THE ROLE OF DIETARY ZINC AND CuZnSOD GENE EXPRESSION IN RESPONSE TO OXIDATIVE STRESS IN THE LUNG AND BRAIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

The Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

Mark A. Levy, M.Sc.

*****

The Ohio State University

2003

Dissertation Committee:

Dr. Tammy Bray, Advisor

Dr. Ning Quan

Dr. Jean T. Snook

Approved by

________________________________

Advisor

Ohio State University Nutrition Program
ABSTRACT

The balance between the antioxidant defenses and the rate of production of reactive oxygen species (ROS), or oxidative stress, is believed to be a critical factor that determines the extent of tissue injury in a number of diseases. The antioxidant defenses, which consist of a network of enzymes, proteins and low molecular weight scavengers, function to protect cellular components against the damaging effects of ROS. This dissertation work focuses on the functional role of two components of the antioxidant defense system: the trace element zinc, and the antioxidant enzyme copper-zinc superoxide dismutase (CuZnSOD). This work was initiated based on accumulating evidence indicating that ROS mediated tissue injury may be a common pathogenic mechanism in several diseases associated with premature birth, including bronchopulmonary dysplasia and cerebral palsy, and that low antioxidant status may be a critical determinant of disease progression. We have hypothesized that low zinc nutritional status and reduced CuZnSOD activity increases the potential for ROS mediated tissue damage in lung and brain tissue. Further, we proposed that exposure to oxygen therapy, a common treatment strategy applied to premature infants, would markedly increase oxidative tissue damage in these tissues. To test this hypothesis we used an animal model to examine the effects of dietary zinc deficiency, reduced CuZnSOD expression and exposure to hyperoxia on oxidative tissue damage in lung and brain tissue.
brain tissue. In addition, we examined the stress response of antioxidant metalloproteins that may play a critical role in maintaining homeostasis under oxidative stress, and have also performed a global analysis of gene expression in the brain of zinc deficient animals using cDNA microarray technology.

Results from the first set of experiments demonstrated that exposure to hyperoxia increased oxidative tissue damage in the lung and also induced the expression of ceruloplasmin and metallothionein, suggesting that these metalloproteins may function as antioxidants in addition to their role in trace metal homeostasis. Subsequent work in brain tissue demonstrated that reduced expression of CuZnSOD increased oxidative tissue damage following exposure to hyperoxia, thus demonstrating the deleterious consequences of a compromised antioxidant defense system. And finally, the microarray analysis demonstrated that in the brain there are a number of genes from different cellular pathways whose expression is changed in response to zinc deficiency.
I wish to extend my sincere gratitude to my advisor and teacher, Dr Tammy Bray. She has taught me, encouraged me, inspired me and guided me. More than that, through her seemingly unlimited patience she has provided me ample opportunity to learn from my mistakes and develop an appreciation for the art of science. I would especially like to thank her for entrusting me with tasks and responsibilities away from the laboratory benchtop that has helped me to develop as a person and gain an appreciation of the machinations of science and life that extend far beyond the test-tube.

I would also like to thank my committee members: Dr. Ning Quan for providing judicious insights in precise measures that truly enhanced my scientific journey, and Dr. Jean Snook for her helpful suggestions, openness and knack for listening when I needed to speak, and speaking when I needed to listen.

I am also very grateful for the many friends and colleagues I’ve worked with along the way. Dr. Mike Noseworthy, for his unassuming nature and his mentoring during the early years that initiated my interest in nutrition research. Dr. Emily Ho for her support and friendship both in and away from the lab. I would also like to thank Dr Yu-Hwai Tsai for her insights, attention to detail and her unwavering dedication to everyone she worked with. Dr. Jordan Li, a former lab member, is a friend and colleague.
whose sense of humor, inquisitive nature and insights into all aspects of life were very much appreciated. And Rich Bruno, Dr. Yoony Lim and Haruhiko Ohtsu have been instrumental in traveling through the ups and downs of research. I’d also like to thank others who have passed through the lab for all their assistance and friendship, including Briana Durica, Tom Boileau, Howard Bingham and Luther Chang. And finally, I would be remiss if I did not acknowledge the help and support of the administrative staff, particularly Ethel Hurley and Rita Compston, who have done so much for me over the years.

And last, but certainly not least, I want to thank my mother and father for all that they have provided in the way of opportunity, encouragement, emotional and yes, financial support, without any of which this process would never have taken place. And I certainly cannot forget my brothers and sisters, nieces and nephews, too many to mention here, who have always supported my endeavors and continue to do so.
VITA

February 25, 1967…………….Born Wolfville, Nova Scotia, Canada

1989………………………….. B.Sc Biology, Acadia University

1995……………………………M.Sc Nutrition Science, University of Guelph.

1996-2002……………………..Research Associate, The Ohio State University

PUBLICATIONS


FIELD OF STUDY

Major field: Ohio State University Nutrition Program
| Chapters                                                                                                                                                                                                 \|   |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------- \|---|
| 1.  Introduction                                                                                                                               \|   |
| 1.1. References                                                                     \|18|
| 2.  Cellular response of antioxidant metalloproteins in Cuznsod transgenic mice exposed to hyperoxia.                                                                                                           \|24|
| 2.1. Summary                                                                      \|25|
| 2.2. Introduction                                                                 \|26|
| 2.3. Materials and Methods.                                                         \|29|
| 2.4. Results                                                                       \|36|
| 2.5. Discussion                                                                   \|40|
| 2.6. References                                                                    \|54|
| 3.  The effect of zinc deficiency and exposure to hyperoxia on oxidative stress in the brain.                                                                                                             \|58|
| 3.1. Summary                                                                      \|59|
| 3.2. Introduction                                                                 \|60|
| 3.3. Materials and Methods.                                                         \|62|
| 3.4. Results                                                                       \|68|
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------- \|---|
3.5. Discussion ..............................................................71
3.6. References ............................................................80

4. Expression of heme-oxygenase-1 (ho-1) in the brain of Sod1 transgenic and
knockout mice exposed to hyperoxia ........................................82

4.1. Summary ...............................................................83
4.2. Introduction ...........................................................84
4.3. Materials and Methods .............................................87
4.4. Results .................................................................93
4.5. Discussion ............................................................96
4.6. References ............................................................107

5. Zinc sensitive genes in the mouse brain: results from: a high-density
oligonucleotide microarray analysis ........................................111

5.1. Summary ...............................................................112
5.2. Introduction ...........................................................113
5.3. Materials and Methods .............................................115
5.4. Results .................................................................120
5.5. Discussion ............................................................124
5.6. References ............................................................132

6. Conclusions ............................................................136

List of References ..........................................................139
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>($^1\Delta gO_2$)</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper-zinc superoxide dismutase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OH$^-$</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SOD1</td>
<td>Copper-zinc superoxide dismutase</td>
</tr>
<tr>
<td>SOD2</td>
<td>Manganese superoxide dismutase</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Examples of ROS mediated oxidative stresses</td>
<td>17</td>
</tr>
<tr>
<td>3.1. Effect of dietary zinc deficiency and exposure to hyperoxia on body weight and plasma zinc level</td>
<td>78</td>
</tr>
<tr>
<td>3.2. Effects of zinc deficiency and 2-day or 4-day hyperoxia (85% O2) on the activity of glutamine synthetase and cytosolic or mitochondrial aconitase and reduced thiol levels in brain tissue</td>
<td>79</td>
</tr>
<tr>
<td>4.1. Effects of SOD1 gene expression and hyperoxia (85% O2) on the activity of glutamine synthetase, cytosolic and mitochondrial aconitase in brain tissue</td>
<td>105</td>
</tr>
<tr>
<td>4.2. Activity or expression level of primary antioxidant defense enzymes in brain tissue following exposure to air (21%O2) or hyperoxia (85% O2) for five days</td>
<td>106</td>
</tr>
<tr>
<td>5.1. Zinc status indicators for zinc adequate (ZnAL) and zinc deficient (ZnDF) mice</td>
<td>129</td>
</tr>
<tr>
<td>5.2. List of genes that are down-regulated in brain tissue of zinc-deficient mice</td>
<td>130</td>
</tr>
<tr>
<td>5.3. List of genes that are up-regulated in brain tissue of zinc-deficient mice</td>
<td>131</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

2.1. CuZnSOD protein expression in the lung (A) and liver (B) of Sod1 transgenic (+++), homozygous (+/+), heterozygous (+/-) and knock-out (-/-) mice exposed to air (21% O₂) or hyperoxia (85% O₂) for five days...............47

2.2 GSH-Px and catalase protein level in the lung (A, B) and liver (C, D) of Sod1+++, Sod1+/+, Sod1+- and Sod1- mice exposed to 21% oxygen or 85% oxygen for 5 days........................................................................48

2.3 GSH-Px and catalase activity in the lung (A, B) and liver (C, D) of Sod1+++, Sod1+/+, Sod1+- and Sod1- mice exposed to 21% oxygen or 85% oxygen for 5 days........................................................................49

2.4. Ceruloplasmin protein levels and copper concentration in the lung (A,B) and liver (C,D) of Sod1+++, Sod1+/+, Sod1+- and Sod1- mice exposed to air (21% oxygen) or hyperoxia (85% oxygen) for five days........................................50

2.5. Metallothionein protein levels and zinc concentration in the lung (A,B) and liver (C,D) of Sod1+++, Sod1+/+, Sod1+- and Sod1- mice exposed to air (21% oxygen) or hyperoxia (85% oxygen) for five days........................................51

2.6 Ferritin protein levels and iron concentration in the lung (A,B) and liver (C,D) of Sod1+++, Sod1+/+, Sod1+- and Sod1- mice exposed to air (21% oxygen) or hyperoxia (85% oxygen) for five days........................................52

2.7 Circulating trace mineral levels and serum ceruloplasmin activity in Sod1+++, Sod1+/+, Sod1+- and Sod1- mice exposed to air (21% oxygen) or hyperoxia (85% oxygen) for five days........................................53

3.1. Effect of zinc deficiency and 2-day (A) or 4-day (B) hyperoxia exposure on total SOD activity in the brain........................................73

3.2. CuZnSOD protein levels in brain tissue of rats exposed to air (21% O₂) or hyperoxia (85% O₂) for 2 days (A) or 4 days (B)........................................74
3.3. MnSOD protein levels in brain tissue of rats exposed to air (21% O₂) or hyperoxia (85% O₂) for 2 days (A) or 4 days (B)…………………………………………………………75

3.4. Effect of zinc deficiency and 2-day (A) or 4-day (B) hyperoxia exposure on GSH-Px activity in the brain………………………………………………………………………………76

3.5. Effect of zinc deficiency and 2-day (A) or 4-day (B) hyperoxia exposure on catalase activity in the brain………………………………………………………………………………77

4.1. SOD1 protein levels (A) and total SOD activity (B) in the brain of SOD1 transgenic (SOD1^{+++}), homozygous (SOD1^{++}), heterozygous (SOD1^{+-}) and knockout (SOD1^{-/-}) mice exposed to air (21% O₂) or hyperoxia (85% O₂) for five days………………………………………………………………………………101

4.2. Effects of SOD1 gene expression and hyperoxia (85% O₂) on brain glutathione status……………………………………………………………………………………………………102

4.3. Western blot analysis of HO-1 in the brain of SOD1 transgenic (SOD1^{+++}), homozygous (SOD1^{++}), heterozygous (SOD1^{+-}) and knockout (SOD1^{-/-}) mice exposed to air (21% O₂) or hyperoxia (85% O₂) for five days…………….103

4.4. Correlation between HO-1 protein expression and the GSH disulfide (GSSG/2GSH) redox potential in the brain………………………………………………………………………………………………104

5.1. Range of the average fold change of differentially expressed genes in the brain of ZnAL and ZnDF mice……………………………………………………………………………………………………128
CHAPTER 1

INTRODUCTION

Oxidative stress occurs when the generation of oxidants exceeds the detoxifying capacity of the antioxidant defense system and may be a common pathogenic mechanism of numerous disease states. The term oxidant refers to any of a number of compounds, including free radicals and reactive oxygen species (ROS), which may react with and damage many integral cellular structures. Free radicals, for example superoxide (O$_2^-$) and the hydroxyl radical (OH·), are compounds that contain one or more unpaired electrons and are therefore highly reactive and capable of extracting electrons from neighboring molecules. Such reactions can damage a variety of biological molecules, including enzymes, membranes, DNA and RNA. ROS, a term often used interchangeably with free radical, in fact refers to oxygen-centered free radicals as well as other reactive oxygen compounds such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen ($^1$ΔgO$_2$) which, although not free radicals, are reactive compounds that may participate in oxidative reactions.

Under conditions of oxidative stress, oxidant generation may be increased, antioxidant defenses may be compromised, or both may occur simultaneously. Regardless of the cause, oxidative stress leads to the progressive accumulation of oxidative damage in
tissues, resulting in a sequence of events that may lead to the dysfunction of specific cells, tissues or organs. Not surprisingly, oxidative stress has been associated with chronic degenerative diseases such as atherosclerosis, cancer, neurodegenerative disease and aging (Table 1.1). The appealing aspect of the oxidative stress hypothesis is that it can account for the delayed onset, cumulative damage and progressive nature of chronic disease. However, there is also a growing body of evidence that oxidative stress may play a critical role in diseases of the young, particularly diseases associated with premature birth. Indeed, the greater susceptibility of premature infants to diseases such as retinopathy of prematurity, bronchopulmonary dysplasia and hypoxic-ischemic brain injury may be due to their increased exposure to oxygen free radicals as a result of drug or oxygen therapy, or as a consequence of a weakened antioxidant defense system due to their biochemical immaturity and precarious nutritional state.

Endogenous Oxidative Stress

Organisms that live in an aerobic environment utilize oxygen to oxidize glucose, amino acids and fatty acids to generate biological fuel, ATP. Oxygen is also essential for various other biological processes such as hormone and xenobiotic metabolism. Although necessary to sustain life, these oxidative processes have some disadvantages because they produce ROS as by-products. As a consequence of the ubiquitous nature of oxygen in oxidative processes, oxygen metabolites such as O$_2^\cdot$ and H$_2$O$_2$ are normally produced from metabolic processes. Notably, the most reactive oxygen-centered free radical is the hydroxyl radical (OH$^\cdot$), generated from O$_2^\cdot$ and H$_2$O$_2$ via the Fenton reaction catalyzed by transition metals such as iron (Fe) and copper (Cu) (1). Although
considered to arise primarily during ‘leakage’ from metabolic processes, ROS are also generated in specific metabolic pathways. For example, cells of the immune system (e.g. macrophages, neutrophils) produce substantial quantities of ROS during the respiratory burst as a host defense mechanism against infection (2).

There are many sites of ROS production during normal cellular metabolism. Mitochondrial aerobic respiration is one of the major sources of ROS leakage during the electron transport pathway of oxidative phosphorylation. It has been estimated that as much as 15% of cellular ROS production occurs in the mitochondria, a rate that increases during periods of increased energy metabolism and oxygen consumption under various physiological conditions. The smooth endoplasmic reticulum (SER, isolated as microsomes), site of xenobiotic metabolism (NADPH-dependent-cytochrome P450) and prostaglandin synthesis (cyclooxygenase and lipoxygenase), produces large quantities of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ (3). It has been estimated that in hepatocytes, microsomes generate more than 50% of total cellular ROS during the oxidative metabolism of xenobiotics and the synthesis of prostaglandins and leucotrienes (3). In fact, it appears that $\text{H}_2\text{O}_2$ and lipid peroxides are necessary to maintain prostaglandin synthesis (4).

Another cellular organelle, the peroxisome, has been estimated to produce 30% of cellular ROS during normal metabolism. Peroxisomes contain several ROS generating enzymes including glucose oxidase, amino acid oxidase, xanthine oxidase, glycollate and urate oxidase, as well as flavoprotein oxidases (5). The major by-product of these oxidases is $\text{H}_2\text{O}_2$. There is therefore little wonder that catalase, the enzyme that detoxifies $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$, is found in large quantities in peroxisomes.
In phagocytic cells such as macrophages and neutrophils, the respiratory burst of the immune response is characterized by production of large amounts of microbicidal ROS products such as H$_2$O$_2$, O$_2$•-, •OH, and the strongly oxidizing and highly microbicidal hypohalous acids (e.g. HOCl) by the membrane enzyme myeloperoxidase (6). In addition to ROS production, macrophages also produce nitric oxide (NO•) through inducible nitric oxide synthase (iNOS) as part of the immune response. NO• can be cytotoxic either alone or following reaction with O$_2$•- to form the even more toxic compound peroxynitrite (ONOO•⁻) (7).

**Exogenous Oxidative Stress**

In addition to the cellular generation of ROS as a normal consequence of living in an aerobic environment, oxidative stress can also be imposed exogenously through exposure to compounds or treatment strategies that may elevate ROS and free radical production. As noted previously, free radicals and ROS are derived from the metabolism of xenobiotics – compounds that are foreign to the body such as drugs, pollutants and cigarette smoke. In fact, the toxicity of many xenobiotics is associated with their bioactivation to free radical intermediates, a necessary step leading ultimately to their detoxification and excretion. Hyperoxia exposure, in which the fractional content of inspired oxygen air is elevated above the normal value of 21%, is also an example of an exogenous oxidative stress that leads to increased ROS generation. In particular, premature infants often require treatment with hyperoxia in order to insure adequate oxygen exchange between the atmosphere and the pulmonary blood supply, and evidence
indicates that this treatment strategy leads to increased ROS production, specifically $O_2^-$ to $H_2O_2$, in several tissues including the lung and brain (8).

Hence, there are numerous sources of cellular ROS generation, and each is a normal consequence of living under an oxidative environment. However, ROS generation does not normally pose an overwhelming threat because organisms are endowed with a series of antioxidant enzymes and compounds that function to prevent oxidative tissue damage.

**Antioxidant Defenses**

As outlined above, ROS are produced by the body as both an essential and inescapable aspect of aerobic metabolism. However, antioxidant defenses have evolved which provide cells with the ability to utilize aerobic pathways while minimizing the potential for ROS induced oxidative tissue damage. Antioxidant defenses can be separated into at least three broad categories: antioxidant enzymes, antioxidant proteins, and antioxidant compounds. Each of these components of the defense system is unique, site specific, and complements each other functionally. For example there are five enzymes localized within tissues which function to convert $O_2^-$ to $H_2O$ through an $H_2O_2$ intermediate. Three isoforms of the antioxidant enzyme superoxide dismutase (SOD) convert $O_2^-$ to $H_2O_2$; manganese SOD (MnSOD) is located in the mitochondria and removes $O_2^-$ produced during electron transport; copper-zinc (CuZnSOD) is found in the cytosol and detoxifies $O_2^-$ generated from SER activity, and extracellular SOD (ECSOD) dismutates $O_2^-$ in extracellular fluids such as lymph and plasma. Two $H_2O_2$
metabolizing enzymes, glutathione peroxidase (GSH-Px) in the cytosol and catalase in peroxisomes, compliment SOD activity by reducing H$_2$O$_2$ to H$_2$O.

In addition to antioxidant enzymes, a second form of defense is provided by antioxidant proteins. These proteins generally serve dual roles, i.e. they function as metal transport and storage proteins and as chelators by binding transition metals and inhibiting their free radical generating potential (e.g. transferrin and ferritin for Fe, ceruloplasmin for Cu) (9). Metallothionein, a zinc containing protein, has also been postulated to function as an antioxidant protein (10, 11). Although the mechanism of metallothionein antioxidant activity is still unknown, it is thought to be a function of its sulphydryl group content as cysteine comprises fully 1/3 of the total protein (12).

The third component of the antioxidant defense system includes a broad array of compounds obtained from food, including essential nutrients such as vitamin E and C, zinc and selenium, as well as GSH, a cysteine-containing tripeptide. Some non-nutritive food components also display antioxidant properties, including carotenoids, isoflavonoids, and other seemingly benevolent factors in our food. In fact functional foods, a significant segment of the health food industry, have come into existence primarily on the basis of the antioxidant properties of their non-nutritive components. In general, antioxidant compounds prevent or delay oxidative damage to cellular components through a chain-breaking mechanism. For example, vitamins C and E, carotenoids, isoflavones, phytochemicals and other antioxidant compounds stabilize free radicals by scavenging (13) or quenching (14) ROS and therefore preventing further oxidative reactions with cellular components.
Together, the antioxidant defenses form a network that provides a protective barrier against ROS produced during aerobic metabolism. Hence, the balance between ROS generation and antioxidant defense is the crucial determinant in the development of ROS-mediated tissue damage. Indeed, a thorough understanding of the antagonism between antioxidant defense and oxidative stress has significant clinical relevance, particularly regarding the treatment of ailments and conditions for which oxidative stress is deemed to be a critical component of disease pathogenesis.

**Oxidative Stress In Premature Infants**

Bronchopulmonary dysplasia (BPD) and cerebral palsy are the two most prominent examples of a diverse array of disorders collectively referred to as diseases of prematurity (15). Although many factors may contribute to the development of these diseases, exposure to hyperoxia continues to be a variable closely associated with their incidence. Because of this, it has been hypothesized that oxidative stress may account for many of the complications associated with prematurity. Indeed, increased ROS generation has been documented in both lung and brain tissue in vivo during exposure to hyperoxia (16, 17). Notably, BPD was first described in infants treated with hyperoxia for severe hyaline membrane disease (18). Although oxygen therapy and pulmonary immaturity are strongly associated with BPD, little is known about the cellular basis of the disorder. Indeed, many other risk factors, including pulmonary edema, antioxidant deficiency and patent ductus arteriosis, have also been implicated. However, histopathological similarities between hyperoxic lung injury and BPD has led to the view that oxygen toxicity and oxidative stress may lead to the development of BPD. It has been
hypothesized that oxygen therapy, particularly in the presence of pulmonary immaturity and low antioxidant status, leads to damage of pulmonary epithelia and increased resistance to alveolar oxygen exchange (15).

Cerebral palsy, unlike many other neuro-developmental disorders, is associated with abnormalities of birth, particularly ‘birth asphyxia’ and low birth weight (19). In fact, premature delivery is the single most important antecedent of cerebral palsy, and the increase in survival of very-low birth weight infants may strengthen this relationship in the future. Of the estimated 50,000 live premature births in the U.S. annually, approximately 5-15% of premature infants exhibit cerebral palsy, characterized by spastic motor deficits. Another 25-50% of premature infants exhibit less severe disturbances of cognition and mobility (20). The specific role of ROS in the development of cerebral palsy is still unknown. However, we do know that the brain is susceptible to oxidative stress for several reasons. First, it contains large amounts of polyunsaturated fatty acids (PUFA’s) which are vulnerable to free radical attack because their double bond structure allows for easy removal of hydrogen atoms by ROS such as ·OH (21). Second, the brain does not appear to be well endowed with either enzymatic or non-enzymatic antioxidant defenses. For example, the activity of catalase and GSH-Px is relatively low in the brain compared to other organs such as the liver and lung. Moreover, this may be very problematic in premature infants as it has been demonstrated that they have very marginal or deficient stores of many antioxidant compounds including vitamin E, β-carotene, GSH, ceruloplasmin, selenium and zinc (22, 23). Typically, vitamin E, β-carotene, selenium and zinc accumulate at an appreciable rate only in the last trimester in the human fetus, and hence stores of antioxidant nutrients decline in proportion to the
degree of prematurity (24). Similarly, the activity of antioxidant enzymes such as GSH-Px and catalase has been found to increase markedly throughout gestation and is much lower in preterm infants than in term infants (15). And last, the brain consumes a disproportionate amount of the body’s oxygen supply, accounting for only ~2% of total body weight, but utilizing ~20% of the total inspired O\textsubscript{2} in a resting individuals (25, 26). With such a high metabolic rate, the inevitable leakage of incompletely reduced oxygen, estimated to be 2-3% of total oxygen consumption, potentiates the development of ROS mediated tissue damage. Hence, this combination of factors may indicate that the brain is highly susceptible to ROS mediated damage, higher probably than elsewhere in the body (21, 27). With regard to premature infants, the use of hyperoxia to increase oxygen exchange between the lung and atmosphere vastly increases the potential for oxidative stress, as hyperoxia increases brain levels of ROS (28, 29). Nevertheless, the mechanism by which oxidative stress may induce brain damage in premature infants has not been established.

**Physiological Responses to Oxidative Stress**

As noted previously, ROS, whether produced endogenously as a consequence of normal cell function or derived exogenously from external sources, pose a constant threat to cells living in an aerobic environment as they can lead to damage to DNA, protein, and lipids. It has become increasingly apparent that oxidative stress plays a significant role in the pathogenesis of many diseases. Although cells contain a number of antioxidant defenses to minimize the deleterious effects of ROS, in instances where ROS generation exceeds the cell's antioxidant capacity, cell death or survival depends upon the ability of
cells and tissues to adapt to or resist the stress. Numerous stress response mechanisms have evolved for these purposes and are activated in response to oxidative insults. Describing and understanding these pathways may offer important avenues for therapeutic interventions aimed at limiting oxidative damage and attenuating pathologies associated with oxidative stress.

It has been proposed that the antioxidant enzymes CuZnSOD, MnSOD, GSH-Px and catalase act in a coordinated manner, since the first two enzymes detoxify $O_2^-$ to $H_2O_2$, whereas the latter two catabolize $H_2O_2$ to water. In fact, these enzymes are viewed as complimentary in that CuZn- and MnSOD reduce $O_2^-$ and are inactivated by $H_2O_2$, whereas catalase and GSH-Px catabolize $H_2O_2$ but are inactivated by $O_2^-$ (30). Evidence for a coordinated regulation has been demonstrated in lung tissue following exposure to ozone. Rats exposed to 0.7 ppm ozone for 5 days exhibited increased activity of CuZnSOD, MnSOD, catalase, and glutathione peroxidase compared to air-breathing rats. The greater enzyme activity occurred with a higher concentration of the mRNA for each enzyme, thus indicating that the increased expression was probably mediated at the level of transcription (31). Similarly, exposure of rats to hyperoxia (85% oxygen) exhibited heightened activities of CuZn- and MnSOD in lung tissue (32). Notably, the induction of antioxidant enzyme activity in the lung has been associated with increased tolerance to oxidative stress. For example, rats exposed to sublethal doses of hyperoxia (85% O2) or ozone for 3 – 7 days survive subsequent exposure to 100% oxygen that is lethal to untreated controls (33, 34). This tolerance has been attributed in part to increased antioxidant enzyme activity. Similarly, low dose endotoxin administration confers tolerance to hyperoxia. Nearly 100% survival of endotoxin treated rats was observed
after exposure to 95% O$_2$ for 72hrs, compared to 30% survival in non-treated rats, with significant increases in the activity of MnSOD, GSH-Px and catalase observed in the endotoxin treated rats (35).

There is also evidence that antioxidant enzyme activity responds to oxidative stress in brain tissue. Both catalase and GSH-Px activity have been shown to be increased in brain tissue following exposure to 0.25 and 0.7 ppm ozone for 5 days (36). As well, it has been demonstrated that a small dose LPS treatment can protect mice against ischemia, with the beneficial effect being attributed to a compensatory increase in MnSOD activity by LPS. However, there was no change in the protein content or activity of CuZnSOD or catalase (37-39). Thus, the precise role of the antioxidant enzyme network in response to oxidative stress remains equivocal. For example, it has been reported that the activity of SOD in the whole brain of oxygen-exposed rats was lowered significantly compared with the normal rats, and that there was no significant change in the activity of catalase (40). However, evidence that the activities of SOD and GSH-Px is increased under hyperoxia has also been demonstrated (41). These discrepancies are presumed to be a function of the differences in experimental design, since rats in the former study were exposed to 100% oxygen for 24 hours, compared to a 48 hour exposure in the latter study. Regardless, these observations may in fact illustrate that the global response to oxidative stress extends beyond the regulation of antioxidant enzymes as an endpoint and that oxidative stress elicits a heterogeneous response that may encompass many different families of protective proteins.
Acute Phase Response

Antioxidant enzymes have long been regarded as the primary means by which cells and tissues protect themselves from ROS toxicity. However, a role for acute phase proteins as antioxidants has emerged which indicates that they may play an equally vital role in maintaining cellular function and stability during periods of oxidative stress. Most notable among this family are ferritin, ceruloplasmin and metallothionein. Collectively, these metalloproteins are well known for their critical role in metal homeostasis, storage and transport of the essential trace metals copper, iron and zinc. Recent evidence, however, indicates that they are induced during periods of oxidative stress and this has led to speculation that they may blunt the deleterious effects of ROS.

Ceruloplasmin is the major copper-containing protein found in plasma (42). In addition to copper transport, ceruloplasmin also exhibits ferroxidase activity, catalyzing the deposition of iron into ferritin (43). Hence, it is an example of a protein that exceeds the one gene-one structure-one function concept. Moreover, recent in vitro evidence suggests that ceruloplasmin destroys H2O2, potentially indicative of yet another functional attribute of this protein in vivo (44). As previous studies have shown that ceruloplasmin is a powerful plasma antioxidant, inhibiting lipid peroxidation by oxidizing ferrous iron (45), and observations that it exhibits GSH-Px-like activity, it is reasonable to speculate that ceruloplasmin may also function as an antioxidant. Recently, both the brain and the lung have been identified as sites of extrahepatic ceruloplasmin synthesis (46, 47). Taken together, these observations may point to a critical secondary role of ceruloplasmin as an antioxidant during periods of oxidative stress.
Ferritin is characterized primarily as an iron storage protein, binding up to 4500 atoms of iron per molecule (48). Thus, iron homeostasis is its best-studied and regarded as its primary function. However, there is growing evidence that it may also exhibit antioxidant functions. For example, induction of ferritin synthesis in endothelial cells reduced the cytotoxicity of subsequent hydrogen peroxide exposure (49). Similarly, sensitivity to oxidants was found to be inversely correlated with ferritin protein levels in tumor cells (50). Notably, ferritin mRNA levels have been shown to increase in the lung of rats exposed to hyperoxia (51). Ferritin protein levels have also been shown to increase in mouse lung tissue following hyperoxia (52). Although these results are consistent with observations that ferritin acts as an iron storage reservoir and thus increased ferritin levels reduce the low molecular weight ("labile" or "regulatory") iron pool (53), they are also consistent with observations that a reduction in ferritin sensitizes cells to ROS toxicity (54), and that overexpression of ferritin reduces ROS levels in cells challenged with oxidative stress (55).

Metallothionein, like ceruloplasmin and ferritin, is a cysteine rich, transition metal binding protein that predominantly binds zinc, but may also bind copper, cadmium and silver (56). Indeed, metallothionein was initially characterized as a heavy metal-sequestering protein (57). An antioxidant role has also been proposed for metallothionein because it is a redox-sensitive gene (58) that is elevated under various forms of oxidative stress (59). Moreover, cells that overexpress metallothionein, either following induction of gene expression or through genetic manipulation, exhibit increased resistance to oxidative stress (60, 61), whereas cells with decreased metallothionein expression exhibit enhanced sensitivity to ROS (62). Recently, in vitro work has demonstrated that
overexpression of metallothionein in lung epithelial cells confers resistance to both \( \text{H}_2\text{O}_2 \) and hyperoxia exposure. Conversely, \( \text{H}_2\text{O}_2 \) exposure has been shown to induce metallothionein expression, suggesting that it is responsive to oxidative stress (63). However, at present, there is little direct in vivo evidence of an antioxidant role of metallothionein. A notable exception includes metallothionein overexpressing mice that exhibit marked resistance to oxidative injury in cardiac tissue compared to non-transgenic littermates (64). Thus, there is a preponderance of evidence consistent with an antioxidant role of metallothionein. This, and the ubiquitous expression of metallothionein, including its expression in lung and brain tissue, may be indicative of its role as an antioxidant that may play a significant role in protecting against oxidative stress in these two organs.

At present, researchers have been highly reliant upon in vitro analysis to discern the potential roles of metalloproteins as part of a stress response. As a result, the contribution of these proteins to the antioxidant defense network in vivo is far from clear. Moreover, little attention has been directed towards their tissue specific expression or their impact on trace metal homeostasis following exposure to oxidative stress. Indeed, loss of trace metal homeostasis may be a significant factor in the pathogenesis of diseases associated with oxidative stress, particularly respiratory and neurological diseases associated with prematurity such as BPD and cerebral palsy (65, 66).

The Interaction of Nutritional Deficiency, Gene Expression and Oxidative Stress

Premature infants are born with antioxidant nutrient stores and antioxidant enzyme activities that a substantially reduced in comparison to term infants (15, 67). It
has also been demonstrated that hyperoxia, a therapeutic treatment strategy often employed to insure adequate oxygen delivery to tissues and organs of premature infants, significantly elevates ROS formation in both the lung and the brain (17, 68). These factors may play a significant role in the development in a myriad of diseases associated with prematurity, collectively referred to as the diseases of prematurity, particularly bronchopulmonary dysplasia and cerebral palsy. Indeed, it has been suggested that many of the clinical and pathological conditions associated with premature infants result from an imbalance of antioxidant defense and ROS production.

Manipulations of dietary zinc in animals can provide a useful model to study the effects of the low zinc nutrition status that has been described in premature infants (24). In particular, it can provide an effective means to examine the influence of zinc status on both the antioxidant defense system as well as host response to an exogenous oxidative stress. Notably, zinc is an antioxidant that functions primarily in biomembranes, but is also a component of CuZnSOD (69). The antioxidant function of zinc may be mediated by two mechanisms: 1) zinc protects sulphydryl groups, essential for protein structure and function, from oxidation, and 2) zinc prevents OH⁻ and O₂⁻ production by competing with transition metals for chelation of cysteine and histidine ligands. Zinc therefore inhibits the formation of iron- and copper-oxygen-enoic acid complexes that are proposed to initiate lipid peroxidation (70).

The development of transgenic and knockout mice has also provided a useful tool to study the effects of variable antioxidant enzyme expression in an animal model. Specifically, CuZnSOD transgenic and knockout mice provide an invaluable tool to examine the role of CuZnSOD, a central component of the primary antioxidant defense
system. By manipulating the expression level of CuZnSOD in mice, we can examine the effects of variable antioxidant enzyme expression as well as host response to an exogenous oxidative stress in a manner that is directly relevant to premature infants that invariably inherit a compromised antioxidant defense system.

An important goal of research is to understand how the balance between ROS generation and antioxidant defense affects disease development and progression. Therefore, the overall goal of this research was to examine the effects of antioxidant nutrient deficiency and variable antioxidant enzyme expression on host response to oxidative stress. Specifically, we have examined the effects of zinc deficiency, variable expression of the antioxidant enzyme CuZnSOD and exposure to hyperoxia on oxidative tissue damage and stress response in an in vivo animal model. We have hypothesized that under normal conditions zinc nutrition and CuZnSOD expression protects target organs against the deleterious effects of oxidative stress. Based on this hypothesis, we speculate that sub-optimal zinc nutrition or reduced expression of CuZnSOD significantly compromises the antioxidant defense system and potentiates hyperoxia-mediated ROS tissue damage in the lung and brain. This research will contribute significantly to our understanding of ROS mediated tissue pathology and have particular clinical relevance to premature infants, a population that appears to be especially sensitive to oxidative stress due to their low nutritional status and the developmental immaturity of their antioxidant defense system.
<table>
<thead>
<tr>
<th>Endogenous Stresses</th>
<th>Exogenous Stresses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic processes (oxygen breathing)</td>
<td>Radiation</td>
</tr>
<tr>
<td>Mitochondrial electron transport</td>
<td>Ozone</td>
</tr>
<tr>
<td>Aging</td>
<td>Nitrogen dioxide</td>
</tr>
<tr>
<td>Transition metal catalyzed reactions</td>
<td>Cigarette smoke</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Xenobiotics</td>
</tr>
<tr>
<td>Physical Exercise</td>
<td>Burn Injury</td>
</tr>
<tr>
<td>Disease States</td>
<td>Paraquat</td>
</tr>
<tr>
<td>Ischemia/reperfusion</td>
<td>Acetaminophen</td>
</tr>
<tr>
<td>Stroke</td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td></td>
</tr>
<tr>
<td>Acute respiratory distress syndrome</td>
<td></td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td></td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td></td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td></td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1 Examples of ROS Mediated Oxidative Stresses.


70. Goldstein S, Czapski G. The role and mechanism of metal ions and their complexes in enhancing damage in biological systems or in protecting these systems from the toxicity of O2. J Free Radic Biol Med 2:3-11, 1986.
CHAPTER 2

CELLULAR RESPONSE OF ANTIOXIDANT METALLOPROTEINS IN CuZnSOD TRANSGENIC MICE EXPOSED TO HYPEROXIA

Mark A. Levy,¹ Yu-Hwai Tsai,¹ Andrew Reaume,² and Tammy M. Bray¹

¹Department of Human Nutrition, The Ohio State University, Columbus OH
and ²Cephalon Inc., West Chester PA
2.1 SUMMARY

Ceruloplasmin, metallothionein and ferritin are metal binding proteins with potential antioxidant activity. Despite evidence of that they upregulated in pulmonary tissue following oxidative stress, little is known regarding their influence on trace metal homeostasis. In this study we have used CuZnSOD transgenic overexpressing and gene knockout mice and hyperoxia to investigate the effects of chronic and acute oxidative stress on the expression of these metalloproteins and to identify their influence on copper, zinc and iron homeostasis. We found that the oxidative stress mediated induction of ceruloplasmin and metallothionein in the lung had no effect on tissue levels of copper, iron or zinc. However, CuZnSOD expression had a marked influence on hepatic copper and iron as well as circulating copper homeostasis. These results suggest that ceruloplasmin and metallothionein may function as antioxidants independent of their role in trace metal homeostasis and that CuZnSOD functions in copper homeostasis via mechanisms distinct from its superoxide scavenging properties.
2.2 INTRODUCTION

In a number of pulmonary diseases, the balance between the antioxidant defenses and the rate of production of reactive oxygen species (ROS) is believed to be the critical factor that determines the extent of tissue injury (4, 22, 29, 41). For example, bronchopulmonary dysplasia (BPD) often develops in premature infants exposed to high levels of oxygen. Exposure to hyperoxia leads to increased production of ROS (18, 20) that can presumably overwhelm the immately developed antioxidant defense system and lead to oxidative tissue damage within the pulmonary tissue (8, 42, 43). The antioxidant defenses, which consist of a network of enzymes, proteins and low molecular weight ROS scavengers, function to protect cellular structures against the damaging effects of ROS. Although antioxidant enzymes such as the superoxide dismutases (CuZnSOD, MnSOD), glutathione peroxidases (GSH-Px) and catalase are generally considered to play the major role in antioxidant defense, this view is being expanded as our knowledge of cellular antioxidant defense response to different forms of oxidative insult continues to advance.

Metalloproteins such as ceruloplasmin, metallothionein and ferritin are well known for their critical role in metal homeostasis and function as storage reservoirs and/or chaperones to essential trace metals such as copper, zinc and iron. However,
evidence indicates that these proteins are induced during the acute phase response (27,
37) and under oxidative stress (13, 40), leading to speculation that they may function to
ameliorate the deleterious effects of ROS. The antioxidant properties of these proteins
have been attributed primarily to their function of binding the redox active metals copper
and iron, thus minimizing their capacity to catalyze ROS production via the Fenton
reaction. Yet the contribution of these metalloproteins to the antioxidant defense network
in vivo is still not clearly defined. Although many investigators have focused on
identifying the antioxidant properties of the induced metalloproteins, little attention has
been paid to their tissue specific expression or their influence upon metal homeostasis
following exposure to acute or chronic oxidative stress. Disruption in trace-element
homeostasis may further exacerbate the pathogenesis of pulmonary diseases associated
with oxidative stress such as BPD and adult respiratory distress syndrome (ARDS) (10,
21, 33).

With the development of transgenic and knockout mice, researchers have
developed a powerful tool to manipulate the antioxidant defense status in vivo. In our lab,
we have used mice that express variable levels of the antioxidant enzyme CuZnSOD and
exposure to hyperoxia to systematically evaluate the in vivo consequences of chronic and
acute oxidative stress on trace mineral metabolism. CuZnSOD protects against oxidative
stress by catalyzing the dismutation of superoxide (O$_2^{-}$) generated under normal aerobic
metabolism. Thus, gene knockout of this enzyme presumably leads to a chronic increase
in endogenous levels of oxidative stress, whereas overexpression of CuZnSOD would
have the opposite effect and result in decreased levels of oxidative stress. In contrast,
exposure to exogenous oxidative stresses, for example exposure to a high level of oxygen,
is known to generate an acute state of oxidative stress by elevating ROS production (18). Hence in this study we have used mice with variable CuZnSOD expression and hyperoxia exposure to investigate the effects of chronic and acute oxidative stress on the expression of the antioxidant metalloproteins ceruloplasmin, metallothionein and ferritin, and to identify the influence of these metalloproteins on copper, zinc and iron homeostasis.
2.3 MATERIALS AND METHODS

CuZnSOD overexpressing and knock-out mice. CuZnSOD overexpression (Sod1<sup>++++</sup>) mice (TGN(SOD1)3Cje, Jackson Labs, Bar Harbor, ME) contain multiple inserts of the human CuZnSOD gene (15). Mice homozygous for the human CuZnSOD insert were identified by SOD activity gel (5) and pedigree analysis. CuZnSOD knockout mice (Sod1<sup>−/−</sup>) were a generous gift of Cephalon Inc. (West Chester, PA). Construction of the Sod1<sup>−/−</sup> gene targeted mice was performed by Cephalon Inc., as previously described (35). In our laboratory, heterozygotes (Sod1<sup>+/−</sup>) were mated to produce knockouts, heterozygotes and wild-type controls (Sod1<sup>+/+</sup>). Mouse strain was similarly confirmed in the laboratory by SOD activity gel (5) and pedigree analysis. All procedures involving animals were approved by the OSU Institutional Laboratory Animal Care Committee.

SOD activity gel electrophoresis. Lung homogenate (~ 5-10 µg protein) was electrophoresed through a 30% polyacrylamide gel for 2-3 hours at 100 V. Staining for SOD activity was done as described (5), except that gels were soaked for 10 minutes in nitroblue tetrazolium followed by a 15-minute soak in phosphate buffer containing
riboflavin. Clear bands appear in the gel that represent either human SOD, mouse SOD, or mouse-human SOD. Absence of a band is indicative of a CuZnSOD knock-out.

**Oxygen exposure of mice.** Adult male mice (25-35 g) expressing either of the four different levels of CuZnSOD gene (Sod1^{+++}, Sod1^{+/-}, Sod1^{+/+} and Sod1^{-/-}) were used in all experiments and randomly assigned to either the control or hyperoxia treatment group. All animals were placed in an airtight Plexiglas chamber and exposed to either room air or 85% oxygen continuously for 5 days (120 hours). 85% oxygen was supplied to the chamber at a flow rate of 3-4 L/min by mixing compressed air and oxygen with a Bird 3800 Microblender (Bird Products Corporation, Palm Springs, CA). The oxygen concentration of the chamber was continuously monitored using a Servomex Oxygen Analyzer OA 570 (Servomex Limited, Crowborough, Sussex, UK). Chamber temperature was maintained at 25-27°C, and the relative humidity was 60-70%.

**Tissue collection and preparation.** Tissue samples were collected after the mice had been exposed to either air or 85% oxygen for 5 days. Mice were killed by decapitation, and trunk blood was collected in heparinized tubes, centrifuged to obtain plasma, and stored at –80°C until further use. The lung and liver was removed, weighed, rinsed in ice-cold saline, immersed in liquid nitrogen and stored at –80°C. Prior to biochemical analysis, frozen tissues were homogenized on ice with a PowerGen homogenizer (Fisher Scientific, Pittsburgh, PA) in 4 volumes of sucrose buffer (0.25M sucrose, 1.0mM EDTA, pH 7.4) containing the following protease inhibitors to prevent protein degradation during sample preparation: aprotinin (0.5μg/ml), leupeptin (0.5 μg/ml), pepstatin (0.7 ug/ml) and
phenylmethylsulfonyl fluoride (40 ug/ml) (30). A fraction of the homogenate (1 ml) was then removed for trace mineral analysis. The remainder of the homogenate was centrifuged at 600 x g for ten minutes to remove the nuclei, and the cytosol was obtained from the post-nuclear supernatant by centrifugation at 5000 x g for 20 minutes.

**Measurement of enzyme activity.** Total superoxide dismutase (SOD) activity was measured as the percent inhibition in the cytochrome c-xanthine-xanthine oxidase assay (11). SOD activity was measured in the presence of 10 uM KCN to remove interference due to cytochrome c oxidase activity. To distinguish the contributions of CuZnSOD and MnSOD, MnSOD activity was measured in the presence of 1 mM KCN, which inhibits CuZnSOD activity. CuZnSOD activity was then determined by subtracting MnSOD activity from total SOD activity. One unit of SOD activity is defined as the amount of protein required to inhibit the rate of oxidation of cytochrome c by 50%. GSH-Px activity was measured as described (32). Tissue homogenate (100 µl) was added to 900 µl HEPES buffer (100mM, pH 7.5) containing EDTA (0.1 mM), GSH (1mM), NADPH (0.11 mM) and GSH Reductase (0.6 U). The disappearance of NADPH was monitored at 340 nm for 5 minutes after the addition of 20 µl t-butyl hydroperoxide in methanol (0.25 mM). All concentrations are expressed as final molarity within the cuvette. Catalase activity was measured as the decomposition of hydrogen peroxide (H₂O₂) (9). Briefly, 333 µl of tissue homogenate was diluted in one volume of phosphate buffer (50mM K₂HPO₄, 50mM KH₂PO₄, pH 7.0), transferred immediately to a cuvette containing 1 volume of 30 mM H₂O₂, and the change in absorbance at 240 nm was recorded for 1
minute. One unit of catalase activity is defined as 1 µmol of H₂O₂ consumed per minute per mg of tissue protein.

Ceruloplasmin activity was measured using p-phenylenediamine (PPD) as a substrate (34). Serum samples (2 mg protein) were added to cuvettes containing 0.6 ml acetate buffer (0.1M, pH = 6.0) and 0.3 ml 0.25% PPD in acetate buffer. Acetate buffer and PPD solution were equilibrated at 37°C for 5 minutes prior to sample addition. The change in absorbance at 530 nm was monitored for 30 min after allowing for a 10 min lag phase. Blank samples containing serum (2 mg protein), 0.3 ml acetate buffer, 0.3 ml 0.25% PPD buffer, and 0.3 ml of 0.1% NaN in 0.1M acetate buffer were prepared for each sample. Ceruloplasmin activity was determined using standard solutions of Bandrowski’s base, where 1 enzyme unit is equivalent to 1 µmol of Bandrowski’s base formed per minute per liter of serum (36).

**Western blot analysis.** CuZnSOD, GSH-Px and catalase as well as ceruloplasmin and ferritin proteins were separated by PAGE under standard conditions (26) using a Bio-Rad Mini Gel apparatus (Bio-Rad, Hercules, CA). For CuZnSOD and catalase, samples containing 15 µg protein were run on 10% polyacrylamide gels for 1.5-2 hours at 100 V. For GSH-Px, 20 µg protein was separated over 2 hours in a 12% polyacrylamide gel at 100V. Separation of ceruloplasmin and ferritin was accomplished by loading 25 µg tissue sample protein on an 8% and 12% polyacrylamide gel run at 100 V for 1.5-2 hours. After proteins were separated, they were electrophoretically transferred overnight at 4°C and 7 V/cm² to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech Inc., Piscataway, NJ) using a Mini Trans-Blot Transfer Cell (Bio-Rad). Membranes were
blocked with 5% non-fat dry milk in phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 4.3mM Na$_2$HPO$_4$, 1.4mM KH$_2$PO$_4$, pH 7.4) at room temperature for 4 hours and then washed 4 times in PBS containing 0.2% Tween-20 for 5 minutes. Membranes were then incubated with their respective primary antibodies (i.e. CuZnSOD, GSH-Px, catalase, ceruloplasmin: sheep anti-human; ferritin: mouse anti-human) at a 1:1000 dilution at 37°C for 2 hours in PBS containing 2% non-fat dried milk. All primary antibodies were supplied by Biodesign International (Kennebunk, ME). After incubation, each membrane was washed a second time as outlined above and then incubated with horseradish peroxidase-conjugated rabbit anti-sheep or rabbit anti-mouse (Sigma, St Louis, MO) antisera at a dilution of 1:2000, for 2 hours at 37°C. Blots were developed in 3.4 mM 4-chloro-1-naphthol, 22% methanol, 0.018% H$_2$O$_2$, 15.6 mM Tris-HCl (pH 7.4) and 120.2 mM NaCl. Development was stopped by immersion of membranes in distilled water.

Western blot analysis of metallothionein was accomplished using a modified procedure (28). Tissue homogenate (20 µg total protein) was diluted 1:1 with sample buffer (10mM Tris-HCl, 10mM EDTA, 20% (v/v) glycerol, 1.0% (w/v) SDS, 0.005% (w/v) bromophenol blue, 100mM dithiothreitol) and then separated in an 18% polyacrylamide gel at 100 V for 2 hours. Membrane transfer was performed in CAPS buffer (10mM 3-cyclohexylamino-1-propane sulfonic acid, 2 mM CaCl$_2$, 10% methanol, pH 10.8) followed by membrane incubation in 2.5% glutaraldehyde in water for 1 hour at room temperature. Glutaraldehyde treated membranes were then washed 3 times for 10 minutes in phosphate buffer (8.1 mM Na$_2$HPO$_4$, 1.2 mM KH$_2$PO$_4$, 2.7 mM KCl, pH 7.4); 50 mM monoethanolamine was added to the third wash to quench residual glutaraldehyde.
reactivity. Membranes were blocked in 3% BSA (Sigma). Application of primary (mouse anti-human; Zymed Laboratories, San Francisco, CA) and secondary antibodies (rabbit anti-mouse; Sigma) as well as all subsequent washing steps and color development was performed as outlined above except that 2% BSA was substituted for 2% non-fat dried milk.

Trace mineral analysis. Lung and liver copper, zinc and iron levels were measured by atomic absorption spectrophotometry (AAS) using a Varian Spectra AA-5 (Varian Techtron Pty Limited, Mulgrave, Australia). Briefly, 1 ml of homogenate was mixed with 4 ml of 2N HCl and incubated with gentle agitation for 24 hours. The suspension was then centrifuged at 7000 x g for 30 minutes, and the supernatant used for direct measurement of each trace mineral. Standard reference material (SRM 1577, National Institute of Standards and Technology, Gaithersburg, MD) was analyzed simultaneously to insure validity of the method.

Protein assay. Lung homogenate protein concentration was determined using the Bradford method (6). Coomassie brilliant blue G-250 staining reagent (Bio-Rad) was used for protein analysis, with bovine serum albumin (BSA; Sigma) prepared as standards.

Statistical methods. All results are expressed as means ± SEM. Data were analyzed by the general linear models procedure to determine significant main effects and by the least
significant means test to determine differences between means (39). A p-value < 0.05 was taken as the level of significance for all analysis.
2.4 RESULTS

CuZnSOD protein levels in the lung (A) and liver (B) of transgenic mice with variable levels of expression (\(Sod1^{++} \), \(Sod1^{+/+} \), \(Sod1^{+/−} \) and \(Sod1^{−/−} \)) are depicted in Figure 2.1. As anticipated, CuZnSOD protein level was well correlated with the copy number of genes in the lung and liver of each \(Sod1\) strain of mouse. Exposure to hyperoxia appears to induce CuZnSOD protein expression in the lung and liver of \(Sod1^{+/+}\) and \(Sod1^{+/−}\) mice, although it did not reach statistical significance. CuZnSOD enzyme activity was also measured and found to mirror protein analysis (data not shown).

The effect of variable \(Sod1\) expression and hyperoxia exposure on the antioxidant enzyme levels of GSH-Px and catalase in the lung and liver are presented in Figure 2.2. In the lung, GSH-Px protein level increased as CuZnSOD protein levels decreased. However, hyperoxia did not further induce GSH-Px protein levels as expected (Figure 2.2A). The inverse relationship between GSH-Px and CuZnSOD protein level was not as apparent in the liver (Figure 2.2C). Figure 2.2B and 2.2D demonstrate that CuZnSOD protein levels did not affect catalase expression in either the lung or liver tissue. However in the lung, an unanticipated result demonstrated that hyperoxia significantly reduced catalase protein levels in all \(Sod1\) strains of mice (Figure 2.2B), yet catalase protein levels remained unaffected in the liver (Figure 2.2D).
The activity of GSH-Px and catalase was then analyzed (Figure 2.3) to determine if variable CuZnSOD expression affected other antioxidant enzymes during hyperoxia exposure. Notably, GSH-Px activity in the lung (Figure 2.3A) did not reflect protein levels (Figure 2.2A) as measured by western blot in mice with variable CuZnSOD expression following hyperoxia. As a result, the previously noted inverse relationship between Sod1 gene expression and GSH-Px protein levels did not exist between CuZnSOD gene expression (Figure 2.1A) and GSH-Px activity (Figure 2.3A).

Moreover, GSH-Px activity in Sod1+/+ and Sod1+/− mice was increased following exposure to hyperoxia without a corresponding increase in GSH-Px protein levels. In the liver, neither Sod1 genotype or hyperoxia exposure influenced the activity of GSH-Px (Figure 2.3C). In contrast with GSH-Px activity, the activity of catalase was significantly reduced following exposure to hyperoxia in the lung of all Sod1 genotypes (Figure 2.3B), a result that mirrored the effects of hyperoxia on catalase protein expression (Figure 2.2B). In the liver, catalase activity was not affected by hyperoxia exposure. Puzzlingly however, catalase activity in Sod1+++ mice was significantly reduced by more than 50% compared to all Sod1 genotypes (Figure 2.3D), yet the level of protein expression was equal to all other strains (Figure 2.2D).

Figure 2.4 illustrates the induction of ceruloplasmin protein in the lung (Figure 2.4A) of Sod1 mice after exposure to hyperoxia, results that were not found in the liver (Figure 2.4C). Notably ceruloplasmin synthesis was not influenced by CuZnSOD gene expression, as there were no observable differences in protein level based on Sod1 genotype in the lung or liver. However, ceruloplasmin synthesis was found to be responsive to the oxidative challenge of hyperoxia in the target tissue, as protein levels
were elevated in the lung, but remained unchanged in the liver, following five days of hyperoxia exposure. The significant increases in ceruloplasmin protein levels in the lung did not, however, affect copper concentrations (Figure 2.4B). In contrast with the lung, liver copper concentrations exhibited substantial variability (Figure 2.4D), yet ceruloplasmin levels remained constant across all treatments (Figure 2.4B). Surprisingly, copper levels were found to correlate with CuZnSOD expression rather than ceruloplasmin protein levels, i.e. copper levels were highest in Sod1++/+ mice, intermediate in Sod1+/+ and Sod1+/- mice, and lowest in Sod1-/- mice.

In contrast with ceruloplasmin, metallothionein synthesis was found to be responsive to the oxidative challenge of hyperoxia in both the target and non-target tissue of all Sod1 strains, with statistically significant increases observed in the lung of Sod1++/+ and Sod1-/- and the liver of Sod1-/- mice (Figure 2.5A and 2.5C). The significant increases in metallothionein protein levels in the lung and liver did not, however, affect zinc concentrations (Figure 2.5B and 2.5D). In the lung, zinc concentrations were slightly reduced by hyperoxia in all Sod1 genotypes whereas in the liver, zinc levels were increased by hyperoxia in Sod1++/+ and Sod1-/- mice. Liver zinc concentrations were also affected by CuZnSOD expression, i.e. zinc levels were highest in Sod1++/+ mice, intermediate in Sod1+/+ and Sod1+/- mice, and lowest in Sod1-/- mice (Figure 2.5D).

Ferritin protein levels and iron concentrations in the lung and liver of each strain of Sod1 mice following exposure to hyperoxia is depicted in Figure 2.6. Lung and liver ferritin protein levels (Figure 2.6A and 2.6C) exhibited little fluctuation in response to either CuZnSOD gene expression or hyperoxia exposure. In the lung there is a trend towards a decrease in iron levels following exposure to hyperoxia, with significant
reductions observed in Sod1+/+ and Sod1−/− mice (Figure 2.6C). Although liver iron concentrations were not affected by exposure to hyperoxia, iron concentration was influenced by Sod1 genotype, as Sod1 overexpressors had significantly lower iron levels (Figure 2.6D). Significantly lower iron levels were also observed in the lung of Sod1+++ mice (Figure 2.6B).

Figure 7 depicts circulating trace mineral levels and serum ceruloplasmin activity in each strain of Sod1 mice following exposure to hyperoxia. Circulating iron levels were significantly elevated in Sod1+/+, Sod1+/− and Sod1−/− mice following hyperoxia (Figure 2.7A). Hyperoxia did not, however, have any effect on circulating copper (Figure 2.7B) or zinc (Figure 2.7C) levels, nor did it influence serum ceruloplasmin activity (Figure 2.7D). Notably, both circulating copper levels as well as serum ceruloplasmin activity was markedly influenced by overexpression of CuZnSOD, as each was significantly reduced in Sod1+++ mice compared to all other genotypes.
2.5 DISCUSSION

In this study we have used a unique model to examine the effects of acute and chronic stress within a physiologically relevant condition. This model is representative of the precarious situation experienced by premature infants that have inherited compromised antioxidant defenses and also require oxygen therapy. By using CuZnSOD overexpressor and knockout transgenic mice, we have manipulated the endogenous antioxidant defense system and produced a model in which animals are chronically subject to graded levels of oxidative stress. We have also employed hyperoxia exposure, a clinically relevant therapeutic treatment, to exert an acute exogenous oxidative stress. This model has allowed us to systematically examine the impact of oxidative stress on tissue specific responses of metalloproteins as part of the cellular defense system and to identify their role in trace element homeostasis.

Results from this study provides in vivo evidence that under acute oxidative stress, the metalloproteins ceruloplasmin and metallothionein may function as antioxidants independent of their role in trace element homeostasis. Specifically, our results demonstrate that ceruloplasmin protein may function as an important antioxidant in vivo, as lung tissue levels were markedly induced after acute exposure to hyperoxia (Figure 2.4A) without significant changes in tissue copper levels (Figure 2.4B). In the
liver, the non-target organ of hyperoxia exposure, ceruloplasmin levels were not affected by hyperoxia (Figure 2.4C). Our results extend earlier observations of others that showed tissue specific induction of ceruloplasmin mRNA in rat lung tissue during acute inflammation and hyperoxia (16). Furthermore, it is noted that tissue ceruloplasmin expression was induced by acute stress only, and was not responsive to chronic oxidative stress imposed by CuZnSOD knockout.

Similarly, metallothionein protein was induced in the lung (Figure 2.5A) following hyperoxia without significantly affecting zinc concentrations (Figure 2.5B). Previous studies have shown that metallothionein mRNA levels (MT-I and MT-II) are increased 10-12 fold in the liver of Sod1-/- mice. This induction was specific to the liver, as no significant induction was observed in the kidney, spleen, heart or lung of these mice (19). Our study extends these findings by demonstrating that under hyperoxia exposure, metallothionein protein levels are significantly induced not only in the liver, but also in the lung of each Sod1 strain of mice. In particular, induction of metallothionein expression was highest in the lung and liver of Sod1-/- mice compared to the other Sod1 strains (Figure 2.5A and 2.5C). Furthermore, metallothionein appears to be more responsive to oxidative stress than ceruloplasmin, as metallothionein was elevated in both the lung and liver under hyperoxic conditions, whereas ceruloplasmin was elevated in the lung only. In addition, liver metallothionein was also induced in response to chronic oxidative stress imposed by diminished CuZnSOD expression (Figure 2.5C). Together, these results demonstrate that under acute oxidative stress, ceruloplasmin and metallothionein may function as antioxidant metalloproteins independent of their role in trace metal homeostasis.
The mechanism by which the induction of these metalloproteins protects against acute oxidative stress is still unknown. It is interesting to speculate on the functional role of ceruloplasmin in lung tissue. Notably, the lung has been recently identified as a major site of extra-hepatic ceruloplasmin synthesis (45). Ceruloplasmin protein has been shown \textit{in vitro} to exhibit GSH-Px like activity by removing \( \text{H}_2\text{O}_2 \) and lipid hydroperoxides (31). Moreover, Atanasiu et al. (2) have demonstrated \textit{in vitro} that ceruloplasmin was an effective peroxyl radical scavenger as well as an effective chain-breaking antioxidant independent of its iron regulatory ferroxidase activity. Collectively, the characterization of GSH-Px like activity and radical scavenging abilities of ceruloplasmin, as well as the hyperoxia induced decline in pulmonary catalase protein (\textbf{Figure 2.2B}) and activity (\textbf{Figure 2.3B}) observed in our experiments, clearly suggests a critical role for ceruloplasmin as a compensatory antioxidant protein that is induced in the lung during acute oxidative stress. Indeed, the absence of hyperoxia induced increases in ceruloplasmin in the liver or circulation may point to the tissue specificity of ceruloplasmin synthesis during oxidative stress. This proposed antioxidant function of ceruloplasmin under acute oxidative stress occurs independent of its function in binding copper and preventing Fenton catalyzed ROS generation. In fact, our experiments demonstrate that the hyperoxia induced increase in ceruloplasmin protein levels did not significantly alter lung tissue copper levels.

Similar to ceruloplasmin, the mechanism by which metallothionein exerts its antioxidant function is not clear. \textit{In vitro} evidence reveals that cysteine-rich metallothionein reacts directly with ROS (1, 44). Metallothionein may also function as an antioxidant indirectly by releasing zinc which may then inhibit metabolic activity,
protect free thiols from oxidation as well as stabilize biomembranes (7). However, results from our experiments do not support this hypothesis, since the significant induction in metallothionein protein levels in the lung and liver under acute oxidative stress were not accompanied by an increase in zinc concentration in either tissue. It is well documented that metallothionein is a redox sensitive gene that is elevated during various forms of oxidative stress, including the administration of endotoxin, heavy metals, cytokines and glucocorticoids (19). Metallothionein antioxidant properties may be indirectly executed through redox signaling, but the precise mechanisms remain unknown. Nevertheless, metallothionein protection against oxidative injury has been explored extensively using metallothionein overexpressing transgenic mouse models. For example, under both acute and chronic conditions of oxidative stress, cardiac tissue of metallothionein overexpressing mice displayed a marked resistance to oxidative injury compared to wild-type control animals (24).

The third metalloproteins we examined, ferritin, is characterized primarily as an iron storage protein (3). There is a growing body of evidence that the role of this protein may extend beyond metal storage and include, among other functions, a direct role in free radical defense. For example, mRNA encoding the ferritin light chain, which is involved in long term iron storage, was increased several fold in the lung tissue of rats exposed to hyperoxia (38). Ferritin protein levels were also found to increase in mice following exposure to 95% oxygen for 3 days (14). Our results, however, do not support these findings, since we did not detect any increase in lung or liver ferritin levels either in response to hyperoxia or Sod1 gene dosage (Figure 2.6A and 2.6C). Moreover, our
results provide no evidence to support previously characterized in vitro interactions between ferritin and ceruloplasmin in oxidative defense (13, 23).

It is generally recognized that SOD, GSH-Px and catalase form a network that provides the first line of cellular defense against oxidative stress. Thus it is speculated that manipulation of one component may result in compensatory adjustments in one or more components of the defense network. We therefore measured the effects of CuZnSOD expression on the expression as well as the activity of GSH-Px and catalase in both lung and liver tissue. First, we observed that CuZnSOD protein levels were not responsive to acute oxidative stress, since neither lung nor liver CuZnSOD levels were significantly affected by exposure to hyperoxia (Figure 2.1A and 2.1B). Similar observations have been reported by others (17, 25). Subsequently, we observed that in the lung, GSH-Px protein levels were increased as CuZnSOD expression decreased, presumably as a compensation for the loss of CuZnSOD protein (Figure 2.2A). However, enzyme analysis revealed that GSH-Px activity did not reflect GSH-Px protein levels (Figure 2.3A). However, we did observe increased GSH-Px activity following hyperoxia in Sod1+/+ and Sod1+/− animals, findings that are consistent with previous research (25). Finally, we measured the activity and expression of catalase, an enzyme located primarily within peroxisomes. The results demonstrated, quite unexpectedly, that both the protein level and the activity of this enzyme were suppressed by more than 50% under the acute oxidative stress of hyperoxia (Figure 2.2B and 2.3B). To our knowledge, this is the first time that this phenomenon has been reported. In the liver, the non-target organ, catalase activity was reduced by 50% in mice that overexpress CuZnSOD. The reason for this decline is not readily apparent and requires further study.
However, it is interesting to note that the expression and activity of catalase, a heme-containing enzyme, is lowest in the liver of \textit{Sod1}^{+++} mice, results that coincide with significantly lower tissue iron levels in this same group (\textbf{Figure 2.6D}). Nevertheless, our studies did not demonstrate a close, compensatory linkage between the three primary antioxidant enzymes CuZnSOD, GSH-Px and catalase. Hence, compartmentalization and sub-cellular localization may be a more critical factor in determining the expression and activity of these enzymes than the relative quantity or activity of each individual component.

Copper is an integral component of both CuZnSOD as well as ceruloplasmin. It is generally recognized that ceruloplasmin is the primary extracellular copper storage protein, particularly when excess copper is present. Our study demonstrated that liver CuZnSOD plays a critical role in copper homeostasis. This is indicated by the parallel increase in hepatic copper levels and hepatic CuZnSOD expression (\textbf{Figure 2.4D and Figure 2.1B}). Moreover, the increased expression of CuZnSOD in \textit{Sod1}^{+++} mice coincides with a significant reduction in circulating ceruloplasmin and copper levels (\textbf{Figure 2.6D and 2.6B}). Hence, the demand for increased liver copper brought about by elevated CuZnSOD protein levels was met, at least in part, by the supply of copper stored in circulating ceruloplasmin. These results provide in vivo evidence that ceruloplasmin functions as a reservoir that supplies copper for CuZnSOD synthesis, findings consistent with previous work demonstrating that, in vitro, ceruloplasmin donates copper to CuZnSOD (12).

This study has employed a unique in vivo model to describe the homeostatic relationship between the induction of antioxidant metalloproteins and trace mineral
metabolism under chronic and acute oxidative stress using transgenic animals. Understanding the regulatory link between antioxidant defense system and trace mineral homeostasis under normal and pathophysiological conditions may provide insights for future therapeutic strategies, for example in premature infants exposed to hyperoxia as well as other forms of respiratory diseases associated with excessive oxidative stress.
Figure 2.1. CuZnSOD protein expression in the lung (A) and liver (B) of Sod1 transgenic (+++), homozygous (+/+) heterozygous (+/-) and knock-out (-/-) mice exposed to air (21% O₂) or hyperoxia (85% O₂) for five days. A representative Western blot has been inserted in (A) and (B) that illustrates the presence of mouse and human CuZnSOD in Sod1 +++ mice, as well as the absence of CuZnSOD in Sod1 -/- mice. Treatment of animals, preparation of lung homogenates and assays of CuZnSOD protein and activity are described in MATERIALS AND METHODS. Data are presented as means ± SEM. Bars with different letters are significantly different (P < 0.05). ND; not detected.
Figure 2.2  GSH-Px and catalase protein level in the lung (A, B) and liver (C, D) of Sod1\textsuperscript{+++}, Sod1\textsuperscript{+/+}, Sod1\textsuperscript{+-} and Sod1\textsuperscript{-/-} mice exposed to 21\% oxygen or 85\% oxygen for 5 days. Data are presented as means ± SEM. Bars with different letters are significantly different (\(P < 0.05\)). Example Western blots of GSH-Px and catalase protein are depicted for the lung (E,F) and liver (G,H) of mice exposed to air (lanes 1,3,5 and 7) or hyperoxia (lanes 2,4,6 and 8). Each lane represents tissue homogenate prepared from a single animal.
Figure 2.3  GSH-Px and catalase activity in the lung (A, B) and liver (C, D) of Sod1+++, Sod1+/+, Sod1+/- and Sod1-/- mice exposed to 21% oxygen or 85% oxygen for 5 days. Data are presented as means ± SEM. Bars with different letters are significantly different (P < 0.05). Example Western blots of GSH-Px and catalase protein are depicted for the lung (E,F) and liver (G,H) of mice exposed to air (lanes 1,3,5 and 7) or hyperoxia (lanes 2,4,6 and 8). Each lane represents tissue homogenate prepared from a single animal.
Figure 2.4 Ceruloplasmin protein levels and copper concentration in the lung (A,B) and liver (C,D) of Sod1^{+++}, Sod1^{+/+}, Sod1^{+-} and Sod1^{-/-} mice exposed to air (21% oxygen) or hyperoxia (85% oxygen) for five days. Data are presented as means ± SEM. Bars with an * indicate a significant difference between air and hyperoxia-exposed mice with the same Sod1 genotype. Example Western blots of ceruloplasmin protein are depicted for the lung (A) and liver (C) of mice exposed to air (lanes 1,3,5 and 7) or hyperoxia (lanes 2,4,6 and 8). Each lane represents tissue homogenate prepared from a single animal.
Figure 2.5  Metallothionein protein levels and zinc concentration in the lung (A,B) and liver (C,D) of Sod1+++ , Sod1+/- , Sod1+/- and Sod1-/- mice exposed to air (21% oxygen) or hyperoxia (85% oxygen) for five days. Data are presented as means ± SEM. Bars with an * indicate a significant difference between air and hyperoxia-exposed mice with the same Sod1 genotype. Example Western blots of metallothionein protein are depicted for the lung (A) and liver (B) of mice exposed to air (lanes 1,3,5 and 7) or hyperoxia (lanes 2,4,6 and 8). Each lane represents tissue homogenate prepared from a single animal.
Figure 2.6  Ferritin protein levels and iron concentration in the lung (A,B) and liver (C,D) of Sod1$$^{+++}$$, Sod1$$^{+/+}$$, Sod1$$^{+-}$$ and Sod1$$^{-/-}$$ mice exposed to air (21% oxygen) or hyperoxia (85% oxygen) for five days. Data are presented as means ± SEM. Bars with different letters are significantly different ($$P < 0.05$$). Example Western blots of ferritin protein are depicted for the lung (A) and liver (B) of mice exposed to air (lanes 1,3,5 and 7) or hyperoxia (lanes 2,4,6 and 8). Each lane represents tissue homogenate prepared from a single animal.
Figure 2.7  Circulating trace mineral levels and serum ceruloplasmin activity in 
Sod1+++, Sod1+/+, Sod1+- and Sod1-/- mice exposed to air (21% oxygen) or hyperoxia
(85% oxygen) for five days. A: iron; B: copper; C: zinc; D: ceruloplasmin. Data are
presented as means ± SEM. Bars with different letters are significantly different (P <
0.05).
2.6 REFERENCES


CHAPTER 3

THE EFFECT OF ZINC DEFICIENCY AND EXPOSURE TO HYPOXIA
ON OXIDATIVE STRESS IN THE BRAIN

Mark A. Levy and Tammy M. Bray

Department of Human Nutrition, College of Human Ecology
The Ohio State University Columbus OH, USA
Dietary zinc deficiency has been associated with brain dysfunctions and may be a significant contributor to neurological abnormalities observed in premature infants. Previously we have demonstrated that zinc deficiency increased the permeability of the blood brain barrier (BBB) and when an oxidative stress (85% O\textsubscript{2} exposure) was superimposed upon zinc deficiency, the leakage of the BBB was exacerbated. On the basis of this evidence, we speculated that the protective function of the BBB is compromised during zinc deficiency and that oxidative stress superimposed on zinc deficiency may lead to increased oxidative tissue damage in the brain, a sequence of events by which zinc deficiency may contribute to the development of neuropathology. We examined several markers of oxidative stress and found that oxidative tissue damage was not increased in brain tissue as a result of either zinc deficiency or hyperoxia exposure. Similarly, analysis of the free radical defense system demonstrated that antioxidant enzyme defense was not affected by zinc deficiency or hyperoxia. These findings indicate that despite the significant physiological stress, including reduced weight gain and epidermal lesions, zinc deficiency and hyperoxia did not induce overt signs of oxidative stress, since neither oxidative tissue damage nor antioxidant defense enzymes were affected under these conditions.
3.2 INTRODUCTION

Premature infants develop neurological disorders at a much higher rate than term infants. Among a list of impairments that includes attention deficit disorder and motor development delay, cerebral palsy remains the most frequent major condition (1) and, indeed, premature delivery is the single most important antecedent of this disease (2). Approximately 5-15% of premature infants exhibit cerebral palsy, characterized by spastic motor deficits. Another 25-50% exhibit less severe disturbances of cognition and mobility (2). Notably, reactive oxygen species (ROS) have been proposed to play a prominent role in the development of neurological disorders of premature infants for several reasons. First, the brain contains large amounts of polyunsaturated fatty acids (PUFA’s) which are vulnerable to ROS attack (3). Second, the brain does not appear to be well endowed with either enzymatic or non-enzymatic antioxidant defenses, particularly in premature infants in whom very marginal or deficient stores of many antioxidant compounds have been observed (4, 5). And third, the potential for ROS-mediated tissue pathology may be increased when premature infants are exposed to hyperoxia, as evidence indicates that this treatment strategy leads to increased ROS production, specifically $O_2^-$ to $H_2O_2$, in the brain (6).
In addition to ROS toxicity, low zinc status has been proposed to be a key variable that may contribute to the neuropathologies observed in premature infants (7). Notably, zinc accumulation in the fetus occurs at an appreciable rate only in the last trimester (8, 9), and there is evidence that late prenatal and early postnatal zinc deficiency may lead to numerous neurological development abnormalities (8). However, the mechanism by which dietary Zn deficiency leads to pathology in the brain, an organ highly susceptible to oxidative damage is not yet clear.

Recently, breakdown of the blood brain barrier (BBB), the specialized capillary structure that serves to protect the brain from the external environment, has been observed in zinc deficiency. Moreover, exposure to hyperoxia has been shown to exacerbate BBB breakdown during zinc deficiency (10). Because zinc has only one valence state and does not readily participate in oxidation-reduction reactions, it is considered an antioxidant that functions in membrane stabilization and in the protection of protein thiol groups (11). These and other observations have led us to hypothesize that the antioxidant properties of zinc protect the brain against ROS mediated tissue damage and that premature infants with low zinc status are susceptible to ROS-mediated oxidative brain damage due to hyperoxia exposure. Therefore in this study we examined the effects of zinc deficiency and exposure to hyperoxia on biomarkers of oxidative stress in brain tissue. In particular, we examined the effects of zinc deficiency and exposure to hyperoxia on free radical generation, free radical defense and oxidative tissue damage in the brain.
3.3 MATERIALS AND METHODS

*Animals and diets.* Male Wistar weanling rats (Harlan Company, Indianapolis, IN)) with initial weights of 55-65 g were housed individually in suspended stainless steel mesh cages in a temperature and humidity controlled room with light from 0800 to 2000 h. The zinc-adequate (ZnAD, 50ppm zinc as ZnCO$_3$) and zinc-deficient (ZnDF <1ppm ZnCO$_3$) diet for all experiments was prepared commercially (Dytes, Philadelphia PA) based on the *AIN-93A* diet for rats in which spray-dried egg white low in zinc concentration was used as the protein source. Vitamin and mineral contents of the diet are outlined in Table 1.

After a two-day acclimation period, animals were divided into three diet treatment groups (n=10 per dietary treatment group). Zinc ad libitum animals (ZnAL) received free access to zinc adequate diet, and zinc deficient (ZnDF) animals received free access to zinc deficient diet. A third group, the zinc pair-fed (ZnPF) animals were used to control for the reduced dietary intake exhibited by ZnDF rats. That is, ZnPF animals received an aliquot of zinc-adequate food equivalent to the quantity of zinc-deficient diet consumed by the ZnDF animals in the previous 24 hour period. All animals were given free access to deionised water. All experiments and procedures were reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University.
At the end of the 18 day feeding period, rats from each dietary treatment group were randomly assigned to either the control (n=5) or hyperoxia treatment group (n=5). All animals were placed in an airtight Plexiglas chamber and exposed to either room air or 85% oxygen continuously for 4 days (96 hours). 85% oxygen was supplied to the chamber at a flow rate of 3-4 L/min by mixing compressed air and oxygen with a Bird 3800 Microblender (Bird Products Corporation, Palm Springs, CA). The oxygen concentration of the chamber was continuously monitored using a Servomex Oxygen Analyzer OA 570 (Servomex Limited, Crowborough, Sussex, UK). Chamber temperature was maintained at 25-27°C, and the relative humidity was 60-70%.

**Tissue collection and preparation.** Tissue samples were collected after the rats had been exposed to either air or 85% oxygen for 2 or 4 days. Rats were killed by decapitation, and trunk blood was collected in heparinized tubes, centrifuged to obtain plasma, and stored at –80°C until further use. The brain was removed, weighed, rinsed in ice-cold saline, immersed in liquid nitrogen and stored at –80°C. Prior to biochemical analysis, frozen tissues were homogenized on ice with a PowerGen homogenizer (Fisher Scientific, Pittsburgh, PA) in 4 volumes of sucrose buffer (0.25M sucrose, 1.0mM EDTA, pH 7.4) containing the following protease inhibitors to prevent protein degradation during sample preparation: aprotinin (0.5ug/ml), leupeptin (0.5 ug/ml), pepstatin (0.7 ug/ml) and phenylmethylsulfonyl fluoride (40 ug/ml) (12). A fraction of the homogenate (1 ml) was then removed for trace mineral analysis. The remainder of the homogenate was centrifuged at 600 x g for ten minutes to remove the nuclei, and the cytosol was obtained from the post-nuclear supernatant by centrifugation at 5000 x g for 20 minutes.
**Plasma zinc analysis.** Plasma zinc was determined by atomic absorption spectrophotometry (AAS) using a Varian Spectra AA-5 (Varian Techtron Pty Limited, Mulgrave, Australia). Briefly, 1 ml of plasma was mixed with 4 ml of 2N HCl and incubated with gentle agitation for 24 hours. The suspension was then centrifuged at 7000 x g for 30 minutes, and the supernatant used for direct measurement of zinc. Standard reference material (SRM 1577, National Institute of Standards and Technology, Gaithersburg, MD) was analyzed simultaneously to insure validity of the method.

**Measurement of enzyme activity.** Total superoxide dismutase (SOD) activity was measured as the percent inhibition in the cytochrome c-xanthine-xanthine oxidase assay (13). SOD activity was measured in the presence of 10 uM KCN to remove interference due to cytochrome c oxidase activity. To distinguish the contributions of CuZnSOD and MnSOD, MnSOD activity was measured in the presence of 1 mM KCN, which inhibits CuZnSOD activity. CuZnSOD activity was then determined by subtracting MnSOD activity from total SOD activity. One unit of SOD activity is defined as the amount of protein required to inhibit the rate of oxidation of cytochrome c by 50%. GSH-Px activity was measured as described (14). Tissue homogenate (100 µl) was added to 900 µl HEPES buffer (100mM, pH 7.5) containing EDTA (0.1 mM), GSH (1mM), NADPH (0.11 mM) and GSH Reductase (0.6 U). The disappearance of NADPH was monitored at 340 nm for 5 minutes after the addition of 20 µl t-butyl hydroperoxide in methanol (0.25 mM). All concentrations are expressed as final molarity within the cuvette. Catalase activity was measured as the decomposition of hydrogen peroxide (H₂O₂) (15). Briefly,
333 µl of tissue homogenate was diluted in one volume of phosphate buffer (50mM K₂HPO₄, 50mM KH₂PO₄, pH 7.0), transferred immediately to a cuvette containing 1 volume of 30 mM H₂O₂, and the change in absorbance at 240 nm was recorded for 1 minute. One unit of catalase activity is defined as 1 µmol of H₂O₂ consumed per minute per mg of tissue protein.

Aconitase activity was determined by the coupled reaction of aconitase and isocitrate dehydrogenase. The appearance of NADPH at 25°C was measured at 340 nm in a 1 ml reaction vessel containing tris-HCl buffer (50 mM, pH = 7.4) (16), sodium citrate (5 mM), MnCl₂ (0.6 mM), NADP⁺ (0.2 mM), 1 unit isocitrate dehydrogenase and 20-100 ug of tissue protein. One unit of aconitase activity was defined as the amount of enzyme required to catalyze the formation of 1 µmol of NADPH per minute. Fumarase activity was measured at 25°C in 1 ml of phosphate buffer (0.1M, pH 7.5) containing L-malate (50 mM) and EDTA (0.1mM) (17). Enzyme activity was determined by monitoring the accumulation of fumarate at 240 nm following the addition of 20-80 ug of tissue protein. One unit of fumarase activity is defined as the synthesis of 1 umol of fumarate in 1 min at the specified conditions. The activity of glutamine synthetase was determined using the method of Miller et al (18). In this method enzyme activity is measured by the rate of formation of g-glutamyl monohydroxymate production when L-glutamine and hydroxylamine are used as substrates. The resulting product reacts with ferric chloride to produce a brown colour that can be measured spectrophotometrically at 505 nm.

Thiol oxidation was measured by the method of Suzuki et al (19). Samples were incubated in 50mM Tris-HCl (pH=8.0) containing 0.5% SDS and 10mM DTNB for 30
minutes. The reaction mixture was then read at 412nm. A reduction in absorbance was interpreted as thiol oxidation.

**Western blot analysis.** CuZnSOD, GSH-Px and catalase proteins were separated by PAGE under standard conditions (20) using a Bio-Rad Mini Gel apparatus (Bio-Rad, Hercules, CA). For CuZnSOD and catalase, samples containing 15 µg protein were run on 10% polyacrylamide gels for 1.5-2 hours at 100 V. For GSH-Px, 20 µg protein was separated over 2 hours in a 12% polyacrylamide gel at 100V. After proteins were separated, they were electrophoretically transferred overnight at 4°C and 7 V/cm² to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech Inc., Piscataway, NJ) using a Mini Trans-Blot Transfer Cell (Bio-Rad). Membranes were blocked with 5% non-fat dry milk in phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.4mM KH₂PO₄, pH 7.4) at room temperature for 4 hours and then washed 4 times in PBS containing 0.2% Tween-20 for 5 minutes. Membranes were then incubated with their respective primary antibodies (i.e. CuZnSOD, GSH-Px, catalase: sheep anti-human) at a 1:1000 dilution at 37°C for 2 hours in PBS containing 2% non-fat dried milk. All primary antibodies were supplied by Biodesign International (Kennebunk, ME). After incubation, each membrane was washed a second time as outlined above and then incubated with horseradish peroxidase-conjugated rabbit anti-sheep or rabbit anti-mouse (Sigma, St Louis, MO) antisera at a dilution of 1:2000, for 2 hours at 37°C. Blots were developed via enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ).
**Statistical analysis.** Data were analyzed by the general linear models procedure of SAS, SAS Institute (1985) to determine significant (p < 0.05) main effects due to atmosphere, diet or day of hyperoxia exposure and their interactions. When the probability of getting a larger t value was less than 0.05 the differences were considered significant. Values were expressed as means ± SEM of 5 animals in each treatment.
3.4 RESULTS

**Assessment of zinc status.** The characteristic features of zinc deficiency, namely reduced weight gain, alopecia and skin lesions, were observed in rats fed a zinc-deficient diet. Table 3.1 displays the parameters measured to assess zinc status in each treatment group. The final body weight of the ZnDF and ZnPF rats was lower than the ZnAL groups in both the 2-day and 4-day hyperoxia study. These results indicate that the dietary regimen was successful in producing zinc deficient rats. Notably, plasma zinc levels exhibited a significant decline in both ZnPF and ZnAL rats following a 4-day exposure to hyperoxia. However, brain zinc levels were not different between groups regardless of hyperoxia exposure.

**Response of the antioxidant defense system.** The effects of hyperoxia and zinc deficiency on the primary antioxidant defense system were determined following both a 2-day and 4-day period of hyperoxia exposure. Work of other authors has demonstrated that exposure to hyperoxia modulates the activity of antioxidant enzymes in lung tissue (21). It was hypothesized that as part of the stress response, antioxidant enzyme activity would be induced in brain tissue. However, in these experiments we did not observe any changes in the activity or expression of the primary antioxidant enzymes in brain tissue in
response to either hyperoxia exposure or zinc deficiency. Total SOD activity was not different between dietary treatment groups before or after exposure to hyperoxia (Figure 3.1A and 3.1B). Western blot analysis revealed that CuZnSOD expression levels remained constant in each of the treatment groups. Representative Western blots are shown in Figure 3.2A and 3.2B. Similarly, we performed Western blot analysis of manganese SOD (MnSOD), an antioxidant enzyme previously demonstrated to be induced under oxidative stress. However, MnSOD protein levels were not altered in response to zinc deficiency or hyperoxia exposure (Figure 3.3).

Further analysis of antioxidant enzyme activity revealed that the activity of GSHPx and catalase were not affected by zinc deficiency or hyperoxia exposure. Previously, we have demonstrated significant reductions in catalase activity following exposure to hyperoxia (22). However, as demonstrated in Figure 3.4A and 3.4B, GSHPx activity remained constant in the brain tissue regardless of dietary treatment or hyperoxia exposure. Similarly, catalase activity in the brain was not affected by either zinc deficiency or hyperoxia exposure (Figure 3.5A and 3.5B).

In order to determine if either zinc deficiency or hyperoxia resulted in oxidative stress in brain tissue, we measured the activity of both cytosolic and mitochondrial aconitase as well as glutamine synthetase. These enzymes have been shown to be sensitive indicators of oxidative stress and are inactivated by ROS. Table 3.2 illustrates that in the brain, neither cytosolic nor mitochondrial aconitase activity was affected by dietary zinc status or exposure to hyperoxia. Similarly, glutamine synthetase was not affected by either a 2-day or 4-day exposure to hyperoxia or zinc deficiency.
Subsequently, we measured reduced thiol levels, as zinc has been shown to protect thiol groups from oxidation. However, reduced thiol levels were not altered in response to either zinc deficiency or hyperoxia exposure.
Dietary zinc deficiency has been associated with brain dysfunction and has recently been shown to increase the permeability of the blood brain barrier. Exposure to hyperoxia has been shown to further increase BBB permeability in zinc deficient animals. These and other observations have led us to hypothesize that neuropathologies associated with zinc deficiency may be attributable to an increased level of oxidative stress in the brain. Confirmation of this hypothesis would provide a critical link between zinc deficiency and neurological diseases associated with zinc deficiency. Moreover, it would contribute significantly to our understanding of the mechanistic roles of zinc in the etiology of brain disorders and lead to the development of better strategies for disease prevention.

Analysis of several physiological parameters throughout the course of this study demonstrated that in this study, we were able to impose zinc deficiency through a strict dietary regimen. Reduced body weight gain, alopecia, dermal lesions and dramatically reduced levels of circulating zinc indicate that zinc deficiency was imposed upon these animals and that the animals fed a zinc deficient diet were in fact zinc deficient. However, results from this study clearly indicate that the brain is very resistant to zinc deficiency as well as to hyperoxia exposure.
To estimate oxidative tissue damage in brain tissue as a result of zinc deficiency or hyperoxia exposure, we examined the activity of several indicator enzymes previously demonstrated to be inactivated by oxidative stress. We also examined the oxidation of protein thiol groups, macromolecular structures that require zinc for in vivo stability (11). However, we did not observe any changes in the activity of the indicator enzymes or in the level of thiol oxidation. Specifically, we examined the activity of both cytosolic and mitochondrial aconitase, two enzymes that are inactivated by O$_2^-$; however, the activity of these enzymes was not affected by zinc deficiency or hyperoxia. We also examined the activity of glutamine synthetase, an enzyme demonstrated to be inactivated by hyperbaric hyperoxia (23), but did not observe any change in activity in our experiments. Taken together, these results indicate that despite its apparent limitations in antioxidant defense, the brain is well protected from oxidative stress.

Antioxidant enzyme activity and expression has been shown to be upregulated in brain tissue in response to oxidative stress, whereas we have previously demonstrated that in lung tissue, oxidative stress can significantly reduce both the expression and activity of antioxidant enzymes. However, results from these experiments demonstrated that the primary antioxidant defenses were not affected in brain tissue in response to either zinc deficiency or hyperoxia. That is, neither the expression or activity of CuZnSOD or MnSOD, nor the activity of GSH-Px or catalase was affected by zinc deficiency or exposure to hyperoxia for 2 or 4 days.
Figure 3.1. Effect of zinc deficiency and 2-day (A) or 4-day (B) hyperoxia exposure on total SOD activity in the brain. Values are means±SEM from n=5 rats per treatment. ZnAL: zinc ad libitum; ZnPF: zinc pair-fed; ZnDF: zinc deficient.
Figure 3.2. CuZnSOD protein levels in brain tissue of rats exposed to air (21% O₂) or hyperoxia (85% O₂) for 2 days (A) or 4 days (B). Lane 1: ZnAL, 21% O₂. Lane 2: ZnAL, 85% O₂. Lane 3: ZnPF, 21% O₂. Lane 4: ZnPF, 85% O₂. Lane 5: ZnDF, 21% O₂. Lane 6: ZnDF, 85% O₂.
Figure 3.3. MnSOD protein levels in brain tissue of rats exposed to air (21% O₂) or hyperoxia (85% O₂) for 2 days (A) or 4 days (B). Lane 1: ZnAL, 21% O₂. Lane 2: ZnAL, 85% O₂. Lane 3: ZnPF, 21% O₂. Lane 4: ZnPF, 85% O₂. Lane 5: ZnDF, 21% O₂. Lane 6: ZnDF, 85% O₂.
Figure 3.4. Effect of zinc deficiency and 2-day (A) or 4-day (B) hyperoxia exposure on GSH-Px activity in the brain. Values are means±SEM from n=5 rats per treatment. ZnAL: zinc ad libitum; ZnPF: zinc pair-fed; ZnDF: zinc deficient.
Figure 3.5. Effect of zinc deficiency and 2-day (A) or 4-day (B) hyperoxia exposure on catalase activity in the brain. Values are means±SEM from n=5 rats per treatment. ZnAL: zinc ad libitum; ZnPF: zinc pair-fed; ZnDF: zinc deficient.
<table>
<thead>
<tr>
<th>Biochemical Parameter</th>
<th>21% O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>85% O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>21% O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>85% O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>21% O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>85% O&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 Day Hyperoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial Body Weight (g)</td>
<td>43±1.9</td>
<td>40±2.0</td>
<td>40±2.3</td>
<td>43±1.8</td>
<td>41±2.1</td>
<td>47±3.7</td>
</tr>
<tr>
<td>Final Body Weight(g)</td>
<td>141±4.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>125±4.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>75±3.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>80±3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66±1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma Zinc (ug/mL)</td>
<td>1.80±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.83±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.51±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.56±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.695±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.630±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brain Zinc (ug/g)</td>
<td>6.86±0.09</td>
<td>6.55±0.11</td>
<td>6.54±0.10</td>
<td>6.43±0.09</td>
<td>6.43±0.08</td>
<td>6.78±0.11</td>
</tr>
<tr>
<td><strong>4 Day Hyperoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial Body Weight (g)</td>
<td>51±3.0</td>
<td>49±3.5</td>
<td>45±2.0</td>
<td>46±1.8</td>
<td>48±2.3</td>
<td>50±1.0</td>
</tr>
<tr>
<td>Final Body Weight (g)</td>
<td>162±5.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>133±3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74±2.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>76±1.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70±2.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>74±2.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma Zinc (ug/mL)</td>
<td>1.78±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.32±0.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.74±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.01±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.467±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.425±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brain Zinc (ug/g)</td>
<td>6.43±0.12</td>
<td>6.61±0.13</td>
<td>6.51±0.05</td>
<td>6.67±0.08</td>
<td>6.62±0.10</td>
<td>6.80±0.12</td>
</tr>
</tbody>
</table>

**Table 3.1.** Effect of dietary zinc deficiency and exposure to hyperoxia on body weight and plasma zinc level. Values are means±SEM from n=5 rats per treatment. Within each row, values with different letters are significantly different (P<0.05) as determined by the least squares mean procedure of SAS. ZnAL: zinc ad libitum; ZnPF: zinc pair-fed; ZnDF: zinc deficient.
<table>
<thead>
<tr>
<th>Biochemical Parameter</th>
<th>21% O₂</th>
<th>85% O₂</th>
<th>21% O₂</th>
<th>85% O₂</th>
<th>21% O₂</th>
<th>85% O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Day Hyperoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosolic aconitase</td>
<td>0.11±0.01</td>
<td>0.12±0.01</td>
<td>0.12±0.02</td>
<td>0.13±0.01</td>
<td>0.12±0.02</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>Mitochondrial aconitase</td>
<td>0.014±0.001</td>
<td>0.015±0.001</td>
<td>0.015±0.001</td>
<td>0.013±0.001</td>
<td>0.013±0.001</td>
<td>0.013±0.001</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>601±38</td>
<td>630±27</td>
<td>685±53</td>
<td>630±24</td>
<td>647±13</td>
<td>642±32</td>
</tr>
<tr>
<td>Reduced thiols</td>
<td>64.1±3.4</td>
<td>60.6±2.7</td>
<td>65.1±1.7</td>
<td>67.2±3.0</td>
<td>64.0±1.6</td>
<td>63.6±0.8</td>
</tr>
<tr>
<td>4 Day Hyperoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosolic aconitase</td>
<td>0.13±0.02</td>
<td>0.10±0.01</td>
<td>0.10±0.01</td>
<td>0.10±0.02</td>
<td>0.09±0.02</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td>Mitochondrial aconitase</td>
<td>0.016±0.002</td>
<td>0.017±0.002</td>
<td>0.017±0.001</td>
<td>0.015±0.001</td>
<td>0.015±0.002</td>
<td>0.014±0.002</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>610±28</td>
<td>565±39</td>
<td>587±22</td>
<td>569±47</td>
<td>587±46</td>
<td>572±51</td>
</tr>
<tr>
<td>Reduced thiols</td>
<td>62.1±2.7</td>
<td>68.0±2.9</td>
<td>65.9±3.1</td>
<td>67.1±1.9</td>
<td>62.2±2.2</td>
<td>63.8±3.2</td>
</tr>
</tbody>
</table>

Table 3.2. Effects of zinc deficiency and 2-day or 4-day hyperoxia (85% O₂) on the activity of glutamine synthetase and cytosolic or mitochondrial aconitase and reduced thiol levels in brain tissue. Values are means±SEM, n=4. Within each row, means not sharing the same letter differ significantly, P<0.05. Glutamine Synthetase activity expressed as units/mg protein, where one unit = 1 pmol glutamyl monohydroxymate produced per minute. Cytosolic and mitochondrial aconitase activity expressed as units/mg protein, where one unit = 1 nmol NADPH produced per minute.
3.6 REFERENCES


CHAPTER 4

EXPRESSION OF HEME-OXYGENASE-1 (HO-1) IN THE BRAIN OF
SOD1 TRANSGENIC AND KNOCKOUT MICE
EXPOSED TO HYPEROXIA

Mark A. Levy and Tammy M. Bray

Department of Human Nutrition, College of Human Ecology
The Ohio State University Columbus OH, USA

82
4.1 SUMMARY

Hyperoxia and reduced antioxidant defense capacity have been proposed as key factors in the development of neuropathologies associated with premature birth. Heme-oxygenase 1 (HO-1) is a stress response protein induced by a variety of oxidative challenges. In this study, we examined HO-1 expression, antioxidant defense enzyme activity and markers of oxidative stress in SOD1 transgenic and knockout mutant mice exposed to hyperoxia for five days. Reduced SOD1 expression and exposure to hyperoxia increased glutathione oxidation and increased HO-1 expression, and there was a significant correlation between tissue glutathione redox potential and HO-1 expression. The activity and expression of catalase, GSH-Px and SOD2 were not significantly altered by exposure to hyperoxia, and GSH-Px was the only antioxidant enzyme affected by SOD1 gene expression. Markers of oxidative stress, including aconitase and glutamine synthetase activity, were not altered by SOD1 expression or hyperoxia exposure. These results indicate that induction of HO-1 by hyperoxia and loss of SOD1 expression may be a general response to oxidative stress, and could therefore provide a major defense mechanism against oxidative damage in the brain. They also demonstrate that HO-1 induction is a useful marker of oxidative stress in brain tissue.
4.2 INTRODUCTION

Reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$) have been implicated in the development of many age related neurologic disorders (1), but have also been linked to the development of neurologic dysfunction common to infants born prematurely (2). As in all tissues, ROS can induce cellular damage in the brain by oxidizing macromolecules (e.g. proteins, lipids, nucleic acids) and initiating a cascade of events that lead ultimately to cell death. The susceptibility of premature infants to ROS mediated brain damage may be two-fold in nature. First, antioxidant defenses are considerably less well developed in premature infants than in term infants (2-4). Second, therapeutic treatment strategies such as oxygen and drug therapy may increase oxidative stress by increasing ROS generation (5, 6). In particular, oxygen therapy, or hyperoxia, while often necessary to increase oxygen delivery to tissues and sustain life, may play a significant role in the development of neurological disorders as it also increases ROS production, particularly O$_2^-$ and H$_2$O$_2$, in brain tissue (7, 8).

In biological systems, an array of antioxidant defenses have evolved to protect against the deleterious effects of ROS. For example, copper-zinc superoxide dismutase (SOD1), a ubiquitous enzyme and key component of the antioxidant defense system,
protects against ROS toxicity by catalyzing the dismutation of \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \) and oxygen (9). Hydrogen peroxide is subsequently converted to water by catalase and glutathione peroxidase, two other enzymes considered to be integral components of the antioxidant defense system. Notably, tolerance to ROS has been demonstrated to occur through the induction of antioxidant enzymes (10) as well as other stress-response proteins. In particular, up-regulation of heme oxygenase 1 (HO-1) has been found to be protective against ROS-mediated stresses such as hypoxia (11) endotoxic shock (12) and inflammation (13). HO-1, also known as heat-shock protein-32, is a stress response protein and the rate limiting enzyme in heme degradation to carbon monoxide (CO) and the antioxidant bilirubin (14).

There has been a wealth of research investigating the roles of antioxidant defense, stress response and hyperoxia in bronchopulmonary dysplasia and retinopathy of prematurity, two prominent diseases associated with premature birth [For reviews see (15-17)]. However, to date there have been few in vivo experiments performed to examine the role of antioxidant defense, stress response and hyperoxia in the development of neuropathologies in premature infants. Therefore, to elucidate the potential deleterious consequences of variable antioxidant enzyme expression and exposure to hyperoxia in the brain of premature infants, we have used a mouse model to examine the effects of a five day exposure to hyperoxia and overexpression or gene knockout of SOD1 on oxidative damage and the stress response in the brain. It is noteworthy that SOD1 knockout mice are apparently normal and do not exhibit a
particular sensitivity to hyperoxia. We therefore reasoned that exposure to hyperoxia and
SOD1 gene deletion may induce HO-1 expression, and that this protein may function as
an antioxidant to scavenge ROS.
4.3 MATERIALS AND METHODS

**Animals.** Mice transgenic for and overexpressing the human SOD1 gene (TGN(SOD1)3Cje) were obtained from Jackson Laboratories, Bar Harbor, ME. Mice homozygous for the human gene insert (SOD1++++) were identified by SOD activity gel electrophoresis (18). SOD1 knockout mice (SOD1-/-) were a generous gift of Cephalon Inc. (West Chester, PA). Construction of the SOD1-/- gene targeted mice was performed by Cephalon Inc., as previously described (19). In our laboratory, mice heterozygous (SOD1 +/-) for the knockout gene were mated to produce knockouts, heterozygotes and wild-type controls (SOD1+/+). Mouse strain was confirmed in the laboratory through polymerase chain reaction (PCR) of mouse tail DNA and SOD activity gel analysis as previously outlined (20).

**Oxygen exposure of mice.** Adult male mice (25-35 g) expressing any of the four different levels of SOD1 gene (SOD1++++, SOD1+/+, SOD1+/- and SOD1-/-, n=8 for each genotype) were used in all experiments. Four mice of each genotype were then randomly assigned to either the control or hyperoxia treatment group and placed in an airtight Plexiglas chamber and exposed to either room air (21% oxygen, normoxia) or...
85% oxygen (hyperoxia) continuously for 5 days (120 hours). 85% oxygen was supplied to the chamber at a flow rate of 3-4 L/min by mixing compressed air and oxygen using a Bird 3800 Microblender (Bird Products Corporation, Palm Springs, CA). The oxygen concentration of the chamber was continuously monitored using a Servomex Oxygen Analyzer OA 570 (Servomex Limited, Crowborough, Sussex, UK). Chamber temperature and relative humidity was maintained at 25-27°C and 60-70%, respectively. Mice were given free access to purified rodent diet (Dyets Inc, Bethlehem, PA) and RO water throughout the duration of the study. All procedures involving animals were approved by the OSU Institutional Laboratory Animal Care Committee.

**Tissue Collection and Preparation.** Following exposure to normoxia or hyperoxia for 5 days, mice were killed by cervical dislocation and brains were collected, weighed, rinsed in ice-cold saline, immersed in liquid nitrogen and stored at -80°C. Prior to biochemical analysis, frozen brain tissue was homogenized in a Dounce ground-glass tissue homogenizer (Fisher Scientific, Pittsburgh, PA) on ice in 4 volumes of sucrose buffer (0.25M sucrose, 1.0mM EDTA, pH 7.4). The buffer contained the following protease inhibitors to prevent protein degradation during sample preparation: aprotinin (0.5ug/ml), leupeptin (0.5 ug/ml), pepstatin (0.7 ug/ml) and phenylmethylsulfonyl fluoride (40 ug/ml) (21). Prior to separation of the nuclear, mitochondrial and cytosolic fractions, 1 ml of the homogenate (1 ml) was removed for trace mineral analysis. The remainder of the homogenate was centrifuged at 600 x g for ten minutes to remove the nuclei, and the supernatant was obtained from the post-nuclear supernatant by centrifugation at 10,000 x
g for 20 minutes. After collecting the supernatant, the pelleted material containing the
mitochondria was washed twice by resuspending the pellet in sucrose buffer followed by
a 20 minute centrifugation at 10,000 x g. After the second wash the pellet was
resuspended in 4 volumes of sucrose buffer and stored as the mitochondrial fraction at –
80°C until analyzed. Contamination of either the mitochondrial or cytosolic fraction was
determined by measuring the activities of lactate dehydrogenase (22) and succinate
dehydrogenase as outlined (23).

**Enzyme Analysis.** Total superoxide dismutase (SOD) activity was measured as the
percent inhibition in the cytochrome c-xanthine-xanthine oxidase assay (24). SOD
activity was measured in the presence of 10 uM KCN to remove interference due to
cytochrome c oxidase activity. To distinguish the contributions of CuZnSOD and
MnSOD, MnSOD activity was measured in the presence of 1 mM KCN, which inhibits
CuZnSOD activity. CuZnSOD activity was then determined by subtracting MnSOD
activity from total SOD activity. One unit of SOD activity is defined as the amount of
protein required to inhibit the rate of oxidation of cytochrome c by 50%. Aconitase
activity was determined by the coupled reaction of aconitase and isocitrate
dehydrogenase (25). The appearance of NADPH at 25°C was measured at 340 nm in a 1
ml reaction vessel containing tris-HCl buffer (50 mM, pH = 7.4), sodium citrate (5 mM),
MnCl₂ (0.6 mM), NADP⁺ (0.2 mM), 1 unit isocitrate dehydrogenase and 20-100 ug of
tissue protein. One unit of aconitase activity was defined as the amount of enzyme
required to catalyze the formation of 1 µmol of NADPH per minute. Fumarase activity
was measured at 25°C in 1 ml of phosphate buffer (0.1M, pH 7.5) containing L-malate (50 mM) and EDTA (0.1mM) (26). Enzyme activity was determined by monitoring the accumulation of fumarate at 240 nm following the addition of 20-80 ug of tissue protein. One unit of fumarase activity is defined as the synthesis of 1 umol of fumarate in 1 min at the specified conditions.

**Western Blot Analysis.** Protein levels of CuZnSOD, MnSOD, HO-1 and HO-2 was determined by Western blot analysis according to previously described methods (27) using a Bio-Rad Mini Gel apparatus (Bio-Rad, Hercules, CA). Post-mitochondrial supernatant was used for denaturing polyacrylamide gel electrophoresis (SDS-PAGE). All samples were normalized for total protein before running on SDS-PAGE, and 50-75ug protein was run on 10% polyacrylamide gels for 1.5-2 hrs at 100 volts. After electrophoresis, band separated proteins were transferred to a nitrocellulose membrane, and blocked overnight in 5% non-fat dry milk in PBS (137 mM NaCl, 2.7mM KCl, 4.3 mM Na$_2$HPO$_4$, pH 7.4). Membranes were then washed 4 times in PBS containing 0.05% Tween 20 for 5 minutes and incubated with their respective primary antibodies (i.e. sheep anti-human CuZnSOD, MnSOD, HO-1 or HO-2) at room temperature for 3 hrs at a 1:1000 dilution in PBS containing 2% non-fat dry milk. Membranes were then washed a second time as outlined above and incubated with horseradish peroxidase-conjugated rabbit anti-sheep secondary antibody at a dilution of 1:2000 for 2 hrs at 37°C. All antibodies were obtained from Stressgen Biotechnology (Victoria, BC Canada). Blots
were visualized with a chemiluminescence system (ECL Plus Kit, Amersham Pharmacia) and densitometry performed using NIH Image software (NIH Image Version 1.61).

**GSH and GSSG.** Tissue levels of GSH and GSSG were determined by the method of Melnyk et al. (28) employing an HPLC-electrochemical detection system (Enviromental Sciences Associates, Inc. Chelmsford, MA) along with two Shimadzu solvent delivery systems, a reverse phase C18 column (5 µm; 4.6 x 150 mm; mobile crystalline material, Tokyo, Japan) and an autosampler. Samples were prepared by homogenizing brain tissue in 10% meta-phosphoric acid buffer, followed by centrifugation at 10,000 RPM for 30 minutes at 4°C. GSH eluted 4 min after sample was autoinjected into the system, whereas GSSG eluted at 10 min. Sample vials were placed in a temperature-controlled (4°C) tray to minimize potential artifacts in the GSH assay. The specificity of GSH and GSSG peak and the reliability of the assay were determined by adding GSH and GSSG standards of known concentrations to the sample.

The tissue glutathione reduction potential, based on the GSSG/2GSH couple, was determined from the Nernst equation (29):

\[ E_{hc} = -240 - \frac{59.1}{2} \log \left( \frac{[\text{GSH}]^2}{[\text{GSSG}]} \right) \text{ mV at } 25^\circ C, \text{ pH 7.0} \]

**Protein assay.** Lung and liver homogenate protein concentration was determined using the Bradford method (30). Coomassie brilliant blue G-250 staining reagent (Bio-Rad)
was used for protein analysis, with bovine serum albumin (BSA; Sigma) prepared as standards.

**Statistical Analysis**  All results are expressed as means ± SEM. Data were analyzed by the general linear models procedure to determine significant main effects and by the least significant means test to determine differences between means. A p-value < 0.05 was taken as the level of significance for all analysis. Pearson’s correlation was determined between HO-1 expression level and the GSSG/2GSH redox potential. All statistical analysis was performed using SAS software (31).
4.4 RESULTS

To verify that SOD1 activity and protein expression correlated with SOD1 gene dosage in the brain, we measured total SOD activity and performed western blot analysis of SOD1 protein levels in whole brain homogenates in each of the four strains of mice. Figure 4.1 illustrates that, similar to previous findings in lung and liver tissue (20), total SOD activity and SOD1 protein level was approximately 3.5-fold greater in SOD1+++ genotype of mice than in wild-type controls (SOD1+/+). Conversely, SOD1 protein was not detected, and total SOD activity was dramatically reduced to approximately 15% of wild-type controls in SOD1-/- mice. As expected, the heterozygous SOD1+/- mice exhibited an approximate 50% reduction in SOD1 protein and total SOD activity compared to wild-type controls. The effects of hyperoxia exposure were also examined and did not affect either SOD1 expression or SOD activity in any of the four SOD1 genotypes.

In an effort to determine if the level of SOD1 expression or exposure to hyperoxia induced oxidative stress in brain tissue, we first measured tissue levels of GSH and GSSG. Figure 4.2A demonstrates that SOD1 expression level did not affect brain GSH levels. However, exposure to hyperoxia significantly reduced GSH levels in each of the four SOD1 genotypes. Analysis of GSSG levels revealed that oxidative stress was higher
in the mice with reduced SOD1 levels following exposure to hyperoxia (Figure 4.2B).

That is, exposure to hyperoxia significantly increased brain GSSG levels in SOD1+/- and Sod1-/- mice, but did not affect brain GSSG levels in SOD1+++ or SOD1+/+ mice. These observations were similarly confirmed by analysis of the GSSG:GSH ratio (Figure 4.2C).

To further examine the level of oxidative stress in brain tissue, we measured the activity of aconitase, both the cytosolic and mitochondrial forms, and glutamine synthetase. Each of these enzymes have been shown to be inactivated by ROS and are considered to be sensitive indicators of oxidative stress. Table 4.1 illustrates that in the brain, neither cytosolic nor mitochondrial aconitase activity was affected by SOD1 gene dosage or exposure to hyperoxia. Similarly, glutamine synthetase was not affected by the five-day exposure to hyperoxia in any of the SOD1 genotypes of mice. Curiously, glutamine synthetase activity was higher in the SOD1+++ mice than in all other SOD1 strains.

We next explored the possibility that as part of a stress response, the expression or activity of other ROS scavenging enzymes might be affected by variable SOD1 gene expression or exposure to hyperoxia. We examined GSH-Px, catalase and MnSOD, three enzymes of the primary antioxidant defense system. Table 4.2 illustrates that there was a small but significant decrease in GSH-Px activity in SOD1-/- mice. These were unexpected findings, and to our knowledge it is the first time that these results have been reported. However, there was no fluctuation in the remaining antioxidant enzymes, as neither catalase activity nor MnSOD protein levels were altered in response to SOD1 gene expression or hyperoxia.
We next sought to determine if the expression of HO-1, a heat shock protein induced in response to a variety of oxidative stresses, was affected by SOD1 gene expression or exposure to hyperoxia in brain tissue. The data in Figure 4.3 illustrate that HO-1 expression varied inversely with SOD1 gene expression. That is, HO-1 expression was lowest in SOD1+++ mice, but highest in SOD1-/- mice. Exposure to hyperoxia led to increased HO-1 expression in mice with reduced SOD1 gene expression only (i.e. SOD1+/+ and SOD1-/- mice). These findings are consistent with the hypothesis that reduced SOD1 activity and exposure to hyperoxia increases oxidative stress in brain tissue and that HO-1 expression is increased as part of the stress response. In fact, the link between oxidative stress and HO-1 expression is further illustrated in Figure 4.4, which demonstrates that a higher reduction potential (more oxidized tissue redox environment) was positively correlated with increased HO-1 protein expression for all mice in the study (r=0.682, P<0.0001, n=28).
In the present study we have established that normobaric hyperoxia increases oxidative stress in brain tissue as demonstrated by significant decreases in GSH levels in each of the 4 SOD1 genotypes of mice (Figure 4.2A). Moreover, the results from this work show that a compromised antioxidant defense system exacerbates the effects of an exogenous oxidative stress, as demonstrated by the significantly increased levels of GSSG in the SOD1+/- and SOD1-/- mice exposed to hyperoxia (Figure 4.2B). Previous studies have shown that normobaric hyperoxia can have deleterious consequences in brain tissue in vivo (32, 33). However, this is the first work to report not only that exposure to hyperoxia can significantly decrease brain GSH levels, but also that the combined effects of an exogenous and an endogenous stress (i.e. hyperoxia exposure and loss of SOD1 gene expression) are additive, resulting in greater tissue levels of GSSG than occurs when either stress is imposed independently.

In this study we have also demonstrated that a compromised antioxidant defense system can induce a stress response under normal physiological conditions, as we show for the first time that HO-1 expression is significantly upregulated in brain tissue of SOD1-/- mice compared to wild-type controls. This observation indicates that loss of SOD1 protein expression is an oxidative stress and argues against the perception that
absence of SOD1 activity does not manifest any phenotypic changes under normal physiological conditions (34). We also demonstrated that the level of HO-1 protein was increased in brain tissue following exposure to hyperoxia in both SOD1-/- and SOD1+/+ mice. Notably, HO-1 was the only protein that was significantly induced in the brain of mice exposed to hyperoxia, clearly demonstrating that it is induced as a compensatory mechanism to detoxify ROS. Although earlier studies have shown that HO-1 is induced in brain tissue, for example in response to ammonia toxicity (35) and traumatic brain injury (36), this is the first study to show that HO-1 is induced in response to decreased SOD1 expression and exposure to hyperoxia.

Although several redox independent pathways are involved in HO-1 gene regulation, decreased GSH levels have been shown to induce HO-1 expression in vitro (37) and in rat brain tissue (38). However, the work in this report demonstrated that significant decreases in GSH levels were not sufficient to increase HO-1 expression. Specifically, exposure to hyperoxia resulted in comparable declines in brain GSH levels in all SOD1 genotypes of mice, yet HO-1 expression was increased in SOD1+/+ and SOD1-/- mice only. This apparent discrepancy was resolved by examining the tissue glutathione redox potential ($E_{hc}$). That is, the GSSG/2GSH redox potential has been proposed to serve as a better indicator of tissue redox status than either the concentration of GSH or the [GSSG]/[GSH] ratio, as the latter two measures do not reflect the correct ratio of oxidized and reduced glutathione (i.e. $GSSG + 2H^+ + 2e^- \rightarrow 2GSH$) (29). Thus, analysis of all treatment groups demonstrated a strong correlation between the glutathione redox potential and HO-1 expression (Fig. 5), whereas no correlation was found between tissue GSH levels and HO-1 expression (data not shown). Notably, the
correlation between the reduced tissue GSH levels and increased HO-1 expression observed in the work of others could be attributed to protocols resulting in substantial GSH depletion of more than 60% (37, 38), whereas in the work presented herein, GSH levels declined by only ~10-15%.

Based upon our examination of the expression and activity of several proteins commonly utilized as indicators of oxidative stress, and which differ in their cellular as well as their sub-cellular localization, it is interesting to speculate that HO-1 induction is indeed functioning as a cytoprotectant throughout brain. In particular, we first measured the activity of glutamine synthetase (GS), an enzyme highly sensitive to and inactivated by ROS in vitro and in brain tissue in vivo (21, 39). However, GS activity was not altered by hyperoxia (Table 4.1), although this could be attributed to its location exclusively within glial cells which, in comparison to neurons, are particularly resistant to ROS. Accordingly, we examined the expression of manganese superoxide dismutase (SOD2), an enzyme localized predominantly within neurons of brain tissue (40) and which is upregulated under numerous stresses (41-43). Again, however, our data indicated that the expression of this protein remained unchanged in response to either hyperoxia or SOD1 gene expression (Table 4.1). Finally, we examined the activity of cytosolic and mitochondrial aconitase, enzyme isoforms that are inactivated by ROS, are expressed uniformly throughout the brain, but which differ in their subcellular localization. Nevertheless, the activity of these enzymes was not affected by either hyperoxia or SOD1 expression (Table 4.2). Thus, despite significant decreases in GSH levels in response to hyperoxia, and despite substantial increases in GSSG levels due to hyperoxia and loss of SOD1 gene expression, the lack of effect either stress had on the
activity and expression of these indicator proteins may in fact point to the functional role of HO-1 induction as a protective response to oxidative stress. Indeed, these results are consistent with the proposition that the product of HO-1 enzymatic activity, biliverdin, is an integral component of a redox cycle of significant physiological consequence (44).

Oxidative stress and tissue oxidation have been proposed to contribute to the development and progression of neurodegeneration, and thus a battery of tests have been developed to evaluate ROS-mediated tissue damage. However, there is evidence that prior to the accumulation of oxidized macromolecules, a shift in cellular redox status and the initiation of a stress response may constitute part of an early reaction to oxidative stress. Indeed, the results presented herein demonstrate that even in the absence of oxidized indicator proteins (e.g. glutamine synthetase, cytosolic and mitochondrial aconitase), cellular redox balance was altered and HO-1 expression was significantly upregulated. These data clearly indicate that in brain tissue, thiol redox balance and HO-1 expression levels are more sensitive indicators of oxidative stress than other conventional markers.

Knockout of the SOD1 gene decreased GSH-Px activity, but did not affect the activity of catalase. Remarkably, both catalase and GSH-Px catabolize H₂O₂ and are inactivated by O₂⁻ (45), yet they are not similarly affected by SOD1 gene knockout. This may be attributable to the cellular location of each enzyme; both SOD1 and GSH-Px are cytosolic, therefore increased O₂⁻ levels as a result of SOD1 knockout would be expected to impact directly on GSH-PX activity, whereas the peroxisomal location of catalase may afford this enzyme some measure of protection. However, it should be noted that a current hypothesis regarding the toxicity of O₂⁻ in vivo is that it oxidizes exposed [4Fe-
4S] clusters in enzymes causing enzyme inactivation, release of iron and promoting "free iron" catalyzed oxidative reactions in the cell. However, our data indicate that the activity of cytosolic and mitochondrial aconitase, each an [4Fe-4S] containing enzyme, were not affected by SOD1 knockout. Thus our results are not consistent with a O$_2^\cdot$ mediated inactivation of GSH-Px and point to a need for further analysis to clarify this apparent discrepancy. Notably, recent work has demonstrated that hyperoxia increases 'NO production in brain tissue in vivo (46). Moreover, increased fluxes of 'NO have been proposed to consume large quantities of O$_2^\cdot$, particularly in the absence of SOD, producing peroxynitrite (ONOO$^-$) and reducing O$_2^\cdot$ mediated tissue damage (47). Since GSH-Px is much more readily inactivated by ONOO$^-$ than catalase (48), it is plausible that these pathways may predominate in SOD1-/- mice.

In summary, HO-1 expression in brain was found to be upregulated in response to hyperoxia and reduced SOD1 gene expression and was strongly correlated to tissue redox status. Our findings corroborate the concept that the stress response protein HO-1 is not only highly sensitive to oxidative stress but is finely regulated by tissue redox status. Thus, increased HO-1 expression may not only be an important neuroprotective agent during periods of oxidative stress, but may also be a sensitive marker of oxidative stress in the central nervous system.
Figure 4.1. SOD1 protein levels (A) and total SOD activity (B) in the brain of SOD1 transgenic (SOD1+++), homozygous (SOD1++/-), heterozygous (SOD1+/-) and knockout (SOD1-/-) mice exposed to air (21% O₂) or hyperoxia (85% O₂) for five days. Data are presented as means ± SEM. Bars with different letters are significantly different ($P < 0.05$). nd: not detected.
Figure 4.2. Effects of SOD1 gene expression and hyperoxia (85% O₂) on brain glutathione status. Tissue levels of GSH (A), GSSG (B) and the GSSG:GSH ratio in the brain of SOD1 transgenic (SOD1+++) homozygous (SOD1+/+), heterozygous (SOD1+/-) and knockout (SOD1-/-) mice exposed to air (21% O₂) or hyperoxia (85% O₂) for five days. A more positive reduction potential (mV) indicates a more oxidized tissue redox environment. Data are presented as means ± SEM. Bars with different letters are significantly different (P < 0.05).
Figure 4.3. Western blot analysis of HO-1 in the brain of SOD1 transgenic (SOD1\(^{+++}\)), homozygous (SOD1\(^{+/+}\)), heterozygous (SOD1\(^{+/−}\)) and knockout (SOD1\(^{−/−}\)) mice exposed to air (21% O\(_2\)) or hyperoxia (85% O\(_2\)) for five days. Data are presented as means ± SEM. Bars with different letters are significantly different (\(P < 0.05\)).
Figure 4.4. Correlation between HO-1 protein expression and the GSH disulfide (GSSG/2GSH) redox potential in the brain. Data points are the values obtained from each of the individual mice from all SOD1 genotypes; n=28. There was a significant inverse correlation between HO-1 protein level and brain tissue reduction potential ($r=0.682$, $P<0.0001$).
Table 4.1. Effects of SOD1 gene expression and hyperoxia (85% O2) on the activity of glutamine synthetase, cytosolic and mitochondrial aconitase in brain tissue. Values are means±SEM, n=4. Within each row, means not sharing the same letter differ significantly, P<0.05. Glutamine synthetase activity expressed as units/mg protein, where one unit = 1 pmol glutamyl monohydroxymate produced per minute. Cytosolic and mitochondrial aconitase activity expressed as units/mg protein, where one unit = 1 nmol NADPH produced per minute.
<table>
<thead>
<tr>
<th>SOD1 Genotype</th>
<th>SOD1+++, 81% O₂</th>
<th>SOD1+/-, 85% O₂</th>
<th>SOD1+/-, 85% O₂</th>
<th>SOD1-/-, 85% O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSHPx</td>
<td>1.57±0.11 a</td>
<td>1.44±0.03 a</td>
<td>1.72±0.05 a</td>
<td>1.63±0.09 a</td>
</tr>
<tr>
<td>Catalase</td>
<td>9.73±0.87</td>
<td>9.67±0.87</td>
<td>9.82±0.65</td>
<td>9.02±1.14</td>
</tr>
<tr>
<td>SOD2</td>
<td>1.05±0.33</td>
<td>1.01±0.20</td>
<td>0.90±0.08</td>
<td>1.12±0.17</td>
</tr>
</tbody>
</table>

Table 4.2. Activity or expression level of primary antioxidant defense enzymes in brain tissue following exposure to air (21% O₂) or hyperoxia (85% O₂) for five days. Values are means±SEM, n=4. Within each row, means with not sharing the same letter differ significantly, P<0.05. GSHPx activity expressed as units/mg protein, where one unit = 1 nmol NADPH consumed per minute. Catalase activity expressed as units/mg protein, where one unit = 1 umol H₂O₂ consumed per minute at maximal activity. MnSOD measured as relative protein density by Western blot analysis.
4.6 REFERENCES


CHAPTER 5

ZINC SENSITIVE GENES IN THE MOUSE BRAIN: RESULTS FROM A HIGH-DENSITY OLIGONUCLEOTIDE MICROARRAY ANALYSIS

Mark A. Levy and Tammy M. Bray

Department of Human Nutrition, College of Human Ecology

The Ohio State University Columbus OH, USA
The detrimental effects of zinc deficiency on brain development and brain function have been established, but the underlying mechanisms remain unknown. In this study, we have used a high density oligonucleotide array to comprehensively screen the mouse brain for genes modulated by dietary zinc in vivo. We found that approximately 0.6% of expressed genes were differentially expressed in the brains of zinc deficient mice. Predominant among these were gene products that participate in RNA and protein biosynthesis (e.g. RNA polymerase I), embryogenesis and development (e.g. Hoxa-5) and cell adhesion and cell signaling (e.g. Ncam2). These observations provide evidence that zinc deficiency is associated with specific transcriptional alterations in brain tissue that may account for some of the distinct phenotypic maladies observed in zinc deficiency.
5.2 INTRODUCTION

Zinc exhibits a broad array of molecular functions in many physiological systems. This includes the central nervous system (CNS), where the importance of zinc in brain function and neurobiology is widely acknowledged. Although many human neurological diseases have been linked to zinc deficiency, the mechanisms by which dietary zinc deficiency may lead to pathology in the brain are not yet clear. Given that zinc deficiency continues to be a substantial health problem in both developing (1) and developed countries (2), knowledge of the relationship between zinc status and brain function is of critical importance.

Zinc, an essential trace element, is a constituent of hundreds of proteins involved in a wide array of physiological processes including intermediary metabolism, hormonal regulatory pathways and immune defense (3). It is also essential for normal brain function where it plays a central role in neurological structure, metabolism and signaling pathways (4). Notably, a critical physiological role of zinc as an antioxidant has been proposed (5), and indeed many neurological disorders linked to zinc have been associated with elevated levels of oxidative tissue damage. Recently it has been hypothesized that the neuroprotective effects of zinc are mediated by its antioxidant properties (6). However, the broad spectrum of pathologies associated with zinc deficiency, in brain as
well as other tissues (7), indicate that its physiological function extends well beyond its role as an antioxidant.

Regulation of gene expression by vitamins, minerals and macronutrients has been well established, however in most cases this influence has only been documented for a small number of genes (8). Several examples of the role of zinc as a regulator of gene expression has been documented (9), including its role as a regulator of metallothionein gene expression (10). However the specific pathways that become dysfunctional during zinc deficiency have not been identified, particularly in the brain. In this paper we utilized a high-density oligonucleotide microarray approach to screen the brain transcriptome during zinc deficiency. Through this technique we have identified 42 genes that are responsive to zinc deficiency in the brain in vivo. The observed changes provide a starting point for defining the pathological sequelae of zinc deficiency related brain dysfunction.
5.3 MATERIAL AND METHODS

**Zinc deficient diet studies.** Young adult (20±4 g, ~ 4 weeks old) male mice from our breeding colony were maintained individually in hanging stainless steel cages on a 12 hour light:dark cycle with free access to deionized water. After weaning at 3 weeks of age, mice were maintained on an AIN76g-based pelleted diet containing 40 mg Zn/kg diet (Dyets Inc, Bethlehem, PA) for 5-7 days. Mice were then randomly assigned to one of two dietary groups, i.e. zinc deficient (ZnDF, <1 mg Zn/kg) or zinc adequate (40 mg Zn/kg), and fed ad libitum. After a 4 week feeding period, between 0900-1200 hours, mice were killed by decapitation and trunk blood collected for subsequent serum zinc measurement by flame atomic absorption spectrophotometry. The brain and pancreas was removed, weighed, rinsed in ice-cold saline, immersed in liquid nitrogen and stored at –80°C until further analysis.

**Plasma zinc analysis** Plasma zinc was determined by atomic absorption spectrophotometry (AAS) using a Varian Spectra AA-5 (Varian Techtron Pty Limited, Mulgrave, Australia). Briefly, 1 ml of plasma was mixed with 4 ml of 2N HCl and incubated with gentle agitation for 24 hours. The suspension was then centrifuged at 7000 x g for 30 minutes, and the supernatant used for direct measurement of zinc.
Standard reference material (SRM 1577, National Institute of Standards and Technology, Gaithersburg, MD) was analyzed simultaneously to insure validity of the method.

**Metallothionein protein measurement.** Prior to biochemical analysis, frozen pancreas was homogenized on ice with a Dounce ground-glass tissue homogenizer (Fisher Scientific, Pittsburgh, PA) in 4 volumes of sucrose buffer (0.25M sucrose, 1.0mM EDTA, pH 7.4) containing the following protease inhibitors to prevent protein degradation during sample preparation: aprotinin (0.5ug/ml), leupeptin (0.5 ug/ml), pepstatin (0.7 ug/ml) and phenylmethylsulfonyl fluoride (40 ug/ml) (11). Homogenate was centrifuged at 600 x g for ten minutes to remove the nuclei, and the cytosol was obtained from the post-nuclear supernatant by centrifugation at 5000 x g for 20 minutes. Western blot analysis of metallothionein was accomplished using a modified procedure (12). Pancreas homogenate (20 µg total protein) was diluted 1:1 with sample buffer (10mM Tris-HCl, 10mM EDTA, 20% (v/v) glycerol, 1.0% (w/v) SDS, 0.005% (w/v) bromophenol blue, 100mM dithiothreitol) and then separated in an 18% polyacrylamide gel at 100 V for 2 hours. Membrane transfer was performed in CAPS buffer (10mM 3-cyclohexylamino-1-propane sulfonic acid, 2 mM CaCl₂, 10% methanol, pH 10.8) followed by membrane incubation in 2.5% glutaraldehyde in water for 1 hour at room temperature. Glutaraldehyde treated membranes were then washed 3 times for 10 minutes in phosphate buffer (8.1 mM Na₂HPO₄, 1.2 mM KH₂PO₄, 2.7 mM KCl, pH 7.4); 50 mM monoethanolamine was added to the third wash to quench residual glutaraldehyde reactivity. Membranes were blocked in 3% BSA (Sigma). Membranes were then incubated with mouse anti-human primary antibody (Zymed Laboratories, San Francisco,
CA) at a 1:1000 dilution at 37°C for 2 hours in PBS containing 2% non-fat dried milk. After washing 3 times in 0.01% Tween 20 in PBS, membranes were incubated with a secondary antibody (rabbit anti-mouse; Sigma) at a dilution of 1:2000, for 2 hours at 37°C. Their respective primary antibodies (i.e. CuZnSOD, GSH-Px, catalase: sheep anti-human). After incubation, each membrane was washed a second time as outlined above, and blots were developed via enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ).

RNA isolation and cDNA array analysis. Total RNA was extracted from the brain tissue using TRIZol reagent (Life Technologies, Inc., Gaithersburg, MD). Briefly, frozen brain samples were homogenized in 12 volumes of TRIZol in Dounce ground-glass homogenizers. After a 5 minute incubation, 1.2 mL of chloroform was added to the sample and then centrifuged for 10 minutes at 10,000 x g at 4°C. The upper aqueous layer containing the RNA was then removed, transferred to an eppendorf tube, mixed with an equal volume of isopropanol and centrifuged again for 10 minutes at 10,000 x g at 4°C. The RNA pellet was collected, washed twice with 70% ethanol and resuspended in DNase/RNase free water. A cleanup procedure was then used (RNeasy mini kit, Qiagen, Valencia, CA) to purify the total RNA. Total RNA quantity was measured by determining the optical density at 260nm (1 O.D. equals 40 ug RNA per ml). RNA quality was determined by evaluating the A_{260}/A_{280} ratio (pure RNA yields a ratio of 2.0 in 10mM Tris-HCl, pH 7.5) and examining RNA bands following electrophoresis in a 1.0% agarose gel.
First and second strand cDNA synthesis was performed using the SuperScript Choice System (Life Technologies, Inc.) according to the manufacturers instructions, but using the T7-(dT)24 primer containing the T7 RNA polymerase binding site. Double-stranded DNA (dsDNA) was recovered via phenol:chloroform:isoamyl alcohol extraction. Biotin-labelled cRNA was synthesized \textit{in vitro} using the MEGAscript In Vitro transcription kit (Ambion Inc., Austin, TX). Following the IVT reaction, biotin-labelled cRNA cleanup and removal of unincorporated nucleotides was performed using RNeasy spin columns (Qiagen).

Array hybridization was performed by injecting 10 ug of biotin labeled cRNA into the MG-U74Av2 array chip that contains 12,488 probe sets (MGU74v2A) corresponding to more than 6000 full length sequences and 6000 expressed sequence tags (EST’s) (Affymetrix, Santa Clara, CA). The gene chip and cRNA were incubated at 45°C for 16 hours, and unhybridized cRNA removed by washing. cRNA hybridization was then measured via fluorescence scanning using a Hewlett-Packard GeneArray Scanner (Model 900154, Affymetrix). The presence or absence of specific gene is calculated by Affymetrix software algorithms. Briefly, the software calculates the difference in signal intensity between a perfect match (a 25-oligomer of a specific gene sequence) and a mismatch (a 25-oligomer containing one incorrect base) pair for a single gene. For the MG-U74Av2 array chip there are 16 perfect match/mismatch pairs, each containing a different 25 oligo sequence for each specific gene. These intensity values are integrated into a decision matrix which determines whether or not the specific transcript is detected (present call) or not detected (absent call) in the sample. The
average of the differences between perfect match and mismatch signals is used to estimate relative mRNA transcript levels.

For each treatment group, three independent samples, or replicates, were used. The criteria used to detect differences in gene expression was a fold-change of 1.5 or greater only when the gene was present in all three replicates and statistical analysis revealed a significant difference between treatments (Student’s t test, $P < 0.05$). This arbitrary threshold was chosen because previous work has shown that Northern blot analysis yields even greater fold-change in transcript levels than detected by array analysis (13).
5.4 RESULTS

**Dietary protocol**  Initial diet studies included a third group, a zinc pair-fed group that received zinc deficient diet in a quantity equally to the allotment of the diet consumed by zinc deficient (ZnDF) mice. However, consumption of a zinc-deficient diet did not alter the feed intake of mice during the 4 week feeding period. Consequently, this group was dropped from the experiment. Zinc status was evaluated in the two dietary treatment groups (ZnDF, ZnAL) by two independent methods: serum zinc analysis and pancreatic metallothionein (MT) expression. Table 5.1 demonstrates that serum zinc and pancreatic MT were significantly depressed in ZnDF animals, thus verifying that zinc deficiency was successfully produced in these animals. Notably, brain zinc concentration did not change between ZnAL and ZnDF animals, consistent with previous work in our lab.

**Overview of gene expression in ZnAL and ZnDF.**  Of the 12,488 probe sets, 6391 (51.2%) were expressed in the ZnAL group and 6432 (51.5%) were expressed in the ZnDF group. Data indicated that the majority of genes remained unchanged in zinc deficiency (Figure 5.1). A total of 488 genes (3.9%) exhibited more than a 1.5 –fold change in expression in the ZnDF group compared to the ZnAL group. After filtering for present calls in all 3 replicates for at least one of the diet treatment groups and a
statistically significant difference between groups of P<0.05, this number was further reduced to 42; 17 probe sets were found to be down-regulated in zinc deficiency, and 25 were up-regulated (Table 5.2 and Table 5.3).

Zinc deficiency down-regulates genes encoding biosynthetic and metabolic proteins. Several genes involved in biosynthetic pathways such as protein synthesis, polyamine metabolism and RNA splicing were down-regulated in zinc deficiency (Table 5.2). These include RNA polymerase 1-1 (Rpo1-1), an enzyme located in the nucleolus that transcribes ribosomal RNA and is therefore integral to protein synthesis pathways (14), and ornithine decarboxylase antizyme (Oaz1), the only protein thus far recognized that regulates ornithine decarboxylase and prevents toxic accumulation of polyamines (15).

Genes involved in RNA splicing were also down-regulated, including splicing factor proline/glutamine rich (Sfpg), an essential pre-mRNA splicing factor (16), neural-salient serine/arginine-rich (NssR) protein which functions in ribonucleotide complexes and exhibits RNA splicing activity in vitro (17), and RNA binding motif protein (Rbmx), an RNA binding protein involved in gene splicing (18). These findings imply that important pre-mRNA processing and metabolic pathways might be disrupted in brain tissue during zinc deficiency.

Zinc deficiency down-regulates genes encoding structural proteins and proteins associated with memory formation. Zinc deficiency also led to the down-regulation of genes involved in structural roles (Table 5.2), including catenin src, a prominent protein of synapses that serves as a component of adherens junctions (19), and integrin alpha 3
(Itga3), which in addition to other roles, plays an important role in building and maintaining synaptic structure (20). In addition to its structural role, the gene product of Itga3, as well as Rho-associated coiled-coil forming kinase 1 (Rock1), have been associated with memory formation (21). These results may have relevance to animal studies demonstrating that during a period of zinc deprivation in animals, learning, attention and memory are adversely affected (22).

**Zinc deficiency up-regulates genes involved in development.** Several genes involved in regulating developmental processes were upregulated in zinc deficiency (Table 5.3). Hox 1.3 (proposed new name, Hoxa-5), which has been shown to be actively taken up in neurons (23), and enhancer trap locus 1 (Etl1) were upregulated in zinc deficiency. Similarly, Unc4.1 homeobox (Uncx4.1) which has been detected in the developing spinal cord, hindbrain, mesencephalon, and telencephalon (24), and neural cell adhesion molecule 2 (Ncam2) which, in addition to its role in cell adhesion, also functions in developmental events such as cell migration, proliferation and differentiation (25), were also upregulated in the brain of zinc deficient animals. These data contrasts with recent work in mouse embryo’s demonstrating a significant reduction in the expression of homeobox gene’s in zinc deficiency (26)

**Zinc deficiency results in up-regulation of genes associated with tumorigenesis.**

Three genes associated with tumor development and progression were found to be upregulated in brain tissue of zinc deficient mice (Table 5.3). Matrix metalloproteinase 15 (MMP-15; MT2-MMP) is a member of a group of membrane-bound MMPs that degrade
extracellular matrix proteins and may play a major role in tumor cell invasion and metastasis (27). N-myc protein (N-myc) is a member of the myc family of transcription factors involved in many aspects of cell physiology including cell proliferation and apoptosis (28). Notably, N-myc is amplified in about 25% of neuroblastomas, an overexpression of this gene is associated with the development of tumors in childhood (29). Finally, although less well characterized, the gene ets variant gene 6 (Etv6) has been identified in both infantile fibrosarcoma and cellular mesoblastic nephroma and there is strong evidence that this gene may play a primary role in breast carcinoma (30).
5.5 DISCUSSION

In this study we have used a microarray expression profiling approach to isolate a number of transcripts with significant differential expression in the brain of zinc deficient mice compared with mice fed a zinc adequate diet. In so doing, we have provided the first global assessment of zinc sensitive genes in the brain. We have also generated a catalogue of genes that exhibit differences in expression and which may contribute to the neuropathologies associated with zinc deficiency. Indeed, these findings suggest that gene expression profiling may be a valuable tool for identifying the molecular mechanisms that mediate pathologies associated with zinc deficiency.

In these experiments, we have identified 42 genes that exhibit significantly altered expression patterns in the brain of zinc deficient mice; 25 were up-regulated and 17 were down-regulated. Notably, many of these genes share similar functional characteristics. For example, decreases in mRNA level for genes intimately involved in nucleic acid and protein synthesis were observed in zinc deficiency. In particular, the down-regulation of genes specifically involved in RNA processing, including \textit{Rpo1-1}, \textit{Sfpg}, \textit{NssR} and \textit{Rbmx}, illustrate the deleterious effects of zinc deficiency on protein synthesis. That is, alterations in RNA processing have major implications in mRNA translation and subsequent protein synthesis in neurons during zinc deficiency. Notably, suppression of
protein synthesis is a common response of cells to severe stress (31). However, early recovery of protein synthesis has been shown to be a critical determinant for the prevention of neuronal injury in a mouse model of brain trauma (32). A critical interaction between zinc, brain function and protein synthesis was recently illustrated in a human study of closed head injury patients (33). In this trial, patients supplemented with zinc during the recovery period exhibited improved neurological recovery rates compared to non-supplemented controls. Moreover, improvements in neurological function tests coincided with evidence of enhanced protein synthesis in the zinc-supplemented patients. Although clearly this data does not establish a cause and effect relationship, it is nevertheless consistent with zinc functioning as a critical intermediary between protein synthesis and brain function.

Impaired nucleic acid and protein synthesis may also impair memory formation in zinc deficiency. Specifically, the formation of long-term memory (LTM) is dependent upon mRNA and protein synthesis at critical time points around the time of training (34), unlike short-term memory that is protein and RNA synthesis-independent. In the data presented herein, zinc deficiency down-regulated several genes that may play a pivotal role in long-term memory formation. For example inhibition of Rock1, a protein kinase that regulates dendrite and axon morphology, has been shown to impair long-term memory in rats (35). Down-regulation of Itga3 in zinc deficiency may also adversely affect memory formation, as Itga3-containing integrins are localized at synapses and are needed to establish long-term memory (21). Notably, a recent series of studies have demonstrated that zinc supplementation in children with low zinc status markedly
improved several indices of psychomotor performance, including recognition memory (36, 37).

Zinc deficiency also led to the up-regulation of 25 genes. Among these are 4 genes, *Uncx4.1, Hoxa5, Est1l* and *Ncam*, that function in cell fate determination and differentiation during development (38). Curiously, two of these genes have also been associated with a nondifferentiated state, cellular proliferation and tumorigenesis. For example, overexpression of *Hoxa5* leads to transformation and tumorigenicity in two fibroblast cell lines (39), and overexpression of other members of the Hoxa family have been implicated in the enhanced growth of metastatic melanomas (40). Recently, a positive association between Ncam expression and metastasis has been observed in a human study of malignant melanoma (41). However, it should be noted that these observations represent potential function, since examples of induction of differentiation can also be cited, and further studies are required to fully elucidate the role of these genes in cellular proliferation and differentiation.

Several other genes associated with tumor development were also found to increase in zinc deficiency. The gene *Nmyc* belongs to the *myc* gene family that functions in cell proliferation and differentiation, but which has also been implicated in tumorigenesis as a functional consequence of overexpression (42). Amplified expression of *Nmyc* occurs in about 25% of neuroblastomas, and overexpression of this gene causes neuroblastoma in transgenic mice (43). Up-regulation of matrix metalloproteinase 15 (MMP-15 or MT2-MMP) has also been linked tumor formation, and a recent study has demonstrated that MT2-MMP is expressed predominantly in glioblastoma tissue and that its expression level increases as tumor grade increases (44). However, a causal
relationship between zinc deficiency and tumorigenesis is tenuous. Nevertheless, epidemiological evidence demonstrates decreased zinc status in cancer patients compared with healthy controls (45), and carcinogen induced esophageal cancers develop with a significantly increased frequency in zinc-deficient compared to control animals (46). Recently, low intracellular zinc was found to induce oxidative DNA damage, disrupts p53 tumor suppressor gene function, and affects DNA repair in a rat glioma cell line in a manner consistent with tumor promotion (47). These and other data suggest that zinc deficiency may play a significant role in cancer development and that it may be a significant factor in tumorigenesis in brain tissue.

This study demonstrates the feasibility of global gene expression profiling of brain tissue and lays the foundation for future work investigating the role of zinc in brain function. We speculate that the differential expression of genes involved in RNA and protein synthesis, memory formation, tumorigenesis as well as developmental processes reflect the mechanisms associated with zinc deficiency-mediated neuropathologies. While there is no doubt that gene expression profiling is highly speculative in nature, it is also true this line of work will provide new avenues for studying nutrient-gene interactions in the brain and further our understanding of the role of nutrition in brain function.
Figure 5.1. Range of the average fold change of differentially expressed genes in the brain of ZnAL and ZnDF mice. Pair-wise comparisons were generated from 3 replicates in each group. Average fold changes were calculated for both the up-regulated and down-regulated genes in response to zinc deficiency.
## Table 5.1. Zinc status indicators for zinc adequate (ZnAL) and zinc deficient (ZnDF) mice.

Mice were fed either ZnAL (40mg Zn/kg diet) or ZnDF (<1 mg Zn/kg diet) for 4 weeks. Values are mean ± SD, n=3 per treatment. *Significantly different from ZnAL treatment group (p<0.005).

<table>
<thead>
<tr>
<th>Variable</th>
<th>ZnAL</th>
<th>ZnDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum zinc (umol/L)</td>
<td>1.233±0.14</td>
<td>0.667±0.12*</td>
</tr>
<tr>
<td>Pancreas metallothionein (ug/g protein)</td>
<td>183.7±31.3</td>
<td>14.3±7.80*</td>
</tr>
<tr>
<td>Brain zinc (ug/g tissue)</td>
<td>5.45±0.18</td>
<td>5.36±0.20</td>
</tr>
<tr>
<td>GenBank No.</td>
<td>Gene description</td>
<td>Fold Change</td>
</tr>
<tr>
<td>------------</td>
<td>------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>AA690583</td>
<td>splicing factor</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>proline/glutamine rich</td>
<td></td>
</tr>
<tr>
<td>AI835044</td>
<td>DNA segment, Chr 14, Wayne State University 146</td>
<td>2.6</td>
</tr>
<tr>
<td>AI848793</td>
<td>distal-less homeobox 6</td>
<td>1.5</td>
</tr>
<tr>
<td>U63720</td>
<td>caspase 3</td>
<td>1.5</td>
</tr>
<tr>
<td>Z17804</td>
<td>catenin src</td>
<td>2.0</td>
</tr>
<tr>
<td>AI840339</td>
<td>Neural-salient serine/arginine-rich</td>
<td>1.7</td>
</tr>
<tr>
<td>AJ237847</td>
<td>RNA binding motif protein,</td>
<td>1.9</td>
</tr>
<tr>
<td>AW125751</td>
<td>RIKEN cDNA 2210412D01</td>
<td>1.6</td>
</tr>
<tr>
<td>AI840339</td>
<td>ribonuclease, RNase A family 4</td>
<td>1.9</td>
</tr>
<tr>
<td>AA245183</td>
<td>mab-21-like 2</td>
<td>1.8</td>
</tr>
<tr>
<td>AF026524</td>
<td>inhibitor of kappaB kinase beta</td>
<td>8.4</td>
</tr>
<tr>
<td>AW228036</td>
<td>Traf and Tnf receptor associated protein</td>
<td>6.6</td>
</tr>
<tr>
<td>AV212241</td>
<td>ornithine decarboxylase antizyme</td>
<td>13.1</td>
</tr>
<tr>
<td>AV148041</td>
<td>RNA polymerase 1</td>
<td>1.7</td>
</tr>
<tr>
<td>D13867</td>
<td>integrin alpha 3</td>
<td>8.6</td>
</tr>
<tr>
<td>U58512</td>
<td>Rho-associated coiled-coil forming kinase 1</td>
<td>1.6</td>
</tr>
<tr>
<td>D32137</td>
<td>oviductal glycoprotein 1, 120kD</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 5.2. List of genes that are down-regulated in brain tissue of zinc-deficient mice.
<table>
<thead>
<tr>
<th>GenBank No.</th>
<th>Gene description</th>
<th>Fold Change</th>
<th>P value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>M59821</td>
<td>immediate early response 2</td>
<td>2.7</td>
<td>0.027</td>
<td>Cell growth &amp; communication</td>
</tr>
<tr>
<td>X69942</td>
<td>enhancer trap locus 1</td>
<td>5.0</td>
<td>0.046</td>
<td>ATP-binding</td>
</tr>
<tr>
<td>U08337</td>
<td>growth differentiation factor 5</td>
<td>1.5</td>
<td>0.046</td>
<td>Cell growth &amp; communication</td>
</tr>
<tr>
<td>AF001287</td>
<td>neural cell adhesion molecule 2</td>
<td>11.8</td>
<td>0.001</td>
<td>Cell adhesion &amp; communication</td>
</tr>
<tr>
<td>AA717740</td>
<td>RIKEN cDNA 4930434H03</td>
<td>1.5</td>
<td>0.037</td>
<td>Unknown</td>
</tr>
<tr>
<td>AI465845</td>
<td>similar to CG4946 gene</td>
<td>1.5</td>
<td>0.004</td>
<td>Unknown</td>
</tr>
<tr>
<td>U91933</td>
<td>adaptor-related protein Ap3s2</td>
<td>1.8</td>
<td>0.018</td>
<td>Cell growth &amp; maintenance</td>
</tr>
<tr>
<td>U89993</td>
<td>lymphocyte-specific adaptor protein Lnk</td>
<td>2.0</td>
<td>0.011</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>D86332</td>
<td>matrix metalloproteinase 15</td>
<td>2.4</td>
<td>0.014</td>
<td>Extracellular matrix metalloproteinase</td>
</tr>
<tr>
<td>AW121327</td>
<td>RIKEN cDNA 2610022K04</td>
<td>35.1</td>
<td>0.043</td>
<td>Unknown</td>
</tr>
<tr>
<td>M15268</td>
<td>aminolevulinic acid synthetase 2,</td>
<td>2.5</td>
<td>0.025</td>
<td>Metabolism</td>
</tr>
<tr>
<td>AJ001116</td>
<td>Unc4.1 homebox</td>
<td>13.1</td>
<td>0.040</td>
<td>Development</td>
</tr>
<tr>
<td>Y09974</td>
<td>TATA box binding protein</td>
<td>4.9</td>
<td>0.003</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>AV244715</td>
<td>multiple PDZ domain protein</td>
<td>2.5</td>
<td>0.027</td>
<td>Unknown</td>
</tr>
<tr>
<td>AV232610</td>
<td>Hermansky-Pudlak syndrome 1 protein</td>
<td>1.7</td>
<td>0.025</td>
<td>Cell trafficking</td>
</tr>
<tr>
<td>AV276689</td>
<td>GPI-anchored membrane protein 1</td>
<td>1.6</td>
<td>0.047</td>
<td>Unknown</td>
</tr>
<tr>
<td>AA016517</td>
<td>RIKEN cDNA 1500005I02 gene</td>
<td>1.7</td>
<td>0.006</td>
<td>Unknown</td>
</tr>
<tr>
<td>AI845538</td>
<td>ets variant gene 6</td>
<td>1.8</td>
<td>0.017</td>
<td>Cell growth, cell cycle regulation</td>
</tr>
<tr>
<td>Y00208</td>
<td>Mouse Hox 1.3 gene</td>
<td>2.5</td>
<td>0.016</td>
<td>Development</td>
</tr>
<tr>
<td>M12731</td>
<td>N-myc protein</td>
<td>4.1</td>
<td>0.013</td>
<td>Cell growth, cell cycle regulation</td>
</tr>
<tr>
<td>AW121646</td>
<td>similar to KIAA1404</td>
<td>1.6</td>
<td>0.043</td>
<td>Unknown</td>
</tr>
<tr>
<td>M24377</td>
<td>Early growth response 2</td>
<td>2.0</td>
<td>0.049</td>
<td>Cell growth &amp; communication</td>
</tr>
<tr>
<td>AF062484</td>
<td>sorting nexin 12</td>
<td>1.9</td>
<td>0.004</td>
<td>Cell communication &amp; transport</td>
</tr>
<tr>
<td>AI846345</td>
<td>synaptobrevin 2 binding protein</td>
<td>2.6</td>
<td>0.006</td>
<td>Cell growth &amp; communication</td>
</tr>
</tbody>
</table>

**Table 5.3.** List of genes that are up-regulated in brain tissue of zinc-deficient mice.
5.6 REFERENCES


132


CHAPTER 6

CONCLUSIONS

Bronchopulmonary dysplasia (BPD) and cerebral palsy are the two most prominent manifestations of a diverse array of disorders collectively referred to as diseases of prematurity. Although many factors may contribute to the development of these diseases, low antioxidant status and exposure to hyperoxia continue to be variables closely associated with their incidence. Because of this, it has been hypothesized that oxidative stress may account for many of the complications associated with prematurity. Indeed, increased ROS generation has been documented in both lung and brain tissue in vivo during exposure to hyperoxia. It is noteworthy that at present, the World Health Organization does not have specific guidelines regarding the resuscitation and implementation of oxygen therapy of preterm infants, despite the much greater need of this population for such treatments. Moreover, the incidence of both bronchopulmonary dysplasia and cerebral palsy, the two most prominent disease manifestations associated with premature birth, have not changed in the last two decades. Obviously there is an urgent need to develop a better understanding of the mechanisms that lead to the development of these diseases if effective treatment and prevention strategies are to be developed.
In these experiments we have examined the effects of low zinc nutritional status, reduced antioxidant enzyme (i.e. CuZnSOD) activity and exposure to hyperoxia on oxidative tissue damage in lung and brain tissue as a model of the precarious situation encountered by premature infants. In addition, we examined the stress response of antioxidant metalloproteins that may play a critical role in maintaining homeostasis under oxidative stress, and have also performed a global analysis of gene expression in the brain of zinc deficient animals using cDNA microarray technology.

In this dissertation research, it was found that exposure to hyperoxia significantly impaired the antioxidant defense system, reducing the activity of the primary antioxidant enzyme catalase approximately two-fold in the lung. However, an increase in the expression of ceruloplasmin and metallothionein in lung tissue, without affecting tissue levels of iron, copper or zinc, was also observed. These results suggest that these metalloproteins may act as antioxidants independent of their role in trace metal homeostasis and indicate that strategies designed to induce their expression during periods of stress may provide clinical benefit.

It was also demonstrated that CuZnSOD is a critical antioxidant defense enzyme in the brain, as oxidative stress, as measured by glutathione oxidation, increased progressively with declining CuZnSOD activity in mice exposed to hyperoxia. Moreover, the expression of HO-1 was found to be up-regulated in response to hyperoxia and reduced CuZnSOD gene expression. These findings corroborate the concept that the stress response protein HO-1 is highly sensitive to oxidative stress and that it may serve as an important neuroprotective agent during periods of oxidative stress.
In the deficiency studies, we did not observe any increased oxidative tissue damage in the brain as a result of either zinc deficiency or exposure to hyperoxia. However, in the gene array study we observed significant changes in the expression of genes involved in nucleic acid and protein synthesis as well as genes associated with memory consolidation and tumorigenesis. These observations provide evidence that zinc deficiency is associated with specific transcriptional alterations in brain tissue and provides the groundwork for future investigations into the role of zinc in brain function.

In summary, this work has moved us forward in our understanding of the roles of antioxidant defense and oxidative stress, critical determinants in the development of diseases associated with premature birth. Collectively, the results of these experiments demonstrate that reduced antioxidant enzyme activity and exposure to hyperoxia increase oxidative tissue damage in both lung and brain tissue, and that in the brain, a significant number of genes were differentially expressed in response to zinc deficiency. There is little doubt that in the future, advances in molecular biology techniques, gene expression technology and even behavior analysis will further our ability to understand the interplay between nutrition and gene expression. This will unquestionably enhance our understanding of the mechanistic processes that prevail in disease states and thereby lead to better treatment strategies.


