NITRATION AND INACTIVATION OF MANGANESE SUPEROXIDE DISMUTASE PLAYS A CRITICAL ROLE IN METABOLIC SWITCH

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ABSTRACT OF DISSERTATION

Muthuswamy Anantharaman

The Graduate School
University of Kentucky
2008
NITRATION AND INACTIVATION OF MANGANESE SUPEROXIDE DISMUTASE
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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Graduate School at the University of Kentucky

By
Muthuswamy Anantharaman

Director: Dr. Daret St. Clair, Professor, Graduate Center for Toxicology
University of Kentucky, Lexington, Kentucky
2008
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ABSTRACT OF DISSERTATION

NITRATION AND INACTIVATION OF MANGANESE SUPEROXIDE DISMUTASE PLAYS A CRITICAL ROLE IN METABOLIC SWITCH

Alzheimer’s disease (AD) is a multifactorial, progressive, age-related neurodegenerative disease. Oxidative stress hypothesis is most prevalent and is gaining significant support. Inspite of the progress achieved on oxidative stress related damages in AD brain; the modification occurring on the various cellular antioxidant enzymes antioxidant has not been identified. Tyrosine nitration, a marker for peroxynitrite induced oxidative damage to protein is widespread in AD brain and Manganese superoxide dismutase (MnSOD), primary mitochondrial antioxidant enzyme is prone to peroxynitrite induced nitration and inactivation. Nitration of proteins involved in energy metabolism has been demonstrated in AD brain, which may explain the altered glucose metabolic status existing in AD brain. In the present study, we investigated the effect of tyrosine nitration of MnSOD on energy metabolism by the use of AD mouse model and cultured neuronal cells. The AD mouse model was generated from a double homozygous knock-in mouse, designated as APP/PS-1 mice, by incorporating the Swedish familial AD mutations in APP and P264L familial AD mutation in PS – 1. These animals develop age dependent increase in Aβ deposition beginning at 6 months along with an increase in insoluble Aβ1-40/Aβ1-42 levels. Genotype and age associated increase in nitration of MnSOD without any change in protein levels was also observed. MnSOD activity and mitochondrial respiration was decreased in APP/PS-1 mice. There was also concomitant increase in levels of lactate, an index of glycolytic activity in APP/PS-1 mice. To directly investigate the role of MnSOD inactivation in mitochondrial function and subsequent alteration in glycolytic activity, SH-SY5Y neuroblastoma cells line was used and treated with peroxynitrite. Enhanced nitration and reduction in the activity of MnSOD was observed upon peroxynitrite treatment. Peroxynitrite treatment also induced mitochondrial dysfunction, but MnSOD was inactivated at a concentration of peroxynitrite 10 times lower than that required to inhibit mitochondrial respiration. Mitochondrial dysfunction was alleviated by SOD mimetic and reproduced by MnSOD siRNA. The decline in mitochondrial function did not result in decreased ATP levels but was accompanied by an up-regulated glycolysis signified by high levels of lactate and lactate dehydrogenase activity but decreased activity of pyruvate dehydrogenase. These
changes were prevented by SOD mimetic and were promoted by MnSOD siRNA. Specific reduction of MnSOD in MnSOD heterozygous knock-out mice resulted in decreased RCR and complex I activity with increased lactate levels. Taken together, these data demonstrate a critical role of MnSOD in influencing the mitochondrial function and thereby the switch in the energy metabolism switch that might occur under the pathological condition of MnSOD deficiency.

KEYWORDS: Alzheimer’s disease, Mitochondria, MnSOD, Nitration, Energy Metabolism

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for my friends and family
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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii

LIST OF TABLES ................................................................................................................... viii

LIST OF FIGURES ................................................................................................................. ix

CHAPTER ONE

NEURODEGENERATIVE DISEASE, ALZHEIMER’S DISEASE, MANGANESE SUPEROXIDE DISMUTASE AND PEROXYNITRITE

Introduction ......................................................................................................................... 1

Neurodegeneration and Neurodegenerative diseases (NDD) ............................................. 2

Alzheimer’s Disease and its Prevalence .............................................................................. 4

Neuritic plaques .................................................................................................................. 6

Neurofibrillary tangles and AD ......................................................................................... 8

Classification of Alzheimer’s disease ................................................................................ 9

Etiopathogenesis of Alzheimer’s disease ......................................................................... 10

Oxidative Stress ................................................................................................................. 13

Antioxidant systems ......................................................................................................... 14

Manganese superoxide dismutase ...................................................................................... 16

Nitration of MnSOD and nitrotyrosine in Alzheimer’s disease ........................................ 17

Peroxynitrite ...................................................................................................................... 19

Energy metabolism .......................................................................................................... 20

Altered metabolism in Alzheimer’s disease ................................................................... 24

Research Objectives ......................................................................................................... 25

CHAPTER TWO

β - AMYLOID MEDIATED NITRATION OF MANGANESE SUPEROXIDE DISMUTASE - Implication for Oxidative Stress in a APPNLh/NLh X PS-1P264L/P264L Double Knock-In Mouse Model of Alzheimer’s Disease

Abstract ............................................................................................................................. 27

Introduction ....................................................................................................................... 28
Materials and Methods

Mutant mouse lines ......................................................... 30
Genotyping of mice ......................................................... 30
Immunocytochemistry for Aβ .................................................. 31
Tissue Preparation for Aβ Enzyme-Linked Immunosorbent Assay (ELISA) ...................................................... 31
Sandwich ELISA to Determine the Levels of Insoluble Brain Aβ1-40 and Aβ1-42 .................................................. 31
Mitochondrial Isolation ...................................................... 32
Measurement of Mitochondrial Respiration ................................ 32
Immunoprecipitation ......................................................... 33
Western Blot Analysis ....................................................... 33
MnSOD Activity Assay ....................................................... 34
Statistical Analysis ......................................................... 34

Results

Aβ Deposition in APP/PS-1 Mice ........................................... 35
Increased Levels of Aβ1-40 and 1-42 Fractions in APP/PS-1 Mice ...... 35
Unchanged MnSOD Protein Levels in WT and APP/PS-1 Mice ........ 35
Increased Nitration of MnSOD in APP/PS-1 Mice .................... 39
Decreased SOD Activity in APP/PS-1 Mice ......................... 39
Decreased Mitochondrial Respiration in APP/PS-1 Mice .......... 39

Discussion ............................................................................ 45

CHAPTER THREE

PEROXYNITRITE INDUCED NITRATION OF MnSOD REVEALS A NOVEL MECHANISM FOR THE REGULATION OF METABOLIC SWITCH IN NEURONAL CELLS

Abstract ............................................................................. 52
Introduction ........................................................................ 53

Materials and Methods

Materials ................................................................. 56
Cell Culture ............................................................ 57
Animals ................................................................. 57
Isolation of mitochondria .................................................. 57
Immunoprecipitation of Nitrated MnSOD ............................. 58
Assessment of ATP ................................................... 58
Results

Peroxynitrite induced nitration and inactivation of MnSOD in SH-SY5Y cells……………………………………………………………………………………………………64
Alteration in mitochondrial function in SH-SY5Y cells is associated with MnSOD activity……………………………………………………………………………………………………64
SOD mimetic (MnIIIITE-2-PyP5+) protects against peroxynitrite –induced mitochondrial dysfunction……………………………………………………………………………………………………67
MnSOD activity is sensitive to peroxynitrite induced inactivation………67
Peroxynitrite has no effect on cellular ATP levels…………………………………………………………………………………………………………………………………………72
Peroxynitrite mediated increase in glycolytic activity……………………………………………………………………………………………………………………………………………………76
Suppression of MnSOD enhances glycolytic activity…………………………………………………………………………………………………………………………………………………………………………76
Decreased mitochondrial respiration and increased glycolytic activity in sod 2 +/- mice……………………………………………………………………………………………………………………………………………………………………………………………………………………………………79

Discussion…………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………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LIST OF TABLES

Table 1.1 Mechanisms leading to protein oxidation observed in AD..........................11
LIST OF FIGURES

Figure 1.1, Processing of Amyloid precursor protein (APP).........................................7
Figure 1.2, Scheme for production of superoxide and its detoxification..........................15
Figure 1.3, Biochemistry of Peroxynitrite: reaction and fate...........................................21
Figure 2.1, Age-related Aβ deposition in the brains of APP/PS-1 mice...........................36
Figure 2.2, Increased levels Aβ1-40 and 1-42 fractions in APP/PS-1 mice.........................38
Figure 2.3, Unchanged MnSOD protein levels in WT and APP/PS-1 mice.........................40
Figure 2.4, Increased nitration and decreased activity of MnSOD in APP/PS-1 mice..........41
Figure 2.5, Decline in mitochondrial respiration via complex I in APP/PS-1 mice..............44
Figure 3.1, Treatment with peroxynitrite induces nitration and reduced activity of MnSOD in SH-SY5Y cells.................................................................65
Figure 3.2, Peroxynitrite reduces mitochondrial complex I activity and mitochondrial function in SH-SY5Y cells.................................................................68
Figure 3.3, SOD mimetic and MnSOD siRNA alter mitochondrial complex I activity….....70
Figure 3.4, MnSOD activity is more sensitive to peroxynitrite than mitochondrial respiration.................................................................73
Figure 3.5, Peroxynitrite treatment does not alter ATP levels in SH-SY5Y cells..............75
Figure 3.6, Peroxynitrite increases the levels of lactate and activity of lactate dehydrogenase in SH-SY5Y cells.................................................................77
Figure 3.7, Peroxynitrite increases the levels of glucose and suppresses the activity of pyruvatedehydrogenase in SH-SY5Y cells.................................................................78
Figure 3.8, Suppression of MnSOD enhances the levels of lactate and activity of lactate dehydrogenase.................................................................81
Figure 3.9, SOD mimetic (MnIII-TE-2-PyPS+) (1000pg/ml) suppresses the level of lactate and increases the activity of pyruvate dehydrogenase.........................82
Figure 3.10, MnSOD deficiency leads to alteration in oxygen consumption, complex I activity and levels of lactate.................................................................83
Figure 4.1, Increased lactate levels in APP/PS-1 mice.......................................................96
Figure 4.2, Increased activity of lactate dehydrogenase in APP/PS-1 mice.......................97
Figure 4.3, Decreased activity of pyruvate dehydrogenase in APP/PS-1 mice.................98
Figure 5.1, Hypothetical model suggested from the data obtained.................................106
LIST OF ABBREVIATIONS

ATP…………………………..Adenosine triphosphate
Aβ…………………………..Amyloid-beta
APP…………………………..Amyloid precursor protein
AD…………………………..Alzheimer’s disease
bp…………………………..Base pair
CNS………………………..Central nervous system
mM…………………………..Milli molar
μmoles/mg protein…………..micromoles/milligram of protein
μmoles/μg protein…………..micromoles/microgram of protein
μg/ml………………………microgram/milliliter
MnSOD…………………….Manganese superoxide dismutase
nmoles/min/mg protein…….nano moles/minute/milligram of protein
3-NT………………………3 - nitrotyrosine
NDD……………………….Neurodegenerative disease
NFT………………………..Neuro fibrillary tangles
ONOÖ………………….Peroxynitrite
PET……………………….Positron emission tomography
ROS………………………Reactive oxygen species
siRNA………………….Small interfering ribonucleic acid
TPP……………………….Thiamine pyrophosphate
WT………………………….Wild-type
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CHAPTER ONE

NEURODEGENERATIVE DISEASE, ALZHEIMER’S DISEASE, MANGANESE SUPEROXIDE DISMUTASE AND PEROXYNITRITE

INTRODUCTION

The mammalian brain occupies a relatively small percentage of body weight (2%) but consumes an inordinate fraction (20%) of total body oxygen consumption. Oxygen supply is essential for the maintenance of cellular respiration and the production of energy in the brain. ATP produced in the mitochondria, by complete oxidation of glucose to carbon-dioxide and water in the presence of oxygen, is the obligate energy source of the brain. In the process of production of energy in mitochondria, incomplete reduction of oxygen can form superoxide radicals and subsequent reactive oxygen species (ROS) capable of damaging macromolecules. Brain is particularly sensitive to ROS owing to its high level of oxygen consumption and inadequate anti-oxidant defenses (Floyd and Carney, 1992). Oxidative stress hypothesis has been proposed and is gaining momentum towards unraveling the pathogenesis of neurodegenerative diseases including Alzheimer’s disease (AD) (Markesbery, 1997). Oxidative stress is evidenced by protein oxidation, lipid peroxidation (Butterfield and Lauderback, 2002), 3-nitrotyrosine (3-NT) levels (Castegna et al., 2003; Smith et al., 1997), advanced glycation end products and DNA/RNA oxidation products (Mecocci et al., 1994). Peroxynitrite is a consistent mediator of 3-NT formation (Smith et al., 1997; Yamakura et al., 1998). Manganese superoxide dismutase (MnSOD), the primary anti-oxidant enzyme in the mitochondria is highly sensitive to peroxynitrite induced nitration of its tyrosine residues and thereby becomes inactivated (MacMillan-Crow et al., 1996; Yamakura et al., 1998). Because mitochondria are the major source of superoxide production under physiological conditions, the function of MnSOD in catalyzing the dismutation of superoxide to hydrogen and molecular oxygen is vital in preventing any accumulation of superoxide and thereby oxidative stress. Studies from our lab have demonstrated that MnSOD protects mitochondrial function (Yen et al., 1999). Thus, inactivation of MnSOD
presumably would lead to a compromised mitochondrial function and thereby reduction in cellular energy production from oxidative phosphorylation. Interestingly, in tumors and transformed cells, glycolysis is known to compensate for the loss of mitochondrial function in producing energy for cellular processes (Gatenby and Gillies, 2004). However, whether similar compensation will occur in non-tumor brain tissue is unclear. The following review will focus on the existence of oxidative stress in Alzheimer’s disease, its consequence on MnSOD activity, mitochondrial function and glycolysis and a possible role for MnSOD in these alterations.

NEURODEGENERATION AND NEURODEGENERATIVE DISEASES (NDD)

It is estimated that almost 400 million individuals suffer from neurological diseases in the world (Welsh, 2006b). The word “Neurodegeneration”, is etymologically comprised of ‘neuro’, designating nerve cells, and ‘degeneration’, referring to a process of losing structure or function. Thus, it refers to any pathological condition primarily affecting neurons (Przedborski et al., 2003). NDD are a heterogeneous group of disorders characterized by gradual progression, selectively affecting specific subsets of neuronal populations in specific functional anatomic systems (Lin and Beal, 2006). The inciting or the causative factors of NDD are not clearly understood, but NDD are known to progress relentlessly and are always related to neurons. So, neoplasm, edema, hemorrhage and trauma of the nervous system are not neurodegenerative diseases and neither is multiple sclerosis, which does not affect neurons but affects the myelin sheath.

There are hundreds of known NDD and many of these overlap with each other clinically and pathologically rendering the classification challenging (Burn and Jaros, 2001). To avoid confusion, the classification is based on predominant clinical feature or topography of the predominant lesion. Accordingly, NDD of the CNS is grouped into those of the cerebral cortex, the basal ganglia, the brain stem and cerebellum. But each group is represented by more than one disease, so within each group, a given disease is further classified based on its main clinical features. For example, the cerebral cortex group includes dementing and non-dementing disorders, Alzheimer’s disease being the predominant dementing disorder. Diseases affecting the basal ganglia are characterized
by abnormal movements classified as hypokinetic or hyperkinetic. Parkinson disease is hypokinetic disease while Huntington disease is an example of a hyperkinetic disorder. Cerebellar diseases comprise three main neuropathological types: cerebellar cortical atrophy, pontocerebellar atrophy and Friedreich ataxia. Spinal cord NDD includes Amyotrophic lateral sclerosis and spinal muscular atrophy (Przedborski et al., 2003). With the dawn of molecular biology and advent of state-of-the-art techniques in the past two decades, the classification of NDD, presently is based on molecular characteristics. Neuropathological entities that formerly belonged to very different categories are now grouped together because of common molecular defects have been identified. This classification has given rise to the groups of trinucleotide-repeat diseases (Cummings and Zoghbi, 2000), prion diseases (Prusiner, 1998), synucleinopathies (Galvin et al., 2001) and tauopathies (Goedert and Spillantini, 2001).

Though a great many NDD have been identified, no clear cut picture about the causes has emerged. A risk factor that is consistently linked to the development of NDD is ageing. The probability of developing a neurodegenerative disorder dramatically increases in the sixth, seventh and eighth decades of life. There is a high probability that a person who lives to the age of 85 years will suffer from Alzheimer’s disease (AD). Parkinson’s disease (PD) is most common in those above the age of 70 years and the probability of developing amyotrophic lateral sclerosis (ALS) rises sharply above the age of 40 years (Mattson and Magnus, 2006). The relative contribution of genetic and environmental factors to the etiology of NDD has been a subject of intense research. The genetic basis was suggested based on findings of familial occurrence of NDD. The genetic causation can be autosomal dominant trait (Huntington disease), autosomal recessive trait (familial spastic paraparesis), an X-linked trait (spinal and bulbar muscular atrophy) or a maternally inherited trait. Sporadic experienced are also being thoroughly studied. Oxidative stress has emerged as an important factor in mediating the effect of many environmental and pathological conditions.
ALZHEIMER’S DISEASE AND ITS PREVALENCE

Despite enjoying the longevity nowadays, people have to be aware of the fact that the chances of suffering from dementia would be higher in their senior years. Dementia may be caused by more than 60 different diseases, and affects a significant proportion of the elderly population (Welsh, 2006b). Alzheimer’s disease (AD), the most common of the dementias, is estimated to account for approximately 60 percent of all dementia (Small et al., 1997). Alzheimer’s disease, named for Alois Alzheimer, who first described in 1907, the findings of senile plaques and neurofibrillary tangles in the hippocampus and neocortex of a middle aged woman with memory deficits and progressive loss of cognitive function. Estimates of numbers affected worldwide are greater than 18 million (http://www.alzheimer’s.org), with over 5 million people living in the USA. The epidemic of AD is expected to quadruple by the year 2047. The greatest risk factor for AD is aging with the prevalence of this disease doubling every 5 years after age 65 and approaches 50% by age 85. Although AD is not caused by aging, nor is it an inevitable part of the aging process, it is age-related (Welsh, 2006b). With the proportion of elderly people in the population increasing steadily, and an expected three quarters of the world population over 60 years of age by 2025 (http://www.who.int/ageing/ActiveAgingPolicyFrame.pdf), the financial, societal and emotional costs of AD will be staggering.

AD is a complex age-related neurodegenerative disorder, with retrograde progressive amnesia that is mentally and physically completely debilitating (Dawbarn and Allen, 2007). AD is a slow disease, starting with mild memory loss and ending with severe dementia. As the neuronal cells die slowly, the individual’s ability to function worsens progressively leading eventually to a loss of most functional skills, including the ability to read, reason, communicate and understand spatial relationships. The course the disease takes and speed of progression vary from person to person. On an average, AD patients live from 8 to 10 years after they are diagnosed, though the disease can last for as many as 20 years. Brain regions involved in learning and memory processes, including the temporal and frontal lobes, are reduced in size in AD patients as the result of degeneration of synapses and death of neurons. Because there can be other causes of
memory loss, definitive diagnosis of AD requires postmortem examination of the brain, which must contain sufficient numbers of "plaques" and "tangles" to qualify as affected by AD (Mattson, 2004). These are the classical hallmark pathologies of Alzheimer’s disease as observed by Alois Alzheimer (1864 – 1915) and subsequent researchers (Dickson, 2001). Apart from this, selective neuronal loss and shrinkage, atrophy of the brain, synapse loss, neuropil thread formation and amyloid angiopathy are also manifested in AD brain. A definitive diagnosis cannot be made without neuropathological confirmation. The core of neuritic plaques is composed of β-amyloid (Aβ) that has aggregated into densely packed, insoluble, β-pleated sheets (Peskind, 1996), with dystrophic neurites, abnormal synapses, activated microglia and fibrous astrocytes located on the periphery. Neurofibrillary tangles (NFT) mainly consist of paired helical filaments that are predominantly composed of abnormally phosphorylated tau protein (Peskind, 1996).

Aβ is normally dissolved in the extracellular fluid of the brain and cerebrospinal fluid. But under certain circumstances, it forms into small fibrils which adopt a β-sheet formation, that precipitate out of solution as fibrillar aggregates and are deposited extracellularly as plaques. Aβ plaques vary in size from about 5 to 200 μm across and can be divided as diffuse or neurotic plaques based on their structural appearance. Diffuse plaques consist of homogeneous deposits of fibrillar material without any local reactive glial cells or abnormal neuritic processes. Neuritic plaques have a more heterogeneous sculpted appearance with a central dense core, with peripheral halo. The main component of diffuse plaques is Aβ42 whereas Aβ40 predominates in neuritic plaques. The halo contains additional elements in the form of glial and abnormal swollen neuritic processes. The glial component consists mainly of astrocytic processes and of microglial cells. The formation of Aβ plaques is not entirely specific for AD but also occur in normal aging, Down’s syndrome and some other neurodegenerative diseases. The essential difference between AD and normal aging with respect to plaques is that they are more numerous in AD. Although neuritic plaques are composed predominantly of Aβ, many other molecules are also seem to be concentrated in them. Some of the proteins include enzymes acetylcholinesterase (Mesulam and Asuncion Moran, 1987) and
\( \alpha_1 \) – antichymotrypsin (Abraham et al., 1988), complement components (Eikelenboom and Stam, 1982), apolipoproteins and growth factors and their receptors.

**NEURITIC PLAQUES**

The amino acid sequence of A\( \beta \), the predominant content of neuritic plaque, was first obtained from cerebrovascular amyloid (Glenner and Wong, 1984). The plaque core of amyloid protein from the cerebral cortex of AD brain was also purified and characterized and found to be similar to that of amyloid of cerebrovascular origin found in the brains of Down’s syndrome patients. In all cases A\( \beta \) was found to consist of 40 – 42 amino acid sequence. In 1987, A\( \beta \) peptide was shown to be derived from a larger precursor, amyloid precursor protein (APP) (Figure 1.1) (Goldgaber et al., 1987) and the gene was later localized to the long arm of chromosome 21. APP is synthesized in the endoplasmic reticulum (ER) and glycosylated within the ER and Golgi apparatus. This is a type I transmembrane glycoprotein with a relatively long N-terminal extracellular domain and a short C-terminal cytoplasmic domain. Of the 18 exons which encode for APP, exons 7, 8 and 15, encoding the extracellular domain, are subject to alternative splicing. There are seven known splice variants of the protein; major isoforms being 695, 751 and 770 amino acids (aa) in length. APP is expressed in all tissues, but the exclusive isoform expressed in neurons of the central and peripheral nervous system is APP695 (Dawbarn and Allen, 2007), whereas 751 aa and 770 aa transcripts are expressed both in neural and non-neural cells (Selkoe, 1996). A battery of functions has been attributed to APP including neurite outgrowth, cell adhesion molecule, synaptogenesis and cell survival (Magara et al., 1999; Perez et al., 1997).

A\( \beta \) domain is present between the amino acids 672 and 712/714 of APP which can undergo concerted endoproteolytic cleavage by several proteases namely \( \alpha, \beta \) and \( \gamma \) secretase in either a non-amyloidogenic pathway (NAP) or amyloidogenic pathway (AP) depending on the sequence of action of secretases. In NAP, the APP is first cleaved by \( \alpha \)-secretase, whose activity is ascribed to the members of ADAM (a disintegrin and metallopeptase) family of proteases (ADAM 17, ADAM 10 and ADAM 9) (Dawbarn and Allen, 2007), resulting in secretion of an N – terminal ectodomain fragment called
Figure 1.1  Processing of Amyloid precursor protein (APP)
sAPPα, and the retention of an 83 aa C- terminal fragment called C83 tethered to the membrane. α-secretase cleaves APP at amino acids 687/688, thereby precluding the formation of Aβ. sAPPα may be involved in the enhancement of synaptogenesis, neurite outgrowth and neuronal survival, and is considered to be neuroprotective.

In AP the APP is cleaved by β-secretase between 671/672 aa producing a N terminal fragment called sAPPβ and a C terminal fragment of C99 attached to the membrane. A sequential cleavage of C99 by γ-secretase leads to the formation of Aβ of length 40 or 42 aa (Aβ40 or Aβ42). Under normal conditions very small amounts of Aβ1-40 and Aβ1-42 are formed in the cell; in both CSF and cell culture Aβ1-40 is the major component (50% - 70%), with Aβ1-42 as the minor component (5% - 20%) (Murphy et al., 1999). Neuronal and non-neuronal cells process APP differently. The α-secretase pathway predominates in non-neuronal cells and so little of Aβ is produced.

NEUROFIBRILLARY TANGLES (NFT) AND AD

The primary component of NFTs is the Tau protein. Tau is a microtubule associated protein (MAP) that maintains the microtubule network in neurons for axonal transport. Tau is expressed throughout the central nervous system, but predominantly in neuronal axons. MAP1A/MAP1B, and MAP2 are the other proteins required to promote assembly and stabilization of microtubules. Tau is the smallest, with a biological activity depending on phosphorylation of normally present 2-3 mole of phosphate/mole of protein for optimal activity (Kopke et al., 1993). To date, the most established and the most compelling cause of dysfunctional tau in AD is the abnormal hyperphosphorylation of tau leading to accumulation of tau as intraneuronal tangles of paired helical filaments (PHFs), twisted ribbons and/or straight filaments (SF) (Iqbal et al., 2000). The hyperphosphorylation of tau can be due to a conformational change (Jicha et al., 1997) in diseased brain, making it a better substrate for phosphorylation and/or a worse substrate for dephosphorylation. The most important factor for the hyperphosphorylation of tau is the imbalance between tau kinases and/or phosphatases in the favor of the former (Pei et al., 2003). Abnormally hyperphosphorylated tau isolated from AD brain polymerizes into PHF/SF tangles in vitro at pH 6.9 under reducing conditions (Alonso et al., 2001),
consistent with findings in AD brain. The hyperphosphorylated tau sequesters normal tau and other MAPs causing inhibition and disassembly of microtubules and microtubular network leading to neurodegeneration.

CLASSIFICATION OF ALZHEIMER’S DISEASE

Classification of AD is based on the age of onset and hereditary characteristics. Two main types have been identified, Familial AD (FAD) and Late-Onset AD (LOAD). The onset of FAD is early (< 60 years) and demonstrates an autosomal dominant pattern of inheritance. The age of onset of LOAD is over 60 years of age and is characterized by a complex pattern of inheritance. Mutations in three genes, APP, presenilin 1 (PS-1) and PS-2 are causatively linked with the pathogenesis of FAD by affecting the production of Aβ (Goate et al., 1991; Selkoe, 1997; Sherrington et al., 1995). The presenilins PS1 and PS2 most likely, together with three additional subunits (Nicastrin, Anterior pharynx defective 1 homologue and Presenilin enhancer 2 homologue), provide the catalytic subunit of γ-secretase (Kimberly et al., 2003) that is involved in processing of APP to Aβ. Most of the mutations that cause FAD increase production of both Aβ40 and Aβ42 or selectively increase the production of Aβ42 (Selkoe, 1997). FAD represents less than 1% of all AD cases.

Goate et al., (Goate et al., 1991) reported first that mutations in APP cause FAD, followed by identification of 19 missense mutations in this gene to account for a small portion of FAD cases (about 10%). Most of these mutations occur at the β or γ secretase cleavage sites. Mutation at γ secretase cleavage site increases the ratio of Aβ42 to Aβ40 (De Strooper and Annaert, 2000), whereas mutation at β secretase cleavage site (Swedish mutation) results in an increase in total Aβ species without altering the ratio. The PS1 gene contains 12 exons and is located in the long arm of chromosome 14, while PS2 is located on chromosome 1 and also has 12 exons. After the initial identification of 5 missense mutations on PS-1 by Sherrington et al., (Sherrington et al., 1995), over 130 mutations have been reported accounting for a large proportion of FAD cases. Mutations in PS1 increase the ratio of Aβ42 to Aβ40 and result in very early onset of AD (26 - 60
years) (Dawbarn and Allen, 2007). In contrast, only eight FAD mutations have been identified on PS2 and result in late onset AD (40 – 75 years).

Although a clear picture seems to exist about the genetic risk factors for FAD, the majority of AD cases are late onset. To date, APOE is the only known genetic risk factor and has been consistently associated with risk for LOAD in many studies (Finckh, 2003). Of the three isoforms (APOE2, APOE3, APOE4), the presence of APOE4 increases the risk in a dose dependent manner. Nearly all individuals homozygous for the APOE4 will develop AD by 80 years of age (Corder et al., 1993).

ETIOPATHOGENESIS OF AD

Narrowing down to a single unique causative factor for AD pathogenesis is a challenge, as being a multifactorial disorder well defined genetic factors as well as numerous potential environmental variables play contributory roles (Welsh, 2006a). For the naked eye, the brain in cases of AD appears atrophied and externally the brain usually shows gyral narrowing and sulcal widening, while an increase in ventricular size is noticed in cerebral slices (Dawbarn and Allen, 2007). Even though the single most important factor which accounts for the clinical symptoms of AD is loss of neuronal synapses and neuronal cell death, understanding of the causation of neuronal loss is confounded by the selective neuronal vulnerability exhibited by the neurons of neocortex and hippocampus. Characteristically a 40% reduction in the pyramidal neuron density of neocortex, 68% loss in hippocampal CA1 neurons, 40-70% loss of neurons in basal nucleus and 40% and 55% loss of neurons in dorsal raphe and locus coeruleus respectively, have been reported (Arendt et al., 1983; Terry et al., 1981). However, the exact pathomechanism leading to neuronal death is not clear. As excessive deposition of Aβ as senile plaques throughout the cerebral cortex is one of the pathological hallmarks of AD, much effort has been made to understand the mechanisms of AD neuropathology and the role of APP processing into Aβ. Being one of the key pathological features of AD, Aβ plaque has been proposed to be a primary cause for neuronal cell loss.

This proposal gained credence by the observations of Aβ plaques surrounded by degenerating neurons (Katzman and Saitoh, 1991), Aβ peptides being toxic to neurons in
culture (Aksenov et al., 1998; Varadarajan et al., 1999; Yankner et al., 1989) and the increased deposition of Aβ due to mutations in APP and presenilin genes. Multiple mechanisms have been advanced for Aβ induced cell death. They are inflammatory response and release of neurotoxic cytokines, excessive release of excitatory amino acids like glutamate from glial cells, inhibition of axonal and dendritic transport and synaptic dysfunction and loss (Bossy-Wetzel et al., 2004). Though these do not point out precisely the mechanism of Aβ induced cell death, current literature suggests that one of the main points of commonality amongst these is that oxidative stress is the mediator of neuronal death or apoptosis. Mechanisms others than oxidative stress mediating neuronal cell death have also been under intense investigation, but evidence of oxidative stress in AD pathology are abundant and compelling.

AD brain is under intense oxidative stress manifested by increased protein oxidation, lipid peroxidation, increased levels of 4 – hydroxynonenal (HNE) (Lovell et al., 1997), free radical formation (Butterfield and Lauderback, 2002), increased levels of isoprostanes (Montine et al., 1998), 3-nitrotyrosine (3NT) levels (Castegna et al., 2003; Smith et al., 1997), dityrosine (Hensley et al., 1998; Smith et al., 1997), advanced glycation end products and DNA/RNA oxidation products (Mecocci et al., 1994). The neurotoxic trace element hypothesis in AD is relevant to the oxidative stress observed in AD (Markesbery, 1997). Aluminium, mercury and iron have received most attention, of these, iron (Fe) has the most pathophysiologic role as catalyst for free radical generation. This is by virtue of its loosely bound electron, ability to exist in more than one valence state (Markesbery, 1997) and higher levels of Fe in brain (Floyd, 1999). Some of the reactions that lead to production of oxidants are illustrated in Table 1 [Adopted from (Welsh, 2006a)]. Aβ, in ways that are inhibited by free radical antioxidants like vitamin E, cause brain cell protein oxidation, lipid peroxidation and ROS formation, among other oxidative stress responses, suggesting that this peptide is a source of oxidative stress in AD brain (Butterfield et al., 2001). The findings that Aβ is central to the pathogenesis of AD (Selkoe, 2001) and that the AD brain is under significant oxidative stress were united into a comprehensive model for neurodegeneration in the brain in AD based on Aβ-associated free radical generation (Butterfield and Lauderback, 2002).
Table 1.1 Mechanisms leading to protein oxidation observed in AD, adopted from Welsh, 2006a

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>Oxidant</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenton reaction</td>
<td>Hydroxyl radical</td>
<td>$\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{Fe}^{3+} + \text{OH}^-$</td>
</tr>
<tr>
<td>Haber-Weiss reaction</td>
<td>Hydroxyl radical</td>
<td>$\text{O}_2^- + \text{H}_2\text{O}_2 + \text{Fe}^{3+}/\text{Fe}^{2+} \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2$</td>
</tr>
<tr>
<td>Nitration</td>
<td>Peroxynitrite</td>
<td>$\text{O}_2^- + \text{NO}^- \rightarrow \text{ONOO}^- \text{and}$  [ ONOO^- + \text{H}^+ \rightarrow \text{ONO}^- \text{OH} \rightarrow \text{OH}^- + \text{NO}_2 ]</td>
</tr>
<tr>
<td>Metal catalyzed oxidation (MCO)</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>$2\text{e}^- (\text{metal e}^- \text{donor}) + \text{O}_2 + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2$ and $\text{H}_2\text{O}_2 + \text{protein (lys) ligated Fe}^{2+} \rightarrow \text{protein alkyl radicals}$</td>
</tr>
<tr>
<td>NADPH oxidation</td>
<td>Superoxide</td>
<td>$2\text{O}_2 + \text{NADPH} \rightarrow 2\text{O}_2^- + \text{NADP}^+ + \text{H}^+$</td>
</tr>
<tr>
<td>Glycation</td>
<td>Glucose and reactive intermediates ($\text{H}_2\text{O}_2$ and $\text{OH}^-$)</td>
<td>Protein products + glucose $\rightarrow$ Schiff base + Maillard + glycated protein advanced glycation end products</td>
</tr>
<tr>
<td>Tyrosine oxidation</td>
<td>Tyrosyl radical ($\text{TyrO}^-$) and tyrosine peroxide ($\text{TyrOOH}$)</td>
<td>Peroxide [ 2\text{Tyr} + \text{H}_2\text{O}_2 \rightarrow 2\text{TyrO}^- + 2 \text{H}_2\text{O} ] [ 2\text{TyrO}^- + \text{O}_2^- + \text{H}^+ \rightarrow 2\text{TyrOOH} ]</td>
</tr>
<tr>
<td>Chlorination</td>
<td>Hypochlorous acid</td>
<td>$\text{H}_2\text{O}_2 + \text{Cl}_2 \rightarrow \text{HOCl}$</td>
</tr>
</tbody>
</table>
**OXIDATIVE STRESS**

Aerobic organisms use O\textsubscript{2} to facilitate the synthesis of energy in the form of ATP mainly by two routes, glycolysis in cytosol and oxidative phosphorylation on the inner mitochondrial membrane (Kadenbach, 2003). Oxidative phosphorylation comprises a respiratory chain consisting of four proton pumps or complexes and produces the majority of ATP required for the cell. Aerobic organisms use O\textsubscript{2} as the terminal electron acceptor for optimal energy release by mediating the transfer of single electrons (Nohl et al., 2004) through these complexes. The electrons from reduced substrates are passed from complexes I and II of the electron transport chain through complexes III and IV to oxygen, forming water and causing protons to be pumped across the mitochondrial inner membrane (Brand et al., 2004). As oxygen is predisposed for univalent reductions (Fridovich, 1999), electrons may leak from the respiratory chain and react inappropriately with oxygen to form superoxide radical (O\textsubscript{2}^\textsuperscript{-}). The activity of mitochondrial respiratory chain complexes, particularly complex I, have been shown to be reduced by the accumulation of O\textsubscript{2}^\textsuperscript{-} (Yen et al., 1999; Yen et al., 1996). Superoxide radical can be considered as the parent radical from which other oxygen radicals and non-radicals are derived (Nohl et al., 2004). Apart from O\textsubscript{2}^\textsuperscript{-}, other superoxide derived reactive radicals include oxygen radicals like hydroxyl radical and peroxyl radicals, and nonradical molecules like singlet oxygen and hydrogen peroxide (Oberley, 2001), and are collectively called reactive oxygen species (ROS). A vast majority of cellular ROS, approximately 90% can be traced back to oxidative phosphorylation in the mitochondria (Balaban et al., 2005). Initial observations suggested that up to 5% of the total molecular oxygen utilized by mammalian mitochondria was converted into ROS (Boveris and Chance, 1973), but more recent analysis estimates about 1-2% to be converted into O\textsubscript{2}^\textsuperscript{-} anion (Cadenas and Davies, 2000). Superoxide and other secondary reactive oxygen species it generates (Brand et al., 2004) can damage proteins, lipids and DNA directly. To prevent the detrimental effects of ROS-induced injury, cells are equipped with an elaborate antioxidant defense system that is comprised of agents that catalytically remove ROS, agents that decrease ROS formation, proteins that protect biomolecules against
oxidative damage by other mechanisms, the physical quenching of ROS, the replacement of molecules susceptible to oxidative damage by molecules resistant to it and the sacrificial agents that are preferentially oxidized by ROS to preserve more important biomolecules (Halliwell and Gutteridge, 2007). ROS can also be generated by other systems existing in endoplasmic reticulum, peroxisomes, intracellular membrane-associated oxidases, plasma membrane-associated oxidases and auto-oxidation of small molecules like dopamine, epinephrine, flavins and epinephrine (Thannickal and Fanburg, 2000). Any disturbance in the pro-oxidant and anti-oxidant balance in favor of the former is defined as oxidative stress (Sies, 1985). Brain as a result of consumption of an inordinate fraction (20%) of the total oxygen consumption for its relatively small weight (2%), its abundance of easily peroxidizable lipid content (Floyd, 1999), and the relative paucity of antioxidant enzymes compared to other tissues (Markesbery, 1997), is especially vulnerable to oxidative stress.

ANTIOXIDANT SYSTEMS

There are three major types of primary interacting (Chow et al., 2002) intracellular antioxidant enzymes in mammalian cells that can remove ROS: superoxide dismutase (SOD), catalase (CAT), peroxidase, of which glutathione peroxidase (GPx) (Oberley, 2001) and peroxiredoxins (Prx) (Halliwell and Gutteridge, 2007) are the most prominent. The SODs catalyze the dismutation of $O_2^-$ into hydrogen peroxide ($H_2O_2$) and molecular oxygen. Hydrogen peroxide is further detoxified by CAT, GPx (Halliwell and Gutteridge, 1989) and Prx to water. Thus, two reactive species - $O_2^-$ and $H_2O_2$ are converted to water (Oberley, 2001) and molecular oxygen. Among the antioxidant systems, SOD is considered the first line of defense against oxidative stress (Halliwell, 1999; Ho et al., 1998). Three distinct SODs are found in human cells: a homodimeric cytosolic copper zinc SOD (CuZnSOD) (McCord and Fridovich, 1969), a extra-cellular homotetrameric glycosylated CuZnSOD (Marklund, 1982) and mitochondrial matrix homotetrameric manganese SOD (MnSOD) (Figure 1.2) (Weisiger and Fridovich, 1973).
Figure 1.2 Scheme for production of superoxide and its detoxification
MANGANESE SUPEROXIDE DISMUTASE (MnSOD)

MnSOD is a critical antioxidant enzyme in aerobic organisms because under physiological conditions superoxide is mainly generated on the matrix side of the inner mitochondrial membrane where MnSOD is located (Balzan et al., 1999) in high concentrations (Van Remmen et al., 1999). Thus MnSOD can protect mitochondria from oxidative damage (Van Remmen et al., 1999).

MnSOD from different organisms are homologous and has a manganese ion in the active site. While Human MnSOD is a homotetramer with identical 24-kd subunits (Matsuda et al., 1990), *E. coli* MnSOD is a dimer (Beyer et al., 1991). It is a single-copy gene consisting of five exons separated by four introns (Wan et al., 1994). The human, bovine and mouse MnSOD cDNAs share more than 90% similarity (Meyrick and Magnuson, 1994). The gene is characterized by lack of a TATA or CAAT box and the presence of a GC-rich region containing SP-1 binding sites (Wan et al., 1994). The mature protein is strictly a mitochondrial enzyme compartmentalized in the mitochondrial matrix, although it is encoded by the nuclear *sod2* gene, localized in chromosome 6q25 (Matsuda et al., 1990). Studies performed by us and others have shown that the imbalance between antioxidants and oxidants leads to various pathological conditions which can be effectively prevented or alleviated by increased MnSOD (Keller et al., 1998; St Clair et al., 1991; Yen et al., 1996). The over-all importance of this enzyme is evident from transgenic animal studies, wherein MnSOD “knock-out” usually dies within 10 days after birth, with cardiac abnormalities, fat accumulation in liver and skeletal muscle (Lebovitz et al., 1996; Li et al., 1995), and metabolic acidosis (Lebovitz et al., 1996) with animals surviving longer than 10 days soon succumbing to severe anemia and neurodegeneration (Melov et al., 1998). It has been demonstrated that MnSOD heterozygous knock-out mice show altered mitochondrial function as illustrated by decreased respiration by complex I and an increase in the sensitivity of the permeability transition pore induction (Van Remmen et al., 2001), while overexpression of MnSOD protected complex I inactivation (Yen et al., 1999). Thus, mitochondrial function and oxidative phosphorylation, necessary machinery for the ATP production in the cell are likely to be influenced by MnSOD function. Further, recent studies suggest that MnSOD
plays a protective role during AD development. For example, MnSOD deficiency increased β-amyloid levels and amyloid plaque burden, and accelerated the onset of behavioral alteration in APP transgenic mice (Esposito et al., 2006; Li et al., 2004)

**NITRATION OF MnSOD AND NITROTYROSINE IN AD**

Post-translation modification like nitration of tyrosine residues present on the subunits of MnSOD can compromise its activity (Ischiropoulos et al., 1992). Of the nine tyrosine residues in each subunit of the homotetrameric protein MnSOD, tyrosines 34, 45 and 193 have been shown to be nitrated, with nitration of tyrosine residue 34 causing inactivation of MnSOD (MacMillan-Crow et al., 1998). Nitration of biological macromolecules can be mediated by nitric oxide, nitrogen dioxide, nitrous acid, hypochlorous acid, nitrated aliphatic reactive intermediates and peroxynitrite (Ischiropoulos, 1998). But only peroxynitrite has been demonstrated to nitrate tyrosine 34 of MnSOD leading to its inactivation (MacMillan-Crow et al., 1998; Yamakura et al., 1998). Residues 45 and 193 are prone for nitration because of their location with proximal glutamate residues. The apparent ability of glutamate residues to direct peroxynitrite-mediated nitration to specific tyrosine residues is implicated in this situation (MacMillan-Crow et al., 1998). Even though tyrosine 34 has no proximal glutamate, it is the most susceptible residue for nitration. This is probably because of its positioning closest to the active site, only 5 Å from the manganese ion. The attraction of peroxynitrite to the active site, by the basic residues in the channel entrance, and its reaction with manganese ion leading to the formation of nitrating species makes tyrosine 34 more prone for nitration, and thereby inactivating MnSOD (Quijano et al., 2001). The experiments carried out with *E. coli* MnSOD, in the absence of manganese ion demonstrated a reduced nitration on treatment with peroxynitrite suggesting that the metal center could be involved in nitration possibly by the formation of nitrating species like nitronium ion or an oxo-manganese complex plus nitrogen dioxide (Quijano et al., 2001).

NT is a stable adduct involving a carbon – nitrogen bond that is difficult to remove chemically. The routes of removing NT *in vivo* are not yet known, but
proteolysis is likely to a major route. NT can be excreted in human urine as an amino acid and as decarboxylated and deaminated products (Beckman, 1996). Tyrosine can be nitrated by several pathways in vitro; it is a common modification made by protein chemists to probe the function of tyrosine residues (Beckman, 1996).

In AD, the levels of 3-NT has been shown to be consistently elevated in hippocampus, neocortical regions and cerebrospinal fluid of AD patients (Hensley et al., 1998). 3-NT levels correlates positively with decreased cognitive functions (Tohgi et al., 1999). The formation of 3-NT by addition of a nitro group onto the tyrosine phenols is a post-translational modification with potentially significant biological implications (Beckman, 1996; Ischiropoulos, 1998; Smith et al., 1996). The functional consequence of 3-NT formation may be causally linked to pathogenesis of various diseases through interference of enzyme function, structural protein assembly or modulation of signaling cascades (Berlett et al., 1998; Estevez et al., 1999; Go et al., 1999; MacMillan-Crow et al., 1998). Although there is no consensus either about the activity level of MnSOD or the nitration status of MnSOD in AD, there have been reports of reduction of SOD activity in AD frontal cortex, hippocampus and cerebellum (Marklund et al., 1985).

Extensive data exist to implicate peroxynitrite as a consistent mediator of 3-NT formation (MacMillan-Crow et al., 1996; Smith et al., 1996; Yamakura et al., 1998; Yamamoto et al., 2002). Of relevance to AD is the finding of increased peroxynitrite production in the synaptosomes exposed to Aβ (Keller et al., 1997). Although one of the major oxidative modifications of proteins resulting from peroxynitrite is carbonyl formation from side-chain and peptide-bond cleavage, perhaps the best known property of ONOO− is its ability to nitrate free tyrosine and tyrosine residues in proteins (Ischiropoulos et al., 1992; Souza et al., 1999). Importantly, MnSOD, has been shown to be nitrated and inactivated in chronic rejection of human renal allograft, which was proposed to be a general mechanism for the amplification of ONOO− mediated oxidative damage (MacMillan-Crow et al., 1996). Overall, the presence of 3-NT has been suggested to be a signature of peroxynitrite involvement (Smith et al., 1997).
PEROXYNITRITE

Peroxynitrite has been the subject of intense investigation by researchers loyal to inorganic peroxide chemistry, radiation, and photochemistry. Since 1990, peroxynitrite caught the attention of a larger scientific community because of its implications to biological systems (Beckman et al., 1990). Peroxynitrite is a powerful oxidant and cytotoxic agent formed by the reaction between the free radicals nitric oxide (\(^{\cdot}\)NO) and superoxide (\(O_2^{-}\)) at rates approaching diffusion limit – 6.7 x 10\(^9\) M\(^{-1}\)s\(^{-1}\) (Beckman, 1996; Huie and Padmaja, 1993). As superoxide is short lived and has restricted diffusion across membranes, peroxynitrite formation has to be spatially associated with the sources of superoxide (plasma membrane NAD(P)H oxidases or the mitochondrial respiratory complexes). Nitric oxide produced from cytosolic NOS can diffuse into mitochondria. This capacity of nitric oxide along with the reports on the presence of nitric oxide synthase inside mitochondria (Tatoyan and Giulivi, 1998), make the intramitochondrial formation of peroxynitrite possible. Targets of peroxynitrite inside mitochondria including MnSOD has been observed (MacMillan-Crow et al., 1996).

In AD brain, nitric oxide can be produced due to activation of inducible nitric oxide synthase (iNOS) by A\(\beta\) surrounding the amyloid plaques (Combs et al., 2001) or from neuronal nitric oxide synthase (nNOS) induced by A\(\beta\) (Stepanichev et al., 2008). The rate of production of peroxynitrite \textit{in vivo} in specific compartments has been estimated to be as high as 50 – 100 \(\mu\)M per minute (Szabo et al., 2007). The short half life of peroxynitrite (~ 10 ms) is compensated for by its ability to cross cell membranes with a permeability coefficient comparable to water (Marla et al., 1997). Thus it could influence surrounding targets cells within one to two cell diameters (~ 5-20 \(\mu\)m). Externally added ONOO\(^-\) has been shown to diffuse lipid bilayers passively (Denicola et al., 1998) over distances of 1 - 10 \(\mu\)m (Radi, 1998).

Peroxynitrite itself is not a free radical because the two unpaired electrons on superoxide and nitric oxide have combined to form a new bond. Peroxynitrite is a isomer of nitrate (Beckman, 1996). It is relatively stable in alkaline solution and can be stored in millimolar concentrations at -80\(^\circ\)C for months (Beckman et al., 1994). The biochemistry
and the reaction of peroxynitrite and/or peroxynitrite-derived with their targets is illustrated in the Figure 1.3.

Many biomolecules are oxidized and/or nitrated by peroxynitrite/peroxynitrite-derived species, including tyrosine residues, thiols, DNA and unsaturated fatty-acid containing phospholipids (Szabo et al., 2007). 3 – nitrotyrosine (3-NT), 3’, 3’ – dityrosine and 3, 4’ – dihydrophenylalanine are formed by nitration, dimerization and hydroxylation respectively, by the action of peroxynitrite on tyrosine residues (Radi, 2004). Apart from tyrosine, cysteine, methionine, phenylalanine are also oxidized (Beckman and Koppenol, 1996; Ischiropoulos and al-Mehdi, 1995). Thiols can be oxidized by one–electron reactions by peroxynitrite-derived radicals (Quijano et al., 1997). In DNA, purine nucleotides are vulnerable to oxidation and adduct formation, with 8 – oxo and 8 – nitroguanine being two of the major products. Peroxynitrite is also capable of causing deoxyribose oxidation and strand breaks (Szabo et al., 2007). Peroxynitrite-derived radicals react with lipids leading to peroxidation (Radi et al., 1991), and formation of nitrito-, nitro-, nitrosoperoxo- and/or nitrated lipid oxidation adducts (Szabo et al., 2007).

**ENERGY METABOLISM**

ATP is the universal currency for biological energy, and the predominant source of cellular energy is glucose. Energy is required mainly for three purposes: (1) for the maintenance of body structure (2) for work activities (3) for thermogenesis (Kadenbach, 2003). ATP can be generated by oxidizing several metabolic fuels, but carbohydrates and fats are especially important. ATP is generated from fatty acids after their conversion to acetyl CoA by beta – oxidation in the mitochondria. Three metabolic pathways are involved in the production of ATP from fatty acids. These are the beta – oxidation, Krebs cycle and the respiratory chain. Like that from glucose, the acetyl CoA formed by beta – oxidation goes through Krebs cycle and respiratory chain to produce ATP. In brain, the ATP produced from fatty acid is of little value as the activity of ketoacetyl Co-A thiolase, a key enzyme involved in beta – oxidation is 125 times lower compared to other tissues (Yang et al., 1987). So glucose is the obligatory energy source.
Figure 1.3 Biochemistry of Peroxynitrite: reaction and fate, adopted from Szabo et al., 2007
for the brain. The ATP from glucose is derived through three major pathways producing carbon dioxide and water as the end products. In aerobic organisms, glucose is metabolized through glycolysis occurring in the cytoplasm, Kreb’s cycle and electron transport chain in the mitochondria to produce energy in the form ATP.

First process in the utilization of glucose for energy production is glycolysis. In this process, one molecule of glucose is broken down in a series of ten enzymatically catalyzed reactions to two molecules of pyruvate. In the priming phase of glycolysis, two ATPs are utilized to produce fructose 1,6-bisphosphate, followed by the energy producing phase, where fructose 1,6-bisphosphate is converted stepwise to pyruvate with the production four molecules of ATP and two molecules of NADH. As two ATP molecules were consumed in the priming phase, there is a net gain of two ATP molecules. The overall reaction can be depicted as follows:

\[
\text{Glucose} + 2 \text{P}_i + 2 \text{ADP} + 2 \text{NAD}^+ \rightarrow 2 \text{Pyruvate} + 2 \text{ATP} + 2 \text{NADH} + 2 \text{H}^+ + 2 \text{H}_2\text{O}
\]

After the glycolysis takes place in the cell’s cytoplasm, the pyruvate molecules travel into the interior of the mitochondrion. Carbon dioxide is enzymatically removed from each three-carbon pyruvate molecule to form acetic acid. The enzyme then combines the acetic acid with an enzyme, coenzyme A, to produce acetyl coenzyme A, also known as acetyl CoA. Once acetyl CoA is formed, the Krebs cycle begins. This important step of converting pyruvate to acetyl CoA is catalyzed by the enzyme pyruvate dehydrogenase (PDH). The Krebs cycle consists of eight steps, catalyzed by various enzymes, resulting in the production of 2 ATP molecules, 6 NADH molecules, 2 FADH\(_2\) and 4 carbon dioxide molecules. The overall reaction is as follows:

\[
2 \text{Acetyl CoA} + 4 \text{H}_2\text{O} + 6 \text{NAD} + 2 \text{FAD} \rightarrow 6 \text{NADH} + 2 \text{FADH}_2 + 4 \text{CO}_2 + 2 \text{ATP}
\]

The electron transport chain comprises of three proton pumps namely, NADH dehydrogenase or complex I, cytochrome c reductase or cytochrome bc\(_1\) complex or complex III and cytochrome c oxidase or complex IV; and an enzyme complex not pumping protons – succinate dehydrogenase or complex II (Kadenbach, 2003). In addition to these complexes, two mobile carriers are also involved: ubiquinone, and cytochrome c. Other key components in this process are NADH/FADH\(_2\) and the electrons from them, hydrogen ions, molecular oxygen, water, and ADP and Pi, which combine to form ATP. Electron transport chain transfers electrons from NADH and FADH\(_2\) to complexes I and
II respectively through complexes III and IV to oxygen, forming water and causing protons to be pumped across the mitochondrial inner membrane (Brand et al., 2004).

At the start of the electron transport chain, two electrons are passed from NADH into the NADH dehydrogenase complex. Coupled with this transfer is the pumping of one hydrogen ion for each electron. Next, the two electrons are transferred to ubiquinone. Ubiquinone is called a mobile transfer molecule because it moves the electrons to the cytochrome bc₁ complex. Each electron is then passed from the cytochrome bc₁ complex to cytochrome c. Cytochrome c accepts each electron one at a time. One hydrogen ion is pumped through the complex as each electron is transferred.

The next major step occurs in the cytochrome oxidase complex. This step requires four electrons. These four electrons interact with a molecular oxygen molecule and eight hydrogen ions. The four electrons, four of the hydrogen ions, and the molecular oxygen, are used to form two water molecules. The other four hydrogen ions are pumped across the membrane. The energy from the transfer of electrons along the chain transports protons across the membrane and creates an electrochemical gradient. The potential energy in this gradient is used by ATP synthase to generate ATP from ADP and inorganic phosphate. The final reaction of electron transport chain is as follows

\[ 4 \text{H}^+ + \text{O}_2 + 4 \text{e}^- \rightarrow 2 \text{H}_2\text{O} \]

Total ATP produced from electron transport chain is 32, which is as outlined below

1. Each NADH produced in Glycolysis is worth 2 ATP (2 x 2 = 4). In actuality, NADH is worth 3 ATP, but it costs an ATP to transport the NADH into the mitochondria, so there is a net gain of 2 ATP for each NADH produced in glycolysis
2. Each NADH produced in the conversion of pyruvate (2 NADH) to acetyl CoA and Krebs Cycle (6 NADH) is worth 3 ATP so 8 NADH x 3 ATP = 24 ATP
3. Each FADH₂ is worth 2 ATP so 2 FADH₂ x 2 ATP = 4 ATP
4. 4 from NADH of glycolysis + 24 from NADH of Kreb’s cycle + 4 FADH₂ of Kreb’s cycle = 32 ATP

Over all, if we add the 4 ATPs produced from glycolysis and Kreb’s cycle (2 each), a total of 36 ATPs are produced from a single molecule of glucose.
Under physiological conditions, the amount of ATP supplied by glycolytic pathway is meager. But in many tumors having impaired mitochondrial function and thereby reduced ATP supply from oxidative phosphorylation pathway, there can be up regulation (faster) of glycolytic capacity. It has been proposed that glycolysis can proceed 100 times faster than respiration, which in turn yield 18-fold more ATP (Bartrons and Caro, 2007), thus acting as a compensatory response for the impaired mitochondrial function.

**ALTERED METABOLISM IN AD**

As glucose is the obligatory energy source for the brain, its utilization has been increasingly investigated in AD. AD patients exhibit reduced glucose metabolism in parietal cortex and temporal gyri when studied longitudinally for one year (Hirono et al., 2004). Specifically, the reduced activity of pyruvate dehydrogenase complex was observed as early as 1980 in the cerebral cortex of AD patients (Perry et al., 1980). The enzymes involved in tricarboxylic cycle also show altered activity in prefrontal cortex of AD patients (Bubber et al., 2005). In addition to AD patients, transgenic mice Tg2576 exhibiting Alzheimer plaque pathology also show reduced activity of phospho fructokinase (PFK) (Bigl et al., 2003). Further, Aβ treated hippocampal neurons have showed significant decrease in glucose uptake (Prapong et al., 2002). Further, report on nitration of alpha-enolase, glyceraldehyde-3-phosphate dehydrogenase, ATP synthase alpha chain, voltage-dependent anion channel – 1 and carbonic anhydrase, enzymes involved in energy production, in AD brain hippocampus (Sultana et al., 2006) have opened up a new area of AD research. This alteration of the enzymes involved in energy production is consistent with that of AD patients having reduced activity of mitochondrial electron chain components, particularly cytochrome oxidase (Kish et al., 1999; Maurer et al., 2000). These findings suggest an environment of altered energy production. Decreased function of electron transport chain components could cause diversion of electrons from their normal pathway into reaction with molecular oxygen in the neocortex and hippocampus of AD, resulting in increased superoxide (O$_2^-$) formation, exacerbating the oxidative stress (Markesbery, 1997). Thus it is possible that nitration of proteins...
involved in energy production and/or protection of mitochondrial function, may be responsible for the observed metabolic impairment encountered in AD.

**RESEARCH OBJECTIVES**

Although, it is documented that, overpression of MnSOD protects against Aβ induced neurotoxicity, there is no consensus about the activity of MnSOD in AD brain. In this regard, this dissertation addresses the following hypotheses:

1. **MnSOD is nitrated and inactivated in association with Aβ deposition.**

   To address this hypothesis, in chapter two, a homozygous knock-in APP<sup>NLh/NLh</sup> x PS-<sup>P264L/P264L</sup> mouse that simulates a natural progression of Aβ plaque pathology observed in AD (Reaume et al., 1996) was used to investigate the correlation between Aβ plaque deposition and nitration of MnSOD. An increased and accelerated deposition of Aβ and increased levels of Aβ 1-40/1-42 along with increased nitration and subsequent inactivation of MnSOD in association with age, was observed in APP<sup>NLh/NLh</sup> x PS-<sup>P264L/P264L</sup> mice. This change was also associated with impaired mitochondrial respiration.

2. **Nitrative inactivation of MnSOD plays an important role in the impairment of mitochondrial respiration resulting in alteration of energy metabolism.**

   This hypothesis is addressed in chapter three, where the SH-SY5Y neuroblastoma cell line was treated with peroxynitrite to nitrate MnSOD. MnSOD was nitrated upon peroxynitrite treatment with an associated decrease in activity. An impairment of mitochondrial respiratory function was also observed. Inactivation of MnSOD was an early event in peroxynitrite-induced impairment of mitochondrial function. Mitochondrial dysfunction was ameliorated by SOD mimetic and reproduced by MnSOD deficiency. The impairment of mitochondrial respiratory function was compensated by
increased glycolytic activity, which was prevented by SOD mimetic and enhanced by MnSOD deficiency.

1. The impairment of mitochondrial respiration observed in APP/PS-1 mice was also associated with an increase in glycolytic activity.

This is addressed in chapter four, where we demonstrate increase in lactate levels, an indicator of enhanced glycolytic activity and increase in activity of lactate dehydrogenase in homozygous knock-in APP^{NLh/NLh} x PS-1^{P264L/P264L} mouse. This was also associated with decrease in pyruvate dehydrogenase activity which can exaggerate the impaired mitochondrial function.

These results demonstrate a critical role for MnSOD in maintaining mitochondrial respiratory function and reveal the role of glycolysis in sustaining cellular energy requirements under pathological conditions associated with MnSOD deficiency.
CHAPTER TWO

β - AMYLOID MEDIATED NITRATION OF MANGANESE SUPEROXIDE DISMUTASE

Implication for Oxidative Stress in a APPNLh/NLh X PS-1P264L/P264L Double Knock-In Mouse Model of Alzheimer’s Disease

ABSTRACT

Alzheimer’s disease is a multifactorial, progressive, age-related neurodegenerative disease. In familial Alzheimer’s disease, Aβ is excessively produced and deposited because of mutations in the amyloid precursor protein, presenilin - 1, and presenilin - 2 genes. Here, we generated a double homozygous knock-in mouse model that incorporates the Swedish familial Alzheimer’s disease mutations and converts mouse Aβ to the human sequence in amyloid precursor protein and had the P264L familial Alzheimer’s disease mutation in presenilin - 1. We observed Aβ deposition in double knock-in mice beginning at 6 months as well as an increase in the levels of insoluble Aβ1-40/1-42. Brain homogenates from 3-, 6-, 9-, 12-, and 14-month-old mice showed that protein levels of manganese superoxide dismutase (MnSOD) were unchanged in the double knock-in mice compared to controls. Genotype-associated increases in nitrotyrosine levels were observed. Protein immunoprecipitation revealed MnSOD as a target of this nitration. Although the levels of MnSOD protein did not change, MnSOD activity and mitochondrial respiration decreased in knock-in mice, suggesting compromised mitochondrial function. The compromised activity of MnSOD, a primary antioxidant enzyme protecting mitochondria, may explain mitochondrial dysfunction and provide the missing link between Aβ-induced oxidative stress and Alzheimer’s disease.
Alzheimer’s disease (AD) is a multifactorial, progressive age-related neurodegenerative disease that affects more than four million persons in the United States (Evans et al., 1989). The pathological hallmarks of AD are extracellular Aβ deposits, neurofibrillary tangles, synaptic loss, and neuronal degeneration. Aβ plaques are composed of 40- and 42-mer peptides (Aβ1-40 and Aβ1-42) that are proteolytically produced from amyloid precursor protein (APP) (Iwatsubo et al., 1994). Three genes, presenilin 1 (PS-1), PS-2, and APP are causatively linked with the pathogenesis of early onset familial AD (FAD). The findings that Aβ is central to the pathogenesis of AD (Selkoe, 2001) and that the AD brain is under significant oxidative stress were united into a comprehensive model for neurodegeneration in the brain in AD based on Aβ-associated free radical generation (Butterfield and Lauderback, 2002). The brain in AD has marked oxidative damage as manifested by increased protein oxidation, lipid peroxidation, free radical formation (Butterfield and Lauderback, 2002), 3-nitrotyrosine (3-NT) levels (Castegna et al., 2003; Smith et al., 1997), advanced glycation end products, and DNA/RNA oxidation products (Mecocci et al., 1994; Nunomura et al., 1999).

Manganese superoxide dismutase (MnSOD) is a homotetramer consisting of identical 24-kd subunits. The translated precursor in cytosol contains an N-terminal 24-amino acid sequence signaling mitochondrial compartmentalization. The mature protein protects the cells against cytotoxic O2−. The importance of this enzyme is evident from MnSOD knockout mice that suffer from a defect of mitochondrial iron-sulfur centers, a modification proving lethal to newborns (Lebovitz et al., 1996; Li et al., 1995). Activity of this antioxidant enzyme declines in the aging process (Wei and Lee, 2002). Because the AD brain is under intense oxidative stress, any dysfunction of MnSOD may lead to progression of the disease.

Peroxynitrite anion (ONOO−) is a potent biological oxidant that has been implicated in diverse forms of free radical-induced tissue injury (Wiseman and Halliwell, 1996). Peroxynitrite is produced by the reaction of O2− and NO, and this peroxynitrite can compromise the functional and/or structural integrity of target proteins (Beckman, 1996). Increased levels of nitrotyrosine (Smith et al., 1997) and 4-hydroxynonenal
Montine et al., 1997) are associated with degenerating neurons in AD, suggesting pathogenic roles of peroxynitrite and membrane lipid peroxidation in this disease.

Mutations in the APP gene and PS-1 gene lead to increased levels of Aβ, which appear to contribute to the disease process. One study demonstrated altered levels of Aβ1-40 and Aβ1-42 distinguish AD from normal aging (Games et al., 1995). Recently, several transgenic animals have been shown to have increased Aβ peptide deposition and some of the pathological characteristics similar to AD patients (Wang et al., 1999). Crossing APP-Tg mutant mice and PS-1-Tg mutant mice resulted in increased Aβ production and accelerated amyloid deposition in the brains of these animals (Borchelt et al., 1997). However, increased Aβ levels in these models may, in part, be attributable to an increase in copy number of the transgene.

The aim of this study was to use a mouse model that resembles the natural progression of Aβ pathology similar to that observed in AD patients and to gain insight into potential causes of the mitochondrial alterations that occur during the progression of AD. The model that we used is a homozygous knock-in APPNLh/NLh X PS-1P264L/P264L (APP/PS-1) (Reaume et al., 1996; Siman et al., 2000). The results presented here demonstrate that these mice have an age-dependent accumulation of Aβ in the brain, consistent with an increase in both Aβ1-40 and Aβ1-42 and an accelerated decline in mitochondrial function associated with a decrease in activity of MnSOD because of nitration. These results suggest a novel Aβ-mediated nitrative inactivation of MnSOD and inhibition of mitochondrial function in AD.
MATERIALS AND METHODS

Mutant Mouse Lines

\[ \text{APP}^{NLh/NLh} \times \text{PS-1}^{P264L/P264L} \]

mutant mice were generated using the Cre-lox knock-in technology (Reaume et al., 1996; Siman et al., 2000). The APP strategy introduced the Swedish FADK670N/M671L mutations and changed the mouse sequence for Aβ to be identical to the human sequence (NLh). These mice demonstrate proper cleavage of the APP protein to generate the Aβ peptide. The PS-1 mutation targeted codons 264 and 265 of the mouse PS-1 gene to introduce the proline to leucine (P264L) mutation. When PS-1 mutant mice (P264L) are crossed with the mutant APP mice (NLh), the mutations are driven by the endogenous promoters of the APP and PS-1 genes, and expression is limited to the replacement of these two endogenous genes and not by the expression of multiple transgenes.

Genotyping of Mice

APP/PS-1 mice were maintained on a CD-1/129 background. Wild-type (WT) mice were obtained from heterozygous APP/PS-1 matings and maintained as a separate line for use as controls. The APP/PS-1 mutant mice were monitored for maintenance of the knock-in gene by PCR analysis of tail snip DNA (Reaume et al., 1996). The APP NLh mutation was screened with primers spanning the loxp sequence in intron 15 of the targeted locus (5'-CACACCAAGAAGTACAATAGAGGG-3' and 5'-CCTGGGTTGTAGGGACTGTACTTG-3') (Invitrogen, Carlsbad, CA). WT mice showed a single band at 214 bp, whereas the homozygous mutant mice had a single band at 298 bp. The PS-1 P264L mutation was identified using primers spanning exon 8 (5'-CCCGTGGAGGTACAGAAGTCAG-3' and 5'-TTACGGGTGTAGCCATGAATG-3') (Siman et al., 2000) (Invitrogen). WT mice showed a single band at 142 bp, whereas the homozygous PS-1 knock-in mutant mice showed a single band at 219 bp. The mice used in all experiments were either WT or homozygous for APP/PS-1 mutation.
Immunocytochemistry for Aβ

To determine the deposition of Aβ in APP/PS-1 mice, one brain hemisphere from 3-, 6-, 9-, 12-, and 14-month-old mice was fixed in 4% formaldehyde, processed in the standard manner, embedded in paraffin, and sectioned at 10-µm thickness. The sections were deparaffinized, hydrated, and immersed in 88% formic acid for 3 minutes and washed in distilled water. After blocking with 15% filtered horse serum in automation buffer (Biomedica Corp., Foster City, CA) for 1 hour at room temperature, the sections were immunostained with 10D-5 monoclonal antibody (1:100) (NCL-B-amyloid) and a biotinylated anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA). The sections were developed using an ABC reagent kit (Vector Laboratories) and counterstained with hematoxylin.

Tissue Preparation for Aβ Enzyme-Linked Immunosorbent Assay (ELISA)

Cerebral cortices were serially extracted with Tris-buffered saline, pH 7.4, followed by RIPA buffer [0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, and 5 mmol/L ethylenediaminetetraacetic acid in Tris-buffered saline] and finally in 70% formic acid. Protease inhibitor cocktail (pepsatin A, leupeptin, TPCK, TLCK, soybean trypsin inhibitor, 1 µg/ml of each in 5 mmol/L ethylenediaminetetraacetic acid) was added during Tris-buffered saline and RIPA extraction. To measure the levels of Aβ1-40 and Aβ1-42, the supernatant obtained from the SDS extraction step was analyzed by ELISA.

Sandwich ELISA to Determine the Levels of Insoluble Brain Aβ1-40 and Aβ1-42

The sandwich ELISA used here was performed as described previously (Skovronsky et al., 1998; Suzuki et al., 1994; Turner et al., 1996), and the absorbencies falling within the standard curve for each assay were converted to pmol. The sandwich ELISA consisted of a capture monoclonal antibody (mAb, BAN50) that was specific for the first 10 amino acids in Aβ and was used in conjunction with one of two different
detection antibodies, BA27 mAb specific for Aβ species ending at amino acid 40 or BC05 mAb specific for Aβ species ending at position 42(43). This ELISA had a detection limit of 3 to 6 fmol/well for Aβ1-40 and 1-42/43. The mAbs BAN50, BA27, and BC05 were prepared as described previously, and each ELISA result was normalized for the dilution and tissue weight (pmol/µg wet tissue).

Mitochondrial Isolation

Mice were euthanized; brains (excluding cerebellum) of three mice from each age group were pooled, homogenized in 5 ml of ice-cold mitochondrial isolation buffer containing 0.225 mol/L d-mannitol, 0.075 mol/L sucrose, 20 mmol/L HEPES, 1 mmol/L EGTA, and 1% bovine serum albumin, pH 7.2, in a Dounce homogenizer with a glass pestle. The homogenized brains were then diluted with isolation buffer to a final volume of 10 ml, centrifuged at 1500 x g for 5 minutes. The supernatant was kept on ice, and the pellet resuspended in 3 ml of isolation buffer, homogenized, and centrifuged at 1500 x g for 5 minutes. The supernatants were combined and centrifuged at 13,500 x g for 10 minutes. The brown mitochondrial pellets were resuspended in 100 µl of isolation buffer and kept on ice, and the protein concentration was determined by Bradford assay.

Measurement of Mitochondrial Respiration

Mitochondrial proteins were resuspended in buffer containing 0.25 mol/L sucrose, 50 mmol/L HEPES, 2 mmol/L MgCl2, 1 mmol/L EGTA, 10 mmol/L KH2PO4, and 0.5% bovine serum albumin, pH 7.4. Oxygen consumption was measured using a Clark-type electrode oxygraph (Hansatech Inc., Norfolk, UK) with 10 mmol/L pyruvate and 5 mmol/L malate as substrate in the absence of exogenous ADP (state 2 respiration) and after addition of 300 mmol/L ADP (state 3 respiration). The ATPase inhibitor oligomycin (100 µg/ml) was then added to inhibit mitochondrial respiration. In normally coupled mitochondria, the addition of oligomycin slows respiration to a rate similar to that of state 2, whereas in uncoupled mitochondria oligomycin inhibition is reduced.
Respiratory control ratio (RCR) was calculated as the ratios between state 3 and state 2 respirations.

**Immunoprecipitation**

Isolated mitochondrial protein (200 µg) was resuspended in 200 µl of RIPA buffer (9.1 mmol/L Na₂HPO₄, 1.7 mmol/L NaH₂PO₄, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% v/v Nonidet P-40, and 0.1% SDS, pH 7.2). Polyclonal nitrotyrosine antibody (anti-rabbit, 2 µg/ml; Cayman Chemical, Ann Arbor, MI) was added and incubated overnight at 4°C. Protein A/G agarose (20 µl) (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the mixture and incubated overnight. Immunocomplexes were collected by centrifugation at 1000 x g for 5 minutes at 4°C, followed by washing with RIPA buffer, four times. Immunoprecipitated samples were recovered by resuspending in 2X sample loading buffer, immediately fractionated by reducing SDS/polyacrylamide gel electrophoresis (PAGE), and analyzed by Western blot.

**Western Blot Analysis**

Equal amounts of brain homogenated proteins were resuspended in 2X sample loading buffer and separated on 12.5% SDS-PAGE. After separation by SDS/PAGE proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell, Dassell, Germany) and blocked with 5% nonfat dried milk in 50 mmol/L Tris, pH 7.9, 150 mmol/L NaCl, and 0.05% (v/v) Tween-20. After blocking, the blots were incubated overnight at 4°C with appropriate primary antibody (rabbit, anti-MnSOD IgG, dilution 1:10,000; Upstate Technology, Lake Placid, NY), followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Probed membranes were washed three times, and immunoreactive proteins were detected using enhanced chemiluminescence (Amersham Corp., Piscataway, NJ).
MnSOD Activity Assay

SOD activity in the homogenized brain sample was measured by the nitro blue tetrazolium (NBT)-bathocuproine sulfonate reduction inhibition method (Spitz and Oberley, 1989). This is an assay based on the competition reaction between SOD and the indicator molecule NBT. When increasing amounts of protein (containing SOD activity) were added to the system, the rate of NBT reduction was progressively inhibited. Potassium cyanide at 5 mmol/L was used to inhibit Cu/ZnSOD and thus measured only MnSOD activity. The assay mixture also contained catalase to remove H$_2$O$_2$ and diethylenetriaminepentaacetic acid to chelate metal ions capable of redox cycling and interfering with the assay system. One unit of SOD activity was defined as the amount of SOD protein that caused a 50% reduction in the background rate of NBT reduction.

Statistical Analysis

Statistical significance was analyzed by two-way analysis of variance, followed by Newman-Keuls multiple comparison test when applicable. The experiments were repeated at least three times, and the graphs were drawn using Graph Pad Prism, version 3.02.
RESULTS

Aβ Deposition in APP/PS-1 Mice

To determine the deposition of Aβ, we examined brain sections that were immunostained with 10D-5 antibody. The brains from WT mice contained no plaques at any age. The APP/PS-1 animals showed no deposition of Aβ at the age of 3 months (Figure 2.1A). At the age of 6 months (Figure 2.1B), a few microscopic, scattered, small Aβ plaques were found in the frontal cortex. By 9 months (Figure 2.1C) there were a few larger Aβ plaques and more scattered smaller Aβ deposits. By 12 months (Figure 2.1D), Aβ deposition was more prominent in the neocortex and spread of plaques to the hippocampus occurred, with many larger confluent Aβ plaques and many small and moderate size plaques. By 14 months, there were larger numbers of Aβ deposits and Aβ in small blood vessel walls in brain and leptomeninges (Figure 2.1E). These results suggest that there was an age-related, regional dependence to Aβ deposition in APP/PS-1 mice.

Increased Levels of Aβ1-40 and 1-42 Fractions in APP/PS-1 Mice

The accumulation of diverse species of Aβ peptides in amyloid plaques is a multistep process including the conversion of soluble Aβ into insoluble derivatives that assemble into amyloid fibrils and aggregate in extracellular deposits (Games et al., 1995). We examined whether the APP/PS-1 mice model showed an increase in levels of SDS-soluble fractions of Aβ1-40 and 1-42 in cerebral cortical tissue. APP/PS-1 mice exhibited a trend of age-related increase in the load of both species of Aβ (Figure 2.2A & B).

Unchanged MnSOD Protein Levels in WT and APP/PS-1 Mice

Expression of MnSOD is highly inducible by oxidative stress-inducing agents. To determine whether increases in Aβ produced increases in MnSOD enzyme levels, Western blot analyses of brain homogenate from animals of different ages of both WT
Figure 2.1 Age-related Aβ deposition in the brains of APP/PS-1 mice
Figure 2.1 Age-related Aβ deposition in the brains of APP/PS-1 mice. Sections of frontal cortex from APP/PS-1 mice immunostained with 10D-5 antibody for Aβ.

A. 3-month old animal showing no amyloid immunostaining in cortex.

B. 6-month old mouse showing rare small deposits of Aβ (arrows).

C. 9-month old mouse showing increased Aβ deposits.

D. 12-month old mouse demonstrating numerous variable size deposits of Aβ.

E. 14-month old mouse showing numerous diffuse Aβ deposits in cortex.

F. 14-month old mouse demonstrating many Aβ deposits in cortex in parenchymal and leptomeningeal vessels (arrows). Original magnifications, ×100.
Figure 2.2 Increased levels Aβ1-40 and 1-42 fractions in APP/PS-1 mice.
Aβ1-40 and Aβ1-42 levels were measured by ELISA from different groups (n = 5/group) as described in Materials and Methods section. Both species of Aβ showed an increasing trend in the levels associated with age (R² = 0.8072 for Aβ1-40 and 0.702 for Aβ1-42).
and APP/PS-1 mutant mice were performed. The protein levels of MnSOD did not change when compared between genotypes or between different ages within a genotype (Figure 2.3A & B).

**Increased Nitration of MnSOD in APP/PS-1 Mice**

MnSOD is susceptible to peroxynitrite-induced inactivation (MacMillan-Crow et al., 1996). To determine whether Aβ-induced oxidative stress was associated with nitration and inactivation of MnSOD, nitration of MnSOD was detected by Western blot analysis of SDS/PAGE-fractionated mitochondrial proteins, which had been immunoprecipitated using a polyclonal anti-nitrotyrosine antibody. Immunodetection with polyclonal anti-MnSOD demonstrated detectable levels of nitrotyrosine in WT mice at all ages tested. In contrast, the APP/PS-1 mice showed an increasing trend in the level of immunoreactive-nitrated MnSOD (Figure 2.4A & B), but the increase was not statistically significant at \( p < 0.05 \). The increase was significant when compared between the two genotypes (\( p < 0.01 \)).

**Decreased SOD Activity in APP/PS-1 Mice**

MnSOD catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen, and this conversion was used to estimate the activity of MnSOD. The activity of MnSOD in APP/PS-1 mice was significantly decreased (\( p < 0.0001 \)) when compared to the WT mice. WT mice showed a significant (\( **p < 0.05 \)) decrease in MnSOD activity at 12 and 14 months when compared to 3-month-old mice of their own genotype (Figure 2.4C). There was also a significant decrease (\( *p < 0.05 \)) in activity of MnSOD in APP/PS-1 mice when compared to their age-matched WT controls (Figure 2.4C).
Figure 2.3 Unchanged MnSOD protein levels in WT and APP/PS-1 mice. Representative (out of three) immunoblot (A) and densitometry analysis (B) showing the levels of MnSOD. Protein (30 μg) extracted from brain specimens of APP/PS-1 knock-in and wild type mice were run on a 12.5% SDS-polyacrylamide gel electrophoresis. Protein levels of MnSOD did not show significant age and genotype associated alterations. β-actin was used to normalize protein loading. WT – Wild type; HO – APP/PS-1.
Figure 2.4 Increased nitration and decreased activity of MnSOD in APP/PS-1 mice
Figure 2.4 Increased nitration and decreased activity of MnSOD in APP/PS-1 mice.

Immunoprecipitation of nitrotyrosine with MnSOD. Isolated mitochondrial protein (200 µg) was precipitated with polyclonal nitrotyrosine antibody and analyzed by Western blotting using MnSOD antibody.

A. Nitrotyrosine coimmunoprecipitated with MnSOD showed that MnSOD is nitrated. WT – Wild type; HO – APP/PS-1; Positive control – Brain homogenate + Peroxynitrite (2 µM), provided by Dr. Timothy R. Miller, Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky; Pellet – Pellet of the isolated mitochondrial protein (200 µg) precipitated with pre-immune IgG, analyzed by Western blotting using MnSOD antibody; Supernatant of the isolated mitochondrial protein (200 µg) precipitated with pre-immune serum, analyzed by Western blotting using MnSOD antibody.

B. Densitometric analysis and subsequent statistical analysis by 2-way ANOVA showed significant differences (*, p < 0.01) when compared between genotypes.

C. MnSOD activity in the brain specimen was measured by the nitroblue tetrazolium (NBT)-bathocuproine sulfonate (BCS) reduction inhibition method. Statistical analysis by 2-way ANOVA showed significant age- and genotype-dependent decreases (p < 0.0001) in activity of MnSOD. APP/PS-1 mice, at all ages, showed significant decreases (*, p < 0.05) in MnSOD activity when compared with age-matched WT mice. WT mice at 12- and 14-months showed significant decrease (**, p < 0.05) in MnSOD activity when compared with 3-month old WT mice. Immunoprecipitation and MnSOD activity was carried out in three sets of animals.
Decreased Mitochondrial Respiration in APP/PS-1 Mice

MnSOD is a primary antioxidant enzyme protecting mitochondria from oxidative injury. To determine whether a reduction of MnSOD activity would affect mitochondrial respiratory function, oxygen consumption by isolated mitochondria was measured as an indicator of the mitochondrial respiration activity. Pyruvate and malate were used as substrates to determine the function of brain mitochondria from WT and APP/PS-1 mice via complex I respiration. The results showed that the RCR of mitochondria was significantly (*p < 0.01) decreased in 9- and 12-month-old APP/PS-1 mice when compared to age-matched WT mice and also when compared to 3-month-old mice of both genotypes (**p < 0.001) (Figure 2.5). The results suggest that in APP/PS-1 mice there is inhibition of NAD-linked state 3 respiration rate, which is mediated through complex I of the mitochondrial electron transport chain. WT mice showed a small decrease in mitochondrial respiration with age; however, it was not statistically significant.
Figure 2.5 Decline in mitochondrial respiration via complex I in APP/PS-1 mice. Oxygen consumption was measured using a Clark-type electrode oxygraph. Respiratory control ratio (RCR) was calculated as the ratios between state 3 and state 2 respirations. APP/PS-1 mice at 9 and 12 months of age showed a significant decrease (*, p < 0.01) in mitochondrial respiration when compared to its age-matched WT mice and a significant decrease (**, p < 0.001) compared to 3-month old mice of both genotypes. Statistical analysis – two-way ANOVA followed by Newman-Keuls multiple comparisons test.
DISCUSSION

Numerous reports have described attempts to recapitulate the hallmark pathologies of AD in the rodent brain by overexpression of human APP or APP fragments in transgenic models (Games et al., 1995; LaFerla et al., 1995; Lamb et al., 1993); examples include, Tg2576 mice that overexpress human APP695 (Hsiao et al., 1996) with Swedish FAD mutations, mice obtained by crossing Tg2576 mice and transgenic PS1-P264L mice (Holcomb et al., 1998; McGowan et al., 1999), and the crosses of other Swedish APP transgenic mice with transgenic FAD mutant PS-1 mice (Borchelt et al., 1997). In this study, we used a double gene-targeted APP\textsuperscript{NLhNLh}/PS-1\textsuperscript{P264L/P264L} mouse model for amyloid deposition without the overexpression of APP (Flood et al., 2002).

Humanization of the mouse A\textbeta gene sequence results in an approximate threefold increase in amyloidogenic processing of recombinant APP in rat hippocampal neurons infected with Semliki Forest virus expression constructs (De Strooper et al., 1995), and single mutant PS-1 allele was sufficient to elevate the concentration of A\textbeta42 in brain and speed the onset of amyloid deposition and reactive astrogliosis (Siman et al., 2000). Our results using mice homozygous for APP/PS-1, which completely lack WT APP or PS-1 and expressed both mutant FAD APP and PS-1 at natural levels, showed age-dependent increases in amyloid pathology (Figure 2) in accordance with the results obtained from APP 695\textsubscript{SWE} transgenic mice having PS-1 P264L knock-in mutation (Flood et al., 2002; Siman et al., 2000). The observed accelerated A\textbeta deposition in APP/PS-1 mice also seems to have region dependence in relation to age, with deposition seen first in frontal cortex and later encompassing other cortical regions and hippocampus. Thus, this humanized mouse model should serve as a useful model to study the A\textbeta-induced pathology in human AD.

Measurement of A\textbeta1-40 and 1-42 showed that there was an increasing trend in both species in APP/PS-1 mice. Our findings are in concordance with that of Wang and colleagues who demonstrated an increase in average levels of A\textbeta1-40 and A\textbeta1-42 in AD. Our finding of increased quantity of A\textbeta1-40 and A\textbeta1-42, is consistent with the possibility that A\textbeta1-42 serves as the initial seeding event for plaque formation and that increased
levels of Aβ1-40 play a role in growing plaques and may be mechanistically linked to the onset and progression of AD (Wang et al., 1999).

The AD brain is under pronounced oxidative stress, as manifested by protein oxidation, lipid peroxidation, DNA and RNA oxidation, widespread peroxynitrite-induced damage, advanced glycation end products, and altered antioxidant enzyme expression. Via mechanisms that are inhibited by antioxidants such as vitamin E, Aβ causes brain cell protein oxidation, lipid peroxidation, and ROS formation, among other oxidative stress responses, suggesting that this peptide is a source of oxidative stress in the brain (Butterfield et al., 2001). Other sources of oxidative stress in AD are likely, ranging from altered mitochondrial function, trace metal ion imbalances to binding of altered metal ion to biomolecules (Markesbery, 1997). Our results indicating increased nitrination of MnSOD protein signify an increase in oxidative stress in the brain of the APP/PS-1 mice and suggest a compromise in mitochondrial function of APP/PS-1 mice attributable to increased oxidative stress.

The mitochondrion, a major subcellular source of ROS (Dugan et al., 1995) plays a pivotal role in apoptosis (Piantadosi and Zhang, 1996). However, the mitochondrion is also a site of cellular protection against ROS that involves an elaborate antioxidant defense system, especially MnSOD. Our studies indicate that the expression pattern of MnSOD in APP/PS-1 mice remained unaltered in all age groups, but SOD activity in APP/PS-1 mice was significantly reduced when compared to age-matched WT mice, suggesting that the protein was inactivated. Our finding is in accordance with that reported by Macmillan-Crow and colleagues (MacMillan-Crow et al., 1996) in a chronic rejecting renal model. The decreased activity of MnSOD is attributable to nitration of tyrosine residues (Smith et al., 1996; Smith et al., 1997) that occurs on the MnSOD protein. Tyrosine nitration (3-nitrotyrosine, 3-NT) is an in vivo posttranslational protein modification with potentially significant biological implications (Beckman, 1996; Ischiropoulos, 1998) and has been detected in a number of human and animal models of disease (Kroemer et al., 1997). 3-NT is increased in the hippocampus and cerebral cortex of aged rats (Shin et al., 2002), the cerebrospinal fluid of aged humans (Tohgi et al., 1999), and the subcortical white matter of aged monkeys (Sloane et al., 1999). 3-NT formation is the hallmark of reactive peroxynitrite (ONOO⁻), and this modification can
compromise the functional and/or structural integrity of target proteins (Smith et al., 1996), especially MnSOD (Ischiropoulos et al., 1992). After diffusing into mitochondria, \( \cdot \)NO can inhibit oxygen consumption by complex IV. Inhibition by \( \cdot \)NO is reversible; however, this process decreases electron transport and could potentially increase the concentration of \( \text{O}_2^- \) (MacMillan-Crow et al., 1996). MnSOD has a function to eliminate \( \text{O}_2^- \) from the mitochondrial matrix space. But, the concentration of \( \cdot \)NO required to inhibit complex IV is sufficient to compete effectively with MnSOD for \( \text{O}_2^- \) by a rapid reaction generating peroxynitrite, which can nitrate tyrosine residues and inactivate MnSOD to increase further the intramitochondrial level of \( \text{O}_2^- \) (MacMillan-Crow et al., 1996). \( \text{O}_2^- \) reacts with \( \cdot \)NO faster than with MnSOD (Hsu et al., 1996); therefore, if mitochondria contain inactive MnSOD, this peroxynitrite can further nitrate MnSOD and inactivate MnSOD to increase the intramitochondrial level of superoxide. Consequently this peroxynitrite-mediated amplification cycle would result in a progressive increase of mitochondrial levels of peroxynitrite, which can induce additional cytotoxic effects (MacMillan-Crow et al., 1996) including inactivation of complexes I and II in the mitochondrial respiratory chain (Cassina and Radi, 1996).

Myeloperoxidase (MPO), one of the principle hemoproteins stored in the azurophilic granules of neutrophils and monocytes, is also a catalyst of nitrotyrosine formation via nitrite oxidation to the potent nitrating species nitrogen dioxide (\( \cdot \text{NO}_2 \)) (Baldus et al., 2001). It has been demonstrated in vascular tissues that MPO significantly contributes to nitrotyrosine formation \textit{in vivo} (Baldus et al., 2002; Baldus et al., 2001). Further, it has been shown that MPO \(-/-\) animals have reduced nitrotyrosine immunoreactivity than WT mice (Baldus et al., 2002). MPO is not only present in neutrophils and monocytes, but also in microglia. Microglia are quiescent in normal brain but can become activated in response to neuronal damage or various other stimuli, including aggregated A\( \beta \) (El Khoury et al., 1998; Meda et al., 1995). In AD, MPO has been shown to be co-localized around the A\( \beta \) plaques and also in microglia-macrophages that are present around the plaques. It has also been shown that A\( \beta \) treatment of the mouse microglia cell line BV-2 resulted in strong induction of MPO mRNA expression (Reynolds et al., 1999). From these observations and the fact that senile plaques are
surrounded by activated microglia, it can be cautiously speculated that MPO may also contribute to the nitration of MnSOD.

Despite major advances in the study of nitration of proteins, the effect of nitration on protein turnover and the pathways that degrade the nitrated proteins have not been fully elucidated. There are reports about faster degradation of proteins treated with peroxynitrite or a generator of nitric oxide and superoxide by 20S proteasome (Grune et al., 1998). Consistent with this, Souza and colleagues (Souza et al., 2000) reported that a single nitrating event is sufficient to target proteins for degradation by the proteasome. Although protein nitration is generally viewed as an irreversible event, activities that appear to specifically repair nitrated proteins have been reported in human and rat tissues (Gow et al., 1996; Kamisaki et al., 1998). Thus it tempting to speculate that nitration of MnSOD may be reversible. In this regard, our APP/PS-1 mouse model is ideal for testing pharmacological intervention by mitochondrially targeted antioxidants such Mito-Q or Mito-Vit E.

Mito-Q [mixture of mitoquinol-10-(6'-ubiquinolyl)de-cytriphenylphosphonium and mitoquinone-10-(6'-ubiquinonyl)decytriphenylphosphonium] (Kelso et al., 2001) and MitoVit E [2-[2-(triphenylphosphonio)ethyl]-3,4-dihydro-2,5,7,8-tetra-methyl-2H-1-benzopyran-6-ol bromide] have been found to destroy superoxide in the mitochondrial matrix (Echtay et al., 2002). Mito-Q (Mito-Q10) has been shown to be an effective antioxidant against lipid peroxidation, peroxynitrite, and superoxide (James et al., 2005). Pretreatment with Mito-Q and Mito Vit-E have been shown to 1) significantly abrogate the lipid peroxide-induced 2′-7′-dichlorofluorescein fluorescence and protein oxidation in bovine aortic endothelial cells; 2) inhibit cytochrome c release, caspase-3 activation, and DNA fragmentation; 3) inhibit H2O2 and lipid peroxide-induced inactivation of complex I and aconitase, thus preventing the production of superoxide; 4) inhibit TfR overexpression and mitochondrial uptake of 55Fe, thereby inhibiting apoptosis; and 5) restore the mitochondrial membrane potential and proteasomal activity (Dhanasekaran et al., 2004). Further, the pro-oxidant effect or superoxide production by Mito-Q10 was found to be insufficient to cause any damage but led to hydrogen peroxide production and nitric oxide consumption (James et al., 2005). Despite these results, the effect of these
compounds on the reversal of damage that has already been caused by oxidative stress has not been investigated.

The antioxidant efficacy of Mito-Q₁₀ is due to its conversion to ubiquinol by complex II of the mitochondrial respiratory chain, but its reoxidation back to ubiquinone by complex III is ineffective. In ubiquinol form, Mito-Q₁₀ quenches ONOO⁻ and becomes oxidized, which can be reduced by complex II to ubiquinol, making it available to quench more ONOO⁻ (James et al., 2005). Mito-Q₁₀ may be effective in preventing the nitration of MnSOD and may serve as an effective therapeutic intervention to slow the progression of AD.

Mitochondrial respiratory dysfunction and oxidative stress have been associated with many neurodegenerative diseases (Beal, 1995). Results obtained in this study on mitochondrial respiration using pyruvate plus malate as substrate demonstrate that in APP/PS-1 mice there is inhibition of NAD-linked state 3 respiration rate, which is mediated through complex 1 of the mitochondrial electron transport chain. RCR represents functional integrity of isolated mitochondria (Yen et al., 1999). A high value of RCR indicates the utilization of substrates is tightly coupled to the production of ATP. The respiration injury would predict a low coupling efficiency of the mitochondrial electron transport and an increased likelihood of electron leakage during respiration, leading to O₂⁻ radical formation and increased oxidative stress. Our finding is consistent with that of Kokoszka and colleagues (Kokoszka et al., 2001) who reported a decrease in state III respiratory states and the RCR of liver mitochondria from both homozygous and heterozygous knock-out mice for the gene encoding the MnSOD protein, sod₂. Cardiac mitochondria from sod₁⁻/⁻ mice, which have 50% reduction in MnSOD activity, showed altered mitochondrial function as exemplified by decreased respiration by complex I and an increase in the sensitivity of the permeability transition pore induction (Van Remmen et al., 2001). Thus, mitochondria from the heart (sod₁⁻/⁻) and liver (sod₀⁻ and sod₂⁺/⁻) show evidence of increased oxidative damage compared with mitochondria isolated from sod₂⁺/⁺ mice. Yen and colleagues (Yen et al., 1999) showed that MnSOD selectively protected state 3 respiration activity through complex I substrates and prevented complex I inactivation in heart mitochondria treated with the anthracyclin antibiotic, adriamycin. These results indicate that MnSOD plays a critical role in oxidative stress.

49
responses and in maintenance of mitochondrial respiration and the lack or reduced activity that leads to increased sensitivity of the animals to oxidative stress-induced mitochondrial damage. The decreased activity of MnSOD in APP/PS-1 mice can be implicated as a cause of decreased state 3 respiration rate seen in these mice. Our study, which shows a decline in mitochondrial respiration in aged (9 and 12 months) WT mice, also implicates the effects of mitochondrial ROS production in the process of aging. Our findings are consistent with studies that demonstrate a decline in respiratory function of mitochondria with age (Wei and Lee, 2002; Yen et al., 1989) and decreased mitochondrial respiratory function in aged or aging mice, due to deficiencies in the MnSOD (Kokoszka et al., 2001). Our results also suggest that nitrative inactivation of MnSOD may be, at least in part, responsible for the decline in the mitochondrial function of aging AD mice. Biochemical analyses of brain specimens from patients with AD have shown abnormalities in the components of electron transport chain, particularly in the activity of cytochrome oxidase (COX) (Kish et al., 1999; Maurer et al., 2000). Inhibition of COX could cause depressed ATP synthesis and bioenergetic impairment in AD. In addition, the decreased COX function could cause diversion of electrons from their normal pathway into reaction with molecular oxygen in the neocortex and hippocampus in AD, resulting in increased $O_2^-$ (Markesbery, 1997). Superoxide can contribute to mitochondrial impairment by generating additional reactive species, particularly hydroxyl radical, and peroxynitrite that can inactivate mitochondrial proteins leading to further decline in mitochondrial respiration, decreased ATP production, and ultimately neuronal cell death (Eckert et al., 2003).

Although there is no consensus about the activity level of MnSOD in AD, there have been reports of reduction of SOD activity in AD frontal cortex, hippocampus, and cerebellum (Richardson, 1993) and elevation of SOD activity in the caudate nucleus of AD (Marklund et al., 1985). However, there are reports of increased nitrotyrosine immunoreactivity in neurons of AD (Good et al., 1996; Vodovotz et al., 1996). To our knowledge the identity of the nitrated proteins in the AD brain is not known. Our results showing decreased activity of MnSOD in relation to age and nitration represents the first study directed to the identification of nitrated proteins in AD brain.
The mouse model used in our study has the sequence of Aβ identical to the human sequence. Further, the mutations introduced are driven by endogenous promoters of APP and PS-1 genes, and expression is limited to the replacement of these two endogenous genes and not by the expression of multiple transgenes. The amyloid pathology observed in this model is similar to that found in AD. Thus, the results obtained from this model may prove useful in unraveling the pathogenesis of AD-induced oxidative stress.

In conclusion, in this study we demonstrate an increased and accelerated deposition of Aβ in APP/PS-1 mice, increased levels of Aβ1-40/1-42, increased nitrotyrosine and subsequent inactivation of MnSOD, and impaired mitochondrial respiration in APP/PS-1 mice in association with age. The increased levels of Aβ1-40/1-42, essential for aggregation and subsequent neurotoxic properties, are observed from 6 months on. It is tempting to speculate that the increased Aβ may be the cause of alterations seen in MnSOD and the associated decrease in mitochondrial function. These changes may lead to altered function of mitochondrial permeability transition pore (Yen et al., 1989), which increases the propensity to undergo apoptosis. This is important because findings indicate that neuronal cell death associated with Aβ peptide are apoptotic in nature (Dickson, 2004). Thus, our studies which indicate mitochondrial dysfunction, an age-associated increase in nitration of MnSOD, and its concomitant decrease in antioxidant activity, may provide the missing link between Aβ-induced oxidative stress and progression of AD.

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CHAPTER THREE

PEROXYNITRITE INDUCED NITRATION OF MnSOD REVEALS A NOVEL MECHANISM FOR THE REGULATION OF METABOLIC SWITCH IN NEURONAL CELLS

ABSTRACT

The energy demand of a cell is met predominantly by oxidative phosphorylation and glycolysis, both capable of mutual compensation. Because the mitochondrial electron transport chain can generate superoxide radicals during the process of ATP production, which leads to mitochondrial dysfunction, we investigated whether inactivation of Manganese superoxide dismutase (MnSOD), a critical antioxidant enzyme in mitochondria, would lead to an increase in glycolysis for ATP production. Here we report that exposure of SH-SY5Y cells to 250 μM peroxynitrite treatment leads to MnSOD nitration and subsequent reduction in activity. MnSOD activity was inactivated at a concentration of peroxynitrite 10 times lower than that required to inhibit mitochondrial respiration. Compromised mitochondrial function was demonstrated by the reduced activity of complex I and inhibition of MTT reduction. The loss of complex I activity was alleviated by SOD mimetic and was reproduced by MnSOD siRNA. The decline in mitochondrial function of peroxynitrite treated cells did not result in decreased ATP levels but was accompanied by an up-regulated glycolysis signified by high levels of lactate and lactate dehydrogenase activity but decreased activity of pyruvate dehydrogenase. These changes were reversed by SOD mimetic and were promoted by MnSOD siRNA, linking the effect of reduced MnSOD activity to a switch in the cell’s energy production mechanism. Specific reduction of MnSOD in MnSOD heterozygous knock-out mice led to decreased RCR and complex I activity with increased lactate levels, further supporting the role of MnSOD in regulating energy metabolism switch. Our results suggest that glycolysis plays a role in sustaining cellular energy requirements under pathological conditions with MnSOD deficiency.
INTRODUCTION

Aerobic organisms use O₂ to facilitate the synthesis of energy in the form of ATP mainly by two routes, glycolysis in cytosol and oxidative phosphorylation in the inner mitochondrial membrane (Kadenbach, 2003). Oxidative phosphorylation comprises a respiratory chain consisting of three proton pumps or complexes and produces the majority of ATP that cells require. Aerobic organisms use O₂ as a terminal electron acceptor for optimal energy release by mediating the transfer of single electrons (Nohl et al., 2004) through these complexes. The electrons from reduced substrates are passed from complexes I and II of the electron transport chain through complexes III and IV to oxygen, forming water and causing protons to be pumped across the mitochondrial inner membrane (Brand et al., 2004). But in circumstances of energy deficiency caused by compromised mitochondrial function, enhanced glycolytic capacity exists, even in the presence of high O₂ concentration (Pedersen, 2007; Schoonen et al., 1990; Warburg, 1956; Wu et al., 2007). Mitochondrial dysfunction can occur due to accumulation of oxygen-derived reactive species such as hydroxyl radical and peroxyl radicals, and nonradical molecules such as singlet oxygen and hydrogen peroxide (Oberley, 2001), collectively called reactive oxygen species (ROS). ROS are formed by the nature of oxygen, which is predisposed for univalent reductions (Fridovich, 1999). Electrons can leak from the respiratory chain and react with oxygen to form superoxide radicals (O₂⁻). Thus, superoxide radicals can be considered the parent radical from which other oxygen radicals and non-radicals are derived (Nohl et al., 2004). Approximately 90% of cellular ROS can be traced to oxidative phosphorylation in mitochondria (Balaban et al., 2005). Initial observations suggest that up to 5% of the total molecular oxygen utilized by mammalian mitochondria was converted into ROS (Boveris and Chance, 1973), but more recent analysis estimates that about 1-2% was converted into O₂⁻ anion (Cadenas and Davies, 2000). Superoxide and other oxygen-derived reactive species (Brand et al., 2004) directly or indirectly can damage proteins, lipids and DNA. To prevent ROS-induced injury, cells are equipped with an elaborate repertoire of antioxidant defense systems (Halliwell and Gutteridge, 2007). There are three major primary intracellular antioxidant enzymes in mammalian cells that can remove ROS: superoxide dismutase...
(SOD), catalase (CAT), and peroxidase, of which glutathione peroxidase (GPx) (Oberley, 2001) and peroxiredoxin (Prx) (Halliwell and Gutteridge, 2007) are the most prominent. The SODs catalyze the dismutation of \( \text{O}_2^- \) into hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and molecular oxygen. Hydrogen peroxide is further detoxified by CAT, GPx (Halliwell and Gutteridge, 1989) and Prx to water. Thus, two reactive species--\( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \)--are converted to water (Oberley, 2001) and molecular oxygen. Any disturbance in the pro-oxidant and anti-oxidant balance in favor of the former is defined as oxidative stress (Sies, 1985).

Among the antioxidant systems, SOD is considered the first line of defense against oxidative stress (Halliwell, 1999; Ho et al., 1998). Four distinct SODs are found in human cells: a homodimeric cytosolic copper zinc SOD (CuZnSOD) (McCord and Fridovich, 1969), an mitochondrial inter-membrane space CuZnSOD (Okado-Matsumoto and Fridovich, 2001), an extra-cellular homotetrameric glycosylated CuZnSOD (Marklund, 1982) and a mitochondrial matrix homotetrameric manganese SOD (MnSOD) (Weisiger and Fridovich, 1973). MnSOD is a critical antioxidant enzyme in aerobic organisms because under physiological conditions superoxide is mainly generated on the matrix side of the inner mitochondrial membrane where MnSOD is located (Balzan et al., 1999). MnSOD is a homotetramer with identical 24-kd subunits and one manganese ion per subunit (Matsuda et al., 1990). The mature protein is strictly a mitochondrial enzyme compartmentalized in the mitochondrial matrix, although it is encoded by the nuclear \textit{sod}2 gene. Studies performed by us and others have shown that an imbalance between antioxidants and oxidants leads to various pathological conditions which can be effectively prevented or alleviated by increased MnSOD (Keller et al., 1998; St Clair et al., 1991; Yen et al., 1996). The over-all importance of this enzyme is evident from transgenic animal studies, wherein the MnSOD knock-out mice usually died within 10 days after birth with cardiac abnormalities, fat accumulation in liver and skeletal muscle (Lebovitz et al., 1996; Li et al., 1995) and metabolic acidosis (Lebovitz et al., 1996); and the animals that survived longer than 10 days soon succumbed to severe anemia and neurodegeneration (Melov et al., 1998). It has been demonstrated that MnSOD heterozygous knock-out mice show altered mitochondrial function, as illustrated by decreased respiration by complex I and increased sensitivity of permeability
transition pore induction (Van Remmen et al., 2001), while overexpression of MnSOD protects complex I from inactivation (Yen et al., 1999). Thus, it is likely that MnSOD function influences mitochondrial function and oxidative phosphorylation, necessary machinery for the production of ATP in cells.

MnSOD activity can be compromised by a post-translation modification, such as nitration of tyrosine residues in its subunits (Ischiropoulos et al., 1992). Of the nine tyrosine residues in each subunit of the homotetrameric MnSOD protein, tyrosine residues 34, 45 and 193 have been shown to be nitrated, with nitration of tyrosine residue 34 causing inactivation of MnSOD (MacMillan-Crow et al., 1998). Nitration of biological macromolecules can be mediated by nitric oxide, nitrogen dioxide, nitrous acid, hypochlorous acid, nitrated aliphatic reactive intermediates and peroxynitrite (Ischiropoulos, 1998). Peroxynitrite has been demonstrated to nitrate tyrosine 34 of MnSOD which leads to its inactivation (MacMillan-Crow et al., 1998; Yamakura et al., 1998). Extensive data implicate peroxynitrite (ONOO\(^-\)) as a consistent mediator of 3-NT formation (MacMillan-Crow et al., 1996; Smith et al., 1996; Yamakura et al., 1998; Yamamoto et al., 2002). Peroxynitrite is a powerful oxidant and cytotoxic agent formed by the reaction between the free radicals nitric oxide (NO) and superoxide (O\(_2^-\)) at rates approaching diffusion limit (Beckman, 1996; Huie and Padmaja, 1993). The half life of peroxynitrite is < 1s, and the homolytic cleavage results in the production of hydroxyl radicals which have a much more deleterious effect than peroxynitrite itself (Beckman, 1996). Peroxynitrite causes oxidative damage to lipids, DNA, carbohydrates, proteins and amino acids, such as cysteine, methionine, phenylalanine and tyrosine (Beckman and Koppenol, 1996; Ischiropoulos and al-Mehdi, 1995). Externally added ONOO\(^-\) has been shown to diffuse passively across lipid bilayers (Denicola et al., 1998) over distances of 1 - 10 \(\mu\)m (Radi, 1998) with a permeability coefficient comparable to water (Marla et al., 1997). Although one of the major oxidative modifications of proteins resulting from peroxynitrite is carbonyl formation from side-chain and peptide-bond cleavage, perhaps the best known property of ONOO\(^-\) is its ability to nitrate free tyrosine and tyrosine residues in proteins (Ischiropoulos et al., 1992; Souza et al., 1999). It has been demonstrated that ONOO\(^-\) nitrates and inactivates MnSOD in chronic rejection human
renal allograft, which has been proposed to be a general mechanism for amplification of ONOO⁻ induced damage (MacMillan-Crow et al., 1996). In addition to nitration of MnSOD, ONOO⁻ has also been shown to mediate inactivation of electron transport chain components and ATPase, thereby inhibiting electron transport and ATP synthesis (Radi et al., 1994) and increasing the rate of glycolysis (Bolanos et al., 1995). However, it is unclear whether nitration of MnSOD is directly relevant to the observed peroxynitrite-induced alteration in energy production.

Bringing together the findings that MnSOD is sensitive to ONOO⁻, the role of MnSOD in maintaining mitochondrial function and the contribution of mitochondrial function to ATP production, we propose that nitrative inactivation of MnSOD is an important event in peroxynitrite-induced alteration in metabolism. Here, we demonstrate that exposure of human dopaminergic neuron SH-SY5Y cells to peroxynitrite (ONOO⁻) leads to nitration and reduced activity of MnSOD. This is accompanied by decreased mitochondrial function and complex I activity, and increased lactate levels, with no decrease in ATP levels. The loss of mitochondrial function and complex I activity and increase in lactate levels are alleviated by the presence of SOD mimetic. These results suggest a novel role of MnSOD in mediating control of mitochondrial function and energy production.

**MATERIALS AND METHODS**

**Materials**

Peroxynitrite was generously provided by Dr. Timothy R. Miller, University of Texas at El Paso. All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of the following: Nitro blue tetrazolium (NBT) was purchased from Fisher Bioreagents (Fairlawn, NJ); protease inhibitor set III from Calbiochem (La Jolla, CA); rabbit poly-clonal anti-nitrotyrosine from Cayman chemical (Ann Arbor, MI); rabbit poly-clonal anti-MnSOD from Upstate Technology (Lake Placid, NY); protein A/G agarose from Santa Cruz Biotechnology (Santa Cruz, CA); minimum
essential medium (MEM) from Gibco (Grand Island, NY); and standard fetal bovine serum from HyClone (Logan, UT). SOD mimetic (Mn^{III}TE-2-PyP^{5+}) was prepared as previously described (Batinic-Haberle, 1999).

Cell Culture

SH-SY5Y, a human neuroblastoma cell line, was grown in MEM, supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin / streptomycin / neomycin), 1% nonessential amino acids and 1 mM sodium pyruvate. Cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Animals

Heterozygous C57BL6 MnSOD knock-out mice (sod 2 +/−) were originally obtained from Dr. Charles J. Epstein, University of California at San Francisco, and maintained as previously described (Van Remmen et al., 1999). The wild-type C57BL6 mice used for generation of sod 2 +/− mice were purchased from Harlan (Indianapolis, IN). Characterization of MnSOD knock-out mice has been previously reported (Van Remmen et al., 1999). A 30 – 80% decrease in MnSOD activity in all tissues (liver, kidney, lung, brain, heart, muscle, stomach and spleen) of the heterozygous offspring has been documented and no alterations in CuZnSOD, glutathione peroxidase or catalase activities were found. All mice used in the experiments were euthanized by intraperitoneal injection with 68 mg/kg of Nembutal followed by cervical dislocation. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Isolation of mitochondria

Three brains (excluding cerebellum) from each group were removed immediately after animals were euthanized and were homogenized in 5 ml of ice-cold mitochondrial buffer, pH 7.2, containing 0.225 mol/L D-mannitol, 0.075 mol/L sucrose, 20 mmol/L
HEPES, 1 mmol/L EGTA and 1% bovine serum albumin in a Dounce homogenizer with a Teflon pestle. The homogenized brain samples were then diluted with isolation buffer to a final volume of 10 ml and centrifuged at 4500 rpm for 5 minutes. The supernatant was transferred to a fresh tube on ice; the pellets were resuspended in isolation buffer, and homogenized and centrifuged at 4500 rpm for 5 minutes. The supernatants were combined and centrifuged at 10,000 rpm for 20 minutes. The brown mitochondrial pellets were resuspended in 200 μl of respiration buffer or phosphate buffered saline, as required. Protein concentration was assessed by Bradford assay. The isolated mitochondria were used for immunoprecipitation of nitrated MnSOD, assessment of oxygen consumption and activity of MnSOD.

**Immunoprecipitation of Nitrated MnSOD**

Isolated mitochondrial protein from the mouse brain or SH-SY5Y cell lysate (300 μg) was resuspended in 200 μl of RIPA buffer (10 mM TRIS, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100 and 0.1% SDS, pH 7.5). Rabbit polyclonal nitrotyrosine antibody (3 μg/ml) was added and incubated overnight at 4°C. Protein A/G agarose beads were added to the mixture and incubated overnight at 4°C. Immunocomplexes were collected by centrifugation at 12,000 rpm for 5 minutes at 4°C, followed by washing the complexes with RIPA buffer three times. The immunoprecipitated complexes were resuspended in 2X sample buffer, boiled for 5 minutes, fractionated by reducing SDS/polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blot using rabbit polyclonal MnSOD antibody.

**Assessment of ATP**

SH-SY5Y cells were plated at a density of 3 x 10^5 cells/well and treated with indicated concentrations of peroxynitrite for 24 hours. The cells were collected in 1X phosphate buffered saline (PBS) after washing twice with 1X PBS. The cells were lysed by three freeze-thaw cycles. The cell lysate was used to assess the ATP level, using the kit developed by Molecular probes (Invitrogen, Eugene, OR) according to the
manufacturer’s instructions, and analyzed by T20/20 luminometer. The tests were repeated three times in triplicate.

**Measurement of Glucose**

Cell lysate prepared as described above for assessment of ATP was used to determine glucose concentration using the Amplex Red glucose/glucose oxidase assay kit developed by Molecular probes (Invitrogen, Eugene, OR) as per the manufacturer’s instructions. The analysis was performed three times in triplicate.

**Determination of Lactate level**

Brains (excluding cerebellum) from MnSOD hetzygous knock-out (sod 2 +/-) mice were collected and washed twice in ice-cold PBS, and homogenized in Dounce homogenizer using Teflon pestle. The homogenate was centrifuged at 5000 rpm for 5 minutes and the supernatant was used to determine the concentration of lactate. The SH-SY5Y cell lysate, as described for assessment of ATP, was used to estimate the lactate level. The reaction mixture contained 2M Tris; pH 7.5 in water with 0.05% sodium azide, stored at room temperature; 60 mM NAD\(^+\) in water, stored at -20\(^\circ\)C; 15 mM INT, prepared in water containing 10% (v/v) acetone; 100 units/ml Dase in 75 mM Tris; pH 7.5 stored at -20\(^\circ\)C; and 50 units/ml of lactate dehydrogenase (LDH). Just prior to the beginning of the assay, Dase was diluted 1:100 with 75 mM Tris, pH 7.5. For the assay, 50 µl of the reaction reagent was added to 20 µl of the cell lysate in a 96-well flat bottom microtitre plate. The reaction was allowed to continue for 40 minutes in a humidified incubator without CO\(_2\). The reaction was stopped by adding 50 µl of 3% acetic acid, followed by a brief agitation. The absorbance was measured at 492 nm using a Spectra Max Gemini plate reader from Molecular Devices. The reaction is based on the production of NADH and pyruvate from NAD and lactate by LDH. This reaction is coupled to Dase-catalyzed reoxidation of NADH to NAD and reduction of INT to red colored formazan which is read at 492 nm. The intensity of the red color is proportional to the concentration of lactate in the sample.
Activity of Lactate Dehydrogenase

Lactate dehydrogenase (LDH) activity was assayed in the cell lysate of SH-SY5Y cells treated with 250 μM peroxynitrite for 24 hours. The cells were washed twice with 1X PBS, collected in 1X PBS and homogenized using a hand homogenizer for 30 seconds. This was repeated three times with 30 second intervals. The homogenized cell lysate was used immediately to determine LDH activity, following the method described by Yen et al. (Yen et al., 1996). Briefly, the cell lysate was added to a solution containing NADH and incubated at room temperature for 20 minutes. Sodium pyruvate (22.7 mM) in 0.1 M potassium phosphate buffer (pH 7.5) was added after 20 minutes. In the assay, the reaction where pyruvate is converted to lactate catalyzed by LDH is followed for two minutes. The decrease in absorbance (dA/min – change in absorbance/minute) at 340 nm that occurs with the oxidation of NADH to NAD is used for calculating the LDH activity using the extinction coefficient of 6.22 mM⁻¹cm⁻¹ for NADH. The activity was measured in three independent experiments, each in triplicate.

Activity of Pyruvate Dehydrogenase

The assay is based on the linking of the NADH produced by PDH to reduction of the dye INT and was performed as described by Elnageh & Gaitonde (Elnageh and Gaitonde, 1988). Briefly, the final reaction mixture of 1 ml volume consisted of 50 mM Tris-HCL, 0.5 mM EDTA and 0.2% (wt/vol) Triton X-100 (pH 7.8), 20 μL of the cell lysate, 2.5 mM NAD, 0.1 mM CoA, 1 mM MgCl₂, 0.1 mM oxalate, 1 mg of bovine serum albumin, 0.6 mM INT, 5-7 units of lipoamide dehydrogenase, 0.2 mM TPP and 5 mM pyruvate. After the addition of 4 mM TPP the contents were kept at 37°C for 5 minutes. A stable baseline at 500 nm was obtained for the reaction mixture. The reaction was started by adding 100 mM pyruvate. The absorbance of the mixture at 500 nm was recorded at the beginning and again after the reaction had been underway for 5 minutes at 37°C. Data was obtained from three independent experiments with triplicate samples.
MTT Assay

The conversion of MTT dye to formazan dye crystals has been demonstrated to be related to mitochondrial respiratory chain function (Musser and Oseroff, 1994). Cells were plated at a concentration of 2 x 10⁴ cells/well and treated with the indicated concentrations of peroxynitrite or SOD mimetic (1000 pg/ml). MTT (1 mg/ml) was added to the cell cultures and incubated for 45 minutes. Cells were then washed three times with PBS, solubilized in dimethylsulfoxide, and the absorbance was quantified at 540 nm using a Spectra Max Gemini plate reader from Molecular Devices. The data was obtained from three independent experiments with six replications each.

Activity of Complex I

Brains (excluding cerebellum) from sod 2 +/- mice were collected and washed twice in ice-cold PBS, and homogenized in Dounce homogenizer using Teflon pestle. The homogenate was used to estimate the activity of complex I. SH-SY5Y cells treated with 250 μM of ONOO⁻ for 24 hours were washed twice in 1X PBS, collected in hypotonic buffer (20 mM potassium buffer, pH 7.2) and lysed by three cycles of fast freeze-thaw. The specific activity of complex I was assayed as described previously (Lash and Jones, 1993), with slight modifications. Briefly, the assay mixture containing 25 mM of potassium phosphate buffer (pH 7.2), 5 mM MgCl₂, 2 mM KCN, 2.5 mg/ml bovine serum albumin (fraction V), 0.13 mM NADH, 65 μM coenzyme Q₅ and 2 μg/ml antimycin A were incubated at 30°C for 1 minute. The cell lysate/brain homogenate was added to initiate the reaction and the initial rate of NADH oxidation was monitored at 340 nm (ε 6.81 mM⁻¹ cm⁻¹) for 1 minute. The complex I specific activity was inhibited by 2 μg/ml of rotenone. Complex I activity was calculated by the differences in dA/min with and without rotenone. Activity was determined from three independent experiments, in triplicate, for cells and from three mice.
**Activity of MnSOD**

SOD activity in the isolated mitochondria from brains of wild-type (WT) mice or from SH-SY5Y cell lysate was measured by the nitro blue tetrazolium (NBT)-bathocuprione sulfonate reduction inhibition method (Spitz and Oberley, 1989). This assay is based on the competitive reaction between SOD and the indicator molecule NBT for superoxide radicals generated by xanthine/xanthine oxidase. The reduction of NBT is progressively inhibited in relation to addition of protein (containing SOD activity). Because potassium cyanide (5 mmol/L) was used to inhibit Cu/ZnSOD, this method measured only MnSOD activity. The assay mixture also contained catalase to remove H$_2$O$_2$ and diethylenetriaminepentaacetic acid to chelate metal ions capable of redox cycling and interfering with the assay system. The reduction of NBT to blue formazan by superoxide with/without cell lysate or isolated brain mitochondria was measured spectrophotometrically at 560 nm at 25°C. The rate of NBT reduction in the absence of cell lysate or isolated brain mitochondria was used as the reference rate (0.02 ± 0.005 absorbance/min). The data were plotted as percent of inhibition of NBT reduction versus protein concentration. One unit of SOD activity was defined as the amount of SOD protein that caused a 50% reduction in the background rate of NBT reduction. The activity of MnSOD was determined in triplicate from three independent experiments.

**Mitochondrial Respiration Assay**

Mitochondrial protein (400 μg) was resuspended in the respiration buffer containing 0.25 mol/L sucrose, 50 mmol/L HEPES, 2 mmol/L MgCl$_2$, 1 mmol/L EGTA, 10 mmol/L KH$_2$PO$_4$ and 0.5% bovine serum albumin, pH 7.4. Oxygen consumption was measured using a Clark-type electrode oxygraph (Hansatech Inc., Norfolk, UK) with 10 mmol/L pyruvate and 5 mmol/L malate as substrate in the absence of ADP (state II respiration), and after addition of 300 mmol/L ADP (state III respiration). The ATPase inhibitor oligomycin (100 μg/ml) was then added to inhibit mitochondrial respiration (state IV respiration). The ratio between the rate of oxygen consumption/min of state III and state IV was used to calculate the respiratory control ratio (RCR).
RNA Interference

siRNA (Thermoscientific Dharmacon, On-TARGETplus SMARTpool) was used to selectively knock-down MnSOD. SH-SY5Y cells were plated at 40% confluence at the time of transfection and transfected with control siRNA or MnSOD siRNA using oligofectamine for 12 hours in a serum-reduced Opti-MEM (Invitrogen). The cells were washed with 1X PBS 48 hours after transfection and lysed by incubating for 30 minutes with lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1, 2 – dianinocyclohexane N, N, N’, N’ – tetraacetic acid, 10% glycerol and 1% Triton X-100). Suppression of MnSOD was analyzed by Western blot. Briefly, equal amounts of cell lysate proteins were resuspended in 2X sample loading buffer and SDS-PAGE was performed on 12.5% polyacrylamide gels. Proteins were transferred electrophoretically (100V, 1 hour) to nitrocellulose membranes (Schleicher & Schuell, Dassell, Germany). Membranes were blocked using 5% nonfat dried milk in 50 mmol/L Tris, pH 7.9, 150 mmol/L NaCl, and 0.05% (v/v) Tween-20. For detection of MnSOD, nitrocellulose membranes were incubated overnight at 4°C with polyclonal MnSOD antibody at a dilution of 1:10000. This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Probed membranes were washed three times, and immunoreactive proteins were detected using enhanced chemiluminescence (Amersham Corp., Piscataway, NJ). Western blot analysis was conducted three times; the representative blot is shown in the manuscript.

Statistical analysis

Data were evaluated using either analysis of variance (ANOVA) with post-hoc Tukey’s multiple comparison test or Student’s t-test. A difference of p < 0.05 was considered significant.
RESULTS

Peroxynitrite induced nitration and inactivation of MnSOD in SH-SY5Y cells

It has been shown that MnSOD is nitrated at tyrosine residue 34 and subsequently inactivated by peroxynitrite (Yamakura et al., 1998). The experimental model used in this study demonstrates nitrated MnSOD and is the first step toward establishing a cell culture model for investigating the effect of MnSOD inactivation on cellular metabolism. SH-SY5Y were treated with 250 μM peroxynitrite and incubated for 24 hours. The presence of nitrated MnSOD was identified by immunoprecipitation using a polyclonal nitrotyrosine antibody followed by Western analysis of MnSOD protein. Immunodetection with polyclonal MnSOD antibody demonstrated an appreciable amount of nitrated MnSOD in cells treated with peroxynitrite (Figure 3.1A).

To verify that the presence of nitrotyrosine in MnSOD is associated with reduced MnSOD activity, we measured MnSOD activity in cells treated with 250 μM peroxynitrite. MnSOD activity in peroxynitrite treated cells was significantly reduced compared to control (Figure 3.1B).

Alteration in mitochondrial function in SH-SY5Y cells is associated with MnSOD activity

To determine whether the decreased activity of MnSOD is associated with decreased mitochondrial function, we performed MTT assay (a measurement of mitochondrial dehydrogenase function) in SH-SY5Y cells 24 hours after treatment with 250 μM peroxynitrite. Quantification of absorbance of the solubilized formazan at 540 nm revealed a significant reduction (p < 0.001) (Figure 3.2A) in the MTT level in cells treated with peroxynitrite.

We have previously demonstrated that overexpression of MnSOD protects complex I from Adriamycin induced inactivation (Yen et al., 1999). To determine whether the observed decrease in MnSOD activity in peroxynitrite treated cells has any
Figure 3.1 Treatment with peroxynitrite induces nitration and reduced activity of MnSOD in SH-SY5Y cells
Figure 3.1 Treatment with peroxynitrite induces nitration and reduced activity of MnSOD in SH-SY5Y cells.

A. Immunoprecipitation (IP) of MnSOD tyrosine nitration. SH-SY5Y cell lysate was precipitated with nitrotyrosine antibody and analyzed by Western blot using MnSOD antibody. Positive control – Isolated brain mitochondria treated with 250 μM peroxynitrite, Negative control – Isolated brain mitochondria precipitated with non-immune rabbit IgG and analyzed by Western blotting using MnSOD antibody. PEL – pellet, SUP – supernatant.

B. MnSOD activity in SH-SY5Y cells treated with 250 μM peroxynitrite was measured by NBT-bathocuprione sulfonate reduction inhibition method. The activity was measured in triplicate at 24 hours post treatment in three sets of independent experiments. *, p < 0.05, compared with the control.
effect on the activity of complex I, we assessed the activity of complex I in SH-SY5Y cells treated with 250 μM peroxynitrite and incubated for 24 hours. As shown in Figure 3.2B, the activity of complex I was significantly reduced (p < 0.05) in comparison with the untreated control.

SOD mimetic (Mn^{III}-TE-2-PyP^{5+}) protects against peroxynitrite–induced mitochondrial dysfunction

Although the reduced MnSOD activity and concurrent reduction in mitochondrial function and complex I activity are interrelated, the interrelationship does not demonstrate that reduction of MnSOD activity is a cause for reduced mitochondrial function. To further establish the role of MnSOD on mitochondrial function, we pre-treated the SH-SY5Y cells for 24 hours with SOD mimetic and measured mitochondrial function after treatment with peroxynitrite. Cells were pre-treated with SOD mimetic (1000 pg/ml) for 24 hours and then exposed to 250 μM peroxynitrite. The results show that the loss of complex I activity was reduced in the cells with SOD mimetic pre-treatment (p < 0.05) (Figure 3.3A).

To verify further that MnSOD indeed contributes to mitochondrial function, we used the siRNA approach. SH-SY5Y cells were transfected with control siRNA or MnSOD siRNA using oligofectamine. Cells were harvested 48 hours after transfection and expression of MnSOD was verified by Western blot. There was a substantial suppression of MnSOD level (Figure 3.3B) in the cells transfected with MnSOD siRNA. These transfected cells were used for determination of complex I activity. The cells transfected with MnSOD siRNA showed significant decrease (p < 0.05) in complex I activity in comparison with untransfected control (Figure 3.3C).

MnSOD activity is sensitive to peroxynitrite induced inactivation

The results demonstrated in Figure 3.2 suggest that reduced activity of MnSOD may contribute to the loss of mitochondrial function. They do not indicate, however, whether MnSOD activity or mitochondrial function is more sensitive to peroxynitrite-
Figure 3.2 Peroxynitrite reduces mitochondrial complex I activity and mitochondrial function in SH-SY5Y cells.
Figure 3.2 Peroxynitrite reduces mitochondrial complex I activity and mitochondrial function in SH-SY5Y cells.

A. Mitochondrial function was assessed by the ability of the mitochondria to uptake and reduce MTT, 24 hours after SH-SY5Y cells were treated with 250 μM peroxynitrite.

B. Complex I activity in SH-SY5Y cells treated with 250 μM peroxynitrite in the same manner described in A. All measurements were performed in triplicate. The data are averages from three sets of independent experiments. **, p < 0.01, *, p < 0.05, compared with the control.
Figure 3.3 SOD mimetic and MnSOD siRNA alter mitochondrial complex I activity.
Figure 3.3 SOD mimetic and MnSOD siRNA alter mitochondrial complex I activity.

A. SH-SY5Y cells were treated with SOD mimetic (1000 pg/ml) for 24 hours, followed by treatment with 250 μM peroxynitrite for an additional 24 hours.

B. Western blot using polyclonal MnSOD and GAPDH antibody, csiRNA – control siRNA, siRNA – MnSOD siRNA.

C. Complex I activity (nmoles/min/mg protein) in SH-SY5Y cells transfected with MnSOD siRNA. The measurements were performed in triplicate. All data represent the average of three independent experimental sets. *, p < 0.05, #, p < 0.05, compared with the control and 250 μM ONOO respectively.
induced inactivation. To answer this question, we conducted a dose-response experiment in mitochondria isolated from mouse brain. The isolated mitochondria were treated with 0, 1, 10, 100 or 250 μM peroxynitrite for 1 minute and then oxygen consumption was measured as an indicator of mitochondrial respiration chain activity using a Clark-type electrode oxygraph with pyruvate and malate as substrates in the absence of ADP (state II respiration), or presence of ADP (state III respiration). The ATPase inhibitor oligomycin was then added to inhibit mitochondrial respiration (state IV respiration). The RCR was not affected and was comparable to the untreated control when exposed to 1 μM peroxynitrite, whereas the RCR was significantly decreased (p < 0.001) at all other concentrations of peroxynitrite (Figure 3.4A). The data obtained from respiration analysis show that 1 μM did not affect the RCR, whereas the activity of MnSOD was significantly reduced (p < 0.01) at this concentration (Figure 3.4B). This result indicates that MnSOD is inactivated by peroxynitrite at concentrations lower than what is required to observe the loss of mitochondrial respiration function.

Concomitant with the reduced activity of MnSOD, immunoprecipitation performed on the mitochondria treated with 1 μM and 10 μM peroxynitrite demonstrated appreciable levels of nitrated MnSOD (Figure 3.4C). This indicates that the observed reduced activity of MnSOD treated with 1 μM is associated with peroxynitrite-induced nitration of MnSOD.

**Peroxynitrite has no effect on cellular ATP levels**

Because treatment with peroxynitrite resulted in a reduction in mitochondrial respiration capacity, we explored the impact of this change on cellular ATP levels. Cells were treated with 250 μM peroxynitrite and the total cellular levels of ATP were compared 24 hours after treatment (Figure 3.5). The level of ATP in peroxynitrite treated cells was comparable to that of control cells. The cells treated with 2 deoxyglucose (2DG), a glycolytic inhibitor used as a positive control, showed significant decrease (p < 0.001) in ATP levels in comparison with both untreated cells and peroxynitrite treated cells (Figure 3.5).
Figure 3.4 MnSOD activity is more sensitive to peroxynitrite than mitochondrial respiration.
Figure 3.4 MnSOD activity is more sensitive to peroxynitrite than mitochondrial respiration.

A. RCR of isolated mitochondria protein from mouse brain tissues. RCR was calculated as the ratio between state 3 and state 4 respirations. Mitochondria were exposed to the indicated concentrations of peroxynitrite for 1 minute prior to the oxygen consumption measurement.

B. MnSOD activity was measured from isolated mitochondrial protein treated with indicated concentrations of peroxynitrite for 1 minute.

C. Nitrotyrosine containing mitochondrial protein was precipitated with nitrotyrosine antibody and analyzed by Western blot using MnSOD antibody. Positive control – Isolated brain mitochondria treated with 250 μM peroxynitrite, Negative control - Isolated brain mitochondria precipitated with non-immune rabbit IgG and analyzed by Western blotting using MnSOD antibody. PEL – pellet, SUP – supernatant. All measurements were performed in triplicate. The data shown is the average of three experimental sets. ***, p < 0.001, **, p < 0.01, compared with the control.
Figure 3.5 Peroxynitrite treatment does not alter ATP levels in SH-SY5Y cells. ATP was measured in SH-SY5Y cells treated with 250 μM peroxynitrite or 5.5 mM 2DG for 24 hours. 2DG was used as positive control. The measurements were performed in triplicate. The data represent an average from three sets of independent experiments. ***, p < 0.001, compared with the control.
Peroxynitrite mediated increase in glycolytic activity

Because the total cellular ATP was maintained in cells with reduced respiratory function, it is possible that glycolysis may compensate for the production of ATP. To address this question, we measured the concentration of lactate 24 hours after treating the SH-SY5Y cells with 250 μM peroxynitrite. The concentration of lactate in cell lysate treated with peroxynitrite was significantly increased (p < 0.01) in comparison with the untreated controls (Figure 3.6A). The activity of lactate dehydrogenase, an enzyme that catalyzes the conversion of pyruvate to lactate, was also significantly increased (p < 0.001) in the cell lysates treated with 250 μM peroxynitrite (Figure 3.6B). These results indicate that glycolytic activity increases in peroxynitrite treated cells.

The increase in glycolytic activity suggests that peroxynitrite treated cells may have a higher level of glucose, which was assessed by the glucose concentrations in the cell lysate. The results indicate that the concentration of glucose was significantly higher (p < 0.05) in peroxynitrite treated cells in comparison with control cells (Figure 3.7A).

Pyruvate dehydrogenase (PDH) catalyzes the conversion of pyruvate to Acetyl-CoA, which then enters the tri-carboxylic cycle. Decreased activity of PDH would lead to accumulation of pyruvate, which can be converted to lactate by lactate dehydrogenase. To probe this possibility, we analyzed the activity of PDH in SH-SY5Y cells 24 hours after treatment with 250 μM peroxynitrite. The results show that the activity of PDH was significantly decreased (p < 0.001) in peroxynitrite treated cells in comparison with the untreated control (Figure 3.7B).

Suppression of MnSOD enhances glycolytic activity

The increase in glycolytic activity obtained in cells treated with peroxynitrite suggests that MnSOD has a role in this process. To test this possibility, we reduced the MnSOD level by transfecting SH-SY5Y cells with control siRNA or MnSOD siRNA. The cells were harvested 48 hours after transfection and suppression of MnSOD was confirmed by Western analysis (data not shown). The corresponding cell lysates were used for measurement of lactate concentration and LDH activity. Both the lactate...
Figure 3.6 Peroxynitrite increases the levels of lactate and activity of lactate dehydrogenase in SH-SY5Y cells.

A. Concentrations of lactate.

B. Activity of LDH.

All these measurements were performed in triplicate. The data are the average of three experimental sets. **, p < 0.01, compared with the control.
Figure 3.7 Peroxynitrite increases the levels of glucose and suppresses the activity of pyruvate dehydrogenase in SH-SY5Y cells.

A. Concentrations of glucose.

B. Activities of PDH.

All these measurements were performed in triplicate. The data are the average of three experimental sets. *, p < 0.05, **, p < 0.01, compared with the control.
concentration (p < 0.01) and LDH activity (p < 0.05) were significantly increased in cells transfected with MnSOD siRNA (Figure 3.8A and B). This increase in lactate levels suggests that increased glycolytic activity compensates for reduced respiration due to reduction of MnSOD activity.

To test this possibility we investigated whether the compensatory increase in glycolytic activity is altered by the presence of SOD mimic. SH-SY5Y cells were pre-treated for 24 hours with SOD mimic (1000 pg/ml) followed by treatment with 250 μM peroxynitrite. Lactate levels were significantly higher (p < 0.05) in the group treated with peroxynitrite alone. However, in the SOD mimic pre-treated group, lactate levels were comparable to those of the control cells and were significantly less (p < 0.05) in comparison with the peroxynitrite treated group (Figure 3.9A). This indicates that SOD mimic protects the mitochondrial function and, thus, there is no compensatory increase in glycolytic activity. The cells treated with mimic showed significantly increased activity of pyruvate dehydrogenase (p < 0.01) in comparison with the group treated with peroxynitrite (Figure 3.9B).

**Decreased mitochondrial respiration and increased glycolytic activity in sod 2 +/- mice**

All the above observations made *in-vitro* suggest that reduction in MnSOD activity is associated with mitochondrial dysfunction and increase in glycolytic activity. To verify that this phenomenon is occurring *in-vivo*, we used sod 2 +/- mice. MnSOD activity in sod 2 +/- mice has been shown to be decreased (30 – 80%) in all tissues examined in comparison with sod 2 +/- mice (Van Remmen et al., 1999). Three mice from both sod 2 +/- and WT mice were euthanized. Brains (excluding cerebellum) were collected and used for analysis of mitochondrial respiration, complex I activity and lactate concentration. The results show that the RCR of sod 2 +/- mice mitochondria was significantly reduced (p < 0.05) in comparison with WT mice (Figure 3.10A).

The activity of complex I in whole brain homogenate was significantly reduced (p < 0.01) in comparison with WT mice (Figure 3.10B). The respiration and activity of complex I indicate that the mitochondrial function in the brain of sod 2 +/- mice was
compromised. To investigate whether glycolytic activity is increased to compensate for this loss of mitochondrial function, the concentration of lactate was evaluated. The concentration of lactate was significantly higher in sod 2 +/- mice (p < 0.01) in comparison with WT mice (Figure 3.10C).
Figure 3.8 Suppression of MnSOD enhances the levels of lactate and activity of lactate dehydrogenase.

A. Concentration of lactate.

B. Activity of Lactate dehydrogenase. All measurements were performed in triplicate. csiRNA – control siRNA, siRNA – MnSOD siRNA.

The data are the average of three experimental sets. *, p < 0.05, **, p < 0.01, compared with the control and csiRNA respectively.
Figure 3.9 SOD mimetic (Mn$^{III}$TE-2-PyP$^{5+}$) (1000 pg/ml) suppresses the level of lactate and increases the activity of pyruvate dehydrogenase.

A. Concentration of lactate.

B. Activity of PDH. All estimates were done in triplicate.

The data are the average of three experimental sets. *, p < 0.05, **, p < 0.01, compared with the control; #, $, p < 0.05, compared with the 250 μM ONOO.
Figure 3.10 MnSOD deficiency leads to alteration in oxygen consumption, complex 1 activity and levels of lactate.
Figure 3.10 MnSOD deficiency leads to alteration in oxygen consumption, complex I activity and levels of lactate.

A. Mitochondrial respiration in isolated mitochondria protein from brains.

B. Complex I activity and lactate levels in whole brain homogenate of sod 2 +/- mice.

C. Levels of lactate in whole brain homogenate of sod 2 +/- mice.

All measurements were performed in triplicate from three animals. *, p < 0.05, **, p < 0.01, compared with the WT.
DISCUSSION

Manganese superoxide dismutase (MnSOD) is the only isoform of SOD found inside the mitochondrial matrix of eukaryotic cells (Quijano et al., 2001) and its presence is essential for the survival of aerobic life (Lebovitz et al., 1996; Li et al., 1995; Melov et al., 1998). Mitochondria are major organelles where most of the cell superoxide is produced. Thus, MnSOD plays a critical role in protecting the cell from superoxide radicals (Fridovich, 1975).

Mitochondria are essential for the production of ATP through oxidative phosphorylation, and it has been shown that peroxynitrite, a strong oxidant that is a product of nitric oxide and superoxide, can nitrate tyrosine 34 of MnSOD, thereby inactivating it (MacMillan-Crow et al., 1998). We therefore asked whether inactivation of MnSOD by nitration influences the mode of energy production in mammalian cells. We treated SH-SY5Y neuroblastoma cells with peroxynitrite to induce nitration and subsequent inactivation of MnSOD. The results demonstrate that there is a link between the shift in energy metabolism and nitrative inactivation of MnSOD in SH-SY5Y cells. The data indicate that reduction of MnSOD activity leads to a reduction in mitochondrial respiration and an increase in glycolytic activity.

Nitration of biological macromolecules can be caused by nitric oxide, nitrogen dioxide, nitrous acid, hypochlorous acid, nitrated aliphatic reactive intermediates and peroxynitrite (Ischiropoulos, 1998). But only peroxynitrite mediates the inactivation of MnSOD by exclusive nitration of tyrosine residue 34. Tyrosine 34 is located at the vertex of the substrate channel and only 5 Å from the active manganese ion site of MnSOD (Yamakura et al., 1998). This makes this residue most susceptible to nitration because peroxynitrite is attracted to the active site by the basic residues in the channel entrance (Quijano et al., 2001) and the ionization state of tyrosine 34 is altered by positively charged manganese atoms making it prone to an electrophilic attack (MacMillan-Crow et al., 1998). Being a major player in removing superoxide, it is possible that nitration and inactivation of MnSOD would result in an increase in mitochondrial superoxide levels leading to enhanced generation of peroxynitrite, thereby
amplifying the nitration/oxidation of mitochondrial proteins and eventually compromising mitochondrial function.

The involvement of MnSOD activity in the maintenance of mitochondrial function has been amply documented by studies using sod 2 +/- mice that show a decrease in mitochondrial function as measured by reduced RCR for the substrates of complex I (Van Remmen et al., 2001). The data obtained from our study of cells treated with peroxynitrite show that decreased activity of complex I is associated with nitration and inactivation of MnSOD. Complex I function can be affected by either direct reaction of superoxide radicals that might accumulate because of reduced MnSOD activity or by direct inactivation of complex I by enhanced production of peroxynitrite (Cassina and Radi, 1996; Rusak et al., 2006). Several lines of experiments indicate that MnSOD activity is important for the protection of mitochondrial function and complex I activity. First, the uptake and reduction of MTT, an indicator for mitochondrial function and complex I activity (Musser and Oseroff, 1994), are reduced. Second, it has been shown that overexpression of MnSOD selectively protects complex I inactivation in transgenic mice treated with adriamycin (Yen et al., 1999) and in lung carcinoma cells exposed to hypoxia-reoxygenation (Powell and Jackson, 2003). Importantly, our data demonstrate that the loss in complex I activity in cells treated with peroxynitrite was alleviated by the presence of a SOD mimetic (Mn^{III}TE-2-PyP^5+) capable of localizing in the mitochondria. It should be noted that this result is confounded by the ability of SOD mimetic (Mn^{III}TE-2-PyP^5+) to be a scavenger of peroxynitrite (Ferrer-Sueta et al., 2003; Spasojevic et al., 2007) but reduced activity of complex I exhibited in SH-SY5Y cells transfected with MnSOD siRNA is consistent with the role of MnSOD in protecting complex I activity.

Since complex I represents a point of entry of electrons from NADH-linked substrates, loss of its activity probably will result in reduced electron flux to the respiratory chain (Radi et al., 1994) and thereby mitochondrial function, and it therefore follows that reduced activity of MnSOD (Van Remmen et al., 2001) and exposure to peroxynitrite (Cassina and Radi, 1996) will result in compromised mitochondrial function. Although there are multiple targets for peroxynitrite in mitochondria (Cassina and Radi, 1996; MacMillan-Crow et al., 1998; Rusak et al., 2006; Yamakura et al., 1998), our result demonstrates that MnSOD is an early target in the process of peroxynitrite-
induced mitochondrial dysfunction. MnSOD was inactivated at 1 µM peroxynitrite, but respiration was inhibited at 10-fold higher concentrations. Given the transient nature of peroxynitrite in biological systems, the early inactivation of MnSOD, an important antioxidant enzyme in the mitochondria, is of immense importance. MnSOD can inhibit peroxynitrite formation in the mitochondria (Radi et al., 1994), but its inactivation can possibly lead to an increase in intramitochondrial superoxide levels and additional generation of peroxynitrite (MacMillan-Crow et al., 1996). This can propagate the initial damaging event that leads to mitochondrial dysfunction (Radi et al., 1994). Further proof of the involvement of MnSOD in protecting mitochondrial function is the decreased activity of complex I and reduced RCR in sod 2 +/- mice. Our results are consistent with the results from an earlier study showing a reduction of RCR and state III respiration in mitochondria isolated from the heart of Sod 2 +/- mice (Van Remmen et al., 2001) and extend to demonstrate the effect of MnSOD in brain mitochondria. Together these data demonstrate an important role for MnSOD in protecting the respiratory function of mitochondria.

Mitochondrial respiration is the most important contributor to the cell’s energy requirement, producing 32 ATPs from one glucose molecule. Thus, dysfunction of respiratory complexes would eventually lead to compromised respiration and thereby loss of ATP generation from oxidative phosphorylation. The ability of cells to shift from oxidative phosphorylation to glycolysis has been observed in many tumors with impaired mitochondrial function (Gatenby and Gillies, 2004). Our data suggest that both SH-SY5Y cells treated with peroxynitrite and brain tissue in MnSOD deficient mice were capable of maintaining their required energy by up-regulating glycolytic activity. The lactate levels in cells treated with peroxynitrite were increased in comparison with control cells, suggesting increased glycolytic activity. These results are consistent with those obtained from platelets (Rusak et al., 2006) and primary neuronal cells (Bolanos et al., 1995) exhibiting decreased mitochondrial respiration when treated with peroxynitrite. The conversion of pyruvate to lactate is catalyzed by the NADH-dependent enzyme, lactate dehydrogenase (LDH). Compared with the untreated cells, peroxynitrite treated cells exhibited a higher activity of lactate dehydrogenase which was similar to the observed increased lactate levels. The activities of mitochondria and LDH are delicately
and mutually regulated at the level of metabolites. They depend on the availability of pyruvate and NADH/NAD⁺. Pyruvate accumulates in the cell under conditions of decreased activity of pyruvate dehydrogenase, which oxidizes pyruvate to acetyl-coA, thereby facilitating the entry of pyruvate into krebs’s cycle. Consistent with this model, we found reduced activity of PDH in cells treated with peroxynitrite. The fast kinetics of LDH ensures that accumulated pyruvate is converted to lactate (Fantin et al., 2006). Pyruvate to lactate conversion utilizes NADH and regenerates NAD⁺ which can be used to drive glycolysis, which produces more lactate. As more and more NADH is consumed for this reaction, less NADH is available for mitochondrial respiration, thereby slowing down the already impaired respiration in cells treated with peroxynitrite. A high rate of conversion of glucose to lactate is coupled to a high rate of glucose uptake and glucose phosphorylation and occurs even in the presence of normal oxygen pressure (Gatenby and Gillies, 2004). In this study, SH-SY5Y cells treated with peroxynitrite also showed significantly increased levels of glucose. Thus, it seems probable that impaired mitochondrial function due to reduced MnSOD activity leads to a compensatory increase in glycolysis. This possibility is further supported by the finding that cells transfected with MnSOD siRNA have increased levels of lactate and lactate dehydrogenase activity. Compensating for SOD activity with a SOD mimetic in the cells treated with peroxynitrite resulted in reduction of lactate concentration and alleviation of PDH activity. Our results from in-vivo experiments with sod 2+/− mice demonstrate increased lactate levels in the brain. Those results further support the likelihood that alteration in MnSOD activity plays a key role in reducing mitochondrial function and subsequently compensating for the energy requirements of cells by the up-regulation of glycolytic activity.

Increased nitrotyrosine has been documented in hippocampus and cerebral cortex of aged rats (Shin et al., 2002), cerebrospinal fluid of aged humans (Tohgi et al., 1999) and in many neurodegenerative disorders (Torreilles et al., 1999). The identities of many proteins modified by nitration have been obtained in human, animal and cellular models of diseases (Ara et al., 1998; Sultana et al., 2006). However, MnSOD was the first protein unequivocally identified as nitrated in chronic rejection of human renal allografts (MacMillan-Crow et al., 1996). A previous study from our lab has shown the presence of
nitrated MnSOD in brains of APP/PS-1 double knock-in mouse model of Alzheimer’s disease (Anantharaman et al., 2006). To our knowledge, this is the first study to implicate the inactivation of MnSOD to the metabolic switch occurring under conditions of mitochondrial dysfunction.

In summary, we demonstrate that MnSOD was nitrated upon peroxynitrite treatment with an associated decrease in activity. Inactivation of MnSOD is an early event in peroxynitrite-induced impairment of mitochondrial function. Mitochondrial dysfunction was compensated for by an increase in glycolytic activity. Mitochondrial dysfunction and increased glycolytic activity were prevented by SOD mimetic and enhanced by MnSOD deficiency. These results demonstrate a critical role for MnSOD in maintaining mitochondrial respiratory function and reveal the role of glycolysis in sustaining cellular energy requirements under pathological conditions associated with MnSOD deficiency.
CHAPTER FOUR

INCREASED GLYCOLYSIS IN THE APP\textsuperscript{NLh/NLh} X PS-1\textsuperscript{P264L/P264L} DOUBLE KNOCK-IN MOUSE MODEL OF ALZHEIMER’S DISEASE

ABSTRACT

Altered energy metabolism is characteristic of many neurodegenerative disorders. In AD patients, memory dysfunction is associated with glucose hypometabolism and neuronal loss in the hippocampus. In addition to the role in the pathogenesis of AD, Aβ peptide has been associated with reduced glucose oxidative metabolism and mitochondrial activity. Here, brains from APP\textsuperscript{NLh/NLh} X PS-1\textsuperscript{P264L/P264L} Double Knock-In Mouse Model of Alzheimer’s disease (APP/PS-1) were used to investigate a possible change in glycolytic activity. We estimated the levels of lactate, the activity of lactate dehydrogenase (LDH) and pyruvate dehydrogenase (PDH) from 3 and 14-16 months old APP/PS-1 mice and wild-type (WT) mice. Significantly increased lactate levels were observed in 14-16 months old APP/PS-1 mice when compared to their age-matched WT counterparts. The activity of LDH was also significantly increased in the APP/PS-1 mice. PDH appears to have age associated decrease in activity and was significantly decreased in APP/PS-1 mice when compared to age-matched WT mice. These findings implicate a possibility of an increased glycolytic activity in APP/PS-1 mice.
INTRODUCTION

Glucose is an essential energy source for the adult human brain. The brain is metabolically the most active organ and is therefore highly dependent on a continuous supply of its fuel. To meet its very high energy demands, the brain (around 1/40 of the body weight) possesses a relatively high blood flow and glucose consumption equal in amount to about one-fifth of the entire resting requirements of the body (Roeder et al., 1982). When the blood glucose levels fall by half from a normal fasting value (to about 2 – 3 mM), there is some cognitive impairment and, at glucose levels below 1 mM, mental confusion is evident and coma may result from sustained glucose deprivation.

Modern humans acquired enhanced longevity accompanied by the expansion of lifetime in nonreproductive ages. However, this prolonged lifetime brought up negative assets: late-onset diseases such as AD. Reported characteristics of AD brain are impaired energy metabolism, and increased oxidative stress (Fukui et al., 2007). Early impairment of memory and learning are characteristic features of AD. These symptoms correspond well to the glucose hypometabolism present even at early stages of the disease when only mild cognitive impairment is evident clinically (de Leon et al., 2001; De Santi et al., 2001). Most of the evidence suggesting an impairment of cerebral energy metabolism has come from in vivo studies as detected by reduced cortical \([^{18}\text{F}]\) deoxyglucose uptake using positron emission tomography (Mielke et al., 1996). Accordingly, activities of key glycolytic enzymes like hexokinase, aldolase, pyruvate kinase, lactate dehydrogenase and glucose – 6 – phosphate dehydrogenase were found to be altered in AD patients (Bigl et al., 1999) along with a reduction in densities of glucose transporter subtypes (GLUT1 and GLUT 3) (Bigl et al., 2003). However, with respect to several other enzymes the results have been contradictory.

Transgenic animals developed to model AD pathology by overexpression of mutated form of APP and PS-1 also demonstrate decreased hippocampal glucose metabolism with a positive correlation to the deposition of A\(\beta\) plaque (Sadowski et al., 2004). Here a APP\(^{NLh/NLh}\) X PS-1\(^{P264L/P264L}\) Double Knock-In Mouse Model of Alzheimer’s Disease (APP/PS-1) carrying a Swedish familial AD mutation in amyloid precursor protein and having the P264L familial AD mutation in presenilin – 1 (Chapter
two) were used to investigate a possible change in glycolytic activity. This mouse has been demonstrated to display increased Aβ deposition from 6 months onwards consistent with an increase in the levels Aβ 1-40 and Aβ 1-42. The finding of increased glycolytic activity in the event of decreased mitochondrial function in SH-SY5Y cells (Chapter three) in association with decreased activity of MnSOD prompted us to investigate whether APP/PS-1 mice with decreased mitochondrial function (Chapter two) (Anantharaman et al., 2006) has an increase in glycolytic activity. A significant increase in lactate levels was observed in 14-16 months old APP/PS-1 mice when compared to age-matched WT mice. The activity of LDH was also significantly increased in the same age group. PDH appears to have an age associated decrease in activity that was significantly decreased in all age groups when compared to age-matched WT mice. These findings implicate a possibility of an increased glycolytic activity in APP/PS-1 mice.
MATERIALS AND METHODS

Determination of Lactate levels

Brains (excluding cerebellum) from six animals from both APP/PS-1 and WT mice of 3- and 14-16 month old were collected and washed twice in ice-cold PBS, and homogenized in Dounce homogenizer using Teflon pestle. The homogenate was centrifuged at 5000 rpm for 5 minutes and the supernatant was used to determine the concentration of lactate. The reaction mixture contained 2M Tris; pH 7.5 in water with 0.05% sodium azide, stored at room temperature; 60 mM NAD⁺ in water, stored at -20°C; 15 mM INT, prepared in water containing 10% (v/v) acetone; 100 units/ml Dase in 75 mM Tris; pH 7.5, stored at -20°C; and 50 units/ml of lactate dehydrogenase (LDH). Just prior to the beginning of the assay, Dase was diluted 1:100 with 75 mM Tris, pH 7.5. For the assay, 50 μl of the reaction reagent was added to 20 μl of the cell lysate in a 96-well flat bottom microtitre plate. The reaction was allowed to continue for 40 minutes in a humidified incubator without CO₂ at 37°C. The reaction was stopped by adding 50 μl of 3% acetic acid, followed by a brief agitation. The absorbance was measured at 492 nm using a Spectra Max Gemini plate reader from Molecular Devices. The reaction is based on the production of NADH and pyruvate from NAD and lactate by LDH. This reaction is coupled to Dase-catalyzed reoxidation of NADH to NAD and reduction of INT to red colored formazan which is read at 492 nm. The intensity of the red color is proportional to the concentration of lactate in the sample.

Activity of Lactate Dehydrogenase

Mice were euthanized, brains (excluding cerebellum) were collected, homogenized and were used immediately to determine LDH activity, following the method described by Yen et al. (Yen et al., 1996). Briefly, the homogenized brain was added to a solution containing NADH and incubated at room temperature for 20 minutes. Sodium pyruvate (22.7 mM) in 0.1 M potassium phosphate buffer (pH 7.5) was added after 20 minutes. In the assay, the reaction where pyruvate is converted to lactate
catalyzed by LDH is followed for two minutes. The decrease in absorbance (dA/min) at 340 nm that occurs with the oxidation of NADH to NAD is used for calculating the LDH activity using the extinction coefficient of 6.22 mM⁻¹cm⁻¹ for NADH. The activity was repeated in three independent experiments, each in triplicate.

**Activity of Pyruvate Dehydrogenase**

The assay is based on the link between the NADH produced by PDH to reduction of the dye INT and was performed as described by Elnageh & Gaitonde (Elnageh and Gaitonde, 1988). Briefly, the final reaction mixture of 1 ml volume consisted of 50 mM Tris-HCL, 0.5 mM EDTA and 0.2% (wt/vol) Triton X-100 (pH 7.8), 100 μg of the brain homogenate, 2.5 mM NAD, 0.1 mM CoA, 1 mM MgCl₂, 0.1 mM oxalate, 1 mg of bovine serum albumin, 0.6 mM INT, 5-7 units of lipoamide dehydrogenase, 0.2 mM TPP and 5 mM pyruvate. After the addition of 4 mM TPP the contents were kept at 37°C for 5 minutes. A stable baseline at 500 nm was obtained for the reaction mixture. The reaction was started by adding 100 mM pyruvate. The absorbance of the mixture at 500 nm was recorded at the beginning and again after the reaction had been underway for 5 minutes at 37°C. Data was obtained from six animals from each genotype.
RESULTS

The concentration of lactate in brain homogenate from 14-16 month old APP/PS-1 mice was significantly increased (p < 0.01) in comparison with its age matched WT mice (Figure 4.1). The activity of lactate dehydrogenase, an enzyme that catalyzes the conversion of pyruvate to lactate, was also significantly increased (p < 0.001) in the brain homogenate of 14-16 month old APP/PS-1 mice, when compared to its age matched WT mice (Figure 4.2). These results indicate that glycolytic activity is increased in APP/PS-1 mice.

Pyruvate dehydrogenase (PDH) catalyzes the conversion of pyruvate to Acetyl-CoA, which then enters the tri-carboxylic cycle. Decreased activity of PDH would lead to accumulation of pyruvate, which can be converted to lactate by lactate dehydrogenase. The activity of PDH was significantly decreased even in 3 month old mice of APP/PS-1 genotype when compared to WT mice. This decrease in activity was further exaggerated at 14-16 months of age in APP/PS-1 mice (Figure 4.3).
Figure 4.1 Increased lactate levels in APP/PS-1 mice.

Brains (excluding cerebellum) were homogenised, centrifuged and the supernatant used for estimation of lactate.

All measurements were performed from six animals. *, p < 0.05, compared with the age-matched WT.
Figure 4.2 Increased activity of lactate dehydrogenase in APP/PS-1 mice.

Brains (excluding cerebellum) were homogenised, centrifuged and the supernatant used for estimation of LDH activity.

All measurements were performed from six animals. ***, p < 0.001, compared with the age-matched WT.
Figure 4.3 Decreased activity of pyruvate dehydrogenase in APP/PS-1 mice.

Brains (excluding cerebellum) were homogenised, centrifuged and the supernatant used for estimation of PDH activity

All measurements were performed from six animals. **, p < 0.01, compared with the age-matched WT.
DISCUSSION

Numerous in vitro studies on various components of cerebral glucose metabolism of AD have revealed a reduced activity of different enzymes involved in glucose and energy metabolism. There is also an impairment of glucose carriers in brain endothelial cells. Therefore reduced glucose uptake and/or metabolism in the brain has been suggested as a reason for neurodegenerative loss of neurons and synaptic connections (Bigl et al., 2003; Bigl et al., 1999). The glucose uptake studies using of [18F] deoxyglucose have demonstrated reduced glucose levels in AD brain. However, these findings do not address whether the decreased deoxyglucose uptake reflects a limited metabolic capacity of the brain or a mere reduction in energy demand of a diseased organ (Bigl et al., 1999).

The increase in lactate levels APP/PS-1 mice at 14-16 months of age suggests an upregulated glycolytic activity. The increased lactate levels implicate a compensation for the reduced mitochondrial function found in APP/PS-1 mice (Chapter two) (Anantharaman et al., 2006). Increase in glycolytic activity in the event of reduction in mitochondrial function is well documented in many tumors (Pedersen, 2007; Schoonen et al., 1990; Warburg, 1956; Wu et al., 2007). Our results are consistent with report of lactate transported from astrocytes by astrocyte-neuron shuttle (ANLSH) (Schurr, 2006) which under the conditions of mitochondrial respiratory inhibition by nitric oxide exhibit high lactate levels (Almeida et al., 2001). Being the most numerous nonneuronal cell type in the central nervous system (Tower and Young, 1973) and also being the first cell to encounter glucose from blood, the possibility of astrocyte being the source of neuronal lactate is highly likely. Although SH-SY5Y cells are cancer cells which are dependent on glucose for energy metabolism, increased lactate levels observed in SH-SY5Y cells (Chapter three), a predominantly neuronal cells line argues that neurons by themselves could be a source of increased lactate observed in APP/PS-1 mice.

The increased lactate levels were consistent with increase in LDH activity in APP/PS-1 mice. This is consistent with the documented findings from the brains of AD patients (Bigl et al., 1999). Although the specific advantage of increased LDH activity in the brain is unclear, a suppression of LDH resulted in decreased tumorigenicity in
malignant cells along with increased mitochondrial respiration (Fantin et al., 2006). LDH utilizes NADH and produces NAD in the process of catalyzing the conversion of pyruvate to lactate. NADH being the reducing equivalent for the mitochondrial electron transport chain, its usage by LDH may lead to depression in mitochondrial respiration and thereby the energy production. Further, as NAD is regenerated, it fuels glycolysis and results in increased pyruvate production.

An additional way by which lactate levels can increase is in the presence of compromised PDH activity. Pyruvate is converted to acetyl-CoA by PDH. In the event of decreased PDH activity as observed in APP/PS-1 mice, pyruvate gets accumulated. The fast kinetics of LDH ensures that pyruvate is converted to lactate (Fantin et al., 2006). PDH is a critical enzyme for carbohydrate oxidation, present in minimal excess in the brain, and is closely involved in synaptic plasticity (Sorbi et al., 1983). A decrease in activity of PDH has been documented in AD (Perry et al., 1977). Acetyl-CoA, the product of pyruvate oxidation by PDH is necessary for the synthesis of acetyl choline, a neurotransmitter known to be involved in learning and memory processes. Therefore, any dysfunction of PDH would lead to decreased acetyl-CoA production and may lead to the cognitive decline associated with AD.
CHAPTER FIVE

SUMMARY AND FUTURE STUDIES

As AD brain is under pronounced oxidative stress, and mitochondria is the major source of superoxide radicals, a dysfunction of MnSOD may exacerbate the progression of the disease. The studies presented in this work were carried out with the goal of adding more knowledge on the status of nitration and thereby the activity of MnSOD in AD brain. The data presented in this dissertation strongly suggest that inactivation of MnSOD contributes to mitochondrial dysfunction and subsequent enhancement of glycolytic activity. Although it is well recognized that impaired mitochondrial function can be compensated by an upregulation of glycolysis, to my knowledge, the data presented here is the first report on the essential role of an anti-oxidant enzyme influencing this process. This is supported by the data obtained when addressing the hypotheses, 1) MnSOD is nitrated and inactivated in association with Aβ deposition. 2) Nitrative inactivation of MnSOD plays an important role in the impairment of mitochondrial respiratory function resulting in alteration of energy metabolism. 3) The impairment of mitochondrial respiration observed in APP/PS-1 mice was also associated with an increase in glycolytic activity.

Nitrative inactivation of MnSOD in the presence of accelerated deposition of Aβ was studied using homozygous knock-in APPNLh/NLh x PS-1P264L/P264L mouse. MnSOD was nitrated with an associated reduction in the activity as Aβ deposition increased in the brains of these mice. Nitration and inactivation of MnSOD showed a strong correlation with age. Aβ deposition was detectable at the age of 6 months and by 14 months deposition was prominent and widespread encompassing neocortex and hippocampus. The finding of increased nitration of MnSOD in an age dependent manner suggested that Aβ deposition may be a cause for MnSOD nitration. This is supported by the findings of increased nitrotyrosine immunoreactivity in cultured cells treated with β-amyloid and in rats subjected to intracerebroventricular infusion of β-amyloid (Mattson et al., 1997; Tran et al., 2003). Further, studies showing that Aβ induced accumulation of mitochondrial peroxynitrite (Keller et al., 1998; Mattson et al., 1997), argues for the
Possibility that Aβ deposition with age is the cause of MnSOD nitration in APP/PS-1 mice. Although the presence of MnSOD nitration by 3 months in APP/PS-1 mice in the absence of any observable Aβ deposits argues against the notion of Aβ induced nitration, more recently, report on increase in levels of protein nitration in inferior parietal lobule (IPL) and hippocampus of mild cognitive impairment (MCI) patients implicate a prominent role for protein nitration during the early events of AD (Butterfield et al., 2007).

Inactivation of MnSOD in association with nitration is consistent with previous studies on peroxynitrite mediated nitration of critical tyrosine residues of MnSOD leading to its inactivation (MacMillan-Crow et al., 1998; Yamakura et al., 1998). In addition, our data from SH-SY5Y cells showing increased nitration of MnSOD upon peroxynitrite treatment suggests that in APP/PS-1 mice, nitrationative inactivation of MnSOD might have occurred by Aβ deposition induced peroxynitrite production. APP/PS-1 mice exhibited a decrease in mitochondrial respiration mediated through complex I, as measured by oxygen consumption of isolated mitochondria. The reduction was significant at 9 and 12 months of age when compared to age-matched WT mice. As APP/PS-1 mice had reduced MnSOD activity, prior to the observed reduction in respiration, it is possible that reduction of MnSOD activity may be a cause for the observed reduction in respiration. This is supported by previous data from our lab demonstrating that MnSOD selectively protects state 3 respiration activity through complex I substrates and prevented complex I inactivation (Yen et al., 1999). Further, Kokoszka et al., (Kokoszka et al., 2001) demonstrated in sod 2 +/−, a impaired mitochondrial respiration adding credence to the possible role of reduced activity of MnSOD in APP/PS-1 in decreasing the mitochondrial respiration. The data that we obtained from SH-SY5Y cells, treated with peroxynitrite, demonstrating reduced complex I activity and mitochondrial function, which was ameliorated by SOD mimetic and reproduced by MnSOD siRNA strongly suggest a predominant role for MnSOD in altering respiration. Although there are multiple targets for peroxynitrite in mitochondria (Cassina and Radi, 1996; MacMillan-Crow et al., 1998; Rusak et al., 2006; Yamakura et al., 1998), our result demonstrates that MnSOD is an early target in the process of peroxynitrite-induced mitochondrial dysfunction. MnSOD was inactivated at 1 μM.
peroxynitrite, but respiration was inhibited at 10-fold higher concentrations. Given the transient nature of peroxynitrite in biological systems, the early inactivation of MnSOD, an important anti-oxidant enzyme in the mitochondria, is of immense importance. MnSOD can inhibit peroxynitrite formation in the mitochondria (Radi et al., 1994), but its inactivation can possibly lead to an increase in intramitochondrial superoxide levels and additional generation of peroxynitrite (MacMillan-Crow et al., 1996). This can propagate the initial damaging event that leads to mitochondrial dysfunction (Radi et al., 1994). Our results suggested that MnSOD is inactivated followed by respiratory impairment. However, the efficient functioning of the mitochondrial respiratory chain depends on the supply of reducing equivalents (NADH & FADH\(_2\)) chiefly from tricarboxylic cycle. Any dysfunction of the enzymes involved in Kreb’s cycle would be expected to result in decreased production of reducing equivalents and an impaired mitochondrial respiration. Peroxynitrite being a potent oxidant might also affect the enzymes of Kreb’s cycle. Effect of peroxynitrite on the activity of isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase, malate dehydrogenase and succinate dehydrogenase involved in production of NADH and FADH\(_2\) needs to be investigated.

A compensatory upregulation of glycolytic activity has been observed in the event of decreased mitochondrial function. In APP/PS-1 mice, we observed increased levels of lactate, an index of increased glycolysis. This, when taken together with the data of reduced activity of MnSOD, decreased mitochondrial respiration in APP/PS-1 mice and the finding of MnSOD in maintaining the mitochondrial function suggest that MnSOD is influencing the metabolic switch to glycolysis in APP/PS-1 mice. This notion is supported by the data from SH-SY5Y cells which showed increased levels of lactate on peroxynitrite treatment, preventable by SOD mimic and reproduced by MnSOD siRNA. Severe impairment of the ability to oxidize substrates through pyruvate dehydrogenase (PDH), tricarboxylic acid cycle or electron transport chain are likely to lead to accumulation of lactate. In this study, we encountered a decreased activity of PDH and impaired mitochondrial function in APP/PS-1 mice and also in SH-SY5Y cells treated with peroxynitrite. These might have resulted in increased lactate levels, without an actual increase in glycolytic activity. Lactate also arises from amino acid alanine, which is converted to pyruvate by alanine amino transferease. But, in our system there is
reduced activity of PDH. Hence, pyruvate cannot enter the TCA cycle and is instead converted to lactate. Acetyl CoA formed from beta oxidation of fatty acids can also contribute to increased lactate levels by inhibiting PDH. This inhibition would lead accumulation of pyruvate that is converted to lactate. In this dissertation work, we have not addressed the contribution from these respective sources. Therefore, to add credence to our finding of increased glycolysis, the contribution to lactate levels by amino acids and fatty acids should be quantified. Further, by redox proteomics approach, enzymes like enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown to be nitrated in the brains of AD patients. If this is true, we should have encountered a decrease in glycolytic activity, which we did not. Perhaps, the treatment regimen that was followed in this dissertation (24 hours treatment of SH-SY5Y cells with peroxynitrite) was not sufficient to cause nitration of glycolytic enzymes or probably glycolytic enzymes are in abundance, to keep the glycolysis active, even in the presence of nitrated and possibly inactivated proteins.

As mitochondrial function is a prerequisite for the majority of ATP produced in a cell, any dysfunction would be expected to result in a decreased ATP levels. A compensatory increase in glycolytic activity may offset ATP loss, which was the case, as we observed comparable ATP levels in control and peroxynitrite treated SH-SY5Y cells. As in many tumors, where an increase in glycolytic activity is supported by enhanced glucose uptake into the cells, peroxynitrite treated SH-SY5Y cells showed significantly increased concentrations of glucose when compared to untreated cells. Further, our observation of decreased respiration, decreased complex I activity and increased lactate in the MnSOD heterozygous knock-out mice adds credence to the hypothesis that decreased activity of MnSOD is contributing to a switch from oxidative phosphorylation to glycolysis in AD.

The finding of enhanced glycolysis in our AD mouse model is in contrast to that of human AD patients, where an impaired glucose metabolism has been reported. The following reasons could explain the apparent inconsistency between these studies,

1. The human data is based on glucose uptake studies using PET imaging. This does not clearly indicate whether there is a true limited metabolic capacity of the brain or a mere reduction in energy demand of a diseased organ (Bigl et al., 1999).
1. Further, the neuronal loss encountered in AD may also account for reduced glucose uptake or probably the loss of neurons is so abundant that a compensatory enhancement of glycolytic activity in the remaining neurons may not be sufficient to manifest as an increase.

2. The age group of APP/PS-1 mice (14 - 16 months) used to analyze the lactate levels may not be comparable to aged human AD patients. The increase in glycolytic activity is perhaps occurring at a very early age in humans, which if found might be utilized as a potential target for modulating the progression of AD.

3. APP/PS-1 mice have not been shown to develop neurofibrillary tangles, which may be necessary to reproduce the metabolic changes occurring in humans.

To further elucidate the role of MnSOD in altering the mitochondrial function and glycolytic activity, it would be worthwhile to investigate in vivo by injecting SOD mimetic in APP/PS-1 mice. Primary neurons from APP/PS-1 mice exhibit increased colocalization of nitrotyrosine with MnSOD, decreased mitochondrial function and increased susceptibility to Aβ treatment (Sompol et al., 2008). So it would be interesting to investigate whether these primary neurons have increased glycolytic activity. Further, APP/PS-1 mice do not develop neurofibrillary tangles and show neuronal loss, it does not recapitulate the complete AD pathology encountered in human beings. Therefore, to make the findings of this study more relevant to human AD, a triple transgenic mouse exhibiting all the pathological hallmarks of human AD could be utilized to study the effect of MnSOD on glycolysis.
Figure 5.1 Hypothetical model suggested from the data obtained
Figure 5.1 Hypothetical model suggested from the data obtained.

Nitric oxide produced by surrounding microglia or astrocytes or by the neurons itself, diffuse into the mitochondria and inhibit complex IV. Inhibition of complex IV lead to electron leak and superoxide production. Superoxide can react with nitric oxide to result in peroxynitrite formation. MnSOD is nitrated and inactivated at low concentrations of peroxynitrite produced leading to accumulation of superoxide and peroxynitrite. Increasing concentrations of these can inhibit respiratory chain and thereby the mitochondrial respiration. Inhibition of mitochondrial respiration leads to increase in glycolytic activity to offset any ATP loss. Black dashed lines and words in black – known in the literature; red arrows and words in red – data obtained from this dissertation; yellow box with words in black – indicated in the existing literature but not proven.
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