

BAX AND OXIDATIVE STRESS IN THE AGING MOUSE BRAIN

by

KYNDRA COTTINGHAM STOVALL

(Under the Direction of James L. Franklin)

ABSTRACT

The primary goal of this research was to determine the effect of the pro-apoptotic protein, Bax, on chronic endogenous oxidative stress. This was accomplished by measuring reactive species (RS) production in cortical neuron cell culture and also by assessing markers of oxidative damage in the aging mouse cortex. It is postulated that the accumulation of RS contributes to the aging process. RS are produced as a natural byproduct of mitochondrial respiration and are normally kept in balance by an extensive antioxidant system. When the RS produced overwhelms this antioxidant system, oxidative stress can occur. The oxidative stress theory of aging proposes damage arising from this process causes aging to occur. The process of aging is compounded by an increasing probability of acquiring age-related disorders, such as Alzheimer's and Parkinson's diseases. Neurons are especially vulnerable to RS damage because these cells are post-mitotic, have a high oxygen demand, and relatively low antioxidant capacity. The literature also suggests that apoptosis is involved with the aging process as well. Bax is a pro-apoptotic Bcl-2 family member that is known to enhance RS production in several cell culture models. Cortical neurons are a target for damage in many neurodegenerative diseases as well as in normal aging. It was the goal of this research to understand the effects of Bax on RS production in cortical neurons. A further goal of this project was to understand these effects on

oxidative damage in the aging nervous system. Understanding the role of Bax in the aging process may lead to improved therapies for age-related neurodegenerative disease.

INDEX WORDS: Reactive species; oxidative stress; neuronal apoptosis; aging; Bax; Sod2

BAX AND OXIDATIVE STRESS IN THE AGING MOUSE BRAIN

by

KYNDRA COTTINGHAM STOVALL

BS, Spelman College, 2004

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2010

© 2010

Kyndra Cottingham Stovall

All Rights Reserved

BAX AND OXIDATIVE STRESS IN THE AGING MOUSE BRAIN

by

KYNDRA COTTINGHAM STOVALL

Major Professor: James L. Franklin

Committee: Julie Coffield
Brian Cummings
Susan Fagan
Shelley Hooks

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2010

DEDICATION

To my amazing family: I could not have been a luckier person by having your support and love.

The definition of success for some may be the acquisition of money, or fame, or fortune. I define it as love – and I have a lot of it in my life. This is for parents who have always been a source of unwavering support. This is for my brothers who are always there for me and are proud of me – it goes both ways. This is for my grandmother who told me stories of life back when my grandfather sought his PhD, God rest his soul. This is for my loved ones that are no longer with me; I know they are looking down on me. This is for the most incredible little girl in the entire world, Kiana, mommy loves you. This is for my unborn son; you are like a light that beams through me, helping me understand that every day is a new day. This is for my husband, Nick; if you can love the stressed-out, miserable, depressed person that I have undoubtedly been through the course of seeking this degree – we will make it through anything. I love you.

ACKNOWLEDGEMENTS

“We all die. The goal isn't to live forever; the goal is to create something that will.”

~Chuck Palahniuk

It is said that behind every successful person there are a lot of unsuccessful years. This journey has certainly exemplified this and has been a humbling experience. This program has taught me that not every project is a slam-dunk, that “simple” assays are not-so-simple, that “beautiful hypotheses” often are ruined by “ugly facts,” and that failure is an integral part of life. A person can choose to give up or to push through the set-backs and hopefully accomplish something worthwhile. To my advisor who is probably the most knowledgeable person I have met, your guidance and vision have shaped my graduate experience. To my committee members, Drs. Coffield, Cummings, Hooks, and Fagan – even though I was terrified every time we met, I appreciate your comments and your belief in me. Also to Dr. Cummings, thank you for your support and expertise as I embarked upon the analysis of lipids. Thank you to the University of Georgia Graduate School for the Graduate Research Opportunities fellowship and to the Department of Pharmaceutical and Biomedical Sciences for economic support over the years. Thank you also to the Fellowships in Research and Science Teaching program at Emory University for giving me a place to enhance my professional skills after receiving my degree. Of course I cannot go without thanking the ones who helped me keep a grip on reality. Thank you to my extended family, my in-laws, and my friends. I have also made the most amazing new friends in the PBS department, ones that have lent a shoulder through the many mini-nervous breakdowns that have plagued me through this experience. Thank you to my graduate school

comrades Dr. Bridg'ette, Dr. Brooks, Dr. Shawn, and the future Drs. Pam and Vivia for being there – you all are the greatest! And to the best lab-mate anyone could ever ask for, Dr. Meagan “you know what goes in these quotes” McManus – thank you for the constant uplifting and joyous spirit.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
LIST OF TABLES	ix
LIST OF FIGURES	x
CHAPTER	
1 INTRODUCTION	1
Neuronal apoptosis, mitochondria and Bax	1
Free radicals and reactive species (RS)	8
Antioxidants	10
Oxidative stress and aging	14
References	19
2 BAX PREVENTS RS BURST IN CORTICAL NEURONS	29
Introduction	29
Methods	30
Results	35
Discussion	39
References	55
3 BAX PREVENTS OXIDATIVE DAMAGE IN AGING BRAIN	61
Introduction	61
Methods	63
Results	70

	Discussion	74
	References	85
4	BAX AND OXIDATIVE DAMAGE IN AGING TISSUES	89
	Introduction	89
	Methods	90
	Results	94
	Discussion	96
	References	106
5	CONCLUSIONS	109
	References	114

LIST OF TABLES

	Page
Table 1.1: Oxidative damage in the aging brain	18
Table 3.1: Body and Brain Weights	76
Table 4.1: Organ Weights	98

LIST OF FIGURES

	Page
Figure 1.1: Mechanisms of neuronal apoptosis	5
Figure 1.2: The formation of RS from mitochondrial leakage of electrons.....	11
Figure 1.3: Oxidative damage induced by RS	15
Figure 2.1: RS production	42
Figure 2.2: RS production after staurosporine treatment.....	43
Figure 2.3: Nitric oxide-related RS detected following staurosporine treatment	44
Figure 2.4: Mitochondria-derived RS detected during staurosporine treatment.....	45
Figure 2.5: Effect of Bax on RS during apoptosis	46
Figure 2.6: Effect of Bax gene dosage on protein levels in cortical neuron cell culture	47
Figure 2.7: Removal of Sod2 does not affect Bax expression in cortical cultures	48
Figure 2.8: Effect of Bax on RS during apoptosis	49
Figure 2.9: Effect of Bax on cortical neurons.....	50
Figure 2.10: Effect of Sod2 on cortical neurons in non-apoptotic neurons	51
Figure 2.11: RS is lowered with the deletion of Bax in neurons with reduced Sod2	52
Figure 2.12: Nitric oxide-related RS detected under chronic oxidative stress conditions in non-apoptotic neurons	53
Figure 2.13 Survival in cell culture.....	54
Figure 3.1: Bax protein levels in the aging wild-type brain	77
Figure 3.2: Sod2 protein levels in the aging wild-type.....	78
Figure 3.3: Effect of lowering Sod2 levels on Bax protein levels in the aging brain	79

Figure 3.4: The effect of Bax deletion on Sod2 levels in the aging brain	80
Figure 3.5: DNA damage is prevented by Bax deletion	81
Figure 3.6: Lipid peroxidation is prevented by Bax deletion	82
Figure 3.7: Shotgun lipidomic analysis	83
Figure 3.8: Analysis of arachidonic acid	84
Figure 4.1: Lipid peroxidation in the aging liver	99
Figure 4.2: Lipid peroxidation in the aging heart	100
Figure 4.3: Lipid peroxidation in the aging kidney	101
Figure 4.4: DNA damage by comet assay	102
Figure 4.5: DNA damage induced by chronic oxidative stress is prevented by Bax deletion in the aging liver	103
Figure 4.6: DNA damage induced by chronic oxidative stress is prevented by Bax deletion in the aging heart.....	104
Figure 4.7: DNA damage induced by chronic oxidative stress is prevented by Bax deletion in the aging kidney.....	105
Figure 5.1: Conclusions	113

Chapter 1

Introduction

Neuronal apoptosis, mitochondria, and Bax

The brain is extremely complex; containing approximately 100 billion neurons, each linked by up to 10,000 synaptic connections each. Equating to around 100 trillion connections, the brain is responsible for controlling body temperature, heart rate, breathing, physical motion, accepting information about the surrounding world, and allowing you to think, reason, dream and remember. The cortex is important for higher learning, as it is the main part of the brain responsible for cognitive abilities. The cortex comprises the largest area of the brain and is essential for reasoning, reading, writing, and storing information. With few exceptions, new neurons are created before birth. Many of these neurons will die by neuronal apoptosis prenatally and during the early postnatal period. During gestation, an overgrowth of neurons occurs, and approximately half of these neurons die via apoptotic mechanisms. Up to half of the remaining neurons in some parts of the brain die within a few weeks after birth (Wright et al., 1983; Pressley and McCormick, 2007). This loss of neurons is important in sculpting the developing nervous system. Much of this is regulated by the availability of sufficient neurotrophic factors, provided by target tissues. Neurons are post-mitotic and, therefore, do not multiply or divide, but continue to mature and interact with each other. This includes the formation of synapses, pruning of dendrites and the myelination of neurons. Neurogenesis does occur in some parts of the brain throughout life; such as the olfactory bulb and the dentate gyrus of the hippocampus, however (Gross, 2000; Bhardwaj et al., 2006; Nowakowski, 2006).

When neurons die, there is generally no replacement. Understanding how and why neuronal cell death occurs is therefore very important. The two main forms of cell death are apoptosis and

necrosis, although there are others such as autophagic cell death. Apoptosis was the term given to the phenomenon of programmed cell death first analyzed by Kerr, Wylie and Currie in 1972. They chose the term apoptosis, from the Greek word meaning “falling off” as in leaves of a tree (Kerr et al., 1972). Apoptosis is a highly conserved mechanism of programmed cell death, characterized by chromatin condensation, and cell shrinkage. The nucleus may also fragment. Membrane-enclosed processes can break off cells and become structures known as apoptotic bodies. Apoptotic cells and bodies are then engulfed by phagocytic cells, preventing inflammation and damage to nearby cells and tissues (Hetts, 1998). It differs from necrotic death in that there is no rupture of the cell membrane or inflammation, and that necrosis is generally not isolated to a single cell. Apoptosis is generally advantageous to the organism as it appropriately eliminates damaged or unhealthy cells. Necrosis results from acute injury and is a more traumatic form of cell death. The characteristics of apoptosis are seen in many, if not all cell death that occurs by physiological means. It also appears to be involved in several pathological disorders as well. Apoptosis is of widespread biological significance. It is involved in development, differentiation, immune system regulation, and removal of defective or harmful cells (Sanchez et al., 1996; Papaliagkas et al., 2007). The dysfunction of the apoptotic pathway is implicated in the pathologies of several disorders, both by depressing apoptosis (e.g. autoimmune disorders and cancer) and by enhancing apoptosis (e.g. neurodegenerative disorders; Hetts 1998).

Apoptosis occurs via two pathways, the extrinsic pathway and the intrinsic pathway (Figure 1.1). The executors of death in both pathways are cysteine-dependent aspartate-directed proteases (caspases). Caspases carry out the execution of cell death by activating DNases, cleaving DNA repair enzymes and degrading nuclear protein. They also play a role in the

regulation of RS (Masahiro et al., 1998). The cell extrinsic pathway is mediated by death receptors like the tumor necrosis factor (TNF) receptor superfamily. In this form of apoptosis, the receptor (e.g. TNFR or Fas) interacts with its ligand (e.g. TNF- α or Fas-ligand) and triggers the formation of the death-inducing signaling complex (DISC), which recruits procaspase-8. Active caspase-8 is formed by autoproteolytic cleavage of procaspase-8 that then goes on to activate effector caspases (Putcha et al., 2002). The cell intrinsic pathway is mediated by mitochondria and is initiated from within the cell.

Mitochondria are the manufacturing plants for the major cellular energy store, adenosine triphosphate (ATP). ATP is produced by oxidative phosphorylation, a process that occurs in mitochondria. The electron transport chain (ETC) is important to this process, and is a series of mitochondrial enzymes that transfer electrons from one complex to the next, with the energy resulting from this process used to pump protons (H^+) from the matrix to the intermembrane space. This results in the formation of a proton-gradient across the inner mitochondrial membrane, creating the mitochondrial membrane potential ($\Delta\psi_m$). Over 95% of the oxygen we breathe is utilized in complex IV (cytochrome *c* oxidase) of the ETC (Cadenas and Davies, 2000). Cytochrome *c* oxidase is the terminal electron acceptor in the ETC. Four electrons are removed from cytochrome *c* and transferred to O_2 producing two molecules of water ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$). In this process, four protons are pumped across the membrane, contributing to the $\Delta\psi_m$. H^+ then flow back into the mitochondrial matrix, acting as the driving force of the motor in complex V (ATP synthase) that converts adenosine diphosphate (ADP) to ATP.

The mitochondria are also home to several proteins involved in the intrinsic apoptotic cascade. Key to the induction or suppression of this pathway of apoptosis is the Bcl-2 family of proteins, named after the first to be discovered, B-cell lymphoma 2 (Bcl-2; Adams and Cory,

1998; Gross et al., 1999). This family of apoptotic regulators contains thirty members with either pro-apoptotic or anti-apoptotic functions. The pro-apoptotic members can further be subdivided into two groups; the Bax subfamily of pro-apoptotic proteins, and the limited homology BH3-only subfamily of pro-apoptotic proteins. Anti-apoptotic proteins all contain three or four regions of Bcl-2 homology, known as Bcl-2 homology domains (BH1-4). These proteins, such as Bcl-2, Bcl-XL, and Mcl-1, function by sequestering BH3-only proteins, and by forming heterodimers with the Bax subfamily of pro-apoptotic proteins, rendering them inactive. The Bax subfamily of pro-apoptotic proteins contain two or three Bcl-2 homology domains and include Bax, Bak and Bok. They are essential for the initiation of the apoptotic pathway by associating with the outer mitochondrial membrane (OMM) and causing the release of apoptotic factors from the mitochondrial intermembrane space (IMS). BH3-only pro-apoptotic proteins (e.g. Bid, Bim, Bik, Bad, Bmf, Puma, Noxa, Hrk, and Blk) share one Bcl-2 homology domain, BH3, which is essential for binding to anti-apoptotic members (Huang et al., 2000, Pong et al., 2001).

There is debate over the mechanism of action of Bcl-2 family members, but data suggests that it is a set of complex and selective interactions between pro- and anti-apoptotic proteins on the OMM that defines a cell's susceptibility to death (Sedlak et al., 1995). While anti-apoptotic members normally reside on the OMM, it is impermeable to the pro-apoptotic proteins that reside in the cytosol. Apoptotic stimulus causes the translocation of these proteins to the OMM. Many of the members can homodimerize and can also form heterodimers between pro- and anti-apoptotic members (Hengartner, 2000). Heterodimerization can act to neutralize opposing proteins. Therefore the balance between pro- and anti-apoptotic proteins on the OMM determines the apoptotic fate of the cell. When the number of pro-apoptotic proteins on the

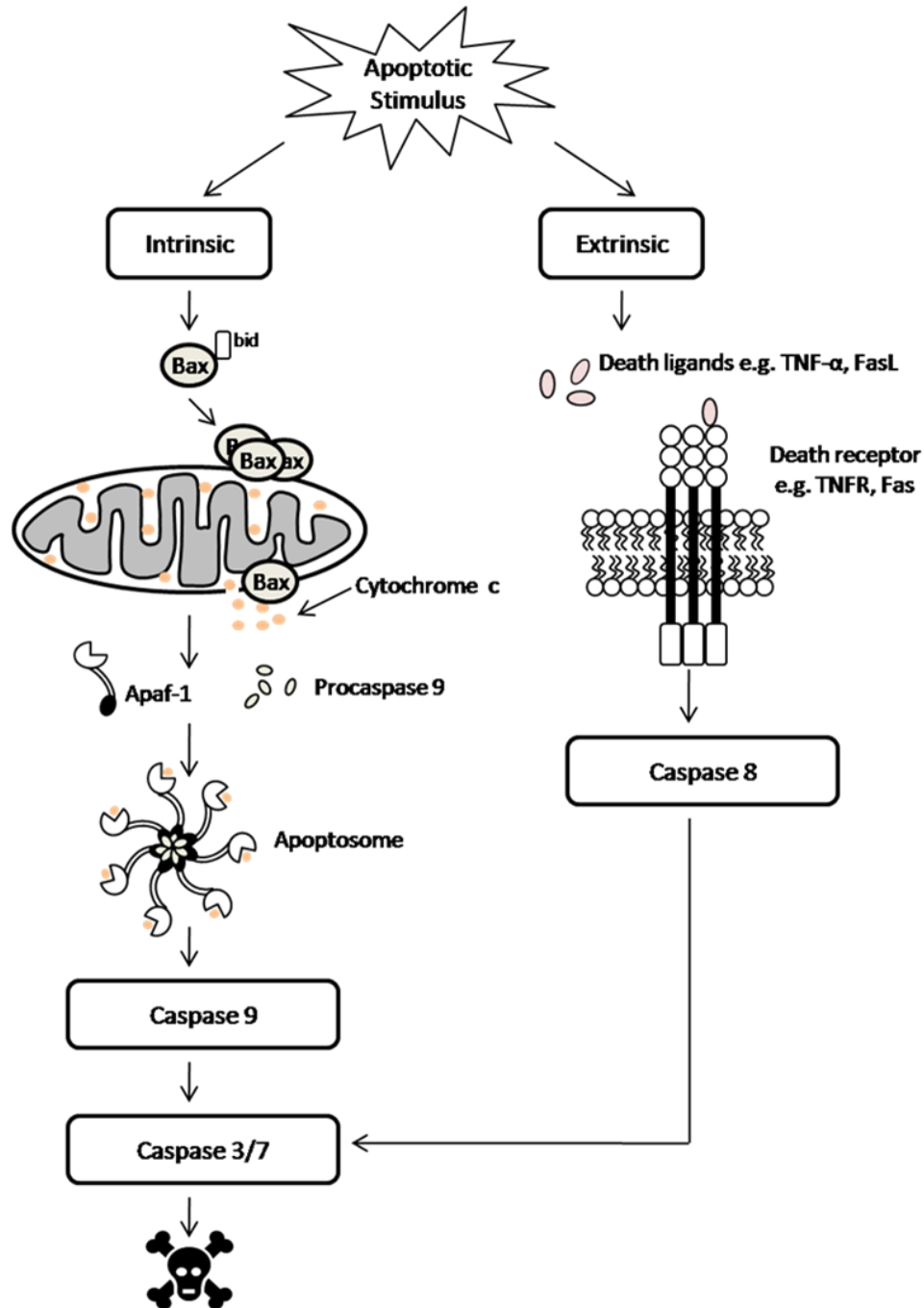


Figure 1.1 Mechanisms of neuronal apoptosis

Apoptosis occurs via cell intrinsic or extrinsic pathways, resulting in the activation of caspases that execute the apoptotic program. Pictured on the left is the cell intrinsic pathway/mitochondrial pathway, mediated by the Bcl-2 family of proteins. Of importance is the translocation of pro-apoptotic members such as Bax to the outer mitochondrial membrane, where it causes release of pro-apoptotic factors such as cytochrome c, and initiates apoptosis. Apoptosis can also occur via the extrinsic activation of a death-receptor such as Fas, pictured on the right.

OMM outweighs the anti-apoptotic proteins, apoptosis can initiate. Upon receipt of apoptotic stimuli, the members of the Bax subfamily of pro-apoptotic proteins insert into the OMM and cause the formation of pores in the membrane. While the mechanism by which Bax family members permeabilize the OMM is under debate, there are several recognized possibilities. Oligomerized Bax has the ability to form pores itself, which can allow the passage of cytochrome *c* (Jürgensmeier et al., 1998). Bax also interacts with other proteins, such as the voltage-dependent anion channel (VDAC) or the adenine nucleotide translocator (ANT; Shimizu et al., 1999, Marzo et al., 1998). The release of cytochrome *c* is followed by the execution of the apoptotic program as described above. Cytochrome *c* is a protein loosely associated with the inner mitochondrial membrane. It is involved in the mitochondrial ETC where it transfers electrons from respiratory complex III to IV. Cytochrome *c* is released through OMM pores during apoptosis and then associates with apoptotic protease activating factor-1 (Apaf-1) and procaspase 9 to form a structure known as the apoptosome. Upon formation of the apoptosome, procaspase 9 is capable of self-processing into active caspase-9, an initiator caspase. Caspase 9 then activates the effector caspases, including caspase 3, that execute the apoptotic program of cell death (Danial and Korsmeyer, 2004).

The Bax subfamily of pro-apoptotic proteins is of key importance in the regulation of apoptosis, especially Bax and Bak. The action of Bok is restricted to reproductive organs, although expression has been reported in other tissues (Hsu et al., 1997; Inohara et al., 1998). Inactivation of both Bax and Bak in the mouse results in the dramatic impairment of apoptosis during development in many tissues, including the brain. The neurons in these Bax-and-Bak-null cells are also resistant to all tested intrinsic death pathway stimuli, including staurosporine (kinase inhibitor), etoposide (topoisomerase II inhibitor), ultraviolet (UV) radiation, and growth

factor deprivation (Wei et al., 2001; Danial and Korsmeyer, 2004). Both Bax and Bak are widely distributed throughout the body and research suggests that they are redundant, pro-apoptotic proteins (Lindsten et al., 2000). Both proteins are able to promote cell death in non-neuronal cells, but this function in post-natal cortical neurons, as well as other major neuronal populations, is attributed to Bax, as these cells express a BH3-only isoform of Bak (N-Bak), instead of the full length version (Sun et al., 2001; Uo et al., 2005). BH3-only proteins are incapable of executing apoptosis on their own, but rather function as activators of Bax-like proteins or inhibitors of anti-apoptotic proteins (Willis and Adams, 2005). This provides explanation for reports describing complete apoptosis prevention by Bax-deficiency (Deckwerth et al., 1996; Miller et al., 1997; Kirkland et al., 2002).

Bax KO mice (*bax*^{-/-}) appear healthy and survive to adulthood. However, males are infertile as a result of arrested spermatogenesis (Knudson et al., 1995). Young females breed poorly, but this improves over time, as the removal of Bax extends ovarian function (Perez et al., 2007). Bax deletion also eliminates the naturally occurring neuronal cell death in the pre-natal period in most peripheral ganglia, including sympathetic neurons, and the trigeminal brainstem nuclear complex. Reduction of apoptotic death in the cerebellum and hippocampus also occurs in Bax-deficient mice (Knudson et al., 1995; Deckwerth et al., 1996, White et al., 1998). Young Bax KO mice have similar abilities to learn as their WT littermates as judged by behavioral screening of basic sensory, motor, and cognitive tasks. Fear conditioning, however, was impaired (Kim, et al. 2009). WT animals have been shown to display an increase in anxious behavior with age, which are not observed in KO animals. As in young animals, middle-aged animals have shown impairments in contextual fear memory. These animals showed no differences in cued memory, however, as compared to WT (Perez et al., 2006).

Free Radicals and Reactive Species

RS are implicated in the induction of apoptosis (Sanchez et al., 1996; Valencia and Morán, 2001; Kirkland et al., 2002). Basal levels of RS are, however, continually produced as a byproduct of normal respiration and from other endogenous sources, such as NADPH oxidases (Liu *et al.* 2002, Serrano *et al.* 2009, Shelat *et al.* 2008). The electrons shuttled through the ETC can leak out and be transferred directly to oxygen, resulting in the formation of superoxide radicals ($O_2^{\bullet -}$), which can be converted into several other RS (Figure 1.2). RS include both reactive oxygen species (ROS) and reactive nitrogen species (RNS) and can include free radicals and also non radicals. Free radicals are molecules or atoms with at least one unpaired electron in the outermost shell, such as $O_2^{\bullet -}$. These oxidants can be formed exogenously or endogenously. Several ROS are formed in the body, the most common being $O_2^{\bullet -}$, the hydroxyl radical ($\bullet OH$), singlet oxygen (1O_2), and hydrogen peroxide (H_2O_2). RNS include nitric oxide ($\bullet NO$) and molecules derived from it such as peroxynitrite ($\bullet OONO$) and nitrogen dioxide (NO_2). RS are ubiquitous and necessary for many physiological reactions. They are involved in enzyme-catalyzed reactions, signal transduction, and gene expression (Hancock et al., 2001). In contrast, they can also lead to negative consequences, such as the induction of oxidative damage to molecules, cells, and tissues as well as having implications in aging and disease (Kokoszka et al., 2001; Halliwell and Gutteridge, 2007; Lee and Wei, 2007).

Superoxide and reactions with superoxide

An estimated 2-5% of the total oxygen intake has the ability to form $O_2^{\bullet -}$. The major source of $O_2^{\bullet -}$ is the activity of the ETC in mitochondria, in which electrons can leak and react with diatomic oxygen (O_2) directly. O_2 itself exists as a diradical, as it has two unpaired electrons in its outermost shell. However, due to laws of quantum physics, it can only accept one electron at

a time. The acceptance of one electron results in the formation of $O_2^{\cdot-}$. Superoxide dismutase (Sod) is the enzyme that catalyzes the dismutation of superoxide to H_2O_2 . It reacts with two molecules of $O_2^{\cdot-}$ to form H_2O_2 , which is then further detoxified to water by the enzymes glutathione peroxidase (Gpx) or catalase. While H_2O_2 itself is not very reactive, it has the ability to convert into the destructive $\cdot OH$ in the presence of transition metals via the Fenton Reaction. $O_2^{\cdot-}$ can also form $\cdot OH$ via the Haber-Weiss reaction, which is a 2 step process involving both $O_2^{\cdot-}$ and H_2O_2 in the net reaction of $\cdot O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$. $\cdot OH$ is considered one of the most damaging free radicals. It has a very short half-life, reacting with any nearby molecule and has the capability of initiating lipid peroxidation and causing damage to DNA and proteins.

The only known biological molecule to be able to compete with endogenous Sod in the reaction with $O_2^{\cdot-}$ is $\cdot NO$ (Huie and Padmaja, 1993; Beckman and Koppenol, 1996). $\cdot NO$ is a molecule used in cell signaling and plays a role in smooth muscle contraction. It easily passes through membranes and is generated from l-arginine by nitric oxide synthases (NOS). The reaction of $\cdot NO$ with $O_2^{\cdot-}$ produces the toxic $\cdot OONO$ that, like $\cdot OH$, can initiate lipid peroxidation and other forms of oxidative damage (Radi et al., 1991).

Bax and RS

A burst in RS is seen during apoptotic death (Sanchez et al., 1996; Valencia and Morán, 2001; Kirkland et al., 2002). In several cell types, including Jurkat cells, yeast, mouse embryonic fibroblasts, sympathetic neurons and cerebellar granule neurons, Bax lies upstream from this RS burst and the deletion of Bax prevents both death and the increase in RS (Xiang et al., 1996; Ligr et al., 1998; Kirkland et al., 2002; Poliakova et al., 2002; Jiang et al., 2008).

Antioxidants

In a healthy, functioning cell, damage by reactive species is mitigated by a comprehensive antioxidant system. The major enzymatic antioxidant systems in the brain include Sod and Gpx. There are also non-enzymatic antioxidants such as vitamin E, glutathione, and metal-binding proteins.

Enzymatic Antioxidant Defenses

Sod

$O_2^{\cdot-}$ is the first free radical produced by leakage of electrons from the ETC in mitochondria. $O_2^{\cdot-}$ will dismutate spontaneously to H_2O_2 , but this reaction is catalyzed at a rate 1000 times faster by Sod (McCord and Fridovich, 1969). In humans, there are three forms, distinguished by the metal in their active site and their compartmental location. It is necessary to have Sod in various compartments because $O_2^{\cdot-}$ does not readily cross biological membranes. Therefore, $O_2^{\cdot-}$ anions must be destroyed within close proximity to their site of generation.

Sod1 contains copper and zinc (Cu/Zn) and is found in the cytoplasm and in the mitochondrial IMS. Sod1 is the predominant Sod in most tissues, accounting for 70-80% of cellular Sod activity. Sod1 is located primarily in astrocytes throughout the CNS, but is also detectable in neurons (Huffman and Chapman, 2009). Sod1 impairment is linked to amyotrophic lateral sclerosis (ALS), age-related muscle loss, early development of cataracts, and shortened lifespan (Muller et al., 2007).

Sod2 is the mitochondrial version and contains manganese (Mn) at its reactive center. Sod2 is a homotetramer of 24 kDa, located in the mitochondrial matrix of all mitochondria-containing cells. This form accounts for 10-20% of total Sod activity, but due to its location in the mitochondria, it serves as a major endogenous antioxidant by converting $O_2^{\cdot-}$ produced by

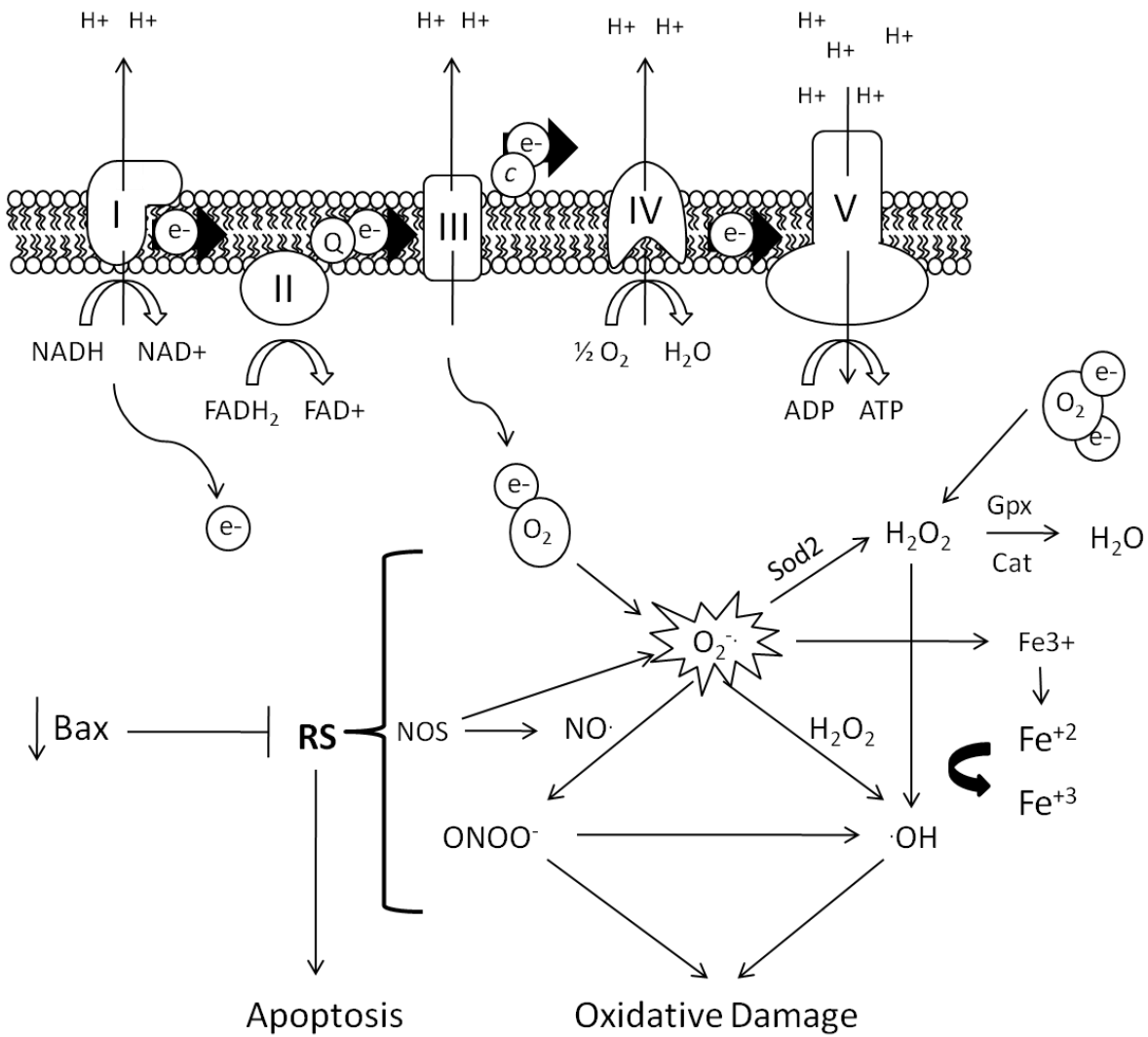


Figure 1.2 The formation of RS from mitochondrial leakage of electrons.

Electron leakage can occur from Complexes I and III of the ETC. These leaked electrons can react with O₂ to form O₂^{•-}. This radical can undergo several different reactions, which can result in the formation of harmless water or the damaging hydroxyl or peroxynitrite radicals, inducers of oxidative damage. A burst in RS can lead to apoptosis, but can be attenuated by the reduction of Bax in a variety of cells.

the ETC (Van Remmen et al., 2003). Mice lacking each of the 3 Sods have been evaluated, and it has been concluded that mice deficient in Sod2 have the most severe phenotype, with complete lack of Sod2 proving lethal within the first week of life (Li et al., 1995). Impaired Sod2 activity in humans has been associated with several diseases, including type I diabetes and ovarian cancer (Nomiya et al., 2003). Mice with half of the normal Sod2 gene dosage have a normal lifespan, but do show increased signs of oxidative stress, such as DNA damage (Van Remmen et al., 1999; Van Remmen et al., 2003; Jang and Remmen, 2009).

Sod3 contains copper and zinc (Cu/Zn) similar to Sod1, but is located in the extracellular matrix. Sod3 is only found in a limited number of tissues, such as the lung, kidney and smooth muscle, and therefore constitutes a minor form of this enzyme. Sod3 provides protection against vascular dysfunction and impaired Sod3 has been associated with coronary artery disease and myocardial infarction (Grammer et al., 2009).

Gpx

GPx converts hydroperoxides to H₂O. There are several different isozymes, which vary by cellular location and substrate specificity. The most abundant version, GPx1, is found in the cytoplasm of most mammalian tissues with H₂O₂ as its preferred substrate. Mice deficient in GPx1 have a normal lifespan, but have early development of cataracts (Wolf et al., 2005, Perez et al. 2009). GPx4 prefers lipid hydroperoxides and is found in much lower levels than GPx1. GPx4 KO mice die during embryonic development (Yant et al., 2003). There is some evidence, however, of GPx4 reduction (*GPx4^{+/-}*) causing an increase in mouse life expectancy (Ran et al., 2007).

Nonenzymatic defenses

Glutathione (GSH)

Glutathione exists in reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate an electron to unstable molecules such as RS. This electron donation increases the reactivity of glutathione, however, which readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). GSSG can be reduced to GSH by glutathione reductase. More than 90% of glutathione exists as the reduced form in healthy tissues. Oxidation to GSSG is considered an indicator of oxidative stress, and depletion of GSH can cause mitochondrial damage in the brain (Maellaro et al., 1990; Jain et al., 1991). The oxidation of GSH is catalyzed by GPx in the following reaction: $2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}$. Glutathione reductase then reduces this oxidized glutathione to complete the cycle in the following reaction: $\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+$.

Metal-binding proteins and chelators

Metals such as iron and copper can participate in the Fenton reaction, which is the conversion of H_2O_2 to $\cdot\text{OH}$. Metal-binding proteins such as ferritin, transferrin, and ceruloplasmin tightly bind transition metals, keeping them from participating in Fenton chemistry. There have been reports showing the benefits of iron chelators, such as desferrioxamine, in the treatment of brain edema (Okauchi et al., 2009).

Vitamins

Large amounts of Vitamin C (ascorbic acid) are found in the brain. At high concentrations, it will reduce free radicals in a reaction producing dehydroascorbate. However, lower concentrations may favor the reaction with transition metals to generate free radicals, as is the case with the reaction of diphosphate-complexed ferric iron (Fe^{3+}) forming the catalytically

active ferrous form (Fe^{2+}). Vitamin E (α -Tocopherol) is located within membranes in several tissues near production sites of free radicals. It serves to block the propagation of peroxidation along a membrane by scavenging the intermediate peroxy radical that is produced in the lipid peroxidation reaction (Kelly, 1988).

Oxidative stress and Aging

The state in which the amount of RS produced overwhelms the antioxidant system is termed oxidative stress. The brain places a large demand on available resources, given its relatively small size. It represents only 2% of the total body weight, yet requires 15% of the cardiac output and 20% of the total oxygen consumed (Lassen, 1959; Lau et al., 2005). This high oxygen consumption, coupled with the long life span of neurons makes the brain particularly vulnerable to damage induced by RS. In addition, the brain is very lipid-rich and contains high levels of iron and copper, two traits that make it an ideal environment for lipid peroxidation. In addition to damage to lipids, excessive RS can also cause damage to DNA (Figure 1.3).

Lipid peroxidation refers to oxidative degradation of lipids and most often affects polyunsaturated fatty acids (PUFAs). More than 20% of the total fatty acids in the brain are unsaturated fatty acids. The detection and measurement of lipid peroxidation is the evidence most frequently cited to support the involvement of RS-related reactions in toxicology and disease (Halliwell and Chirico, 1993). Lipid peroxides are unstable and decompose to form more complex and reactive compounds such as malondialdehyde (MDA). This byproduct is a generally accepted marker of oxidative stress.

Oxidative damage to DNA can produce a multitude of DNA modifications, including base and sugar fragmentation products, strand breaks, and DNA-protein cross-links. Research shows that oxidative DNA damage plays an important role in several disease processes, such as cancer

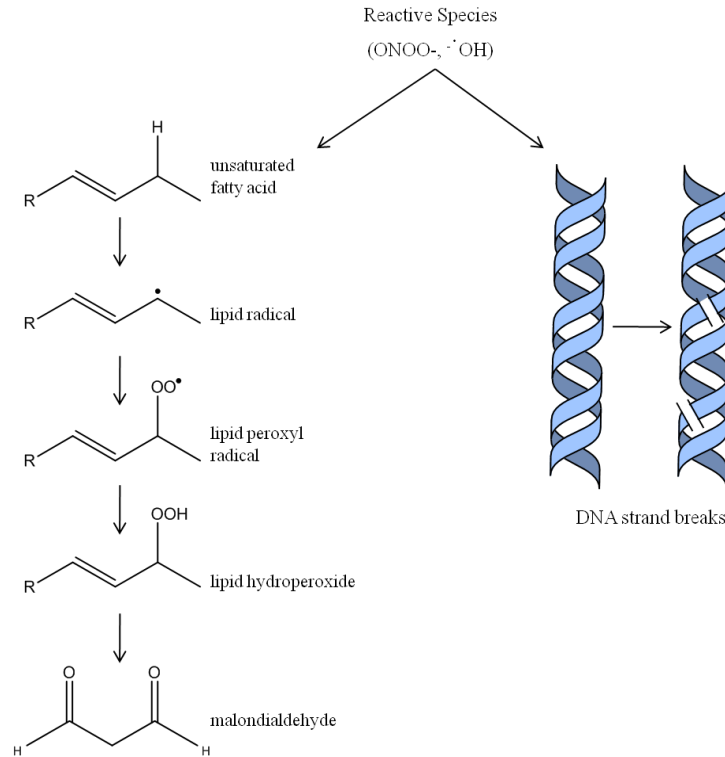


Figure 1.3: Oxidative damage induced by RS

RS such as the $\cdot\text{OH}$ and peroxynitrite can induce the production of oxidative damage to DNA and lipids. They can initiate lipid peroxidation reactions which can create terminal products such as malondialdehyde (MDA), detectable by TBARS assay. They also can interact directly with DNA, inducing strand breaks that can then be measured via the comet assay.

and neurodegenerative diseases. The accumulation of damage in non-dividing cells such as neurons is thought to contribute to the aging process and to age-related diseases. Damage to DNA is mitigated by extensive endogenous repair mechanisms and is also less susceptible to oxidative damage than lipids (Duthie et al., 2005). Oxidative stress is implicated in several disorders, but also in the normal aging process.

Aging is defined as the accumulation of changes in the structure and function of an organism over time that do not result from disease or accidents and that leads to the increased probability

of death. In aging, there is a gradual impairment of normal function initiated at the cellular level, having a direct impact on the functional ability of the organism. Several theories of aging exist, including the free radical theory of aging, first introduced by Harman in 1956. This theory, now called the oxidative stress theory of aging, proposes that damage caused by RS accumulate in tissues and serve as a major determinant of aging (Harman, 1992). There have been a multitude of studies supporting this theory. 1) Reports show that levels of oxidative damage to lipids, DNA, and proteins increase with age in several tissues. 2) Mice undergoing caloric restriction show increased resistance to oxidative stress. 3) Increased lifespan of genetic mouse models of longevity have correlated with an increased resistance to oxidative stress. Table 1.1 shows the involvement of oxidative damage with regards to aging in the brain and shows increases in markers of oxidative stress in several species. There are, however, studies showing no significant changes with age.

In addition, removal of the pro-oxidant Bax has a positive effect on aging mice. Bax deficiency has been shown to improve age-related health complications in female mice, including the decrease in the incidence of alopecia, cataracts, and skin wrinkling. These mice also do not express the mouse equivalent of menopause (Perez et al., 2007). In addition, this study noted no increased incidence of cancer as compared to wild-type animals, an observation also previously reported (Ahlemeyer et al., 2001). This is consistent with the hypothesis that Bax may reduce oxidative stress in the aged organism.

The Aging Brain

Neurons are carriers and processors of information and as such are meant to last the lifetime of an organism. The long-term retention of neurons is consistent with the need to retain information throughout life (Nowakowski, 2006). Also, the replacement of neurons is

complicated by the intricate workings of neural networks. The survival of new neurons in the adult brain is dependent on their ability to make functional connections with existing neurons. Approximately half of newly generated adult neurons fail to do so and die (Dayer et al., 2003). With advances in medicine and agriculture, the human lifespan increased over the years (Partridge, 2010). In the last century, the average life expectancy increased from 48 years to over 75 years, but the maximum lifespan has not changed (Austad, 1997). This increase in age requires neurons to remain physiologically intact and functional for longer periods of time. The rise of the human lifespan has led to a correlative rise in age-related neurodegenerative diseases, such as Alzheimer's and Parkinson's Diseases, which affect 2-5% of the population over the age of 65 (Lilienfeld and Perl, 1994). With approximately six million people suffering from these diseases in the United States, the cost to the economy is over \$100 billion annually. Understanding the etiologies of these diseases is important from a medical, social, and economical perspective. These disorders, in addition to being age-related, have pathologies suggesting a role for apoptosis and oxidative stress in their etiology (Antonsson, 2001). Thus, understanding the neurobiology of aging with regards to oxidative stress and apoptotic factors may help develop future therapies.

Table 1.1: Oxidative damage in the aging brain

Animal Model	DNA Damage	Lipid Peroxidation	Aging Biomarkers (e.g. skin wrinkling, cataract formation) *compared to wild-type
Wild-type Mouse (C57Bl/6)	<p>↑ (Sohal et al., 1994; Hamilton et al., 2001)</p> <p>↔ (Cardozo-Pelaez et al., 1999)</p>	<p>↑ (Mo et al., 1995)</p> <p>↔ (Massie et al., 1983; Oxenkrug and Requintina, 2003)</p>	<p>↑ with age (Van Remmen et al., 2003; Perez et al., 2007)</p>
Human	<p>↑ (Mecocci et al., 1993)</p>	<p>↑ (Chia et al., 1983; Dei et al., 2002)</p>	
Sod2 het mouse	<p>↑ (Van Remmen et al., 2003)</p>		<p>↔* (Van Remmen et al., 2003)</p>
Bax het mouse	<p>?</p>	<p>?</p>	<p>↓ * (Perez et al., 2007)</p>

References

- Adams JM, Cory S (1998) The Bcl-2 Protein Family: Arbiters of Cell Survival. *Science* 281:1322-1326.
- Ahlemeyer B, Bauerbach E, Plath M, Steuber M, Heers C, Tegtmeyer F, Kriegstein J (2001) Retinoic acid reduces apoptosis and oxidative stress by preservation of SOD protein level. *Free Radic Biol Med* 30:1067-1077.
- Antonsson B (2001) Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. *Cell Tissue Res* 306:347-361.
- Austad SN (1997) Why we age : what science is discovering about the body's journey through life. New York: J. Wiley & Sons.
- Beckman JS, Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol Cell Physiol* 271:C1424-1437.
- Bhardwaj RD, Curtis MA, Spalding KL, Buchholz BA, Fink D, Björk-Eriksson T, Nordborg C, Gage FH, Druid H, Eriksson PS, Frisén J (2006) Neocortical neurogenesis in humans is restricted to development. *Proceedings of the National Academy of Sciences* 103:12564-12568.
- Cadenas E, Davies KJ (2000) Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 29:222-230.
- Cardozo-Pelaez F, Song S, Parthasarathy A, Hazzi C, Naidu K, Sanchez-Ramos J (1999) Oxidative DNA damage in the aging mouse brain. *Mov Disord* 14:972-980.
- Chia LS, Thompson JE, Moscarello MA (1983) Changes in lipid phase behaviour in human myelin during maturation and aging: Involvement of lipid peroxidation. *FEBS Lett* 157:155-158.

- Daniel NN, Korsmeyer SJ (2004) Cell Death: Critical Control Points. *Cell* 116:205-219.
- Dayer AG, Ford AA, Cleaver KM, Yassaee M, Cameron HA (2003) Short-term and long-term survival of new neurons in the rat dentate gyrus. *The Journal of Comparative Neurology* 460:563-572.
- Deckwerth TL, Elliott JL, Knudson CM, Johnson EM, Snider WD, Korsmeyer SJ (1996) BAX Is Required for Neuronal Death after Trophic Factor Deprivation and during Development. *Neuron* 17:401-411.
- Dei R, Takeda A, Niwa H, Li M, Nakagomi Y, Watanabe M, Inagaki T, Washimi Y, Yasuda Y, Horie K, Miyata T, Sobue G (2002) Lipid peroxidation and advanced glycation end products in the brain in normal aging and in Alzheimer's disease. *Acta Neuropathol (Berl)* 104:113-122.
- Duthie SJ, Gardner PT, Morrice PC, Wood SG, Pirie L, Bestwick CC, Milne L, Duthie GG (2005) DNA stability and lipid peroxidation in vitamin E-deficient rats in vivo and colon cells in vitro--modulation by the dietary anthocyanin, cyanidin-3-glycoside. *Eur J Nutr* 44:195-203.
- Er E, Oliver L, Cartron P-F, Juin P, Manon S, Vallette FM (2006) Mitochondria as the target of the pro-apoptotic protein Bax. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1757:1301-1311.
- Grammer TB, Renner W, Hoffmann MM, Kleber M, Winkelhofer-Roob BM, Boehm BO, Maerz W (2009) SOD3 R231G polymorphism associated with coronary artery disease and myocardial infarction. The Ludwigshafen Risk and Cardiovascular Health (LURIC) study. *Free Radic Res* 43:677 - 684.

- Gross A, McDonnell JM, Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 13:1899-1911.
- Gross CG (2000) Neurogenesis in the adult brain: death of a dogma. *Nat Rev Neurosci* 1:67-73.
- Halliwel B, Chirico S (1993) Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr* 57:715S-724.
- Halliwel B, Gutteridge JMC (2007) *Free radicals in biology and medicine*, 4th Edition. Oxford ; New York: Oxford University Press.
- Hamilton ML, Van Remmen H, Drake JA, Yang H, Guo ZM, Kewitt K, Walter CA, Richardson A (2001) Does oxidative damage to DNA increase with age? *Proc Natl Acad Sci U S A* 98:10469-10474.
- Hancock JT, Desikan R, Neill SJ (2001) Role of reactive oxygen species in cell signalling pathways. *Biochem Soc Trans* 29:345-350.
- Harman D (1992) Free radical theory of aging. *Mutation Research/DNAging* 275:257-266.
- Hengartner MO (2000) The biochemistry of apoptosis. *Nature* 407:770-776.
- Hetts SW (1998) To Die or Not to Die: An Overview of Apoptosis and Its Role in Disease. *JAMA* 279:300-307.
- Hsu SY, Kaipia A, McGee E, Lomeli M, Hsueh AJW (1997) Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proc Natl Acad Sci U S A* 94:12401-12406.
- Huffman MA, Chapman CA (2009) *Primate parasite ecology : the dynamics and study of host-parasite relationships*. Cambridge, UK ; New York: Cambridge University Press.
- Huie RE, Padmaja S (1993) The Reaction of NO With Superoxide. *Free Radic Res* 18:195-199.

- Inohara N, Ekhterae D, Garcia I, Carrio R, Merino J, Merry A, Chen S, Nunez G (1998) Mtd, a Novel Bcl-2 Family Member Activates Apoptosis in the Absence of Heterodimerization with Bcl-2 and Bcl-XL. *J Biol Chem* 273:8705-8710.
- Jain A, Mårtensson J, Stole E, Auld PA, Meister A (1991) Glutathione deficiency leads to mitochondrial damage in brain. *Proc Natl Acad Sci U S A* 88:1913-1917.
- Jang YC, Remmen HV (2009) The mitochondrial theory of aging: Insight from transgenic and knockout mouse models. *Exp Gerontol* 44:256-260.
- Jiang J, Huang Z, Zhao Q, Feng W, Belikova NA, Kagan VE (2008) Interplay between bax, reactive oxygen species production, and cardiolipin oxidation during apoptosis. *Biochem Biophys Res Commun* 368:145-150.
- Jürgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC (1998) Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci U S A* 95:4997-5002.
- Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26:239-257.
- Kim WR, Park O, Choi S, Choi S-Y, Park SK, Lee KJ, Rhyu IJ, Kim H, Lee YK, Kim HT, Oppenheim RW and Sun W (2009) The maintenance of specific aspects of neuronal function and behavior is dependent on programmed cell death of adult-generated neurons in the dentate gyrus. *Eur J Neurosci* 29(7):1408-1421.
- Kirkland RA, Windelborn JA, Kasprzak JM, Franklin JL (2002) A Bax-Induced Pro-Oxidant State Is Critical for Cytochrome c Release during Programmed Neuronal Death. *J Neurosci* 22:6480-6490.

- Knudson CM, Tung KSK, Tourtellotte WG, Brown GAJ, Korsmeyer SJ (1995) Bax-Deficient Mice with Lymphoid Hyperplasia and Male Germ Cell Death. *Science* 270:96-99.
- Kokoszka JE, Coskun P, Esposito LA, Wallace DC (2001) Increased mitochondrial oxidative stress in the Sod2 (+/-) mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis. *Proc Natl Acad Sci U S A* 98:2278-2283.
- Lassen NA (1959) Cerebral Blood Flow and Oxygen Consumption in Man. *Physiol Rev* 39:183-238.
- Lau FC, Shukitt-Hale B, Joseph JA (2005) The beneficial effects of fruit polyphenols on brain aging. *Neurobiol Aging* 26:128-132.
- Lee HC, Wei YH (2007) Oxidative stress, mitochondrial DNA mutation, and apoptosis in aging. *Exp Biol Med (Maywood)* 232:592-606.
- Li Y, Huang T-T, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, Wallace DC, Epstein CJ (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 11:376-381.
- Ligr M, Madeo F, Fröhlich E, Hilt W, Fröhlich K-U, Wolf DH (1998) Mammalian Bax triggers apoptotic changes in yeast. *FEBS Lett* 438:61-65.
- Lilienfeld DE, Perl DP (1994) Projected Neurodegenerative Disease Mortality among Minorities in the United States, 1990--2040. *Neuroepidemiology* 13:179-186.
- Lindsten T et al. (2000) The Combined Functions of Proapoptotic Bcl-2 Family Members Bak and Bax Are Essential for Normal Development of Multiple Tissues. *Mol Cell* 6:1389-1399.

Maellaro E, Casini AF, Del Bello B, Comporti M (1990) Lipid peroxidation and antioxidant systems in the liver injury produced by glutathione depleting agents. *Biochem Pharmacol* 39:1513-1521.

Maier CM, Chan PH (2002) Book Review: Role of Superoxide Dismutases in Oxidative Damage and Neurodegenerative Disorders. *Neuroscientist* 8:323-334.

Marzo I, Brenner C, Zamzami N, Jürgensmeier JM, Susin SA, Vieira HL, Prévost M-C, Xie Z, Matsuyama S, Reed JC, Kroemer G (1998) Bax and Adenine Nucleotide Translocator Cooperate in the Mitochondrial Control of Apoptosis. *Science* 281:2027-2031.

Massie HR, Aiello VR, Banziger V (1983) Iron accumulation and lipid peroxidation in aging C57BL/6J mice. *Exp Gerontol* 18:277-285.

Mecocci P, MacGarvey U, Kaufman AE, Koontz D, Shoffner JM, Wallace DC, Beal MF (1993) Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain. *Ann Neurol* 34:609-616.

Miller TM, Moulder KL, Knudson CM, Creedon DJ, Deshmukh M, Korsmeyer SJ, Johnson EM, Jr. (1997) Bax Deletion Further Orders the Cell Death Pathway in Cerebellar Granule Cells and Suggests a Caspase-independent Pathway to Cell Death. *J Cell Biol* 139:205-217.

Mo JQ, Hom DG, Andersen JK (1995) Decreases in protective enzymes correlates with increased oxidative damage in the aging mouse brain. *Mech Ageing Dev* 81:73-82.

Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H (2007) Trends in oxidative aging theories. *Free Radic Biol Med* 43:477-503.

Nomiyama T, Tanaka Y, Piao L, Nagasaka K, Sakai K, Ogihara T, Nakajima K, Watada H, Kawamori R (2003) The polymorphism of manganese superoxide dismutase is associated

- with diabetic nephropathy in Japanese type 2 diabetic patients. *J Hum Genet* 48:0138-0141.
- Nowakowski RS (2006) Stable neuron numbers from cradle to grave. *Proceedings of the National Academy of Sciences* 103:12219-12220.
- Okauchi M, Hua Y, Keep RF, Morgenstern LB, Xi G (2009) Effects of Deferoxamine on Intracerebral Hemorrhage-Induced Brain Injury in Aged Rats. *Stroke* 40:1858-1863.
- Oltvai ZN, Milliman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *74:609-619*.
- Oxenkrug GF, Requentina PJ (2003) Mating Attenuates Aging-Associated Increase of Lipid Peroxidation Activity in C57BL/6J Mice. *Ann N Y Acad Sci* 993:161-167.
- Papaliagkas V, Anogianaki A, Anogianakis G, Ilonidis G (2007) The proteins and the mechanisms of apoptosis: A mini-review of the fundamentals. *Hippokratia* 11:108-113.
- Partridge L (2010) The new biology of ageing. *Philos Trans R Soc Lond B Biol Sci* 365:147-154.
- Perez GI, Jurisicova A, Wise L, Lipina T, Kanisek M, Bechard A, Takai Y, Hunt P, Roder J, Grynpas M, Tilly JL (2007) Absence of the proapoptotic Bax protein extends fertility and alleviates age-related health complications in female mice. *PNAS* 104:5229-5234.
- Perez VI, Bokov A, Remmen HV, Mele J, Ran Q, Ikeno Y, Richardson A (2009) Is the oxidative stress theory of aging dead? *Biochim Biophys Acta*.
- Poliakova D, Sokolikova B, Kolarov J, Sabova Lu (2002) The antiapoptotic protein Bcl-xL prevents the cytotoxic effect of Bax, but not Bax-induced formation of reactive oxygen species, in *Kluyveromyces lactis*. *Microbiology* 148:2789-2795.

- Pong K, Doctrow SR, Huffman K, Adinolfi CA, Baudry M (2001) Attenuation of Staurosporine-Induced Apoptosis, Oxidative Stress, and Mitochondrial Dysfunction by Synthetic Superoxide Dismutase and Catalase Mimetics, in Cultured Cortical Neurons. *Exp Neurol* 171:84-97.
- Pressley M, McCormick C (2007) *Child and adolescent development for educators*. New York: Guilford Press.
- Putcha GV, Harris CA, Moulder KL, Easton RM, Thompson CB, Johnson EM, Jr. (2002) Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice. *J Cell Biol* 157:441-453.
- Radi R, Beckman JS, Bush KM, Freeman BA (1991) Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 288:481-487.
- Ran Q, Liang H, Ikeno Y, Qi W, Prolla TA, Roberts LJ, II, Wolf N, VanRemmen H, Richardson A (2007) Reduction in Glutathione Peroxidase 4 Increases Life Span Through Increased Sensitivity to Apoptosis. *J Gerontol A Biol Sci Med Sci* 62:932-942.
- Sanchez A, Alvarez AM, Benito M, Fabregat I (1996) Apoptosis induced by transforming growth factor-beta in fetal hepatocyte primary cultures: involvement of reactive oxygen intermediates. *J Biol Chem* 271:7416-7422.
- Schinzel A, Kaufmann T, Schuler M, Martinalbo J, Grubb D, Borner C (2004) Conformational control of Bax localization and apoptotic activity by Pro168. *J Cell Biol* 164:1021-1032.
- Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB, Korsmeyer SJ (1995) Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc Natl Acad Sci U S A* 92:7834-7838.

- Shimizu S, Narita M, Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 399:483-487.
- Sohal RS, Agarwal S, Candas M, Forster MJ, Lal H (1994) Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. *Mech Ageing Dev* 76:215-224.
- Sun Y-F, Yu L-Y, Saarma M, Timmusk T, Arumae U (2001) Neuron-specific Bcl-2 Homology 3 Domain-only Splice Variant of Bak Is Anti-apoptotic in Neurons, but Pro-apoptotic in Non-neuronal Cells. *J Biol Chem* 276:16240-16247.
- Uo T, Kinoshita Y, Morrison RS (2005) Neurons Exclusively Express N-Bak, a BH3 Domain-only Bak Isoform That Promotes Neuronal Apoptosis. *J Biol Chem* 280:9065-9073.
- Valencia A, Morán J (2001) Role of oxidative stress in the apoptotic cell death of cultured cerebellar granule neurons. *J Neurosci Res* 64:284-297.
- Van Remmen H, Salvador C, Yang H, Huang TT, Epstein CJ, Richardson A (1999) Characterization of the Antioxidant Status of the Heterozygous Manganese Superoxide Dismutase Knockout Mouse. *Arch Biochem Biophys* 363:91-97.
- Van Remmen H, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, Alderson NL, Baynes JW, Epstein CJ, Huang T-T, Nelson J, Strong R, Richardson A (2003) Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol Genomics* 16:29-37.
- Wei MC, Zong W-X, Cheng EH-Y, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001) Proapoptotic BAX and BAK: A Requisite Gateway to Mitochondrial Dysfunction and Death. *Science* 292:727-730.

- Willis SN, Adams JM (2005) Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* 17:617-625.
- Wolf N, Penn P, Pendergrass W, Van Remmen H, Bartke A, Rabinovitch P, Martin GM (2005) Age-related cataract progression in five mouse models for anti-oxidant protection or hormonal influence. *Exp Eye Res* 81:276-285.
- Wright LL, Cunningham TJ, Smolen AJ (1983) Developmental neuron death in the rat superior cervical sympathetic ganglion: cell counts and ultrastructure. *J Neurocytol* 12:727-738.
- Xiang J, Chao DT, Korsmeyer SJ (1996) BAX-induced cell death may not require interleukin 1 β converting enzyme-like proteases. *Proc Natl Acad Sci U S A* 93:14559-14563.
- Yant LJ, Ran Q, Rao L, Van Remmen H, Shibatani T, Belter JG, Motta L, Richardson A, Prolla TA (2003) The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. *Free Radic Biol Med* 34:496-502.

Chapter 2

The effect of Bax on RS in cortical neurons

Introduction

RS are involved in both physiological and pathological conditions. RS play an important role in the normal physiological state of a cell, as they are involved in enzyme-catalyzed reactions, signal transduction, gene expression, and a host of other processes (Droge 2002, Hancock *et al.* 2001). One specific physiological role of RS is the involvement of $\cdot\text{NO}$ in smooth muscle contraction. RS are produced as a byproduct of normal mitochondrial respiration and from certain other sources, such as NADPH oxidases (Liu *et al.* 2002, Serrano *et al.* 2009, Shelat *et al.* 2008). The principal endogenous RS is $\text{O}_2^{\cdot-}$, produced primarily by leakage of electrons from the mitochondrial electron transport chain (Obonai *et al.* 1998). In the mitochondria, $\text{O}_2^{\cdot-}$ converts into hydrogen peroxide (H_2O_2) via a dismutation reaction catalyzed by Sod2, that is then detoxified into harmless H_2O via the Gpx or catalase pathway (Loschen *et al.* 1974). In the absence of Sod2, this reaction occurs at a much slower rate. Since $\text{O}_2^{\cdot-}$ has a very short half-life, it will then favor more damaging reactions such as the production of the $\cdot\text{OH}$ via the Haber-Weiss reaction or the production of $\cdot\text{ONOO}$ via a reaction with $\cdot\text{NO}$. This can lead to an increase in oxidative stress in the cell (Figure 2.1). RS are normally kept in balance by antioxidants, but when the amount of RS produced outweighs the ability of antioxidants to detoxify them, oxidative stress and subsequent damage can occur (such as is the case with the reduction of Sod2). Damage caused by oxidative stress may contribute to several disorders, including Parkinson's disease, Alzheimer's disease, and others. There are also indications of a role for RS in the changes that occur in the aging brain (Melov 2000, Barja 2002). These processes also show the involvement of apoptotic death (Peers *et al.* 2009). It is therefore

important to study the relationship between apoptosis and RS. There is a naturally occurring basal level of RS found in cells, but an amplification of these RS occurs early in neurons undergoing apoptotic death (Valencia & Morán 2001, Greenlund *et al.* 1995, Whitemore *et al.* 1994, Palluy & Rigaud 1996). The pro-apoptotic protein Bax lies upstream from this RS burst in both sympathetic and cerebellar granule neurons and RS plays an important role in apoptosis in these cells (Kirkland & Franklin 2001, Kirkland *et al.* 2002). Cortical neurons degenerate in a wide variety of pathological conditions, and oxidative stress may have a role in these pathologies (Wines-Samuelson *et al.* , Jackson & Lowe 1996, Jellinger 1991). The role of Bax on RS production in this cell type has not been established in detail. We therefore sought to evaluate the effect of reducing cellular levels Bax in cortical neurons undergoing chronic endogenous oxidative stress. Here we provide evidence that Bax lies upstream from RS in both apoptotic and non-apoptotic cortical neurons under chronic oxidative stress conditions.

Methods

Materials

Neurobasal-A medium, B-27 supplement, Bax antibody and CM-H₂DCFDA were obtained from Invitrogen (Carlsbad, CA). Sod2 antibody was obtained from Stressgen (Assay Designs, Inc., Ann Arbor, MI). Quick Extract was purchased from Epicentre (Madison, WI). All other chemicals were purchased from Sigma or Fisher unless otherwise indicated.

Mice, mating, and genotyping

All animal procedures were reviewed by the Animal Studies Committee at the University of Georgia. The mice were housed in an AAALAC-approved facility under supervision of two ACLAM certified veterinarians. The mice were kept on a 12 hour light/dark cycle and given access to food and water *ad libitum*.

Male Bax KO ($bax^{-/-}$) mice are infertile as a result of arrested spermatogenesis and young females breed poorly (Knudson *et al.* 1995). Sod2 KO ($sod2^{-/-}$) mice do not survive past the first week of life and therefore could not be used in matings. Therefore, mice heterozygous for *bax* and *sod2* (The Jackson Laboratory; Bar Harbor, ME) were mated resulting in the following 9 genotypes: $bax^{+/+}/sod2^{+/+}$, $bax^{+/+}/sod2^{+/-}$, $bax^{+/+}/sod2^{-/-}$, $bax^{+/-}/sod2^{+/+}$, $bax^{+/-}/sod2^{+/-}$, $bax^{+/-}/sod2^{-/-}$, $bax^{-/-}/sod2^{+/+}$, $bax^{-/-}/sod2^{+/-}$, and $bax^{-/-}/sod2^{-/-}$. While young females breed poorly, older females have better breeding performance (Perez *et al.* 2007). Therefore, to improve the probability of $bax^{-/-}/sod2^{+/-}$ and $bax^{-/-}/sod2^{-/-}$ genotypes in latter experiments, older $bax^{-/-}/sod2^{+/-}$ females were mated with $bax^{+/-}/sod2^{+/-}$ males.

DNA preparation:

Genotyping for both *bax* and *sod2* were performed by PCR using isolated DNA from mouse pups or weanlings. Genomic DNA was extracted from each mouse from a tail snip using either the Wizard Prep kit (Promega, Madison, WI) or QuickExtract DNA extraction solution (Epicentre Biotechnologies, Madison, WI).

Polymerase chain reaction (PCR)

In a 0.5 ml microcentrifuge tube, 24 μ l of reaction mixture (9 μ l ddH₂O, 2.5 μ l primers, 12.5 μ l 2X MangoMix solution) and 1.5 μ l of extracted DNA were combined. PCR was performed using a Techne Genius PCR machine (Burlington, NJ).

Bax

The PCR for the *bax* gene was performed as previously described (Kirkland *et al.* 2002). The primers used in the identification of the *bax* gene were: intron reverse 5'-GTT GAC CAG AGT GGC GTA GG-3' and exon forward 5'TGA TCA GAA CCA TCA TG-3' to amplify a 304 pb sequence of the wild-type allele; neo/pgk reverse 5'-CCG CTT CCA TTG CTC AGC GG-3' and

same exon forward to amplify a 507bp sequence of the mutant allele. Cycling conditions were 33 cycles of 94⁰C for 1 min, 55⁰C for 1 min, and 72⁰C for 1 min.

Sod2

The primers used for *sod2* were: 5'GGC CTA CGT GAA CAA CCT CAA C-3' and 5'GAA AGG ACG TTT ATG CGA ACC AAC-3' to amplify a 134 bp sequence of the wild-type allele; 5'CCA GTC TCA GGG GCA ACA AAG ATG-3' and 5'CGC CTA CCG GTG GAT GTG GAA TGT-3' to amplify a 311 bp sequence of the mutant allele. Cycling conditions were 35 cycles of 94⁰C for 30 sec, 59⁰C for 30 sec, and 72⁰C for 30 sec. PCR products were separated on a 2% (w/v) agarose gel and visualized with ethidium bromide using Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software (Fotodyne Inc., New Berlin, WI).

Cortical cell culture

Cortical neurons were cultured using the method of Xiang, *et al.* (1996) with minor modifications. Modifications included shortened trypsinization time, coating the culture dishes with poly-L-lysine instead of poly-D-lysine, and dissociation with a 1 ml eppendorf pipet tip instead of fire-polished pasture pipette. Post-natal day 1 mice were killed by decapitation and cerebrums were rapidly dissected in ice-cold Leibovitz (L-15) medium. Meninges were removed and the tissue minced, transferred to a 1.5ml centrifuge tube and centrifuged for 3 minutes at 4⁰C. The tissue was then digested with trypsin (1mg/ml) in Hank's Balanced Salt Solution without Ca²⁺ and Mg²⁺ (HBSS; Sigma, St. Louis, MO) for 15 minutes at 37⁰C. Trypsin inhibitor (2mg/ml; in HBSS with Ca²⁺ and Mg²⁺; Sigma) containing 30 units of DNase (Sigma) was then added to the tissue to inactivate the trypsin, centrifuged, and the supernatant discarded. The tissue was then rinsed 1X with 1mg/ml trypsin inhibitor and 1X with conditioned culture medium (serum-free Neurobasal-A medium, 2% B-27 serum-free supplement, 1% Pen/Strep,

0.1% L-glutamine). Cells were dissociated by triturating in culture medium. Cells were plated at a density of 350,000 cells/ml on poly-L-lysine coated 35mm dishes and maintained in a 5% CO₂ atmosphere at 37°C.

Bax and SOD2 Western Blot

Cultured cells were washed 1X with Phosphate Buffered Saline (PBS), scraped with a cell scraper, and collected in a 1.5ml centrifuge tube. The cells were centrifuged at 2500xg for 10 minutes to ensure collection of cells. After discarding the supernatant, the cell pellet was resuspended in Mammalian Protein Extraction Reagent (M-PER; Pierce, Rockford, IL) and mixed well. The solution was then centrifuged at 14000xg for 1 minute to remove cell debris and the supernatant transferred to a new 1.5ml centrifuge tube for analysis. Protein concentration was determined using the Coomassie Plus - The Better Bradford Assay kit (Pierce, Rockford, IL). A molecular marker was added to 1 lane of a 12% Tris-HCl pre-cast gel (Bio-Rad, Hercules, CA). The remaining lanes were filled with a mixture of sample containing 10-20µg protein, sample buffer (100mM Tris-HCl, 4% SDS, 10% bromophenol blue, 30% glycerol), DTT, and protease inhibitor cocktail (Calbiochem #539134, La Jolla, CA) and the gel was run for 1 hour at 150V. Proteins were transferred onto PVDF membranes (Millipore, Bedford, MA) at 100V for 1 hour, dried, and blocked with blocking buffer for 1 hour at room temperature with gentle shaking. The membrane was then incubated in mouse anti-Bax or rabbit anti-SOD2 monoclonal antibodies overnight at 4°C with gentle shaking. The next day, the membrane was rinsed with 2 changes of wash buffer (TBS-T), followed by a 15 minute wash with shaking. Secondary anti-mouse or anti-rabbit at a 1:3000 dilution were added for Bax and Sod2, respectively, for 1 hour at room temperature. Washes were repeated, followed by incubation in detection solution (ECL Western Blotting Substrate, Pierce) for 5 minutes. Blot

visualization and analysis were performed using Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software.

Induction of apoptosis

Experiments were performed on 3-5 day-old cultures. Bax-dependent apoptosis was induced by 6 hour treatment with 300nM staurosporine.

Survival

Neurons were characterized as “healthy,” or non-viable, at day 3 and day 6 in culture. “Healthy” neurons were distinguished by a smooth and round or oval-shaped soma and neurites with a relatively uniform diameter and smooth appearance. Neurons with irregular shaped somas and missing or fragmented neurites were considered non-viable (Zhu *et al.* 2000, Mattson *et al.* 1992). Live/Dead staining was also used as another method of quantifying survival (Uysal *et al.* 1989). Neurons were incubated for 3 min with fluorescein diacetate (5µg/ml) and propidium iodide (10µg/ml) to determine live and dead cells, respectively. Excitation of 488-nm results in green fluorescence for live cells and red fluorescence for dead cells. Percent survival was calculated as the ratio between number of live cells and total number of live + dead cells x 100. Results reported as fold change of survival assessed at day 6 versus day 3 *in vitro*.

Confocal imaging of RS

The redox-sensitive dye 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was utilized to determine RS levels in neurons. This dye becomes intensely fluorescent when oxidized by RS and primarily detects H₂O₂-associated ROS as well as RNS (Royall & Ischiropoulos 1993). The characterization of this dye was performed extensively in rat and mouse sympathetic neurons (Kirkland *et al.* 2002, Kirkland & Franklin 2001). 3-5 day old cultures were incubated for 10 minutes at 35°C in cell culture medium containing 10µM of

CM-H₂DCFDA. They were then washed 3X with L-15 medium and left in the last wash for microscopy. A laser scanning confocal microscope (Nikon C1; Southern Micro Instruments, Marietta, GA) attached to a Nikon Eclipse TE300 inverted microscope was used for microscopy experiments. CM-H₂DCFDA fluorescence was excited by the 488-nm laser-line of the microscope. Emission was 515 to 540-nm. Images were captured using EZC1 software and quantified by measuring the raw pixel intensity in the cytoplasm of neuronal somas with the 30µm region tool of MetaMorph software (Molecular Devices, Downingtown, PA). All microscopy was done at room temperature.

Protein determination

Protein concentration was determined using Coomassie Plus - The Better Bradford Assay kit (Pierce, Rockford, IL).

Data Presentation and Statistical Analysis

Unless otherwise indicated, data is presented as fold change versus control. Fold change was calculated as (Experimental / Control) - 1. Statistical analysis and graph design were done using Sigma Plot 11.0 (Systat Software, Inc., San Jose, CA). Appropriate statistical measures were determined for each experiment based on analysis of data distribution. Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparisons post-*hoc* test was used unless otherwise indicated. Error bars are \pm SEM.

Results

Bax deletion inhibited RS formation in staurosporine-treated cortical neurons.

Bax induces the production of mitochondrial RS and apoptosis in sympathetic neurons deprived of nerve growth factor and serum-deprived, repolarized cerebellar granule neurons (Kirkland et al. 2002). The role of Bax in RS production in cortical neurons has not been fully elucidated.

The current study sought to determine the role of Bax on RS production in cortical neurons under both apoptotic and oxidative stress conditions. The broad-spectrum kinase inhibitor, staurosporine, was used to induce apoptosis in cortical neuron cell culture (Koh *et al.* 1995). Staurosporine increases cellular RS content in chick embryonic neurons and rat hippocampal neurons (Krohn *et al.* 1998, Zhou & Arthur 1992, Pong *et al.* 2001, Ahlemeyer *et al.* 2001). Staurosporine-induced apoptosis of cortical neurons is also Bax-dependent (Dargusch *et al.* 2001, Whitemore *et al.* 1994, Palluy & Rigaud 1996). We therefore hypothesized that the deletion of Bax would prevent the burst in RS that occurs in this paradigm. 3-5 day old cultures were treated with 300nM staurosporine that induced apoptosis within 24 hours (Ahlemeyer *et al.* 2001). Figure 2.2 displays the time course of RS increase as measured by increase in intensity of the redox-sensitive dye, CM-H₂DCFDA. This cell-permeant indicator for RS is non-fluorescent in its reduced form, but becomes fluorescent when oxidized by RS. RS peaked at 6 hours post-treatment and remained at this level. Cortical neurons treated with staurosporine increased levels of O₂⁻ when using O₂⁻-specific indicators, such as hydroethidine (Krohn *et al.* 1998). Therefore, it is most likely that the species in our study is O₂⁻-associated. CM-H₂DCFDA is used mainly for the detection of H₂O₂ and downstream products. It can, however, detect RNS as well. In order to have a clearer idea of the species detected, NOS inhibitors were utilized. Co-administration of the NOS inhibitor, N_ω-Nitro-L-arginine (L-NNA), with staurosporine resulted in the complete ablation of RS and a 10 minute treatment of L-NNA significantly decreased dye intensity (Figure 2.3). These results suggest that the species detected during staurosporine treatment are primarily *NO-associated.

Mitochondria are a major source of RS production due to electrons leaking from the ETC. Mitochondrial RS production is highly dependent on the membrane potential (Halliwell &

Gutteridge 2007, Hancock et al. 2001). The protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) is a mitochondrial uncoupler that dissipates the mitochondrial membrane potential (Duchen 1999). In doing this, electrons flow through the ETC with less resistance, leading to a reduced amount of leakage. As a result, RS are suppressed due to the reduced production of O_2^- . The increase in RS following staurosporine treatment was inhibited by FCCP treatment, which provides evidence that the RS were derived from the mitochondrial electron transport chain (Figure 2.4).

The increase in RS levels induced by staurosporine treatment was also prevented by genetically reducing Bax concentrations. Removing Bax prevented the increase. Even a 50% reduction of Bax levels completely blocked the RS increase in these cells (Figure 2.5). To be certain that the effect was due to altering the endogenous concentration of Bax, western blots were performed on 3-5 day old cultures showing that bax heterozygous animals (*bax^{+/-}*) had an approximately 50% reduction in Bax protein. There was no detectable Bax in cultures from Bax KO mice (*bax^{-/-}*, Figure 2.6).

Bax deletion reduces RS levels in neurons under oxidative stress

Sod2 serves as a major endogenous antioxidant, as it catalyzes the conversion of O_2^- to H_2O_2 in the mitochondria, a major source of cellular O_2^- . The removal of Sod2 leads to an increase in O_2^- and, therefore, induces a chronic state of oxidative stress in cells (Li *et al.* 1995, Kokoszka *et al.* 2001). Our studies show that genetically reducing Sod2 levels does not alter the level of Bax protein expression as determined by western blot analysis (Figure 2.7).

The genetic reduction of Sod2 (*sod2^{+/-}*) did not cause a further increase in RS over wild-type staurosporine-treated neurons (Figure 2.8a). The removal of Sod2 (*sod2^{-/-}*), however, caused an almost 2-fold increase in RS levels in staurosporine-treated neurons (Figure 2.8b).

This increase was prevented by the genetic reduction of Bax levels. While evaluating RS, we noticed a surprising effect of Bax on RS in non-apoptotic neurons. In cortical neurons, the reduction of Bax concentration (*bax*^{+/-}) surprisingly led to an increase of basal-level RS. Deletion of Bax had the opposite effect, decreasing RS levels (Figure 2.9).

The reduction of Sod2 levels in untreated cultures led to a dose-dependent increase in RS (Figure 2.10). Lowering levels of Bax opposed the increase in Sod2 heterozygous (*sod2*^{+/-}) and Sod2 KO (*sod2*^{-/-}) neurons. In *sod2*^{+/-} neurons, there was a 0.3-fold increase in RS (Figure 2.11a). This was significantly reduced by the deletion of Bax (*bax*^{-/-}), but not the reduction of Bax (*bax*^{+/-}). In *sod2*^{-/-} neurons, a 1.5-fold increase in RS was observed (Figure 2.11b). Lowering Bax levels by 50% was enough to significantly reduce this increase. Deletion of Bax completely prevented it. Similar to staurosporine-treated neurons, the RS detected in neurons with reduced levels of Sod2 appears to be primarily ^{*}NO-associated, determined by the decrease of RS by L-NNA (Figure 2.12).

Bax expression is required for some of the spontaneous cell death in cortical neuron cultures under chronic oxidative stress

There is a natural attrition that occurs in cortical neuronal cell culture, with the loss of over 50% of neurons occurring during the first 2 days under typical culture conditions (Yankner et al. 1990, Dargusch, et al. 2001). Survival can be increased by culturing under special conditions, such as under low oxygen (Li *et al.* 1998). Bax is required for this spontaneous death, with neurons isolated from *bax*^{-/-} animals have a two-fold increase in survival over wild-type littermates after 4 days in culture (Dargusch et al. 2001). Furthermore, under typical conditions, a rapid die-off of neurons is observed in cultures where Sod2 is lacking (Li et al. 1998). Our studies show that cortical neurons from Sod2 KO animals do not survive in cell culture for more

than 5 days. If this process is caused by a classic apoptotic mechanism, neurons lacking Bax should show increased survival. The removal and reduction of Bax increased survival over the time period studied. In our study, even cultures from Bax-heterozygous animals ($bax^{+/-}$) had increased survival over wild-type (Figure 2.13).

Discussion

Chronic oxidative stress is implicated in several pathologies as well as the normal aging process (Kregel & Zhang 2007, Harman 1992). The current study sought to determine what, if any, effect the proapoptotic protein Bax has on cortical neurons under chronic oxidative stress conditions. Previous studies have determined that Bax is a pro-oxidant that lies upstream from the production of RS, release of cytochrome *c* and activation of caspases in sympathetic neurons (Kirkland & Franklin 2001, Kirkland et al. 2002). Removal of Bax leads to the inhibition of apoptosis and the marked reduction of RS in sympathetic and cerebellar granule neurons (Kirkland et al. 2002). Here, we demonstrate that the removal of Bax leads to the inhibition of death and the reduction of RS in cortical neurons under chronic oxidative stress conditions. First, we generated a time course of RS production after exposure to the kinase inhibitor, staurosporine. We utilized the redox-sensitive dye, CM-H₂DCFDA, in order to determine RS levels in live neurons. Like previous research has shown in rat hippocampal neurons, we saw a rapid increase upon treatment with staurosporine in mouse cortical neurons (Sastre *et al.* 1998). Using mice bred from the mating of heterozygous Bax mice and a 6 hour exposure to staurosporine, we determined that the reduction of Bax was sufficient to suppress this increase of RS. In order to study the effects of chronic oxidative stress, endogenous Sod2 levels were genetically reduced. A total of 9 different combinations of Bax and Sod2 levels were achieved in litters produced from mating $bax^{+/-}/sod2^{+/-}$ x $bax^{+/-}/sod2^{+/-}$ mice. Reducing Sod2 levels

resulted in the increase of mitochondrial RS levels in non-apoptotic neurons and a further increase of RS in staurosporine-treated neurons. Bax deletion opposed this increase. Bax deletion also reduced basal-level RS levels in untreated neurons. A surprising observation, however, was that 50% Bax reduction led to an increase in basal-level RS. This is in contrast to other cell types, including SCG and CG neurons, in which the reduction of RS occurs with the reduction of Bax in a dose-dependent manner. One difference between the cortical neurons in this study and these two cell types is the type of RS detected by CM-H₂DCFDA. In cortical neurons, our data suggests that the RS detected is most likely -ONOO, whereas in SCG and CG neurons, the RS detected was mainly associated with ROS downstream of O₂⁻ (Kirkland et al. 2002).

A plausible explanation for this increase in RS as it relates to cortical neurons isolated from Bax heterozygous mice and not Bax KO mice, is the possible involvement of the pro-apoptotic protein Bak. Both Bax and Bak proteins are widely expressed throughout the body and may have a redundant function. These proteins are both able to promote cell death in non-neuronal cells, but this function in post-natal cortical neurons as well as other major neuronal populations is attributed solely to Bax, as these cells express a BH3-only isoform of Bak (N-Bak), instead of the full length version (Uo *et al.* 2005, Sun *et al.* 2001). BH3-only proteins are incapable of executing apoptosis on their own, but rather function as activators of Bax-like proteins or inhibitors of pro-survival proteins (Willis & Adams 2005). It has also been reported that Bax KO mice have a compensatory increase in the expression of Bak (Gavaldà et al. 2008). The reduction of Bax, therefore, could also induce a compensatory increase in Bak. Since the neurons in the current study only possess a truncated BH3 only form of Bak, it could function to increase the association of Bax with the OMM, leading to the increase in RS. Neurons from Bax

KO mice would still have this increase in Bak, but their RS would not increase since Bax is no longer available for this process to occur. Furthermore, in the conditions of apoptosis and chronic oxidative stress, there would be minimal effect by Bak because the process of Bax translocation and insertion into the OMM would already be occurring.

Staurosporine treatment as well as Sod2 deletion led to an increase in death in cortical neurons. The combination of the two led to the death of most cortical neurons within the 6 hour treatment (*bax*^{+/+}/*sod2*^{-/-} + 6 hour staurosporine treatment; figure 2.8B). Of those that survived, RS levels were very high. This RS increase was inhibited by deletion of Bax. This leads to the suggestion that Bax deletion aids in the protection of cortical neurons and this may occur via its ability to regulate RS levels.

The fact that Bax deletion offers protection during both staurosporine treatment and the removal of Sod2 is not surprising as there are similarities in the pathways involved. Although the mechanism underlying staurosporine-induced apoptosis is not well understood, the treatment of rat hippocampal neurons led to a decrease in the protein levels of both Sod1 and Sod2 (Zhou & Arthur 1992, Ahlemeyer et al. 2001). This would account for the increase in RS levels after treatment. Staurosporine-induced apoptosis is known to be Bax-dependent in cortical neurons (Dargusch *et al.* 2001, Whittemore et al. 1994, Palluy & Rigaud 1996). The current study goes further to show that the removal of Bax inhibits the burst in RS seen in the early stages of death.

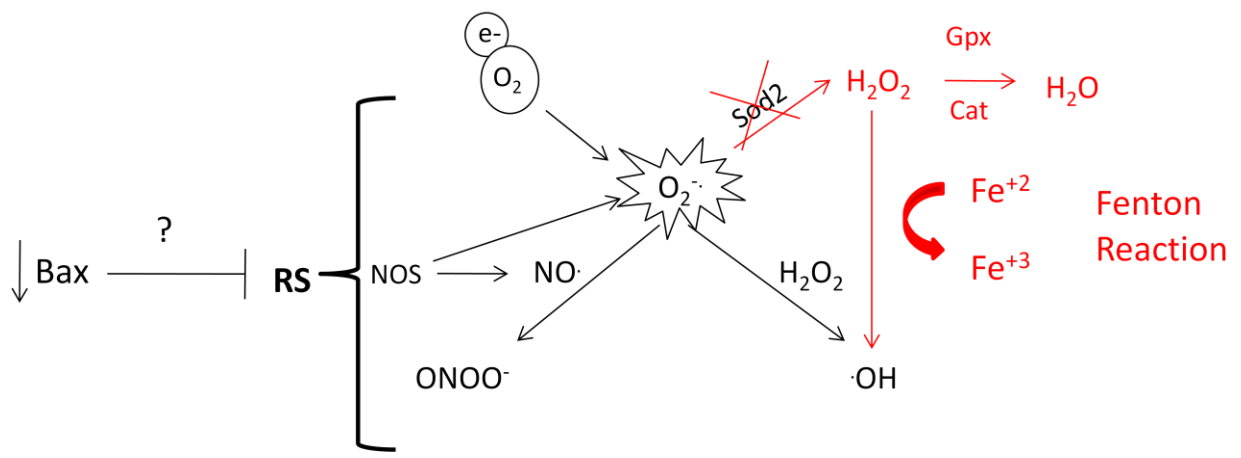


Figure 2.1: RS production

Leakage of electrons from the mitochondrial ETC leads to the formation of superoxide radicals. $O_2^{\cdot-}$ can undergo several reactions in the cell. It quickly is converted into H_2O_2 via Sod2 that then can be detoxified into harmless H_2O or converted into the damaging $\cdot OH$ via the Fenton reaction. $O_2^{\cdot-}$ can also undergo a Haber-Weiss reaction to produce the damaging $\cdot OH$ or react with $\cdot NO$ to produce the damage -ONOO. Both of these pathways result in damage to the cell. Upon reduction or deletion of Sod2, the conversion to H_2O_2 occurs much slower, shifting the reactivity of $O_2^{\cdot-}$ from the red pathway to the black pathways. The pathway in red is favorable in that the outcome is generally the complete detoxification of the radical. $O_2^{\cdot-}$ has a very short half-life, and as such, will favor more damaging reactions.

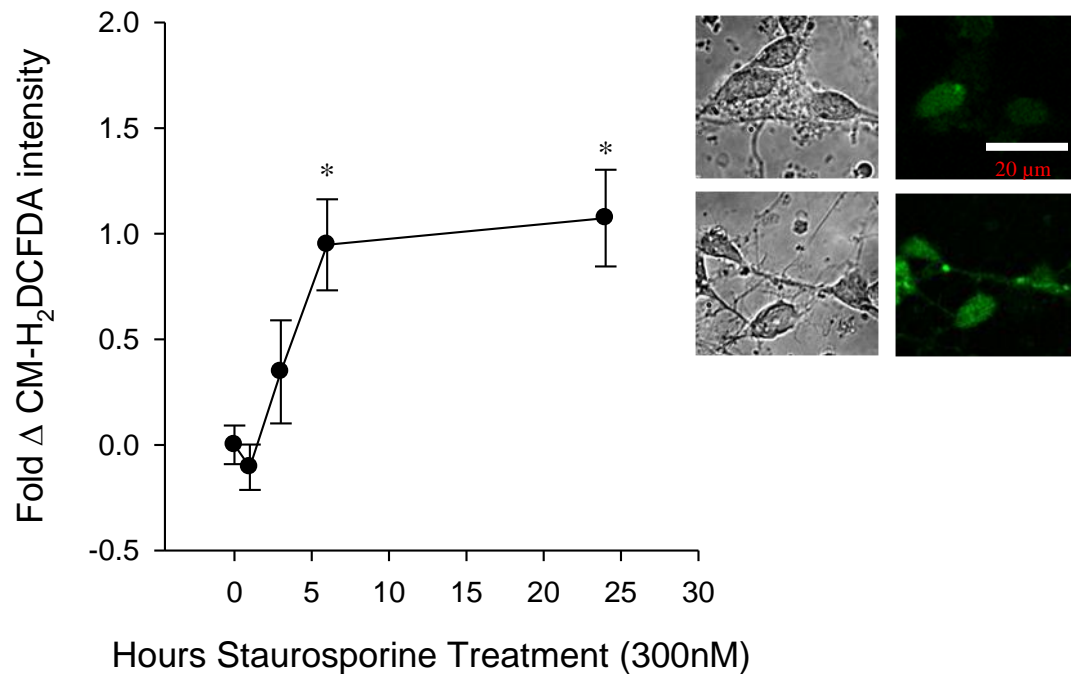


Figure 2.2: RS production after staurosporine treatment.

Left: Wild-type ($bax^{+/+}/sod2^{+/+}$) neurons were treated with 300nM staurosporine for 0-24 hours. RS levels reached maximal levels at 6 hours after treatment. After this point, RS levels reached a plateau and neurons began to succumb to apoptotic death. At the 24 hour period, very few neurons were left to quantify. The results are presented as the mean \pm SEM for at least 3 separate experiments ($n=20-90$ neurons). Data is calculated as fold change relative to untreated cultures. * indicated statistically significant difference from time 0 (ANOVA on Ranks with Dunn's multiple comparisons test, $p<.05$).

Right: Representative micrographs of untreated cultures (top panels) and cultures treated with staurosporine for 6 hours (bottom panels). To the right are cells treated with CM-H₂DCFDA, notice the increase in fluorescence of staurosporine-treated cells (bottom panels).

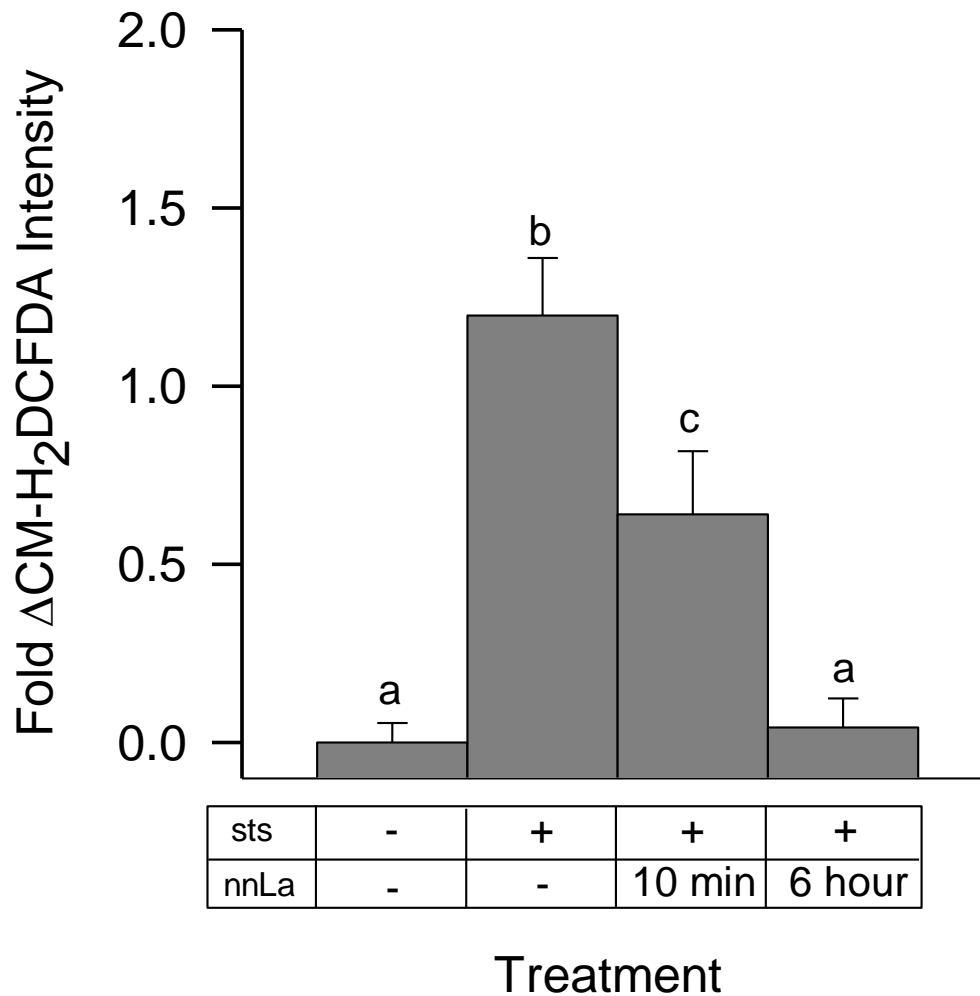


Figure 2.3: Nitric Oxide-related RS detected following staurosporine treatment

RS detected following staurosporine treatment is nitric-oxide associated. 3 day old wt cortical neurons were treated with 300nM staurosporine as outlined in Materials and Methods. Cells were then treated with the nitric oxide synthase inhibitor, N-nitro-L-arginine (NnLa; 0.15M in 1N HCl), for either the duration of the CM-H₂DCFDA treatment (10 minutes) or the duration of the staurosporine treatment (6 hours). Control cultures also contained 500x dilution of 1N HCl. Media changed color slightly, but no significant alteration in pH was observed. 6 hour NOS inhibition completely prevented RS increase and 10 minute inhibition significantly lowered the RS detected. The results are presented as the mean \pm SEM for at least 3 separate experiments ($n=50-150$ neurons). Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test; $p<.05$). Data is calculated as fold change relative to untreated cultures.

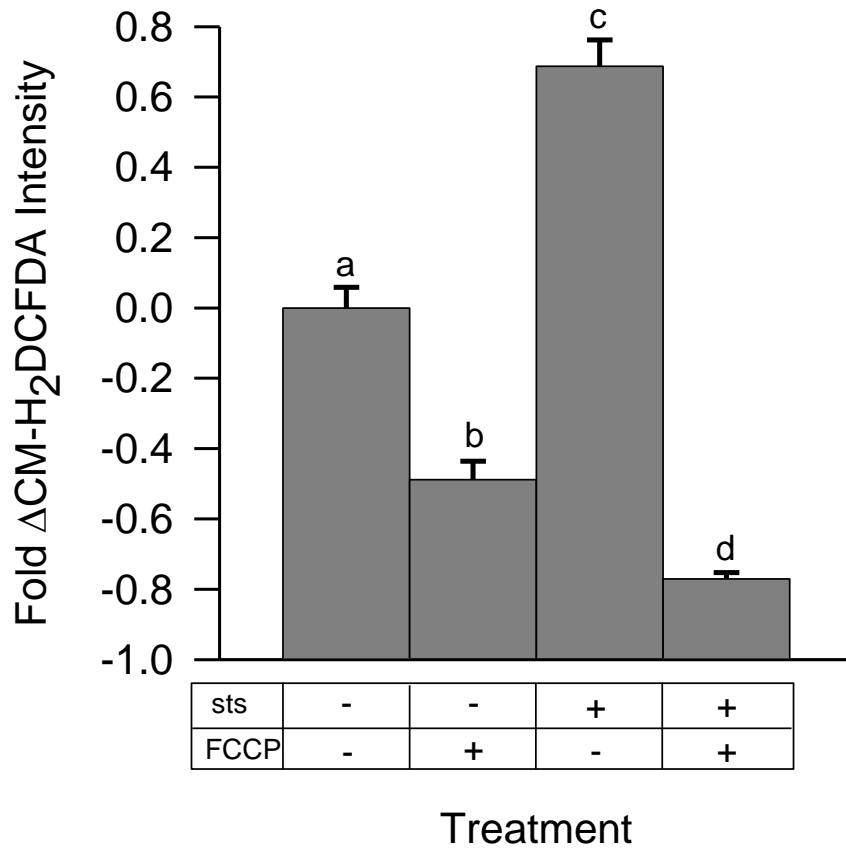


Figure 2.4: Mitochondria-derived RS detected during staurosporine treatment

RS detected following staurosporine treatment is primarily mitochondrial. 3 day old wt cortical neurons were treated with 300nM staurosporine or vehicle as outlined in Materials and Methods. Treatment with the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) significantly lowered the RS detected. The results are presented as the mean \pm SEM for at least 3 separate experiments ($n=50-150$ neurons). Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test $p<.05$). Data is calculated as fold change relative to untreated cultures.

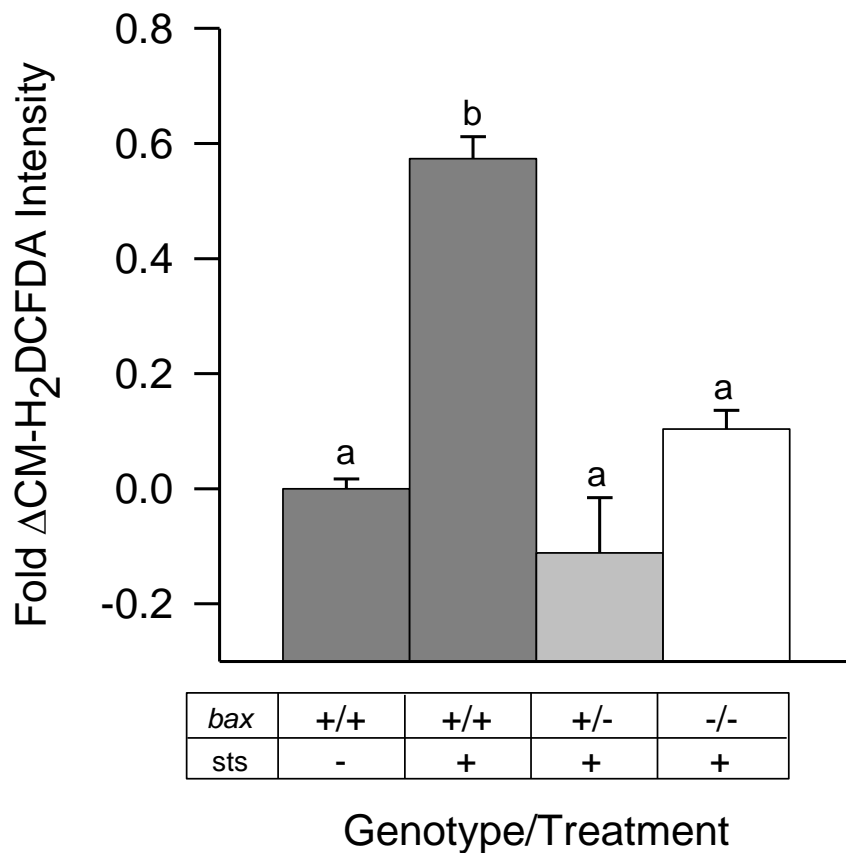


Figure 2.5. Effect of Bax on RS during apoptosis.

Staurosporine treatment (300nM, 6 hours) led to a 0.6-fold increase in RS. Reduced levels of Bax have RS levels not significantly different from untreated wt neurons. The results are presented as the mean \pm SEM for at least 3 separate experiments (n=100-470 neurons). Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test $p < .05$). Data is calculated as fold change relative to untreated wt cultures.

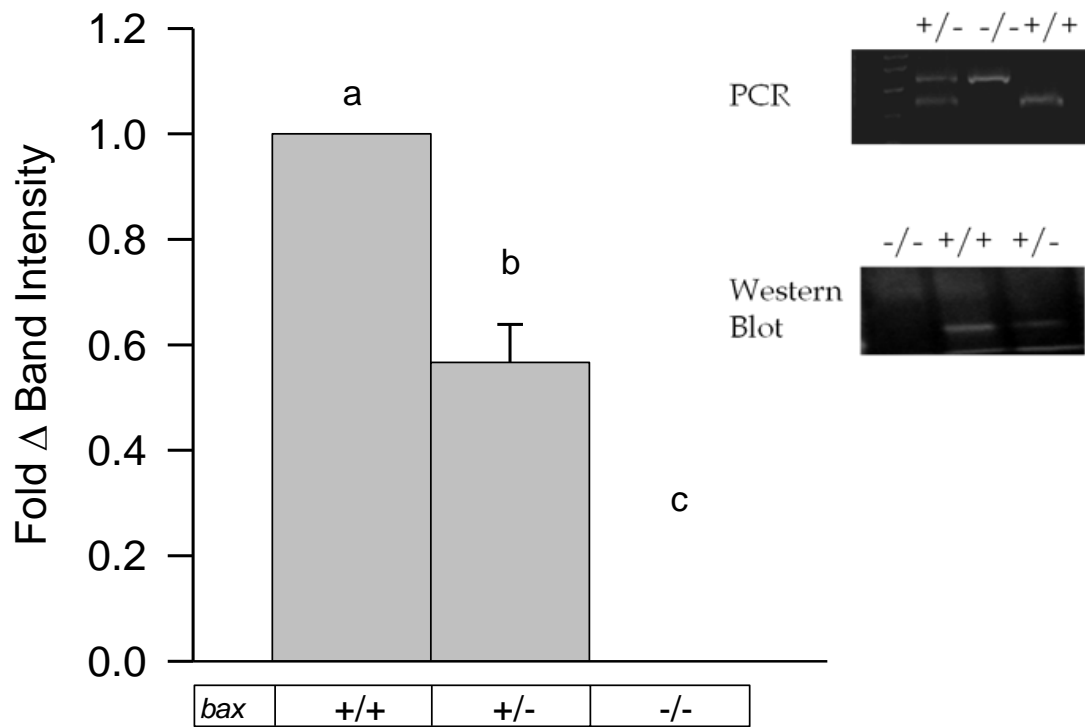


Figure 2.6: Effect of *Bax* gene dosage on *Bax* protein levels in cortical neuron cell culture.

Mice heterozygous for *Bax* show ~50% reduction in *Bax* protein levels as determined by western blot analysis. There are no detectable levels of *Bax* in cultures of KO animals (*bax*^{-/-}). Also pictured is PCR data used to determine genotype of animals used for western blot analysis. The results are presented as the mean \pm SEM for at least 3 separate experiments. Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test; $p < .05$).

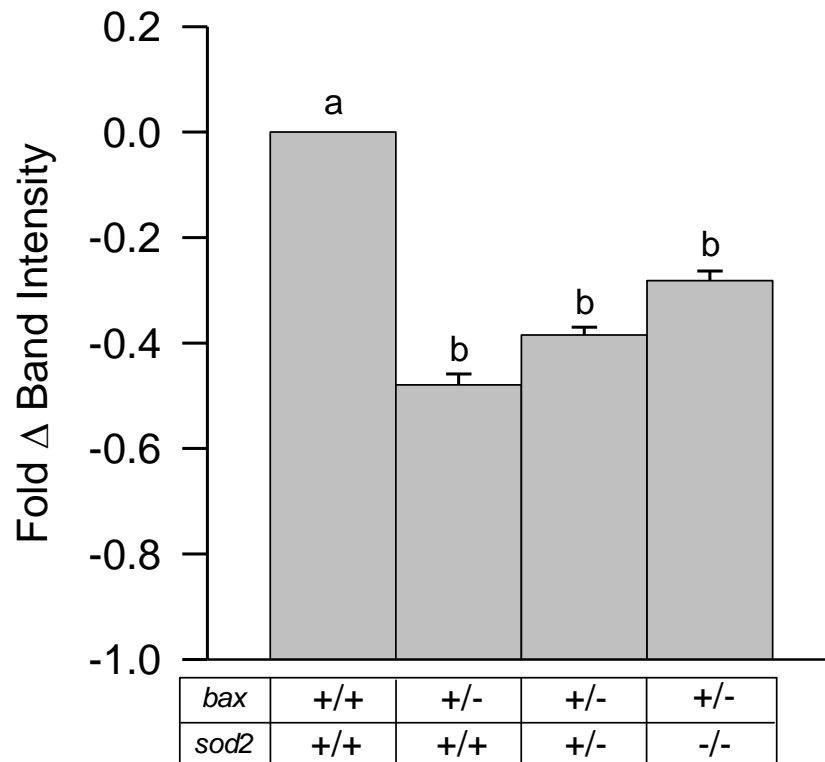


Figure 2.7: Removal of Sod2 does not affect *bax* expression in cortical cultures.

Mice heterozygous for *Bax* ($bax^{+/-}/sod2^{+/+}$) show ~50% reduction in *Bax* protein levels as determined by western blot. The reduction ($sod2^{+/-}$) or removal ($sod2^{-/-}$) does not significantly alter these levels of *Bax*. Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test; $p < .05$). Data is calculated as fold change relative to wt cultures.

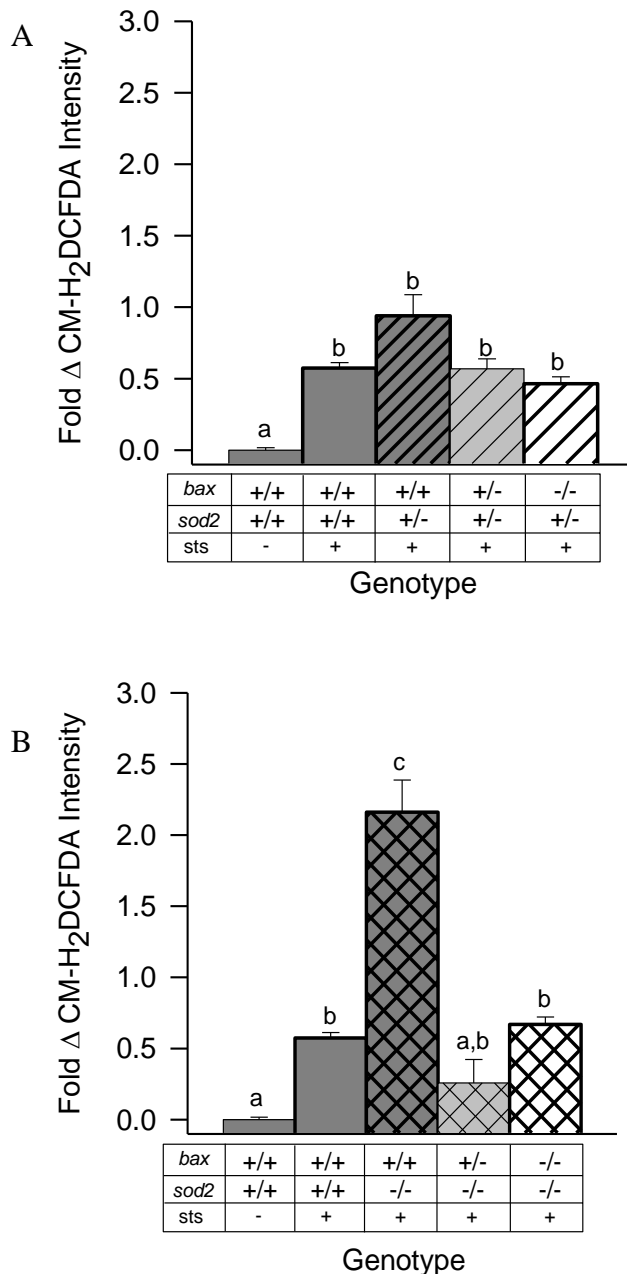


Figure 2.8: Effect of Bax on RS during apoptosis.

Staurosporine treatment (300nM, 6 hours) led to an increase in RS. A: Reduction of Sod2 did not cause further significant increase in RS. B: Deletion of Sod2 caused a 2.2-fold increase in RS. This increase was completely prevented by the reduction of Bax. The results are presented as the mean \pm SEM for at least 3 separate experiments ($n=50-470$ neurons). Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test; $p < .05$). Data is calculated as fold change relative to untreated wt cultures.

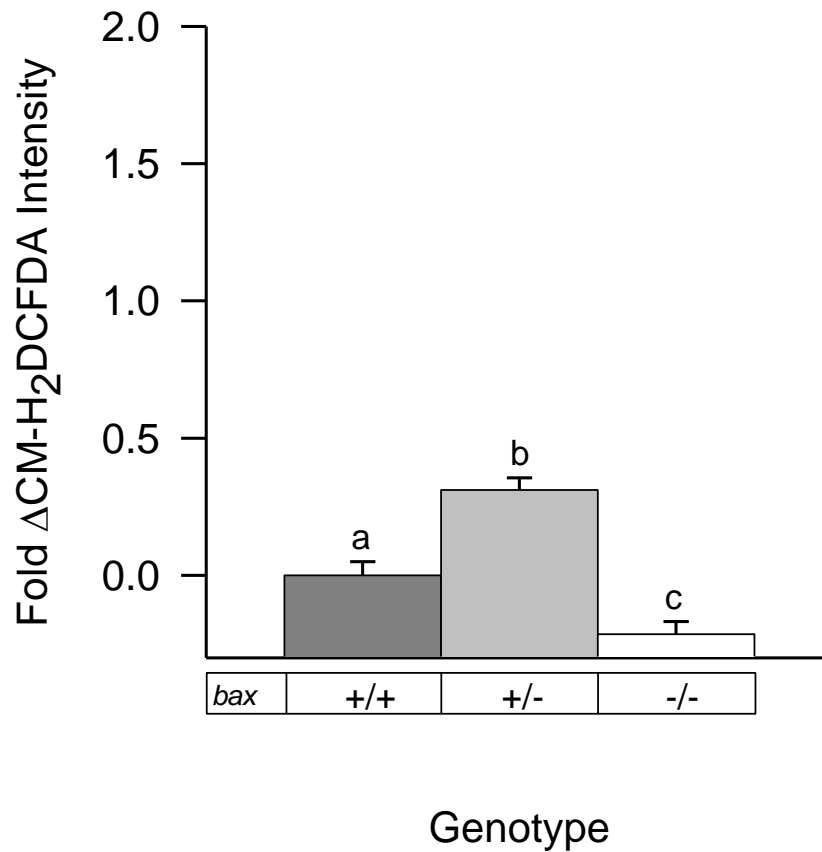


Figure 2.9: Effect of Bax on cortical neurons

The reduction of bax leads to a surprising increase of 0.3-fold in RS in untreated neurons. Deletion of bax led to a non-significant decrease in RS. 3 day old cultures were assessed for RS levels by incubation with CM-H₂DCFDA . Data are expressed as mean \pm SEM for at least 3 separate experiments (n=50-200). Data was calculated as fold change in dye intensity relative to wt neurons. Different letters represent a difference in statistical significance (ANOVA on Ranks with Dunn's multiple comparisons test; $p < 0.05$)

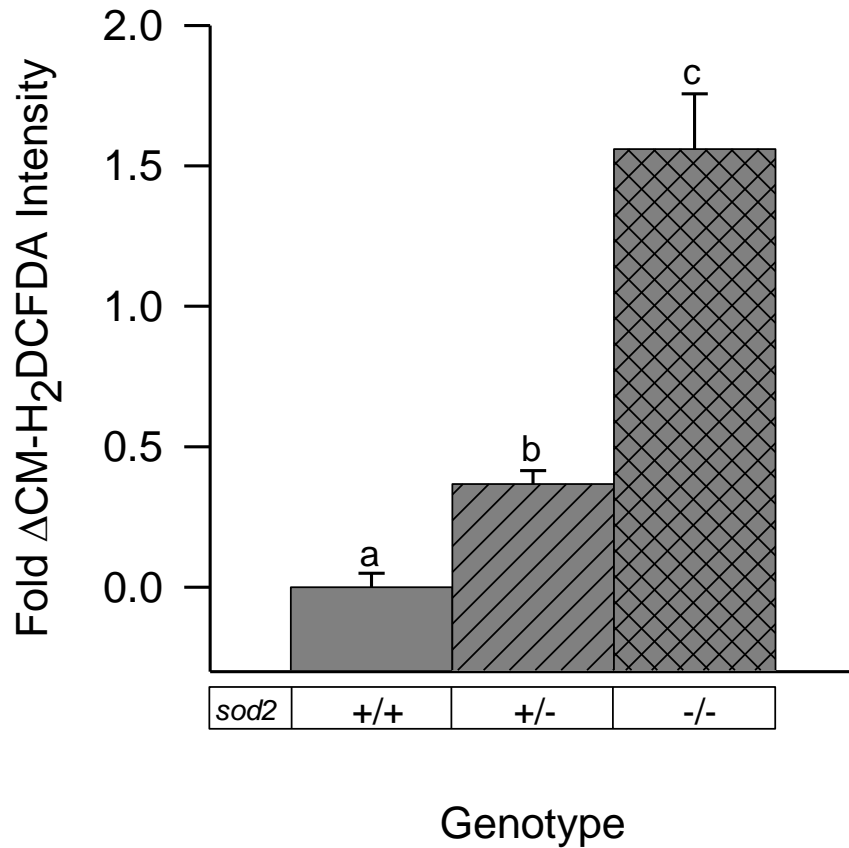


Figure 2.10: Effect of *sod2* on cortical neurons in untreated neurons

The reduction of *sod2* causes a dose-dependent increase in RS in cortical neurons with wt levels of Bax. 3 day old cultures were assessed for RS levels by incubation with CM-H₂DCFDA. Data are expressed as mean \pm SEM for at least 3 separate experiments (n=60-117). Data was calculated as fold change in dye intensity relative to wt neurons. Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test; $p < .05$).

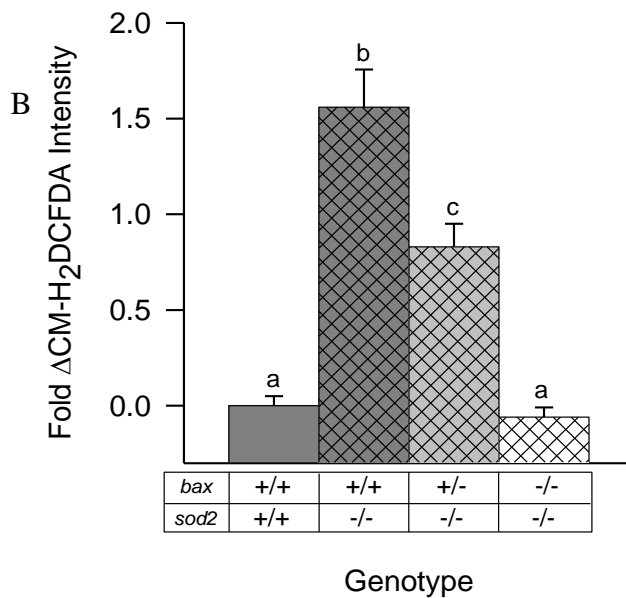
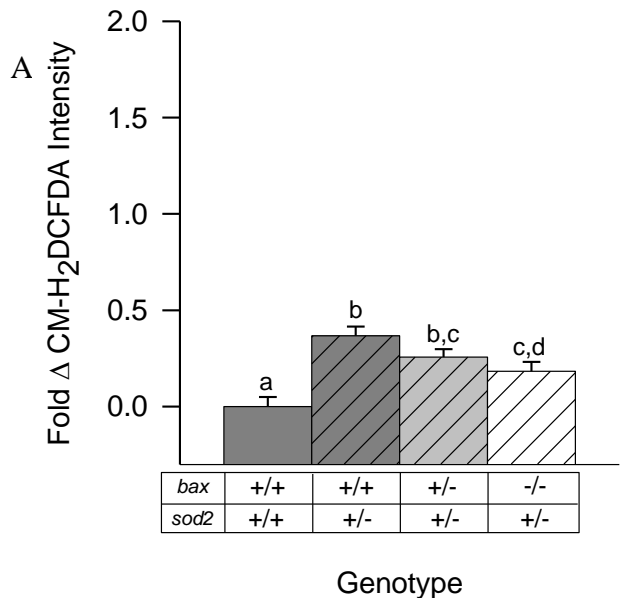


Figure 2.11: RS is lowered with the deletion of Bax in neurons with reduced Sod2

A: The reduction of Sod2 led to a 0.4-fold increase in RS. This was significantly reduced by the deletion of Bax. B: The deletion of Sod2 led to a 1.5-fold increase in RS. This was significantly reduced by the reduction of Bax. Bax deletion caused RS levels to be reduced to wt levels. The results are presented as the mean \pm SEM for at least 3 separate experiments (n=50-217 neurons). Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test; $p < .05$). Data is calculated as fold change relative to wt cultures.

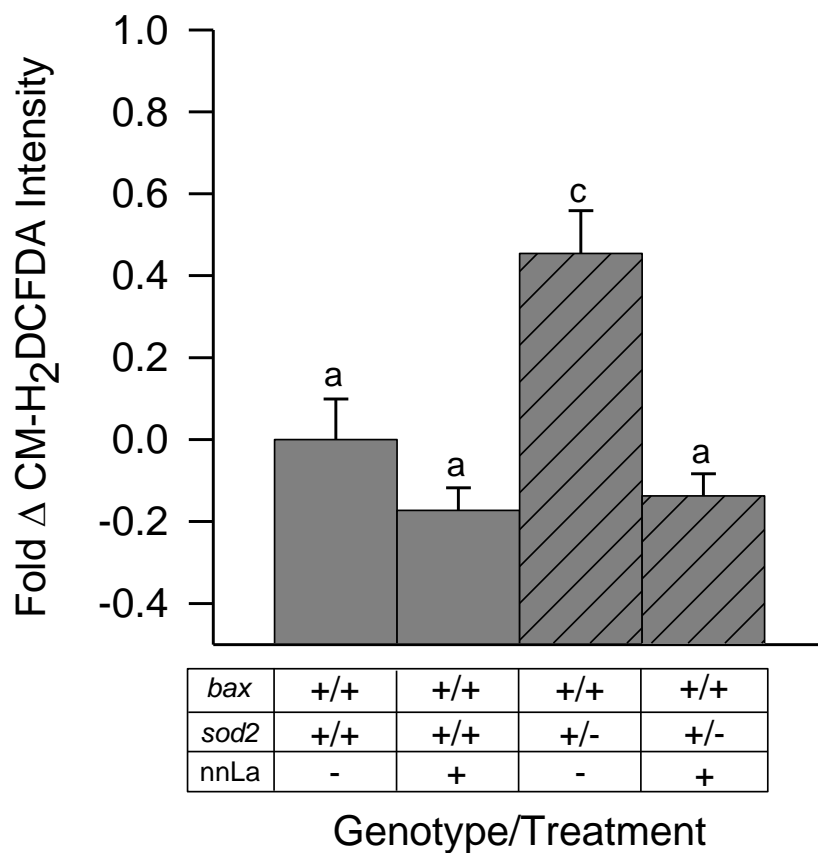


Figure 2.12: Nitric Oxide-related RS detected under chronic oxidative stress conditions in untreated neurons

RS detected under chronic oxidative stress conditions is nitric-oxide associated. 3 day old wt cortical neurons of different genotypes were treated with the nitric oxide synthase inhibitor, nnLA, for the duration of the CM-H₂DCFDA treatment (10 minutes). Inhibiting NOS significantly lowered RS detection in both wt and sod2^{+/-} cultures. The results are presented as the mean \pm SEM for at least 3 separate experiments (n=40-90 neurons). Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons test; $p < .05$). Data is calculated as fold change relative to untreated wt cultures.

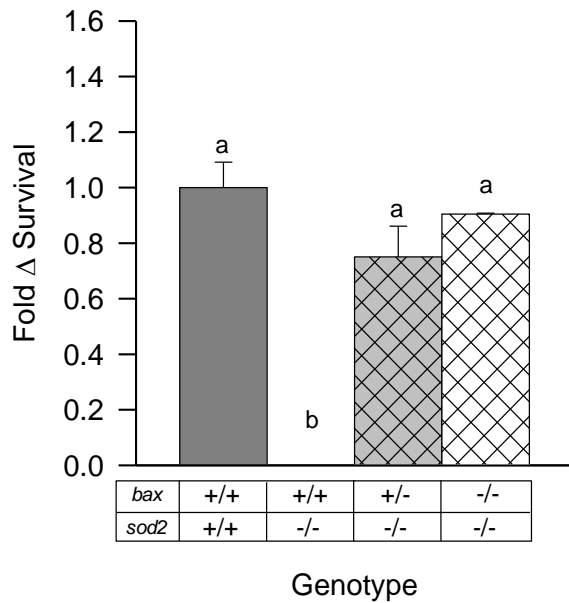
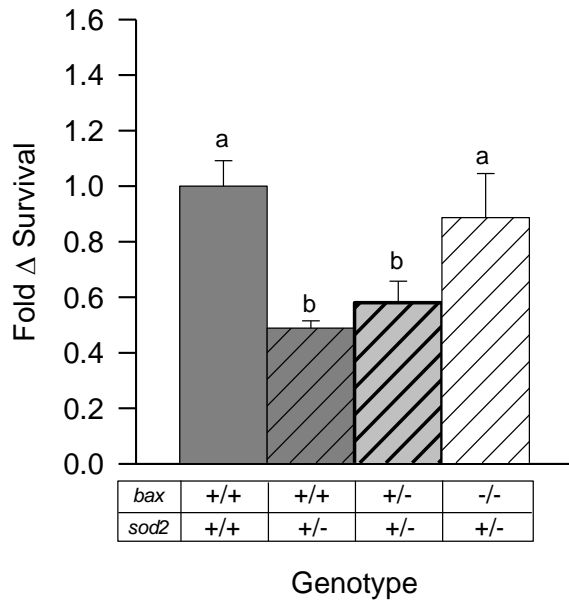


Figure 2.13: Survival in cell culture

Comparison between the number of healthy neurons at day 6 versus day 3 in culture. The results are presented as the mean \pm SEM for at least 3 separate experiments. Different letters represent statistically significant difference ($p < .05$). Data is calculated as fold change relative to untreated wt cultures.

References

- Ahlemeyer, B., Bauerbach, E., Plath, M., Steuber, M., Heers, C., Tegtmeier, F. and Krieglstein, J. (2001) Retinoic acid reduces apoptosis and oxidative stress by preservation of SOD protein level. *Free Radic. Biol. Med.*, **30**, 1067-1077.
- Barja, G. (2002) Endogenous oxidative stress: relationship to aging, longevity and caloric restriction. *Ageing Res. Rev.*, **1**, 397-411.
- Beckman, J. S. and Koppenol, W. H. (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol Cell Physiol*, **271**, C1424-1437.
- Dargusch, R., Piasecki, D., Tan, S., Liu, Y. and Schubert, D. (2001) The role of Bax in glutamate-induced nerve cell death. *J. Neurochem.*, **76**, 295-301.
- Droge, W. (2002) Free Radicals in the Physiological Control of Cell Function. *Physiol. Rev.*, **82**, 47-95.
- Duchen, M. R. (1999) Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. *The Journal of Physiology*, **516**, 1-17.
- Greenlund, L. J. S., Deckwerth, T. L. and Johnson, E. M. (1995) Superoxide dismutase delays neuronal apoptosis: A role for reactive oxygen species in programmed neuronal death. *Neuron*, **14**, 303-315.
- Halliwell, B. and Gutteridge, J. M. C. (2007) *Free radicals in biology and medicine*. Oxford University Press, Oxford ; New York.
- Hancock, J. T., Desikan, R. and Neill, S. J. (2001) Role of reactive oxygen species in cell signalling pathways. *Biochem. Soc. Trans.*, **29**, 345-350.
- Harman, D. (1992) Free radical theory of aging. *Mutation Research/DNAging*, **275**, 257-266.

- Huie, R. E. and Padmaja, S. (1993) The Reaction of NO With Superoxide. *Free Radic. Res.*, **18**, 195-199.
- Jackson, M. and Lowe, J. (1996) The new neuropathology of degenerative frontotemporal dementias. *Acta Neuropathol. (Berl.)*, **91**, 127-134.
- Jellinger, K. (1991) Pathology of Parkinson's disease. *Mol. Chem. Neuropathol.*, **14**, 153-197.
- Jourd'heuil, D., Jourdeuil, F. L., Kutchukian, P. S., Musah, R. A., Wink, D. A. and Grisham, M. B. (2001) Reaction of Superoxide and Nitric Oxide with Peroxynitrite. *J. Biol. Chem.*, **276**, 28799-28805.
- Kirkland, R. A. and Franklin, J. L. (2001) Evidence for redox regulation of cytochrome *c* release during programmed neuronal death: antioxidant effects of protein synthesis and caspase inhibition. *J. Neurosci.*, **21**, 1949-1963.
- Kirkland, R. A., Windelborn, J. A., Kasprzak, J. M. and Franklin, J. L. (2002) A Bax-Induced Pro-Oxidant State Is Critical for Cytochrome *c* Release during Programmed Neuronal Death. *J. Neurosci.*, **22**, 6480-6490.
- Knudson, C. M., Tung, K. S. K., Tourtellotte, W. G., Brown, G. A. J. and Korsmeyer, S. J. (1995) Bax-Deficient Mice with Lymphoid Hyperplasia and Male Germ Cell Death. *Science*, **270**, 96-99.
- Koh, J.-Y., Wie, M. B., Gwag, B. J., Sensi, S. L., Canzoniero, L. M. T., Demaro, J., Csernansky, C. and Choi, D. W. (1995) Staurosporine-Induced Neuronal Apoptosis. *Exp. Neurol.*, **135**, 153-159.
- Kokoszka, J. E., Coskun, P., Esposito, L. A. and Wallace, D. C. (2001) Increased mitochondrial oxidative stress in the Sod2 (+/⁻) mouse results in the age-related decline of

- mitochondrial function culminating in increased apoptosis. *Proc. Natl. Acad. Sci. U. S. A.*, **98**, 2278-2283.
- Kregel, K. C. and Zhang, H. J. (2007) An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **292**, R18-36.
- Krohn, A. J., Preis, E. and Prehn, J. H. M. (1998) Staurosporine-Induced Apoptosis of Cultured Rat Hippocampal Neurons Involves Caspase-1-Like Proteases as Upstream Initiators and Increased Production of Superoxide as a Main Downstream Effector. *J. Neurosci.*, **18**, 8186-8197.
- Li, Y., Copin, J.-C., Reola, L. F., Calagui, B., Gobel, G. T., Chen, S. F., Sato, S., Epstein, C. J. and Chan, P. H. (1998) Reduced mitochondrial manganese-superoxide dismutase activity exacerbates glutamate toxicity in cultured mouse cortical neurons. *Brain Res.*, **814**, 164-170.
- Li, Y., Huang, T. T., Carlson, E. J. et al. (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat. Genet.*, **11**, 376-381.
- Liu, Y., Fiskum, G. and Schubert, D. (2002) Generation of reactive oxygen species by the mitochondrial electron transport chain. *J. Neurochem.*, **80**, 780-787.
- Loschen, G., Azzi, A., Richter, C. and Flohé, L. (1974) Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS Lett.*, **42**, 68-72.
- Mattson, M., Cheng, B., Davis, D., Bryant, K., Lieberburg, I. and Rydel, R. (1992) beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J. Neurosci.*, **12**, 376-389.

- Melov, S. (2000) Mitochondrial oxidative stress. Physiologic consequences and potential for a role in aging. *Ann. N. Y. Acad. Sci.*, **908**, 219-225.
- Obonai, T., Mizuguchi, M. and Takashima, S. (1998) Developmental and aging changes of Bak expression in the human brain. *Brain Res.*, **783**, 167-170.
- Palluy, O. and Rigaud, M. (1996) Nitric oxide induces cultured cortical neuron apoptosis. *Neurosci. Lett.*, **208**, 1-4.
- Peers, C., Haddad, G. G. and Chandel, N. S. (2009) *Hypoxia and consequences : from molecule to malady*, Vol. 1177: Annals of the New York Academy of Sciences,.
- Perez, G. I., Jurisicova, A., Wise, L. et al. (2007) Absence of the proapoptotic Bax protein extends fertility and alleviates age-related health complications in female mice. *Proceedings of the National Academy of Sciences*, **104**, 5229-5234.
- Pong, K., Doctrow, S. R., Huffman, K., Adinolfi, C. A. and Baudry, M. (2001) Attenuation of Staurosporine-Induced Apoptosis, Oxidative Stress, and Mitochondrial Dysfunction by Synthetic Superoxide Dismutase and Catalase Mimetics, in Cultured Cortical Neurons. *Exp. Neurol.*, **171**, 84-97.
- Royall, J. A. and Ischiropoulos, H. (1993) Evaluation of 2',7'-Dichlorofluorescein and Dihydrorhodamine 123 as Fluorescent Probes for Intracellular H₂O₂ in Cultured Endothelial Cells. *Arch. Biochem. Biophys.*, **302**, 348-355.
- Sastre, J., Millan, A., Garcia de la Asuncion, J. et al. (1998) A Ginkgo biloba extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. *Free Radic. Biol. Med.*, **24**, 298-304.

- Serrano, F., Chang, A., Hernandez, C., Pautler, R., Sweatt, J. D. and Klann, E. (2009) NADPH oxidase mediates beta-amyloid peptide-induced activation of ERK in hippocampal organotypic cultures. *Mol. Brain*, **2**, 31.
- Shelat, P. B., Chalimoniuk, M., Wang, J. H., Strosznajder, J. B., Lee, J. C., Sun, A. Y., Simonyi, A. and Sun, G. Y. (2008) Amyloid beta peptide and NMDA induce ROS from NADPH oxidase and AA release from cytosolic phospholipase A2 in cortical neurons. *J. Neurochem.*, **106**, 45-55.
- Sun, Y.-F., Yu, L.-Y., Saarma, M., Timmusk, T. and Arumae, U. (2001) Neuron-specific Bcl-2 Homology 3 Domain-only Splice Variant of Bak Is Anti-apoptotic in Neurons, but Pro-apoptotic in Non-neuronal Cells. *J. Biol. Chem.*, **276**, 16240-16247.
- Uo, T., Kinoshita, Y. and Morrison, R. S. (2005) Neurons Exclusively Express N-Bak, a BH3 Domain-only Bak Isoform That Promotes Neuronal Apoptosis. *J. Biol. Chem.*, **280**, 9065-9073.
- Uysal, M., Seckin, S., Kocak-Toker, N. and Öz, H. (1989) Increased hepatic lipid peroxidation in aged mice. *Mech. Ageing Dev.*, **48**, 85-89.
- Valencia, A. and Morán, J. (2001) Role of oxidative stress in the apoptotic cell death of cultured cerebellar granule neurons. *J. Neurosci. Res.*, **64**, 284-297.
- Whittemore, E. R., Loo, D. T. and Cotman, C. W. (1994) Exposure to hydrogen peroxide induces cell death via apoptosis in cultured rat cortical neurons. *Neuroreport*, **5**, 1485-1488.
- Willis, S. N. and Adams, J. M. (2005) Life in the balance: how BH3-only proteins induce apoptosis. *Curr. Opin. Cell Biol.*, **17**, 617-625.

- Wines-Samuelson, M., Schulte, E. C., Smith, M. J., Aoki, C., Liu, X., Kelleher, R. J., III and Shen, J. Characterization of Age-Dependent and Progressive Cortical Neuronal Degeneration in *Presenilin* Conditional Mutant Mice. *PLoS ONE*, **5**, e10195.
- Yankner BA, Duffy LK, Kirschner DA (1990). Neurotrophic and Neurotoxic Effects of Amyloid β Protein: Reversal by Tachykinin Neuropeptides. *Science* 250:279-282.
- Zhou, X. and Arthur, G. (1992) Improved procedures for the determination of lipid phosphorus by malachite green. *J. Lipid Res.*, **33**, 1233-1236.
- Zhu, H., Fu, W. and Mattson, M. P. (2000) The Catalytic Subunit of Telomerase Protects Neurons Against Amyloid β -Peptide-Induced Apoptosis. *J. Neurochem.*, **75**, 117-124.

Chapter 3

Bax and oxidative stress in the aging brain

Introduction

Many factors are considered important in the modulation of the aging process, including genetic, environmental and dietary factors. Identifying causal factors in normal aging, however, is compounded by the connection of age-related disorders (Halliwell and Gutteridge 1992). In fact, aging and age-related disorders are almost inseparable. Advances in medicine have increased life expectancy over the years, and with this there has also been an increase in age-related disorders. Several theories of aging have been proposed, with the free radical theory of aging at the forefront. Proposed is the idea that free radicals and RS contribute to oxidative damage of tissues and organs leading to their deterioration and ensuing aging of the organism. The theory has recently been expanded after the discovery of $\cdot\text{NO}$ (Drew and Leeuwenburgh 2002). $\cdot\text{NO}$ derived oxidants, reactive nitrogen species (RNS) have been shown to have implications in oxidative damage and aging. A growing number of studies have implicated RNS, in particular $\cdot\text{OONO}$, in oxidative stress and aging (van der Loo et al. 2000, Imam and Ali 2001). $\cdot\text{OONO}$ can initiate transformation of fatty acids and can induce lipid peroxidation. $\cdot\text{OONO}$ can also participate in reactions including one with CO_2 to produce $\cdot\text{OH}$, which is a major player in the induction of lipid peroxidation. The aging process is likely a combination of several factors, but oxidative stress has been identified as having a possible causal effect in this process (Kregel & Zhang 2007, Harman 1992). The brain is especially vulnerable to such damage due to its high oxygen consumption, abundant lipid content, and relative scarcity of endogenous antioxidants. *In vitro* data in our laboratory and others suggests that the pro-apoptotic protein Bax is a pro-oxidant with the removal of Bax eliminating the RS burst in

apoptotic sympathetic (SCG) and cerebellar granule (CG) neurons in cell culture (Kirkland & Franklin 2001, Kirkland *et al.* 2002). *In vitro* data shows that Bax deletion can eliminate RNS production both at basal levels (*sod2*^{+/+}) and in neurons under chronic oxidative stress (*sod*^{+/-}) caused by the reduction of endogenous Sod2 (Chapter 2). In SCG and CG neuronal cell types, Bax deletion has shown that ROS are eliminated under these same conditions as well. The role of Bax as a pro-oxidant may, therefore, contribute to oxidative stress in the aging brain. In addition, animals lacking the *bax* gene (*bax*^{-/-}) have reduced age-related complications in female mice (Perez *et al.* 2007). We, therefore, proposed that the genetic elimination of Bax would reduce oxidative damage over the lifespan of animals subject to chronic oxidative stress.

During normal aging, an increase in damage to DNA, lipids, and proteins has been shown in a variety of different animal models and in humans (Hamilton *et al.* 2001, Uysal *et al.* 1989, Miró *et al.* 2000). This data is somewhat inconsistent, however, with several studies showing decreases or no significant differences in markers of oxidative damage with age (Cand & Verdetti 1989, Zhan *et al.* 1992, Tiana *et al.* 1998).

In addition to several well-established measurements of oxidative damage, alterations in lipid profile can be analyzed. Phospholipids constitute 60% of the lipid mass of eukaryotic cell membranes (Han and Gross, 2004). Phospholipids comprise the majority of the high lipid content of the brain and consist of a polar head group and two fatty acid hydrophobic tails. Polyunsaturated fatty acids (PUFAs) are targets of oxidative damage and therefore phospholipids may be vulnerable to oxidative damage and thus cause a change in lipid profile. While studies demonstrate that oxidative stress correlates to lipid peroxidation in the brain, the exact phospholipid targeted or the mechanisms involved in the alterations in these phospholipids has not been determined.

Sod2 is a major endogenous antioxidant, in that it is considered the first line of defense against $O_2^{\cdot -}$ generated by leakage of electrons from the mitochondrial electron transport chain. Mice lacking Sod2 do not survive past the first week of life and suffer from dilated cardiomyopathy, neurodegeneration, and oxidative damage to mitochondria (Lebovitz *et al.* 1996). Sod2 heterozygous mice live as long as their wild-type counterparts but have increased oxidative damage throughout life, particularly to DNA (Jang & Remmen 2009, Van Remmen *et al.* 1999, Van Remmen *et al.* 2003).

In the current study we observed the effect of Bax reduction on DNA damage and lipid peroxidation in aged mice under chronic oxidative stress, using the Sod2 heterozygous mouse model. Based on *in vitro* results, we expected that the reduction of RS by Bax elimination would aid in the reduction of markers of oxidative stress in these animals.

Methods

Materials

Bax antibody was obtained from Invitrogen (Carlsbad, CA). Sod2 antibody was obtained from Stressgen (Assay Designs, Inc., Ann Arbor, MI). All other chemicals were purchased from Sigma or Fisher unless otherwise indicated.

Mice, mating, and genotyping

All animal procedures were reviewed by the Animal Studies Committee at the University of Georgia. The mice were housed in an AAALAC-approved facility under supervision of two ACLAM certified veterinarians. The mice were kept on a 12 hour light/dark cycle and given access to food and water *ad libitum*.

Male Bax KO (*bax*^{-/-}) mice are infertile as a result of arrested spermatogenesis and young females breed poorly (Knudson *et al.* 1995). Sod2 KO (*sod2*^{-/-}) mice do not survive past

the first week of life and therefore could not be used in matings. Therefore, mice hemizygous for *bax* and *sod-2* (The Jackson Laboratory; Bar Harbor, ME) were mated resulting in the following 9 genotypes: *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-}, *bax*^{+/+}/*sod2*^{-/-}, *bax*^{+/-}/*sod2*^{+/+}, *bax*^{+/-}/*sod2*^{+/-}, *bax*^{+/-}/*sod2*^{-/-}, *bax*^{-/-}/*sod2*^{+/+}, *bax*^{-/-}/*sod2*^{+/-}, and *bax*^{-/-}/*sod2*^{-/-}. Sod2 KO mice could not be used for the aging study. We chose three of the nine genotypes for the current study based on which would give the most dramatic results. The genotypes chosen were *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-}, and *bax*^{-/-}/*sod2*^{+/-}. Three different ages were chosen to evaluate oxidative damage over time, therefore nine separate groups of animals were used in this study. The ages were chosen based on physiological relevance to the human lifespan and were 5 month old “young” animals, 14 month “middle-aged” animals and 22 month “old” animals (Hamilton et al. 2001).

DNA preparation:

Genotyping for both *bax* and *sod2* were performed by PCR using isolated DNA from mouse pups or weanlings. Genomic DNA was extracted from each mouse from a tail snip using either the Wizard Prep kit (Promega, Madison, WI) or QuickExtract DNA extraction solution (Epicentre Biotechnologies, Madison, WI).

Polymerase chain reaction (PCR)

In a 0.5 ml microcentrifuge tube, 24µl of reaction mixture (9µl ddH₂O, 2.5µl primers, 12.5µl 2X MangoMix solution) and 1.5µl of extracted DNA were combined. PCR was performed using a Techne Genius PCR machine (Burlington, NJ).

Bax

The PCR for the *bax* gene was performed as previously described (Kirkland et al. 2002). The primers used in the identification of the *bax* gene were: intron reverse 5'-GTT GAC CAG AGT GGC GTA GG-3' and exon forward 5'TGA TCA GAA CCA TCA TG-3' to amplify a 304 pb

sequence of the wild-type allele; neo/pgk reverse 5'-CCG CTT CCA TTG CTC AGC GG-3' and same exon forward to amplify a 507bp sequence of the mutant allele. Cycling conditions were 33 cycles of 94⁰C for 1 min, 55⁰C for 1 min, and 72⁰C for 1 min.

Sod2

The primers used for *sod2* were: 5'GGC CTA CGT GAA CAA CCT CAA C-3' and 5'GAA AGG ACG TTT ATG CGA ACC AAC-3' to amplify a 134 bp sequence of the wild-type allele; 5'CCA GTC TCA GGG GCA ACA AAG ATG-3' and 5'CGC CTA CCG GTG GAT GTG GAA TGT-3' to amplify a 311 bp sequence of the mutant allele. Cycling conditions were 35 cycles of 94⁰C for 30 sec, 59⁰C for 30 sec, and 72⁰C for 30 sec. PCR products were separated on a 2% (w/v) agarose gel and visualized with ethidium bromide using Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software (Fotodyne Inc., New Berlin, WI).

Brain Harvesting

Mice were rapidly killed by decapitation and cerebrums dissected in a weigh boat chilled on ice, weighed, and immediately snap-frozen in a 70% ethanol/dry ice bath and stored at -80⁰C until analysis. Prior to dissection, mice were weighed for total body weight. There were no significant alterations in brain or body weights either with age or genotype (Table 3.1)

Bax and Sod2 Western Blot

Frozen tissue was homogenized in 10% (w/v) ice-cold RIPA buffer with protease inhibitors using a glass dounce homogenizer. Homogenate was then sonicated for 30s and centrifuged for 10 minutes at 10000xg. The supernatant was transferred to a new 1.5ml centrifuge tube for analysis. Protein concentration was determined using the Coomassie Plus - The Better Bradford Assay kit (Pierce, Rockford, IL). A molecular marker was added to 1 lane of a 12% Tris-HCl pre-cast gel (Bio-Rad, Hercules, CA). The remaining lanes were filled with a mixture of sample

containing 10-20µg protein, sample buffer (100mM Tris-HCl, 4% SDS, 10% bromophenol blue, 30% glycerol), DTT, and protease inhibitor cocktail (Calbiochem #539134, La Jolla, CA) and the gel was run for 1 hour at 150V. Proteins were transferred onto PVDF membranes (Millipore, Bedford, MA) at 100V for 1 hour, dried, and blocked with blocking buffer for 1 hour at room temperature with gentle shaking. The membrane was then incubated in mouse anti-Bax or rabbit anti-SOD2 monoclonal antibodies overnight at 4°C with gentle shaking. The next day, the membrane was rinsed with 2 changes of wash buffer (TBS-T), followed by a 15 minute wash with shaking. Secondary anti-mouse or anti-rabbit at a 1:3000 dilution were added for Bax and Sod2, respectively, for 1 hour at room temperature. Washes were repeated, followed by incubation in detection solution (ECL Western Blotting Substrate, Pierce) for 5 minutes. Blot visualization and analysis were performed using Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software.

DNA Damage

Oxidative damage to DNA is to known to cause modification to purine and pyrimidine bases, single and double-stranded breaks, and cross-linking to other molecules (Croteau *et al.* 1999). DNA damage, as assessed by the appearance of DNA strand breaks, in the aging mouse brain tissue was assessed using single cell gel electrophoresis, known as the comet assay (Fairbairn *et al.* 1995). 25mg of frozen cortex was thawed on ice and dissociated in 1ml ice-cold PBS. This cell suspension was embedded in a 1% (v/v) solution of low melting point agarose and placed on agarose-coated glass slides (prepared 24 hours in advance). After gelling, the slides were immersed in lysis buffer (2.5M NaCl, 100mM EDTA, 10mM EDTA, 10mM Trizma base, pH 10) for 1 hour. Following 3 washes of ice-cold dH₂O, slides were immersed in electrophoresis buffer (300mM NaOH, 1mM EDTA) for 25 minutes and subject to electrophoresis at 25mV

(400mA) for 25 minutes. Slides were dried overnight. Slides were visualized by staining with 20mM ethidium bromide for 30 minutes and images were acquired using fluorescence microscopy and MetaMorph software. Cells were scored using TriTek CometScore™ (TriTek Corp., Sumerduck, VA) software and results calculated as %DNA in tail, and reported as fold change, normalized to young *bax*^{+/+}/*sod2*^{+/+} animals.

Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS) assay:

Oxidative stress can result in the formation of highly reactive and unstable lipid peroxides that decompose into more stable products such as malondialdehyde (MDA). MDA can be quantified using the TBARS assay, a technique based on the reaction of MDA with thiobarbituric acid (TBA). Although there has been much controversy in the literature regarding the specificity of TBARS, it remains the most widely employed assay in the determination of lipid peroxidation (Armstrong and Browne 1994). Frozen tissues were homogenized in 10% w/v in ice-cold PBS, sonicated for 30s, and centrifuged at 800xg for 10 minutes at 4°C. The supernatant was used for protein determination and TBARS assay. The supernatant was added to a 15% solution of trichloroacetic acid (TCA) at a ratio of 2:1 and left on ice for 15 minutes followed by centrifugation. The supernatant was added to a 0.67% solution of TBA at a 1:1 ratio, vortexed and heated in a boiling water bath for 10 minutes. After cooling, a 150µl aliquot was used to measure the absorbance at 532nm using a Molecular Devices SpectraMax M2 spectrophotometer. Each sample was performed in triplicate. The concentration of lipid peroxides was expressed as nmols TBARS per mg protein, calculated by a malondialdehyde standard curve, prepared from 0-50µM of tetraethoxypropane. Data was normalized to young *bax*^{+/+}/*sod2*^{+/+} animals.

Protein determination

Protein concentration was determined by the Bradford Assay using the Pierce Coomassie Plus Bradford Assay (Thermo Fisher Scientific, Rockford, IL.).

Lipid Profiling

Bligh-Dyer lipid extraction

Phospholipids were extracted using chloroform and methanol according to the method of Bligh and Dyer (Bligh & Dyer 1959). ~50 mg brain tissue was homogenized in 500µl PBS and sonicated for 30 seconds. The homogenate was mixed with an equal volume methanol: water (2.0: 0.8 v/v). The liquid was transferred to a glass test tube and 1 ml chloroform was added. Tubes were vortexed for 30 seconds and allowed to sit for 5 minutes. Tubes were centrifuged at 213 x g for 10 minutes and the bottom chloroform layer was transferred to a new test tube. The extraction steps were repeated and the chloroform layers combined. The combined chloroform layers were dried under nitrogen, reconstituted with 100 µl of chloroform:methanol (2:1 v/v), and stored at -80°C.

Lipid phosphorus assay

In order to normalize the amount of lipid in extracts, lipid phosphorus was quantified using malachite green (Zhou & Arthur 1992). 10 µl of lipid extract was dried down under argon in a test tube. 200 µl of perchloric acid was added to the tube, and heated at 130°C for 2-3 hours. After this time, 1 ml of dH₂O was added to the tube followed by vortexing. 1.5 ml of reagent C (4.2 g Ammonium molybdate tetrahydrate in 100 ml 5 N HCl and 0.15 g malachite green oxalate in 300 ml ddH₂O) was added and vortexed. 200 µl of 1.5% v/v Tween 20 was then added and vortexed. After 25 minutes of sitting at room temperature, a 200 µl aliquot was used to measure the absorbance at 590 nm.

Characterization of phospholipids by electrospray ionization-mass spectrometry (ESI-MS):

Shotgun lipidomics is the quantification of individual molecular species of most major and many minor classes of lipids directly from biological lipid extracts without purification by chromatographic methods. This analytical method utilizes ESI-MS (Han and Gross 2005). Lipid extract samples (100 pmol/ μ l) were prepared by dilution in chloroform:methanol (2:1, v/v). Samples were analyzed using an ion trap mass spectrometer equipped with an electrospray ion source (LC/MSD Trap XCT Ultra; Agilent, Foster City, CA). 5 μ l of sample (20pM) was introduced into the ESI source via autosampler at a rate of 0.2 ml/min. The solvent was acetonitrile:methanol:water (2:3:1, v/v/v) containing 0.1% (w/v) ammonium formate (pH 6.4). The mass spectrometer was operated in the positive ionization mode, scanning 120 to 2200 m/z. The flow rate of nitrogen drying gas was 8 L/min at 350°C and 30psi. Qualitative identification of individual phospholipid molecular species was based on their calculated theoretical monoisotopic mass values (m/z) and quantification was done by normalization to the total ion count (Taguchi et al., 2000, Peterson et al., 2008). Results are reported as fold change from young *bax*^{+/+}/*sod2*^{+/+} animals.

High Performance Thin-Layer Chromatography (HPTLC)

HPTLC provides a tool for the extraction of lipids based on their phospholipid head group. This extraction was performed in order to separate cardiolipin from total lipid extracts in order to analyze the data without interference of overabundant lipid peaks in the shotgun lipidomic analysis. 20 μ l of lipid extract and 2 μ l of cardiolipin standard were spotted onto a 5x5 cm HPTLC plate. The lipid spots were allowed to dry under cool air. The plate was run in the first dimension using chloroform:methanol:ammonium solvent (80:35:8). The plate was thoroughly dried and run in the second dimension using a solvent of chloroform:methanol:acetic acid:acetone:water (50:10:20:10:5). The plate was again thoroughly dried under cool air and the

lipid spots were visualized with exposure to iodide vapor. The lipid spot corresponding to cardiolipin was circled and scraped from the HPTLC plate into a glass tube. The cardiolipin was then extracted from the silica gel with a solution of chloroform:methanol:water (10:5:1). Tubes were vortexed for 30 seconds and allowed to sit for 5 minutes. Tubes were centrifuged at 213 x *g* for 10 minutes and the bottom chloroform layer was transferred to a new test tube. The extraction steps were repeated and the chloroform layers combined. The combined chloroform layers were dried under nitrogen, reconstituted with 100 μ l of chloroform:methanol (2:1 v/v), and stored at -80°C until MS analysis.

Data Presentation and Statistical Analysis

Unless otherwise indicated, data is presented as fold change versus control. Fold change was calculated as (Experimental / Control) - 1. Statistical analysis and graph design were done using Sigma Plot 11.0 (Systat Software, Inc., San Jose, CA). Appropriate statistical measures were determined for each experiment based on analysis of data distribution. Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparisons post-*hoc* test was used unless otherwise indicated. Error bars are \pm SEM.

Results

Body and Brain Weights

Bax deletion can lead to an increase in certain neuronal populations, such as sympathetic neurons and others (Knudson et al., 1995; Deckwerth et al., 1996, White et al., 1998). There has been no correlation with the deletion of Bax and an increase in brain size or neuronal numbers with age, however. Brain and body weights were analyzed for the mice used in this study, consisting of nine separate groups of animals with differing ages and genetic combinations. The ages were chosen based on physiological relevance to the human lifespan and were 5 month old "young"

animals, 14 month “middle-aged” animals and 22 month “old” animals (Hamilton et al. 2001). The genotypes studied were wild-type ($bax^{+/+}/sod2^{+/+}$), those under chronic oxidative stress ($bax^{+/+}/sod2^{+/-}$), and those under chronic oxidative stress but lacking Bax ($bax^{-}/sod2^{+/-}$). There were no significant changes noted in brain or body weights of the mice, regardless of age or genotype (table 3.1).

Bax and Sod2 protein levels in the aging brain

Bax is known to play a major role in developmental apoptosis. Bax levels decline shortly after birth, which raises the question of whether or not Bax will have any effect on aged organisms. A rapid decrease in Bax is seen in the early postnatal period. Bax levels in the mouse brain remain low throughout life, but are still detected even in old age (Figure 3.2). Sod2 levels show a rapid increase following birth and remain relatively constant throughout life (Figure 3.4).

Also determined was whether a decrease in *sod2* affects Bax protein levels and vice-versa. A 50% reduction in *sod2* showed a non-significant decrease in Bax levels at all ages studied. Bax KO brains had no detectable protein levels, regardless of Sod2 status. When Sod2 was genetically reduced, there was approximately a 50% decrease in Sod2 protein levels at all ages studied. Genetic reduction of Bax increased these levels in the brains of $sod2^{+/-}$ mice.

Bax deletion reduces oxidative damage to DNA in the aging mouse brain

The oxidative stress theory of aging posits that the accumulation of oxidative damage over time is a causal factor in aging. Single-cell gel electrophoresis, known as the Comet assay, is widely used in the determination of DNA damage (Piperakis *et al.* 1999, Collins 2004). Undamaged DNA exists as a tightly coiled package in the cellular nucleus. If damaged, this organization becomes more relaxed, allowing breaks in the DNA strands to expand outside of this circular cavity. The addition of an electric field allows for the migration of these strand breaks

proportional to the amount of damage in the cell, forming a comet-shaped “tail”. Undamaged DNA strands are too large to migrate out of the comet “head” and thus remain in a compact circular shape. This can then be analyzed using software specific for comet parameters, including percent DNA in tail.

Our results show an approximate 0.4-fold increase in DNA damage from young to middle aged and 0.3-fold increase between young and old wild-type brains (Figure 3.5). There was no significant decrease in damage seen between middle age and old wild-type brains. The Sod2 heterozygous mouse brain had higher levels of damage at both young and middle-ages as compared to wild-type. Brains of old animals show approximately the same amount of damage. The increases were nullified by the deletion of Bax, showing that Bax deletion decreased oxidative stress. In fact, brains of *bax^{-/-}/sod2^{+/-}* mice had lower levels of damage than even wild-type (*bax^{+/+}/sod2^{+/+}*) animals at all ages studied. This suggests that Bax may have an even a higher impact on oxidative stress in normally-aged animals, in that it reduces more than just the increase in damage caused by chronically increased oxidative stress.

Lipid peroxidation in the aging mouse brain

Lipid peroxidation is a well-established product of oxidative stress. It leads to an increase in lipid peroxides and can be measured by more stable end-products, such as malonaldehyde (MDA) that is derived from the breakdown of PUFAs. Measurement of MDA therefore provides an estimation of lipid peroxidation that is considered reputable. The thiobarbituric acid reactive substances (TBARS) assay was utilized in order to study MDA in the aging brain. There was a 0.6 fold increase in damage from the brains of young to middle age wild-type animals (Figure 3.6). This damage was prevented at middle age by the genetic deletion of Bax. Old animals appeared to have similar amounts of damage compared to young animals, regardless of genetic

background. There were no other differences of statistical significance detected with this measure of lipid peroxidation. However, the brains of Bax KO animals are slightly lower than wild-type at all ages studied.

Phospholipid Analysis in the aging mouse brain

Another way to assess lipid peroxidation is to analyze the compositional alterations of brain phospholipids (Lopez, et al. 1995). Several studies have noted changes in phospholipid profiling during aging. Among these findings have been global increases in phosphatidylserine (PS) and decreases in both phosphatidylethanolamine (PE) and phosphatidylcholine (PC; Delion, 1997). Also reported is the loss of both arachidonic acid and cardiolipin (Mgahon *et al.* 1997). Still others report no significant change in phospholipid or fatty acid composition with respect to normal aging (Söderberg *et al.* 1991). Using extracted lipids from brain tissues, we evaluated the lipid profile of the aging brain under normal and chronic oxidative stress conditions as well as the effect of Bax deletion on chronic oxidative stress. Shotgun lipidomic analysis identified few significant alterations in lipids with respect to age. These corresponded to lipids with m/z ratios of 708 (PS or PE), 824 (PC or PS), 848 (PC or PS) and 850 (PC or PS) and all represented increases from young to old animals (Figure 3.7). There appeared to be no overt influence of either Sod2 or Bax on the relative abundance of these lipids (data not shown). We specifically wanted to look at arachidonic acid (AA), a fatty acid that is known to decrease during the aging process. AA is an important PUFA of cell membrane phospholipids. Under physiological conditions, the amount of free AA is small, but upon oxidation, AA can be released from phospholipids thus increasing levels in tissues. AA corresponds to an m/z ratio of 303, but it also showed no significant alterations with age or genotype (Figure 3.8). Another specific lipid of interest was cardiolipin. Cardiolipin levels are also known to decrease with age. The shotgun

lipidomic analysis showed no significant alterations in the major cardiolipin species (data not shown). These peaks, however, could have been overshadowed by the intense peaks corresponding to more abundant phospholipids, such the phosphatidylcholine species. HPTLC was therefore used to separate and concentrate the cardiolipin species from the total lipid extract. The HPTLC separation and extraction, however, resulted in the loss of all major cardiolipin species and was no longer pursued. In addition, results of this analysis in the current study would need to be taken cautiously since whole brain homogenates were used instead of isolated mitochondria. Reduced levels of cardiolipin could simply reflect reduction in mitochondrial mass, a phenomenon also seen in aging.

Discussion

The goal of the current study was to evaluate the role of Bax on an important aspect of aging, oxidative stress. To date, this is the first study to look at the effects of Bax on oxidative damage in the aging brain caused by endogenous oxidative stress. Despite some controversy, the oxidative stress theory of aging remains the most popular of the aging theories (Sanz & Stefanatos 2008). It was first introduced by Harmon in 1956 and proposes that aging occurs due to the accumulation of free radical-produced oxidative damage. The free radical theory of aging has been challenged over the past several years, noting that mice lacking specific endogenous antioxidants (e.g. GPx1) survive as long as their wild-type counterparts (Perez *et al.* 2009). Also, heterozygous Sod2 animals show higher levels of oxidative damage, particularly to DNA, over time, but this is not enough to shorten lifespan. The literature suggests, however, that while RS and oxidative stress may not be the sole cause of aging, they have a major role in the process as well as in age-related diseases (Finkel & Holbrook 2000, Wallace 1999, Simonian & Coyle 1996). The heterozygous Sod2 mouse is a model of chronic oxidative stress, in that tissues have

reduced levels of this major endogenous antioxidant. Sod2 heterozygous mice are associated with increases in damage to DNA and lipids. Protein carbonyls and F₂ isoprostanes have been shown to not be affected in this mouse model (Mansouri et al., 2006). Removal of the pro-apoptotic protein Bax leads to decreased production of RS *in vitro*. Since RS are involved in the production of oxidative damage, the hypothesis arose that removal of Bax would aid in the reduction of such damage. In addition, a study highlighted the ability of Bax deletion to alleviate age-related complications such as cataract formation and skin wrinkling (Perez et al. 2007). The current study evaluated damage to DNA and lipids in wild-type mice and those under chronic oxidative stress as well as determining whether or not genetic deletion of Bax would aid in reducing damage. The results showed that at middle ages, there was an increase in damage in the brains of Sod2 heterozygous animals. Bax deletion had an opposing effect. This suggests that Bax, in part, does aid in the alleviation of damage caused by endogenous stress. From western blot analysis, it was also observed that in reducing Sod2 levels, there was a slight decrease in Bax protein levels. This may reflect some compensatory action in order to reduce the level of oxidative stress present. Bax deletion eliminates the increase in RS caused by chronic oxidative stress *in vitro* (Chapter 2). Since the formation of RS is integral to the development of oxidative damage, it is a logical outcome that Bax deletion would aid in the reduction of this damage. In addition, the genetic elimination of Bax increased protein levels of Sod2 in those mice with reduced sod2 levels (*bax*^{-/-}/*sod2*^{+/-}) to levels similar to wild-type mice. This provides further explanation for the beneficial effect on oxidative damage due to the elimination of Bax.

Table 3.1: Body and Brain Weights

Body weights were recorded prior to dissection of mouse organs. Brains were weighed following dissection. Recorded are brain weights normalized to the total body weight of each animal. Total number of animals per group ranged from 3 to 19. No significant differences were observed in the body weights of the animals studied, with respect to either age or genotype.

Body Weights

Age	<i>bax</i>^{+/+}/<i>sod2</i>^{+/+}	<i>bax</i>^{+/+}/<i>sod2</i>^{+/-}	<i>bax</i>^{-/-}/<i>sod2</i>^{+/-}
Young	29.38 ± 0.5	30.97 ± 0.4	28.74 ± 1.4
Middle-age	28.21 ± 0.5	31.05 ± 0.4	31.71 ± 0.5
Old	26.78 ± 1.5	29.98 ± 0.3	31.77 ± 0.5

Brain Weights (normalized to body weight)

Age	<i>bax</i>^{+/+}/<i>sod2</i>^{+/+}	<i>bax</i>^{+/+}/<i>sod2</i>^{+/-}	<i>bax</i>^{-/-}/<i>sod2</i>^{+/-}
Young	0.009 ± 0.0012	0.008 ± 0.0008	0.0121 ± 0.0007
Middle-age	0.012 ± 0.0012	0.0010 ± 0.0004	0.013 ± 0.0010
Old	0.011 ± 0.0008	0.0101 ± 0.0003	0.008 ± 0.0008

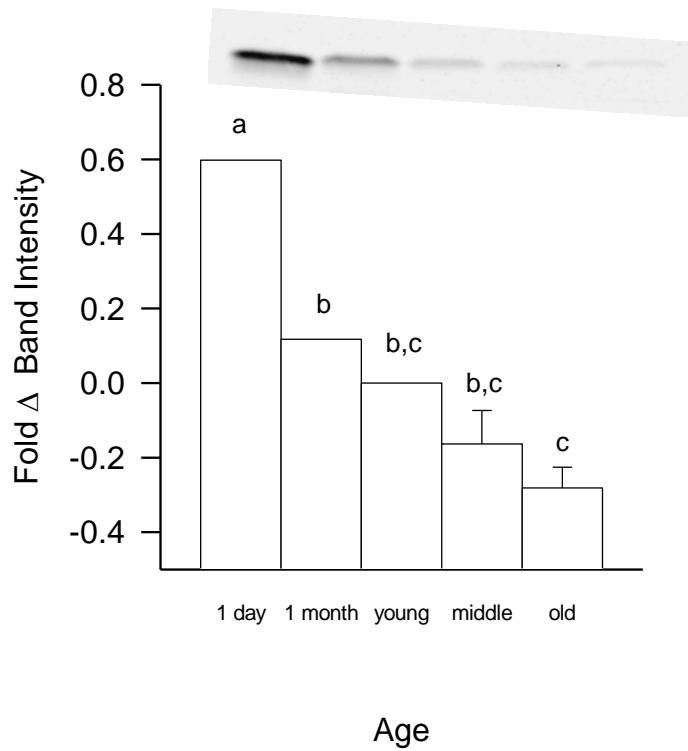


Figure 3.1: Bax protein levels in the aging wild-type brain

There is a rapid decrease in Bax levels following birth and levels remain low. Bax is still detected, however, even into old age. Data is presented as mean \pm SEM using the brains of 3 mice per group. Data was normalized to young animals. Different letters represent statistical significance (ANOVA on Ranks with Dunn's multiple comparisons, $p < .05$).

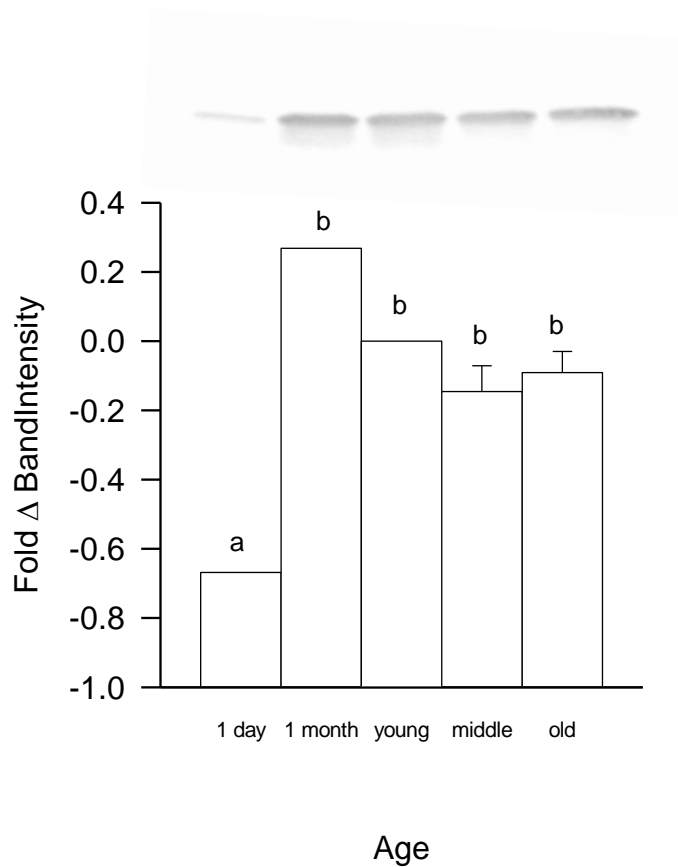


Figure 3.2: Sod2 protein levels in the aging wild-type brain

There are low levels of Sod2 in newborn brains, but this increases and remains constant throughout life. Data is presented as mean \pm SEM using the brains of 3 mice per group. Data was normalized to young animals. Different letters represent statistical significance (ANOVA on Ranks with Dunn's multiple comparisons, $p < .05$).

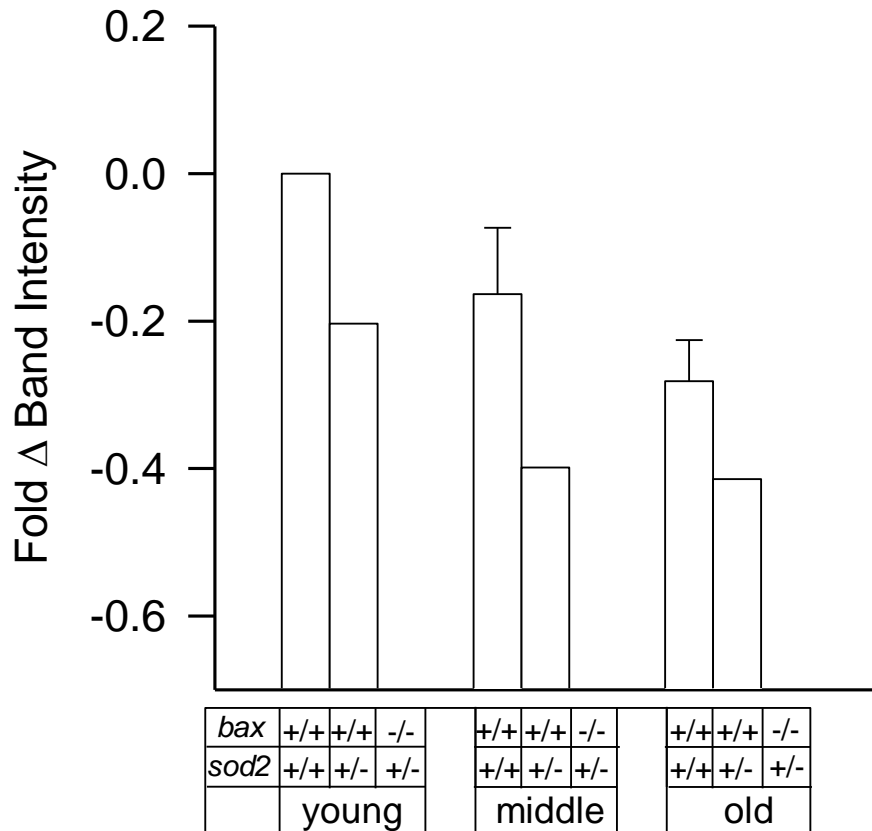


Figure 3.3: Effect of lowering *Sod2* levels on *Bax* protein levels in the aging brain

Sod2 has a modest effect on *Bax* protein levels at each of the ages studied. A 50% reduction in the *sod2* gene had no significant decrease in *Bax* protein levels as determined by *Bax* western blot. *Sod2* has no effect on *Bax* levels in mice lacking the *bax* gene (*bax*^{-/-}), where *Bax* was not detectable. Data is presented as mean \pm SEM using the brains of 3 mice per group. Data was normalized to young wild-type animals.

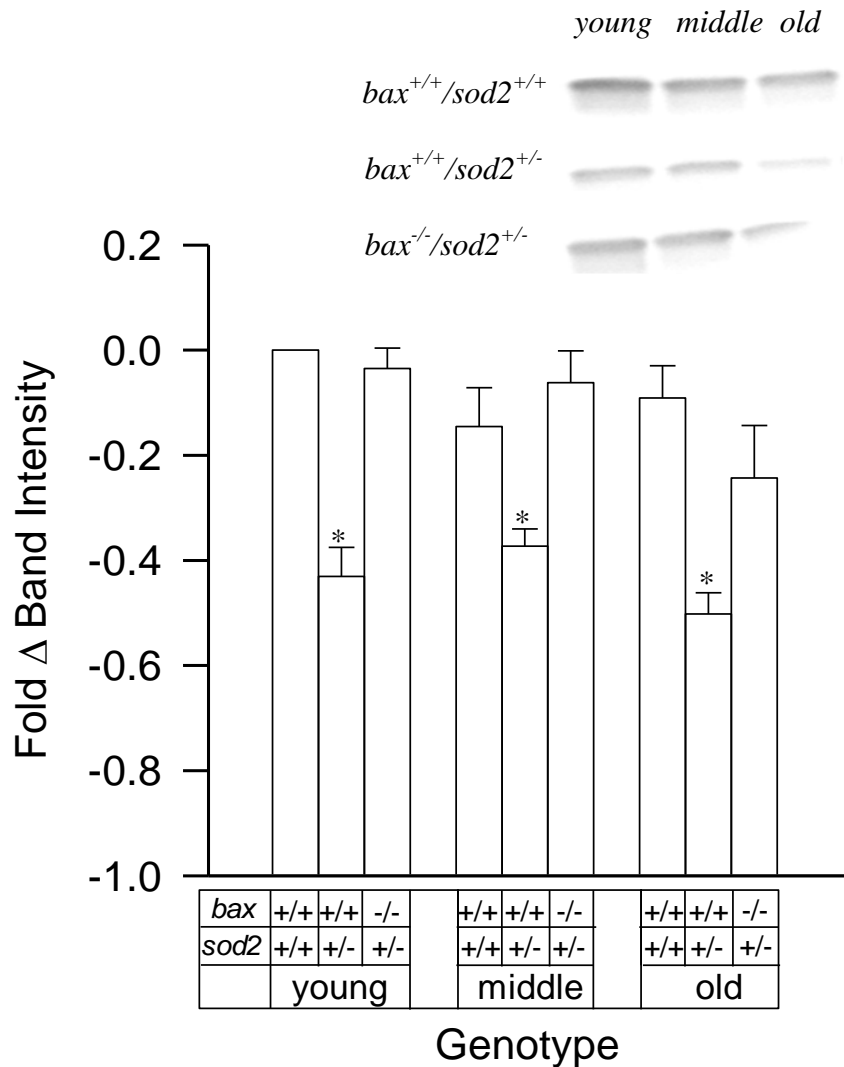


Figure 3.4: The effect of Bax deletion on Sod2 levels in the aging brain

Genetic reduction of *Sod2* led to a 0.4-0.5 fold decrease in *Sod2* protein levels as determined by *Sod2* western blot. Genetic deletion of *Bax* restored these levels to that of wild-type mice. Data is presented as mean \pm SEM using the brains of 3 mice per group. Data was normalized to young wild-type animals. *Statistically significant difference vs. *bax*^{+/+}/*sod2*^{+/+} and *bax*^{-/-}/*sod2*^{+/-} (ANOVA on Ranks with Dunn's multiple comparisons, $p < .05$).

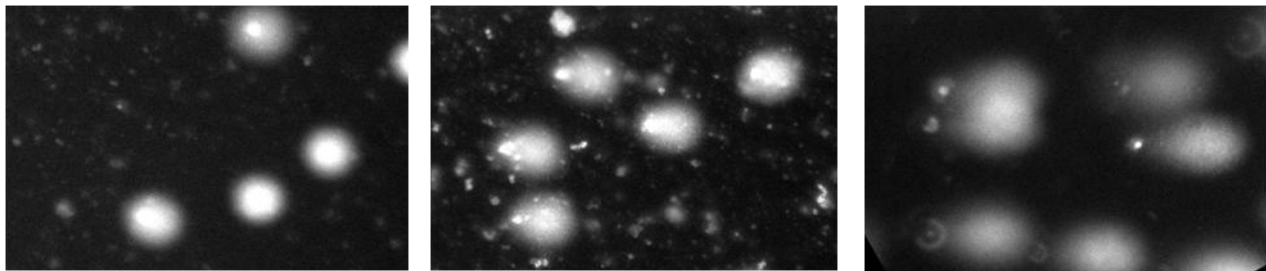
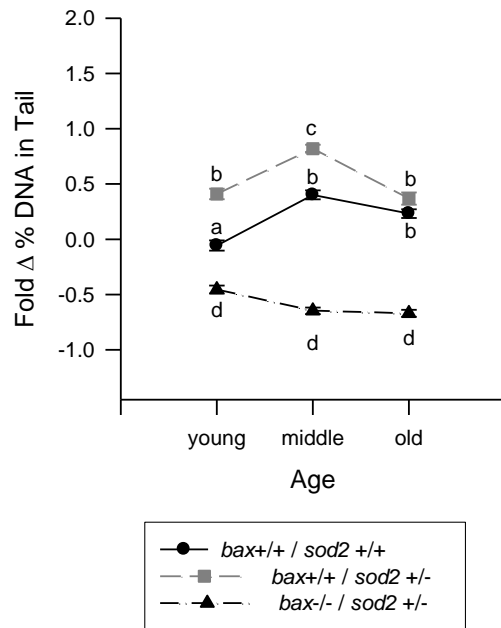


Figure 3.5: DNA damage is prevented by Bax deletion

Comet assay was performed on young, middle-age, and old mice representing *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-} and *bax*^{-/-}/*sod2*^{+/-} genotypes. **Top:** An increase occurred over time in wild-type animals. There was an increase in damage as represented by a higher percentage of DNA in the comet tail in *Sod2* heterozygous animals with wt Bax, which was prevented by the deletion of Bax. **Bottom:** Representative comets of increasing levels of damage as from left to right. The results are presented as the mean \pm SEM for 5 separate experiments. Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons, $p < .05$). Data is calculated as fold change relative to young wt animals based on percent DNA in tail.

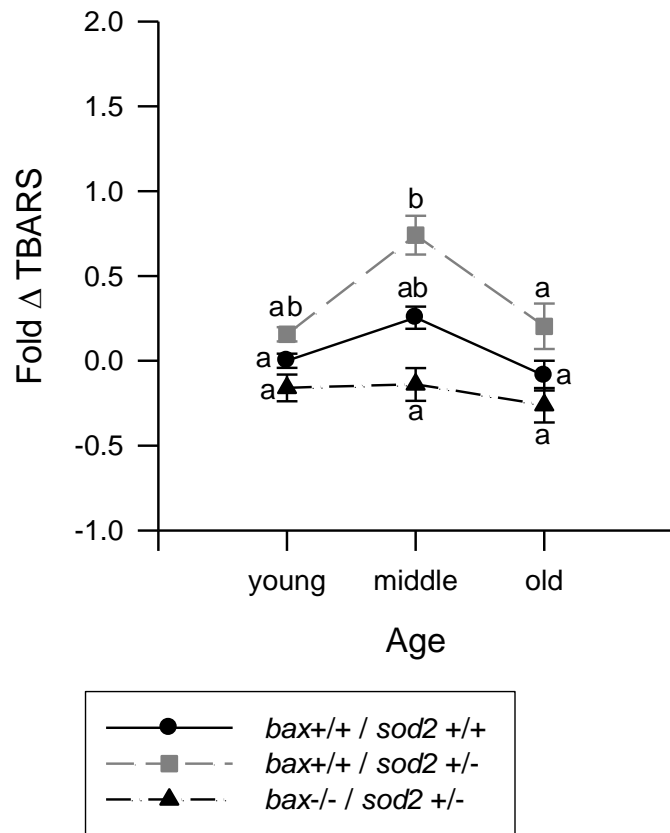


Figure 3.6: Lipid peroxidation is prevented by Bax deletion

TBARS assay was performed on young, middle-age, and old mice representing *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-} and *bax*^{-/-}/*sod2*^{+/-} genotypes. There was a slight, but not significant increase in damage in wild-type animals from young to middle age. Sod2 reduction caused an increase in damage over wild-type at middle age. Bax removal prevented this increase. Results are presented as the mean ± SEM for 5 separate experiments. Different letters represent statistically significant difference (ANOVA on Ranks with Dunn's multiple comparisons, *p* < .05). Data is calculated as fold change relative to wild-type young animals, TBARS levels were calculated as μM MDA/mg protein.

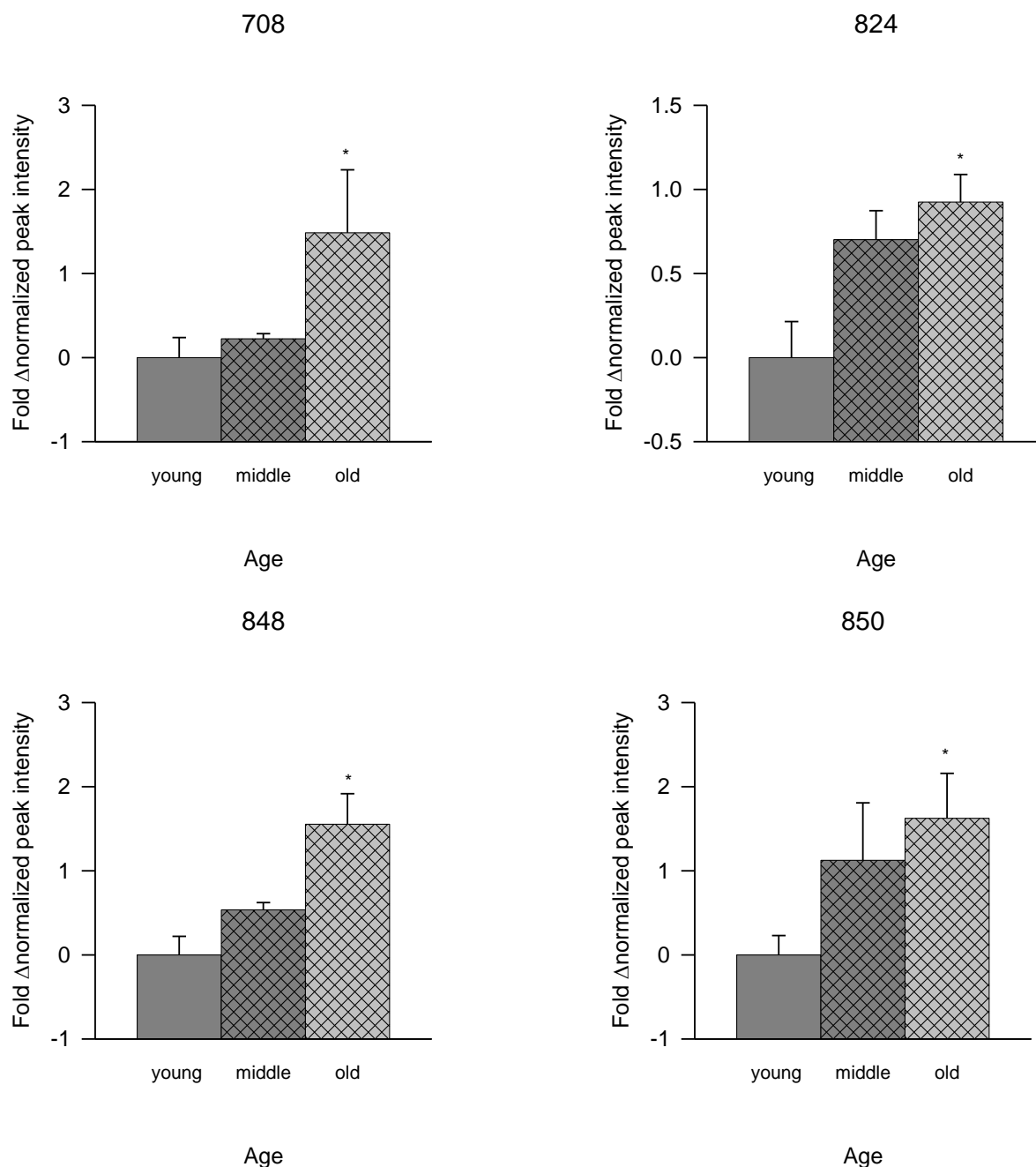


Figure 3.7: Shotgun lipidomic analysis

*Shotgun lipidomic analysis identified few significant alterations in lipids with respect to age. These corresponded to lipids with m/z ratios of 708 (PS or PE), 824 (PC or PS), 848 (PC or PS) and 850 (PC or PS) and all represented increases from young to old animals. Results are presented as the mean \pm SEM for 2 separate experiments (n=3-7 mice per group). * represents statistically significant difference from young animals (p<.05). Data is calculated as fold change relative to wild-type young animals, calculated by peak intensity, normalized to total ion count.*

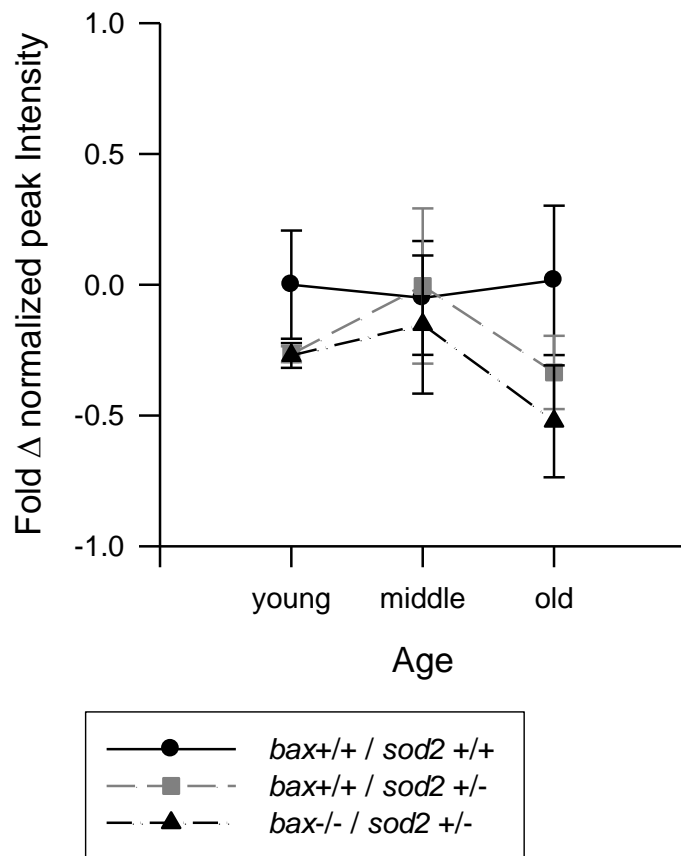


Figure 3.8: Analysis of arachidonic acid

There were no significant changes in arachidonic acid levels with respect to either age or genotype. Results are presented as the mean \pm SEM for 2 separate experiments ($n=3-7$ mice per group). Data is calculated as fold change relative to wild-type young animals, calculated by peak intensity, normalized to total ion count.

References

- Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*, **37**, 911-917.
- Cand, F. and Verdetti, J. (1989) Superoxide dismutase, glutathione peroxidase, catalase, and lipid peroxidation in the major organs of the aging rats. *Free Radic. Biol. Med.*, **7**, 59-63.
- Collins, A. (2004) The comet assay for DNA damage and repair. *Mol. Biotechnol.*, **26**, 249-261.
- Croteau, D. L., Stierum, R. H. and Bohr, V. A. (1999) Mitochondrial DNA repair pathways. *Mutation Research/DNA Repair*, **434**, 137-148.
- Fairbairn, D. W., Olive, P. L. and O'Neill, K. L. (1995) The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology*, **339**, 37-59.
- Finkel, T. and Holbrook, N. J. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature*, **408**, 239-247.
- Hamilton, M. L., Van Remmen, H., Drake, J. A., Yang, H., Guo, Z. M., Kewitt, K., Walter, C. A. and Richardson, A. (2001) Does oxidative damage to DNA increase with age? *Proc. Natl. Acad. Sci. U. S. A.*, **98**, 10469-10474.
- Han X and Gross RW (2005) Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom Rev* 24:367-412.
- Harman, D. (1992) Free radical theory of aging. *Mutation Research/DNAging*, **275**, 257-266.
- Ho, Y.-S., Magnenat, J.-L., Bronson, R. T., Cao, J., Gargano, M., Sugawara, M. and Funk, C. D. (1997) Mice Deficient in Cellular Glutathione Peroxidase Develop Normally and Show No Increased Sensitivity to Hyperoxia. *J. Biol. Chem.*, **272**, 16644-16651.

- Jang, Y. C. and Remmen, H. V. (2009) The mitochondrial theory of aging: Insight from transgenic and knockout mouse models. *Exp. Gerontol.*, **44**, 256-260.
- Kirkland, R. A. and Franklin, J. L. (2001) Evidence for redox regulation of cytochrome *c* release during programmed neuronal death: antioxidant effects of protein synthesis and caspase inhibition. *J. Neurosci.*, **21**, 1949-1963.
- Kirkland, R. A., Windelborn, J. A., Kasprzak, J. M. and Franklin, J. L. (2002) A Bax-Induced Pro-Oxidant State Is Critical for Cytochrome *c* Release during Programmed Neuronal Death. *J. Neurosci.*, **22**, 6480-6490.
- Knudson, C. M., Tung, K. S. K., Tourtellotte, W. G., Brown, G. A. J. and Korsmeyer, S. J. (1995) Bax-Deficient Mice with Lymphoid Hyperplasia and Male Germ Cell Death. *Science*, **270**, 96-99.
- Kregel, K. C. and Zhang, H. J. (2007) An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **292**, R18-36.
- Lebovitz, R. M., Zhang, H., Vogel, H., Cartwright, J., Dionne, L., Lu, N., Huang, S. and Matzuk, M. M. (1996) Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.*, **93**, 9782-9787.
- Mgahon, B., Clements, M. P. and Lynch, M. A. (1997) The ability of aged rats to sustain long-term potentiation is restored when the age-related decrease in membrane arachidonic acid concentration is reversed. *Neuroscience*, **81**, 9-16.
- Miró, Ò., Casademont, J., Casals, E., Perea, M., Urbano-Márquez, Á., Rustin, P. and Cardellach, F. (2000) Aging is associated with increased lipid peroxidation in human hearts, but not with mitochondrial respiratory chain enzyme defects. *Cardiovasc. Res.*, **47**, 624-631.

- Perez, G. I., Jurisicova, A., Wise, L. et al. (2007) Absence of the proapoptotic Bax protein extends fertility and alleviates age-related health complications in female mice. *Proceedings of the National Academy of Sciences*, **104**, 5229-5234.
- Perez VI, Bokov A, Remmen HV, Mele J, Ran Q, Ikeno Y, Richardson A (2009) Is the oxidative stress theory of aging dead? *Biochim Biophys Acta*.
- Piperakis, S. M., Visvardis, E.-E., Tassiou, A. M. and Lester, P. (1999) Comet assay for nuclear DNA damage. In: *Methods Enzymol.*, Vol. Volume 300, pp. 184-194. Academic Press.
- Sanz, A. and Stefanatos, R. K. (2008) The mitochondrial free radical theory of aging: a critical view. *Curr Aging Sci*, **1**, 10-21.
- Simonian, N. A. and Coyle, J. T. (1996) Oxidative Stress in Neurodegenerative Diseases. *Annu. Rev. Pharmacol. Toxicol.*, **36**, 83-106.
- Söderberg, M., Edlund, C., Kristensson, K. and Dallner, G. (1991) Fatty acid composition of brain phospholipids in aging and in Alzheimer's disease. *Lipids*, **26**, 421-425.
- Tiana, L., Caib, Q. and Wei, H. (1998) Alterations of Antioxidant Enzymes and Oxidative Damage to Macromolecules in Different Organs of Rats During Aging. *Free Radic. Biol. Med.*, **24**, 1477-1484.
- Uysal, M., Seckin, S., Kocak-Toker, N. and Öz, H. (1989) Increased hepatic lipid peroxidation in aged mice. *Mech. Ageing Dev.*, **48**, 85-89.
- Van Remmen, H., Ikeno, Y., Hamilton, M. et al. (2003) Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol. Genomics*, **16**, 29-37.

- Van Remmen, H., Salvador, C., Yang, H., Huang, T. T., Epstein, C. J. and Richardson, A. (1999) Characterization of the Antioxidant Status of the Heterozygous Manganese Superoxide Dismutase Knockout Mouse. *Arch. Biochem. Biophys.*, **363**, 91-97.
- Wallace, D. C. (1999) Mitochondrial Diseases in Man and Mouse. *Science*, **283**, 1482-1488.
- Zhan, H., Sun, C.-P., Liu, C.-G. and Zhou, J.-H. (1992) Age-related change of free radical generation in liver and sex glands of rats. *Mech. Ageing Dev.*, **62**, 111-116.
- Zhou, X. and Arthur, G. (1992) Improved procedures for the determination of lipid phosphorus by malachite green. *J. Lipid Res.*, **33**, 1233-1236.

Chapter 4

The effect of Bax deletion on oxidative damage in aging tissues

Introduction

During normal aging, an increase in damage to DNA, lipids, and proteins is reported in a variety of different animal models and in humans (Hamilton *et al.* 2001, Uysal *et al.* 1989, Miró *et al.* 2000). This data is somewhat inconsistent, with studies showing decreases or no significant differences in markers of oxidative damage with age in organs such as the heart, kidney, liver, and brain (Cand & Verdetti 1989, Zhan *et al.* 1992, Tiana *et al.* 1998).

Sod2 is a major endogenous antioxidant that serves to detoxify $O_2^{\cdot-}$, which can be produced from leakage of electrons from the mitochondrial ETC. Mice lacking Sod2 do not survive past the first week of life and suffer from dilated cardiomyopathy, neurodegeneration, and oxidative damage to mitochondria. Sod2 heterozygous mice live as long as their wild-type counterparts but have increased oxidative damage throughout life, particularly to DNA (Jang & Remmen 2009, Van Remmen *et al.* 1999, Van Remmen *et al.* 2003).

Bax is a pro-oxidant and is involved in the apoptotic processes in a wide variety of tissues, including the brain, heart, liver, and kidney (Hou & Hsu 2005, Gerard 1997, Pothana & Manjeri 2003). The deletion of Bax appears to have a positive effect in reducing damage to DNA and lipids caused by chronic oxidative stress in the aging brain (Chapter 3). This is most likely due to its ability to reduce levels of RS and also by the increase in levels of Sod2 in the mice that have reduced Sod2 levels. Apoptosis in many neuronal cell types is mediated solely by the pro-apoptotic protein, Bax. This is due to the expression of a truncated version of the other major pro-apoptotic protein, Bak (N-Bak; Uo *et al.* 2005). In addition, studies of the aging rat brain have shown that Bak levels are reduced shortly after birth and are barely detectable throughout

life (Shimohama *et al.* 2001). The liver, heart, and kidneys all possess full length versions of Bax and in these organs, Bax may have less of an effect due to the compensatory activity of Bak (Yoon & Gores 2002, Ing *et al.* 1999, Krajewski *et al.* 1996). In addition, the degree of cellular turnover is higher in these organs compared to the brain. The current study sought to investigate whether the deletion of Bax would lead to a reduction of oxidative damage caused by endogenous oxidative stress in organs other than the brain (Higami *et al.* 1997, Zajicek *et al.* 1985). In the current study we observed the effect of Bax reduction on DNA damage and, to a lesser extent, lipid peroxidation in aged mice under chronic oxidative stress, using the Sod2 heterozygous mouse model.

Methods

Materials

Bax antibody was obtained from Invitrogen (Carlsbad, CA). Sod2 antibody was obtained from Stressgen (Assay Designs, Inc., Ann Arbor, MI). All other chemicals were purchased from Sigma or Fisher unless otherwise indicated.

Mice, mating, and genotyping

All animal procedures were reviewed by the Animal Studies Committee at the University of Georgia. The mice were housed in an AAALAC-approved facility under supervision of two ACLAM certified veterinarians. The mice were kept on a 12 hour light/dark cycle and given access to food and water *ad libitum*.

Male Bax KO (*bax*^{-/-}) mice are infertile as a result of arrested spermatogenesis and young females breed poorly (Knudson *et al.* 1995). Sod2 KO (*sod2*^{-/-}) mice do not survive past the first week of life and therefore could not be used in matings. Therefore, mice hemizygous for *bax* and *sod-2* (The Jackson Laboratory; Bar Harbor, ME) were mated resulting in the following

9 genotypes: $bax^{+/+}/sod2^{+/+}$, $bax^{+/+}/sod2^{+/-}$, $bax^{+/+}/sod2^{-/-}$, $bax^{+/-}/sod2^{+/+}$, $bax^{+/-}/sod2^{+/-}$, $bax^{+/-}/sod2^{-/-}$, $bax^{-/-}/sod2^{+/+}$, $bax^{-/-}/sod2^{+/-}$, and $bax^{-/-}/sod2^{-/-}$. Nine separate groups of animals; including three different ages and three different genetic combinations. The ages were chosen based on physiological relevance to the human lifespan and were 5 month old “young” animals, 14 month “middle-aged” animals and 22 month “old” animals (Hamilton *et al.* 2001).

DNA preparation:

Genotyping for both *bax* and *sod2* were performed by PCR using isolated DNA from mouse pups or weanlings. Genomic DNA was extracted from each mouse from a tail snip using either the Wizard Prep kit (Promega, Madison, WI) or QuickExtract DNA extraction solution (Epicentre Biotechnologies, Madison, WI).

Polymerase chain reaction (PCR)

In a 0.5 ml microcentrifuge tube, 24 μ l of reaction mixture (9 μ l ddH₂O, 2.5 μ l primers, 12.5 μ l 2X MangoMix solution) and 1.5 μ l of extracted DNA were combined. PCR was performed using a Techne Genius PCR machine (Burlington, NJ).

Bax

The PCR for the *bax* gene was performed as previously described (Kirkland *et al.* 2002). The primers used in the identification of the *bax* gene were: intron reverse 5'-GTT GAC CAG AGT GGC GTA GG-3' and exon forward 5'TGA TCA GAA CCA TCA TG-3' to amplify a 304 pb sequence of the wild-type allele; neo/pgk reverse 5'-CCG CTT CCA TTG CTC AGC GG-3' and same exon forward to amplify a 507bp sequence of the mutant allele. Cycling conditions were 33 cycles of 94⁰C for 1 min, 55⁰C for 1 min, and 72⁰C for 1 min.

Sod2

The primers used for *sod2* were: 5'GGC CTA CGT GAA CAA CCT CAA C-3' and

5'GAA AGG ACG TTT ATG CGA ACC AAC-3' to amplify a 134 bp sequence of the wild-type allele; 5'CCA GTC TCA GGG GCA ACA AAG ATG-3' and 5'CGC CTA CCG GTG GAT GTG GAA TGT-3' to amplify a 311 bp sequence of the mutant allele. Cycling conditions were 35 cycles of 94⁰C for 30 sec, 59⁰C for 30 sec, and 72⁰C for 30 sec. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide using Fotodyne Foto/Analyst Dual-Light Luminary Workstation with TotalLab software (Fotodyne Inc., New Berlin, WI).

Organ Harvesting

Mice were rapidly killed by decapitation and livers, kidneys, and hearts were dissected in a weigh boat chilled on ice, weighed, and immediately snap-frozen in a 70% ethanol/dry ice bath and stored at -80⁰C until analysis. The effect of genotype and age on organ weights is described in table 4.1.

DNA Damage

DNA damage in the aging mouse brain tissue was assessed using single cell gel electrophoresis, known as the comet assay (Fairbairn *et al.* 1995). 25mg of frozen cortex was thawed and dissociated in 1ml ice-cold PBS. This cell suspension was embedded in a 1% (w/v) solution of low melting point agarose and placed on agarose-coated glass slides (prepared 24 hours in advance). After gelling, the slides were immersed in lysis buffer (2.5M NaCl, 100mM EDTA, 10mM EDTA, 10mM Trizma base, pH 10) for 1 hour. Following 3 washes of ice-cold dH₂O, slides were immersed in electrophoresis buffer (300mM NaOH, 1mM EDTA) for 25 minutes and subject to electrophoresis at 25mV (400mA) for 25 minutes. Slides were dried overnight. Slides were visualized by staining with 20mM Ethidium Bromide for 30 minutes and images were acquired using fluorescence microscopy and MetaMorph software. Cells were scored using

TriTek CometScore™ (TriTek, Sumerduck, VA) software and results reported as %DNA in tail, normalized to young *bax*^{+/+}/*sod2*^{+/+} animals.

Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS) assay:

Oxidative stress can result in the formation of highly reactive and unstable lipid peroxides that decompose into more stable products such as malondialdehyde (MDA). MDA can be quantified using the TBARS assay, based on the reaction of MDA with thiobarbituric acid (TBA).

Although there has been much controversy in the literature regarding the specificity of TBARS, it remains the most widely employed assay in the determination of lipid peroxidation (Armstrong and Browne 1994). Frozen tissues were homogenized in 10% (w/v) in ice-cold PBS, sonicated for 30s, and centrifuged at 800xg for 10 minutes at 4°C. The supernatant was used for protein determination and TBARS assay. For TBARS, supernatant was added to a 15% (v/v) solution of trichloroacetic acid (TCA) at a ratio of 2:1 and left on ice for 15 minutes followed by centrifugation. The supernatant was added to a 0.67% (w/v) solution of TBA at a 1:1 ratio, vortexed and heated in a boiling water bath for 10 minutes. After cooling, a 150µl aliquot was used to measure the absorbance at 532nm using a Molecular Devices SpectraMax M2 spectrophotometer. Each sample was performed in triplicate. The concentration of lipid peroxides was expressed as nmols TBARS per mg protein, calculated by a malondialdehyde standard curve, prepared from 0-50µM of tetraethoxypropane. Protein concentration was determined by the Bradford Assay. Data was normalized to young *bax*^{+/+}/*sod2*^{+/+} animals.

Protein determination

Protein concentration was determined by the Bradford Assay using the Pierce Coomassie Plus Bradford Assay (Thermo Fisher Scientific, Rockford, IL.).

Data Presentation and Statistical Analysis

Unless otherwise indicated, data is presented as fold change versus control. Fold change was calculated as (Experimental / Control) – 1. Statistical analysis and graph design were done using Sigma Plot 11.0 (Systat Software, Inc., San Jose, CA). Appropriate statistical measures were determined for each experiment based on analysis of data distribution. Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparisons post-*hoc* test was used unless otherwise indicated. Error bars are \pm SEM.

Results

Organ Weights

Organ weights were analyzed for the mice used in this study, consisting of nine separate groups of animals with differing ages and genetic combinations. The ages were chosen based on physiological relevance to the human lifespan and were 5 month old “young” animals, 14 month “middle-aged” animals and 22 month “old” animals (Hamilton et al. 2001). The genotypes studied were wild-type ($bax^{+/+}/sod2^{+/+}$), those under chronic oxidative stress ($bax^{+/+}/sod2^{+/-}$), and those under chronic oxidative stress but lacking Bax ($bax^{-}/sod2^{+/-}$). There were few alterations in the organ weights between the groups of mice (table 4.1). There was an increase in the heart weight of $bax^{+/+}/sod2^{+/-}$ mice at old age. Kidney weights of wild-type mice also increased at middle and old ages and increased at middle age in $bax^{-}/sod2^{+/-}$ mice. Liver weights of $bax^{+/+}/sod2^{+/-}$ mice increased at old age as well.

Bax deletion reduced oxidative damage to lipids in aging mouse organs

The thiobarbituric acid reactive substances (TBARS) assay was utilized in order to study lipid peroxidation in the aging brain. The same groups of mice were used as in the DNA damage study. There were few significant changes to TBARS levels in any of the organs studied. However, there were trends towards an increase in lipid peroxidation with higher levels of endogenous oxidative stress and a trend toward the decrease of this damage with Bax deletion (Figure 4.1).

Bax deletion reduced oxidative damage to DNA in aging mouse organs

The free radical theory of aging posits that the accumulation of oxidative damage over time is a causal factor in aging. Single-cell gel electrophoresis, known as the Comet assay, is widely used in the determination of DNA damage (Piperakis *et al.* 1999, Collins 2004). We compared the amount of DNA in the comet tails of nine separate groups of animals; including three different ages and three different genetic combinations. The ages were chosen based on physiological relevance to the human lifespan and were 5 month old “young” animals, 14 month “middle-aged” animals and 22 month “old” animals (Hamilton *et al.* 2001).

Representative comet images are shown in Figure 4.4. There was no significant difference in DNA strand breaks with age in wild-type animals in any of the organs in this study (Figure 4.3). In livers and hearts, there were increases in damage in Sod2 heterozygous animals with wild-type Bax at middle and old ages, which was prevented by the deletion of Bax. In the kidney, there was an increase in damage in Sod2 heterozygous animals with wt Bax at all ages studied, which was prevented by the deletion of Bax.

Discussion

The goal of the current study was to evaluate whether the beneficial effect of Bax deletion on oxidative stress is a global phenomenon or is specific to the aging mouse brain. Oxidative stress plays an important role in aging. The free radical theory of aging was first introduced by Harmon in 1956 and proposes that aging occurs due to the accumulation of free radical-produced oxidative damage. However, this theory has been challenged by the observations no decreased survival in mice lacking specific endogenous antioxidants (Perez et al. 2009). Also, heterozygous Sod2 animals show higher levels of oxidative damage, particularly to DNA, over time, but this is not enough to shorten lifespan. It is suggested by the literature, however, that while RS and oxidative stress may not be the sole cause of aging, they play a major role in the process as well as in age-related diseases (Finkel & Holbrook 2000, Wallace 1999, Simonian & Coyle 1996). The heterozygous Sod2 mouse is a model of chronic oxidative stress, in that tissues have reduced levels of this major endogenous antioxidant. Sod2 heterozygous mice are associated with increases in damage to DNA and lipids. Age-related protein carbonyls and F₂ isoprostanes have been shown to not be affected in this mouse model (Mansouri et al., 2006). Removal of the pro-apoptotic protein Bax led to decreased production of oxidative damage to DNA and lipids to the mouse cerebral cortex *in vivo* (Chapter 3). In addition, a study highlighted the ability of Bax deletion to alleviate age-related complications such as cataract formation and skin wrinkling (Perez et al. 2007). This suggests that the deletion of Bax would have effects on several organs of the mouse. The current study evaluated damage to DNA and lipids in wild-type mice and those under chronic oxidative stress as well as determining whether or not genetic deletion of Bax would aid in reducing damage in livers, hearts, and kidneys. These organs have several differences to the mouse brain. First, the cellular turnover rate is higher in these organs,

which may lead to lower overall accumulation of oxidative damage. Second, these organs all possess full-length Bak, another pro-apoptotic protein with a suggested redundant function to Bax. The presence of Bak may counteract the beneficial effect of Bax deletion. This was not the case, however. Damage to DNA in all of the tissues examined showed similar results to the damage in the aging brain. There were increases to damage caused by endogenous oxidative stress and these increases were eliminated by the genetic deletion of Bax. While lipid peroxidation did not show significant effects with age or genotype, a similar trend was observed. This suggests that Bax, in part, does aid in the alleviation of damage caused by endogenous stress and that this effect is widespread throughout the body.

Table 4.1: Organ Weights

Livers, hearts and kidneys were weighed following mouse dissection. Between 3 and 19 mice were used in each group. Each organ weight was normalized to the total body weight of each animal. No significant differences were seen in the liver weights with respect to either age or genotype.

Liver Weights

Age	<i>bax</i> ^{+/+} / <i>sod2</i> ^{+/+}	<i>bax</i> ^{+/+} / <i>sod2</i> ^{+/-}	<i>bax</i> ^{-/-} / <i>sod2</i> ^{+/-}
Young	0.039 ± 0.003	0.033 ± 0.006	0.034 ± 0.004
Middle-age	0.046 ± 0.007	0.029 ± 0.003	0.034 ± 0.004
Old	0.046 ± 0.006	0.055 ± 0.006**	0.029 ± 0.004

Statistical significance with age: **vs middle-age

Heart Weights

Age	<i>bax</i> ^{+/+} / <i>sod2</i> ^{+/+}	<i>bax</i> ^{+/+} / <i>sod2</i> ^{+/-}	<i>bax</i> ^{-/-} / <i>sod2</i> ^{+/-}
Young	0.0068 ± 0.0003	0.0056 ± 0.0002	0.0065 ± 0.0008
Middle-age	0.0066 ± 0.0006	0.0062 ± 0.0007	0.0065 ± 0.0003
Old	0.0091 ± 0.0008	0.0086 ± 0.0004**	0.0056 ± 0.0002

Statistical significance with age: **vs middle-age

Kidney Weights

Age	<i>bax</i> ^{+/+} / <i>sod2</i> ^{+/+}	<i>bax</i> ^{+/+} / <i>sod2</i> ^{+/-}	<i>bax</i> ^{-/-} / <i>sod2</i> ^{+/-}
Young	0.0062 ± 0.0002	0.0074 ± 0.0009	0.0070 ± 0.0002 [†]
Middle-age	0.0085 ± 0.0007*	0.0088 ± 0.0010	0.0124 ± 0.0042*
Old	0.0099 ± 0.005*	0.0094 ± 0.0006*	0.0080 ± 0.0003

Statistical significance with age: *vs young

Statistical significance with genotype: [†]vs *bax*^{+/+}/*sod2*^{+/+}

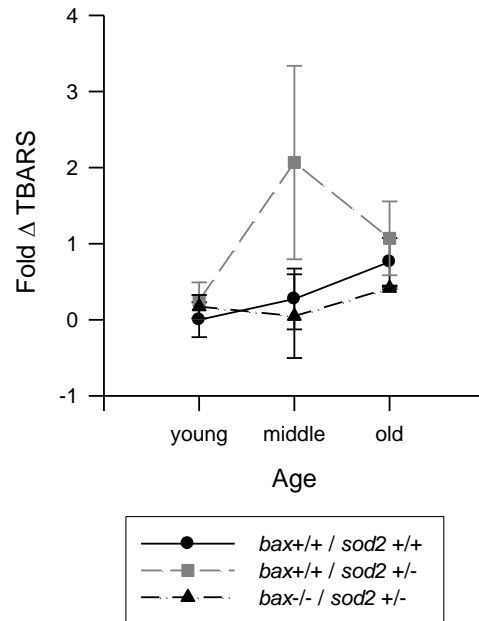


Figure 4.1: Lipid peroxidation in the aging liver

TBARS assay was performed on young, middle-age, and old mice representing *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-} and *bax*^{-/-}/*sod2*^{+/-} genotypes. Results are presented as the mean ± SEM for 3 separate experiments. Data is calculated as fold change relative to wild-type young animals, *TBARS* levels were calculated as μM MDA/mg protein.

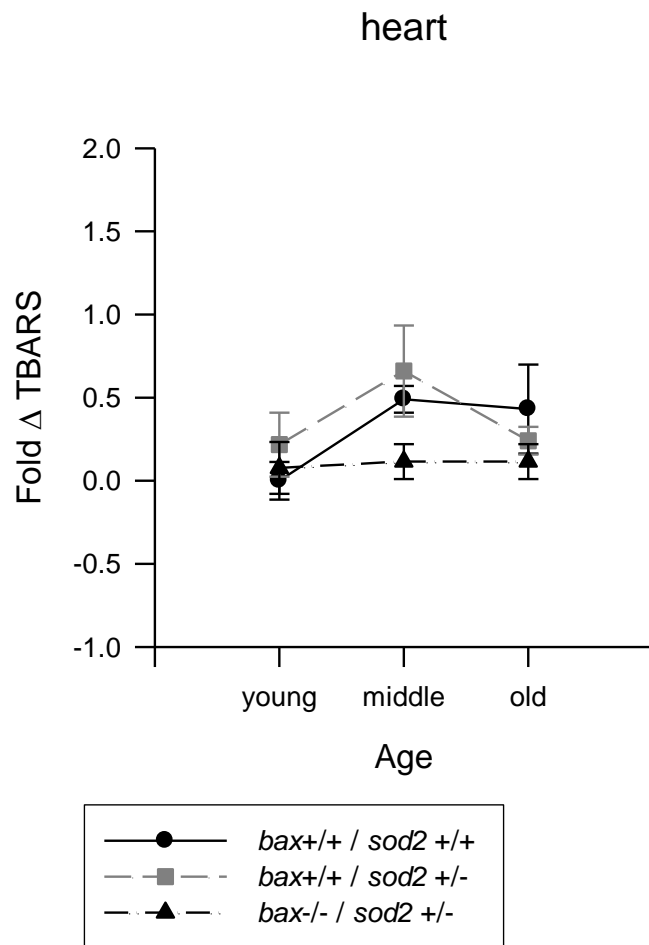


Figure 4.2: Lipid peroxidation in the aging heart

TBARS assay was performed on young, middle-age, and old mice representing *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-} and *bax*^{-/-}/*sod2*^{+/-} genotypes. There were no significant changes in *TBARS* levels with respect to either age or genotype, although the deletion of *Bax* showed lower levels of damage than either wild-type or chronic oxidative stress animals. Results are presented as the mean ± SEM for 3 separate experiments. Data is calculated as fold change relative to wild-type young animals, *TBARS* levels were calculated as μM MDA/mg protein.

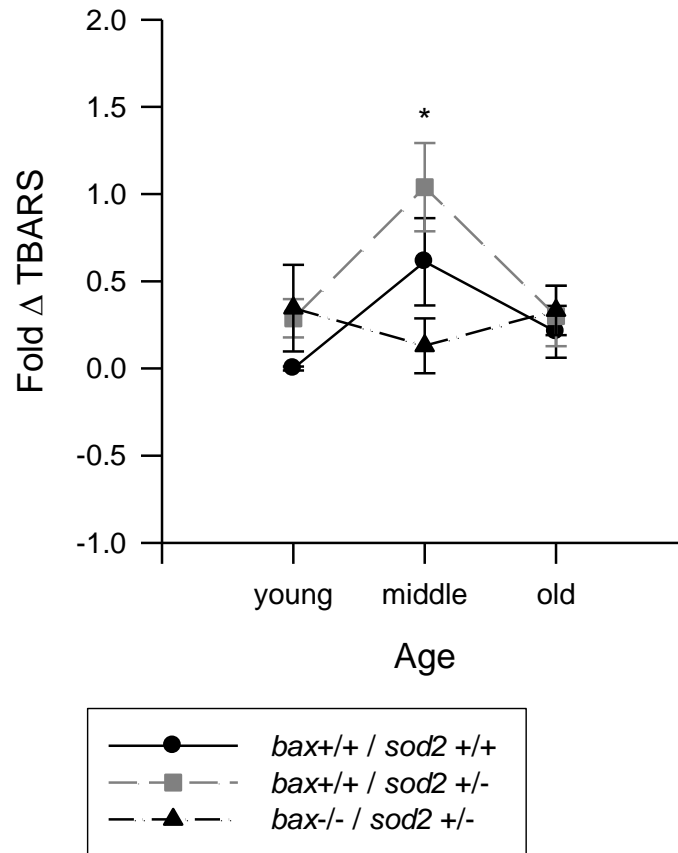


Figure 4.3: Lipid peroxidation in the aging kidney

TBARS assay was performed on young, middle-age, and old mice representing *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-} and *bax*^{-/-}/*sod2*^{+/-} genotypes. * represents statistically significant difference as compared to middle-aged *bax*^{-/-}/*sod2*^{+/-} animals. There were no significant changes in *TBARS* levels with respect to either age or genotype, although the deletion of *Bax* showed lower levels of damage than either wild-type or chronic oxidative stress animals at middle age. Results are presented as the mean \pm SEM for 3 separate experiments. Data is calculated as fold change relative to wild-type young animals, *TBARS* levels were calculated as μ M MDA/mg protein.

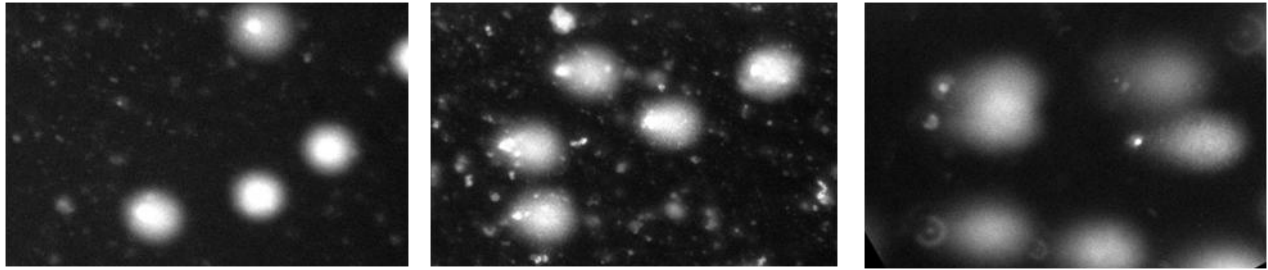


Figure 4.4: DNA damage by comet assay

Representative comets of increasing levels of damage as from left to right. The comet assay is a measure of DNA damage in single cells by quantifying the amount of strand breaks in DNA. Strand breaks cause a loosening of the tight coil of DNA that is present in healthy cells. These breaks can then migrate away from the comet “head” forming a “tail” by applying an electric field. The negatively charged DNA migrates toward the anode of the electrophoresis chamber. Undamaged DNA strands are too large to migrate and stay in the comet “head”. DNA was quantified using ethidium bromide staining and evaluation using CometScoreTM software.

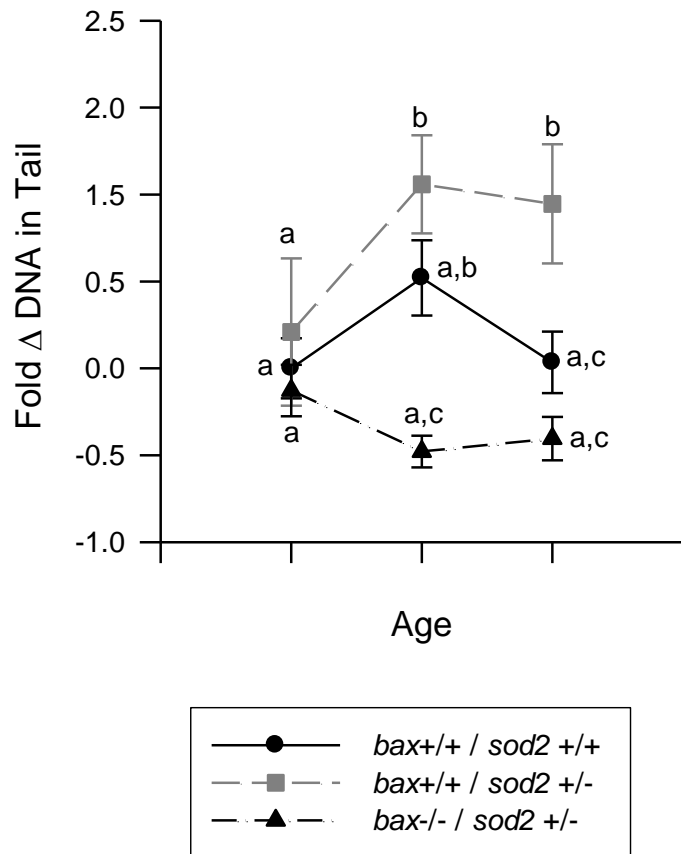


Figure 4.5: DNA damage induced by chronic oxidative stress is prevented by Bax deletion in the aging liver

Comet assay was performed on young, middle-age, and old mice representing *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-} and *bax*^{-/-}/*sod2*^{+/-} genotypes. There was no significant difference in DNA strand breaks with age in wild-type animals. There was an increase in damage in *Sod2* heterozygous animals with wt Bax at middle and old ages, which was prevented by the deletion of Bax. The results are presented as the mean ± SEM for 3 separate experiments. Different letters represent statistically significant difference ($p < .05$). Data is calculated as fold change relative to young wt animals based on percent DNA in tail.

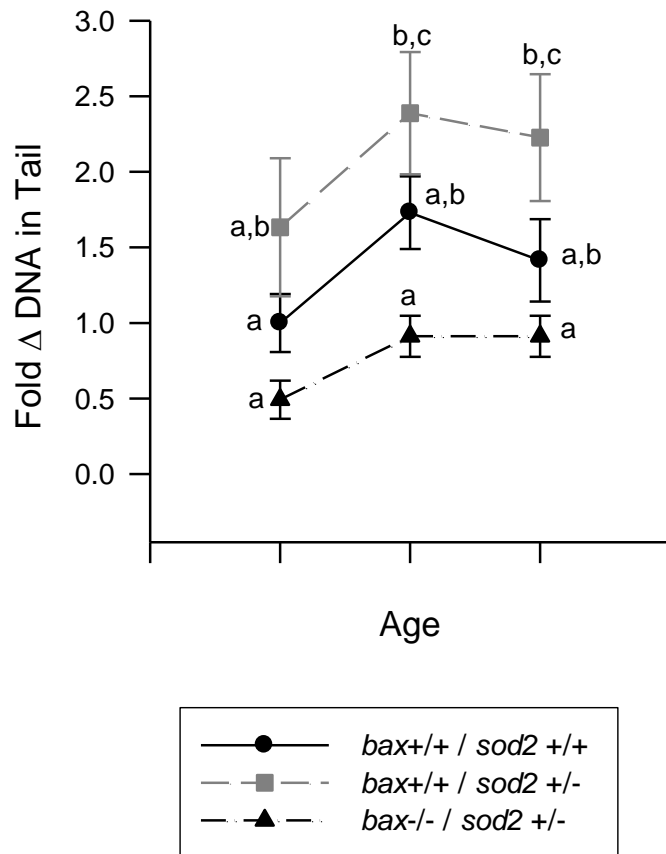


Figure 4.6: DNA damage induced by chronic oxidative stress is prevented by Bax deletion in the aging heart

Comet assay was performed on young, middle-age, and old mice representing *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-} and *bax*^{-/-}/*sod2*^{+/-} genotypes. There was no significant difference in DNA strand breaks with age in wild-type animals. There was an increase in damage in *Sod2* heterozygous animals with wt Bax at middle and old ages, which was prevented by the deletion of Bax. The results are presented as the mean ± SEM for 3 separate experiments. Different letters represent statistically significant difference (p < .05). Data is calculated as fold change relative to young wt animals based on percent DNA in tail.

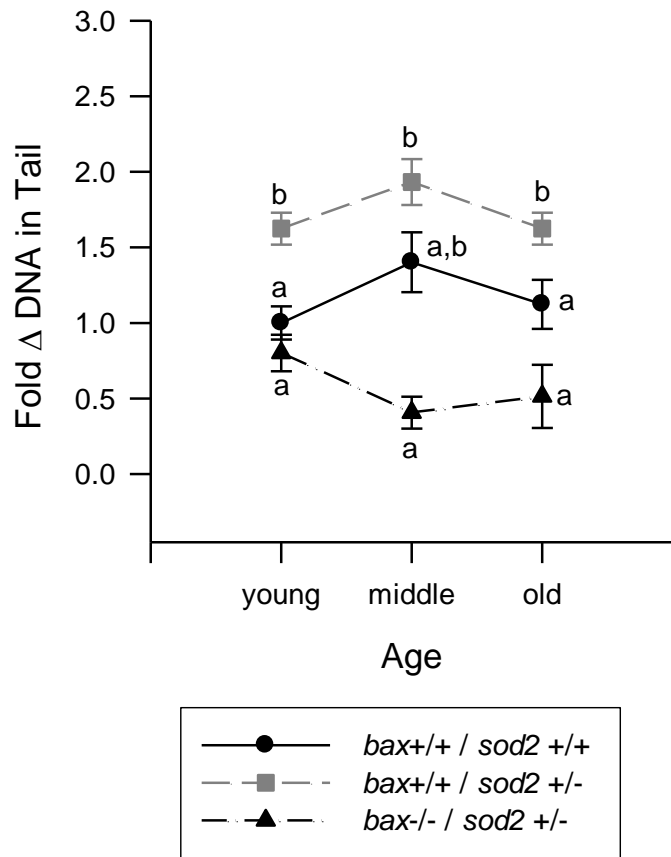


Figure 4.7: DNA damage induced by chronic oxidative stress is prevented by Bax deletion in the aging kidney

Comet assay was performed on young, middle-age, and old mice representing *bax*^{+/+}/*sod2*^{+/+}, *bax*^{+/+}/*sod2*^{+/-} and *bax*^{-/-}/*sod2*^{+/-} genotypes. There was no significant difference in DNA strand breaks with age in wild-type animals. There was an increase in damage in *Sod2* heterozygous animals with wt Bax at all ages studied, which was prevented by the deletion of Bax. The results are presented as the mean ± SEM for 3 separate experiments. Different letters represent statistically significant difference (p < .05). Data is calculated as fold change relative to young wt animals based on percent DNA in tail.

References

- Cand, F. and Verdeti, J. (1989) Superoxide dismutase, glutathione peroxidase, catalase, and lipid peroxidation in the major organs of the aging rats. *Free Radic. Biol. Med.*, **7**, 59-63.
- Collins, A. (2004) The comet assay for DNA damage and repair. *Mol. Biotechnol.*, **26**, 249-261.
- Fairbairn, D. W., Olive, P. L. and O'Neill, K. L. (1995) The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology*, **339**, 37-59.
- Gerard, F. (1997) Liver apoptosis. *J. Hepatol.*, **26**, 1-11.
- Hamilton, M. L., Van Remmen, H., Drake, J. A., Yang, H., Guo, Z. M., Kewitt, K., Walter, C. A. and Richardson, A. (2001) Does oxidative damage to DNA increase with age? *Proc. Natl. Acad. Sci. U. S. A.*, **98**, 10469-10474.
- Higami, Y., Shimokawa, I., Okimoto, T., Tomita, M., Yuo, T. and Ikeda, T. (1997) Effect of aging and dietary restriction on hepatocyte proliferation and death in male F344 rats. *Cell Tissue Res.*, **288**, 69-77.
- Hou, Q. and Hsu, Y.-T. (2005) Bax translocates from cytosol to mitochondria in cardiac cells during apoptosis: development of a GFP-Bax-stable H9c2 cell line for apoptosis analysis. *Am. J. Physiol. Heart Circ. Physiol.*, **289**, H477-487.
- Ing, D. J., Zang, J., Dzau, V. J., Webster, K. A. and Bishopric, N. H. (1999) Modulation of Cytokine-Induced Cardiac Myocyte Apoptosis by Nitric Oxide, Bak, and Bcl-x. *Circ. Res.*, **84**, 21-33.
- Jang, Y. C. and Remmen, H. V. (2009) The mitochondrial theory of aging: Insight from transgenic and knockout mouse models. *Exp. Gerontol.*, **44**, 256-260.

- Kirkland, R. A., Windelborn, J. A., Kasprzak, J. M. and Franklin, J. L. (2002) A Bax-Induced Pro-Oxidant State Is Critical for Cytochrome *c* Release during Programmed Neuronal Death. *J. Neurosci.*, **22**, 6480-6490.
- Knudson, C. M., Tung, K. S. K., Tourtellotte, W. G., Brown, G. A. J. and Korsmeyer, S. J. (1995) Bax-Deficient Mice with Lymphoid Hyperplasia and Male Germ Cell Death. *Science*, **270**, 96-99.
- Krajewski, S., Krajewska, M. and Reed, J. C. (1996) Immunohistochemical Analysis of in Vivo Patterns of Bak Expression, a Proapoptotic Member of the Bcl-2 Protein Family. *Cancer Res.*, **56**, 2849-2855.
- Miró, Ò., Casademont, J., Casals, E., Perea, M., Urbano-Márquez, Á., Rustin, P. and Cardellach, F. (2000) Aging is associated with increased lipid peroxidation in human hearts, but not with mitochondrial respiratory chain enzyme defects. *Cardiovasc. Res.*, **47**, 624-631.
- Navarro, A. and Boveris, A. (2004) Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **287**, R1244-1249.
- Obonai, T., Mizuguchi, M. and Takashima, S. (1998) Developmental and aging changes of Bak expression in the human brain. *Brain Res.*, **783**, 167-170.
- Perez VI, Bokov A, Remmen HV, Mele J, Ran Q, Ikeno Y, Richardson A (2009) Is the oxidative stress theory of aging dead? *Biochim Biophys Acta*.
- Piperakis, S. M., Visvardis, E.-E., Tassiou, A. M. and Lester, P. (1999) Comet assay for nuclear DNA damage. In: *Methods Enzymol.*, Vol. Volume 300, pp. 184-194. Academic Press.
- Pothana, S. and Manjeri, A. V. (2003) Role of apoptosis in hypoxic/ischemic damage in the kidney. *Semin. Nephrol.*, **23**, 511-521.

- Shimohama, S., Tanino, H. and Fujimoto, S. (2001) Differential Expression of Rat Brain Caspase Family Proteins during Development and Aging. *Biochem. Biophys. Res. Commun.*, **289**, 1063-1066.
- Tiana, L., Caib, Q. and Wei, H. (1998) Alterations of Antioxidant Enzymes and Oxidative Damage to Macromolecules in Different Organs of Rats During Aging. *Free Radic. Biol. Med.*, **24**, 1477-1484.
- Uo, T., Kinoshita, Y. and Morrison, R. S. (2005) Neurons Exclusively Express N-Bak, a BH3 Domain-only Bak Isoform That Promotes Neuronal Apoptosis. *J. Biol. Chem.*, **280**, 9065-9073.
- Uysal, M., Seckin, S., Kocak-Toker, N. and Öz, H. (1989) Increased hepatic lipid peroxidation in aged mice. *Mech. Ageing Dev.*, **48**, 85-89.
- Van Remmen, H., Ikeno, Y., Hamilton, M. et al. (2003) Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol. Genomics*, **16**, 29-37.
- Van Remmen, H., Salvador, C., Yang, H., Huang, T. T., Epstein, C. J. and Richardson, A. (1999) Characterization of the Antioxidant Status of the Heterozygous Manganese Superoxide Dismutase Knockout Mouse. *Arch. Biochem. Biophys.*, **363**, 91-97.
- Yoon, J. H. and Gores, G. J. (2002) Death receptor-mediated apoptosis and the liver. *J. Hepatol.*, **37**, 400-410.
- Zhan, H., Sun, C.-P., Liu, C.-G. and Zhou, J.-H. (1992) Age-related change of free radical generation in liver and sex glands of rats. *Mech. Ageing Dev.*, **62**, 111-116.

Chapter 5

Conclusions

The principle goal of this research was to evaluate the effect of the pro-apoptotic protein, Bax, on chronic oxidative stress. This was accomplished by measuring RS production in cortical neuron cell culture and also and by assessing markers of oxidative damage in the aging mouse cortex.

Bax and RS in both apoptotic and non-apoptotic neurons

In cortical neuron cell culture, there was a burst in RS associated with the induction of apoptosis with staurosporine treatment. This burst in RS was inhibited by the genetic reduction and deletion of Bax, suggesting that Bax plays a key role in this process. This is in line with studies of RS in the apoptotic induction of other neuronal populations, including sympathetic neurons (isolated from the superior cervical ganglia; SCG) deprived of nerve growth factor and serum-deprived, repolarized cerebellar granule neurons (CG). One major difference between cortical neurons and these other neuronal cultures was the type of RS evaluated, however. In all of these studies, the redox-sensitive dye, CM-H₂DCFDA, was utilized that detects both ROS and RNS. In cortical neurons, the species detected was associated with [•]NO and most likely was [•]OONO. In both SCG and CG neurons, the species detected was not [•]NO-associated but instead was a ROS downstream of O₂^{•-}.

In non-apoptotic neurons, the difference between cortical neurons and both SCG and CG neurons is more apparent. RS are continually being generated as a natural byproduct of mitochondrial respiration. This basal-level RS also appears to be regulated by Bax, suggesting that this protein has a larger role in cellular function than just in apoptosis induction. In both SCG and CG neurons, both the genetic reduction and deletion of Bax lead to a reduction in basal-level RS. In cortical neurons, however, there is a reduction of RS observed with the

deletion of Bax, but not Bax reduction. The genetic reduction of Bax caused an unexpected increase in RS production. One plausible explanation is a possible involvement of n-Bak in cortical neurons. Removal of Bax is known to cause a compensatory increase in Bak (Gavaldà *et al.* 2008). Since Bak in post-natal cortical neurons is a BH3 only version, it may cause Bax to associate with the mitochondrial OMM and increase levels of RS. N-bak promotes Bax-dependent apoptosis in cortical neurons, but not SCG neurons (Uo *et al.* 2005, Sun *et al.* 2001). This could account for the disparity.

Bax and RS in chronic oxidative stress

Oxidative stress is associated with many disease processes. It is therefore of great importance to study proteins involved in chronic oxidative stress paradigms. Sod2 is a major endogenous antioxidant and its reduction leads to an increase in RS. In cortical neurons, the reduction of Sod2 led to a dose-dependent increase in RS in non-apoptotic neurons. These RS, similarly to staurosporine treatment, were likely \cdot OONO species. In apoptotic cortical neurons, the increase in RS was enhanced over the RS produced during staurosporine treatment. The genetic reduction and deletion of Bax counteracted this increase in RS in both apoptotic and non-apoptotic cortical neurons, suggesting that Bax is involved in RS production under chronic oxidative stress conditions. Bax also provided protection of the apoptotic death of staurosporine-treated neurons lacking Sod2, adding to its function in both apoptotic and non-apoptotic capacities.

Bax and oxidative damage in the aging mouse brain

Bax deletion lowered RS in non-apoptotic cortical neurons and in neurons under chronic oxidative stress. This, in conjunction with the role of Bax in these processes in other neuronal populations, prompted the hypothesis that Bax deletion may aid in lowering oxidative damage

caused by endogenous oxidative stress in the aging brain. In order to evaluate this hypothesis, markers of oxidative damage in three sets of mice were compared: wild-type mice ($bax^{+/+}/sod2^{+/+}$), mice with reduced levels of Sod2 (Sod2 het, $bax^{+/+}/sod2^{+/-}$), and Sod2 het mice with genetically deleted Bax ($bax^{-/-}/sod2^{+/-}$). It was discovered that chronic oxidative stress led to an increase in DNA damage at young and middle ages and an increase in lipid peroxidation at middle age. These increases in oxidative stress markers were eliminated by the deletion of Bax. In addition, Bax deletion generally reduced oxidative damage to levels lower than even wild-type mice. This suggests that Bax plays a very important role in this process and that Bax deletion in wild-type neurons may decrease oxidative damage in the aging brain even further. If so, it would mean a far broader role for Bax in the aging brain.

Better without Bax? (Adler 2007)

In the current study, it was established that Bax deletion had a beneficial effect of reducing oxidative damage in the aging brain. In neurons, Bax deletion alone is sufficient to both suppress apoptosis and to inhibit the formation of RS and is largely independent of the other pro-apoptotic protein, Bak. This is most likely due to the discovery that fully-functional Bak is absent in neurons. Other cell types, however, do not possess this BH3 only version of Bak and instead have the full-length, functioning version. Bax and Bak both are widely distributed throughout the body and Bax deletion also prompts the up-regulation of Bak (Krajewski *et al.* 1994). These facts suggest that Bax deletion may not have as large a role in the regulation of RS and oxidative damage in other tissues as it does in the brain. In 2007, however, an article was published that described the benefit of Bax deletion on ovarian function with the added effect of attenuating age-related complications in female mice (Perez *et al.* 2007). Several of the age-related biomarkers used in this study have oxidative stress components suggested in their

pathologies, including cataract formation and skin wrinkling (Nachbar & Korting 1995, Chiba *et al.* 1999, Spector 1995). These conclusions, along with the wide tissue distribution of Bax throughout the body prompted the hypothesis that Bax deletion may promote the reduction of oxidative stress markers in organs other than the brain. This is precisely what was discovered in the heart, liver, and kidneys of the aging mouse. While the results were not as statistically significant as in the brain, all 3 organs showed a beneficial effect of Bax deletion on both DNA damage and lipid peroxidation. This suggests that the deletion of Bax has a more global effect on the regulation of oxidative damage. The hypothesis proposed in this research project was that the deletion of the pro-apoptotic protein Bax would inhibit RS in cortical neurons and thus lead to an overall decrease in oxidative damage in animals under chronic oxidative stress. What was observed was that Bax deletion did in fact reduce RS levels in cortical neurons and that markers of oxidative stress were reduced in multiple organs (figure 5.1).

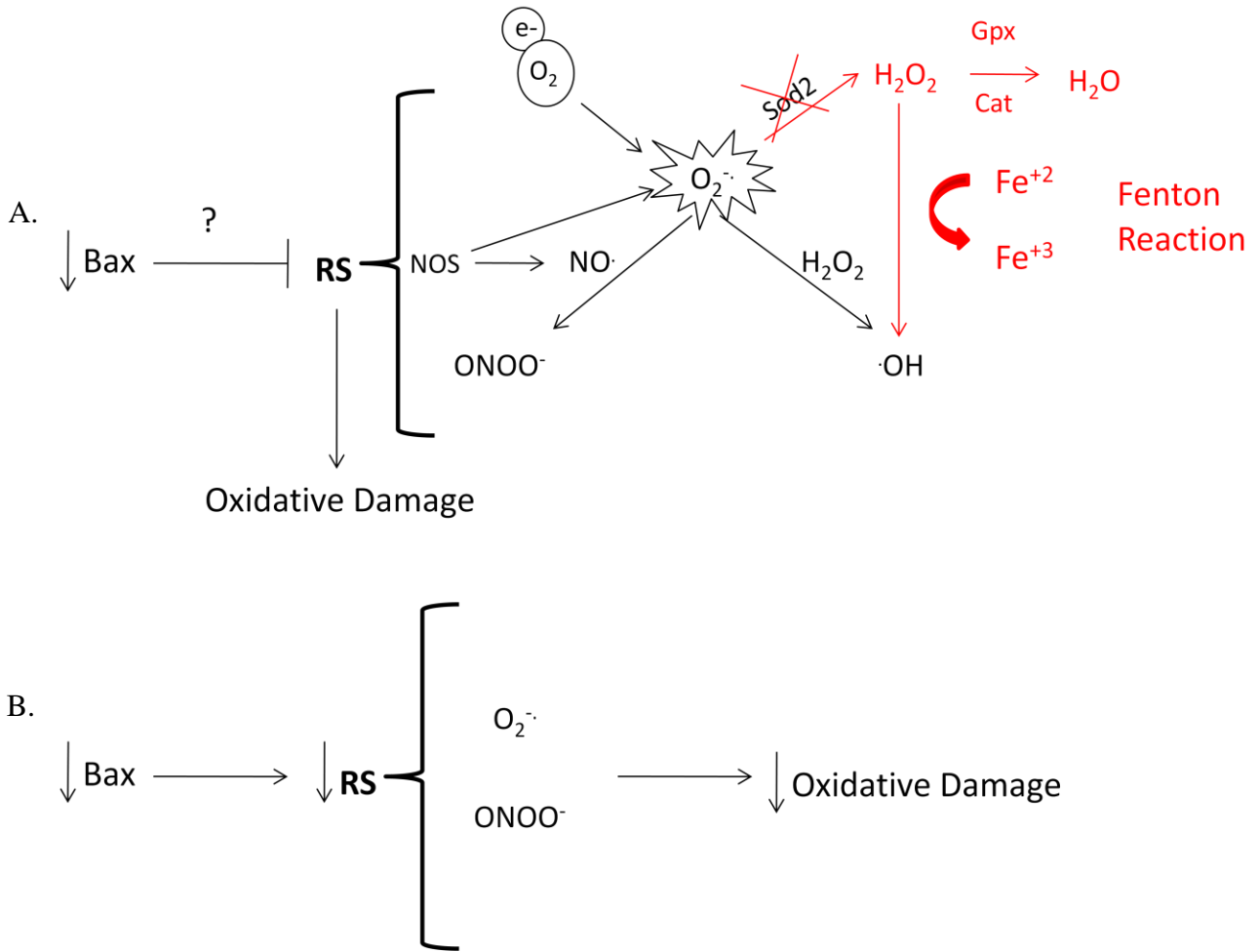


Figure 5.1: Conclusions

This project sought to investigate whether or not the genetic reduction of Bax would lead to a decrease in RS production in cortical neurons and whether this would have any effect on oxidative damage in aging animals under chronic oxidative stress (A). Conclusions drawn from experimental data suggest that reducing levels of Bax does in fact lower RS levels in cortical neurons and that there is a correlative decrease in oxidative damage seen in animals under chronic oxidative stress(B).

References

- Adler, E. M. (2007) Better Without Bax? *Sci. STKE*, **379**, tw98.
- Chiba, K., Sone, T., Kawakami, K. and Onoue, M. (1999) Skin roughness and wrinkle formation induced by repeated application of squalenemonohydroperoxide to the hairless mouse. *Exp. Dermatol.*, **8**, 471-479.
- Gavaldà, N., Pérez-Navarro, E., García-Martínez, J. M., Marco, S., Benito, A. and Alberch, J. (2008) Bax deficiency promotes an up-regulation of BimEL and Bak during striatal and cortical postnatal development, and after excitotoxic injury. *Molecular and Cellular Neuroscience*, **37**, 663-672.
- Krajewski, S., Krajewska, M., Shabaik, A., Miyashita, T., Wang, H. and Reed, J. (1994) Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. *Am. J. Pathol.*, **145**, 1323-1336.
- Kregel, K. C. and Zhang, H. J. (2007) An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **292**, R18-36.
- Nachbar, F. and Korting, H. C. (1995) The role of vitamin E in normal and damaged skin. *J. Mol. Med.*, **73**, 7-17.
- Perez, G. I., Jurisicova, A., Wise, L. et al. (2007) Absence of the proapoptotic Bax protein extends fertility and alleviates age-related health complications in female mice. *Proceedings of the National Academy of Sciences*, **104**, 5229-5234.
- Spector, A. (1995) Oxidative stress-induced cataract: mechanism of action. *FASEB J.*, **9**, 1173-1182.

Sun, Y.-F., Yu, L.-Y., Saarma, M., Timmusk, T. and Arumae, U. (2001) Neuron-specific Bcl-2 Homology 3 Domain-only Splice Variant of Bak Is Anti-apoptotic in Neurons, but Pro-apoptotic in Non-neuronal Cells. *J. Biol. Chem.*, **276**, 16240-16247.

Uo, T., Kinoshita, Y. and Morrison, R. S. (2005) Neurons Exclusively Express N-Bak, a BH3 Domain-only Bak Isoform That Promotes Neuronal Apoptosis. *J. Biol. Chem.*, **280**, 9065-9073.