metallothionein, MT-III, a form found primarily in the brain$^{139,140}$. Though this molecule is typically attributed different functions from those of MT I + II$^{141}$, its structural similarities might contribute to the regulation of MT-I + II synthesis through MTF-1 modulation.

In combination with a number of studies on the mechanisms of nitric oxide signaling$^{123,128,129,131,133}$, our data support a role for metallothioneins I + II as sensors of increased nitric oxide and superoxide during delayed isoflurane-mediated protection. In Figure 4.4, we present a possible model for this pathway. The pathway begins with the generation of nitric oxide by iNOS$^{40,50,142}$ eNOS$^{52,143}$, or a combination of the two following the administration of the preconditioning agent. In the presence of superoxide, nitric oxide will react to form peroxynitrite$^{11}$. Nitric oxide alone$^{122,128,144}$, superoxide alone$^{127,145}$ and peroxynitrite$^{146}$ have each been shown to react with the cysteines that make up a third of the ~60 amino acids that make up metallothioneins I + II. This reaction triggers a confirmational change in metallothionein that results in the release of zinc from one or both its two zinc-binding domains$^{146,147}$. Zinc then complexes with cytoplasmically localized MTF-1, causing the metal response element-binding transcription factor to translocate to the nucleus$^{123,148}$. This translocation may rely on a cytoplasmic interaction with hypoxia-inducible transcription factor-1$^{149}$. Once in the
nucleus, MTF-1 binds the metal response element, inducing the production of apo-metallothionein I + II (aka, thionein)\textsuperscript{85,45}. Apo-metallothionein and the zinc-bound metallothionein then have a broad range of described activities that potentially play a role in preconditioning-mediated protection including buffering cellular zinc concentrations\textsuperscript{150}, buffering cellular redox\textsuperscript{151}, acting extracellularly to activate cellular survival pathways\textsuperscript{121,152}, suppressing local inflammatory response\textsuperscript{91}, modulating mitochondrial respiration\textsuperscript{153}, modulating enzyme activity through zinc modulation\textsuperscript{154}, and protecting mitochondria against oxidative damage\textsuperscript{85}.

This study presents evidence of an important role for metallothioneins I + II as sensors of oxidative stress in anesthetic-mediated delayed preconditioning. Further studies will be important to unravel the mechanisms by which metallothioneins are able to contribute to protection against toxic ischemia.
Figure 4.1. The nitric donor SNAP and the superoxide generator paraquat precondition wild type cultures and induce nuclear localization of MTF-1. A.) 2hr treatment with sublethal doses of the nitric oxide donor SNAP or the superoxide generator paraquat provide significant protection against 4hr oxygen-glucose deprivation 72 hours following preconditioning. * Significantly different from control (p < 0.05). ** Significantly different from fresh SNAP (p < 0.05). B.) SNAP and paraquat, but not the degraded form of SNAP induce increased nuclear localization of MTF-1. C.) Semiquantitative analysis of nuclear MTF-1 levels from Western blots (percent above control ± SEM, n=3).
Figure 4.2. Isoflurane preconditioning corresponds with increased MTF-1 localization. A.) Preconditioning with isoflurane provided significant protection from 4hr oxygen glucose deprivation in wild type-derived cultures. Co-incubation with the inducible nitric oxide inhibitor, aminoguanidine, diminished isoflurane mediated protection to non-significant levels. The superoxide dismutase mimetic MnTBAP also diminished isoflurane mediated protection. * Significantly different from control p < 0.05. ** Significantly different from isoflurane treatment alone (p< 0.05) B.) 2 hr isoflurane preconditioning increased nuclear levels of the metal-responsive transcription factor-1. Aminoguanidine and MnTBAP, abrogated isoflurane-mediated increases in nuclear MTF-1. C.) Semi-quantitative nuclear MTF-1 levels from Western blots (percent above or below control ± SEM, n=3).
Figure 4.3. Metallothionein I + II knockout abrogates protection from isoflurane, nitric oxide and superoxide preconditioning, but does not block MTF-1 translocation. A.) Cultures from MT-I + II knockouts showed no significant protection from preconditioning with isoflurane, nitric oxide or superoxide. B.) Knockout of metallothioneins I + II attenuated nuclear translocation of MTF-1 in isoflurane, nitric oxide and superoxide treatment treated cultures. C.) Semi-quantitative nuclear MTF-1 levels (percent above control ± SEM, n=3) from Western blots. Wild-type data from Figures 4.1 B and 4.2 B are included for comparison with MTKO results.
Figure 4.4. Simplified model for nitric oxide signaling involving metallothioneins.

1.) iNOS, eNOS or both activity increases in response to isoflurane treatment 40,50,52,142,143

2.) Nitric oxide will combine with superoxide if it is present 41.

3.) Nitric oxide, superoxide and/or peroxynitrite 146 react with metallothionein cysteines to form sulphydryl groups. In the process, metallothionein conformation is changed such that it releases zinc ions 146,147.

4.) Metal responsive transcription factor-1 binds with zinc and translocates to the nucleus 123,148. This process may require interaction with hypoxia-inducible factor 1 149.

5.) Once in the nucleus, MTF-1 binds to metal response element, triggering the transcription of metallothionein I + II genes 85.

6.) APO metallothionein (aka thionein) in the cytoplasm will bind with labile zinc to form metallothionein and confers protection against OGD by a yet to be determined mechanism. This process may require interaction with hypoxia-inducible factor 1 149.

5.) Once in the nucleus, MTF-1 binds to metal response element, triggering the transcription of metallothionein I + II genes 85. 6.) APO metallothionein (aka thionein) in the cytoplasm will bind with labile zinc to form metallothionein and confers protection against OGD by a yet to be determined mechanism.
CHAPTER 5

FUTURE DIRECTIONS

In the course of this dissertation, I was not able to address what may end up being one of the more interesting questions of the metallothionein/preconditioning story: What is the mechanism by which metallothioneins are able to protect against ischemic injury? The road to answer to this question will undoubtedly be interesting, but also challenging. It has been ~50 years since Margoshes and Vallee isolated and Kagi and Vallee characterized metallothioneins derived from horse kidney that had been exposed to high levels of cadmium\(^{155}\). Since that time metallothioneins have been extensively studied, and despite thousands of studies, a definitive and comprehensive role for metallothioneins in either normal or pathological physiology has yet to emerge\(^ {156}\). Yet, as reviewed by Penkowa\(^ {85}\), metallothioneins have been shown to participate in a number of processes involved with cellular protection in the brain. Major biological roles for metallothioneins, as depicted in Figure 5.1, include modulation of inflammation, control of cellular oxidation levels, support of tissue repair and remodeling, angiogenesis, cell cycle and proliferation, and neuronal survival and plasticity\(^ {85}\). And while the above roles are described for brain, metallothioneins I + II have also been shown to be protective in other tissues including heart\(^ {157}\), liver\(^ {158}\), kidney\(^ {112}\) and intestine\(^ {159}\), suggesting the possibility that preconditioning induced metallothioneins may play an important role in the protection of other tissues.

One of the more interesting questions in regards to metallothionein-mediated protection, especially in the brain, is the provenance of the metallothioneins that provide
protection. While the vast majority of the literature describing metallothionein-mediated protection focuses on intracellular metallothioneins, there is a growing literature supporting a protective role for extracellular metallothioneins. Exogenous metallothioneins have been shown to reduce inflammation, inhibit apoptosis, and improve remyelination in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. Furthermore, exogenous MT I + II administration was beneficial in an experimental model of traumatic brain injury. The acute injuries sustained in the core of the traumatic lesion by the MT I + II–treated and control groups were largely the same. However, in animals treated with subcutaneous MT I + II, the tissue surrounding the core lesion showed less oxidative stress, neurodegeneration and apoptotic cell death in the days and weeks that followed. MT-I + II treatment also improved cellular repair, with increased expression of growth factors, improved astrogliosis, and angiogenesis. Furthermore, the necrotic lesion cavity showed enhanced reorganization in MT treated subjects than in controls. Recent work by Amborn et al., suggests a possible mechanism for exogenous metallothionein-mediated protection. They found that extracellular metallothioneins interact with plasma membrane megalin receptors to trigger cellular protection through a signaling pathway that involved extracellular signal-regulated kinase, protein kinase B and c-AMP response element binding protein. Finally, they found exogenous metallothioneins inhibited apoptosis by reducing expression of the proapoptotic B-cell leukemia/lymphoma-2 interacting member of cell death (Bim(S)). In order to understand how metallothioneins are protective in the context of delayed anesthetic preconditioning, it will be important to determine where metallothioneins I + II are acting. If
metallothioneins are acting intra-cellularly, then approaches to increase metallothionein expression might prove beneficial for prevention of ischemic injury in settings where there is a known risk. On the other hand, since exogenous xenobiotic metallothioneins appear to be well-tolerated, the finding that extra-cellular metallothioneins can protect against ischemic injury would open the door to a range of potential treatment avenues including metallothioneins, metallothionein mimetics and so forth.

Other questions that will be interesting to pursue include, but are not limited to:  
1.) How does metallothionein’s role in zinc metabolism influence cellular signaling and protection? Is it metallothionein or its modulation of available zinc, or both that determine protection? 2.) How do metallothionein’s antioxidant characteristics contribute to preconditioning. Do they act to simply detoxify existing oxidants, or do they act to inhibit the production of them in the first place? 3.) Can exogenous metallothioneins be used to protect against ischemic injury both in vitro and in vivo? Can synthetic MT mimetics be used to greater effect? 4.) In the context of preconditioning, what is the role of metallothioneins in modulating inflammatory response? and 5.) What role do metallothioneins I + II play in preconditioning-mediated protection of other tissues?

In the work described above, we have established an important role for metallothioneins I + II in delayed anesthetic mediated protection against ischemic injury. In the years to come, there is much work to be done to better understand how metallothioneins act both on their own, and in concert with other molecules involved in preconditioning. By improving this understanding of these processes, we hope to contribute to improved clinical outcomes for patient subject to ischemic injury.
Figure 5.1. Summary of the major biological roles of metallothioneins I + II. Figure modified from Penkowa.
CHAPTER 6

CONCLUSION

Delayed anesthetic preconditioning is a growing field of study for protection against ischemic injury due to its promise of a multi-pathway approach, multi-tissue protection and the relative safety of administration. This dissertation described three projects aimed at furthering our knowledge of the mechanisms involved in phenomenon.

In our first study, we hypothesized that, given the complexity of the ischemic cascade, it was likely that there were molecules and pathways involved in preconditioning that had not yet been identified. Using DNA microarray we were able to identify genes that were differentially expressed in rat liver, kidney, and heart 90min following a clinical exposure to the commonly used volatile anesthetic, isoflurane. Given that preconditioning has been reported in a wide range of tissues and organisms, we were able to combine and reduce the individual tissue lists to reflect those genes that were differentially regulated in more than one tissue and select for genes potentially involved in conserved endogenous protection pathways. This combined approach yielded a list of 34 genes that we subjected to a more in-depth literature review. Using this approach we were able to identify a number of interesting genes for further study including metallothioneins I + II, basigin, and interferon regulatory factor 1.

For the second study, I selected metallothioneins I + II for further study, two genes that were both highly differentially regulated in multiple tissues and had literature support for potential as protective agents. In this study we established the viability of using primary cultures in concert with oxygen-glucose deprivation to study delayed
anesthetic preconditioning. From these experiments it was determined that our *in vitro* model displayed levels of protection and protection time-courses that were very similar to those reported for *in vivo* models. Once the model was established, I was able to: 1.) establish a time-course for metallothionein I + II expression in response to isoflurane treatment, 2.) show that both neurons and glia are protected during delayed APC and 3.) show that neurons were both more susceptible to oxygen-glucose deprivation toxicity and showed greater protection from preconditioning. 4.) establish, through the use of knockdown and knockout studies, that metallothioneins are integrally involved in isoflurane-mediated delayed protection and 5.) demonstrate through direct transfection of exogenous metallothionein protein that metallothioneins likely act as end effectors of protection against ischemic injury.

Finally, in the third study, I addressed the mechanism of metallothionein action in the context of delayed preconditioning. Dirnagl, in his 2008 review of delayed preconditioning, categorized molecules involved in preconditioning as being sensor, transducer, or effector molecules 91. From our investigations in the second study, it seemed likely that metallothioneins act as effector molecules whose action is directly involved in protection. Although the knockdown and knockout studies could arguably support any of these roles for metallothionein, the experiment in which cells were transfected and protected by exogenous metallothionein immediately before OGD supports a likely role for MT I + II as an end effector. The literature however, indicated another possible role for metallothioneins as sensors of oxidative stress 123,128,129,161,163. In order to determine if metallothioneins I + II occupy this role in the context of delayed preconditioning, I correlated isoflurane, nitric oxide and superoxide-mediated protection
with the nuclear translocation of the metallothionein transcription factor MTF-1. From these studies, I was able to determine that: 1.) blocking NO or \( \cdot O_2 \) production during APC decreased both protection and nuclear localized MTF-1. 2.) The NO and \( \cdot O_2 \) generators alone were sufficient to precondition mixed neuronal cultures and that this too, corresponded with increased nuclear localization of MTF-1. 3.) Finally, knockout of metallothionein I + II genes not only eliminated protection induced by NO and \( \cdot O_2 \) but also diminished preconditioning-mediated increases in nuclear MTF-1 levels. Together, these data supported a role for metallothioneins I + II in delayed preconditioning as both sensors of oxidative stress and as end effectors of protection.

In conclusion, the three studies described above contribute to our understanding of the mechanisms of delayed anesthetic preconditioning. These studies, along with future experiments will lead to a better understanding of the mechanisms involved in endogenous protection and give us clues as to how to harness these pathways to provide improved clinical outcomes.


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