

Quantitative proteomic analysis of differentially expressed proteins in A β (17-42) treated synaptosomes

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Abstract: Oxidative stress has been associated in the pathogenesis of numerous diseases such as various neurodegenerative disorders, ischemia, and cancer. The brain is susceptible to oxidative stress due to its high content of peroxidizable unsaturated fatty acids, high consumption of oxygen, and elevated levels of free radicals. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can react with biomolecules such as proteins, lipids, carbohydrates, DNA, and RNA, which can lead to oxidative damage, cellular dysfunction, and ultimately cell death. Down syndrome (DS) is caused by trisomy of chromosome 21, a genetic abnormality in which an extra copy of the chromosome is present. DS patients have extensive deposition of A β (17-42) peptide, which could contribute to their increased rate of developing Alzheimer's disease (AD), which is consistent with current research. Since AD cannot be properly diagnosed until autopsy, development of a novel Down syndrome model using A β (17-42) could be beneficial in determining oxidative stress levels and their relationship to mild cognitive impairment (MCI), the earliest form of AD. This work will demonstrate the use of a novel Down Syndrome model and its correlation to oxidative stress. We have found a significant difference between oxidative stress levels in A β (17-42) treated synaptosomes and control. By using proteomics, we have also identified several biomarkers including aldehyde dehydrogenase, aldolase, α -enolase, heat shock cognate 71, peptidyl-prolyl cis-trans isomerase, and ATP synthase α chain. Our present findings suggest the role of A β (17-42) as one of the contributing factors in mediating oxidative stress in DS and AD brain leading to neurodegeneration. This novel DS model may have potential applications as a diagnostic tool to identify biomarkers that may contribute to Alzheimer's disease.

Keywords: oxidative stress; Down syndrome; Alzheimer's disease, amyloid beta peptide, neurodegeneration.

Introduction

Oxidative stress has been associated in the pathogenesis of numerous diseases such as neurodegenerative disorders, ischemia, and cancer. Under oxidative stress conditions, the balance between the pro-oxidant and antioxidant levels is impaired. Certain environmental factors, stressors, or disease may cause an imbalance and as a result, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced. ROS and RNS have the ability to react with biomolecules including carbohydrates, proteins, lipids, DNA and RNA which leads to oxidative damage and ultimately cellular dysfunction¹. The brain is susceptible to oxidative stress due to its high content of peroxidizable unsaturated fatty acids, high consumption of

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oxygen, elevated levels of free radicals, and comparatively low levels of antioxidant defense systems².

The typical markers of oxidative stress commonly studied to determine the oxidative stress levels include protein carbonyls, 3-nitrotyrosine (3-NT), free fatty acid release, 4-hydroxy-2-nonenal (HNE), acrolein, advanced glycation end products for carbohydrates, iso- and neuroprostane formation, 8-OH-2'-deoxyguanosine, and altered DNA repair mechanisms¹. Amyloid beta (A β), a 40-42 amino acid peptide, is formed by the proteolytic cleavage of amyloid precursor protein (APP) via β and γ -secretases. It has been discovered that mutations in APP, presenilin-1, or presenilin-2 lead to increased production of A β (1-42) and the early onset of Alzheimer's disease (AD)¹. However, if APP is cleaved by α - and γ -secretases, the A β (17-42) fragment, also known as the p3 fragment, is formed (Figure 1).

Down syndrome (DS) is the most common genetic cause of mild to moderate mental retardation occurring in newborn infants³. DS is characterized by a trisomy at chromosome 21, the location of APP. DS persons also demonstrate elevated levels of oxidative stress and A β (17-42) deposition⁴⁻⁷. They age prematurely and develop Alzheimer's like brain changes during their 30s or 40s leading to dementia throughout their life. DS persons have a higher risk for AD and develop this debilitating disorder at a younger age compared to typical AD onset.

Protein expression analysis can potentially elucidate pathways involved in the pathogenesis of AD; therefore depicting the mechanistic progression of AD⁸. Protein oxidation can lead to loss of protein function, abnormal protein turnover, imbalance of cellular redox potential, interference with cell cycle and eventual cell death which is observed in AD⁹. Oxidation of proteins that are involved in biosynthesis, cytoskeletal dynamics, energy production, and signal transduction may lead to their loss of function². Previous literature states that the proteins that are oxidized in DS and AD brain affect various cellular functions including energy metabolism, proteasome function, glutamate uptake and excitotoxicity, neuritic connections, and neuronal communication^{5,9}.

Proteomics deals with the systematic study of proteins that help to provide the complete view of the structure, function, and regulation of a given cell, tissue, or organism. Protein expression is found to be altered in disease conditions; hence proteomic studies can serve as a sensitive technique to expand insight into a host of biologic processes and phenotypes of both diseased and normal cells⁸. Since there is an underlying correlation between Down syndrome and Alzheimer's disease and definitive AD diagnosis can only be conducted at autopsy, the need for an AD diagnostic system is great. The goal of this work is to develop a novel model of Down syndrome using A β (17-42) peptide that may have potential applications as a diagnostic tool to identify biomarkers that may contribute to Alzheimer's disease.

Results and Discussion

To assess whether there were any changes in the proteomic profile between the control and A β (17-42) treated synaptosomes, differential protein expression was determined by measuring the differences in densitometric intensities on the 2D gels using the PDQuest software package (Bio-Rad, Hercules, CA, USA). Five proteins showed increased expression, while one protein showed decreased expression between the experimental and control set. Mass spectrometry analysis allowed for the identification of these differentially expressed proteins. The following enzymes were identified: aldehyde dehydrogenase, aldolase, ATP synthase, alpha enolase, dihydrolipoyllysine acetyltransferase, peptidyl-propyl cis-trans

isomerase (Pin-1), and heat shock protein 71 (Table 1). Slot blot analysis was performed for control and A β (17-42) treated synaptosomes. Statistical analysis using Student's t-test was conducted and probability (p) values of less than 0.05 were considered to be significant. Levels of protein carbonyls (Figure 2), protein nitration (Figure 3), and HNE (Figure 4) were calculated and a significant difference was found between the control and the experimental set for each oxidative parameter. For each data set, the standard error of mean (SEM) was analyzed as well. After determining the differentially expressed protein spots via PDQuest software, these spots were excised from the 2D gel and in-gel trypsin digestion was performed. Table 1 gives an overall summary of the proteins identified by mass spectrometry.

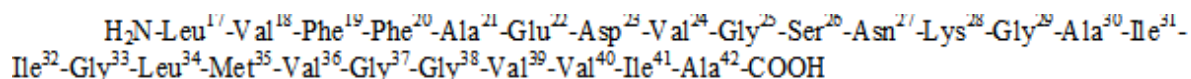


Figure 1. Primary structure for the A β (17-42) peptide.

Table 1: Expression profile of significantly differential expressed proteins.

Protein Identification	Peptide matches *	pI	MW (kDa)	Protein expression	P value **
Aldehyde dehydrogenase	2/3	7.53	56.5	Upregulated	<0.005
Aldolase	2/2	8.30	39.3	Upregulated	<0.005
Alpha enolase	3/5	6.37	47.1	Upregulated	<0.0003
ATP synthase	3/4	9.22	59.7	Downregulated	<0.002
Heat shock cognate 71	10/17	5.37	70.8	Upregulated	<0.003
Peptidyl-prolyl cis-trans isomerase	2/2	5.38	64.7	Upregulated	<0.002

*denotes the number of peptide matches to those available in the NCBI database. (ex. Aldehyde dehydrogenase has two peptide sequences that match the three found in the database). ** Values below 0.05 denote significance based on Student's t- test.

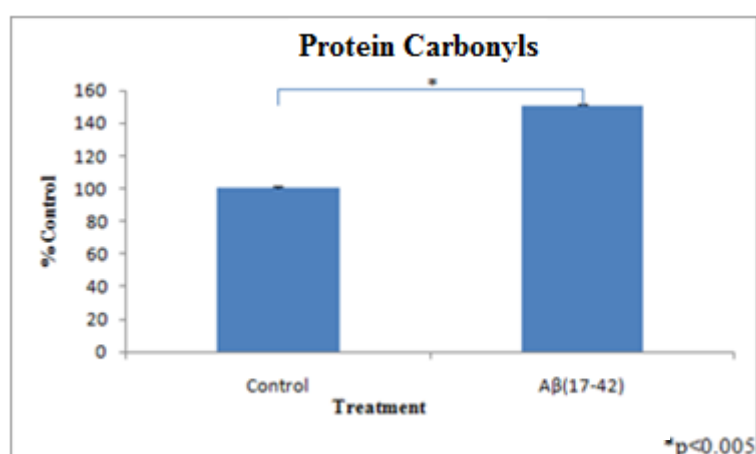


Figure 2. Levels of protein carbonyls in A β (17-42) treated synaptosomes compared to control. A 0.5mg/mL concentration of A β (17-42) was preincubated at 37°C for 24 h prior to incubation with synaptosomes. Error bars indicate the SEM for each group measured (n=6). SEM = standard error of mean.

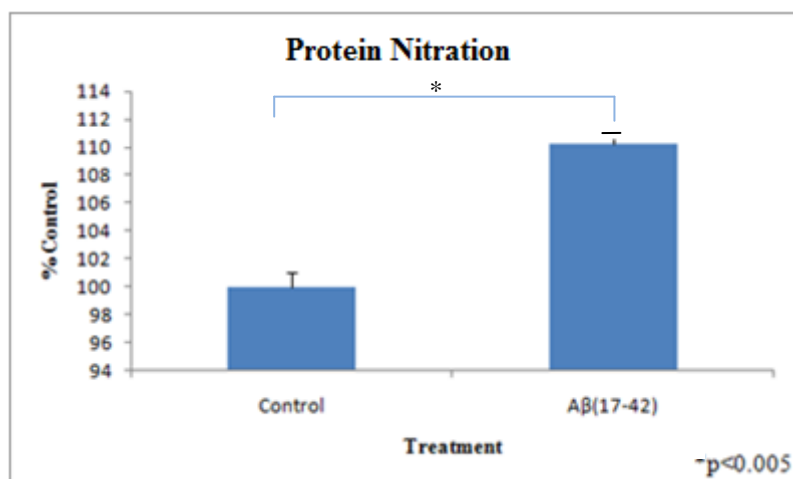


Figure 3. Protein nitration levels in Aβ(17-42) treated synaptosomes versus control. A 0.5mg/mL concentration of Aβ(17-42) was preincubated at 37°C for 24 h prior to incubation with synaptosomes. Error bars indicate the SEM for each group measured (n=6).

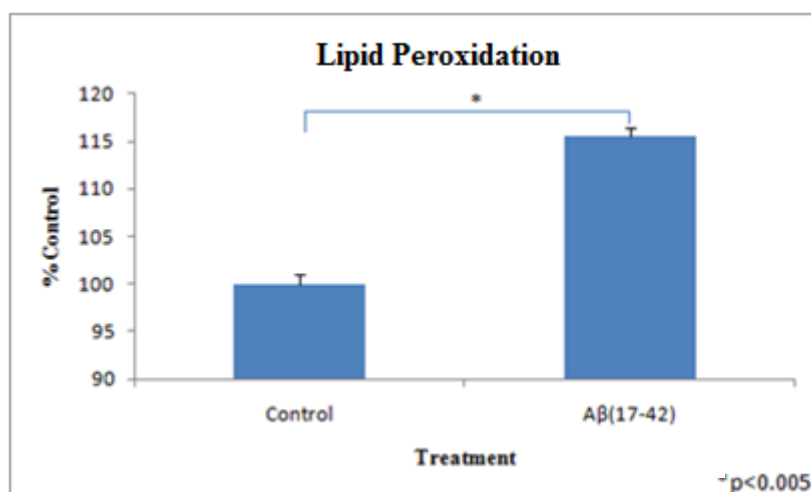


Figure 4. Protein bound HNE levels in Aβ(17-42) treated synaptosomes compared to control. A 0.5mg/mL concentration of Aβ(17-42) was preincubated at 37°C for 24 h prior to incubation with synaptosomes. Error bars indicate the SEM for each group measured (n=6).

Protein expression refers to the presence and abundance of proteins in the proteome. Proteins can be downregulated or upregulated. Overexpression of proteins may cause molecular crowding, which can result in changes in protein conformation. This may also lead to protein aggregation, inhibition of protein degradation, and production of protein formulations such as plaques and fibrillary structures, which can promote pathological processes. Overexpression cannot be balanced by a down regulation of other proteins in the cell; the cell may enter a disease state due to imbalance of homeostasis¹⁰. It has been described that in Huntington's disease, Parkinson's disease, and other neurodegenerative disorders, mere overexpression of disease related proteins causes disease progression¹¹. The proteins found to be significantly upregulated are aldehyde dehydrogenase, aldolase, alpha enolase, heat shock cognate 71, and peptidyl-prolyl cis-trans isomerase (Figures 5a and b).

Differential protein expression is always context dependent. They occur within a specific context of a tissue, organ, environmental conditions, and individual fate¹². Proteins that are expressed less are considered to be downregulated. The only protein found to be expressed

less is ATP synthase using mass spectrometric analysis (Figures 5a and 5b). Although, dihydrolipoyllysine acetyltransferase is an essential member of the pyruvate dehydrogenase complex, which converts pyruvate to acetyl CoA, thereby establishing a bridge between glycolysis and the Krebs cycle. This enzyme was found to be differentially expressed, but it was not determined to be significantly upregulated or downregulated; therefore it is not discussed in this work. Enzyme assays were carried out for alpha enolase and aldolase in control and A β (17-42) treated synaptosomes. Although a reduction in enzymatic activity was observed in both enzymes, it was deemed to be not statistically significant ($p < 0.25$ for enolase (Figure 6) and $p < 0.1$ for aldolase (Figure 7)).

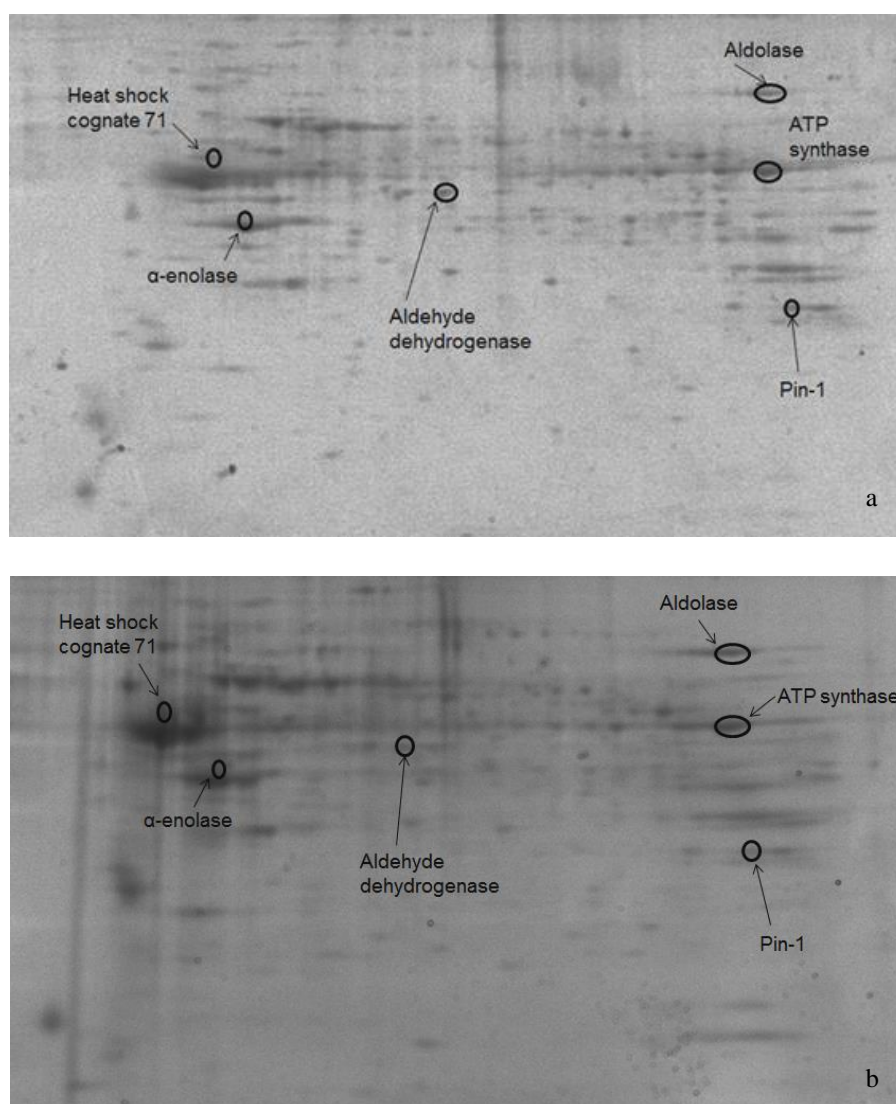


Figure 5. 2D gel map of synaptosomal proteins identified by mass spectrometry for control (a) and A β (17-42) treated synaptosomes (b). Proteins showing differential expression are shown as the circled spots.

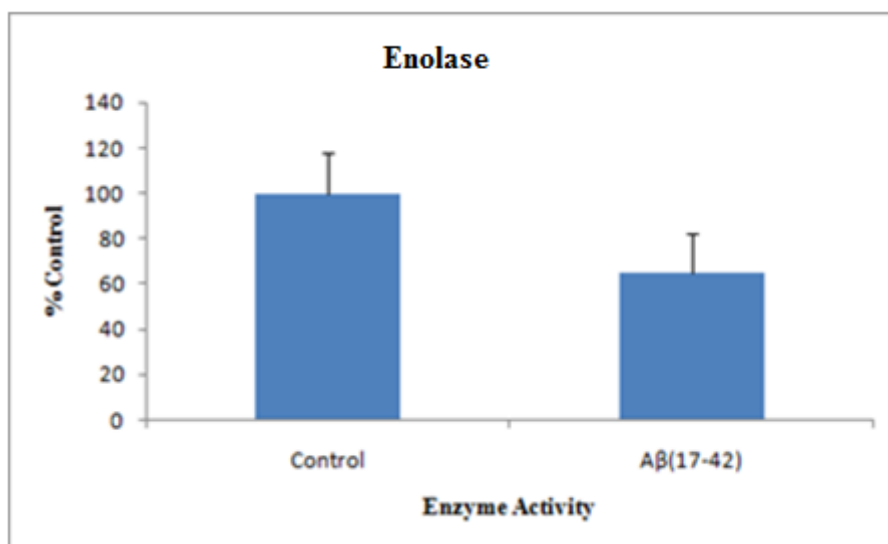


Figure 6. Enzyme activity of enolase A β (17-42) treated synaptosomes versus control. A 0.5mg/mL concentration of A β (17-42) preincubated at 37°C for 24 h prior to incubation with synaptosomes was used. Error bars indicate the SEM for each group measured (n=6).

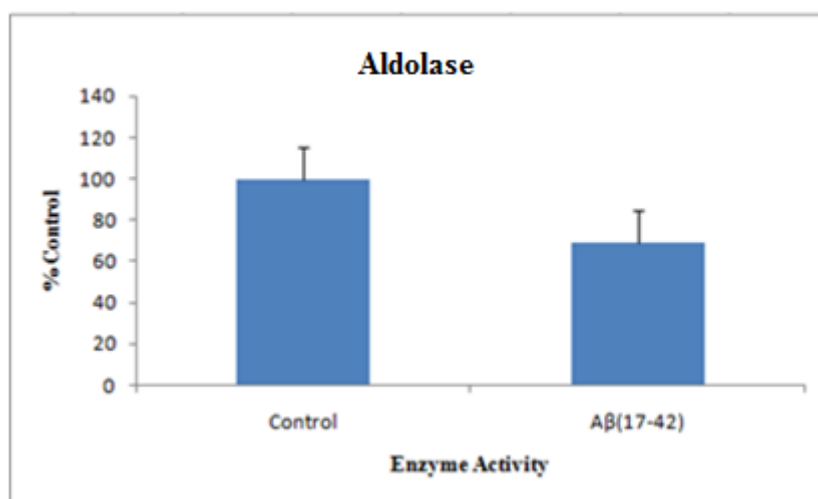


Figure 7. Enzyme activity of aldolase in A β (17-42) treated synaptosomes versus control. A 0.5mg/mL concentration of A β (17-42) preincubated at 37°C for 24 h prior to incubation with synaptosomes was used. Error bars indicate the SEM for each group measured (n=6).

The p3 fragment is extensively deposited in DS and observed in AD. Individuals with DS have a trisomy at chromosome 21, the location of amyloid precursor protein. Overexpression of the amyloid precursor protein (APP) is related to the deposition of amyloid in the brain of DS individuals and it appears to be critical to the development of AD in DS individuals¹³. As a result of this dementia, the brain regions responsible for thought, memory, and language are affected which leads to further serious cognitive decline and the inability to carry out normal daily activities.

In this study, we examined the specific oxidative stress effects of A β (17-42) on synaptosomes. As A β (17-42) is found both in DS and AD, it could contribute to the development of AD at an earlier age in DS patients. The oxidative stress induced by A β (17-42) in our study suggests that it could be one of the contributing factors in the

pathogenesis of AD. It has been shown that the Met35 residue A β (1-42), is a key amino acid residue involved in amyloid beta peptide mediated toxicity, and consequently, the pathogenesis of AD¹⁴. Parallels can be drawn that the Met35 residue found in the A β (17-42) could also contribute to the oxidative stress induced by this peptide.

Several proteins were differentially expressed in our Down syndrome model and identified by mass spectrometry. These proteins include aldehyde dehydrogenase, aldolase, alpha enolase, ATP synthase, heat shock cognate 71, and peptidyl-prolyl cis-trans isomerase. The majority of these proteins are directly or indirectly involved in energy related metabolic processes. We found a reduction in enzyme activity in several enzymes suggesting A β (17-42) induced toxicity contributes to decreased enzymatic activity.

Enolase, a key glycolytic enzyme, is one of the most abundantly expressed cytosolic proteins found in many organisms. α -enolase is a metalloenzyme that catalyzes the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate in the glycolytic pathway. This enzyme belongs to a new class of surface proteins that do not possess classical machinery for surface transport, but are transported on the cell surface by an unknown mechanism¹⁵. Enolase has been recently reported to be a multifunctional protein, having such important roles as a neurotrophic factor¹⁶, a hypoxic stress protein, and a strong plasminogen binding protein¹⁷. It has also been identified as one of the most frequently identified differentially expressed brain proteins in human and animal tissues¹⁸. α -enolase is one of the most consistently upregulated and oxidatively modified proteins in early-onset AD, amnesic mild cognitive impairment, and late stage AD brain¹⁹. Taken together, all of these findings suggest that enolase may possess one or more additional functions critical to brain cell survival along with its role in glucose metabolism. Furthermore, enolase could be integral to both normal and pathological brain function and may possess other functions necessary to preserve brain function. Upregulation and loss of enolase's enzymatic activity is considered a significant factor for disease progression and is consistent with research showing that glycolytic enzymes are functionally altered in neurodegenerative disorders^{6,7}.

Glucose is the primary source of energy in the brain²⁰. A decrease in ATP production could cause disturbances in ion homeostasis, cholesterol homeostasis, cholinergic defects, altered protein synthesis and transport, protein degradation, and reduced synaptic transmission; all of which could be detrimental to cell viability¹⁹. These changes may expose phosphatidylserine to the outer leaflet of the plasma membrane triggering a loss of phospholipid asymmetry, an early signal of synaptosomal apoptosis²¹.

Fructose 1,6-bisphosphate aldolase cleaves fructose 1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, an important ATP generating step in glycolysis²². Aldolase exists in three isoforms: A, B, and C. Aldolases A and C are preferentially involved in the glycolytic pathway and are predominantly expressed in muscle and brain respectively. Aldolase B is typically expressed in liver and is also reportedly involved in gluconeogenesis²³. As this is a glycolytic enzyme, aldolase is vitally important in the brain for energy production. Identification of this enzyme, in addition to other glycolytic enzymes, suggests impairment in energy metabolism could lead to a decrease in ATP production. ATP is very crucial at nerve terminals for maintaining intact neuronal connections. Consequently, an upregulation of aldolase may lead to synapse loss and lowered synaptic function, both of which may promote memory loss. Additionally, reduced ATP production may result in cholinergic defects, improper pump maintenance, disturbances in cholesterol homeostasis and signal transduction, and alterations in glucose and glutamate transporters ultimately leading to cell death and cognitive decline¹.

Aldehyde dehydrogenases (ALDH) are a class of detoxification enzymes that remove excess aldehydes present in the body²⁴. Aldehyde dehydrogenase catalyzes the oxidation of various aldehydes (i.e. carbonyls) to carboxylic acids and is known to play an important role in xenobiotic and endobiotic metabolism²⁵. ALDH belongs to a family of NADP-dependent enzymes that have common structural and functional features and also catalyze the oxidation of a broad spectrum of aliphatic and aromatic aldehydes. Each class is thought to oxidize various substrates that may be derived from endogenous sources (i.e. amino acids, biogenic amines, lipids) or exogenous sources, such as aldehydes derived from xenobiotic metabolism²⁶. Three classes of ALDH (ALDH1, ALDH2, and ALDH3) have been studied with respect to cytotoxic aldehyde metabolism. ALDH1 is present in cytosol, while ALDH2 is found in the mitochondrial matrix and is responsible for acetaldehyde metabolism. ALDH3 is found extensively in the lung and stomach²⁷. All three classes of ALDH enzymes metabolize HNE and utilize NAD⁺ as a cofactor²⁸. Acetaldehyde is considered a neurotoxic product and is produced during valine and threonine metabolism. It is also believed that accumulation of acetaldehyde or aldehyde derivatives could affect the development of AD²⁹. Upregulation of this protein would result in reduced detoxification capacity of the cell and an increase in protein carbonyls demonstrated in Figure 2.

Cells respond to sublethal heat stress by synthesis and accumulation of several members and compartmentally distinct families of heat shock proteins (Hsp). These proteins include Hsp70, Hsp90, Hsp60, and Hsp27. Some of these proteins have been found to be thermotolerant and resistant to other environmental stresses. Heat shock response has a cytoprotective role in a variety of metabolic disturbances and injuries, such as hypoxia, epilepsy, stroke, cell and tissue trauma, aging, and neurodegenerative diseases³⁰. The brain consumes a high rate of oxygen, as it has abundant lipid content and a relatively low availability of antioxidant enzymes when compared with other body tissues. This makes the brain highly susceptible to oxidative stress. To overcome this vulnerability, the brain has developed networks to combat oxidative stress. One such cellular stress response is heat shock proteins, which protect cells from various forms of stress. Heat shock proteins serve as molecular chaperones which exist in various types including Hsp32 (also known as heme oxygenase-1), Hsp60, and Hsp72 all of which have been shown to play a protective role in the brain in regard to oxidative stress³¹. Heat shock cognate (Hsc71), an isoform of Hsp73, is employed by the cell as a primary defense against unfavorable conditions. Hsc71 is specifically involved in the degradation of misfolded proteins. It can bind to a specific peptide region and label it for proteolysis thereby preventing protein aggregation³². Upregulation of oxidized Hsc71 can result in lowered cytoprotection which leads to an increase in protein aggregation thus triggering proteosomal overload which has been observed in Down syndrome individuals³³.

The α and β subunits of membrane bound ATP synthase complex bind ATP and ADP. The α subunit is involved in the regulation of ATP synthase activity, while the β subunit contributes to catalysis. The ATP synthase complex plays a pivotal role in energy transduction in living cells³⁴. ATP synthase, also known as complex V of the mitochondrial electron transport chain, helps in proton transport needed for the phosphorylation of ADP to produce ATP⁹. The α chain of ATP synthase, present in the inner mitochondrial membrane, plays an important role in energy production. ATP synthase promotes ATP synthesis and the release of ATP. It produces ATP by complex rotational movements of its subunits, and coupling the proton gradient generated by the respiratory chain³⁵. The ATP synthase complex is associated with proton transport necessary for the phosphorylation of ADP. The

mitochondrial complex may become inactivated due to the oxidation of ATP synthase. Failure of ATP synthase may decrease the activity of the entire electron transport chain and could contribute to impaired ATP production⁹. Notably, this is the only enzyme to be downregulated in this DS model. Therefore, ATP will not be generated at normal levels which can alter overall energy metabolism, which is a highly regarded theory of Alzheimer Disease.

Peptidyl-prolyl cis-trans isomerase (Pin-1) supports the formation of correct disulfide bonds during protein folding. Pin-1 is a chaperone protein that catalyzes the isomerization of the peptide bond between pSer/Thr-Pro in proteins, thereby controlling their biological functions including protein assembly, folding, intracellular signaling, intracellular transport, transcription, cell cycle progression, and apoptosis³⁶. This alteration can cause remarkable structural modification, which can affect the properties of targeted proteins. In most cells, including neurons, Pin-1 is mostly nuclear and its activity is required for the checkpoint of DNA replication. Conventionally, Pin-1 phosphorylates cytoskeletal proteins, such as tau. The targeting of the tau protein by an unregulated Pin-1 protein can result in hyperphosphorylation, the main component of neurofibrillary tangles, which is a hallmark of AD³⁷ and observable in DS brain. Several studies have shown that hyperphosphorylation of tau protein may occur due to improper activation of mitotic events in the cell cycle, thereby playing an important role in the progression of Alzheimer's disease³⁶.

Conclusion

In this study, we identified six biomarkers that were differentially expressed in A β (17-42) treated synaptosomes compared to control. The proteins that were found to be upregulated include aldehyde dehydrogenase, aldolase, alpha enolase, heat shock cognate 71, and peptidyl-prolyl cis-trans isomerase. The only protein found to be downregulated was ATP synthase. It has been shown that protein oxidation may lead to protein conformational changes³⁸ and loss of function^{39,40}. Based on this concept, oxidation and successive loss of function of the proteins identified in our study would lead to the disruption of neuronal communication and diminished energy metabolism. Taken together, the oxidative stress induced by A β (17-42) in synaptosomes in this current study is similar to the oxidative stress induced by A β (1-42) found in AD brain⁹.

Our present findings, suggest the role of A β (17-42) as one of the contributing factors in mediating oxidative stress in DS and AD brain leading to neurodegeneration. Protein oxidation observed in our study suggests that oxidative stress may be an early event in the progression of neurodegenerative diseases. The loss of enzyme activity by oxidative modification may contribute to abnormal energy production found in many neurodegenerative disorders. Furthermore, these findings support the role of A β (17-42) as a mediator of oxidative stress and a causative agent in the pathogenesis of Down syndrome and Alzheimer's disease.

Acknowledgements

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Experimental Section

All chemicals were of the highest purity and obtained from Sigma Aldrich (St. Louis, MO, USA). A β (17-42) peptide was obtained from AnaSpec (San Jose, CA, USA) with HPLC and MS purity verification. The peptide was stored at -20°C until used. The OxyBlot protein oxidation detection kit was obtained from Chemicon International (Temecula, CA, USA). SYPRO Ruby stain was obtained from Bio-Rad (Hercules, CA, USA).

Synaptosomes are isolated terminals of neurons, prepared by homogenization and fractionation of nerve tissue. First isolated by Hebb and Whittaker in 1958⁴¹, they were later identified by electron microscopy as detached synapses⁴². They contain all the components necessary to store, release, and retain neurotransmitters. Synaptosomes also contain mitochondria for ATP production and active energy metabolism⁴³. For our study, synaptosomes were isolated from the brain tissue of Mongolian gerbils. A total of twelve Mongolian gerbils (6 control and 6 experimental) were used in this experiment. These animals were used because their synaptosomes have been extensively characterized and employed in neuroscience⁹. Synaptosomes were used to study consequences of protein oxidation by incubating the experimental set A β (17-42) peptide (AnaSpec, San Jose, CA, USA). Amyloid beta peptide A β (17-42) was dissolved in phosphate buffered saline (PBS), to a final concentration of 0.5mg/mL and preincubated at 37°C for 24 h prior to incubation with synaptosomes. This is done to aggregate the A β peptide and increase its solubility. The experimental set of synaptosomal preparations was incubated with A β (17-42) for 6 h at 37°C to induce oxidative stress. Following approval of animal protocols by the University of Kentucky Animal and Use Committee, the animals were fed standard Purina rodent laboratory chow and housed in the University of Kentucky's Central Animal Facility in a 12 hour light/dark phase. Synaptosomes were isolated from three month old Mongolian gerbils at the University of Kentucky in their Division of Laboratory Animal Research Center. Briefly, the animals were anaesthetized and sacrificed. Their brains were immediately isolated and dissected following sacrifice. The brain was homogenized with a Wheaton tissue homogenizer placed in ice containing 0.32M sucrose isolation buffer (4 μ g/ml leupeptin (4 μ g/ml), pepstatin (5 μ g/ml), aprotinin (5 μ g/ml), 2mM ethylene glycol-bis(tetraacetic acid (EGTA), 2mM ethylenediaminetetraacetic acid (EDTA), 20mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 20 μ g/ml trypsin inhibitor, and 0.2mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4). The tissue was homogenized by 20 passes in the tissue homogenizer. The homogenate obtained was centrifuged at 1500 g for 10 minutes. The supernatant was retained and the pellet was discarded. The supernatant was then centrifuged at 20,000 g for 10 minutes. Subsequently, the pellet was retained and the supernatant was discarded. The pellet obtained was resuspended in 1 ml of 0.32M sucrose isolation buffer. Sucrose solutions (0.85M pH 8, 1.0M pH 8 and 1.18M pH 8.5) were prepared and layered in plastic centrifuge tubes using 18 gauge syringe needles carefully to form a discontinuous sucrose gradient. The resuspended pellet was layered over the sucrose gradient (0.85M pH 8, 1.0M pH 8 and 1.18M pH 8.5 sucrose solutions each having 2mM EDTA, 10mM HEPES, and 2mM EGTA) and centrifuged by using an ultracentrifuge at 82,000 g for 60 minutes at 4°C. Synaptosomes were collected from the sucrose interface of 1.0M/1.18M layer and washed in Locke's buffer (154mM sodium bicarbonate, 5mM glucose, 5mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4) twice for 10

min at 32,000 g. The synaptosomes obtained were assayed to determine protein concentration by using Pierce Bicinchoninic acid assay method¹⁴.

Briefly, 150 μ g of the protein sample was precipitated by adding 100% cold trichloroacetic acid (TCA) to achieve a final concentration of 15% TCA and was placed on ice for 10 minutes. The precipitated proteins were centrifuged at 4000 g for 2 min at 4°C. The resulting protein pellets were washed three times with 1ml of 1:1 (v/v) ethanol: ethyl acetate solution. The protein samples were dissolved in 200 μ L of rehydration buffer (8M urea, 20mM dithiothreitol, 2M thiourea, 2.0% (w/v) CHAPS, 0.2% Biolytes and bromophenol blue). The proteins were separated based on their isoelectric point during isoelectric focusing. This was performed by using an IEF cell and 110-mm pH 3-10 immobilized pH gradients (IPG) strips both obtained from Bio-Rad (Hercules, CA, USA). 200 μ L of sample solution was added to each 110-mm IPG strip and constant voltage (50V) was applied for 1 h. Two milliliters of mineral oil was added to the top of each strip to prevent the evaporation. The IPG strips were then actively rehydrated for 16 hours at 50V. Isoelectric focusing of proteins was performed at 20°C under the following conditions: 300V for 2 h linearly, 500V for 2 h linearly, 1000V for 2 h linearly, 8000V for 8 h linearly, and 8000V for 10 h rapidly. The strips were then stored at -80°C until future use.

Two dimensional (2D) gel electrophoresis separates a mixture of proteins into single easily detectable protein spots based on isoelectric point and molecular migration (M_r). The 2D gel map helps to compare and match different sets of samples in order to identify isoforms, mutants, and post-translationally modified proteins for statistical analysis. This technique gives high reproducibility and resolution. The proteins are first separated based on their isoelectric point (pI) in the first dimension and are separated based on their sizes in the second dimension. High molecular weight proteins travel slower and low molecular weight proteins travel faster during the second dimension of electrophoresis. As a result of separation of proteins on the 2D gel, each individual spot on the gel represents a unique protein.

In SDS-PAGE, the gel strips obtained after isoelectric focusing were allowed to thaw. The thawed strips were then equilibrated for 10 minutes in equilibration buffer (50mM Tris-HCl (pH 6.8) containing 6M urea, 0.5% dithiothreitol (DTT), 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol). After 10 minutes, they were re-equilibrated in the same buffer for 10 minutes but DTT was replaced by 4.5% iodoacetamide (IA). For 2D gel electrophoresis, 8-16% precast Criterion Tris-HCl gels were used (Bio-Rad, Hercules, CA, USA). Control and A β (17-42) strips were placed on 8-16% precast Criterion gels and in turn placed in the 2D gel electrophoresis gel apparatus (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed for 65 min at 200V.

Following second dimension electrophoresis, the gels were incubated with fixing solution (10% (v/v) methanol and 7% (v/v) acetic acid) for 20 minutes. The gels were then stained with 50mL of SYPRO Ruby gel stain (Bio-Rad, Hercules, CA, USA) for two hours on a slow continuous rocker. After staining, the gels were placed in 70mL distilled water overnight for destaining.

Following SYPRO ruby staining, each 2D gel was placed under a UV transilluminator (EDVOTEK, Bethesda, MD, USA) to visualize different protein spots. The UV transilluminator (λ_{ex} =470nm, λ_{em} =618 nm) captures the fluorescent gel image with a built in digital camera. Gels were stored in distilled water at 4°C until spot excision for in-gel trypsin digestion. The images were analyzed for significant differences between the images in terms of protein spots by using PDQuest 2-D image analysis software (Bio-Rad, Hercules, CA,

USA). This software matches and analyzes visualized protein spots among different gels and compares protein intensity between control and experimental gel images. It has powerful, automatching algorithms that identify and accurately match gel protein spots in terms of their intensity and determines if the spots are significantly different. After the images were analyzed and matched by the PDQuest software, the normalized intensity of each protein spot from individual gels was compared between groups for statistical analysis using Student t-test. P values of less than 0.05 were considered to be significant. For each data set, the standard error of mean (SEM) was analyzed as well.

For mass spectrometric analysis, the significant protein spots were excised from the gels by the method described by Thongboonkerd⁴⁴. Briefly, the significant protein spots from the 2D gels were excised by using a clean sharp razor blade and were transferred into new clean microcentrifuge tubes. The gel pieces were incubated with 0.1M ammonium bicarbonate (NH_4HCO_3) for 15 minutes at room temperature. To the same tube, acetonitrile was added to the gel pieces containing ammonium bicarbonate and incubated at room temperature for 15 minutes. Ammonium bicarbonate and acetonitrile solvents were removed after 15 minutes and gel pieces were allowed to dry in laminar flow hood for 30 minutes. The gel pieces were rehydrated with 20 μL of 20mM dithiothreitol (Bio-Rad, Hercules, CA, USA) prepared in 0.1M NH_4HCO_3 and incubated at 56°C for 45 minutes. The DTT solution was removed and 20 μL of 55mM iodoacetamide (Bio-Rad, Hercules, CA, USA) prepared in 0.1M NH_4HCO_3 was added to the gel pieces and incubated for 30 minutes in the dark at room temperature. The iodoacetamide solution was removed and replaced with 200 μL 50mM NH_4HCO_3 and incubated for 15 minutes at room temperature. To the same tube, 200 μL of acetonitrile was added and incubated at room temperature for 15 minutes. The mixture of ammonium bicarbonate and acetonitrile solutions were removed from the tube and the gels were allowed to dry in a laminar flow hood for 30 minutes. Following drying in the laminar flow, the gel pieces were rehydrated with 20ng/ μL of modified trypsin (Promega, Madison, WI, USA) in 50mM NH_4HCO_3 with the minimal volume to cover the gel pieces and allowed to incubate overnight (~18 hours) with shaking at 37°C.

Four buffers: A (5% acetonitrile, 0.1% formic acid), B (95% acetonitrile, 0.1% formic acid), C (100 % acetonitrile), and D (50% acetonitrile, 0.1% formic acid) were prepared. The protein digest from the gel pieces was obtained after 18 hours of incubation following shaking. The digest was removed and sixty microliters of buffer A was added to the gel pieces until the gel piece was submerged in the buffer. The tubes were then sonicated in a waterbath for 15 minutes at room temperature. Ninety microliters of buffer B was added to the tubes and was allowed to sonicate for 15 minutes. The resulting supernatant obtained was mixed with the original peptide extract present in the already labeled microcentrifuge tubes. The tubes were centrifuged by using a high speed vacuum centrifuge until the peptide extract was concentrated to a volume of 10 μL .

A Supelco Zip tip consists of a 10 μL pipette tip with a micro-volume bed of chromatography media fixed at its end. Attached peptides are transferred from the chromatographic column into new set of microcentrifuge tubes for mass spectrometric analysis. Ten microliters of buffer C was drawn up into the Zip tip (Millipore Corporation, Billerica, MA, USA) and emptied to waste five times. The same Zip tip was reequilibrated with 10 μL of buffer A five times and discarded. The above Zip tip was used to draw up and gently expel the peptide

extract present in the microcentrifuge tubes. This aspiration and release of sample was done for ten times in the microcentrifuge tubes. The sample was washed in the Zip tip with buffer A five times by drawing up 10 μ L of buffer A and aspirating it. Finally, 10 μ L of buffer D was drawn with the same Zip tip and the resulting solution was transferred into a new labeled microcentrifuge tube. The eluent was drawn and gently expelled several times to remove the sample from the Zip tip column completely. The tubes containing the peptides were sent to the University of Louisville's Core Mass Spectrometry Facility for mass spectrometric analysis and protein identification.

All mass spectra were recorded at the University of Louisville Core Mass Spectrometry Facility on a LTQ Orbitrap XL mass spectrometer connected with a nanospray nanomate ionization source. The LTQ-Orbitrap combines high-resolution, high mass accuracy, and high sensitivity in a compact and robust instrument⁴⁵. Nanoelectrospray (nanoESI) technology offers the ability of increased sensitivity and lower sample consumption compare to that of conventional electrospray ESI. A nanoESI chip-based system, the NanoMate contains an array of 100 individual ESI nozzles on a single chip and a robotic arm which is capable of delivering the samples from a 96-well plate⁴⁶. The major advantages of this system include multiple-sample capability, low sample consumption, no sample carryover, and high sample throughput.

Tryptic peptides were analyzed with an automated nanospray Nanomate Orbitrap XL MS/MS platform. The Orbitrap MS was operated by trained technicians in a data-dependent mode whereby the 8 most intense parent ions measured in the FT at 60,000 resolution were selected for ion trap fragmentation with the following conditions: injection time 50 ms, 35% collision energy, MS/MS spectra were measured in the FT at 7500 resolution, and dynamic exclusion was set for 120 seconds. Each sample was acquired for a total of ~2.5 minutes. MS/MS spectra were searched against the ipi_Rodent. Database using SEQUEST with the following criteria: Xcorr > 1.5, 2.0, 2.5, 3.0 for +1, +2, +3, and +4 charge states, respectively, and P-value (protein and peptide) < 0.01. IPI accession numbers were cross-correlated with SwissProt accession numbers for the final protein identification.

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