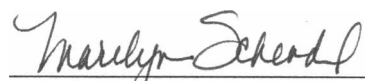


Differential Expression of Mitochondrial Proteins in Alzheimer's Disease Patients
Compared to Age-Matched Controls

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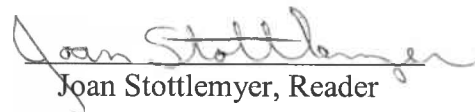
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Abstract

To date, the pathology of Alzheimer's remains unknown, and many researchers have focused on the mitochondria as playing a significant role in its progression. In earlier studies, it was found that mRNA levels of mitochondrial proteins of Alzheimer's Disease patients were altered in mouse and human models. Additional research is warranted to determine if the mRNA differences are translated to the protein level. It has been shown that mitochondrial genes are differentially expressed in Alzheimer's patients when compared to age-matched controls. I propose the levels of the translated gene product will also be altered. To test this hypothesis a proteomic approach utilizing 2-D electrophoresis to separate mitochondrial proteins and compare expression between diseased and control samples was used. These results are preliminary and further trials will cement the results.

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Introduction

Typical aging and Alzheimer's Disease share a connection, but both are independent occurrences. Development of Alzheimer's Disease does not go hand-in-hand with old age; however, age can be considered a risk factor for this disease. It has been hypothesized that mitochondria play an integral role in the aging process. The brain depends on the production of ATP for energy, and the main source of production of ATP depends on the metabolic pathways requiring oxygen found in the mitochondria. Because the brain depends heavily on aerobic metabolism, the normal function of the mitochondria are thought to play an integral role in proper functioning of the brain cell (Ojaimi *et al.*, 2001).

Research on Alzheimer's Disease (AD) has primarily focused on the pathogenesis of the accumulation of extracellular amyloid precursor protein and the intracellular neurofibrillary tangle (Smith *et al.*, 2000). Consequently, other hypotheses concerning Alzheimer's have received less attention, resulting in a lack of knowledge about the mechanism of AD. Pathogenic studies are difficult, if not impossible, to carry out because brain samples from AD patients are available only after a person passes away (Selkoe, 2001). Because the progression of the disease is of importance, researchers turn to murine models to study the stages of AD. Despite these setbacks, recent research points to the significance of oxidative imbalance in AD. In a healthy cell, antioxidants work to keep the oxygen radicals formed by metabolic processes in balance. (Smith *et al.*, 2000).

Mitochondria have their own set of DNA which contributes 13 components to the respiratory chain. The oxidative stress hypothesis proposes that unknown factors allow for and perhaps even favor the formation of reactive oxygen species that cause damage to

nucleic acids and proteins found in the mitochondria, some in the respiratory chain (Bonilla *et al.*, 1999). Smith *et al* (2000) showed that evidence exists for the hypothesis that oxidative stress plays an important role in the pathogenesis of AD. It has been proposed that the pathological hallmarks associated with AD, neurofibrillary tangles, and amyloid plaques, may actually serve a defensive role in response to oxidative damage, and it has also been observed that neurons with these hallmarks actually display reduced oxidative damage (Perry *et al.*, 2000).

Data suggest that irregularities of cerebral metabolism are present in many neurodegenerative diseases such as AD. These irregularities have been attributed to modifications in mitochondrial enzymes. Much evidence has accumulated over the years indicating that AD patients experience an inhibited rate of cerebral metabolism. This occurrence precedes the stage in which the neuropsychological or neuroanatomic characteristics of the disease are observed. A study completed by Hoyer and colleagues noted that changes in the pattern of the substrates metabolized in the brain go hand-in-hand with the initial stages and signs of AD (Gibson *et al.*, 1998). Castegna *et al* (2002) showed that three enzymes important in cellular metabolism, collapsing mediator protein 2, heat shock cognate 71, and α -enolase, when oxidized as a result of free-radical alteration, play a significant role in AD and therefore suggest that oxidation of proteins serves a role in the mechanism of neurodegeneration in AD. The impaired brain function of AD patients can be attributed to the lessened metabolism (Gibson *et al.*, 1998).

Mitochondrial mutations, which are inherited maternally, have been linked to many human diseases including Alzheimer's Disease, and there is increasing evidence suggesting that altered or impaired mitochondria play a role in the progression of AD

(Bonilla *et al.*, 1999). Recent studies have shown that many neurodegenerative diseases have been associated with mitochondrial gene mutations that manifest in mtDNA or nDNA (that are responsible for coding the proteins necessary for mitochondria function (Wallace, 2001)). Recent evidence provides support for the hypothesis that a mutation in a gene responsible for synthesizing a mitochondrial protein may contribute to the development of AD in some patients (Gibson *et al.*, 1998). Mutations involving deletions have been found accumulated in specific regions of the brain, such as the cerebral cortex, from aging patients (Bonilla *et al.*, 1999).

The pathophysiology of AD has been linked to impaired function of the proteins associated with the electron transport system found in mitochondria. Hirai *et al.* (2001) found mitochondrial abnormalities in the neurons of AD patients in the form of increased levels of mitochondrial DNA and protein in lipofuscin, a vacuole that has been associated with the breakdown of mitochondria. These findings suggest that the neurons involved in AD have more degradation products indicating either an increased turnover of mitochondria by autophagy or a decrease of proteolytic turnover which would cause mitochondrial DNA and proteins to build up. (Hirai *et al.*, 2001).

It has been shown that reactive oxygen species modify proteins and decrease their function to a greater extent in AD patients compared to controls (Castegna *et al.*, 2002). Their studies have shown that the oxidation of brain proteins is evident in AD patients and these studies propose that these altered proteins play an integral role in impairing metabolism and the process of neuronal defects. Researchers have found increased protein carbonyl levels, an indicator of protein oxidation, in more than one region in the brains of AD patients (Castegna *et al.*, 2002). Oxidative damage results

when the balance of reactive oxygen species to anti-oxidant defenses is altered and the production of free radicals surpasses the amount of anti-oxidant present (Smith *et al.*, 2000). This type of damage to proteins impacts cells by impairing protein function, depleting the cellular redox balance, interrupting the cell cycle, and ultimately causing death (Castegna *et al.*, 2002).

Reddy (2003) showed that mRNA levels of mitochondria genes were altered in AD patients when compared to age-matched controls. In this study, I hypothesized that mitochondrial proteins are differentially expressed in Alzheimer's patients compared to their age-matched controls. Mitochondria isolated from areas of the brain known to show the pathological signs of AD were selected from diseased patients and from control brain specimens and were compared. The mitochondrial proteins were then subjected to 2D electrophoresis for analysis.

Materials and Methods

Isolation of Mitochondrial Proteins. Mitochondria were prepared from human and murine brain tissue (150-220 mg) and stored at -80°C. Cells were lysed using a Teflon-glass homogenizer and 5 ml homogenization buffer (0.5 µg/mL pepstatin, 0.5 µg/mL leupeptin, 1 mM PMSF, 1 mM EDTA, 250 mM sucrose, 1 mM EGTA, 10 mM HEPES/NAOH pH 7.4. The 5 ml homogenate was centrifuged at 1500g for 10 min and this process of homogenization and centrifugation was repeated twice more. The supernatants were collected, pooled, and centrifuged for 15 min at 9000g to pellet mitochondria. For larger amounts of tissue, subsequently more centrifugation was necessary. The mitochondrial pellet was resuspended in 5 mL wash buffer (0.5 µg/mL pepstatin 0.5 µg/mL leupeptin, 1mM PMSF, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 10 mMTris/HCl, pH 7.5) and centrifuged again at 9,000g. The pellet was resuspended in 200-400 µL wash buffer and stored at -80°C until protein assays could be performed.

BCA Protein Assay. For sample quantification, a standard curve was prepared following the instructions given in Pierce's BCA Protein Assay Kit. The mitochondrial wash buffer was used as the diluent. Mitochondrial and nuclear proteins were prepared in an approximate 10:1 dilution in the wash buffer from isolation and compared to a standard curve of known concentration for sample quantitation.

2D Gel Electrophoresis. Mitochondrial proteins (200 μ g-1.5 mg) were suspended in rehydration buffer (8M urea, 2% CHAPS, 50 mM DTT, 0.2% ampholytes), and loaded onto Biorad's 11 cm Ready Strip. The strips were placed into trays and actively rehydrated overnight for 16 hours at 50 V in the PROTEAN IEF Cell. The first dimension was run according to conditions provided by Biorad (<http://www.proteomeworks.bio-rad.com>). After the first dimension, the strips were equilibrated in Biorad's Equilibration Buffer I (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT) for 10 min on an orbital shaker. Strips were moved to another lane in the casting tray and covered with Biorad's Equilibration Buffer II (6M urea, 0.375M Tris, pH 8.8, 2% SDS, 20 % glycerol, 2.5 % (w/v) iodoacetamide) for 10 min on an orbital shaker. Strips were then dipped in 1x SDS running buffer. For the second dimension, the Ready Strip IPG strip was loaded onto a Criterion gel from Biorad with 0.5 % agarose overlay and electrophoresed for 30 minutes at 200V. Gels were removed from casting trays and set to stain using silver stain (<http://www.proteomeworks.bio-rad.com>).

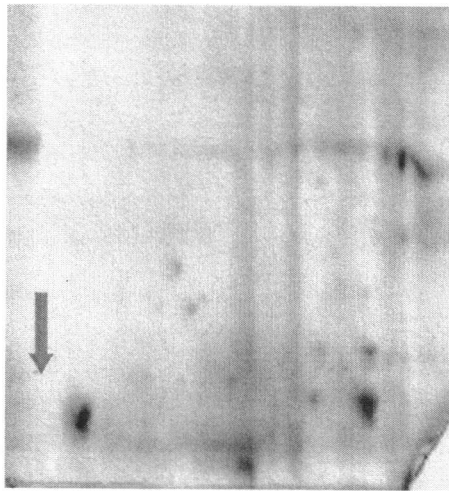
Staining. Gels were stained according to the procedure provided by Biorad (<http://www.proteomeworks.bio-rad.com>). After second dimension electrophoresis, gels were placed in the Fixative Enhancer Solution (Reagent grade methanol (50% v/v), reagent grade acetic acid (10% v/v), Fixative enhancer concentrate (10% v/v), and deionized distilled water (30% v/v)) for 20 min. This solution was decanted and the gel was gently rinsed in 400 mL H₂O for 10 min on an orbital shaker and again for another 10 min with fresh H₂O. To stain and develop the gel, 35 mL H₂O were mixed in the following order with 5 mL Complex Solution, 5 mL Reduction Moderator Solution, and

5 mL Image Development Solution. Immediately before applying to the gel, 50 mL of the room temperature Development Accelerator Solution were added, and the gel was placed to stain in a container rinsed with nitric acid with gentle agitation on an orbital shaker. After 10 min the reaction was stopped with a 5% acetic acid solution. The gel was then rinsed in double distilled water for 5 min. In later experiments the pellet was subjected to a Biorad protein purification kit (<http://www.proteomeworks.bio-rad.com>

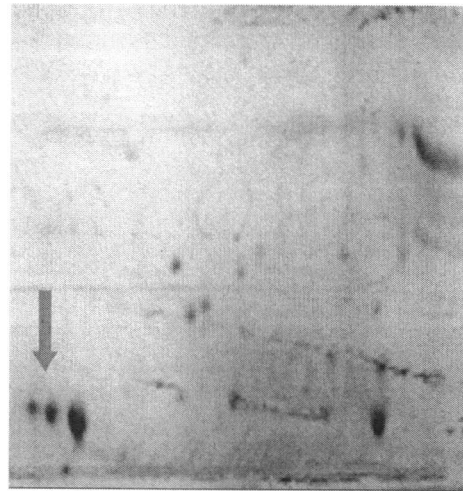
Results

2-D Gel Electrophoresis was performed on the brain mitochondrial isolates of Alzheimer's Disease (AD) patients and their age-matched controls. Figure 1 shows isolated mitochondrial proteins run on a second dimension gel without being subjected to a purification kit. The arrow shows the absence of two proteins in the control sample, but present in the mitochondrial sample.

In the later experiments, the mitochondrial isolate went through a purification kit to increase resolution and clean some proteins from the membranes. Figure 2 shows two purified samples run on 2D gels. In figure 2, these three landmark proteins are present in both the control and AD sample after they have been run through a purification kit. In addition to the three landmark proteins, other spots of protein differ in their intensities. The darker arrows in the upper left-hand corner show a more intense signal in the control sample. Equal amounts of protein were loaded of each sample, and differences of intensity are evident when comparing proteins of the control to AD patients.

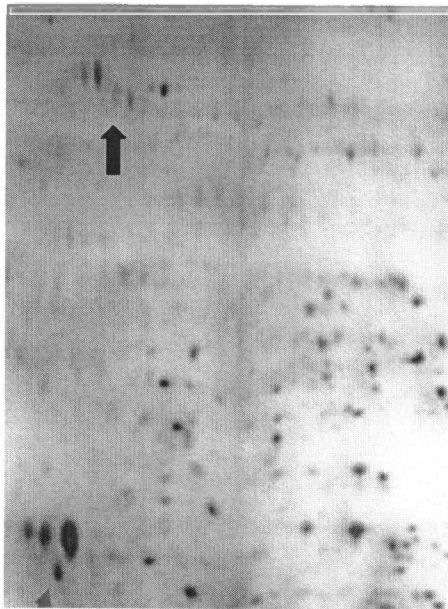


1.6 mg control mitochondrial protein

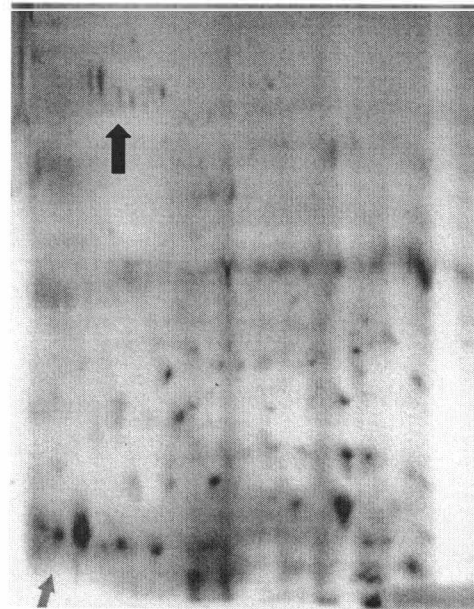


1.6 mg AD mitochondrial protein

Figure 1. Silver Staining of 2D gel without purification. These gels compare proteins isolated from equal amounts of brain tissue of control and Alzheimer's Disease patients run on a 2-D gel without purification. The arrow shows where two proteins are absent in the control model. The intensity of other proteins is also evident.



1.6 mg control mitochondrial protein



1.6 mitochondrial AD protein

Figure 2. Silver Staining of 2D gel with purification kit. These gels compare equal amounts of brain tissue isolated from control and Alzheimer's Disease patients after being run through a purification kit. The increased resolution allows one to compare the intensities (amount) of the proteins present.

Discussion

The purpose of this study was to test if the mitochondrial proteins in Alzheimer's Disease patients are altered compared to age-matched controls. In the first trial, two of the three landmark proteins, located in the lower left hand corner, are absent in the control sample when compared to the sample taken from an AD patient. We then hypothesized that these proteins were altered (upregulated) in AD patients. These spots, however, appear in both the AD and control samples after being run through a purification kit. The first trial, therefore, perhaps had an error leading to the degradation of these proteins or failure to isolate them. The three landmark spots absent in the first mitochondrial sample may not have absorbed into the first dimension if they are membrane proteins because it has been noted that membrane proteins are difficult to solubilize in the first dimension. The 2D gel approach poses problems when working with membrane proteins, such as many in the electron transport chain proteins in the mitochondria, because many are not observed due to problems with precipitation and absorption (Taylor *et al.*, 2002).

In addition to the absence or presence of protein spots, differing intensities also indicate that a protein is altered in one sample compared to another. In Figure 2, several spots are shown to have differing intensities. The arrows in the upper right-hand corner of both the control and AD sample show the differences in intensity. The proteins appear to be more intense in the control sample. Figure 2 represents the culmination of our efforts. These are the clearest gels obtained from our trials, but further consistent studies to confirm this data are needed as well as identification of the proteins in question by mass spectrometry.

It has been shown that mRNA levels are altered in Alzheimer's Disease patients compared to age-matched controls by microchip array analysis (Reddy, 2003). Our observations of differing protein intensities in 2-D profiles suggests that like the mRNA, the protein products are altered as well.

To date, much evidence has been gathered supporting the hypothesis that mitochondria are somehow involved in Alzheimer's Disease. More research, however, is needed to strengthen this theory. Our results are preliminary and must be analyzed and repeated in order to conclude that the differences in mRNA are carried through to the protein level.

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