ABSTRACT

Title of Dissertation: Sulforaphane inhibits redox-regulated permeability transition and provides neuroprotection following cardiac arrest

Tiffany Greco, Doctor of Philosophy, 2011

Dissertation Directed by: Dr. Gary Fiskum, Ph.D. Matjasko Professor for Research in Anesthesiology and Vice-Chair for Research, Department of Anesthesiology, Department of Biochemistry and Molecular Biology and Department of Pharmacology and Experimental Therapeutics

Program in Molecular Medicine

Exposure of mitochondria to oxidative stress and elevated $\text{Ca}^{2+}$ promotes opening of the mitochondrial permeability transition pore, resulting in mitochondrial membrane depolarization, metabolic failure, and necrotic cell death. Pharmacologic activation of the Nrf2/ARE pathway of antioxidant gene expression by sulforaphane provides cytoprotection, but little is known about the effects of this pathway on mitochondrial responses to stress. This study tested two hypotheses: 1. Administration of sulforaphane increases the resistance of mitochondria from cells and tissues to permeability transition pore opening caused by oxidative stress. 2. Treatment of animals with sulforaphane following cardiac arrest reduces hippocampal neuronal death and improves neurologic outcome. Hypothesis 1. Sulforaphane or drug vehicle was administered to rats
intraperitoneally 40 hr prior to isolation of brain or liver mitochondria. Cultured PC12 cells were exposed to sulforaphane or vehicle for 24 hr. Fluorescent measurements of mitochondrial Ca^{2+} uptake and subsequent release induced by the oxidant tert-butyl hydroperoxide were performed using isolated brain and liver mitochondria and digitonin permeabilized PC12 cells, while simultaneously monitoring NAD(P)H autofluorescence. Sulforaphane treatment substantially reduced the rate of cyclosporin A-sensitive Ca^{2+} release induced by tert-butyl hydroperoxide by 25% in brain mitochondria and 50% in both liver mitochondria and PC12 cells, but only inhibited NAD(P)H oxidation in liver mitochondria. Sulforaphane treatment significantly increased peroxide detoxification, glutathione, and expression of glutathione peroxidase, malic enzyme, and thioredoxin in liver mitochondria. Hypothesis 2. Anesthetized beagles were subjected to 10 min cardiac arrest followed by resuscitation and critical care. At 30 min reperfusion, dogs received either sulforaphane or drug vehicle, intravenously. At 23 hr animals were awakened and a neurologic deficit test performed. Animals were euthanized at 24 hr; hippocampal neurons and markers of oxidative stress were quantified. Sulforaphane treatment improved neurologic outcome and reduced neuronal death and DNA oxidation. We conclude that sulforaphane administration after cardiac arrest significantly reduces hippocampal neuronal death and oxidative stress and improves short-term neurologic outcome. As permeability transition pore opening plays an important role in many examples of acute cell death, the ability of sulforaphane to confer resistance of mitochondrial to permeability transition pore opening may contribute to the cytoprotective properties of sulforaphane.
Sulforaphane inhibits redox-regulated permeability transition and provides neuroprotection following cardiac arrest

By
Tiffany Greco

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LIST OF ABBREVIATIONS

Aβ: amyloid beta
ABG: arterial blood gas
AD: Alzheimer’s disease
ANT: adenine nucleotide translocase
ARE: antioxidant response element
BBB: blood brain barrier
BHA: butylated hydroxytoluene
CG5N: calcium green 5N
CNS: central nervous system
CO: carbon monoxide
CSA: cyclosporine A
CyD: cyclophilin D
ER: endoplasmic reticulum
ETC: electron transport chain
FFA: free fatty acid
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GPX: glutathione peroxidase
GSH: reduced glutathione
GSSG: oxidized glutathione
GST: glutathione s-transferase
HCV: hepatitis C virus
HO-1: heme oxygenase 1
IDH: isocitrate dehydrogenase

iTRAQ: isobaric tag for relative and absolute quantitation

MAPK: mitogen activated protein kinase

ME3: malic enzyme 3

MMP9: matrix metalloprotease 9

NCX: sodium calcium exchanger

NDS: neurologic deficit score

NMDA: N-Methyl-D-aspartate

NO: nitric oxide

NQO1: NAD(P)H:quinone oxidoreductase 1

NRF2: NF-E2-related factor 2

O2⁻: superoxide

OAA: oxaloacetate

OGD: oxygen glucose deprivation

ONOO⁻: peroxynitrite

PARP1: Poly [ADP-ribose] polymerase 1

PD: Parkinson’s disease

PDHC: pyruvate dehydrogenase complex

PhAsO: phenylarsine oxide

PI3K: phosphoinositide 3-kinase

PiC: phosphate carrier

PKC: protein kinase C

PTP: permeability transition pore
RCR: respiratory control ratio

ROS/RNS: reactive oxygen/nitrogen species

SFP: sulforaphane

sMAF: small musculoaponeurotic fibrosarcoma oncogene homolog

SOD: superoxide dismutase

TBHQ: tert-butylhydroquinone

tBOOH: tert-butylhydroperoxide

TMRM: tetramethylrhodamine methyl ester

TH: transhydrogenase

TRX: thioredoxin

VDAC: voltage dependent anion channel
CHAPTER 1: INTRODUCTION

Citation: Adapted from Greco, T., Fiskum, G. J Bioenerg Biomembr., 2010; 42 (6):491-497.

I. OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTION IN NEURODEGENERATION

Figure 1.1 summarizes many of the known targets of reactive oxygen and nitrogen species (ROS/RNS) that are located in mitochondria or that are extramitochondrial but have strong secondary effects on mitochondrial bioenergetic or apoptotic activities. The most acute influence of ROS/RNS on mitochondria is mediated by oxidative modifications of proteins present in the electron transport chain (ETC) (Brown and Borutaite, 2004; Keeney et al., 2006) other metabolic proteins, e.g., pyruvate dehydrogenase and aconitase (Poon et al., 2006; Richards et al., 2006), and the permeability transition pore (PTP) (Battaglia et al., 2010; Petronilli et al., 1994). Oxidation of cardiolipin, a phospholipid primarily located in the mitochondrial inner membrane, can also cause rapid mitochondrial functional alterations, including stimulated release of mitochondrial apoptotic proteins (Bayir et al., 2006; Ott et al., 2007). Oxidation of mitochondrial mRNA can limit the expression of any one of the 13
polypeptides coded for by the mitochondrial genome, resulting in delayed bioenergetic impairment. (Nunomura et al, 2009)

Figure 1.1: Mitochondrial targets of reactive oxygen and nitrogen species and their effects on mitochondrial bioenergetic and apoptotic activities.

Metabolic depression and increased ROS production also occurs in response to mitochondrial DNA oxidation, which is associated with neurodegenerative disorders and normal aging (Lovell and Markesbery, 2007). Finally, ROS/RNS are known to stimulate the activity of poly-ADP ribose polymerase 1 (PARP1) and the expression of P53, which in turn can cause release of mitochondrial apoptotic proteins and poly-ADP ribosylation of mitochondrial proteins (Chipuk et al., 2004; Lai et al., 2008; Mattson, 2003; Wang et al., 2009).
Several lines of evidence support the hypothesis that oxidative stress and associated mitochondrial bioenergetic dysfunction and activation of apoptosis are common etiological factors in many neurodegenerative diseases and acute disorders of the central nervous system (CNS). Descriptive experimental support includes the findings that mitochondrial morphology is altered, that metabolic activities are depressed and that mitochondrial pro-apoptotic proteins are released to the cytosol prior to the death of neurons and other brain cells in both animal and cell culture models of neurodegeneration (de la Monte and Wands, 2006; Gogvadze et al., 2009; Knott and Bossy-Wetzel, 2008; Moreira et al., 2010; Swerdlov, 2009; Yap et al., 2009). Biochemical markers of oxidative stress often exhibit close tempo-spatial relationships with these indicators of mitochondrial dysfunction (Kauppinen and Swanson, 2007; Niizuma et al., 2009; Nunomura et al., 2009; Pope et al., 2008). Moreover, agents or conditions that either decrease the production of reactive oxygen or nitrogen species or increase their detoxification both ameliorate the mitochondrial functional anomalies and provide protection against subsequent cell death and neurologic impairment. Such agents include novel antioxidants that are both lipophilic and that have a net positive charge, enabling them to be selectively accumulated within energized mitochondria, which possess a negative inside membrane potential (Murphy, 2008; Sheu et al., 2006).

Ambient oxygen is one fundamental environmental factor that influences mitochondrial oxidative stress. For example, hyperoxic reperfusion immediately after global cerebral ischemia increases protein and lipid oxidation, impairs mitochondrial respiration and hippocampal cerebral energy metabolism, exacerbates delayed neuronal death, and worsens neurologic outcome (Balan et al., 2006; Fiskum et al., 2008; Richards
et al., 2007). In contrast to interventions utilizing exogenous antioxidants, avoiding unnecessary hyperoxia under conditions where cells are particularly vulnerable to oxidative stress likely improves outcome by reducing the production of ROS/RNS, by simply restricting the concentration of O$_2$ available for reactions that produce superoxide and nitric oxide (Allen et al., 2009). Hypoxia can also promote mitochondrial oxidative stress when the concentration of O$_2$ is below the level necessary for sustaining normal respiration. Hypoxic oxidative stress is promoted by nitric oxide, which competes with O$_2$ at cytochrome oxidase, the terminal reaction of the ETC. This form of respiratory inhibition causes a reduced shift in the oxidation/reduction state of ETC redox centers capable of reducing O$_2$ to superoxide, increasing the production of this free radical and its metabolites, resulting in oxidative stress, even at very low O$_2$ levels (Moncada and Bolanos, 2006).

In addition to the correlative evidence provided by comparisons between markers of oxidative stress, mitochondrial dysfunction, and cell death or neurologic outcome, genetic manipulation of proteins involved in both the production and detoxification of ROS/RNS and important mitochondrial targets of oxidative stress has provided independent evidence for their pathophysiological importance. For instance, overexpression of certain mitochondrial uncoupling proteins appears to both reduce production of ROS and provide neuroprotection (Conti et al., 2005; Kim-Han and Dugan, 2005; Mattiasson et al., 2003; Sullivan et al., 2004); however, a direct link between respiratory uncoupling and these two activities has not been proven conclusively (Johnson-Cadwell et al., 2007). Stronger molecular mechanistic evidence comes from knockouts and overexpression of the mitochondria-specific manganese superoxide
dismutase (SOD2). Genetically modified mice that overexpress or are deficient in SOD2 display resistance or vulnerability, respectively, to both oxidative stress and neurodegeneration in models of Alzheimer’s and Parkinson’s disease and stroke (Andreassen et al., 2001; Chan, 2005; Esposito et al., 2006). An example of a critically important mitochondrial target of oxidative stress is the inner membrane permeability transition pore (Mazzeo et al., 2009), as discussed in more detail below. Genetically modified mice that do not express cyclophilin D (CyD), a protein that promotes activation of the PTP, are resistant to neuronal death in models of stroke and multiple sclerosis (Forte et al., 2007; Schinzel et al., 2005). Moreover, cells from CyD knockout mice are much more resistant to death induced by hydrogen peroxide than those from wild-type animals, indicating a role for CyD and the PTP in cell death induced by oxidative stress (Schinzel et al., 2005).

Examples of experimental manipulation of antioxidant genes and mitochondrial targets of oxidative stress that confirm the role of oxidative stress in neurodegenerative disease are modest compared to the large scale of antioxidant and other cytoprotective genes that are both induced by endogenous oxidative stress and by exposure of cells or animals to certain drugs and environmental conditions. A master regulator of this system is the transcriptional activating factor, Nrf2.

II. THE NRF2/ARE SYSTEM

A. Nrf2/ARE regulation

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a nuclear transcription factor that belongs to the “cap-‘n collar” family that share a conserved basic leucine zipper (bZip) structure (Jaiswal, 2004). Under normal conditions, Nrf2 is kept inactive by being
bound to Kelch like ECH-associated protein (KEAP 1). KEAP 1 is localized in the cytosol where it is bound to the actin cytoskeleton and targets Nrf2 for proteosomal degradation by being a Cul3-based E3 ligase adaptor. In the presence of ROS/RNS or certain electrophilic organic compounds, specific cysteine residues on KEAP 1 are oxidized, causing a conformational change in KEAP 1 and the release of Nrf2 into the cytosol. Oxidative stress also activates specific protein kinases, e.g., protein kinase C, which serine phosphorylates Nrf2, facilitating dissociation from KEAP1 and enabling transport of Nrf2 into the nucleus. Electrophiles can also oxidize critical cysteine sulfhydryl groups present on Nrf2, masking the nuclear export signal sequence and allowing Nrf2 to remain within the nucleus long enough to activate gene transcription. Within the nucleus, Nrf2 forms heterodimers with sMAF proteins that also stabilize nuclear retention. These heterodimers bind to antioxidant response element (ARE) sequences and recruit transcriptional enzymes and other proteins to these locations (Li et al., 2008). Since an ARE sequence is located proximal to the Nrf2 gene, Nrf2 activation acts in a positive, feed-forward manner (Yu and Kensler, 2005). To prevent continuous transcriptional activation of this and many other genes, nuclear tyrosine kinases phosphorylate Nrf2, stimulating its translocation out of the nucleus back to the cytosol (Yu and Kensler, 2005).

**B. Transcriptional regulation by Nrf2**

While Nrf2 is widely known for its regulation of phase II detoxification enzymes, it has transcriptional control over many genes that include an ARE sequence in their promoter region. Some of the commonly known genes that Nrf2 regulates include NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), glutathione S-
transferases (GST), glutathione synthetic enzymes, antioxidant enzymes, and NADPH regenerating enzymes like glucose-6-phosphate dehydrogenase and malic enzyme (Thimmulappa et al., 2002). In various proteomic studies, Nrf2 has been shown to control genes involved in immunity, membrane transport, cell adhesion, cell cycle, and energy metabolism, among others (Hu et al., 2006b). Numerous kinases and phosphatases are also under the regulation of Nrf2, including mitogen activated protein kinase (MAPK), serine kinases and tyrosine phosphatases (Hu et al., 2006b). Several Nrf2-regulated genes code for important regulators of cellular energy metabolism, including the mitochondrial enzymes pyruvate dehydrogenase (lipoyamide) β and pyruvate dehydrogenase kinase. Nrf2 also controls expression of aquaporin 4, multiple classes of ATPases, chloride, calcium and potassium channels and the folate transporter (Hu et al., 2006b; Thimmulappa et al., 2002).

III. INDUCTION OF THE NRF2/ARE PATHWAY

A. Pharmacologic activation of the Nrf2/ARE pathway

This field of research grew from the observation that when mice were fed with chow containing both carcinogens plus the electrophilic preservative butylated hydroxyanisole (BHA), they were protected from stomach cancer that was induced by the carcinogens in the absence of BHA (Wattenberg et al., 1980). Subsequently, it was determined that this inducible xenobiotic response was mediated by phase II detoxification enzymes and that the Nrf2/ARE pathway was responsible for inducing the expression of genes coding for these proteins (Yu and Kensler, 2005). This discovery lead to the search for and identification of compounds that could safely activate the
Nrf2/ARE pathway of gene expression (Ramos-Gomez et al., 2001; Yu and Kensler, 2005).

**B. Electrophilic activation**

Sulforaphane is a compound derived from a glucosinolate found in cruciferous vegetables such as broccoli and kale in the *Brassica* genus. When these foods are consumed, the salivary enzyme myrosinase converts the inactive glucosinolate, glucoraphanin, into its active isothiocyanate form, sulforaphane (see Fig. 1.2).

**Figure 1.2:** Structure of sulforaphane and its activation of Nrf2/antioxidant response element (ARE) pathway of gene expression.

Two primary mechanisms of activation include oxidation of specific cysteine sulfhydryl groups on KEAP1, e.g., by reaction with sulforaphane, and serine phosphorylation of Nrf2 by enzymes including protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K).
The central carbon of its electrophilic cyanate group, –N=C=S, reacts with nitrogen-, sulfur- and oxygen-based nucleophilicities (Fahey and Talalay, 1999). Following rapid uptake of sulforaphane into tissues and cells, this cyanate group reacts rapidly with three critical cysteines, C\textsuperscript{151} C\textsuperscript{273} C\textsuperscript{288}, on KEAP 1, causing a conformational change that reduces its affinity for Nrf2. Nrf2 is released into the cytoplasm, enters the nucleus, and activates ARE-driven genes (Dinkova-Kostova et al., 2002). While traditional focus has been on KEAP 1 as the redox sensor mediating Nrf2 translocation, Nrf2 itself may be the key redox sensor and thus control its own nuclear translocation (Li and Kong, 2009). Central to this model is a single cysteine, C\textsuperscript{183}, present within the nuclear export signal sequence located in the transactivation domain (NES\textsubscript{TA}) of Nrf2. Experiments using expression of a glial fibrillary acidic protein-tagged, truncated Nrf2 containing the NES\textsubscript{TA} demonstrate dose-dependent translocation of the protein by SFP. When this cysteine is mutated to an alanine, the protein is unable to translocate to the nucleus, thus identifying C\textsuperscript{183} as a critical redox sensor on Nrf2. In summary, SFP oxidatively modifies KEAP 1 and reduces its affinity for Nrf2. Nrf2 released into the cytosol is then oxidized by sulforaphane, promoting its entry into the nucleus (Li and Kong, 2009). Other electrophilic compounds that appear to act by the same mechanism as that of sulforaphane include carnosic acid and curcumin (Li and Kong, 2009).

C. Activation of Nrf2 by upstream phosphorylation events

In addition to redox modulation of KEAP1 and Nrf2, phosphorylation of Nrf2 facilitates its stabilization and translocation into the nucleus (Jaiswal, 2004). Treatment of various cell lines with either tert-butyl hydroquinone (TBHQ) (Jaiswal, 2004) or plumbagin (Son et al., 2010), a pigment extracted from the Plumbaginaceae family of
flowering plant that contains anti-microbial properties, results in activation of the PI3K/AKT signaling cascade, causing phosphorylation and activation of Nrf2 (Son et al., 2010). If PI3K inhibitors wortmannin or LY294002 are present, Nrf2 translocation is abrogated (Son et al., 2010; Wang et al., 2008). Protein kinase C (PKC) mediates phosphorylation of Nrf2 at serine 40 (Kaspar et al., 2009). While phosphorylation of serine 40 is not required for translocation of Nrf2 into the nucleus, it plays a critical yet still undefined role in the interaction between KEAP1 and Nrf2 (Kaspar et al., 2009). One model states that phosphorylation of Nrf2 is absolutely required for release of Nrf2 from KEAP1 (Kaspar et al., 2009). An alternative model contends that phosphorylated free Nrf2 is unable to bind KEAP1 therefore stabilizing Nrf2 and contributing to the increase of free Nrf2 in the cytoplasm (Niture et al., 2009; Yu and Kensler, 2005).

IV. ROLE OF NRF2 IN NEUROPROTECTION

A. Pharmacologic activation of Nrf2 is neuroprotective

On average, the brain consumes 20% of the O₂ consumed by the human body, despite representing only 2% of the adult body weight (Bader, 2006). This extraordinary high rate of aerobic energy metabolism is associated with a relatively high rate of ROS formation (Dringen et al., 2000). Normally the ROS formation is balanced under normal conditions and detoxified by endogenous antioxidants. During brain injury or neurodegenerative disease there is increased ROS production that overwhelms the endogenous detoxification systems and creates oxidative stress (Piantadosi and Zhang, 1996). Neurons with their high metabolic need and low levels of endogenous antioxidants are particularly vulnerable (Hogler et al., 2010). ROS damage lipids, proteins and DNA (Uttara et al., 2009), potentially leading to cell death by apoptosis or necrosis (Lin and
Beal, 2006). Past approaches toward inhibiting such damage have used exogenous antioxidants capable of detoxifying a single free radical specie (Uttara et al., 2009). New pharmacologic approaches aim to target an array of endogenous antioxidant defenses via the Nrf2/ARE pathway. Temporarily enhancing the cell’s endogenous antioxidant defense system should detoxify all species of radicals and restore redox balance to the cell and thus prevent cell death. One compound, SFP, has already shown great promise in boosting endogenous antioxidants. In multiple models of brain injury and neurodegeneration SFP has demonstrated neuroprotective properties (Dash et al., 2009; Siebert et al., 2009; Zhao et al., 2006).

B. Acute CNS injury

Several animal models have demonstrated neuroprotection with post-treatment of sulforaphane following brain injury. In a rat model of intracerebral hemorrhage, administration of sulforaphane was followed by translocation of Nrf2 to the nucleus, an increase in mRNA levels for superoxide dismutase 1 (SOD1), GST and NQO1, and an improvement in neurologic outcome (Zhao et al., 2007b). In a rat focal ischemia stroke model, sulforaphane reduced brain infarct volume and increased the levels of both HO-1 mRNA and protein (Zhao et al., 2006). Similarly, post-treatment of rats with sulforaphane after traumatic brain injury resulted in an increase in mRNAs for various phase II response enzymes and improved memory and cognition (Dash et al., 2009; Zhao et al., 2005; Zhao et al., 2007a). Pre- or post-treatment of cells with SFP using in vitro models of oxygen and glucose deprivation (OGD) with either primary rat cortical astrocytes or mouse hippocampal neurons resulted in improvement in cell survival, reduction in oxidative stress and upregulation of phase II enzymes (Danilov et al., 2009;
Soane et al., 2010). In a spinal cord injury model that utilizes an organotypic spinal cord culture, results showed that pharmacologic upregulation of Nrf2 prevented motor neuron cell death, upregulated both Nrf2 and HO-1, reduced toxic extracellular levels of glutamate and preserved mitochondrial ultra-structure (Liu et al., 2008). In summary, pharmacologic activation of Nrf2-mediated gene expression provides neuroprotection in several in vitro and in vivo models of acute injury to the CNS.

C. Neurodegenerative disease

Oxidative stress has been implicated in mechanisms that contribute to neuronal cell death in many neurodegenerative diseases including Alzheimer’s disease (AD) and Parkinson’s disease (PD) (Uttara et al., 2009). These diseases both exhibit aggregated proteins in affected cells. Modification of proteins by ROS may make them unable to be degraded by the proteasome and may trigger a misfolded protein stress response (Uttara et al., 2009). Also contributing to the oxidative stress in these disorders is the natural decline in endogenous antioxidant defenses that occur with aging (Gilmer et al., 2010). In AD, amyloid β (Aβ) is capable of interacting with metals in the brain and creating more ROS (Hureau and Faller, 2009). In PD, which is characterized by the loss of dopaminergic neurons in the substantia nigra, ROS are produced by dysfunctional mitochondria and abnormal dopamine metabolism which contribute to neuronal cell death (Sherer et al., 2003). Since oxidative stress is a contributing factor in neurodegenerative disorders, enhanced levels of endogenous antioxidants may either halt or slow the progression of these diseases. Pre-treatment of neuroblastoma cell lines with SFP significantly increased neuronal survival against Aβ mediated toxicity. The cytoprotection against Aβ toxicity was attributed to SFP mediated upregulation of
proteosomal subunits. With increased levels of proteasomes, Aβ was able to be digested and not invoke cell death (Park et al., 2009). It has yet to be determined whether SFP treatment enhances endogenous antioxidants in Aβ toxicity models. SFP may also enhance blood brain barrier (BBB) integrity by increasing expression of tight junction proteins (Zhao et al., 2007a), which may also play a role in preventing Aβ toxicity (Chen et al., 2010). In other Aβ toxicity models, TBHQ has been used to upregulate the Nrf2/ARE pathway and in these experiments, endogenous antioxidants are enhanced and are attributed to the neuroprotection observed (Johnson et al., 2008). In in-vitro models of PD, pretreatment of either dopaminergic cell lines or nigrostriatal co-cultures with SFP provided cytoprotection following administration of H2O2 or 6-hydroxydopamine (Siebert et al., 2009; Tarozzi et al., 2009). The increase in NQO1 is able to detoxify the abnormally high levels of quinone produced from aberrant dopamine metabolism. Increased levels of glutathione (GSH) and phase II enzymes allowed cells to detoxify other forms of radicals compared to vehicle treatment (Tarozzi et al., 2009).

V. REDOX REGULATION IN THE MAMMALIAN BRAIN

A. Detoxification mechanisms in the brain

As noted above, the mammalian brain has much higher aerobic energy demand than other organs in the body. A side product of mitochondrial respiration is superoxide. Superoxide is further metabolized to species that are either less or more toxic. Superoxide can react with nitric oxide to produce the stable and highly toxic radical, peroxynitrite. Superoxide generated by mitochondria is detoxified to form the less toxic radical, H2O2, by superoxide dismutase 2 (SOD2) and further detoxified to H2O by glutathione peroxidase (GPx) (Chaudiere and Ferrari-Iliou, 1999). SOD is a key cellular anti-oxidant
and has three isoforms; SOD1 is cytoplasmic, SOD2 is located in the mitochondria and SOD3 is extracellular (Valdivia et al., 2009). Reduced glutathione donates the electrons needed by GPx to reduce H$_2$O$_2$ to H$_2$O and O$_2$. GSH is oxidized to glutathione disulfide (GSSG). GSSG is then reduced to GSH by glutathione reductase (Dringen et al., 2000). The reducing power necessary for this is provided by NAD(P)H-generating enzymes such as mitochondrial and cytosolic isoforms of malic enzyme and isocitrate dehydrogenase (Kowaltowski et al., 2009). The cell has other inducible defenses such as NQO1 and HO-1 (Dringen et al., 2005).

B. Glutathione

Glutathione (GSH) has been long implicated in cellular redox homeostasis. GSH normally exists primarily in the reduced form (GSH), but shifts to its oxidized form, GSSG, during times of cellular stress. The proportion of reduced to oxidized is thought to be about 5:1 (Ostergaard et al., 2004). GSH is maintained by de novo synthesis, cellular GSH uptake and reduction of GSSG. GSH exists in distinct pools in the endoplasmic reticulum (ER), nucleus, cytoplasm and mitochondria (Circu and Aw, 2010). While the percentages vary depending on cell type, GSH buffers ROS generation, mediates redox reactions and keeps critical proteins and lipids in a reduced state. In the ER, GSH assists in protein folding and, when the balance is shifted to the oxidized form, this triggers a misfolded protein stress response (Circu and Aw, 2010). In the nucleus, GSH provides the appropriate reduced environment to prevent DNA and RNA oxidation (Circu and Aw, 2010). In the mitochondria, GSH buffers ROS production, provides reducing power for detoxification enzymes to function properly and to keep critical protein cysteine sulfhydryl groups reduced (Circu and Aw, 2010). Among the most important of these
cysteines are those located on one or more components of the mitochondrial permeability transition pore. Oxidation at these sites promotes pore opening, metabolic failure and cell death (Halestrap, 2009b).

C. Pyridine nucleotides

NAD(P)⁺ and NAD(P)H are traditionally associated with metabolism and ATP production, but are intimately involved in redox regulation of the cell and therefore defense against oxidative stress. Their continuous cycling of oxidation and reduction is also involved in signal transduction (Koch-Nolte et al., 2009), cell death mechanisms (Ying et al., 2003) and regulation of sirtuin proteins (Finkel et al., 2009). Similar to glutathione, pyridine nucleotides are normally maintained in primarily a reduced state. NADPH is critical to antioxidant detoxification and provides the reducing power needed to keep cellular antioxidants such as GSH and thioredoxin (Trx) reduced. In the cytosol, either glucose-6-phosphate dehydrogenase or the pentose phosphate pathway are responsible for maintenance of NADPH. In the mitochondria, NADP⁺ linked enzymes such as malic enzyme 3 (ME3), isocitrate dehydrogenase (IDH) are responsible for NADPH maintenance (Kowaltowski et al., 2009).

D. Cytoplasmic detoxification systems

In addition to being potentially toxic, ROS and RNS also perform physiological functions. H₂O₂ and nitric oxide (NO) are critical second messengers, involved in regulation of the cell cycle, gene expression and signal transduction (Patel and Insel, 2009). ROS and RNS become toxic when there is a substantial imbalance of ROS generation versus detoxification. The cell is therefore dependent on upregulation of detoxification systems during periods of oxidative stress. Two of the best described
inducible antioxidant enzymes are NQO1 and HO-1. NQO1 catalyzes two-electron reduction and detoxification of quinones, resulting in stable hydroquinones that can be removed by conjugation. NQO1 catalysis of quinones is in direct competition to enzymes such as P450 that catalyze one-electron reduction, producing toxic semiquinone radicals (Kowaltowski et al., 2009). At high concentrations, NQO1 can also function as a superoxide scavenger. Heme oxygenase exists in two isoforms, HO-2 which is constitutively expressed in the brain and HO-1 which is constitutively expressed and is inducible (Schipper et al., 2009). The only similarity between the two is that they process heme in a similar manner. However, they are regulated by different mechanisms, have different structures and do not share the same chromosomal location. HO-1, also known as hsp32, is inducible not only by free heme, but by many stimuli including, hypoxia and ischemia (Schipper et al., 2009). HO-1 catalyses the breakdown of heme to carbon monoxide (CO), biliverdin and iron and requires both oxygen and NADPH for reducing power (Schipper et al., 2009). Biliverdin redox cycles to form bilirubin, which is a potent antioxidant. Chodorowski and colleagues have proposed that the CO produced by HO-1 functions as a second messenger and regulates vascular tone after brain injury (Chodorowski et al., 2005). CO production has also been shown to play a role in mitochondrial biogenesis in the heart (Piantadosi et al., 2008). In addition to being upregulated following injury, both NQO1 and HO-1 contain an ARE sequence in their promoter that allows them to be upregulated even further by pharmacokinetic agents that can induce the Nrf2/ARE system. For example, sulforaphane is a potent upregulator of both these genes (Thimmulappa et al., 2002).
E. Mitochondrial detoxification systems

The majority of ROS produced in the mitochondria are a byproduct of the respiratory chain or specific TCA cycle reactions. The primary species generated is the superoxide radical O$_2^\bullet^-$. Under normal conditions, the O$_2^\bullet^-$ is converted to H$_2$O$_2$ by SOD2. While H$_2$O$_2$ alone is not extremely toxic and is involved in cell signaling, if not further metabolized it can react with iron via the Fenton reaction to form hydroxyl radicals (OH$^\bullet$). NO$^\bullet$, a diffusible second messenger will freely interact with O$_2^\bullet^-$ to create peroxynitrite (ONOO$^-$). This relatively stable product can react with tyrosines and cysteines present on metabolic proteins, resulting in enzymatic inhibition and potentially result in metabolic failure. During oxidative stress, these endogenous detoxification systems can become saturated and even oxidatively inactivated. Many of the detoxificants mentioned above contain ARE sequences in their promoter region, suggesting transcriptional control by activation of Nrf2. Increasing mitochondrial resistance to oxidative stress may have the potential to prevent cell death during acute injury and potentially either halt or slow the progression of many neurodegenerative diseases that are promoted by ROS and RNS.

F. Upregulation of Nrf2 following injury and during neurodegenerative disease

While there are multiple examples of Nrf2 being upregulated following renal ischemia reperfusion (Leonard et al., 2006) and in chronic lung inflammatory models (Rangasamy et al., 2009), No in-depth study has yet been published of Nrf2 upregulation following acute injury or its possible upregulation during chronic disease. One study using a traumatic brain injury model in Sprague Dawley rats characterized the activation of Nrf2 and its target genes after injury (Yan et al., 2008). These authors observed a
robust increase in nuclear translocation of Nrf2 but no corresponding increase in Nrf2 mRNA. This suggested that increases observed were due to post-translational modifications of the protein. Tissue from the ipsilateral injured hemisphere, showed significant increase in HO-1 expression compared to undamaged tissue contralateral to the injury. Models of traumatic brain injury using Nrf2 KO mice have demonstrated a significant increase in inflammatory pathways and cell death, suggesting a role of Nrf2 in modulating cell injury (Yan et al., 2008). HO-1 is chronically upregulated in both Alzheimer’s and Parkinson’s disease (Schipper et al., 2009). Whether this is due to chronic Nrf2 upregulation or another stimulus factor has yet to be determined, but chronic oxidative stress leading to chronic Nrf2 upregulation is plausible. Introduction of compounds that can further upregulate the Nrf2/ARE system may provide the additional capacity for restoring redox balance that inhibits cell injury and death.

All tissues possess redundant antioxidant defense systems for detoxifying the ubiquitous ROS and RNS that play important roles as intracellular and transcellular regulatory signals. Considerable effort has been made to characterize these systems in the brain, as oxidative stress plays a major role in most forms of acute CNS injury and in chronic neurodegenerative disorders. Gene expression for many of the antioxidant associated proteins is upregulated in response to oxidative and other forms of stress. For instance, HO-1 and SOD2 gene induction occurs rapidly following focal cerebral ischemia (Mattson, 1997; Nimura et al., 1996). These observations, taken together with those demonstrating stress-induced increased expression of anti-apoptotic genes like Bcl-2, led to the concept of genomic preconditioning against brain injury. Thus cells and tissues can become relatively resistant to injury by exposure to levels of stress that are
sufficient to activate cytoprotective gene expression but that are also below the threshold for inducing significant cell dysfunction or death.

It now appears that many of the genes induced in the brain following both toxic and sub-toxic levels of stress may be activated via the Nrf2/ARE system (Kaspar et al., 2009). Products of these genes include SOD1 (cytosolic), SOD2 (mitochondrial), and SOD3 (extracellular) (Shukla et al., 2008; Valdivia et al., 2009) and the peroxide detoxifying enzymes glutathione peroxidase (Dohare et al., 2008) and catalase (Al-Omar et al., 2006; Dringen et al., 2005). Nrf2 also mediates induction of glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase, modifier subunit (GCLM), both of which are necessary for glutathione biosynthesis (Lavoie et al., 2009). NAD(P)H is critical to antioxidant detoxification as it provides the reducing power needed to maintain reduced glutathione. Several of the antioxidant proteins and NAD(P)H generating enzymes mentioned in previous sections are also upregulated. These include ME, HO-1 and NQO1 (Thimmulappa et al., 2002).

VI. MITOCHONDRIAL DYSFUNCTION IN BRAIN PATHOPHYSIOLOGY

A. Dysfunction of mitochondria during acute injury and chronic oxidative stress

Mitochondrial dysfunction has been strongly implicated as a major contributor to the pathophysiology of brain injury (Fiskum et al., 1999; Starkov et al., 2004a). Mitochondrial dysfunction can lead to a variety of outcomes, including metabolic failure, promotion of apoptosis, oxidative stress and loss of Ca\(^{2+}\) homeostasis. A common cause of injury to the mitochondria is oxidative stress. A primary target of oxidative stress and ischemia/reperfusion is Complex I of the ETC, which catalyzes the oxidation and reduction of ubiquinone and is considered the rate limiting step of the ETC (Hillered and
Ernster, 1983; Sims, 1991). Also involved in inhibiting ETC activity is cytochrome c release from the intermembrane space of the mitochondria into the cytosol, resulting in the initiation of apoptotic cascades. Oxidative stress and ischemia/reperfusion promote cytochrome c release by the formation of pores initiated by two critical apoptotic proteins, Bax and Bak (Perier et al., 2005). Inhibition of the ETC is not just limited by the decreased activity of its chain components (Bogaert et al., 1994; Richards et al., 2006; Vereczki et al., 2006). Oxidative stress may also target many mitochondrial enzymes and membrane transporters. Impaired alpha-ketoglutarate dehydrogenase, pyruvate dehydrogenase and aconitase by oxidative stress have all been implicated in metabolic failure following ischemia/reperfusion (Brown and Borutaite, 1999). In addition to the direct effects of ROS on the structure and activity of these proteins oxidative stress could also affect their metabolic co-factor, NAD(H) which is necessary for Krebs cycle enzymes, the pyruvate dehydrogenase complex (PDHC) and other reactions involved in amphibolic metabolism.

Oxidative stress can cause an oxidized shift of the redox state in the mitochondria. An oxidized shift of redox state in the presence of abnormally high concentrations of $\text{Ca}^{2+}$ activate the inner membrane permeability transition pore (PTP), which results in transmembrane equilibration of small ions and molecules of up to approximately 1500 Da (Halestrap et al., 2002). Opening of the PTP results in inability of the mitochondria to maintain membrane potential leading to metabolic failure and eventual cell death and has been repeatedly implicated in brain injury. When CyD, the only known protein associated with the PTP, is knocked out in mice, the mice become resistant to ischemia/reperfusion injury (Baines et al., 2005).
B. Regulation of the permeability transition pore

1. Permeability transition pore components

The permeability transition pore is a nonselective channel of unknown components that allows for the transfer of ions and molecules <1500 Da in and out of the mitochondrial inner membrane (Halestrap et al., 2002). Until recently the pore was thought to be comprised of the voltage dependent anion channel (VDAC), adenine nucleotide transporter (ANT) and CyD; however, studies have shown animals lacking VDAC or ANT show no resistance to calcium induced PTP opening (Halestrap, 2010). In the case of CyD knockout mice or cells, pore opening is more resistant to activation by \( \text{Ca}^{2+} \) indicating that activation of CyD an important regulator but not a necessary component of the PTP (Baines et al., 2005; Nakagawa et al., 2005). Another recent PTP component candidate is the phosphate carrier (PiC), which can transform into a pore upon exposure to high levels of \( \text{Ca}^{2+} \). Bernardi (Di and Bernardi, 2009) and Halestrap’s (Leung et al., 2008) groups have shown that phenylarsine oxide (PhAsO) binds to and crosslinks thiols on the PiC and results in pore opening. Oxidized ubiquinone, an inhibitor of the PTP, prevents PhAsO binding to PiC and inhibits PhAsO induced pore opening. While these studies provide strong evidence for PiC involvement, genetic studies utilizing a PiC knockdown are necessary to confirm an essential role for the PiC.

2. Induction of PTP opening by calcium and oxidative stress

As already discussed, the permeability transition pore is associated with the cell death seen in both acute injury and chronic disease. However, the way that pore opening is regulated differs in acute versus chronic injury. Under physiologic conditions, \( \text{Ca}^{2+} \) functions as a messenger that positively regulates mitochondrial respiration, allowing
modification of ATP production to keep up with cellular demand (Brookes et al., 2004). Under pathologic conditions however, excessive Ca$^{2+}$ uptake by mitochondria leads to inhibition of respiratory complexes, ROS/RNS production and PTP activation. During ischemia, ATP depletion results in intracellular accumulation of Ca$^{2+}$ due to reversal of the Na$^+$/Ca$^{2+}$ antiporter (heart, liver, kidney) or entry through NMDA channels (brain). Upon restoration of circulation, mitochondria are reenergized and begin to take up the Ca$^{2+}$ (Halestrap, 2010). Excessive Ca$^{2+}$ initially stimulates mitochondrial respiration in turn increasing the amount of superoxide production at complex I (Sadek et al., 2004). Ca$^{2+}$ will also result in oxidation of cytochrome c resulting in inhibition of complex III (Iwase et al., 1998). Ca$^{2+}$ also activates nitric oxide synthetase (NOS) activity (Fleming et al., 1997) leading to the production of NO$^\bullet$ that can inhibit complex IV. In addition, Ca$^{2+}$ and NO$^\bullet$ can work cooperatively to inhibit complex I. In addition to ROS/RNS production Ca$^{2+}$ activates CyD activity and begins the cascade which facilitates PTP opening. The ROS/RNS produced by excessive Ca$^{2+}$ uptake may also be a necessary component for pore opening. Mice or cells lacking CyD are resistant to PTP opening but are still able to undergo PTP opening albeit at much higher concentrations of Ca$^{2+}$. PiC has critical thiol residues that modulate its activity and as described above, is another candidate PTP protein (Halestrap, 2010). ROS/RNS may alter these thiol groups either directly or indirectly, thereby modulating PiC activity and possibly contributing further to PTP opening.

While exposure of mitochondria to excessively high Ca$^{2+}$ alone can induce PTP, the permeability transition can be activated by exposure to relatively low levels of Ca$^{2+}$ in the additional presence of oxidative stress or pro-oxidant chemicals. Excessive
oxidative stress can exceed mitochondrial antioxidant capacity and deplete mitochondria of co-factors (GSH, NAD(P)H) necessary for both keeping detoxification systems operating and keeping critical PTP-regulating thiol groups in a reduced redox state.

Irrespective of the mechanism by which the PTP is activated, opening of the PTP leads to the collapse of membrane potential, disruption of Ca^{2+} homeostasis, loss of antioxidants and cofactors, bioenergetic failure, mitochondrial osmotic swelling, outer membrane rupture and necrotic cell death. While PTP opening per se does not cause apoptosis, evidence suggests that it may promote mitochondrial pathways of programmed cell death (Gogvadze et al., 2010).

C. Targeting the Nrf2/ARE pathway to mitochondria to prevent PTP opening

The majority of ROS that are produced in the mitochondria are byproducts of the respiratory chain and specific dehydrogenases, e.g., α-ketoglutarate dehydrogenase (Lambert and Brand, 2009; Murphy, 2009; Starkov et al., 2004b; Starkov, 2008; Stowe and Camara, 2009; Tretter and Adam-Vizi, 2004). The primary species generated is the superoxide radical O_{2}^{-}. As shown in Fig. 1.3, under normal conditions, the O_{2}^{-} is converted to H_{2}O by antioxidant systems discussed in the mitochondrial detoxification section. Oxidative stress occurs within the mitochondria, as it does in other cellular locations, when the rate of ROS production and ROS detoxification are unbalanced. Increased production of toxic superoxide and consequently hydrogen peroxide can exceed the rate of detoxification, resulting in toxic metabolites like peroxynitrite and hydroxyl radicals that can cause mitochondrial dysfunction through the oxidation of various proteins, lipids and nucleic acids as described earlier (see Fig. 1.1). However, growing evidence suggests that protection against ROS accumulation can occur through
increased expression of mitochondrial antioxidant enzymes like SOD2 or proteins responsible for the biosynthesis of glutathione and for the reduction of NADP⁺. Levels of SOD2 and mitochondrial glutathione peroxidase and reductase and glutathione are elevated by treatment of aortic smooth muscle cells and cardiac myocytes with sulforaphane (Zhu et al., 2008) or dithiolethione, another organosulfur compound (Zhu et al., 2009). While these effects have not yet been reported for mitochondria present within neurons or the CNS, results from our lab support the hypothesis that activation of Nrf2 increases mitochondrial antioxidant capacity (Chapter 4) and demonstrates that mitochondria from the brains (Greco and Fiskum, 2010) and livers of rats (Chapter 4) treated with sulforaphane are resistant to t-butyl hydroperoxide-induced opening of the PTP.

Possible explanations for this indirect effect of sulforaphane include increased expression of mitochondrial enzymes that reduce NADP⁺ to NADPH (Fiskum and Pease, 1986) and increased expression of the anti-apoptotic mitochondrial protein, Bcl-2. Cardiac Bcl-2 levels are elevated in rats maintained on a high broccoli diet (Mukherjee et al., 2008). Moreover, we have shown that mitochondria within Bcl-2 overexpressing neural cell lines are resistant to PTP opening (Kowaltowski et al., 2000). Whatever the mechanism by which sulforaphane inhibits PTP opening, this effect could contribute to the cytoprotective effects of this drug and others that act through the Nrf2/ARE system, since PTP opening contributes to bioenergetic failure and cell death in many models of acute and chronic neurodegeneration and in cell death that occurs in the cardiovascular, renal, and other systems (Bernardi and Rasola, 2007; Halestrap, 2009a; Robertson et al., 2009).
Figure 3: Redox regulation of the mitochondrial permeability transition pore (PTP).

Opening of the PTP is promoted by elevated intramitochondrial Ca$^{2+}$ and by conditions that promote protein sulfhydryl oxidation, including the presence of peroxynitrite (ONOO$^{-}$) and hydroxyl radical (OH) and a relatively low ratio of reduced to oxidized glutathione (GSH/GSSG). The glutathione redox state is determined by the balance between its reduction by the NADPH-dependent glutathione reductase (GR) and its oxidation, e.g., by glutathione peroxidase (GPX). Intramitochondrial NADP$^{+}$ is reduced to form NADPH by several enzymes including the inner membrane potential-driven transhydrogenase and cell-type selective isoenzymes, including malic enzyme 3 and NADP$^{+}$-dependent isocitrate dehydrogenase glutamate dehydrogenase. The presence of elevated peroxides, including hydrogen peroxide (H$_2$O$_2$) generated e.g., by mitochondrial superoxide dismutase promote PTP opening by shifting the mitochondrial redox state, including that of glutathione, to a more oxidized level. PTP opening is subject to pharmacological inhibition, e.g., by the interaction of cyclosporin A with cyclophilin D, a protein that regulates but is not equivalent to the PTP.
VII. SUMMARY

Mitochondria are both important producers of ROS/RNS and very sensitive to damage caused by these molecules either directly or through the oxidative stress associated with their metabolism. Exogenous antioxidants, including those that are targeted to mitochondria, can increase mitochondrial resistance to oxidative damage and consequently provide neuroprotection for neurologic disorders and diseases. Another approach, which is also showing great promise, is pharmacologic activation of endogenous antioxidant gene expression, including that which is controlled by the Nrf2/ARE system. Preliminary evidence indicates that this strategy can protect mitochondria against oxidative damage and bioenergetic dysfunction; however, the molecular mechanisms responsible for this defense have yet to be elucidated.
CHAPTER 2: BRAIN MITOCHONDRIA FROM RATS TREATED WITH SULFORAPHANE ARE RESISTANT TO REDOX-REGULATED PERMEABILITY TRANSITION

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I. ABSTRACT

Oxidative stress promotes Ca\(^{2+}\)-dependent opening of the mitochondrial inner membrane permeability transition pore, causing bioenergetic failure and subsequent cell death in many paradigms, including those related to acute brain injury. One approach to preconditioning against oxidative stress is pharmacologic activation of the Nrf2/ARE pathway of antioxidant gene expression by agents such as SFP. This study tested the hypothesis that administration of SFP to normal rats increases resistance of isolated brain mitochondria to redox-sensitive PTP opening. SFP or DMSO vehicle was administered intraperitoneally to adult male rats at 10 mg/kg 40 hr prior to isolation of non-synaptic brain mitochondria. Mitochondria were suspended in medium containing a respiratory substrate and were exposed to an addition of Ca\(^{2+}\) below the threshold for PTP opening. Subsequent addition of \( \tau \)BOOH resulted in a cyclosporin A-inhibitable release of accumulated Ca\(^{2+}\) into the medium, as monitored by an increase in fluorescence of Calcium Green 5N within the medium, and was preceded by a decrease in the autofluorescence of mitochondrial NAD(P)H. SFP treatment significantly reduced the...
rate of tBOOH-induced Ca\textsuperscript{2+} release but did not affect NAD(P)H oxidation or inhibit PTP opening induced by the addition of phenylarsine oxide, a direct sulfhydryl oxidizing agent. SFP treatment had no effect on respiration by brain mitochondria and had no effect on PTP opening or respiration when added directly to isolated mitochondria. We conclude that SFP confers resistance of brain mitochondria to redox-regulated PTP opening, which could contribute to neuroprotection observed with SFP.

II. INTRODUCTION

Mitochondrial sensitivity to oxidative stress is strongly implicated in the pathophysiology of many diseases and disorders, including those affecting the central nervous system (Fiskum et al., 1999; Navarro and Boveris, 2009; Niizuma et al., 2009; Starkov et al., 2004a). Mitochondrial targets of oxidative stress include metabolic enzymes, proteins involved in electron transport and oxidative phosphorylation, DNA and RNA, membrane lipids, and a Ca\textsuperscript{2+}-activated, non-selective pore in the inner membrane known as the permeability transition pore. Opening of the normally quiescent PTP results in transmembrane equilibration of small ions and molecules of up to approximately 1500 Da (Halestrap et al., 2002; Lemasters et al., 2009; Rasola and Bernardi, 2007), therefore causing membrane depolarization and uncoupling of oxidative phosphorylation. PTP opening also results in release of mitochondrial metabolites, including pyridine nucleotides and glutathione, which are necessary for energy metabolism and defense against oxidative stress. Cyclophilin D is at this juncture the only mitochondrial protein unequivocally associated with the PTP and appears responsible for mediating both Ca\textsuperscript{2+}-induced pore opening and inhibition of pore opening by the cyclophilin drug, cyclosporin A. Cyclophilin D knock-out mice are relatively resistant to ischemia/reperfusion injury to
both the heart and brain (Baines et al., 2005; Schinzel et al., 2005), illustrating the importance of PTP opening in pathophysiology.

While abnormally high intramitochondrial Ca\(^{2+}\) is the primary stimulus for PTP opening, oxidative stress greatly increases the sensitivity of PTP opening to Ca\(^{2+}\) (Akao et al., 2003). Oxidative stress promotes PTP opening either by direct oxidation of mitochondrial proteins and possibly lipids by reactive O\(_2\) and nitrogen species, e.g., superoxide and nitric oxide, or by causing an oxidized shift in the mitochondrial redox state, such as occurs during metabolism of peroxides by the glutathione peroxidase / reductase system (Kowaltowski et al., 2000; Navet et al., 2006; Petronilli et al., 2009; Petrosillo et al., 2009). Dynamics of the cellular redox state during and following ischemia/reperfusion show a hyperoxidized state indicating this may be a contributing factor to PTP activation (Perez-Pinzon et al., 1997). This oxidized shift in redox state can be monitored through measurements of NAD(P)H autofluorescence (Duchen, 1992) and indirectly results in PTP opening through impaired reduction of oxidized macromolecules (Catisti and Vercesi, 1999).

The levels of gene products that are responsible for both detoxification of reactive O\(_2\) species and for the reducing power that drives their detoxification are controlled through transcriptional regulation employing ARE that interact with transcriptional activating factors such as Nrf2 (Thimmulappa et al., 2002). One mechanism by which the Nrf2/ARE pathway of antioxidant- and other cytoprotective-gene expression is activated is oxidation of critical cysteine sulfhydryl groups located on KEAP1, a cytoplasmic Nrf2 binding protein. Upon oxidation of KEAP1, Nrf2 is released and translocates to the nucleus where it binds to AREs (Jaiswal, 2004).
SFP, an isothiocyanate derived from a glucosinolate found in cruciferous vegetables, e.g., broccoli, forms mixed disulfide bonds with KEAP1, and is a well-studied pharmacologic activator of Nrf2-mediated gene expression (Kensler et al., 2000; Zhang et al., 1992). SFP demonstrates neuroprotection in several rat models of acute brain injury, e.g., stroke (Zhao et al., 2006) and head trauma (Zhao et al., 2005), in which evidence for mitochondrial PTP involvement exists (Kristian and Siesjo, 1998; Okonkwo and Povlishock, 1999). Although effects of SFP on mitochondrial PTP have not been reported, rats fed a broccoli-enriched diet exhibit significant increases in aortic smooth muscle mitochondrial proteins that could influence PTP opening, including thioredoxin, thioredoxin reductase, glutathione reductase, glutathione, and superoxide dismutase and catalase enzyme activities (Mukherjee et al., 2008). This study tested the hypothesis that PTP opening by isolated brain mitochondria is inhibited by treatment of rats with sulforaphane.

III. MATERIALS AND METHODS

A. Chemicals and reagents

R,S-Sulforaphane was purchased from LKT Laboratories, Inc (St. Paul, MN). Mannitol, sucrose, EGTA, HEPES, Tris, potassium phosphate dibasic (K₂HPO₄), magnesium chloride (MgCl₂), malate, glutamate, oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), adenosine 5′-diphosphate (ADP), bovine serum albumin (BSA), percoll, succinate, rotenone, calcium, dimethylsulfoxide (DMSO) and tert-butyl hydrogen peroxide were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ultra-pure potassium chloride was obtained from EM Sciences (Fort Washington, PA, USA).
B. Treatment of rats with sulforaphane

All experimental procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals. Male 300 g Fischer 344 rats (Charles River) were injected intraperitoneally with sulforaphane at 10 mg/kg in 200 µL solution containing 40% DMSO and 60% isotonic saline. Animals received DMSO plus saline as the vehicle control.

C. Isolation of non-synaptic brain mitochondria

Rats were euthanized by decapitation and their forebrains rapidly removed, chopped and homogenized in ice-cold isolation buffer (225 mM mannitol, 25 mM sucrose, 10 mM Hepes, 1 mM EGTA, pH 7.4, at 4°C). The homogenate was centrifuged at 1330 g for 3 min. The pellets were re-suspended and re-centrifuged at 1330 g for 3 min. The combined supernatants were centrifuged at 21,200 g for 10 min. The pellets were re-suspended in 15% Percoll and layered on a 40%/23% Percoll gradient. The tubes containing the Percoll gradients were then centrifuged at 31,700 g for 10 min. The mitochondrial fraction located at the interface between the 40% and 23% layers was removed, diluted with isolation buffer and centrifuged at 16,700 g for 10 min. The purified mitochondrial pellet was re-suspended in isolation buffer and de-fatted bovine serum albumin was added to the suspension at 10 mg/mL. After a final centrifugation at 9000 g for 10 min, the mitochondria were re-suspended in approximately 20 µL of isolation medium not containing EGTA or BSA. Mitochondrial protein concentrations were measured using a Lowry DC kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin used as concentration standards.
D. Mitochondrial oxygen consumption

The respiratory activities of isolated brain mitochondria were measured polarimetrically with a Clark-type oxygen electrode apparatus (Hansatech Instruments, Norfolk, England). Mitochondria were suspended at a protein concentration of 0.5 mg/ml in buffer containing 125 mM KCl, 20 mM HEPES, 2 mM K$_2$HPO$_4$, 0.01 mM EGTA, 1 mM MgCl$_2$ (pH 7.0) at 37°C, plus glutamate (5 mM) and malate (0.1 mM) or succinate (5 mM) plus the electron transport chain complex 1 inhibitor, rotenone (4 µM). Addition of ADP (0.5 mM) was used to initiate State 3 (phosphorylating) respiration. Oligomycin (2.5 µg/ml), an inhibitor of the mitochondrial ATP synthase, was used to induce State 4 (resting) respiration. The use of oligomycin to obtain the rate of respiration limited by the inner membrane proton permeability is necessary when magnesium is present as even small contamination of isolated mitochondria with Mg-dependent ATPases, e.g., the plasmalemmal sodium pump, can result in turnover of mitochondrially-generated ATP, resulting in an artificially high rate of State 4 respiration in the absence of oligomycin. Maximal respiration was initiated with the addition of the protonophore uncoupler, FCCP (54 nM). Rates of oxygen consumption are expressed as nmol O$_2$/mg mitochondrial protein/min). The respiratory control ratio (RCR) is defined as the rate of ADP-stimulated oxygen consumption (State 3) divided by the rate of respiration determined in the presence of oligomycin (State 4).

E. Measurements of mitochondrial Ca$^{2+}$-uptake and release and pyridine nucleotide redox state

Mitochondria were suspended in a cuvette at a protein concentration of 0.5 mg/ml in 2 ml of 125 mM KCl, 2 mM K$_2$HPO$_4$, 1 mM MgCl$_2$, 20 mM Tris-HCl, 5 mM succinate, and
4 μM rotenone, pH 7.4 at 30°C. The use of the electron transport chain complex II substrate succinate in the presence of the complex I inhibitor rotenone allows for the mitochondrial pyridine nucleotide redox state (NAD(P)H/NAD(P)^+ to be varied between totally reduced (no added pro-oxidant) to oxidized (after addition of pro-oxidants), independent of mitochondrial respiration and membrane potential. The medium free Ca^{2+} was measured fluorimetrically in the presence of the Ca^{2+}-sensitive fluorescent dye Calcium Green-5N (0.1 μM), using excitation and emission wavelengths of 506 nm and 532nm. Autofluorescence of reduced mitochondrial pyridine nucleotides (NADH plus NADPH) was measured simultaneously using excitation and emission wavelengths of 350 nm and 460nm. Fluorescence measurements were performed on a Hitachi 2500 spectrofluorimeter, equipped with magnetic stirring and cuvette temperature control. Fluorescent wavelength settings cycled every 2 sec for medium Ca^{2+} and NAD(P)H measurements.

Mitochondria were added to the cuvette and allowed to equilibrate, bioenergetically, for 2 min, as reflected by steady-state Calcium Green 5N (CG5N) and NAD(P)H fluorescence. CaCl_2 was then added to the medium at a level that the mitochondria could completely accumulate with only a very slow rate of subsequent spontaneous release. At 200 sec after the addition of Ca^{2+}, either tert-butyl hydroperoxide or phenylarsine oxide were added as pro-oxidant inducers of PTP opening, as reflected by the release of accumulated Ca^{2+}. In some experiments, the PTP inhibitor, cyclosporin A (1 μM), was present in the medium in which mitochondria were suspended. PTP opening was quantified by measuring the rate of increase in (CG5N) after the addition of pro-oxidants. The effect of these pro-oxidants on the pyridine nucleotide redox state was quantified by measuring the extent to which NAD(P)H autofluorescence was lost after their addition.
F. Immunoblot analysis of cyclophilin D

Isolated mitochondria were lysed in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Calbiochem). Equal amounts of mitochondrial protein (5 µg) from each sample were separated by SDS-PAGE (4-12% Bis-Tris gels) (Invitrogen) and transferred to PVDF membranes (Invitrogen), and then incubated with primary antibodies (overnight at 4°C) with the mouse monoclonal antibodies anti-cyclophilin D antibody 1:500,000 (Mitosciences, Cat. #MSA04) or anti-VDAC antibody 1:500,000 (Mitosciences, Cat. #MSA03). The membranes were then washed with PBST and incubated for 1 hr at room T in HRP-conjugated anti-mouse antibody (Millipore Cat. #12-349) at 1:2000 dilution for 1 hr at room T. The washed blots were then treated with enhanced chemiluminiscence detection reagent (Amersham Bioscience, UK). Densitometric analysis of the protein bands was performed using the Image J software.

G. Data Analysis

Data are expressed as means ± S.E.M. of n different experiments. Differences between data obtained from mitochondria obtained from rats treated with sulforaphane or drug vehicle were assessed by Student’s t-test. For data that were not normally distributed, the Mann-Whitney rank sum test was used. P < 0.05 was considered to be statistically significant.

IV. RESULTS

A. Inhibition of peroxide-induced brain mitochondrial PTP opening by treatment of rats with sulforaphane

Fig. 2.1A provides the results from one typical experiment where pro-oxidant-induced PTP opening was evaluated by monitoring the increase in CG5N that occurs
during release of accumulated mitochondrial Ca\textsuperscript{2+} into the medium. Pilot experiments were first performed to establish a level of added CaCl\textsubscript{2} that when added alone would not induce PTP opening but when followed by the pro-oxidant, \textit{t}BOOH, would induce a measurable rise in CG5N. The immediate increase in fluorescence that occurred upon addition of 50 µM Ca\textsuperscript{2+} (100 nmol/mg protein) was followed by a rapid decrease, with mitochondrial Ca\textsuperscript{2+} uptake reaching completion within 20 sec. In the absence of further additions, the fluorescence remained relatively constant, but increased slowly over the following 300 sec (Fig. 2.1A, Line 1 and 2), using mitochondria isolated from either SFP- or drug vehicle-treated rats. Addition of \textit{t}BOOH (250 µM) at 200 sec after the addition of Ca\textsuperscript{2+} resulted in an immediate rise in CG5N, due to release of accumulated mitochondrial Ca\textsuperscript{2+}, which was slower for mitochondria from SFP-treated rats (Fig. 2.1A, Line 3 and 5). The presence of SFP in the mitochondrial suspensions at concentrations up to 100 µM had no effect on either Ca\textsuperscript{2+} uptake or release (not shown). The presence of the PTP inhibitor, cyclosporin A, virtually eliminated the \textit{t}BOOH-induced Ca\textsuperscript{2+} release (Fig. 2.1A, Line 4). The Ca\textsuperscript{2+} release induced by \textit{t}BOOH was incomplete, as indicated by the fact that the subsequent addition of the Ca\textsuperscript{2+} ionophore, ionomycin, caused a large additional increase in CG5N (Fig. 2.1A, Line 3).
**Figure 2.1: Inhibition of peroxide-induced permeability transition pore (PTP) opening by systemic sulforaphane administration.**

A. Mitochondrial Ca$^{2+}$ uptake and release was measured by monitoring the fluorescence of Calcium Green 5N (CG5N) in the medium. At time zero, 50 µM CaCl$_2$ was added to a suspension of non-synaptic brain mitochondria (0.5 mg/ml) in medium containing succinate as the respiratory substrate in the presence of rotenone, an inhibitor of complex I of the electron transport chain. Spontaneous release of accumulated Ca$^{2+}$ was very slow and not affected by treatment of rats with SFP (Lines 1, 2). The subsequent addition of 250 µM tert-butyl hydroperoxide (tBOOH) (Lines 3, 5) or 30 µM phenylarsine oxide (PhAsO; Line 6) induced PTP opening and release of Ca$^{2+}$, which was inhibitable by the presence of 1 µM cyclosporin A (Line 4). Release of Ca$^{2+}$ by tBOOH was incomplete compared to that observed after subsequent addition of 1 µM ionomycin (Line 3). B. Mitochondrial pyridine nucleotide redox state was measured by monitoring the autofluorescence of NAD(P)H in the absence (Lines 1, 2) or presence of tBOOH (Lines 3, 4), with no apparent effects of SFP treatment.
Comparisons were made between the initial slope of the tBOOH-induced increase in CG5N for mitochondria isolated from 9 rats that had been injected intraperitoneally 40 hr earlier with SFP and 7 rats that were injected 40 hr earlier with the saline/DMSO vehicle. Fig. 2.2A demonstrates that there was a significant, approximately 50% slower rate of tBOOH-induced Ca^{2+} release by mitochondria from the SFP-treated rats. There was no difference, however, between the background release-rate for mitochondria from the two animal groups (n = 4/group).

Previous studies indicate that tBOOH induces opening of the PTP indirectly by oxidation of protein sulphhydril groups due to glutathione oxidation during tBOOH metabolism by glutathione peroxidase and reductase. This shift in mitochondrial redox state can be continuously monitored by following the autofluorescence of NAD(P)H which is lost during NAD(P)H oxidation by glutathione reductase. We therefore followed NAD(P)H redox state along with the medium free Ca^{2+} concentration to determine if mitochondria from SFP-treated rats are relatively resistant to tBOOH-induced oxidation or are resistant to PTP opening caused by NAD(P)H oxidation. As shown in Fig. 2.1B, Line 3 and 4, addition of tBOOH to the mitochondrial suspensions caused a rapid loss of NAD(P)H autofluorescence for mitochondria from both SFP- and vehicle-treated rats, compared to the slow, modest decline in fluorescence that occurred in the absence of tBOOH. The extent to which NAD(P)H was oxidized by tBOOH was not significantly different for mitochondria from the two animal groups (Fig. 2.2B).
Figure 2.2: Effects of systemic sulforaphane administration on peroxide-induced brain mitochondrial calcium release and pyridine nucleotide oxidation.

A. Comparisons of the initial rise in Calcium Green 5N fluorescence after the addition of 250 μM tert-butyl hydroperoxide (tBOOH), 30 μM phenylarsine oxide (PhAsO) or no addition (Ca\(^{2+}\) alone) for brain mitochondria isolated from rats treated 40 hr earlier with either sulforaphane (SFP; 10 mg/kg) or drug vehicle. * p < 0.05, n = 9. B. Comparisons of the extent to which NAD(P)H autofluorescence decreased after the addition of tBOOH or no addition to suspensions of mitochondria from SFP- or drug vehicle-treated rats.
**B. Sulforaphane treatment does not protect against PTP opening induced by direct sulphydryl group oxidation**

Experiments were performed where PTP opening was induced by exposure of isolated brain mitochondria to phenylarsine oxide, a direct sulphydryl group oxidizing agent (Kowaltowski et al., 2000), to determine if treatment of rats with SFP protects against this direct oxidation or just against indirect oxidation induced by peroxide metabolism. Fig. 2.1A, Line 6, demonstrates that phenylarsine oxide induces a greater rate and extent of Ca\(^{2+}\) release than that induced by \(\text{tBOOH}\). Comparison of the release rate for mitochondria from SFP- and vehicle-treated rats shown in Fig. 2.2A indicates no difference between these groups (n = 4/group).

**C. Mitochondrial respiration is unaffected by treatment of rats with sulforaphane**

Sensitivity of mitochondria to PTP opening can be influenced by many factors, including normal mitochondrial bioenergetic activities, e.g., rates of respiration (Mirandola et al., 2010). We therefore compared rates of ADP-stimulated State 3 respiration, oligomycin induced State 4 respiration, and the respiratory control ratios calculated from these rates for mitochondria isolated from rats treated with sulforaphane and drug vehicle. As shown in Table 2.1, there were no significant differences between these rates of respiration and respiratory control ratios for brain mitochondria respiring on NADH-dependent oxidizable substrates, malate plus glutamate, from SFP- and drug vehicle-treated animals. In addition, one experiment was performed to compare rates of respiration on succinate in the presence of rotenone. Similar values were obtained from mitochondria from the SFP- and vehicle-treated rats (State 3 = 87 vs. 114 nmoles \(\text{O}_2/\text{min/mg}\); State 4 = 26 vs. 42 nmoles \(\text{O}_2/\text{min/mg}\); RCR = 3.3 vs. 2.7).
<table>
<thead>
<tr>
<th>Respiration Parameters</th>
<th>DMSO</th>
<th>SFP</th>
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<tr>
<td>State 3</td>
<td>134 ± 18</td>
<td>168 ± 13</td>
</tr>
<tr>
<td>State 4</td>
<td>27 ± 2</td>
<td>26 ± 2.7</td>
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<tr>
<td>RCR</td>
<td>5 ± 0.8</td>
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**Table 2.1.** Respiratory characteristics of non-synaptic brain mitochondria isolated from sulforaphane and drug vehicle-treated rats. ADP-stimulated (State 3) and oligomycin-induced (State 4) respiration was measured in medium containing 5 mM malate plus 5 mM glutamate as oxidizable substrates, as described in Materials and Methods. The respiratory control ratio (RCR) is State 3/State 4. Values represent the means ± S.E. for n = 4 animals per group.
In addition to determining if treatment of rats with SFP affects respiration by isolated brain mitochondria, we also tested for any direct effects of SFP on mitochondria isolated from the brains of normal animals. The presence of SFP at concentrations up to 100 µM had no discernible effect on either State 3 or 4 respiration (not shown), indicating that SFP exhibits no mitochondrial toxicity even at levels that are an order of magnitude higher than the maximal that could be reached within the brain or other tissues at doses that have been used for neuroprotection. Additional experiments tested the effects of phenylarsine oxide on mitochondrial respiration and found no effects at the concentration used to induce PTP in mitochondria pre-loaded with Ca²⁺ (not shown).

D. Mitochondrial cyclophilin D immunoreactivity is unaffected by treatment of rats with sulforaphane

Cyclophilin D is the primary mitochondrial protein whose level is known to affect sensitivity to PTP opening. We therefore used immunoblots for cyclophilin D from mitochondrial protein extracts to determine if differences exist in brain mitochondria from SFP- and vehicle-treated rats. A representative immunoblot for cyclophilin D and the mitochondrial voltage dependent anion channel is shown in Fig. 2.3A. Densitometric ratios for cyclophilin D / VDAC for 7 vehicle-treated rats and 7 SFP-treated rats are shown in Fig. 2.3B. There was no significant difference in these ratios for the two groups, although the ratios tended to be lower in mitochondria from animals treated with SFP.
Figure 2.3: Cyclophilin D immunoreactivity in non-synaptic brain mitochondria from sulforaphane and drug vehicle-treated rats.

A. Sample immunoblots for cyclophilin D (CyD) and the voltage gated anion channel (VDAC) for mitochondria isolated from the brain of a rat treated 40 hr earlier with sulforaphane (SFP) or drug vehicle. B. Densitometric ratios of mitochondrial CyD/VDAC for n = 7 animals/group.
V. DISCUSSION

The primary conclusion reached from these experiments is that intraperitoneal injection of rats with a non-toxic level of sulforaphane results in resistance of isolated non-synaptic brain mitochondria to peroxide-induced PTP opening measured 40 hr later. The potential significance of this conclusion is that if such resistance also occurs to mitochondria as they exist within the brain, treatment of animals with SFP could protect against PTP opening and its metabolically catastrophic consequences that are known to occur in acute brain injury, e.g., caused by ischemia or trauma, and even in neurodegenerative diseases.

Our experiments provide some insight into the mechanisms by which SFP confers resistance to peroxide-induced release of accumulated Ca\(^{2+}\). The complete inhibition of \(t\)BOOH-induced Ca\(^{2+}\) release by cyclosporin A verifies that the SFP is affecting PTP opening rather than some other mechanism of release, which can occur particularly with brain mitochondria, depending on the experimental conditions (Andreyev et al., 1998). Previous \textit{in vitro} experiments comparing mitochondria from wild-type and Bcl2 overexpressing cell lines demonstrated that Bcl2 protects against \(t\)BOOH-induced PTP opening exclusively by protecting against oxidation of NAD(P)H and its redox partners (Kowaltowski et al., 2000).

This mechanism is not responsible for SFP induced resistance to peroxide-triggered PTP opening, as indicated by no significant difference between the extent of NAD(P)H oxidation for mitochondria from SFP- and vehicle treated rats. This finding is important since ingestion of cruciferous vegetables, from which SFP is derived, can increase the level of Bcl2, at least in the heart (Mukherjee et al., 2010). SFP treatment also does not affect rates of respiration by isolated mitochondria and does not have any
direct effects on mitochondrial respiration or PTP opening when added to mitochondrial suspensions at concentrations up to 100 µM. These findings argue against any generalized effects of SFP on mitochondrial bioenergetics, which could affect PTP opening. The level of cyclophilin D immunoreactivity is also not affected by SFP treatment, indicating that the expression of this well-established regulator of PTP activity is not the basis of the SFP effect. It is possible, however, that the relative redox state of cysteine sulfhydryls present on this protein or other PTP affiliated proteins, e.g., the adenine nucleotide translocase, could be the key to understanding PTP resistance conferred by systemic administration of SFP (Linard et al., 2009b; Linard et al., 2009a). While the NAD(P)H-based reducing power appears unaffected by SFP treatment, the mitochondrial components that utilize this power to inhibit PTP opening may be the target of SFP. The finding that SFP administration did not inhibit PTP opening caused by exposure of mitochondria to phenylarsine oxide indicates that SFP does not affect sensitivity of proteins to oxidation but rather affects the process whereby peroxide metabolism elicits oxidative stress and subsequently protein oxidation. Thus experiments are in progress to determine if levels of mitochondrial glutathione or thioredoxin are increased by SFP treatment, as they are affected in other tissues at either the cellular or mitochondrial levels (Angeloni et al., 2009; Crane et al., 2009; Vauzour et al., 2010).

Future determination of which mitochondrial protein or proteins mediate the inhibition of redox-regulated PTP opening by systemic SFP administration will help test the hypothesis that the primary action of SFP is to activate the Nrf2 pathway of cytoprotective gene expression. Numerous studies, including our own with primary cultures of neurons and astrocytes (Danilov et al., 2009; Siebert et al., 2009; Soane et al.,
2010), have provided evidence in favor of this primary mechanism for SFP (Dinkova-Kostova and Talalay, 2008); however, at a dose approximately 10 times higher than that used in our experiments, SFP may exert additional effects, e.g., inhibition of histone deacetylase (Myzak et al., 2006). Irrespective of the specific steps by which SFP confers resistance of mitochondria to peroxide-induced PTP opening, this effect could contribute importantly to the cytoprotection observed by SFP in both in vitro and in vivo models of cell injury and death for the brain, heart, and other vital organs.
CHAPTER 3: SULFORAPHANE PROTECTS AGAINST NEURONAL DEATH AND NEUROLOGIC IMPAIRMENT AFTER CANINE CARDIAC ARREST

INVESTIGATORS: Hazelton J.L., Balan I.S., Greco T., Cotto-Cumba C., Rosenthal R.E., and Fiskum G.

I. ABSTRACT

Oxidative stress and mitochondrial permeability transition pore (PTP) opening contribute to the reperfusion injury of the brain following cardiac arrest. Considering treatment of animals with separate oxygenation protocols altered sensitivity of PTP opening, we tested the ability of sulforaphane (SFP) to be neuroprotective following cardiac arrest. After 10 minutes of V-fib-induced cardiac arrest, chloralose-anesthetized adult female beagles were resuscitated using a hyperoxic or pulse-oximetry guided normoxic ventilation protocol. Thirty minutes later, sulforaphane at 1 mg/kg was infused intravenously over 30 minutes. At hour 23, animals were awoken and a neurologic exam performed. They were then re-anesthetized and the brains removed for measurements of neuronal death and oxidative stress. The neurologic deficit scores (NDS: 0 = normal; 100 = brain dead) of the SFP treated animals were 24 ± 3.8 (hyperoxic) and 24 ± 7.8 (normoxic groups) compared to historical averages of 61 ± 4 and 43 ± 5.0, respectively, for untreated animals. The percentage of cresyl violet-stained hippocampal CA1 neurons that appeared
dead or dying in the SFP-treated animals were 6.8 ± 2.5 (hyperoxic) and 12.1 ± 9.6 (normoxic groups) compared to historical averages of 60.5 ± 3.3 and 35.5 ± 4.3, respectively, for untreated animals. The percent area expressing 8-hydroxydeoxyguanosine staining in the SFP group was 41 ± 5.6 compared to 58 ± 3.5 in the untreated normoxic group. These results suggest that the second phase of oxidative stress mitigates short-term cell viability and neurologic outcome and that sulforaphane is neuroprotective following global cerebral ischemia.

II. INTRODUCTION

Sudden cardiac arrest accounts for over 300,000 deaths per year and is most commonly caused by ventricular fibrillation (Callans, 2004). Time to the beginning of cardiopulmonary resuscitation or use of an automated external defibrillator is a critical prognostic indicator of whether normal cardiac rhythm can be restored (Callans, 2004; Sasson et al., 2010). However, even among that 6% of patients in whom normal cardiac rhythm is restored after arrest, even relatively brief periods of global cerebral ischemia result in significant neurological morbidity. (Culley et al., 2004).

Standard medical practice has been to use 100% O₂ to ventilate patients during resuscitation and recovery. This paradigm continues to be used despite evidence from animal models showing supraphysiologic levels of O₂ are actually harmful to outcome (Balan et al., 2006; Liu et al., 1998; Mickel et al., 1990; Zwemer et al., 1994). Nor are there current guidelines as to how long a patient should be ventilated on 100% O₂, and actual experience can vary tremendously from patient to patient (Kilgannon et al., 2010). Global cerebral ischemia and reperfusion result in the overwhelming production of ROS (Vereczki et al., 2006) in a biphasic response. During the ischemic phase, loss of blood
flow and oxygen results in loss of ATP (Welsh et al., 1982). The drop in ATP causes depolarization of the cell and reversal of the sodium calcium exchanger (NCX) and influx of Ca\(^{2+}\) into the neuron. This Ca\(^{2+}\) further depolarizes the cell causing release of glutamate from synaptic terminals. Free glutamate is then able to bind to NMDA receptors allowing more Ca\(^{2+}\) to flood the cell. These high levels of Ca\(^{2+}\) result in ROS production and activation of endonucleases, ATPases, calpain and phospholipases (Halestrap, 2010; Lemasters et al., 2009). Upon return of circulation, mitochondria are re-energized and begin to take up the excessive Ca\(^{2+}\) in the cell which stimulates oxidative phosphorylation and in turn producing more ROS that will further inhibit the mitochondria and the initiation of cell death pathways (Kristian and Siesjo, 1998).

Over the course of several hours, the combination of oxidative stress and cell death activates various inflammatory pathways meant to clear cellular debris (Block et al., 2001). An unfortunate byproduct of this process is the production of more ROS by inflammatory cells (Hakim, 1993). This secondary wave of oxidative stress, (Siesjo and Siesjo, 1996) following about 6 hours into the reperfusion state, will go on to further inhibit mitochondrial function and oxidize lipids, proteins and DNA/RNA and promote cell death.

It has been shown by our lab and others that this initial burst of ROS production and accompanying depression in cerebral energy metabolism can be inhibited by avoiding excessive O\(_2\) use (Fiskum et al., 2004; Martin et al., 2005; Vereczki et al., 2006). Specifically, our lab has shown using our oximetry protocol, in which animals are initially resuscitated with 100% O\(_2\) but then have O\(_2\) delivery titered down to physiologic levels within 10 minutes, results in preservation of hippocampal cerebral energy
metabolism (Fiskum et al., 2004), neuronal viability, improved short-term neurologic deficit score (Balan et al., 2006), and the elimination of ROS production fueled by supraphysiologic \( \text{O}_2 \) concentrations (Vereczki et al., 2006). However, despite these initial improvements, there is still a significant amount of hippocampal cell death and neurologic impairment compared to sham-treated animals indicating the need for a supplementary treatment for this secondary wave of oxidative stress. Cell death and injury trigger inflammatory cascades which create ROS/RNS by products leading to a secondary event of oxidative stress. Attempts have been made to target oxidative stress with limited success. Commonly used approaches have been to utilize highly specific ROS species scavengers (Silbergleit et al., 1999). The downside to specific scavengers is they are so specific they target a very small percentage of the problem and fail to have a large impact. Based on these findings it is logical to conclude there needs to be development of a compound that can target oxidative stress as a whole.

Recent interest has been focused on the Nrf2 pathway and its involvement in brain injury (Dash et al., 2009; Innamorato et al., 2008; Zhao et al., 2005; Zhao et al., 2006; Zhao et al., 2007b). Nrf2 is a master regulatory protein of many pathways in the cell including those involved in antioxidant expression and inflammation (Hu et al., 2006a). Sulforaphane, an extract from cruciferous vegetables, allows for dissociation of Nrf2 from its inhibitory protein KEAP1 and translocation into the nucleus where it binds to the antioxidant response element located in the promoter of hundreds of genes (Jaiswal, 2004). While reports have separately shown that SFP is able to improve neuronal and neurologic outcome, a recent publication using a neonatal hypoxia/ischemia model showed that SFP inhibited both oxidative stress and microglial activation (Ping et
Clinical inference from this study is limited however, as SFP was administered before the hypoxic/ischemic insult. In a clinically relevant canine cardiac arrest model developed by our laboratory, we tested the hypothesis that post-insult administration of SFP improves neurologic and neuronal outcome and reduces oxidative stress.

III. MATERIALS AND METHODS

A. Surgical preparation (Dr. Cotto-Cumba, Ms. Hazelton and Ms. Greco)

All animal experiments were approved by the University of Maryland Institutional Animal Care and Use Committee. Adult purebred female beagles (aged 2 – 4 years and weighing 10 – 15kg) were used in a well-established model of cardiac arrest and resuscitation. In this model, animals undergo induction of anesthesia with pentobarbital (12.5 mg/kg) through a peripheral intravenous line. Anesthesia is subsequently maintained through preparation and surgery with α-chloralose (75 mg/kg). Fluid support is maintained throughout with intravenous infusion of normal saline (3 ml/kg/h). Animals are endotracheally intubated and mechanically ventilated with room air (21% O₂). Once properly intubated, animals receive ceftriaxone (250mg/10ml) as a prophylaxis. Core body temperature is continuously monitored and maintained at physiological levels with heating blankets and lamps (rectal temperature >37°C and <38.5°C). Once full anesthesia is secured, left femoral arterial and venous catheters are placed for drug delivery, central venous pressure and blood pressure monitoring, and arterial blood gas (ABG) sampling. A left lateral thoracotomy is performed through the fourth intercostal space, and the pericardium is incised and reflected.
B. Experimental cardiac arrest and resuscitation (Dr. Rosenthal)

Ventricular fibrillation (VF) was initiated with a 250-Hz, square-wave DC pulse generated by a Grass stimulator to the right ventricle. The presence of cardiac arrest was verified by EKG rhythm consistent with VF in the presence of systolic arterial pressure of <20 mm Hg. At the initiation of VF, artificial ventilation was discontinued and cardiac arrest was allowed to persist untreated for 10 minutes. At the end of this period, epinephrine (0.02 mg/kg) and sodium bicarbonate (1 mEq/kg) were administered intravenously, simultaneously with the resumption of ventilation with 100% O₂ and the performance of open-chest cardiopulmonary resuscitation for 3 minutes, followed by internal defibrillation at 5 J.

C. Experimental groups (Dr. Rosenthal, Dr. Cotto-Cumba, Ms. Hazelton and Ms. Greco)

1. Oxygenation protocols

All dogs were ventilated with 100% oxygen in room air during CPR and then randomized for resuscitation under 1) an oximetry-guided (normoxic) or 2) a hyperoxic ventilatory protocol immediately following cardiac arrest. Dogs under the hyperoxic protocol were resuscitated with 100% O₂ for 1 hour after resuscitation followed by ABG-guided adjustments to physiological O₂ levels. In the oximetry protocol, hemoglobin O₂ saturation was continuously monitored via pulse oximetry measured on the underside of the tongue. Pulse oximetry measurements were always found to be 99% to 100% immediately after resuscitation; inspired O₂ in this group was then decreased to 50%. Further reductions of 5% of inspired O₂ were performed every 2 minutes until O₂
desaturation (96%) was noted. At this point, ABG was measured, and pulse oximetry was no longer used to guide respiratory parameters.

2. Sulforaphane administration

Animals from both the hyperoxic and oximetry groups were randomized to receive either vehicle (saline) or drug (sulforaphane). 30 minutes following resuscitation, animals were intravenously infused with either saline (30ml) or sulforaphane (1mg/kg) at a rate of 1.15ml/min over the course of 30 minutes.

D. Post-resuscitative care (Ms. Greco and Ms. Hazelton)

All animals received intensive care for 24 hours after resuscitation, according to a standardized protocol. Morphine (1 – 2 mg per hour) provided analgesia during intensive care. Pancuronium (0.1 mg/kg) was used intermittently as needed to facilitate ventilation and only after verification that the animal was adequately sedated. Ventilation was adjusted to maintain a PaCO$_2$ of 30 to 35 and a PaO$_2$ of 80 to 100 torr.

E. Neurologic examination (Dr. Rosenthal, Dr. Fiskum and Dr. Cotto-Cumba)

At hour 20, analgesia was lightened, and animals were weaned from controlled ventilation. At hour 23 morphine was reversed with naloxone (0.4 mg/kg), and the neurological deficit score (NDS) was measured by 2 examiners blinded to treatment protocol. The NDS is a multisystem test of 18 parameters in 5 categories (level of consciousness, respiration, cranial nerve, motor, sensory, behavior), yielding a score between 0 (normal) and 100 (brain death). Animals were reanesthetized with pentobarbital and α-chloralose, intubated, and mechanically ventilated.
F. Tissue preparation (Dr. Rosenthal, Dr. Cotto-Cumba and Ms. Julie Hazelton)

Once deeply anesthetized, a craniotomy was performed and one hemisphere was immersion fixed in 4% paraformaldehyde.

G. Histopathology (Dr. Balan)

Representative sections throughout the hippocampus (1 in 12 series, six sections total) were collected and stained with cresyl violet. The sections were placed sequentially in 50%, 75%, and 95% ethanol for 5 minutes each, and then in 100% ethanol for 6 hours. The sections were then rehydrated in 95%, 75%, and 50% ethanol and water for 5 minutes each, and treated with cresyl violet solution (FD NeuroTechnologies, Baltimore, MD) for 2 minutes. The slides were then placed in 0.1% acetic acid in 75% ethanol for 2 minutes, rinsed in water, dehydrated, and mounted on cover-slips with DPX.

Using Stereo Investigator software (MicroBrightField, Williston, VT), unbiased estimates of the total number of uninjured neurons in the CA1 region were obtained using the optical fractionator technique of stereology. After outlining the boundaries of the CA1 region at low magnification (10×), the software places a set of optical dissector frames (count frame 30×30 μm) in a systematic-random fashion. Shrunken neurons with a condensed nucleus and lacking a nucleolus were classified as injured and/or dying cells. Non-injured neurons were then counted in optical dissectors to 7 μm in depth at 100× magnification. At least 500 cells were sampled to ensure robustness of the data.

H. Immunohistochemistry (Ms. Julie Hazelton)

Markers of oxidative stress were identified in free-floating sections using antibodies against 8 hydroxydeoxyguanosine (clone 15A3). Tissue was rinsed multiple times in 0.05 M KPBS buffer before and after exposure to the following: 1% solution of
sodium borohydride for 20 minutes; primary antibody diluted in 0.05 M KPBS + 0.4% Triton-X for 48 h (15A3, QED Bioscience, 1:2000); biotinylated secondary antibody (1:600) for 1 hour; and avidin biotin complex (1:222) solution for 1 hour. Tissue was then rinsed before and after a 12 minute incubation in NiDAB solution with 0.175 M sodium acetate buffer. A second series of tissue was labeled with the same primary antibody (1:250) and tagged with a fluorescent secondary antibody (1:500; Invitrogen Corp., Carlsbad, CA) for illustration purposes. The slices were mounted on slides, dehydrated, and cover-slipped with DPX mounting media. The sections were examined with a Nikon Eclipse E800 microscope equipped with appropriate filters for the fluorochrome-treated sections.

I. Quantification of 8 hydroxy 2 deoxyguanosine immunostaining (Ms. Julie Hazelton)

Quantitative analyses were performed on NiDAB labeled sections with computer assisted image analysis consisting of a Nikon Eclipse 800 photomicroscope, a Retiga cooled CCD digital camera (Biovision Technologies), a Macintosh G4 computer with IP Spectrum software (Scanalytics, Fairfax, VA). Quantification was performed by an individual blinded to the treatment protocol. Across the septotemporal axis 3 slides per animal were selected for quantitative analyses: 1 at the rostral, 1 in the middle and 1 in the caudal part of the ventral hippocampus. The stage of the microscope was adjusted so that the cell layer was centered in the field and was oriented horizontally in the captured image. At each hippocampal depth, an image from the CA1 region was captured under 10x magnification. Microscope settings for gain, light normalization and contrast were standardized and kept constant across all images from all treatments.
Image J was then used to place three boxes of equal area (128 x 128 µm) across the pyramidal cell layer in each thresholded 10x image to obtain 3 separate measures of density per slide. A total of nine boxes (3 from each slide) were averaged to determine amount of staining per animal. The amount of staining was expressed as the percent area within the ROI occupied by black reaction product representing 8OHdG immunoreactivity.

**J. Statistical analysis**

Data obtained from immunostaining measurements were analyzed (STAT32, SigmaStat) using a standard t-test with a 95% confidence interval for difference of means. A p value of ≤0.05 was considered significant.

**IV. RESULTS**

**A. Neurologic deficit score**

In the standard protocol, at hour 20, one last half dose (0.5mg/ml) of Pavulon was given to facilitate the weaning of the animal from the ventilator. Upon successful independent breathing, the animal is extubated and at hour 23, given naloxone. After successful arousal, two investigators blinded to the randomization group status of the animal then perform the neurologic deficit examination. Figure 3.1 shows the plotted NDS scores from the current study. Consistent with previous data, normoxic animals had a better NDS score compared to hyperoxic animals. At an average score of 60, hyperoxic animals were non-responsive, had poor corneal reflex and exhibited opisthotonos as well as random purposeless movements. Oximetry animals had an average NDS score of 40 and were awake, alert and attempted to right themselves. Whatever the oxygenation
protocol, animals treated with SFP had mean NDS scores of 20 in both oxygenation protocol groups, were awake and alert, could right themselves and walk, albeit ataxically, drink water, and vocalize.

Figure 3.1: SFP improves short-term neurologic outcome.

Comparison of neurologic deficit scores (NDS) from either historical controls or SFP treated animals from either hyperoxic or normoxic oxygenation protocols. For hyperoxic animals, n = 7 – 4 per group, for normoxic animals n = 8 – 4 per group. * p < 0.05
B. Hippocampal cell death

Figure 3.2 shows representative staining for cresyl violet in the CA1 region of the hippocampus. Sham neurons had the round “ghost like” appearances of a typical healthy neuron (Fig. 3.2A). Dying neurons from the hyperoxic animals were shrunken, condensed and contain pyknotic nuclei (Fig. 3.2B). The oximetry protocol reduced the number of positively stained nuclei, but neurons still exhibited abnormal morphology (Fig. 3.2C). SFP treatment further reduced the number of positively stained nuclei and preserved cellular morphology (Fig. 3.2D). Quantification of positively stained nuclei show that the oximetry protocol significantly reduced cell death by 40% compared to hyperoxic resuscitation, while SFP independent of oxygenation significantly protected against cell death in combination with hyperoxic resuscitation and trended towards protection in combination with the oximetry protocol (Fig. 3.3).

![Figure 3.2: SFP reduces hippocampal CA1 neuronal death at 24 hr after canine cardiac arrest.](image)

Representative cresyl violet staining of CA1 hippocampal neurons in Sham (A), Hyperoxic (B), Oximetry/DMSO (C) and Oximetry/SFP (D) animals. (20x)
Figure 3.3: SFP increases hippocampal CA1 neuronal viability.

Comparison of cresyl violent stained CA1 hippocampal neurons from either historical controls or SFP treated animals from either hyperoxic or normoxic oxygenation protocols. For hyperoxic animals, n = 7 – 4 per group, for normoxic animals n = 8 – 4 per group. * p < 0.05
C. Effects of Sulforaphane on markers of oxidative stress

Figure 3.4A/B is representative fluorescent staining for 8-hydroxydeoxyguanosine, a commonly used marker of DNA/RNA oxidation, in the CA1 region of the hippocampus. Percent region of interest covered by immunoreactive product was quantified from three separate regions of interest by an observer blinded to sample identification. Compared to oximetry animals alone oximetry + SFP appeared to reduce DNA/RNA oxidation (Fig. 3.4C).

Figure 3.4: SFP decreases oxidative stress induced DNA damage within the ventral CA1 hippocampus 24 hr after CA.

8OHdG immunostaining is robust in cell bodies of pyramidal neurons and is reduced in an animal treated with SFP (1 mg/kg) at 30 min reperfusion (B) compared to those treated with DMSO vehicle (A). (20x). Quantification of 8OHdG staining (C). n = 4 – 2 animals per group.
V. DISCUSSION

This study suggests that sulforaphane is neuroprotective following cardiac arrest. This is the first study to demonstrate the neuroprotective efficacy of post-injury SFP treatment following global cerebral ischemia. We also gave SFP intravenously, unlike previous work showing neuroprotection, which has only administered the compound intraperitoneally (Chen et al., 2011a; Dash et al., 2009; Hong et al., 2010; Zhao et al., 2005; Zhao et al., 2006; Zhao et al., 2007b). Finally, we showed that SFP is neuroprotective 30 minutes after resuscitation, but the actual therapeutic window has yet to be determined. Specifically, given the knowledge that translational machinery is inhibited following global cerebral ischemia (Martin, V et al., 2001), future studies will need to define the optimal time for administration so as to avoid this immediate-post-injury metabolic down-time but also not sacrifice the potential for neuroprotection by waiting too long. Dosing patterns also need to be explored: is a single dose sufficient or would multiple doses provide additional protection? Human studies show that SFP is almost completely metabolized and excreted from the body within 72 hours (Kensler et al., 2005), but long term outcome studies in rats that undergo global cerebral ischemia demonstrate inflammation up to 30 days after injury (Hazelton et al., 2010), suggesting that multiple doses might increase neuroprotective efficacy.

An additional interesting finding in our work is that the prevention of post-injury hyperoxia with oximetry and the administration of SFP are independently neuroprotective and not dependent on each other. Further, SPF appears to be more neuroprotective in the setting of hyperoxic resuscitation presumably due to the excessive damage cause by hyperoxic resuscitation. It also suggests that the secondary oxidative injury caused by activation of inflammatory pathways may ultimately be responsible for mediating cell
viability. These observations are intriguing but as yet very preliminary and based on a single observation at 24 hours. It is unknown at this point of whether survival time lines of the animals from the hyperoxic + SFP or oximetry + SFP would significantly differ.

Another compounding factor that may regulate SFPs neuroprotective affects is estrogen availability. In our study we used only intact female beagles, although we did not quantified levels of circulating estrogen in the individual animal. Estrogen is known to have some neuroprotective efficacy and activate Nrf2. Catechol estrogens, a by-product of estrogen metabolism, undergo redox recycling and in the process create electrophilic quinones that have been shown to covalently modify KEAP1 and upregulate of Nrf2 associated genes (Lee et al., 2003; Sumi et al., 2009). SFP and estrogen may then either work cooperatively to modify KEAP1 or may be in competition. SFP has also been shown to modify estrogen receptor α expression (Ramirez and Singletary, 2009) and may ultimately affect estrogen’s normal cellular processes. This especially relevant for the population of post-menopausal women participating in various hormone replacement therapies shown to activate Nrf2 transcription by either directly binding to the ARE or by modifying the transactivation of the ARE by estrogen receptor β (Froyen and Steinberg, 2010). Previous work demonstrating the neuroprotective efficacy of SFP treatment in both acute and chronic CNS injury models has only used male animals. The possible interactions of SFP with testosterone have not yet been explored.

Our data supports the role of Nrf2 in the upregulation of cellular antioxidants and protection against oxidative stress. Our data demonstrates significant reduction 8OHdG staining, suggesting that cells were more resistant to oxidative stress. This also supports the idea that cardiac arrest is associated with oxidative stress. This has not yet been
confirmed in humans but is the subject of ongoing investigation (ClinicalTrials.gov identifier: NCT00888966). As noted above, however, Nrf2 also regulates inflammatory pathways, and SFP may also inhibit the inflammatory processes underlying the second wave of oxidative stress (Innamorato et al., 2008). Publications have shown that SFP can inhibit macrophage and microglial activation (Brandenburg et al., 2010; Lin et al., 2008; Ping et al., 2010) as well as inhibit matrix metalloproteinase 9 (MMP9) activity (Rose et al., 2005) which plays a crucial role in macrophage infiltration to the injury site (Bergin et al., 2005). SFP also preserves the expression of tight gap junction proteins (Zhao et al., 2007a) which is particularly relevant in epilepsy models where K+ induced depolarization is propagated by the breakdown of tight gap junctions (Hinterkeuser et al., 2000).

VI. SUMMARY

We conclude that SFP is indeed neuroprotective after acute, time-limited cerebral ischemic injury, but many aspects of clinical importance remain to be explored, not the least of which are the individual endocrine environment and outcomes over more extended periods of observation and observations which include long-term survival issues. In particular, such studies will help tease apart results from our combination treatment groups (hyperoxic + SFP and oximetry + SFP). Future studies will also address current limitations of small sample size and comparison to historical controls and trials will be randomized and NDS observers blinded to treatment protocol.
I. ABSTRACT

Aims: Exposure of mitochondria to oxidative stress and elevated Ca\(^{2+}\) promotes opening of the mitochondrial inner membrane permeability transition pore, resulting in membrane depolarization, release of accumulated Ca\(^{2+}\), and uncoupling of oxidative phosphorylation. These events can lead to metabolic failure and cell death. Pre-conditioning of cells against oxidative stress is a powerful cytoprotective approach that could involve resistance of mitochondria to injury. Prior studies have shown treatment of rats with sulforaphane (SFP) results in brain mitochondria that are resistant to redox-regulated permeability transition. This study tested the hypothesis that treatment of rats with SFP, an agent that activates the Nrf2 pathway of antioxidant gene expression, increases the resistance of liver mitochondria to redox-regulated PTP opening and elevates mitochondrial levels of antioxidants.

Results: Rats were injected with SFP or drug vehicle and liver mitochondria were isolated 40 hr later. Respiring mitochondria accumulated a small addition of Ca\(^{2+}\), which
was then released by exposure to any of several agents that either cause an oxidized shift in the mitochondrial redox state or that directly oxidize protein thiol groups. Mitochondria were also tested for levels of glutathione, antioxidant proteins, and peroxidase activity. SFP treatment of rats inhibited the rate of pro-oxidant-induced mitochondrial Ca$^{2+}$ release and increased expression of the glutathione peroxidase/reductase system and expression of thioredoxin 2.

Innovation and Conclusions: These results are the first to demonstrate that treatment of animals with the Nrf2 activator, SFP, increases the resistance of liver mitochondria to redox-regulated PTP opening and increases mitochondrial antioxidants. This novel form of preconditioning could protect against a wide variety of pathologies that include oxidative stress and mitochondrial dysfunction in their etiologies.

II. INTRODUCTION

Oxidative stress contributes significantly to the pathophysiology of several liver disorders including hepatitis (Bianchi et al., 1997), cirrhosis (Yamamoto et al., 1998), ischemia/reperfusion (Gonzalez-Flecha et al., 1993; Packer and Murphy, 1994), and acetaminophen toxicity (Moore et al., 1985). Mitochondria are a primary target of oxidative stress in these and other pathologies, as reflected by oxidative damage to mitochondrial DNA (Kukielska et al., 1994), proteins (Devi et al., 1994) and lipids (Ponsoda et al., 1995), oxidation and loss of mitochondrial glutathione (Shigesawa et al., 1992), and an oxidized shift in mitochondrial redox state (Thorniley et al., 1995). The combination of mitochondrial oxidative stress with exposure to abnormally high Ca$^{2+}$ concentrations can lead to opening of the mitochondrial permeability transition pore.
(Takeyama et al., 1993), which incapacitates mitochondrial ATP formation (Nieminen et al., 1995), often leading to metabolic failure and cell death (Lemasters et al., 1997).

PTP opening facilitates the flux of solutes of up to 1,500 Da across the inner membrane, resulting in membrane depolarization, uncoupling of oxidative phosphorylation, and mitochondrial osmotic swelling (Halestrap et al., 2002; Rasola and Bernardi, 2007). The molecular identity of the PTP is unknown; however, cyclophilin D is a target through which both Ca\(^{2+}\) and oxidative stress trigger PTP opening (Schinzel et al., 2005). Moreover, mitochondria isolated from the livers of CyD knockout mice are resistant to Ca\(^{2+}\) induced PTP opening (Baines et al., 2005) and CyD -/- hepatocytes are resistant to H\(_2\)O\(_2\) induced necrosis (Nakagawa et al., 2005). Other mitochondrial proteins that are strongly implicated in PTP activity and regulation include the adenine nucleotide translocase (ANT) (Ruck et al., 1998) and the phosphate transporter (Leung et al., 2008). The potential clinical importance of PTP opening within liver cells is exemplified by the finding that CyD knockout mice are resistant to liver damage caused by toxic levels of acetaminophen (Ramachandran et al., 2011).

Numerous antioxidant-based drugs have been used experimentally to reduce damage to the liver and other organs caused by oxidative stress. N-acetylcysteine is one such antioxidant that is also used clinically for acute liver failure (Stravitz and Kramer, 2009) but failed to show efficacy for contrast-induced renal dysfunction (Buyukhatipoglu et al., 2010). While the use of specific antioxidants has been successful in various animal models of disease and injury (Liu et al., 1995), their effectiveness may be limited by their relatively small range of targets compared to the numerous reactive oxygen species and molecular mechanisms of cellular toxicity induced by oxidative stress. An alternative
approach that has emerged recently is the use of chemicals or conditions that stimulate the expression of a wide variety of gene products representing the endogenous antioxidant defense system (Aleksunes and Manautou, 2007). Compounds such as curcumin (Balogun et al., 2003), carnosine, tert-butylhydroquinone (Lee et al., 2001) and SFP (Thimmulappa et al., 2002) can induce such gene expression apparently through the transcriptional activating factor NF-E2-related factor 2 (Nrf2). Activation of Nrf2 involves its release from a binding protein, KEAP1, and Nrf2 serine phosphorylation. Nrf2 then translocates into the nucleus where it binds to antioxidant response elements located upstream of the 5’ region of the promoter of many cytoprotective genes, e.g., NQO1, HO-1 and those that code for enzymes necessary for glutathione biosynthesis (Jaiswal, 2004).

Given that PTP opening is promoted by ROS (Petronilli et al., 1994) and is inhibited by both exogenous antioxidants (Halestrap et al., 1998) and endogenous antioxidant enzymes, we hypothesized that mitochondria from animals treated with an Nrf2 activator would be resistant to redox-regulated PTP opening. We did observe a small inhibition of peroxide-induced PTP opening in mitochondria isolated from the brains of normal rats injected with sulforaphane (Greco and Fiskum, 2010); however, no increase in likely Nrf2-targeted mitochondrial proteins was apparent. Since the blood brain barrier may limit access of sulforaphane to the brain and since only a fraction of brain mitochondria exhibit redox-sensitive PTP opening, we tested the much more redox-sensitive liver mitochondrial PTP for sensitivity to sulforaphane. Our results demonstrate that sulforaphane administration to rats dramatically inhibits redox-regulated PTP
opening by liver mitochondria and also increases immunoreactive levels of mitochondrial antioxidant-related proteins.

III. MATERIALS AND METHODS

A. Chemicals and reagents

R,S-Sulforaphane was purchased from LKT Laboratories, Inc (St. Paul, MN). Mannitol, sucrose, EGTA, HEPES, Tris, potassium phosphate dibasic (K₂HPO₄), magnesium chloride (MgCl₂), malate, glutamate, oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), adenosine 5′-diphosphate (ADP), bovine serum albumin (BSA), succinate, rotenone, calcium, dimethylsulfoxide (DMSO), tert-butyl hydrogen peroxide, phenylarsine oxide, horseradish peroxidase and oxaloacetate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Potassium chloride suprapure was obtained from EM Sciences (Fort Washington, PA). Amplex red was obtained from Invitrogen (Carlsbad, CA).

B. Administration of sulforaphane to rats

All experimental procedures were approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee and conducted in accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals. Male 300 g Fischer 344 rats (Charles River) were injected intraperitoneally with sulforaphane at 10 mg/kg in 200 µL solution containing 40% DMSO and 60% isotonic saline. Animals received DMSO plus saline as the vehicle control.
C. Isolation of liver mitochondria

Liver mitochondria were isolated as previously described (Andreyev and Fiskum, 1999). Rats were fasted overnight and euthanized by decapitation at 40 hr after injection of rats with sulforaphane or drug vehicle. All four lobes of the liver rapidly removed, chopped and homogenized in ice-cold isolation buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, 0.5 mg/ml fatty acid free BSA, 1 mM EGTA, pH 7.2, at 4°C). The homogenate was centrifuged at 2800 rpm for 12 min. Residual fat was aspirated from the top of the centrifuge tubes and the supernatant collected and centrifuged at 9300 rpm for 12 min. The pellets were re-suspended and centrifuged at 12,000 rpm for 12 min. After a final centrifugation at 12,000 rpm for 12 min the pellet was re-suspended in 1 mL isolation media not containing EGTA. Mitochondrial protein concentrations were measured using a Lowry DC kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin used as concentration standards.

D. Mitochondrial oxygen consumption

The respiratory activities of isolated liver mitochondria were measured polarimetrically with a Clark-type oxygen electrode apparatus (Hansatech Instruments, Norfolk, England). Mitochondria were suspended at a protein concentration of 0.5 mg/ml in buffer containing 125 mM KCl (ultra-pure), 20 mM HEPES, 2 mM K₂HPO₄, 0.01 mM EGTA, 1 mM MgCl₂ (pH 7.0) at 37°C, plus glutamate (5 mM) and malate (0.1 mM) or succinate (5 mM) and rotenone (4 µM) as oxidizable respiratory substrates. Addition of ADP (0.5 mM) was used to initiate State 3 (phosphorylating) respiration. Oligomycin (2.5 µg/ml), an inhibitor of the mitochondrial ATP synthase, was used to induce State 4 (resting) respiration.
E. Concurrent measurements of mitochondrial Ca\(^{2+}\)-uptake and release and pyridine nucleotide redox state

Experiments were performed as previously described for isolated brain mitochondria (Greco and Fiskum, 2010). Briefly, rat liver mitochondria were suspended at a protein concentration of 0.5 mg/ml in 2 ml of assay buffer (125 mM supra-pure KCl, 2 mM K\(_2\)HPO\(_4\), 1 mM MgCl\(_2\), 20 mM Tris-HCl, 5 mM succinate, and 4 µM rotenone, pH 7.4 at 30°C). The use of the electron transport chain complex II substrate succinate in the presence of the complex I inhibitor rotenone allows for the mitochondrial pyridine nucleotide redox state (NAD(P)H/NAD(P)\(^{+}\)) to be varied between totally reduced (no added pro-oxidant) to oxidized (after addition of pro-oxidants) independent of mitochondrial respiration and membrane potential. Mitochondria were added to the medium containing succinate and allowed to equilibrate, bioenergetically, based on stabilization of the medium free [Ca\(^{2+}\)] and NADP(H) autofluorescence. CaCl\(_2\) was then added to the medium at a level that the mitochondria were capable of completely accumulating. The rate of spontaneous Ca\(^{2+}\) release, if any, was then quantified in the absence of further additions. In other experiments, the rate of release was measured after the addition of a chemical pro-oxidant. The pro-oxidants used in these experiments were tert-butyl hydroperoxide, which induces oxidation of NAD(P)H by its metabolism via mitochondrial glutathione peroxidase and reductase, oxaloacetate, which drives the oxidation of NADH through the malate dehydrogenase reaction, and phenylarsine oxide, which selectively and directly oxidizes sulfhydryl groups. In a few experiments, cyclosporin A, a permeability transition inhibitor, was present to confirm that mitochondrial Ca\(^{2+}\) release was a consequence of permeability transition pore opening. Respiration-dependent mitochondrial uptake of Ca\(^{2+}\) and subsequent release, either
spontaneous or induced by addition of pro-oxidants, were measured by monitoring the free Ca\(^{2+}\) concentration present in the medium in which the mitochondria were suspended. The medium free Ca\(^{2+}\) was measured fluorimetrically in the presence of the Ca\(^{2+}\)-sensitive fluorescent dye Calcium Green-5N (0.1 µM), using excitation and emission wavelengths of 506 nm and 532 nm, respectively.

**F. Mitochondrial pyridine nucleotide redox state**

Autofluorescence of reduced pyridine nucleotides (NADH plus NADPH) in the mitochondrial suspensions was measured concurrently with the Calcium Green 5N (CG5N) measurements of medium \([\text{Ca}^{2+}]\) using excitation and emission wavelengths of 350 nm and 460 nm, respectively.

**G. Immunoblot analysis**

Freshly isolated liver mitochondria and liver homogenates were lysed in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Calbiochem). Equal amounts of mitochondrial protein from each sample were separated by SDS-PAGE (4-12% Bis-Tris gels) (Invitrogen) and transferred to PVDF membranes (Invitrogen). Membranes then incubated with primary antibodies (overnight at 4°C) with NQO1 1:500 (Santa Cruz), Cyclophilin D 1:30000 (Mitosciences), Glutathione Peroxidase 1:500 (Abcam), Thioredoxin 2 1:150000 (Santa Cruz), Malic Enzyme 3 1:5000 (Sigma), Isocitrate Dehydrogenase 2 1:2000 (Santa Cruz), Manganese Superoxide Dismutase 1:250 (Santa Cruz), GAPDH 1:500000 (Abcam), VDAC 1:50,000 – 1:300,000 (Mitosciences). The membranes were then washed with PBST and incubated for 1 h at RT in HRP-conjugated antibodies at 1:2000 dilution for 1 hr at RT. The washed blots were then treated with an enhanced chemiluminiscence detection reagent (Amersham.
Bioscience, UK). Densitometric analysis of the protein bands was performed using the Image J software.

**H. Peroxidase activity**

Isolated mitochondria were suspended at 0.5 mg/ml in assay buffer and incubated with 1 mM tBOOH for 1 min at 30°C. A 1 µl aliquot was then withdrawn and added to 2 mL of assay buffer at 30°C containing 10 µM Amplex Red and 25 U/mL horseradish peroxidase. Fluorescence of resorufin, the oxidized form of Amplex Red generated by peroxidation of tBOOH, was measured using excitation and emission wavelengths of 563 nm and 587 nm, respectively (Kowaltowski et al., 2004).

**I. Measurements of total reduced glutathione**

Glutathione measurements are measured using ThioGloTM-1 (excitation-388nm emission-500nm) as described elsewhere (Bayir et al., 2007). Glutathione was measured by the resulting fluorescence of the sample combined with cumene hydroperoxide and glutathione peroxidase subtracted from the fluorescence of the sample containing neither.

**J. Data analysis**

Data are expressed as means ± S.E.M. of n different experiments. Differences between groups were assessed by Student’s t-test. For data that are not normally distributed, the Mann-Whitney rank sum test was used. p < 0.05 was considered to be statistically significant.
IV. RESULTS

A. Evidence for Nrf2 activation in livers from rats treated with sulforaphane

NQO1 is an antioxidant enzyme whose gene and protein expression is generally up-regulated by compounds that activate the Nrf2/ARE pathway, including sulforaphane (Dinkova-Kostova and Talalay, 2008). NQO1 activity is elevated in the livers of mice fed broccoli seeds rich in glucosinolates like sulforaphane but does not change in mutant, Nrf2 knockout mice (McWalter et al., 2004). In order to verify that this Nrf2 pathway biomarker protein was elevated in rats 40 hr after intraperitoneal injection of sulforaphane at 10 mg/kg, liver homogenates were used for immunoblot analysis. Fig. 4.1A provides a sample immunoblot for NQO1 and the house-keeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for liver homogenates from a rat treated with drug vehicle and a rat treated with sulforaphane. As expected, the densitometric ratio of NQO1/GAPDH was significantly increased by more than 100% in homogenates from sulforaphane-treated rats (Fig. 4.1B).

![Image](image.png)

Figure 4.1: NAD(P)H quinone oxidoreductase 1 (NQO1) immunoreactivity in liver homogenates from vehicle- and sulforaphane (SFP) treated rats.

A. Representative immunoblots for NQO1 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). B. Densitometric ratios for NQO1/GAPDH for $n = 4 - 6$ animals per group. * $p<0.05$
B. Inhibition of redox-regulated mitochondrial permeability transition pore opening by treatment of rats with sulforaphane

After each mitochondrial preparation, O₂ electrode measurements were made of State 3 (ADP-stimulated) and State 4 (ATP synthase-inhibited) respiration as described in Materials and Methods. There were no significant differences in these rates for mitochondria isolated from the livers of vehicle- and sulforaphane-treated rats (not shown). Figure 4.2A describes the respiration-dependent uptake and peroxide-induced release of Ca²⁺ by mitochondria isolated from the livers of rats inoculated 40 hr earlier with either sulforaphane or drug vehicle. Fig 4.2B describes the concurrent measurements of NAD(P)H autofluorescence as an indicator of mitochondrial redox state. The addition of 25 µM CaCl₂ to the suspension of mitochondria caused an immediate increase in the fluorescence of the Ca²⁺ indicator CG5N, followed by a reduction back to near baseline. Electrophoretic uptake of Ca²⁺ into mitochondria through the Ca²⁺ uniporter is driven by succinate-driven, respiratory generation of the electrochemical gradient of protons, with a positive-outside membrane potential. In the absence of further additions, this mitochondrial load of 50 nmol/mg protein was retained within the mitochondria for at least 8 min with only a very slow rate of release into the medium (Fig. 4.2A, trace 1). There was no change in NAD(P)H autofluorescence during this time (Fig. 4.2B, trace 1), as expected since the presence of the electron transport chain complex I inhibitor rotenone blocks the oxidation of NADH that would otherwise be stimulated by respiration-dependent activities like Ca²⁺ accumulation. Both the CG5N and NAD(P)H fluorescent measurements were very similar for mitochondria from rats treated with vehicle and those treated with sulforaphane (Fig. 4.2A & B, traces 1 and 4).
Figure 4.2: Peroxide-induced permeability pore transition (PTP) opening and pyridine nucleotide oxidation in rat liver mitochondria.

A. Representative traces of respiration-dependent mitochondrial Ca\(^{2+}\) uptake and subsequent PTP-dependent release. Extramitochondrial free Ca\(^{2+}\) was monitored with the Calcium Green 5N fluorescent dye in medium containing succinate as a respiratory substrate and the respiratory chain complex I inhibitor, rotenone. At time 0 mitochondria (0.5 mg/ml) from vehicle-treated or sulforaphane-treated rats were loaded with 25 µM calcium, (Trace 1 & 4). At 250 sec, tBOOH was added at either 5 µM (Trace 2) or 250 µM (Trace 3), or presence of 1 µM cyclosporin A (CsA) (Trace 7). At 450 sec, ionomycin was added. B. Representative tracings of NAD(P)H autofluorescence in the absence and presence of tBOOH. Pyridine nucleotide redox state for mitochondria from either vehicle- or sulforaphane-treated rats remained highly reduced and stable after the addition of Ca\(^{2+}\) alone (Traces 1 & 4). The addition of 5 µM (Trace 2) or 250 µM (Trace 3) tBOOH caused immediate and complete oxidation of NAD(P)H within vehicle control mitochondria. Treatment of rats with SFP inhibited NAD(P)H oxidation at 5 µM tBOOH (Trace 5), but did not inhibit oxidation at 250 µM tBOOH (Trace 6).
Mitochondrial PTP opening is promoted by the presence of peroxides through their metabolism via glutathione peroxidase and subsequent oxidation of reduced glutathione, pyridine nucleotides, and protein cysteine sulfhydryl groups (Toman and Fiskum, 2011). Glutathione peroxidase requires the reduced form of glutathione (GSH) formed from oxidized glutathione (GSSG) via NADPH-dependent glutathione reductase. At rates of peroxidation that exceed maximal rates of NADP+ and GSSG reduction, an oxidized shift in redox state occurs, as reflected by a decrease in NAD(P)H autofluorescence. When a 250 µM final concentration of tBOOH was added to the suspension of mitochondria from a vehicle-treated rat at 250 sec after the addition of Ca2+, a rapid and continuous release of Ca2+ calcium ensued (Fig. 4.2A, tracing 3). This release of Ca2+ was nearly complete, as indicated by an only small further increase in CG5N fluorescence after the subsequent addition of the Ca2+ ionophore, ionomycin (Fig. 4.2A, trace 3). The tBOOH-induced Ca2+ release was due to PTP opening, as evidenced by the fact that 1 µM cyclosporin A completely blocked mitochondrial Ca2+ efflux (Fig. 4.2A, trace 7). The rate at which Ca2+ was released upon addition of tBOOH was slightly slower than the rate at which the NAD(P)H fluorescence declined (Fig. 4.2B, trace 3). In comparison to the mitochondria from vehicle-treated rats, the tBOOH-induced Ca2+ release by mitochondria from sulforaphane-treated rats was substantially slower (Fig. 4.2A, trace 6) even though the rate and extent of NAD(P)H oxidation was very similar (Fig. 4.2B, tracing 3). Even at a much lower, 5 µM addition of tBOOH, the release of Ca2+ and the NAD(P)H oxidation for mitochondria from a vehicle-treated rat were very similar to those observed with 250 µM tBOOH (Fig. 4.2A & B, trace 2). However, the addition of 5 µM tBOOH to mitochondria from sulforaphane-treated rats resulted in only
a transient, minor release of accumulated Ca\(^{2+}\) and transient NAD(P)H oxidation, followed by a re-reduction to a level of maximal fluorescence (Fig. 4.2 A & B, trace 5).

Initial rates of the increase in CG5N fluorescence starting 250 sec after the addition of Ca\(^{2+}\) alone or immediately after the subsequent addition of 5, 50, and 250 µM tBOOH are shown in Fig. 4.3A for \(n = 6\) different mitochondrial preparations from either sulforaphane or vehicle-treated rats. Even though the Ca\(^{2+}\) release rate was very slow in the absence of tBOOH, it was significantly slower for sulforaphane-treated rats. The release rate was also significantly slower at each concentration of tBOOH with the largest reduction (75%) observed at 5 µM tBOOH. The final reductions in NAD(P)H autofluorescence observed at the end of these experiments is shown in Fig. 4.3B. No difference was observed after the addition of Ca\(^{2+}\) alone for mitochondria from vehicle and sulforaphane-treated rats. There were also no significant differences between animal groups for the extent of NAD(P)H oxidation elicited at either 50 or 250 µM tBOOH. At 5 µM, however, there was a significant, approximately 50% decrease in NAD(P)H oxidation observed with mitochondria from sulforaphane-treated rats.
Figure 4.3: Comparison of peroxide induced Ca\(^{2+}\) release rates and pyridine nucleotide oxidation for mitochondria from sulforaphane treated and vehicle treated rats.

A. Initial rates of rise in Calcium Green 5N fluorescence after addition of 0, 5, 50, or 250 µM tBOOH. B. Extent to which NAD(P)H autofluorescence decreased after additions of different concentrations of tBOOH. n = 7 animals per group. * p<0.05
To determine if treatment of rats protects against mitochondrial PTP opening promoted by oxidative stress other than that generated by peroxide metabolism, we measured Ca$^{2+}$ release rates in response to either phenylarsine oxide (PhAsO) or oxaloacetate (OAA). PhAsO induces PTP opening by directly oxidizing protein thiol groups associated with PTP components, e.g., CyD and ANT. Oxaloacetate indirectly causes PTP opening through its reduction to malate via the malate dehydrogenase reaction (Lehninger et al., 1978), which oxidizes NADH directly and NADPH indirectly, thus also causing an oxidized shift in the GSH and protein thiol redox states (Vercesi, 1984). As shown in Fig. 4.4, sulforaphane treatment of rats reduced the rate of OAA-induced Ca$^{2+}$ release by approximately 60%, which is similar to what was observed with the saturating concentrations of tBOOH. Sulforaphane treatment also significantly inhibited the rate of Ca$^{2+}$ release induced by the addition of 30 µM PhAsO, albeit by only 25%. As with tBOOH, Ca$^{2+}$ release elicited by the addition of either OAA or PhAsO was inhibited by the presence of the PTP inhibitor cyclosporin A (not shown).
Figure 4.4: Inhibition of oxaloacetate- or phenylarsine oxide-induced Ca$^{2+}$ release by rat liver mitochondria.

A. Initial rates of rise in Calcium Green 5N fluorescence after the addition of 5 mM oxaloacetate (OAA) or B. 30 µM phenylarsine oxide (PhAsO). n = 4 animals per group. * p<0.05
C. Treatment of rats with sulforaphane does not alter cyclophilin D protein expression

Cyclophilin D is currently the only widely accepted protein associated with regulation of PTP opening (Halestrap, 2010). One possible explanation for inhibition of PTP opening by sulforaphane treatment is down-regulation of its expression, since mitochondria from CyD knockout mice exhibit little if any PTP activity (Baines et al., 2005). A sample immunoblot and densitometric quantification of CyD levels, expressed as the ratio of CyD to the mitochondrial voltage dependent anion channel, are shown in Fig. 4.5A &B. These results indicate that sulforaphane treatment has no effect on levels of CyD in rat liver mitochondria under the conditions used in these experiments.
Figure 4.5: Effects of sulforaphane treatment on immunoreactive levels of mitochondrial proteins related to regulation of permeability transition pore opening.

Representative immunoblots and densitometry of A, B. cyclophilin D (CyD)/voltage dependent anion channel (VDAC), C, D. glutathione peroxidase 1 (GPX1)/VDAC, E, F. malic enzyme 3 (ME3)/VDAC, G, H. isocitrate dehydrogenase 2 (IDH2)/VDAC, I, J. thioredoxin 2 (Trx2)/VDAC, K, L. superoxide dismutase 2 (SOD2)/VDAC. n = 6 - 7 animals per group. * p<0.05
D. Sulforaphane treatment selectively increases expression of proteins involved in mitochondrial defenses against oxidative stress

Sulforaphane could potentially affect the level of several mitochondrial proteins that could contribute to the inhibition of redox-sensitive PTP opening. For instance, an elevation of glutathione peroxidase could increase the rate at which added tBOOH is fully metabolized, resulting in a transient rather than a prolonged oxidized shift in mitochondrial redox state, as demonstrated in Fig. 4.2B. We therefore performed immunoblots for the mitochondria-specific glutathione peroxidase 1 (GPX1). As shown in Fig. 4.5 C & D, sulforaphane treatment caused a significant, approximately 50% increase in GPX1 immunoreactivity present in isolated liver mitochondria. Evidence that this increase in protein is associated with an increase in activity came from measurements of peroxidase activity performed with freshly isolated mitochondria, as described in Materials and Methods. The resorufin fluorescence value shown in Fig. 4.6A represents the fraction of tBOOH present in a mitochondrial suspension after one minute incubation since the fluorescence value in the absence of mitochondria was 1000 units. There was significantly less fluorescence present after incubation with mitochondria from sulforaphane treated compared to vehicle treated rats. Based on the differences between maximal and post-incubation fluorescence of approximately 300 and 600 units for control and sulforaphane mitochondria, respectively, treatment of rats with sulforaphane resulted in an approximate 100% increase in total mitochondrial peroxidase activity.
Figure 4.6: Effects of sulforaphane treatment on mitochondrial peroxidase activity and glutathione content.

A. Liver mitochondria were incubated with 1 mM tBOOH for one minute. An aliquot was added to a cuvette containing horseradish peroxidase (HRP) and Amplex Red. Any residual tBOOH not reduced to H₂O reacts with HRP to oxidize Amplex Red into its fluorescent product, reozurin. n = 7 animals per group. * p<0.05

B. Content of reduced glutathione in liver mitochondria isolated from either vehicle or sulforaphane treated rats. n = 9 – 10 animals per group. * p<0.05.
Glutathione peroxidase activity in situ requires the enzyme, reduced glutathione, glutathione reductase and the NADPH necessary to re-reduce glutathione as it is oxidized. Although antibodies to mitochondrial glutathione reductase were not available, we did measure total mitochondrial reduced glutathione and immunoreactivity for two of the enzymes responsible for reduction of NADP⁺ to form NADPH. Fig. 4.6B demonstrates that there was a small, albeit significant increase in total glutathione present in mitochondria from sulforaphane- compared to vehicle-treated rats. Sample immunoblots and densitometry results for NADP⁺-dependent mitochondrial malic enzyme 3 (ME3) and NADP⁺-dependent isocitrate dehydrogenase 2 (IDH2) indicate that there was a significant, >100% increase in ME3 immunoreactivity in mitochondria from sulforaphane treated rats (Fig. 4.5 E & F) but no difference in IDH2 levels for the two animal groups (Fig. 4.5 G & H).

Mitochondria contain other antioxidant defense enzymes, including thioredoxin 2 (Trx2), thioredoxin reductase, and thioredoxin peroxidase, also known as peroxiredoxin 3 and 5. Thioredoxins in the mitochondria serve a dual function of being able to both scavenge free radicals as well as keep protein thiols reduced. Trx2 protein levels were significantly higher in mitochondria from sulforaphane-treated rats (Fig. 4.5 I & J).

We also tested for the effects of sulforaphane treatment on the immunoreactivity of the mitochondria-specific SOD2, based on the findings that the tBOOH-induced PTP is more active in both liver and heart mitochondria from SOD2 +/- mice (Van Remmen et al., 2001), and that sulforaphane increases total mitochondrial SOD activity in rat aortic muscle cells (Zhu et al., 2008). As shown in Fig. 4.5 K & L, sulforaphane had no effect on rat liver mitochondrial SOD2 immunoreactivity.
V. DISCUSSION

This is the first in-depth study of the regulation of mitochondrial PTP opening and antioxidant-related mitochondrial proteins by a pharmacologic activator of the Nrf2 pathway of cytoprotective gene expression. The primary conclusion reached from this study is that treatment of rats with sulforaphane results in a robust inhibition of PTP opening in rat liver mitochondria triggered by several forms of oxidative stress. These forms include a shift in redox state induced through either glutathione-dependent peroxide metabolism or direct oxidation of pyridine nucleotides by NADH-dependent reduction of oxaloacetate. In addition, sulforaphane treatment also inhibits PTP opening by direct oxidation of sulfhydryl groups mediated by phenylarsine oxide. An additional novel finding from this study is that sulforaphane treatment selectively increases several important mitochondrial proteins and molecules that serve as direct antioxidants, e.g. glutathione peroxidase, thioredoxin, and glutathione, or that generate the reducing power in the form of NADPH (e.g. malic enzyme) that is necessary for driving these antioxidant activities.

A number of findings provide insight into molecular mechanisms responsible for inhibition of PTP opening by treatment of rats with sulforaphane. The > 100% increase in NQO1 immunoreactivity in liver homogenates strongly suggests that sulforaphane activates the Nrf2 pathway of gene expression (Dinkova-Kostova and Talalay, 2010). While the pattern of mitochondrial protein expression that is elevated by sulforaphane treatment supports this conclusion, additional comparisons between mitochondria from Nrf2 +/- and Nrf2 -/- mice will be necessary to prove that Nrf2 activation is necessary for sulforaphane inhibition of PTP opening (Kensler et al., 2007). One mechanism of
action that can be ruled out is a general influence of sulforaphane treatment on basic mitochondrial bioenergetics. Any such effects on the activities responsible for oxidative phosphorylation or the energy coupling between electron transport and ATP synthesis should be reflected by differences in either state 3 or 4 respiration, which were not different for liver mitochondria from sulforaphane- and vehicle-treated rats. The absence of effects on mitochondrial respiration by sulforaphane was also observed with isolated rat brain mitochondria (Greco and Fiskum, 2010). These findings do not, however, address the possibility that sulforaphane and other Nrf2 activators can stimulate mitochondrial biogenesis, as described by Piantadosi et al. (Piantadosi et al., 2008).

The possibility that sulforaphane treatment inhibits PTP opening by reducing the expression of PTP components remains an open question, due mainly to the fact that the identity of these components is unresolved. We focused our measurements on cyclophilin D since the relationship between its expression and PTP activity is best characterized and because it has no apparent direct influence over mitochondrial bioenergetics like other putative components, e.g., the ANT and the phosphate transporter. We found that sulforaphane treatment has no effect on CyD immunoreactivity in rat liver mitochondria, which is consistent with the lack of an effect reported earlier for rat brain mitochondria (Greco and Fiskum, 2010).

Insight into mechanisms that are applicable to the inhibition of PTP opening by sulforaphane comes from comparisons between release of accumulated Ca\(^{2+}\) and oxidization of pyridine nucleotides at different concentrations of \(t\)BOOH. Similar rates of \(t\)BOOH -induced mitochondrial Ca\(^{2+}\) release and complete NAD(P)H oxidation were observed at 5, 50, and 250 \(\mu\)M \(t\)BOOH for mitochondria from vehicle-treated rats (Fig.
3). While Ca\(^{2+}\) release rates and pyridine nucleotide oxidation were also similar at both 50 and 250 µM tBOOH with mitochondria from sulforaphane-treated rats, both were much reduced at 5 µM tBOOH and also much lower than the values obtained with mitochondria from vehicle-treated animals. This comparison is similar to what was reported when comparing redox- sensitive PTP activity for mitochondria from normal rat liver and AS-30D hepatoma mitochondria. Compared to normal liver mitochondria, hepatoma mitochondria are resistant to both tBOOH -induced NAD(P)H oxidation and Ca\(^{2+}\) release (Fiskum and Pease, 1986). It was determined that the cause for this resistance is increased mitochondrial malic enzyme activity, which produces NADPH and increases the intramitochondrial redox buffering power. The fact that ME3 immunoreactivity is more than twice as high in the liver mitochondria from rats treated with sulforaphane (Fig. 4.5) is consistent with the redox buffering power being responsible for resistance to PTP opening, at least at the low tBOOH concentration of 5 µM. Another possible explanation is that tBOOH is metabolized so quickly that the redox state is only temporarily disturbed, resulting in only transient PTP opening. The transient rise in medium [Ca\(^{2+}\)] concentration and reduction in NAD(P)H autofluorescence described in Fig. 2 for mitochondria from sulforaphane-treated rats is consistent with this mechanism. This hypothesis is also supported by the finding that the total peroxidase activity of these mitochondria is significantly greater than that of mitochondria from vehicle-treated rats (Fig. 4.6).

The increase in either malic enzyme or mitochondrial peroxidases could explain sulforaphane-induced resistance of PTP opening at low tBOOH levels but are unlikely to be responsible at concentrations of 50 µM and above. At these higher tBOOH levels,
complete and sustained pyridine nucleotide oxidation occurs with mitochondria from both vehicle- and sulforaphane treated rats, thus overcoming any elevated rates of NADPH production or peroxide metabolism. The increase in mitochondrial total glutathione observed after sulforaphane administration could contribute to increased sulfhydryl buffering power; however the 25% increase is modest (Fig. 4.6). Another possible mechanism may lie distal to redox buffering and be limited kinetically rather than thermodynamically by reduction of protein thiol groups. The increased thioredoxin immunoreactivity observed in mitochondria from sulforaphane-treated rats is consistent with this possibility (Fig. 4.5). There are several other proteins that could also contribute to maintenance of reduced sulfhydryl redox state for PTP associated proteins like CyD and ANT. These include but are not limited to thioredoxin reductase, glutaredoxin and glutaredoxin reductase (Koehler et al., 2006).

VI. INNOVATION

In summary, measurements of peroxide induced release of accumulated Ca$^{2+}$ and pyridine nucleotide oxidation demonstrate that redox-sensitive PTP opening by isolated liver mitochondria is substantially inhibited by treatment of rats with i.p. sulforaphane over one and a half days earlier, particularly at lower levels of added tBOOH that are likely most relevant to levels of oxidative stress that occur in situ within cells. Several mechanisms of action are probably responsible for this inhibition, including increased redox buffering power, more rapid disposition of added peroxide, and more effective use of mitochondrial redox buffering to maintain PTP associated protein sulfhydryls in the reduced redox state. Experiments are in progress to further characterize the relative contribution of each of these mechanisms in this system. Considering the over 100 genes
whose expression are induced by Nrf2 activators like sulforaphane, it is not surprising that the mitochondrion, like the entire cell, responds in multiple ways to Nrf2 activation. This multifactorial response probably confers mitochondria with protection against different forms of oxidative stress and many sequelae, including PTP opening and associated metabolic dysfunction.
CHAPTER 5: NRF2 ACTIVATORS INHIBIT MITOCHONDRIAL PERMEABILITY TRANSITION IN PERMEABILIZED PC12 PHEOCHROMOCYTOMA CELLS

INVESTIGATORS: Greco, T., Fiskum, G.

I. ABSTRACT

Previously we have shown that treatment of rats with SFP supports mitochondrial resistance to redox-regulated permeability transition pore (PTP) opening. While isolated mitochondria represent a direct model for which to test this hypothesis, the isolation procedure introduces mechanical damage that can obscure results. In order to verify our previous experiments that utilized isolated liver and non-synaptic brain mitochondria, we exposed to PC12 cells to either sulforaphane (SFP) or tert-butylhydroquinone (TBHQ) 24 hours prior to digitonin permeabilization to allow access to mitochondria. Mitochondria within digitonin permeabilized PC12 cells were loaded with a non-PTP-activating concentration of Ca\(^{2+}\) followed by the addition of tert-butylhydroperoxide (tBOOH). tBOOH induced rapid oxidation of pyridine nucleotides and release of calcium from the mitochondria. To verify activation of the PTP by tBOOH we simultaneously monitored mitochondrial membrane potential depolarization with tetramethylrhodamine methyl ester (TMRM) induced by tBOOH addition. Treatment of cells with either SFP or TBHQ
significantly inhibited both Ca\textsuperscript{2+} release and depolarization. These results suggest that treatment with compounds known to activate the Nrf2 pathway modify redox regulated mitochondrial PTP activation.

II. INTRODUCTION

Production of reactive oxygen species leads to oxidative stress that inevitably has a negative effect on cell survival (Nagley et al., 2010). The cell is continuously exposed to both endogenous and exogenous sources of ROS that can lead to the oxidation of proteins (Maltecca and Casari, 2010), lipids (Tyurin et al., 2010) and DNA/RNA (Wang et al., 2005), promoting bioenergetic failure and cell death. It is therefore necessary for the cell to have systems available to rid itself of unnecessary levels of ROS.

The cell contains both constitutive and inducible antioxidant systems (Brown et al., 2006) that maintain proper redox balance within the cell and its organelles. However, if these systems are either compromised or overwhelmed by the amount of ROS production, this can lead to the oxidative stress. Oxidative stress is responsible for the pathophysiology and cell death that is associated with many acute and chronic central nervous system diseases.

One of the several yet most important targets of oxidative stress is the mitochondria (Siesjo and Siesjo, 1996). ROS produced during incidences such as ischemia/reperfusion injury will inhibit oxidative phosphorylation (Fiskum et al., 2004) and lead to the activation of the mitochondrial permeability transition pore (PTP) (Halestrap, 2010). The PTP, a megachannel with largely unknown components, is particularly sensitive to oxidative insults. On the PTP are sulfhydryl groups that act as a redox “sensor” that controls the pore opening, with reduced equaling closed and oxidized
equaling open (Linard et al., 2009b). Once activated the pore allows molecules smaller than 1500Da to diffuse freely across the inner membrane, allowing the release of glutathione and NAD$^+$ as well as causing membrane potential collapse. These events further inhibit oxidative phosphorylation and promote ROS production (Lemasters et al., 2009). Eventually, mitochondrial swelling will lead to inner membrane rupture and promote necrotic cell death. Groups have tried with limited success to introduce exogenous ROS scavengers into the mitochondria (Smith et al., 2011). This approach is successful in that it eliminates a specific radical; however in the larger scheme of things eliminating a single species is not enough (Edeas, 2009). A more suitable approach may be to influence several targets at once by utilizing a system both the cell and mitochondria already have, e.g. inducible antioxidant gene expression.

Consequently, attention has been focused on the inducible antioxidant system as a means to either prevent or alleviate oxidative stress. The key component of the system is the Nrf2 protein which is a part of the leucine zipper/cap’n’collar family of protein and has been shown to be the master regulator of inducible antioxidant gene expression (Hu et al., 2006a; Thimmulappa et al., 2002). Nrf2 is constitutively expressed and bound to its repressor, KEAP1 which is a cullin 3 dependent ubiquitin protein ligase complex that target Nrf2 for degradation by the 26s proteasome (Jaiswal, 2004). Upon exposure to either oxidative stress or compounds such as SFP (Hu et al., 2011) or TBHQ (Hong et al., 2005) that are known to modify KEAP1, Nrf2 is released, stabilized and translocated into the nucleus where it binds to the ARE and induces the expression of cytoprotective genes (Jaiswal, 2004).
Our previous work demonstrated that when a compound known to activate Nrf2 was administered to rats, isolated non-synaptic brain and liver mitochondria became resistant to peroxide induced opening of the mitochondrial permeability transition pore (Greco and Fiskum, 2010) (Chapters 2 and 4). Although immunoblots showed significant upregulation of NQO1 in liver homogenates, indirectly suggesting that Nrf2 activation had occurred; it was still unknown whether Nrf2 was truly mediating this effect. Future studies utilizing RNA interference to knock down gene expression of Nrf2 in an in-vitro model are necessary to understand the role of Nrf2 in our paradigm.

III. MATERIALS AND METHODS

A. Cell culture

Rat pheochromocytoma (PC12) cells were grown in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 5% (v/v) heat inactivated fetal bovine serum (FBS), 10% (v/v) heat inactivated horse serum (HS) 10,000 U/mL penicillin G and 10 mg/ml streptomycin. The cultures were grown at 37°C in a humidified atmosphere containing 5% CO2. DMEM was purchased from Cellgro (Manassas, VA), FBS was purchased from Sigma (St. Louis, MO), HS was purchased from Hyclone (Waltham, MA) and the penicillin:streptomycin solution was purchased from Gemini Bio-products (West Sacramento, CA). Vehicle control (DMSO) 0.025%, 50 µM TBHQ or 1 µM SFP were added to the media for 24 hours prior to measurements.

B. Mitochondrial Ca\textsuperscript{2+}-uptake and release

PC12 cells were suspended at 5 x 10\textsuperscript{5} cells in 2 ml of assay buffer (125 mM supra-pure KCl, 2 mM K\textsubscript{2}HPO\textsubscript{4}, 1 mM MgCl\textsubscript{2}, 20 mM Tris-HCl, 5 mM succinate, 4 µM rotenone, and 0.006% digitonin pH 7.4 at 30°C). The use of the electron transport chain complex II
substrate succinate in the presence of the complex I inhibitor rotenone allows for the
mitochondrial pyridine nucleotide redox state (NAD(P)H/NAD(P)⁺) to be varied between
totally reduced (no added pro-oxidant) to oxidized (after addition of pro-oxidants)
independent of mitochondrial respiration and membrane potential. Cells were added to the
medium containing succinate and allowed to equilibrate bioenergetically, based on
stabilization of the medium free [Ca²⁺] and NADP(H) autofluorescence. CaCl₂ (175 µM)
was then added to the medium at a level that the mitochondria were capable of completely
accumulating. The rate of spontaneous Ca²⁺ release, if any, was then quantified in the
absence of further additions. In other experiments, the rate of release was measured after the
addition of a chemical pro-oxidant, rBOOH, which induces oxidation of NAD(P)H by its
metabolism via mitochondrial glutathione peroxidase and reductase. In two experiments,
cyclosporin A (1 µM), a permeability transition pore inhibitor, was present to further
confirm that mitochondrial Ca²⁺ release was a consequence of permeability transition pore
opening. Respiration-dependent mitochondrial uptake of Ca²⁺ and subsequent release, either
spontaneous or induced by addition of pro-oxidants, were measured by monitoring the free
Ca²⁺ concentration present in the medium in which the mitochondria were suspended. The
medium free Ca²⁺ was measured fluorimetrically in the presence of the Ca²⁺-sensitive
fluorescent dye Calcium Green-5N (0.1 µM), using excitation and emission wavelengths of
506 nm and 532nm, respectively.

C. Mitochondrial pyridine nucleotide redox state

Autofluorescence of reduced pyridine nucleotides (NADH plus NADPH) in the
mitochondrial suspensions was measured simultaneously with the change in medium Ca²⁺
using excitation and emission wavelengths of 350 nm and 460nm, respectively.
D. Monitoring of mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was monitored simultaneously with the change in medium Ca^{2+} using the monovalent cationic methyl ester fluorescent dye tetramethylrhodamine (1 µM), using excitation and emission wavelengths of 549 nm and 575 nm, respectively.

E. Data Analysis

Data are expressed as means ± S.E.M. of n different experiments. Differences between groups were assessed by Student’s t-test. For data that are not normally distributed, the Mann-Whitney rank sum test was used. p < 0.05 was considered to be statistically significant.

IV. RESULTS

A. Inhibition of redox regulated opening of the PTP by pre-treatment with SFP

Figure 5.1A diagrams a representative experiment showing mitochondrial uptake and release of calcium as induced by tBOOH and monitored with the extra-mitochondrial dye, CG5N. PC12 cells were treated with SFP (1 µM) 24 hours prior to the experiment. Cells were suspended with the respiratory substrate succinate, CG5N and the detergent digitonin which strips the cell membrane of cholesterol and allows access to the mitochondria. Mitochondrial were loaded with calcium (175 µM) that did not result in the spontaneous activation of the PTP (Fig. 5.1A, Trace 1 and 2). Following uptake and stable retention of calcium, we added tBOOH (250 µM) that resulted in a biphasic release of calcium from the mitochondria (Fig. 5.1A, Trace 3) that is delayed with SFP treatment (Fig. 5.1A, Trace 4). SFP treatment significantly inhibited the time to half maximal calcium release (Fig. 5.1B). We also simultaneously monitored pyridine nucleotide
autofluorescence. There was no spontaneous oxidation with calcium alone (Fig. 5.1C, Trace 1 and 2), but showed rapid oxidation upon addition of tBOOH (Fig. 5.1C, Trace 3 and 4). Similar to our results utilizing non-synaptic brain mitochondria, SFP treatment did not significantly protect against pyridine nucleotide oxidation (Fig. 5.1D).
Figure 5.1: Treatment of PC12 cells with SFP delays PTP activation.

A. Extra-mitochondrial calcium was monitored by calcium green 5N fluorescence and succinate was used as a respiratory substrate. Digitonin permeabilized PC12 cells (0.5 x 10^5/ml in suspension) were loaded with 175 μM Ca^{2+} followed by the addition of 250 μM tBOOH at 400 sec. Spontaneous release of Ca^{2+} was minimal and was not affected by SFP treatment (Traces 1 and 2). The addition of tBOOH induced a biphasic release of Ca^{2+} (Trace 3) that was significantly delayed with SFP treatment (Line 4). B. Comparisons of the time to half maximal release of Ca^{2+} following tBOOH addition. C. mitochondrial pyridine nucleotide redox state was measured by monitoring NAD(P)H auto fluorescence in the absence (Traces 1 and 2) or presence of tBOOH (Traces 3 and 4). D. Comparisons of the extent of pyridine nucleotide oxidation following the addition of tBOOH. n = 4 experiments per group. * p < 0.05.
B. Redox regulated PTP opening in cells pre-treated with TBHQ

Figure 5.2A is the representative image of Ca\(^{2+}\) uptake and release induced by tBOOH in cells that were pre-treated with vehicle or TBHQ 24 hours prior to the experiment. Similar to previous results in our SFP group, mitochondria took up Ca\(^{2+}\) with no spontaneous release (Fig. 5.2A, Traces 1 and 2) and biphasic release of Ca\(^{2+}\) was again observed upon addition of tBOOH (Fig. 5.2A, Trace 3). Unlike SFP treatment, TBHQ resulted in the almost complete inhibition of Ca\(^{2+}\) release from the mitochondria (Fig. 5.2A, Trace 4). Quantification of our traces shows that Ca\(^{2+}\) release from our mitochondria within TBHQ treated cells is minimal compared with the vehicle treatment (Fig. 5.2B). We again monitored pyridine nucleotide autofluorescence and saw no spontaneous oxidation with Ca\(^{2+}\) alone (Fig. 5.2C, Traces 1 and 2) and robust oxidation with addition of tBOOH (Fig. 5.2C, Traces 3 and 4). As in the SFP group, we did not observe any significant protection against pyridine nucleotide oxidation (Fig. 5.2D).

Although an accepted method of monitoring PTP activation, calcium uptake and release is an indirect measure of mitochondrial involvement. To determine if TBHQ treatment indeed affects the mitochondria, we simultaneously monitored mitochondrial membrane potential while observing Ca\(^{2+}\) uptake and release with CG5N.

Digitonin permeabilized PC12 cells were suspended with membrane potential sensitive dye TMRM (1 μM) and succinate as a respiratory substrate. Ca\(^{2+}\) was added which results in minimal mitochondrial depolarization (Fig. 5.2E, Traces 1 and 2). The addition of tBOOH caused rapid depolarization that preceded complete calcium release (Fig. 5.2E, Trace 3) and was attenuated with TBHQ pre-treatment (Fig. 5.2E, Trace 4). When quantified, TBHQ reduced depolarization by 50%.
**Figure 5.2:** Treatment of PC12 cells with TBHQ inhibits PTP activation and membrane depolarization.

A. Extra-mitochondrial calcium was monitored by calcium green 5N fluorescence with succinate as a respiratory substrate. Digitonin permeabilized PC12 cells (0.5 x 10⁵/ml in suspension) were loaded with 175 µM Ca²⁺ (Traces 1 and 2), followed by the addition of 250 µM tBOOH at 300 sec (Traces 3 and 4). B. Comparisons of the percent increase of calcium green 5N (CG5N) fluorescence following addition of tBOOH. n = 6 experiments per group. C. mitochondrial pyridine nucleotide redox state was measured by monitoring NAD(P)H auto fluorescence in the absence (Traces 1 and 2) or presence of tBOOH (Traces 3 and 4). D. Comparisons of the extent of pyridine nucleotide oxidation following the addition of tBOOH. E. Mitochondrial membrane potential was monitored with TMRM. Traces 1 and 2 represent Ca²⁺ alones followed by addition of tBOOH (Traces 3 and 4). F. Comparisons of the initial rise in TMRM fluorescence following addition of tBOOH. n = 2 experiments per group. * p < 0.05.
V. DISCUSSION

Previously we showed that treatment of rats with SFP results in mitochondria that are resistant to redox regulated PTP activation (Greco and Fiskum, 2010). Use of CsA confirmed calcium release is via the PTP and not by alternative mechanisms such as changes in the membrane, reversal of the Ca\(^{2+}\) uniport or by the specific calcium/hydrogen, or sodium antiport system. However our experimental model left open the possibility that SFP simply made the mitochondria more resistant to the mechanical damage during the isolation procedure and in turn made the mitochondria more resistant to calcium overload and oxidative stress. To validate our results with isolated mitochondria we used digitonin permeabilized PC12 cells. In this model, digitonin precipitates cholesterol from the cell membrane, forming pores that release intracellular contents and allow access to the mitochondria without any mechanical damage. Preliminary experiments also confirmed that the concentration of digitonin used did not inhibit mitochondrial respiratory function (data not shown). We individually used two separate compounds, SFP and TBHQ, both known to activate Nrf2, to pre-treat cells prior to experimentation. We were successfully able to reproduce our paradigm previously shown in non-synaptic brain (Greco and Fiskum, 2010) and liver mitochondria (Chapter 4) indicating our previously observed effects were not an artifact of isolation.

We also wanted to verify that our compounds were indeed affecting the mitochondria and the PTP in addition to the use of CsA. To do this, we simultaneously monitored the fluorescence of the membrane potential dye, TMRM, in addition to extramitochondrial calcium. Addition of tBOOH to permeabilized cells loaded with calcium caused rapid mitochondrial depolarization that preceded total calcium release.
from the mitochondria. Depolarization was inhibited with TBHQ treatment, confirming our compounds were indeed having an effect on the mitochondria.

While it has been widely shown that both SFP and TBHQ are potent inducers of Nrf2 and able to induce antioxidant gene expression (Hu et al., 2011; Lee et al., 2001), it is still unknown whether Nrf2 is mediating the effect on PTP redox-regulation. The observation that two different compounds known to activate Nrf2 appear to have the same effect is supportive but is not conclusive evidence.

The logical progression of this experiment would be to knock down Nrf2 gene expression. Several methods exists for this purpose, but based on the time-frame of the experiment as well as the use of an adherent cell line, cationic lipid mediated siRNA transfection is the appropriate approach. Due to feasibility issues of the large scale of the experiment in its current state, it would need to be scaled down to a plate reader format. Of the two dyes, TMRM would be an optimal candidate as it provides a simple yes/no readout, making quantification of the data a simpler task. Initially, experiments would need to be done to optimize transfection conditions and confirm knock down of Nrf2 by monitoring at both the RNA and protein expression levels. Several methods exist to extract both RNA and DNA from the same sample. Ultimately, the goal of the experiment would be to observe in the Nrf2 knock down group, loss of protection against PTP activation with pre-treatment of SFP or TBHQ.
VI. CONCLUSIONS

Understanding the mechanism by which SFP and TBHQ exert their effects will lead to a better understanding of their cytoprotection. They can also be used as tools to determine what proteins/pathways are contributing to injury as well as how certain mechanisms are regulated. We have shown that the PTP is indeed regulated by redox state of the mitochondria. Further studies are necessary to determine if this is due to redox state of the mitochondria as a whole or the redox status of individual thiol groups situated on the PTP thought to act as a redox “switch” for PTP activation. Our work provides evidence that oxidative stress plays a strong role in the promotion of cell death that is seen in several disease and injury states.
CHAPTER 6: SULFORAPHANE TREATMENT CHANGES THE LIVER MITOCHONDRIA PROTEOME

INVESTIGATORS: Greco, T., Dubinsky, W.P., Fiskum, G.

I. ABSTRACT

Sulforaphane, an isothiocyanate extracted from cruciferous vegetables, is a promising cytoprotective compound that is known to activate the Nrf2/ARE pathway of antioxidant gene expression. Activation of Nrf2 leads to the transcription of several antioxidant genes that can eliminate oxidative stress. The oxidative stress that occurs during both acute injury and chronic diseases states is associated with mitochondrial dysfunction and activation of the mitochondrial permeability transition pore. Previously we have shown that treatment of rats with SFP results in mitochondria that are resistant to redox regulated PTP activation. In this study our aim was to determine how SFP affects the mitochondrial proteome such that PTP activation is avoided following oxidative injury. We utilized a shotgun proteomic approach comparing pools of liver mitochondria from either vehicle or SFP treated rats that were labeled with isobaric tags (iTRAQ) analyzed by nano-liquid chromatography tandem mass spectrometry. A total of 1937 iTRAQ labeled proteins were positively identified with 260 proteins analyzed and 80 proteins found to be differentially regulated by SFP treatment.
II. INTRODUCTION

Oxidative stress can either be the primary component (Flora, 1999) or secondary by product of disease (Kojima et al., 2007) and acute injury (Lemasters et al., 1997). Whatever the stage at which ROS are produced, oxidative stress is tightly associated with mitochondrial dysfunction that can promote disease/injury progression and cell death (Lee and Lee, 2005). Common targets of oxidative stress within the mitochondria are ETC proteins (Schild et al., 1997), metabolic proteins (Martin et al., 2005), DNA/RNA oxidation (Kukielska et al., 1994) as well as oxidation of lipids (Ponsoda et al., 1995) and protein (Devi et al., 1994) thiols the latter of which has been shown to regulate much of the activity in the mitochondria (Jones and Go, 2010). Thiol redox status is thought to mainly be involved in cell signaling pathways that utilize nitric oxide as the signaling molecule (Stowe and Camara, 2009). Alteration of the thiol redox status has been shown to regulate respiratory chain function (Beer et al., 2004; Piantadosi, 2011), induction of apoptosis (Herrmann and Riemer, 2010) and activation of the PTP (Greco and Fiskum, 2010; Piantadosi, 2011).

The PTP is a megachannel that, once activated, leads to loss of mitochondrial membrane potential, respiratory inhibition, efflux/influx of molecules smaller than 1500 Da including loss of necessary antioxidants and cofactors all of which contribute to further ROS production (Halestrap, 2010). Ultimately this leads to complete bioenergetic failure and promotes a necrotic cell death pathway. How PTP opening is regulated is an ongoing debate that is complicated by the fact that its components are widely speculated, but not confirmed. Current models suggest activation of the PTP is due to excessive uptake of calcium by the mitochondria that then binds to CyD, the only confirmed
component of the PTP (Baines et al., 2005). This is thought to initiate the assembly and activation of the PTP in combination with ROS. However, other research indicates that oxidative stress alone is capable of activation and that calcium overload is sufficient but not necessary. The primary mechanism thought to regulate oxidative stress induced PTP opening is alteration of thiols located on CyD that can act as a redox “switch” where reduced is closed and oxidized is open (Halestrap et al., 2002). This is supported by work that has shown that when CyD is knocked out, PTP activation is inhibited in a mouse model of focal centrilobular necrosis induced by acetaminophen (APAP) toxicity (Ramachandran et al., 2011) or by direct application of H₂O₂ to primary hepatocytes (Nakagawa et al., 2005).

While targeting mitochondrial oxidative stress is not a novel concept, the potential clinical relevance of this approach has yet to be demonstrated. Work has primarily focused on using vitamin E (Mao et al., 2011) or C (Singh and Rana, 2010) as well as specific species scavengers, targeted at mitochondria (Larosche et al., 2010). These appear to be proficient in eliminating their particular species targets yet fail to have an overall beneficial effect (Steinhubl, 2008). Broad based approaches may offer better outcomes. One such approach is the targeting of endogenous inducible antioxidant pathways that can detoxify multiple species. In particular, activation of Nrf2 appears promising as this protein has been shown to be a master transcriptional regulator of antioxidant gene expression.

Nrf2 normally resides in the cytoplasm where it is bound to its repressor, KEAP1. Under conditions of oxidative stress, ROS modify KEAP1 such that Nrf2 is released and able to translocate into the nucleus where it binds to ARE located in the promoter of
target genes and initiates gene expression (Jaiswal, 2004). In addition to activation by oxidative stress, several electrophilic compounds have also been shown to activate Nrf2 in a similar fashion. One of the more common and potent activators of Nrf2 is SFP. SFP is an isothiocyanate isolated from cruciferous vegetables including broccoli and cauliflower. SFP interacts with KEAP1, forming mixed disulfides bonds that result in conformational change and release of Nrf2 (Hu et al., 2011). Activation of Nrf2 by SFP was cytoprotective in models of ischemia/reperfusion injury (Zhao et al., 2010) and microcystin (Gan et al., 2010) and aflatoxin B(1) toxicity (Gross-Steinmeyer et al., 2010) by reducing amount the oxidative stress.

In this study, naïve rats were treated with either vehicle or SFP for 40 hours prior to isolation of liver mitochondria. We then utilized an iTRAQ based shotgun method of quantitative proteomics to compare expression profiles between vehicle and SFP, with the goal of examining how differences may ultimately provide insight into redox regulation of the PTP.

III. MATERIALS AND METHODS

A. Administration of sulforaphane to rats

All experimental procedures were approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee and conducted in accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals. Male 300 g Fischer 344 rats (Charles River) were injected intraperitoneally with sulforaphane at 10 mg/kg in 200 µL solution containing 40% DMSO and 60% isotonic saline. Animals received DMSO plus saline as the vehicle control.
B. Isolation of liver mitochondria

Liver mitochondria were isolated as previously described (Andreyev and Fiskum, 1999). Rats were fasted overnight and euthanized by decapitation at 40 hr after injection with sulforaphane or drug vehicle. All four lobes of the liver were rapidly removed, chopped and homogenized in ice-cold isolation buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, 0.5 mg/ml fatty acid free BSA, 1 mM EGTA, pH 7.2, at 4°C). The homogenate was centrifuged at 2800 rpm for 12 min. Residual fat was aspirated from the top of the centrifuge tubes and the supernatant collected and centrifuged at 9300 rpm for 12 min. The pellets were re-suspended and centrifuged at 12,000 rpm for 12 min. After a final centrifugation at 12,000 rpm for 12 min the pellet was re-suspended in 1 mL isolation media not containing EGTA. Mitochondrial protein concentrations were measured using a Lowry DC kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin used as concentration standards.

C. Analysis of differential expression of proteins

Differential expression of proteins is determined by mass spectrometry using the iTRAQ (Applied Biosystems) system of isotopic labeling for the identification and quantitation of proteins (Gu et al., 2004; Gygi and Aebersold, 2000; Zhang et al., 2005) (1, 2, 3). An isobaric amino specific reagent is used to labeling of the peptides generated by a tryptic digest of as many as 8 different protein pools (Figure 6.1).
Figure 6.1: iTRAQ Chemistry

A. The iTRAQ reagent is an amino specific label that is modified with stable isotopes to vary the masses of the reporter ion group and the balance group such that the total mass is constant. B. A peptide labeled with the reagent has an increase in its total mass of 145 amu regardless of the distribution of stable isotopes. C. As many as eight pools of peptides can be separately labeled with the eight versions of the reagent. The label adds exactly the same mass to all peptides regardless of the pool, thus identical peptides in the mixture will chromatograph together and appear in the MS-TOF spectrum as a single species. D. Fragmentation of a given peptide in the MSMS analysis yields the peptide sequence and fragments the label to yield the reporter ions identifying which pool from which the peptide originated. The relative concentration of each of the peptides is proportional to the relative intensities of the reporter ions.
A
Isobarcic Tag
Total mass = 145

Reporter Group mass
114-117 (Retains Charge)

Amine specific peptide reactive group (NHS)

Balance Group
Mass 31-28 (Neutral loss)

B

\[ \text{PEPTIDE} \]

\[ m/z\ 114 \ (+1) \quad ^{13}C \]
\[ m/z\ 115 \ (+2) \quad ^{13}C_{2} \quad ^{16}O \ (+2) \]
\[ m/z\ 116 \ (+3) \quad ^{13}C_{2} \quad ^{14}N \quad ^{12}C \ (+1) \]
\[ m/z\ 117 \ (+4) \quad ^{13}C_{2} \quad ^{14}N \quad (+0) \]

C

\[ \text{NHS + PEPTIDE} \]
\[ m/z\ 114 \ (31) \]
\[ m/z\ 115 \ (30) \]
\[ m/z\ 116 \ (29) \]
\[ m/z\ 117 \ (28) \]

Mix \text{NHS PEPTIDE PEPTIDE}

MS/MS

Reporter-Balance-Peptide INTACT
- 4 samples identical m/z

- Peptide fragments EQUAL
- Reporter ions DIFFERENT
An abbreviated overview of the procedure for protein digestion and labeling of the peptides is presented in Figure 6.2. Similar peptides in the mix chromatograph and are observed in the MS-TOF modes as a single ion. In the information-dependent acquisition mode the mass spectrometer automatically performs the fragmentation in the MS/MS mode and yields both the peptide sequence and distinct reporter ions which allows a determination of their relative concentration in the mixture.

**Figure 6.2: Flow Chart for iTRAQ Labeling Procedure**

For clarity, the chart shows the workflow for labeling two pools of proteins. The same steps are used to label up to eight samples.
D. Sample Preparation

Biological fluids, cell extracts or subcellular fractions are readily analyzed by this technique (Braun et al., 2006; Hergenroeder et al., 2008; Streckfus et al., 2008) according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Briefly, a 100 μg protein mixture from each pool was precipitated with 6 volumes -20°C acetone. The precipitate was taken up in dissolution buffer, denatured and disulfides reduced by incubation in the presence of 0.1% SDS and 5 mM TCEP (tris-(2-carboxyethyl)phosphine). Cysteine residues were blocked with MMTS (methyl methane-thiosulfonate) and trypsin was added to the mixture to a protein:trypsin ratio of 10:1. The mixture was incubated overnight at 37°C. The protein digests were labeled by mixing with the appropriate iTRAQ reagent. Following labeling the individual reaction mixtures were combined. Interfering species were removed by a combination of cation exchange and reverse phase chromatography. Prior to MS analysis the samples were fractionated by stepwise elution from the cation exchange column (for a more detailed description see (Hergenroeder et al., 2008).

E. Reverse Phase LCMS/MS

The desalted and concentrated peptide mixtures were quantified and identified by nano-LCMS/MS on a QSTAR Elite mass spectrometer (ABS Sciex Instruments) operating in positive ion mode. The chromatographic system consists of an UltiMate nano-HPLC and FAMOS autosampler (Dionex LC Packings). Peptides were loaded on a 75μm x 10 cm, 3μm fused silica C18 capillary column, followed by mobile phase elution: buffer (A) 0.1% formic acid in 2% acetonitrile/98% Milli-Q water and buffer (B): 0.1% formic acid in 98% acetonitrile/2% Milli-Q water. The peptides were eluted
with a gradient from 2% buffer B to 30% buffer B over 180 minutes at a flow rate of 250 nL/min. The liquid chromatography eluent was directed to a NanoES source for ESI/MS/MS analysis. Using information-dependent acquisition, peptides were selected for collision-induced dissociation (CID) by alternating between an MS (1 sec) survey scan and MS/MS (3 sec) scans (Figure 3). The mass spectrometer automatically chooses the top two ions for fragmentation with a 30 s dynamic exclusion time. The isotope dilution analysis collision energies parameters were optimized based upon the charge state and mass value of the precursor ions.

The accumulated MSMS spectra were converted to .mgf format for analysis by MASCOT for quantitation and peptide identification using a SwissProt fasta database. For more accurate normalization of the data the MASCOT generated .DAT files were further analyzed by IsobariQ which incorporates the statistical package R and performs variance stabilizing normalization of the data (Arntzen et al., 2011). IsobariQ generates a .csv file that is displayed in Microsoft Excel. All protein identifications were in the 95% confidence level.
Figure 6.3: MS/MS Spectrum of an iTRAQ Labeled Peptide.

The upper portion of the Figure is the fragmentation pattern that was used to determine the peptide sequence. The portion of the spectrum used for the quantitation is expanded to show reporter ions for the two pools used in this experiment. The peak intensities were determined and used to estimate the relative concentration of the peptide in the eight mixtures.
IV. RESULTS

A. Effects of SFP on mitochondrial protein expression

Mitochondria from the livers of either vehicle- or SFP-treated rats were subjected
iTRAQ analysis. In this experiment, four replicates from either vehicle or SFP groups
were labeled with different isobaric tags. Of the proteins labeled, 1,937 proteins were
identified with this number being refined to a total of 260 proteins in enough abundance
to be analyzed. Of those, 80 proteins were identified where protein fold changes were ≤
0.77 or 1.30 ≥ and those were considered statistically significant. Among those, 68
proteins were upregulated and 12 were down-regulated following 40 hours of SFP
treatment. We have further categorized SFP-regulated genes based on their functions
(Figure 6.4). The SFP-dependent proteins increased after 40 hours of treatment are
classified in several functional categories including respiratory chain components
(17.6%), ETC regulation (4.4%), antioxidants (5.7%), transporters (10%), TCA cycle
(4.4%), fatty acid and lipid metabolism (8.8%), trafficking proteins (2.9%), structural
proteins (2.9%), xenobiotic metabolism (2.9%), amino acid metabolism (4.4%), signal
transduction (8.8%) and transcriptional proteins (16%). Down-regulated categories
included transcriptional proteins (25%), xenobiotic metabolism (45%), stress response
(9.1%), amino acid metabolism (9.1%) and lipid metabolism (9.1%).
Figure 6.4: Percentage of proteins regulated by SFP according to function

A. Mitochondrial proteins upregulated by SFP. B. Mitochondrial proteins down regulated by SFP. n = 4 animals per group.
B. Influence of SFP on mitochondrial redox status

Treatment with SFP affected functional groups involved with both the production and detoxification of ROS (Table 6.1). SFP increased key mitochondrial antioxidants including glutathione peroxidase 1 (validated by immunoblots in Chapter 4, figure 4.5), microsomal glutathione-S-transferase, glutathione-S-transferase subunit Mu 2 and α-amino adipic semialdehyde dehydrogenase. SFP also increased components of the alternative fuel pathway, fatty acid β-oxidation that can provide the cofactors necessary for antioxidant activity. This includes includes Acyl-CoA binding protein that facilitates transport of acyl-CoA, acyl-CoA synthetase responsible for initiation of β-oxidation and acyl-CoA dehydrogenase (thiolase), responsible for the final cleavage that yields acetyl-CoA. There was also an increase in isocitrate dehydrogenase (NAD⁺ dependent) that provides the mitochondria with NADH. Conversely, SFP decreased several cytochrome P450 enzymes including 2D1, 2D26, 2C11 that produce ROS as a byproduct of xenobiotic metabolism.
Table 6.1 – Mitochondrial proteins that may be involved in PTP regulation

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene ID</th>
<th>Protein Name</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antioxidants</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Q64057</td>
<td>AL7A1 a</td>
<td>alpha-aminoadipic semialdehyde dehydrogenase</td>
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<tr>
<td>P04041</td>
<td>GPX1</td>
<td>Glutathione peroxidase 1</td>
<td>1.84</td>
</tr>
<tr>
<td>P08011</td>
<td>MGST1</td>
<td>Glutathione S-Transferase</td>
<td>1.71</td>
</tr>
<tr>
<td>P08010</td>
<td>GSTM2</td>
<td>Glutathione S-Transferase Mu 2</td>
<td>2.23</td>
</tr>
<tr>
<td><strong>Respiratory Chain Components</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P31399</td>
<td>ATP5H</td>
<td>ATP synthase, subunit d</td>
<td>1.46</td>
</tr>
<tr>
<td>P35434</td>
<td>ATPD</td>
<td>ATP synthase, subunit delta</td>
<td>1.37</td>
</tr>
<tr>
<td>P29418</td>
<td>ATP5E</td>
<td>ATP synthase, subunit epsilon</td>
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<td>P11951</td>
<td>CX6C2</td>
<td>Cytochrome c oxidase subunit 6C-2</td>
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<tr>
<td>P00406</td>
<td>COX2</td>
<td>Cytochrome c oxidase, subunit 2</td>
<td>1.72</td>
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<td>COX5A</td>
<td>Cytochrome c oxidase, subunit 5A</td>
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<td>P62898</td>
<td>CYC</td>
<td>Cytochrome c oxidase, somatic</td>
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<td>NDUS1</td>
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<td>DHSB</td>
<td>Succinate dehydrogenase ubiquinone, iron-sulfur subunit</td>
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<td><strong>Fatty acid and lipid metabolism</strong></td>
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<td>ACBP</td>
<td>Acyl-CoA binding protein</td>
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</tr>
<tr>
<td>P70584</td>
<td>ACDSB</td>
<td>Acyl-CoA dehydrogenase, short/branched chain specific</td>
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<td>Acetyl-CoA acetyltransferase</td>
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<td>RELN</td>
<td>Reelin</td>
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<td>VIGLN</td>
<td>Vigilin</td>
<td>1.7</td>
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<td><strong>Xenobiotic-Metabolism Enzymes</strong></td>
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<tr>
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<td>Cytochrome P450 2C11</td>
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<tr>
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<td>UDB23</td>
<td>UDP-glucuronosyltransferase 2B3</td>
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</tr>
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</table>
V. DISCUSSION

Previous studies including oligonucleotide comparisons of SFP- treated WT and Nrf2 -/- mice as well as studies showing upregulation of expression of individual proteins through immunoblot or enzymatic activity have suggested SFP is able to regulate expression of mitochondrial proteins (Hu et al., 2006a; Thimmulappa et al., 2002). However, no one has yet performed a comprehensive analysis of how SFP alters mitochondrial protein expression. The present study represents the first comparison of the proteome of liver mitochondria between vehicle- and SFP-treated rats.

We have shown that SFP induces expression changes in several different functional groups of proteins within the mitochondria providing us insight into the mechanisms by which SFP reduces oxidative stress and maintains redox balance. This is especially relevant given the role of the liver in the intact organism. Common infections and conditions like morbid obesity/metabolic syndrome (Maher, 2010), alcohol consumption (Bondy, 1992) and hepatitis C (HCV) (Gong et al., 2001), which lead to familiar and well-described chronic and life-threatening liver disease are known to have a large oxidative stress component.

Manifestations of the above diseases all have common components of increased free fatty acid (FFA) production (Clement and Negro, 2007; Mavrelis et al., 1983; Zucker, 1972), impaired transport of lipids from the liver into the bloodstream (Fujita et al., 2011; Kharbanda et al., 2009; Negro, 2006), oxidative stress (Comporti et al., 2010; Krawczyk et al., 2010) and mitochondrial dysfunction (Knockaert et al., 2011; Krawczyk et al., 2010; Simula and De, V, 2010). Increases seen in the fatty acid metabolism pathway and the lipid transport protein vigilin (Table 6.1) following SFP treatment may
reduce the overall levels of FFA and the downstream oxidative stress. Inhibition of the Cytochrome P450 family has the potential to inhibit their lipoxygenase activity, thus reducing the production of ROS. Increased mitochondrial antioxidant capacity can further contribute to SFP-mediated protection, by scavenging ROS and maintaining the appropriate redox balance within the mitochondria.

Interestingly, we also saw upregulation of several ETC proteins, NADH ubiquinone oxidoreductase subunits 75kDa, iron sulfur protein 6 and flavoprotein 2, ATP synthase subunits delta and e, cytochrome c oxidase subunit 2 and 5A and their chaperone protein, prohibitin 2. ETC proteins are targeted and inhibited by ROS, rapid replacement and assembly of proteins could potentially circumvent decreases in oxidative phosphorylation and prevent further ROS production. In addition, while we did not see a significant influence on respiration between vehicle and SFP animals using complex I or II substrates (Chapter 2 and 4), there is a possibility that due to increased respiratory chain components coupled with increased β oxidation that we may see an increase in respiration when using palmityl carnitine as a substrate.

**VI. CONCLUSIONS**

It is clear that oxidative stress is responsible for the damage seen in many pathologies of the liver and is a primary therapeutic target. Our study has shown that a compound known to activate the Nrf2 pathway cause beneficial changes in expression of proteins that can manipulate the redox status of the mitochondria. We also observed upregulation of the β oxidation pathway that can affect the mitochondria in multiple ways. The FFA observed during several liver pathologies are a significant source of oxidative stress that impairs mitochondrial function. An increase in FFA metabolism
could potentially reduce ROS production and prevent mitochondrial damage. In other pathologic settings including ischemia/reperfusion where glucose utilization is impaired, an increase in β oxidation may prove to be cytoprotective as it produces both ketone bodies and acetyl-CoA which are utilized by mitochondrial as fuel sources.
CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

I. CONCLUSIONS

A. Mitochondria as a target of oxidative stress

Oxidative stress plays a central role in a multitude of acute injuries and chronic disease and has many targets within the cell. One of the more critical targets is the mitochondria. Oxidative alterations within the mitochondria have been shown to inhibit activity of ETC components which promotes further ROS production (Chen et al., 2008). ROS/RNS has been shown to inhibit key metabolic enzymes including the pyruvate dehydrogenase complex (PDHC) (Martin et al., 2005). DNA/RNA adduct formations trigger DNA repair enzymes such as poly-ADP ribose polymerase that in turn become over activated and can result in NAD⁺ depletion from the mitochondria causing a blockade in oxidative phosphorylation (Pankotai et al., 2009; Strosznajder et al., 2010). Furthermore, oxidative stress can promote cell death by the oxidation of cardiolipin leading to the release of cytochrome c (Wiswedel et al., 2010) and the initiation of apoptotic cascades. Alternatively, the oxidation of thiol groups that promote the activation of the PTP (Greco and Fiskum, 2010) can result in bioenergetic failure and necrotic cell death.

Mitochondrial impairment due to oxidative stress can determine the extent of injury in acute incidents such as ischemia/reperfusion (Chen et al., 2011b) or traumatic
brain injury (Mazzeo et al., 2009) as well as contribute to the disease progression seen in Parkinson’s disease (Mounsey and Teismann, 2010) and nonalcoholic steatohepatitis (Mantena et al., 2009). Further highlighting the relationship between mitochondria and oxidative stress are studies that demonstrate that when mitochondrial ROS is reduced or eliminated, function is restored and cell death is prevented (Mustafa et al., 2010; Ramirez-Tortosa et al., 2009; Rocha et al., 2010). An alternative to the use of specific ROS scavengers is to manipulate the endogenous inducible antioxidant system within the cell. Genomic screens have shown hundreds of genes are induced by activation of the Nrf2 pathway, several of which are antioxidants or detoxificants (Hu et al., 2006a; Thimmulappa et al., 2002). This approach has the potential to broadly target oxidative stress allowing mitochondria to be more resistant to ROS that is produced extra-mitochondrially, i.e. peroxisomal sources, and within the mitochondria themselves.

B. SFP is neuroprotective

Previous evidence has shown that resuscitation with hyperoxic levels of O₂ results in increased amounts of ONOO⁻ (Vereczki et al., 2006) that directly results in inhibition of the PDHC (Richards et al., 2006) and inhibition of mitochondrial bioenergetics (Richards et al., 2007).

Compared to hypoxic resuscitation, if animals are instead resuscitated with an oximetry guided method initial ROS/RNS observed at 2 hours is ameliorated (Vereczki et al., 2006) and both neurologic outcome and CA1 neuron viability are improved (Balan et al., 2006). However there was still a significant amount of cell death and neurologic impairment observed in these animals, indicating there is a likely a secondary contributing factor to impairment and cell death. One potential mechanism for a second
wave of oxidative stress is activation of inflammatory pathways. Macrophages and microglia meant to rid the tissue of cellular debris release ROS as a byproduct of their function (Bazan et al., 2005), further impairing already damaged and vulnerable mitochondria. This impairment promotes more cell death, creating a futile cycle of ROS production, cell death and inflammatory activation.

We observed that irrespective of oxygenation protocols, administration of SFP following CA improved neurologic outcome and neuronal cell viability to similar extents (Chapter 3). This potentially indicates that over-activation of the inflammatory system is largely responsible for mediating the cell death and neurologic impairment seen at 24 hours following ROSC. One hypothesis is that the oximetry protocol eliminates the initial oxidative injury to mitochondria allowing them to be more resistant to a secondary oxidative insult. Although in the hyperoxic protocol mitochondria are injured during the initial oxidative injury, eliminating that “second hit” may allow them to functionally recover, enabling cell survival. SFP has at least two potential mechanisms of action within our model, the first by increasing both cellular and mitochondrial levels of antioxidants, making them more resistant to previous and on-going ROS production and the second by inhibiting activation of inflammatory processes thereby decreasing further ROS production and mitochondrial impairment. Supporting evidence shows that 8OHdG staining is significantly reduced at 24 hours (Chapter 3) suggesting a decrease in oxidative DNA damage with SFP treatment. Further studies quantifying microglia/macrophage activation will determine the ability of SFP to limit the inflammatory response.
C. Redox regulation of the PTP

One of the major ROS species produced within the mitochondria at complex I is \( \text{O}_2^- \) (Pearce et al., 2001). This can be directly scavenged by thioredoxin 2 (Nordberg and Arner, 2001) or metabolized to \( \text{H}_2\text{O}_2 \) by SOD2 (Melov, 2000) and further detoxified to \( \text{H}_2\text{O} \) by catalase, glutathione peroxidase or peroxiredoxin (Lowes and Galley, 2011). Eventually, excessive ROS formation depletes the mitochondria of the cofactors GSH and NADPH that are necessary for these detoxification systems to operate. In addition to stalling detoxification, loss of these cofactors also leads to inactivation of the enzymes necessary to maintain the reduced state of protein thiols within the mitochondria. This is especially relevant for the activation of the PTP. It is believed that critical thiol groups situated on CyD act as a “redox switch” where when they become oxidized, this promotes pore opening (Linard et al., 2009b). Supporting this are studies showing ablation of CyD results in prevention of oxidative stress mediated PTP activation (Nakagawa et al., 2005; Ramachandran et al., 2011).

In our paradigm we showed that tBOOH induces PTP opening in the presence of calcium. When observing this phenomenon in mitochondria from either rats treated with SFP or PC12 cells treated with SFP or TBHQ, tBOOH induced PTP activation was inhibited. In the case of liver mitochondria, SFP treatment also protected against pyridine nucleotide oxidation. In addition, SFP showed similar protection against OAA and PhAsO induced PTP opening. This finding indicates that SFP can protect against both indirect and direct oxidation of thiol groups. We determined that SFP does not alter the levels of CyD protein, eliminating that as a potential mechanism of protection against PTP opening.
Utilizing a shotgun approach, we examined the effects of SFP on the rat liver mitochondria proteome. We saw increases three important groups of proteins within the mitochondria, ETC components, proteins influencing fatty acid metabolism and several antioxidants including glutathione peroxidase 1. Confirming the results of our proteomics study, we saw significant upregulation of antioxidant proteins and the co-factors necessary for their operation. Of prominent interest was the glutathione peroxidase system as we saw protection against PN oxidation indicating increased peroxide metabolism. Indeed, liver mitochondria from SFP-treated animals metabolized 50% more peroxide compared to control. Further involvement of the GPX system was confirmed by immunoblots of GPX1, ME3 and measurements of GSH that collectively showed significant upregulation in our SFP group. Positive results from three separate methods indicate that two of the mechanisms by which SFP inhibits thiol oxidation in the mitochondria is through both upregulation of necessary antioxidants and cofactors as well as increased activity of this system. While the GPX system could be responsible for the protection against PTP opening observed with rBOOH and OAA, it does not explain protection against direct thiol oxidation with PhAsO. We also saw significant upregulation of Trx2 which is responsible for maintaining the reduced state of protein thiols. This indicates that SFP acts by at least two separate mechanisms to prevent protein thiol oxidation within in the mitochondria. These results also provide strong support that PTP activation is regulated by the redox state of the mitochondria. However it is still to be determined if inhibiting bioenergetics failure translates into a functional cell or is even enough to prevent cell death triggered by dysfunction of other organelles within the cell.
Post-treatment with SFP has been shown to improve neurologic outcome following TBI and CA, providing indirect evidence that surviving cells are indeed functional.

II. FUTURE DIRECTIONS

Two more important and outstanding questions are: 1) are the effects we see on redox regulation of the PTP truly mediated by Nrf2; and 2) does improving mitochondrial function maintain cellular function and improve neurologic outcome?

The first question can be addressed with both in vivo and in vitro methods. As outlined in chapter 6, siRNA can be utilized in a cell culture model to knock down Nrf2 expression and determine if loss of Nrf2 abrogates the protective effect of SFP or TBHQ. That study however, is potentially limited by the fact that only a partial knockdown of Nrf2 expression is technically available. The critical level of Nrf2 expression to abrogate all effects of Nrf2 activation is unknown. An alternative to the knock-down approach would be the use of transgenic Nrf2 KO mice. Similar to prior experiments, both non-synaptic brain and liver mitochondria would be isolated and subjected to calcium uptake and release as induced by tBOOH. Results would be compared to results obtained with WT animals. There is the possibility that Nrf2 may not mediate the effects of SFP as several hundred genes are upregulated in response to SFP in Nrf2 KO mice (Hu et al., 2006a).

It also unknown if sparing mitochondrial dysfunction is enough to prevent cell death and retain cellular function. Data from our global cerebral ischemia experiments as well as TBI experiments from Dash et al. using SFP following injury certainly support this as both models show increased cell viability and improved neurologic outcome. However, as of yet there is no causative link. One potential method to begin to answer
this question is the use of acute brain slices. Currently our cardiac arrest model is terminated at 24 hours and half the brain is immersion fixed for immunohistochemistry and the other half sectioned and frozen for biological assays. In addition to simply dissociating and freezing the hippocampus, it is feasible to generate acute hippocampal slices. It is well established that the hippocampus is selectively vulnerable to ischemia/reperfusion injury (Simon et al., 1984) and represents an important brain region to study. The slices would be part of two separate experiments. One group of slices would have their mitochondrial bioenergetics measured in the Seahorse Bioscience metabolic flux analyzer. The second group of slices would be subjected to electrophysiological studies. Specifically, we can study the ways in which ischemia/reperfusion alters and SFP improves synaptic transmission in synapses between Schäffer collateral/commissural fibres and apical dendrites of CA1 pyramidal neurons. Several procedures including the measurements of field excitatory post-synaptic potentials as well as short-term and long-term potentiation would allow us to observe whether ischemia/reperfusion impairs and SFP improves basal synaptic strength, or short-term or long-term plasticity of synaptic transmission within the CA1 region of the hippocampus. Correlative statistics comparing mitochondrial function and neuronal function could then be done to evaluate the relationship between the two.

III. SIGNIFICANCE

The findings in our studies indicate use of SFP, a known activator of Nrf2, is neuroprotective following cardiac arrest. Further work with isolated mitochondria and digitonin permeabilized PC12 cells indicates one possible mechanism of action is by enhancing mitochondrial antioxidant capacity. Use of a compound that enhances levels of
several different antioxidants has wide reaching applications. These include pre-treatment of patients undergoing cardiac by-pass and treatment of organ donor prior to organ harvesting to prevent ischemic/reperfusion injury associated with transplant. There is also the possibly of combination therapy such as in the case of hepatitis C patients. Several studies already indicate that SFP is cytoprotective following acute injury including stroke, (Zhao et al., 2006; Zhao et al., 2007b) TBI, (Dash et al., 2009) and toxic agents (Gan et al., 2010). SFP is also protective in chronic disease models such as Parkinson’s (Siebert et al., 2009) or Alzheimer’s disease (Park et al., 2009).

The major limitation in the advancement of SFP to the clinic are that no oral or iv pharmacokinetic studies have been completed. Studies in China where participants drank broccoli tea to reduce oxidative stress associated with aflatoxin (B) poisoning showed great variability in bioavailability (Egner et al., 2011; Kensler et al., 2005). Although preliminary studies indicate SFP elicits a safe and cytoprotective response, ultimately human studies are necessary to determine whether the metabolism and mechanism of SFP are similar to that seen in animal models.
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