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Increased Glutathione Metabolic Defense Capabilities in Cultured Alzheimer's Diseased Lymphoblast Cell Lines

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THESIS APPROVAL

The abstract and thesis of Collin M. Shaw for the Master of Science in Biology were presented November 9, 1998, and accepted by the thesis committee and the department.

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ABSTRACT

An abstract of the thesis of Collin M. Shaw for the Master of Science in Biology presented November 9, 1998.

Title: Increased glutathione metabolic defense capabilities in cultured Alzheimer's diseased lymphoblast cell lines.

The hypothesis to be tested states that the pathology of Alzheimer's disease (AD) involves elevated levels of oxidative stress, resulting in elevated levels of cellular oxidative defense mechanisms. If the premise is true, than AD pathologically afflicted cells should have a higher demand for glutathione (GSH) as an innate oxidative defense mechanism hence; greater GSH concentrations, increased GSH resynthesis capabilities, and increased levels of cystathionine gamma-lyase (CNase). Alzheimer diseased and age matched control lymphoblast cells, obtained from OHSU's Oregon Brain Aging Study, were cultured, and GSH biochemistry was subsequently evaluated. GSH was depleted by exposing cells to the GSH depleting agent diethylmaleate (DEM) and the resulting GSH concentrations were measured. GSH resynthesis was measured after depleting GSH

with DEM, to a level of approximately half base GSH concentration, then removing the depleting agent, resuspending the cells in fresh medium (DEM-free), and subsequently measuring GSH levels. GSH concentrations were measured by HPLC, and all data was normalized to cellular protein concentration. Cellular CNase specific activity levels were measured by adding cystathionine, the CNase substrate, and then measuring the amount of cysteine produced by means of the DTNB assay. The AD cell lines showed no increase in base levels of GSH as compared to control cell lines. The AD cell lines showed a statistically significant increase in GSH resynthesis capabilities and cystathionine gamma-lyase specific activity levels. These findings add further weight to the AD oxidative stress hypothesis, which is based on the premise that the causative agent of AD pathogenesis is an increase in the level of cellular free radicals produced.

INCREASED GLUTATHIONE METABOLIC DEFENSE
CAPABILITIES IN CULTURED ALZHEIMER'S DISEASED
LYMPHOBLAST CELL LINES

By

COLLIN M. SHAW

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
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ABBREVIATIONS USED

- (AD) Alzheimer's disease
- (Apo E) Apolipoprotein Epsilon
- (APP) Amyloid beta precursor protein
- (β -AP) Beta-Amyloid protein
- (BCA) Bicinchoninic acid
- (BSA) Bovine Serum Albumin
- (CAT) Catalase
- (cm) Centimeter
- (cDNA) Complimentary DNA
- (CNase) Cystathionine gamma-lyase
- (COX) Cytochrome c Oxidase
- (CO₂) Carbon Dioxide
- (cys⁻) Cysteine Thiolate ion
- (DEM) Diethyl maleate
- (ETC) Electron transport chain
- (e4) Apolipoprotein E epsilon 4
- (FDNB) Fluoro-2, 4-dinitrobenzene
- (g) Gravity
- (g-GG) Gamma-glutamyl glutamate
- (GPX) GSH peroxidase

(GS-) Glutathione Thiolate Ion

(GSH) Glutathione

(GSK3b) Glycogen synthase kinase 3 Beta

(GSSG) Oxidized form of GSH

(GSSG-R) GSH reductase

(GST's) Glutathione transferases

(HNE) E-4-hydroxy-2-nonenal

(HPLC) High Pressure Liquid Chromatography

(KCl) Potassium Chloride

(KOH) Potassium Hydroxide

(KHC03) Potassium Bicarbonate

(MDA) Malondialdehyde

(μ l) Microliter

(μ moles) Micromoles

(mM) Millimolar

(μ M) Micromolar

(μ g) Microgram

(mg) Milligram

(mtDNA) Mitochondrial DNA

(NaOH) Sodium Hydroxide

(N) Normality

(nDNA) Nuclear DNA

(nM) Nanomoles

(NFT) Neurofibrillary tangle

(OBAS) Oregon Brain Aging Study

(OH⁸dG) 8-hydroxy-2'-deoxyguanosine

(OHSU) Oregon Health Sciences University

(PCA) Perchloric acid

(PHF) Paired helical filament

(PHF-t) Hyperphosphorylated tau protein

(PLP) Pyridoxal 5'-phosphate

(RNS) Reactive Nitrogen Species

(ROM) Reactive Oxygen Metabolites

(ROS) Reactive Oxygen Species

(RPMI) Roswell Park Memorial Institute

(SOD) Superoxide Dismutase

(SDS) Sodium Dodecyl sulfate

(SP's) Senile plaques

(TBARS) Thiobarbituric acid-reactive products

(TNF) Tumor Necrosis Factor

(TPKI) Tau protein Kinase I

(TPKII) Tau protein Kinase II

INTRODUCTION

Clinically, Alzheimer's disease (AD) is characterized by gradual progressive intellectual deterioration. Clinical symptoms begin with a subtle, and poorly recognized, degeneration of memory. Slowly memory loss becomes more noticeable and will eventually become incapacitating, resulting in dementia. Other common symptoms include confusion, poor judgement, linguistic difficulties, agitation, withdrawal, and hallucinations. Some patients may even develop Parkinsonian symptoms, seizures, urinary and fecal incontinence, and fluent aphasia. Terminally ill patients are mute, and may develop flexion contractures. Pulmonary and urinary tract infections resulting in systemic sepsis, commonly cause death to afflicted AD patients (*Morrison-Bogorad, 1993*). Pathologically, AD is characterized by the neuronal presence of neurofibrillary tangles (NFT's), senile plaques (SP's), Hirano bodies, granulovacuolar degeneration, synaptic loss and selective neuronal loss.

In 1907 Alois Alzheimer, a German neuropathologist and psychiatrist, initially noted AD. Dr. Alzheimer described the hallmark pathological findings in a 51 year-old woman, who

presented a continuing memory loss, ultimately to the state of dementia. Upon autopsy of the afflicted patient, Dr. Alzheimer described the presence of NFT's, a loss of cerebral cortical neuronal mass, and the presence of numerous SP's (*Markesbery, 1997*). The contemporary pathologists, of 1907, suggested that severe arteriosclerosis was at the heart of these pathological findings. It was not until the 1960's, when researchers and clinicians had a better understanding of neurotransmitters and access to specific cortical maps, that a specific neurological disease (a separate entity from arteriosclerosis) dubbed Alzheimer's Disease was recognized. Since the 1980's, advances in molecular biological approaches have led to the more pathologically descriptive understanding of the cellular and molecular processes involved in AD pathogenesis.

(NEUROFIBRILLARY TANGLES)

Neurofibrillary tangles in Alzheimer's diseased brains are commonly found in the hippocampus, neocortex, and many subcortical nuclei. The hippocampus is involved in the development of short-term memory and the integration of that information into long term memory. The neocortex is associated

with higher cognition, and the size of the neocortex is proportional to increasing evolutionary cognition (*Shephard, 1994a*).

The NFT's consist of abnormally paired helical filaments (PHF's), which are composed of hyperphosphorylated tau (PHF-t) proteins and are laden with small amounts of ubiquitin (*Mori, 1987*). The tau protein is one of the many microtubule-associated proteins and is utilized in promoting the stability of axonal microtubules. Axonal microtubules function essentially, as railroad tracks for intra-neuronal and intra-axonal transport (*Shephard, 1994b*). Tau proteins become hyperphosphorylated throughout the AD pathological process. The enzymes that phosphorylate the tau proteins are thought to be tau protein kinase I (TPK I) and tau protein kinase II (TPK II) (*Imahori, 1997*). Sequence analysis of TPK I complimentary-DNA (cDNA) has provided convincing evidence that the enzyme is actually glycogen synthase kinase-3-beta (GSK3b) (*Ishiguro, 1993*).

The enzymatic sequence of tau phosphorylation by TPK I and II is convoluted. TPK I can only phosphorylate non-native tau proteins that have been abnormally phosphorylated, but cannot phosphorylate tau proteins that have not been abnormally

phosphorylated. TPK II can phosphorylate both native and non-native tau proteins, regardless of whether the protein has previously been phosphorylated (*Imahori, 1997*). It is theorized that TPK I and TPK II act in concert to regulate one another. Once TPK I is activated, the active enzyme converts native tau proteins into PHF-tau proteins (*Imahori, 1997*). What activates TPK I and TPK II? Imahori, et al. theorized that amyloid beta protein (β -AP) occurs in neuronal cells before the phosphorylation of native tau protein is induced (*Imahori, 1997*). They tested this hypothesis by adding 20 μ M β -AP to primary tissue cultures of embryonic rat hippocampal cells. After three hours the cells were stained with anti-TPK I antibody. The results showed that after the introduction of β -AP the statistical quantity and the activity of TPK I increased. These results suggest that upon the introduction of β -AP, the activity of TPK I is induced, leading to the phosphorylation of PHF into PHF-tau proteins. The researchers also analyzed the survival of β -AP cultured cells versus control cells and found only 10% of the β -AP cultured cells survived as compared to control cells. When the same experiment was repeated, this time with the addition of actinomycin-D and/or

cyclohexamide to the medium containing the β -AP, hippocampal cell death was prevented. Application of the anti-transcriptional and anti-translational agents suggests that β -AP induction of TPK I activity induced programmed cell death (apoptosis) to occur within the hippocampal cell culture. The investigators concluded that hyperphosphorylation was ultimately induced by β -AP, which then stimulated oxidative induction (from an unknown molecular species) of TPK II and ultimately TPK I. This would lead to a disruption of axonal transportation, and eventually to cell death by apoptosis.

(BETA-AMYLOID PROTEIN)

Since 1968, there have been direct correlations observed between the density of SP's found within the brain of post-mortem Alzheimer's diseased patients and the severity of clinical dementia presented by those patients prior to death (*Perry, 1978*). Typical SP's, averaging some 50 micrometers in diameter, are found congregated within extracellular neuronal spaces and can be classified into four types (*Iverson, 1995*). The four types vary by morphological characteristics: diffuse, primitive, neuritic, and a compact structure. SP's are spherical extracellular lesions that

were originally identified and classified by the use of silver-based histological stains. The central deposit of the SP's is composed of amyloid fibrils, and is surrounded by dystrophic neurites (dendrites and axon terminals), activated microglia, and reactive astrocytes (*Iverson, 1995*). All four categories of SP's are distributed predominantly within the cerebral cortex, particularly the frontotemporal cortex and the hippocampus.

Neuritic SP's contain a 6-10 nanometer β -AP, which is the predominant component of the amyloid fibrils. The β -AP protein contains 40-43 amino acids and is derived from amyloid beta precursor protein (APP). The APP is a ubiquitous transmembrane glycoprotein. Currently, it is unknown how β -AP becomes involved with senile plaque etiology. One hypothesis is that neuritic SP's are formed following neuronal cell death from the remnants of neurons containing NFT's (*Pike, 1993*). This theory suggests that the SP's are the result of neuronal damage. Other theories suggest that SP deposits are induced by the deposition of β -AP. Alzheimer's disease has been linked to a mutation on the APP sequence or to a mutation on β -AP sequence itself (*Iverson, 1995*) (as described below in AD genetic risk factors). There are

two predominant hypotheses relating β -AP mutations to derivations of SP's. First: β -AP results from a point mutation in the APP cleavage mechanics leading to the catabolic overproduction of β -AP. Second: a mutation in the cleavage mechanism for producing β -AP from APP results in a more insoluble 42 amino acid β -AP.

(GENETIC RISK FACTORS)

Sporadic (non-mendelian) AD and all familial cases of AD appear to have the same phenotypic pathological and clinical hallmarks, although genotypically they differ. Alzheimer's disease can be divided into four main categories based on genetics (*Blacker, 1998 & Dewji, 1996*). Alzheimer's disease associated with Down's syndrome (trisomy 21), occurs in less than 1% of AD patients. Early onset AD occurs before the age of 65, represents 5% of AD patients, and is associated with three different genetic mutations. Late onset AD afflicts 10-25% of all AD patients and is associated with a mutation on chromosome 19, resulting in a mutation of apolipoprotein E. Sporadic AD, occurs in about 75% of all AD afflicted individuals, and has a variety of etiologies, including mitochondrial mutations.

Down's syndrome is caused by an extra (normal) copy of the APP gene, located on chromosome 21 (*Hardy, 1997*). Nearly all persons afflicted with a genetic mutation on chromosome 21, resulting in Down's Syndrome, will eventually develop cognitive decline and will inevitably show all the neuropathological hallmarks of AD by the age of 40 (*Brugge, 1994*). Both AD and Down's syndrome show the common etiological pathology of an increase in the β -AP deposition resulting in cellular death.

Early onset familial AD can be divided into three categories, AD1, AD3, and AD4. Alzheimer's disease 1 afflicts about 10% of the early onset AD patients and is associated with a mutation on chromosome 21, which regulates Apolipoprotein, producing an over abundance of APP. AD3 is caused by mutations in the presenilin 1 gene located on chromosome 14 (*Schellenberg, 1992*). The functions of presenilin are not known as of yet, but it is thought to be an ion channel, a G-coupled receptor, or thought to interact with the metabolism of APP, or β -AP. The mutations in the presenilin genes cause an increased production of insoluble B-AP resulting in neuronal damage. AD4 is caused by a point mutation on chromosome 1, specifically at the site of the

presenilin 2 gene. Again, the mutation on the presenilin 2 gene results in an overproduction of β -AP.

Late onset familial AD, categorized as AD2, affects 10-25% of the AD population and is associated with a mutation on the apolipoprotein E (Apo E) gene on chromosome 19 (Martinez, 1998). There are three different Apo E gene alleles distributed throughout the population: Apo E-e2, -e3 and -e4. A person inherits one form of the alleles from each parent, resulting in six possible genotypes. The Apo E-e4 allele has been theorized to have a dose related effect when affiliated with AD pathology. It was found that persons with two Apo E-e4 alleles had a nine-fold increased risk and an earlier onset distribution of AD than those with no Apo E-e4 alleles (Corder, 1993). Persons with one Apo E-e4 allele had a three-fold increased risk and a younger age of onset than patients with no Apo E-e4 alleles. Currently no relationship as to how Apo E-e4 affects the pathogenesis of AD is understood. Elevated cholesterol and low-density lipoprotein levels have been associated with the presence of Apo E-e4 alleles (Kamboh, 1998).

Sporadic AD represents the majority of AD patients and a non-mendelian genetic epidemiology can be presumed. Sporadic AD can be related to head trauma, cigarette smoking (or other oxidative damaging mechanisms), and environmental factors such as viruses and/or bacteria. Many theories also relate a mutated mitochondrial genome to sporadic AD pathology

Mitochondria are involved in the respiration of individual cells. Mitochondria are responsible for the reduction of oxygen to water and the production of ATP through the employment of a sequence of proteins known collectively as the electron transport chain (ETC). Mitochondria carry their own DNA, which codes for 13 polypeptides integral to the ETC and 24 rRNA molecules utilized in the production of those 13 polypeptides. Mitochondria are replicated strictly by binary fission, meaning that all the mitochondria present within an animal or a plant cell have come directly from the mother's oocyte of that animal or plant cell via binary fission.

Sporadic AD exhibits protein defects within ETC complex 4, specifically the cytochrome c oxidase (COX), which is encoded by the mitochondrial genes CO1 and CO2 (Davis, 1997). COX

activity has been reported to be reduced in blood platelets (*Parker, 1990*), cultured skin fibroblasts (*Petersen, 1986*), and the brain tissue of AD patients (*Kish, 1992*). The ETC mutation gives rise to an increased number of reactive oxygen metabolites (ROM's) within the mitochondria. ROM examples include superoxide radicals, hydroxyl radicals, and hydrogen peroxide. Normal mitochondrion biochemistry results in the discharge of about 2-3% high energy electrons, which ultimately will produce ROM's. A mutation in the ETC ultimately can lead to an even higher discharge of high energy electrons, resulting in the increased production ROM's and consequently to an increase in oxidative damage.

For any familial or sporadic genetic risk factor classifying AD, a common theme can be implied: the increase of insoluble β -AP and/or overall increase in oxidative damage (as described above). If AD is a collection of different molecular pathologies resulting in one common histological presentation, then a common biochemical pathway resulting from many different genetic and environmental insults can be inferred. The most common form of pathology coexisting with AD is an increase in

oxidative damage. The brain is relatively vulnerable to oxidative free radical damage because of its high oxygen consumption rate, abundant lipid content, and relatively insufficient number of antioxidant enzymes (*Coyle, 1993*).

An increase in β -AP elevates oxidative damage (*Behl, 1992*). Yankner and colleagues first showed that β -AP fragments were cytotoxic to cells in 1989. They did this by digesting β -AP into smaller fragments, and applying these digested polypeptides directly to both primary hippocampal neurons and to clonal neuronal cell lines. After the application of digested protein fragments the researchers noted neuronal cell death (*Yankner, 1990*). Behl, et al. (1994) tested rat cortical neurons for damage caused by direct application of β -AP. These researchers described that within an hour after the addition of β -AP the neuronal cell's membranous organelles (golgi and mitochondria) began to show evidence of ultrastructural damage, and/or deterioration. The implication was that free radicals were at the root of neuronal cellular damage, since ROM's cause membrane damage. It has been shown that vitamin E and other lipophilic antioxidants protected the neuronal cell lines from β -AP toxicity (*Behl, 1992*).

The damage inflicted on neuronal cells is caused as β -AP exerts a cytotoxic effect on neurons by binding to microglia's mononuclear phagocyte scavenger receptor A. This binding stimulates microglia to produce neurotoxins, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), tumor necrosis factor alpha (TNF- α), and Apo E (Ikeda, 1998). In addition, β -AP stimulates the activation of the complement cascade proteins. All of these microglial products ultimately lead to neurotoxicity through inflammation and apoptosis (Ikeda, 1998).

The cellular antioxidant glutathione (GSH) was discovered in 1888 and acquired the name *philothion*, which is Greek for 'love-sulfur' (Cooper, 1997). In 1930, the structure of GSH was determined to be a tripeptide composed of glutamate, cysteine, and glycine. The chemical name for GSH is γ -Glutamyl-cysteinylglycine. The γ -glutamyl bond connecting glutamate and cysteine are resistant to the enzymatic cleavage by cellular peptidases, allowing GSH to be relatively stable in both the cellular, and the extra-cellular environments. The majority of cellular GSH is present in the cytosolic compartment while about

20% of GSH can be found within the mitochondria (*Cooper, 1997*). In the mitochondria, GSH serves as an important defense mechanism against ROM's created from the process of electron transport oxidative phosphorylation. Compartmentalization of GSH pools also occurs in the brain. Cerebral GSH pools are segregated in the non-neuronal cells, including the glial cells, astrocytes, and/or in axons and nerve terminals.

Glutathione, which is present ubiquitously in plant, bacterial, and animal cells, plays an essential role in free radical detoxification. It does so by cycling between the reduced thiol form (GSH) and an oxidized form (GSSG) in which two GSH tripeptides are linked together by a disulfide bond. The GSH/GSSG cycling is catalyzed by two enzymes: GSH reductase (GSSG-R) and GSH peroxidase (GPX). The cycling between the reduced and oxidized forms allows GSH to react with and neutralize many harmful molecules enzymatically. Glutathione is reactive towards free radicals by donating its sulfhydryl (-SH) hydrogen atom to most carbon, oxygen (e.g. hydrogen peroxide H_2O_2), and nitrogen free radicals (ROM's), forming GS^- (thiolate ion), which can react with another GS^- to form GSSG.

Glutathione is the substrate for many enzymes; three of great significance relative to the buffering effects of GSH are Glutathione S-transferases (GST), GSSG-R and GPX. The selenium-containing enzyme, GPX enzymatically scavenges toxic peroxides and other free radicals, neutralizing them by reducing them with the hydrogen ion from the GSH sulfhydryl group, as described above.

The oxidation of GSH produces GSSG, then GSSG-R reduces GSSG, with help of the cofactor NADPH, reproducing two reduced GSH molecules.

The GSH sulfhydryl group conjugates to a variety of acceptor molecules that need to be biologically neutralized. The sulfhydryl group is involved in nucleophilic reactions; nucleophilic addition to a double or triple bond, nucleophilic elimination (of a halide), and nucleophilic displacement. Glutathione transferases

are a class of enzymes which catalyze the conjugation of GSH to electrophilic substances (RX), forming the corresponding GSH S-conjugates (RSG). These conjugate molecules include many xenobiotics (such as organophosphates, allylic compounds, and epoxides) all of which become relatively unreactive and soluble when combined with GSH.

When these compounds become neutralized by GSH conjugation, they are no longer highly electrophilic and hence no longer toxic to cells. The GSH S-conjugates can thus be efficiently cleared out of tissues, because of increased solubility and limited reactivity.

Glutathione synthesis is dependent upon the presence of limiting cysteine. Intracellular cysteine levels are usually low, due to the primary sulfhydryl toxicity of cysteine at elevated concentrations. Cysteine toxicity is a consequence of cysteine reactivity with protein mixed disulfides, alpha-keto acids, chelation of metals, and many other properties of this highly reactive amino acid. Cysteine undergoes disulfide exchange with protein mixed disulfides, causing a denaturation of some protein's

native states (*Reed, 1983*). Cysteine combines with alpha-keto acids and forms stable crystalline hemithioketals, resulting in denatured proteins and polypeptides (*Cooper, 1997*).

Cystathionine gamma-lyase (CNase) is capable of providing cysteine for cell use by utilizing the sulfur atom of methionine and the carbon skeleton and amine group of serine, through the transulfuration pathway. Methionine enzymatically loses its gamma methyl group, forming homocysteine. Homocysteine then condenses serine to form the thioether cystathionine, through the catalysis of the enzyme cystathionine synthetase. The pyridoxal 5'-phosphate (PLP) dependent enzyme, CNase, enzymatically cleaves *L*-cystathionine into alpha-ketobutyrate, ammonia, and *L*-cysteine.

Cystathionine is a thioether containing amino acid with no known functions, except to serve as an intermediate in the transulfuration pathway. The transulfuration pathway is a major source of cysteine when the rate of GSH biosynthesis is high, or when cysteine concentrations are limiting (*Reed, 1995*)

The adult brain normally relies almost exclusively on the oxidation of glucose to meet its high energy demands. In the brain, hydrogen peroxide results from oxidative metabolism in the mitochondria, and from a variety of other sources including the monoamine oxidases, *d*-amino acid oxidases, and catecholamine autooxidation.

Other toxic oxygen species produced systemically and in the brain include singlet oxygen ($^1\text{O}_2$), superoxide free radicals (O_2^-), and peroxides (O_2^{2-}) (*Brock, 1984*). Chemical activation from cytochromes and flavins induces $^1\text{O}_2$ formation from biologically ground state triplet oxygen (O_2). Singlet oxygen is also chemically formed by peroxysomal enzyme catalysis. Formed in transiently small amounts during respiration and from light activation, O_2^- is very reactive and can easily cause damage to lipids and other biochemical components. Hydrogen peroxide is an example of O_2^{2-} and peroxides are produced by two electron reduction of ground state O_2 .

Pathologically, the genetic disruptions that occur in AD lead to an overload of free radicals; some created by β -AP activation of

microglia and/or the ETC COX mutation, and some created by normal biological chemistry.

To defend against these oxidative insults, cells have developed cellular defense systems, which include GSH and the enzymes catalase and superoxide dismutase. Catalase is limited to the peroxisomal compartments within cells. Superoxide dismutase converts the superoxide anion radical into the less active but relatively stable species, hydrogen peroxide. Thus the brunt of the free radical detoxification process, defending against and neutralizing hydrogen peroxide, is dependent on GSH's antioxidant capabilities.

(SYNOPSIS OF EXPERIMENTATION)

In these experiments GSH concentration and GSH resynthesis capabilities in AD afflicted lymphoblasts and non-afflicted age matched control lymphoblasts were measured and subsequently evaluated. The theory being tested is that if AD cells are continuously under increased oxidative stress, then the Alzheimer's diseased cell capacity to defend against any oxidative insult should be greater than the defense capacity of comparable

non-afflicted control cells. Control and AD lymphoblasts were used throughout these experiments because their mitochondria are the same as the mitochondria of neurological cells (since direct binary fission from the mother's oocyte's mitochondrial pool, produces all cellular mitochondria).

METHODS&MATERIALS

Roswell Park Memorial Institute medium 1640 (RPMI), Glutamine, Streptomycin and Penicillin, were purchased from Sigma chemical company, St. Louis, MO. Bovine calf serum was purchased from Hyclone laboratories, Logan, UT. Flasks were purchased from Falcon plastics, Boston, MA. Lymphoblasts from Alzheimer's diseased patients and lymphoblasts from control patients were donated from the Oregon Brain Aging Study (OBAS) at Oregon Health Sciences University (OHSU). The AD donors were post mortem histologically confirmed Alzheimer's diseased positive. The age matched control cells were obtained from persons of similar age, sex, lifestyle, geographic area, ethnicity, and trauma backgrounds by the OBAS. The cell lines that were investigated are listed below in Table 1. AD1085, AD864, & AD1137 represent Alzheimer's diseased cell lines and Co152, Co166, & Co203 represents lymphoblast cell lines from the Oregon Brain Aging Study whom were control subjects.

TABLE 1. Lymphoblast cell lines investigated

Cell Line	Sex of donor
AD 1085	Female
AD 1137	Female
AD 864	Male
Co 152	Female
Co 166	Female
Co 203	Male

Anchorage independent lymphoblast cell lines were grown in RPMI 1640 (pH of 7.45) supplemented with 4 mM L-glutamine; (100 µg/ml) streptomycin; (100 units/ml) penicillin G sodium; and 11% bovine calf serum. The cell lines were individually maintained in 500 ml and 1000 ml volume Erlenmeyer flasks with sponge closures. Cell suspensions in the flasks were always kept below a level of one inch, allowing normal oxygen metabolism to thrive. Cell suspensions in volume flasks were incubated at 37°C and a six percent carbon dioxide water saturated atmosphere. Lymphoblast cultures were diluted on a tri-weekly basis to an optimal cell density of 1.5×10^6 cells/ml. Cell density was counted using a Reichert Bright Line hemocytometer and an Olympus optical CK2 10x inverted Phase-contrast light microscope.

Medium was routinely changed by centrifuging cellular suspensions (Beckman TJ-6 centrifuge 5 minutes at 1000 x g), removing the medium superior to the cell pellet, and then replacing with fresh medium. A volume of fresh medium was

added which was necessary to yield an optimal 1.5×10^6 cells/ml.

Cells were grown for at least two weeks in the medium before beginning any studies. This allowed the cells to adapt to the culture conditions.

To prepare for assays, cell density was counted the day before the assay was to be performed. Cells were centrifuged in polypropylene centrifuge tubes at $1,000 \times g$ (for 5 minutes) and resuspended in fresh medium to an optimum cell density of 3×10^6 cells/ml for the GSH depletion and resynthesis assays and 5×10^6 cells/ml for the cystathionase assays. After centrifugation, cells were pre-incubated in RPMI 1640, and placed in 25 cm^2 polystyrene tissue culture flasks. The cells and the flasks were then positioned on their side, in a 37°C incubator, overnight.

GSH Depletion Assay

Cell density was determined the day of the experiment and recorded. Flasks containing cells were removed from the incubator and gently shaken to evenly distribute all cells. A quantity of cell suspension was prepared (13 mls) which would be adequate for six one ml samples, allowing for one extra ml.

Protocol; GSH depletion preparation: A one ml sample of suspension was removed from the flask and added to an appropriately labeled 1.5 ml microfuge tube. The microfuge tube was centrifuged at 13,000 x g in a Fisons micro centaur centrifuge for 20 to 30 seconds. The supernatant liquid was removed and discarded with a disposable pipette. Next 500 µl of 10% perchloric acid (PCA) and 15 µl of 1 µmole/ml gamma-glutamyl glutamate (γ-GG) was added to the cells left from centrifugation. The protocol was repeated twice for each time allotment. Tops were closed on the now prepared microfuge tubes were placed in a rack, at room temperature, until the remainder of the assay was completed.

To the residual cells, remaining in the tissue culture flask, diethyl maleate (DEM) (DEM 1:10 dilution in DMSO) was added to a final concentration of 200 mM DEM. The DEM used was 98% pure. Addition of the DEM was designated as time zero. The tissue culture flasks were then returned to the 37°C incubator. At time points of 15, 30, 45, 60, and 90 minutes tissue culture flasks were removed from the incubator, and a one ml sample of cell suspension was extracted and treated as previously described

for the GSH depletion preparation. Duplicate samples were removed and treated at each time point. The sample microfuge tubes were placed in a tube rack, at room temperature, for the duration of the experiment.

PROTEIN ASSAY AND HPLC PREPERATION

From the GSH depletion assay, microfuge tubes containing AD and control cell lines (immersed in PCA and g-GG) were centrifuged at 12,000 x g for 20 minutes, at 3°C (Fisons microcentaur centrifuge). After centrifugation, microfuge tubes were removed and the supernatant was transferred to the corresponding labeled 4 ml yellow disposable culture tube, and left uncovered.

A quantity 250 µl of 30 mM NaOH in 1% sodium dodecylsulfate (SDS) was added to the precipitated protein pellets in the microfuge tubes. The microfuge tubes were placed in a refrigerator at 4°C until the protein assay could be completed.

Yellow disposable culture tubes were utilized in order to minimize ultra-violet light exposure and inhibit degradation of susceptible compounds. To each yellow tube containing the cell supernatant, 50 µl of 15 mg iodoacetic acid in 1 ml of 0.2 mM

meta-cresol purple was added. Then 500 μ l (added in increments of 100 μ l) of 2 normal (N) potassium hydroxide (KOH) in 2.4 N potassium bicarbonate (KHCO_3) was added to the yellow tubes. Next, the yellow tubes were placed in the dark (at room temperature) for more than 2 hours, to allow CO_2 to escape. After at least 2 hours, 500 μ l of 1% (v/v) fluoro-2,4-dinitrobenzene (FDNB) in 95% ethanol was added to each tube. The tubes were then capped and again placed in the dark (at room temperature) for at least 6 hours, to allow formation of N-DTNB derivatives for measurement of GSH by HPLC.

The glutathione concentration was determined using a method of HPLC (*Fariss, 1987*). Yellow tube HPLC samples were centrifuged at 2000 x g for 10 minutes (Beckman TJ-6 centrifuge). The supernatants were analyzed for GSH, using a Spectra Physics 8700 high-performance liquid chromatograph, equipped with a LDC UV-III detector and a Hewlett Packard 8390A recording integrator. The internal standard, γ -GG (15 μ mole/ml), was used as a reference. All GSH concentrations were derived using quantity/ml data relative to the γ -GG known standard.

Cell extract protein concentrations were determined by the Pierce bicinchoninic acid (BCA) protein assay (*Smith, 1985*). The BCA assay is based on comparing unknown protein concentrations to known protein concentrations of bovine serum albumin (BSA) standards. BSA standards prepared were 10, 20, 30, 40, 50, 60, 80, and 100 µg/1000 µl of 1% SDS in water. A standard curve was created by measuring the absorbance of the known BSA standards at a wavelength of 562 nm. The same quantity of experimental cell protein extract was measured at 562 nm, the absorbance was noted, and the respective µg of protein per ml was calculated. Protein assays were performed in microtiter plates, allowing for multiple samples to be read and hence greater accuracy. The microplate reader was an Anthos ht II labtech. To micro titer plate wells 10 µl of evenly distributed protein pellet sample along with 200 µl of BCA protein reagent was added.

GSH RESYNTHESIS ASSAY

Cell density was determined the day of the experiment using a Reichert bright line hemocytometer. Tissue culture flasks were removed from the incubator and gently shaken to evenly

distribute the cells. A quantity of cell suspension was prepared (12 mls) that would be adequate for the GSH resynthesis assay: 2 one ml samples for the negative time, for time 0 resuspension, and for resuspension times 20, 50, and 90 minutes post resuspension.

Protocol; GSH resynthesis preparation: A one ml sample of cell suspension was removed from the flask and transferred to a 1.5 ml microfuge tube. Duplicate samples were removed at zero time and for each other time point. The microfuge tubes, labeled with their appropriate times ($t = -35$ and -40), were centrifuged at $13,000 \times g$ in a Fisons micro centaur centrifuge for 20 to 30 seconds. The supernatant was removed and discarded, then 500 μ l of 10% PCA and 15 μ l of 1 μ mol/ μ l γ -GG was added to the cell pellet.

To the cell suspension remaining in the flask DEM was added to give a final concentration of 200 mM DEM. The flasks were then returned to the incubator.

The time necessary to deplete GSH to half base GSH concentration was determined from the GSH depletion assays.

For all AD cell lines, GSH depletion time was 35 minutes, and for control cell lines GSH depletion time was 40 minutes.

At the pre-determined GSH depletion time, duplicate one-ml aliquots were prepared, as previously described for GSH resynthesis preparation. The residual cell suspension remaining in the 25 cm² flask was removed from the flask and transferred to a polypropylene centrifuge tube. The suspension was centrifuged at 1000 x g for three minutes in a Beckman model TJ-6 centrifuge. The samples were removed from the centrifuge and the supernatant liquid was discarded. The cells were then returned to the 25 cm² tissue culture flasks and incubated at 37°C, in an equal amount of RPMI. This was designated as resuspension time zero.

At time points of 20, 50, and 90 minutes post cell resuspension, duplicate samples were removed and treated as previously described, (in "GSH resynthesis protocol") to determine GSH concentration by HPLC.

Once the GSH resynthesis assay was complete, the precipitants were saved and assayed for protein concentration as

previously described. The cell extracts were prepared for HPLC as previously described.

DTNB CNase ASSAY

The DTNB (5-5'-dithio bis-2-nitrobenzoic acid) assay measures the quantity of product catalyzed by the enzyme CNase. This is done via colorimetric determination of the quantity of thiol containing cysteine produced from the added substrate, cystathionine (*Flavin, 1962* and *Hargrove, 1987*). The thiol groups produced, bind to DTNB and yields para-nitrothiolate, a structure that highly resonates. Para-nitrothiolate is yellow and readily absorbs at 412 nm. Absorbance was measured, using a LKB Biochrom Ultrospec II spectrophotometer, and results were calculated using Beer's Law, an extinction coefficient of $13,600 \text{ m}^{-1}\text{cm}^{-1}$, and an absorbency of 412 nm. CNase units were then calculated in mmoles of thiol containing cysteine produced per minute per mg of protein.

Prior experiments were conducted, using a variety of cell concentrations, in order to determine what CNase cell concentration would be within the linear slope of the protein standard curve (5×10^6 cells/ml).

Background thiol group concentrations (background control) within the cell were determined by the addition of 200 μ l of 10% SDS to the time zero cell sample. Addition of SDS effectively inhibited all metabolic activity. All other reagents utilized in the CNase assay were then added to the control samples (DTNB, *L*-cystathionine, PLP, and EDTA). The reagent DTNB covalently bonded with all background thiol groups, producing para-nitrothiolate, thus giving the background thiol concentration.

To assay the enzyme CNase, the enzyme's substrate, *L*-cystathionine, was added. *L*-cystathionine, added to a final concentration of 2 mM, was added to AD and control cell lines suspended in 0.1 M Potassium phosphate buffer (pH 7.35 KCL added) at time zero along with PLP and EDTA (*Flavin, 1962* and *Hargrove, 1987*). PLP was added to a final concentration of 0.1 mM and was utilized because it is a cofactor for the CNase enzyme. EDTA was added to a final concentration of 0.5 mM and was used as a chelator of unnecessary divalent metals. At 5 and 10 minutes, 200 μ l of 10% SDS was added to the cells, thus stopping all metabolic reactions. Ten minutes after the addition

of SDS, 50 μ l of 20 mM DTNB (DTNB diluted in 95% ethanol) was added,

Subtracting the control thiol concentrations (time zero experiments) from the experimental thiol concentrations (enzyme allowed to catalyze substrate into product for 5 and 10 minutes) gave the total amount of cysteine produced from the enzyme CNase. For CNase specific activity, the moles of cysteine per ml, was divided by μ g of protein per ml and then divided by time (5 and 10 minutes).

The statistical significance of the results obtained from the GSH (depletion and resynthesis) and CNase assays were determined by the student's *t* test. (*Arkin, 1970*).

RESULTS

The working hypothesis was that AD lymphoblasts should have a higher level of GSH, an increased GSH resynthesis capability after GSH depletion, and an increased activity level of the enzyme CNase. All of these parameters would be indicative of an increased oxidative defense capability, as is suggested by AD pathophysiology.

The cell lines selected for the study were received from the OHSU Oregon Brain Aging Study program. There were three control lymphoblast cell lines (C-1066, C-152, & C-152) and three Alzheimer's diseased lymphoblast cell lines (AD-1085, AD-1137, & AD-864) investigated throughout the experimental process (as shown in Table 1).

The GSH concentration in control cell lines was analyzed and the average concentration was calculated to be 0.1547 nmols GSH/mg of protein. The AD cell lines' base GSH concentration averaged 0.1342 nmoles GSH/mg of protein (Table 2). The standard deviations for AD and control cell GSH concentrations were 4.06×10^{-2} and 1.34×10^{-2} , respectively. Figure 1 shows a graphical comparison between the AD and control cells GSH

concentrations. Analysis of the mean AD and control GSH concentration data through statistical analysis, a t value of 0.4097 was calculated ($n=18$): thus the differences in concentrations calculated were determined not to be significant, ($p>0.3$).

Figures 2 through 4 display the rate of GSH resynthesis for the AD and control lymphoblast cell lines. The rates of GSH depletion for both AD and control cell lines are ostensibly parallel, as shown in Figure 2. The GSH resynthesis data was evaluated twenty minutes after washing off of DEM and resuspension in fresh RPMI (Figure 3). The AD cell lines GSH resynthesis mean slope was 4.225×10^{-4} (nmols GSH/mg of protein/min) and for the control cell lines the mean slope was 2.583×10^{-4} (Figure 3). The GSH resynthesis standard deviation for both the AD and control cell line slope was 1.99×10^{-7} and 0.711×10^{-7} , respectively. A t value was calculated to be 3.29, and therefore synthesis in AD lines was significantly faster, at $P < 0.001$. Figure 4 depicts the GSH resynthesis concentration results relative to the initial GSH concentration. The percentage of GSH relative to initial was averaged for both the AD and control cell lines, and the

slope calculated. The AD mean slope was 0.2643 and the control slope was 0.1703. The standard deviations were 0.1089 for AD and 0.024 for the control. Synthesis rates were significantly different at $p < 0.001$.

Figure 5 displays the significant differences of individual cell lines GSH resynthesis rates. The control cell line Co203 GSH resynthesis rate was significantly different from that of AD1085 ($P < 0.001$) and AD864 ($P < 0.005$). The Co152 resynthesis data statistically differed from AD1085 ($P < 0.001$) and AD864 ($P < 0.005$). Co166 resynthesis data significantly differed from only AD1085 ($P < 0.001$). The AD1085 resynthesis data significantly differed from all five other cell lines ($P < 0.001$ for all control and AD1137) ($P < 0.005$ for AD864). The AD1137 resynthesis results significantly differed from AD864 ($P < 0.005$) and AD1085 ($P < 0.001$). AD864 resynthesis results differed from four other cell lines ($P < 0.005$ compared to control 203, 152, 166, and AD 1085 and 1137), but did not statistically differ from control 166.

Cystathionase assays for the AD and control cell lines are displayed in Figure 6 and are in units of moles of thiol containing cysteine produced/min/mg of cellular protein. The AD cell line

mean CNase production was calculated to be 2.2079×10^{-10} (n=18) while 1.426×10^{-10} (n=18) was obtained for the control cell lines. The standard deviation was 4.4866×10^{-11} and 1.53×10^{-11} for the AD and control cell lines, respectively. A *t* was calculated to be 7.824, indicating that CNase specific activity in AD cell lines was statistically larger than control cell lines ($p < 0.001$).

Figures 7 through 12 represent AD and control cell lines individual GSH depletion and resynthesis concentrations at time points of -35 (AD, Fig 10-12), -40 (Control, Fig 7-9), and at times 0, 20, 50, and 90 minutes (for all 6 cell lines). The graphs also displays the individual standard deviations (SD) for all time points' GSH concentrations.

Figure 13 displays four different HPLC graphs from the AD cell line 1085. Retention time (RT) of 38.10 minutes represents the internal, standard g-GG cellular concentrations, and RT of 42.01 minutes represents GSH cellular concentrations. In Figure 13, four individual HPLC graphs are displayed (-35b, 0a, 50b, and 90a), all at different time points throughout the resynthesis assay. In Figure 13, Graph -35B represents GSH concentration before

the addition of DEM. Graph 0A represents the GSH concentration at the time of cellular resuspension in DEM-free medium. Graph 50B and 90A represents the GSH concentrations at 50 and 90 minutes post-resuspension.

DISCUSSION

The oxidative stress hypothesis has suggested that reactive oxygen species may be of significance in the development of AD. The ultimate question to be investigated is whether AD patients have an increased sensitivity to oxygen free radicals or an increased level of free radical formation. An increased sensitivity to oxygen free radicals would be indicative of a decreased level of free radical defense mechanisms, such as GSH. The alternative, an increased level of free radical formation could be caused by nuclear and mitochondrial mutations (*Davis, 1997*) or by external factors. Intracellular generation of ROM's, such as H_2O_2 and O_2^- , results in increased transcription of the regulatory and catalytic subunits of the GSH synthesizing enzyme g-glutamylcysteine synthetase (*Moellering, 1998*). Also noted, ROM's increase the activity of the transulfuration pathway and the cysteine supplying enzyme, CNase (*Reed, 1995*). If the etiological foundation of AD pathology is based on an increased amount of free radical formation, then an increased level of free radical defense mechanism would be expected throughout all afflicted AD cells.

In these studies, DEM was the GSH depleting agent used throughout the depletion and resynthesis experiments. DEM conjugates to GSH through the enzymatic catalysis of GST's. GSH nucleophilically attacks DEM's carbon-carbon double bond, beta to a keto group, the result is a stable molecule, which has effectively removed GSH.

The results of these experiments showed no statistical difference between AD and control cell lines for resting GSH cellular concentration (Figure 1 and Table 2). This result is in opposition to one of the three parameters investigated throughout this study. These results may be a phenomenon based on GSH's high degree of turn over, or on the feed back inhibition of GSH synthesis. GSH is reported to have a half life of 192 minutes in the rat liver (Meredith, 1986), 240 minute half life in the kidney (Scott, 1993), 114 minutes in resting macrophages (Rouzer, 1982), and 59.5 hours in the brain. (Chang, 1997). With such a quick and varied GSH turnover rate throughout systemic tissues, GSH level could be maintained at an appropriate level for defense against insult. Maintenance of adequate GSH concentrations can be sustained by recycling GSH and GSSG from an oxidized to a

reduced form, and by GSH resynthesis from the amino acid components glycine, glutamate, and cysteine. The quick turnover rate seem to be an appropriate rational in support of the findings that GSH levels were not highly elevated in AD afflicted lymphocytes versus the control lymphocytes tested *in vitro*. The second notable process was that feedback inhibition of GSH synthesis helps to give a rational interpretation for the unelevated GSH concentrations.

Balazs and Leon determined the levels of GSH in 12 regions of the brain from AD patients and age matched control patients (Balazs, 1994). The cells were removed from the AD and control subjects' brains within 7.3 hours postmortem for AD and 5.8 hours postmortem for control. These investigators reported no significant difference in GSH levels in AD brain as compared to control tissue samples. They also reported no overall difference in GSH concentrations between the 12 different brain regions. The investigators used the GSH disulfide reductase DTNB recycling assay and measured DTNB products using the same procedures used in the current work.

The second parameter investigated in this study was the GSH resynthesis capability in AD cell lines as compared to the control cell lines. Figures 2, 3, 4 and 5 display the combined results of GSH resynthesis experiments. Figures 7 through 12 display individual cell lines GSH concentrations at different time points, and also displays relative standard deviations. Figure 2 shows that while the rates of depletion of GSH between AD and control lymphoblasts were indistinguishably parallel, the resynthesis rates differed. The amount of GSH depletion is also displayed in Figure 2. Although the goal was to obtain a 50% depletion concentration (as compared to initial GSH concentration), there were some variations throughout cell lines. Sierra-Rivera, et al. noted that the resynthesis rates of GSH were indistinguishable between GSH depletion concentrations of 20-70% (*Sierra-Rivera, 1994*). Figure 3 represents the GSH resynthesis data from 20 to 90 minutes after DEM washing and cellular resuspension in DEM-free medium. Resynthesis data was analyzed 20 minutes after resuspension, thus allowing all cell lines to have comparable resynthesis parameters. The mean slopes for all three AD and control slopes were averaged and

compared with their standard deviations. A significant difference was calculated differentiating the two mean slopes, with a probability of greater than 99.9% ($P < 0.001$). The AD lymphoblasts showed an increased resynthesis capability as compared to the control cell lines mean resynthesis rate. The AD mean resynthesis slope was 4.225×10^{-7} nmols GSH produced/mg protein/minute, as compared to the control cells 2.583×10^{-7} .

Figure 4 displays the GSH resynthesis capabilities of AD and control cell lines relative to the initial GSH concentrations. The AD and control GSH resynthesis slopes were averaged, and the mean rates were compared. Again, AD lymphoblasts showed an elevated capacity to resynthesis GSH as compared to control cell lines.

Figure 5 displays the significant difference of individual resynthesis slopes, as obtained from figure three data. AD cell lines Ad1085 and 864 were distinguishable from all other cell lines, except AD 864 and control 166 were shown to be of no

significant difference. Ad1137 resynthesis data was similar to all other control cell lines, but the mean resynthesis of all three AD cell lines did statistically differ from the mean of all the control cell lines tested (Figures 3 and 4).

GSH is synthesized intracellularly from three free amino acids in two steps. First, the gamma carboxyl group of glutamate is activated by ATP and forms an amide with the amino group of cysteine with the help of the enzyme gamma-glutamyl cysteine synthetase. The second step involves a similar activation of the cysteine carboxyl group, by ATP, allowing the dipeptide to condense with the free amino acid glycine. The second enzyme, gamma glutamylcysteine synthetase, which catalyzes the condensation of the dipeptide and glycine, is feedback inhibited by GSH. This feedback inhibition could aid in the rationalization of the findings that GSH concentration in AD versus control cells did not statistically differ. Notably, mean GSH resynthesis capability was elevated in AD versus control lymphoblast cell lines.

The limiting reagent in *de novo* GSH synthesis and resynthesis is the amino acid cysteine. CNase is capable of

providing cysteine through the utilization of methionine's sulfur atom and serine's carbon skeleton. The transulfuration pathway, as described in the introduction, provides cysteine when GSH biosynthesis is at elevated levels and when the cysteine concentrations are below glutathione's synthesis demands for the amino acid. Therefore, cystathionine's capabilities to provide cysteine in times of high GSH demand are essential too cellular oxidative defense mechanisms. When cystathionine is cleaved by CNase alpha-ketobutyrate, ammonia and the thiolate ion of cysteine (cys^-) are immediately produced. Immediately after lysis, cys^- can perform disulfide exchange with DTNB. The disulfide exchange with DTNB produces the highly resonating para-nitrothiolate anion. This anion is easily detectable at a wavelength of 412 nm, and can be measured with a spectrophotometer. The CNase experimental assays executed denoted an increased capability of AD lymphoblast cell lines as compared to control cell lines to produce cysteine from cystathionine (Figure 6). The AD lymphoblast cell lines showed 1.45 times the level of CNase specific activity of control

lymphoblast cell lines. This data was confirmed with a greater than 99.9% significant difference ($P < 0.001$).

In addition, it is important to note that the control 166 cell line showed an increased GSH resynthesis capability and an increased CNase level as compared to the other two control cell lines (Figures 3, 4 and 5). The explanation for this atypical cell line can be explained by the possibility that the subject tested is metabolically in a state of increased oxidative damage. This subject may possibly have a potential for AD pathogenesis, although the donor is still disease free.

Marcus, et al. analyzed neurological tissue samples from the brain of post-mortem confirmed AD afflicted individuals and compared those results to tissue samples from age matched control patients (*Marcus, 1998*). The investigators analyzed the activity of oxidative defense enzymes throughout tissue samples from frontal, temporal, and cerebellar brain regions.

The investigators reported an increase in overall lipid peroxidation throughout all three tissues, determined by the production of thiobarbituric acid-reactive products (TBARS). In AD tissue samples, the enzyme superoxide dismutase (SOD) was

reported to be statistically higher in the frontal and temporal brain regions as compared to control cell tissue. A significant decrease in catalase enzymatic activity was reported in the temporal brain region. In contrast, the investigators reported no significant difference in the activity of the enzyme GPX. They reported a 12% increase (AD vs. control) in GPX activity in the frontal region, almost equal enzyme activity in the temporal region, and a 28% decrease in GPX activity in the cerebellum (although none of the data was statistically significant). As stated above, CAT is limited to the peroxisomal compartments within cells and SOD only converts the superoxide anion radicals into hydrogen peroxide, leaving the brunt of hydrogen peroxide to be neutralized by GPX. The fact that they reported relatively no difference in the GPX activity further supports the theory that the AD etiology stems from an increase in oxidative damage and not from a decrease in oxidative defense mechanisms, which is contradictory to their reported theory.

Lovell, et al. determined the levels of the GSH enzymes GPX and of GSSG-R (Lovell, 1995). GSSG-R reduces the oxidized form of GSH, GSSG, allowing the two to cycle between the reduced

thiol form (GSH) and an oxidized form (GSSG) (in which two GSH tripeptides are linked together by a disulfide bond with the help of NADPH). The investigators reported a statistical increase in GPX and GSSG-R activity throughout two regions of AD brain as compared to control brain tissue samples. This evidence is not congruent with the previous study, but does support the theory of increased oxidative defense mechanisms throughout AD tissue.

E-4-Hydroxy-2-nonenal (HNE) is an electrophilic species that can covalently modify cellular macromolecules, and is produced as a by-product of lipid peroxidation. Oxidative stress causes lipid peroxidation, an autocatalytic process that damages lipid containing structures and yields HNE along with malondialdehyde (MDA). The electrophilic species HNE cross-links proteins by interacting with cellular nucleophiles, particularly lysine, histidine, serine, and cysteine residues. The investigators Montine, et al. investigated HNE's cytotoxicity by adding HNE, an HNE analog, and MDA to cultured neuroglia (*Montine, 1996*). They were investigating the mechanism to which HNE causes cell death. Their data showed that HNE can cross link cytoskeletal proteins and is directly cytotoxic to cell cultures. However, the

HNE analog and MDA did not cross link proteins and was not shown to cause cell death. These investigators theorized that HNE might be responsible for the disulfide bond-independent cross linking of the proteins in NFT'S. These experimental results further support the theory that increased oxidative damage is the causative agent of AD. Since HNE is a direct product of lipid oxidative damage (peroxidation), a mechanism for the production of cross-linked cytoskeletal protein from peroxidation damage can be inferred.

Smith, et al. studied overall protein oxidation in AD versus age matched control brain samples (*Smith, 1991*). Tissue samples were taken from the brain frontal lobe and occipital lobe of each subject. The protein oxidation state was determined by measuring brain carbonyl content. Excessive oxidative modification of proteins in AD patients suggests an increase in overall oxidative damage, as is suggested in the oxidative stress hypothesis. The investigators provided statistically significant evidence that AD brain samples had an increased amount of protein oxidation as compared to control tissue samples. Glutamine synthetase is the enzyme that catalyzes the conversion

of glutamate to glutamine. Mixed function oxidation is a site-specific oxidative modification of proteins, occurring near the metal containing catalytic sites of particular enzymes, as is the case with glutamine synthetase. These investigators continued to report a statistical decrease of glutamine synthetase activity in AD brain samples as compared to control tissue samples.

8-Hydroxy-2'-deoxyguanosine (OH^8dG) is a biomarker of oxidative DNA damage. The investigators Morocci, et al. used OH^8dG to differentiate between the DNA oxidation state of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) in AD brain tissue samples as compared to control samples (*Morocci, 1994*). They reported a statistical increase in oxidative damage to the nDNA of AD brain samples as compared to control tissue samples, with an increase throughout all brain regions studied. Furthermore, they reported an even higher level of mtDNA damage in AD as compared to the control brain tissue samples. The mtDNA damage due to oxidation was drastically higher in AD versus control samples as compared to the nDNA oxidative damage. The higher mtDNA versus nDNA damage supports the

theory that an ETC mutation, specifically a COX mutation, has resulted in elevated levels of oxidative stress in AD pathology.

There is an abundance of data supporting the oxidative stress hypothesis. The oxidative stress hypothesis is so appealing as an etiological base for AD because of the fact that an accumulation of oxidative damage over time could account for the late life onset and slow progressive nature of the neurological disorder. Increased amounts of Fe^{++} , Al^{++} , and Hg^{++} have been reported in AD brain tissue (*Markesbery, 1993*), and this is indicative of increased oxidative damage. Many parameters support the increase in oxidative damage theory: increased lipid peroxidation, increased HNE levels, increased protein and DNA oxidation, the presence of NFT's, SP's and β -AP.

As stated in the discussion, β -AP elevates oxidative damage in cells (*Behl, 1992*), and is capable of generating free radicals by stimulating microglia to produce neurotoxins such as ROS, RNS, $\text{TNF-}\alpha$, Apo E. The microglial activation also stimulates the complement cascade proteins activation, further leading to cell death. Because genetic mutations associated with familial AD

result in an ultimate increase in insoluble β -AP, and because of the genetic mutations associated with sporadic AD (COX mutation), an overall increase in oxidative insults are resultantly associated with AD genetic mutations.

The data reported in this study extends evidence which support the oxidative stress hypothesis. Briefly, In this study, AD cell lines exhibited ubiquitously higher levels of GSH resynthesis capabilities and an increase in CNase levels as compared to age matched control cell lines. Data accumulated from this study, taken in context with other experimental data, discussed throughout this paper, provides considerable evidence that increased oxidative insult, not decreased defense mechanisms, are probably at the heart of the Alzheimer's disease pathogenesis.

CELL TYPE	(GSH) nmol/mg cell protein	Standard deviation
Co203 (n=7)	0.116997	0.04617
Co166 (n=6)	0.19778	0.037486
Co152 (n=6)	0.1496	0.033223
Ave Co (n=19)	0.1547	0.0134
Ad1085 (n=10)	0.17355	0.023612
Ad 864 (n=6)	0.14733	0.03538
Ad1137 (n=6)	0.1556	0.04254
Ave Ad (n=22)	0.1342	0.0406

TABLE 2: Alzheimer (Ad) and Control (Co) resting cellular GSH concentration, labeled with relative Standard deviations. Cellular GSH concentration was measured by a method of HPLC and all GSH concentrations were normalized to cellular protein concentrations, as described in "materials and methods". Results show no statistical difference between Ad and Co cell lines.

Figure 1. Glutathione levels compared between Ad and Co lymphoblast cell lines. Alzheimer (Ad) vs. Control (Co). Cellular GSH levels were determined by a method of HPLC as described in "materials and methods". Error bars show mean SD. Results showed no statistically significant difference between Ad and Co GSH concentrations. n displays the number of replications.

Figure 2. Mean GSH depletion and resynthesis concentrations for Ad and Co cell lines. Alzheimer (Ad) and Control (Co) lymphoblast cell lines were treated with 200mM DEM, the GSH depletion agent, then resuspended at time 0 in DEM-free medium (as described in "materials and methods"). Ad and Co cells show parallel glutathione depletion rates. Resynthesis rates differ and individual SD are displayed in Figures 7-12. Concentration of GSH was normalized to cellular protein and GSH was measured by a method of HPLC.

Figure 3. Comparison of GSH resynthesis from 20 to 90 minutes post cellular resuspension in DEM-free medium. Mean Alzheimer's diseased (Ad) and control (Co) lymphoblast cell line GSH concentrations are displayed. The GSH depleting agent, DEM, was removed at time 0 and cell were resuspended in DEM-free medium. Time 20 through 90 minutes represents optimal cellular GSH resynthesis conditions. Concentrations of GSH were normalized to cellular protein and GSH was measured by method of HPLC, as described in "methods and materials". Slope is in units of nmol of GSH/mg protein/min.

Figure 4. Percent of GSH resynthesized relative to initial GSH concentration in Alzheimer's diseased (Ad) and Control (Co) lymphoblast cell lines. Resynthesis of GSH after removal of the GSH depleting agent, DEM. Cellular GSH concentrations were measured by a method of HPLC and GSH concentrations were normalized to cellular protein, as described in "methods and materials".

CELL TYPE						
	Co203	Co152	Co166	Ad1085	Ad1137	Ad864
203 n=7	---	ns	ns	**	ns	*
152 n=6	ns	---	ns	**	ns	*
166 n=6	ns	ns	---	**	ns	ns
1085 n=10	**	**	**	---	**	*
1137 n=6	ns	ns	ns	**	---	*
864 n=6	*	*	ns	*	*	---

Figure 5: Significant difference between Alzheimer's diseased (Ad) and control (Co) lymphoblast cell lines GSH resynthesis rates. Figure displays relavency of individual cell line resynthesis rates, compared with all other cell lines.

(ns) = no significant difference

(*) = Statistically significant difference (P<0.05)

(**)= Statistically significant difference (P<0.001)

Figure 6. Comparison of cystathionase (CNase) activity in Alzheimer's diseased (Ad) and Control (Co) lymphoblast cell lines. After the addition of the CNase substrate, cystathionine, the enzyme activity was measured by colorimetrically determining the amount of product (thiol containing cysteine) produced in a quantity of mols/minute/mg of protein. Procedures of CNase assay are discussed in "materials and methods". Results show a statistically significant difference between the mean AD and Co CNase activity ($P < 0.001$).

Figure 7. GSH depletion and resynthesis graph for the control (Co) 203 lymphoblast cell line. Y error bars represent mean SD for Co 203 GSH concentrations. Cell lines were treated with 200mM DEM and were then allowed to replenish GSH in DEM-free medium. GSH levels were determined by a method of HPLC, as described in "materials and methods". GSH content was normalized to cellular protein concentrations.

Figure 8. GSH depletion and resynthesis graph for the control (Co) 166 lymphoblast cell line. Y error bars represent mean SD for Co 166 GSH concentrations. Cell lines were treated with 200mM DEM and were then allowed to replenish GSH in DEM-free medium. GSH levels were determined by a method of HPLC, as described in "materials and methods". GSH content was normalized to cellular protein concentrations.

Figure 9. GSH depletion and resynthesis graph for the control (Co) 152 lymphoblast cell line. Y error bars represent mean SD for Co 152 GSH concentrations. Cell lines were treated with 200mM DEM and were then allowed to replenish GSH in DEM-free medium. GSH levels were determined by a method of HPLC, as described in "materials and methods". GSH content was normalized to cellular protein concentrations.

Figure 10. GSH depletion and resynthesis graph for the Alzheimer's disease (AD) 1085 lymphoblast cell line. Y error bars represent mean SD for AD 1085 GSH concentrations. Cell lines were treated with 200mM DEM and were then allowed to replenish GSH in DEM-free medium. GSH levels were determined by a method of HPLC, as described in "materials and methods". GSH content was normalized to cellular protein concentrations.

Figure 11. GSH depletion and resynthesis graph for the Alzheimer's disease (AD) 864 lymphoblast cell line. Y error bars represent mean SD for AD 864 GSH concentrations. Cell lines were treated with 200mM DEM and were then allowed to replenish GSH in DEM-free medium. GSH levels were determined by a method of HPLC, as described in "materials and methods". GSH content was normalized to cellular protein concentrations.

Figure 12. GSH depletion and resynthesis graph for the Alzheimer's disease (AD) 1137 lymphoblast cell line. Y error bars represent mean SD for AD 1137 GSH concentrations. Cell lines were treated with 200mM DEM and were then allowed to replenish GSH in DEM-free medium. GSH levels were determined by a method of HPLC, as described in "materials and methods". GSH content was normalized to cellular protein concentrations.

Figure 13: Alzheimer's disease (AD) cell line 1085, high performance liquid chromatography (HPLC). The graphs show an example of AD 1085 HPLC data results. Retention time (RT) of 38.10 minutes represents the internal standard gamma-glutamyl glutamate (g-GG) within the cellular suspension sample. The RT of 42.01 minutes represents the quantity of glutathione (GSH) within the cellular suspension sample. The quantities were normalized to cell protein (μg of cell protein/ ml). Noted on each individual graph is the time of the sample, a-35 represents the sample before the addition of DEM. Time 0 represents the sample at the time of cellular resuspension in DEM-free medium, and times of 50 and 90 minutes represents the times post cellular resuspension in DEM-free medium.

BIBLIOGRAPHY

Arkin,H., Colton,R.R. 1970 Significance of the difference between two means. *Statistical methods*. 5thed. 150-154. Barnes & Noble, Inc. New York.

Balazs,L., Leon,M. Evidence of oxidative challenge in the Alzheimer's diseased brain. *Neurochem. Res.* 1994;**119**,9;1131-1171.

Behl,G., Davis,J.B., Cole.G.M., Schubert,B. Vitamin E protects nerve cells form amyloid beta protein toxicity. *Biochem. Biophys. Res. Commun.* 1992;**186**;944-952.

Behl,C., Davis,J.B., Klier,G., Schubert,D. Amyloid beta peptide induces necrosis rather that apoptosis. *Brain Res.* 1994;**645**;253-264.

Blacker,D., Tanzi,R.E. The genetics of Alzheimer's disease. *Arch. Of Neuro.* 1998;**55**;294-296.

Brock,T.D., Smith,D.W., Madigan,M.T.1984 *Biology of Microorganisms*. 4th edition. Prentice-Hall, Inc., Englewood Cliffs, NJ. 260-265.

Bugge,K.L., Nichols,S.L., Salmon,D.P., Hill,L.R., Delis,D.C., Asron,L., Trauner,D.A. Cognitive impairment in adults with Down's syndrome: Similarities to early cognitive changes in Alzheimer's disease. *Neurology.* 1994;**44**;232-238.

Cooper,A.J.L, Rosenberg,R.N., Prusiner,S.B., DiMiauro,S., Barchi,R.L. 1997a The molecular and genetic Basis of neurological disease. Glutathione in the Brain: Disorders of Glutathione metabolism. 2nd edition. Butterworth-Heinemann. Boston, Oxford, Hohannesburg, Melbourne, NewDelhi, Singapore. 1195-1230.

Chang,M.L., Klaidman,L.K., Adams Jr,J.D. The effects of oxidative stress on *in vivo* brain GSH turnover in young and mature mice. *Mol. Chem. Neuropathol.* 1997;**30**,3;187-197.

Corder,E.H., Saunders,A.M.E., Gaskell,P.C., Small,G.W., Roses, A.D., Haines,J.L., Pericak-Vance,M.A. Gene dose of Apolipoprotien E Type 4 allele and the risk of Alzheimer's disease in late onset families. *Science.* 1993;**261**;921-923.

Coyle,J.T., Puttfarcken,P., Oxidative stress, glutamate, and neruodegenerative disorders. *Science.* 1993;**262**;689-692.

Davis,R.E., Miller,S., Hernstadt,C., Ghosh,S.S., Fahy,E., Shinobu,L.A., Galasko,D., Thal,L.J., Beal,M.F., Howell,N., Parker Jr,D. Mutations in mitochondrial cytochrome c oxidase genes segregate with late onset Alzheimer's disease. *Proc. Nat. Acad. Sci. USA*. 1997;**94**;4526-4531.

Dewji,N.N., Singer,S.J. Genetic clues to Alzheimer's disease. *Science*. 1996;**271**;159-160.

Fariss,M.W., Reed,D. 1987. Methods in Enzymology. High-Performance liquid chromatography of thiols and disulfides: Dinitrophenol derivatives. Academic Press. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto. **143**,101-114.

Flavin,M.J. Microbioal transsulfuration: The mechanism of an enzymatic disulfide elimination reaction. *J. Biol.Chem.*1962;**237**;768-777.

Freeman,M.L., Meredith,M.J., Laszlo,A. Depletion of glutathione, heat shock protein sythesis, and the development of thermotolerance in Chinese hamster ovary cells. *Cancer res.* 1988;**48**;7033-7037.

Hargrove,J.L., Wichman,D.R. A cysteine-dependent inactivator of tyrosine aminotransferase co-purifies with gamma-cystathionase (cysteine desulfurase). *J. Biol. Chem.* 1987;**262**,15;7351-7357.

Hardy,J. The Alzheimer family of diseases:many etiologies, one pathogenesis. *Proc. Natl. Acad. Sci. USA*. 1997;**94**; 2095-2097.

Hoshi,M., Takashima,A., Noguchi,K., Murayama,M., Sato,M., Kondo,S., Saitoh,Y., Ishiguro,K., Hoshino,T., Imahori,K. Regulation of mitochondrial pyruvate dehydrogenase activity by tau protein kinase I/glycogen synthase kinase 3 beta in brain. *Proc. Natl. Acad. Sci. USA*. 1996;**93**;2719-2723.

IkedA,K., Aklyama,H., Nishimura,T. Glial tau pathology in neurodegenerative disease: their nature and comparison with neuronal tangles. *Neurobio. of Aging*. 1998;**19**,1(S);S85-S91.

Imahori,K., Uchida,T. Physiology and Pathology of Tau Protein Kinases in Relation to Alzheimer's disease. *J. Biochem.* 1997;**121**,2;179-188.

Ishiguro,K., Shiratsuchi,A., Sato,S., Omori,A., Arioka,M., Kobayachi,S., Uchida,T., Imahori,K., Glycogen synthase 3 β is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS. Lett.* 1993;**325**,3; 167-172.

Iverson,L.L., Mortishire-Smith,J., Pollack,S.J., Shearman,M.S. The toxicity in vitro of Beta-amyloid protein. *Biochem. J.* 1995;**311**;1-16.

Kamboh,M.I., Ferrell,R.E., DeKosky,S.T. Genetic association studies between Alzheimer's disease and two polymorphisms in a low density lipoprotein receptor-related protein gene. *Neuroscience lett.* 1998;**244**;65-68.

Kish,S.J., Bergeron,C., Rajput,Ali., Dozic,S., Mastrogiacono,F., Chang,L., Wilson,J.M., DiStefano,L.M., Nobrega,J.N. Brain cytochrome oxidase in Alzheimer's disease. *J. Neurochem.* 1992;**59**,2;776-779.

Lovell,M.A., Ehmann,W.D., Butler,S.M., Markesberry,W.R. Elevated Thiobarbituric acid reactive substances and antioxidant enzyme activity in the brain of Alzheimer's patients. *Neurology.*1995;**45**;1594-1601.

Marcus,D.L., Thomas.C., Rodriquez,C., Simberkoff,K., Tsai,J.S., Strafacia,J.A., Freedmann.M.L. Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. *Experimental Neuro.* 1998;**150**;140-144.

Markesberry,W.R. Oxidative stress hypothesis in Alzheimer's disease. *Free Radic. Biol. Med.* 1997;**23**,1; 134-147.

Markesberry,W.R., Ehmann,W.D. 1993 Brain trace elements in Alzheimer's disease. *Alzheimer's disease.* Raven press. New York. 353-367.

Martinez,M., Campion,D., Brice,A., Hannequin,D., Dubois,B., Didierjean,Ol., Michon,A., Thomas-Anterion,C., Puel.M., Frebourg,T., Agid,Y., Clerget-Darpux,F. Apolipoprotein E-e 4 allele and familial aggregation of Alzheimer's disease. *Arch.Neurol.*1998;**55**;810-816.

Meredith,M.J., Reed,D.J. Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J. Biol. Chem.* 1982;**257**,7;3747-3753.

Meredith,M.J., Williams,C.M. Intracellular glutathione cycling by gamma-glutamyl transpeptidase in tumorigenic and non-tumorigenic cultured rat liver. *J. Biol. Chem.* 1986;**261**,11;4986-4992.

Montine,T.J., Ventkataraman,A., Martin,M.E., Strittmatter,W.G., Graham,D.G. E-4-hydroxyl-2-nonenal is cytotoxic and cross links cytoskeletal proteins in P19 neuroglial cultures. *Am. J. Pathol.* 1996;**148**;89-93.

Mori,H., Kondo,J., Ihara,Y. Ubiquitin is a component of paired helical filaments in Alzheimer's disease. *Science*. 1987;235;1641-1644.

Morocci,P., MacGarvey,V., Beal,M.F. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann. Neurol*. 1994;36,5;747-751.

Morrison-Bogard,M., Weiner,M.F., Rosenberg, R.N., Bigio,E., White III, C.L. 1997 The Molecular and Genetic Basis of neurological disease. 2nd edition. Butterworth-Heinemann, Boston, Oxford, Johannesburg, Melbourne, New Delhi, Singapore. Alzheimer's Disease. 581-627.

Parker Jr,W.D., Filey,C.M., Parks,J.K. Cytochrome Oxidase deficiency in Alzheimer's disease. *Neruology*. 1990;40;1302-1303

Pike,C.J., Burdick, D., Walencewicz, A.J., Glabe, C.G., Cotman, C.W. Neurodegeneration induced by β -amyloid peptides in vitro: the role of peptide assembly state. *J. Neuroscience*. 1993;13,4;1676-1687.

Perry,E.K., Tonlinson,M.E., Blessed,G., Bergmann,K., Gibson,P.H., Perry,R.H. Correlation of cholinergic abnormalities with senile plaque and mental test scores in senile dementia. *Br. Med. J*. 1978;25,2(6150);1457-1459.

Peterson,C., Goldman,G.E. Alterations in calcium content and biochemical processes in cultured skin fibroblasts from aged and Alzheimer donors. *Proc. Natl. Acad. Sci. USA*. 1986;83;2758-2762

Richman,P.G., Meister,A. Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. *J.Biol.Chem*. 1975;250,4;1422-1426.

Reed,D.J. 1995. Methods in Enzymology. Cystathionine. Academic Press. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto. 252,92-102.

Reed,D.J., Brodie,A.E., Meredith,M.J. 1983 Cellular heterogeneity in the status and function of cysteine and glutathione. *Functions of glutathione: Biochemical, Physiological, Toxicological, and Clinical aspects*. Raven Press, New York.39-49.

Rouzer,C.A., Scott,W.A., Griffith,O.W., Hamill,A.L., Cohn,Z.A. Glutathione metabolism in resting and phagocytic peritoneal macrophages. *J. Biol. Chem*. 1982;257,4;2002-2008.

Schellenberg,G.D., Bird,T.D., Wijsman,E.M., Orr,H.T., Anderson,L., Nemens,E., White,J.A., Bonnycastle,L., Weber,J.L., Alonso,E., Potter,H., Heston,L.L. Genetic linkage evidence for a familial Alzheimer's disease locus on chromosome 14. *Science*. 1992;258;668-671.

Scott,R.D., Hughey,R.P., Curthoys,N.P. Role of apical and basolateral secretion in turnover of glutathione in LLC-PK1 cells. *Am. J. Physiol.* 1993;265,5pt2; F723-728.

Shephard,G.M. 1993a Neurons and Glia. *NeuroBiology*. 3rd edition. Oxford University Press. New York, Oxford.53-57.

Shephard,G.M. 1993b Learning and Memory. *NeuroBiology*. 3rd edition. Oxford University Press. New York, Oxford.618-637.

Sierra-Rivers,E., Meredith,M.J., Summar,M.L., Smith,M.D., Voorhees,G.J., Stoffer,C.M., Freeman,M.L. Genes regulating glutathione concentrations in X-ray-transformed rat embryo fibroblasts: changes in γ -glutamylcysteine synthetase and γ -glutamyltranspeptidase expression. *Carcinogenesis*. 1994;15,7;1301-1307.

Smith,C.D., Carney,T.M., Stark-Reed,P.E., Oliver,C.N. Stadman,E.R., Floyd,R.A., Markesbery,W.R. Excess protein oxidation and enzyme dysfunction in normal aging and Alzheimer's disease. *Proc.Nat.Acad.Sci.USA*. 1991;88,23;10540-10543

Smith,P.K., Krohn,R.I., Hermanson,G.I., Mallia,A.K., Gartner,F.H., Provenzano,M.D., Fujimoto,E.K., Goeke,N.M., Olson,B.J., Klenk,D.C. Measurement of Protein Using Bicinchoninic Acid. *Anal. Biochem.* 1985;150;78-85.

Yankner,B.A. Duffy,L.K., Kirschner,D.A. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science*. 1990;250;279-282.