PROTEOMICS ANALYSIS IN ALZHEIMER'S DISEASE: NEW INSIGHTS INTO MECHANISMS OF NEURODEGENERATION

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I. Introduction

Alzheimer's disease (AD) is a progressive, age-related neurodegenerative disorder associated with cognitive decline and aging that is estimated to affect more than 5 million Americans and more than 15 million people worldwide (Katzman and Saitoh, 1991). Oxidative stress is associated with the pathogenesis of the disease (Marksberry, 1997). Protein carbonyl formation, lipid peroxidation, 3-nitrotyrosine (3-NT), and DNA oxidation are among the oxidative stress markers reported for AD (Butterfield, 2002; Butterfield and Lauderback, 2002; Castegna et al., 2003; Lovell et al., 2001; Smith et al., 1997).

Amyloid β-peptide (1-42) (Aβ[1-42]) has been implicated as a causative agent in AD. Evidence supporting a central role for Aβ(1-42) in the pathogenesis of AD has been provided by the genetic mutations resulting in overexpression of this peptide in cases of familial Alzheimer's disease (FAD). For example, mutations of presenilin-1 (PS1), presenilin-2 (PS2), and amyloid precursor protein (APP) result in overexpression of Aβ(1-42) and subsequent inheritance of AD. APP, the transmembrane glycoprotein from which Aβ(1-42) is proteolytically cleaved, is
encoded by chromosome 21. Consequently, individuals with Down syndrome, or trisomy 21, who have three copies of chromosome 21, typically have an increased load of Aβ(1-42) and will develop AD if they live long enough. Likewise, in rodent models of AD, overexpression of human APP and tau lead to the development of plaques prior to neurofibrillary deposits (Selkoe, 2001a,b; Selkoe and Podlisny, 2002). Aβ(1-42) induces protein carbonylation, lipid peroxidation, and formation of 3-NT (Butterfield, 2003; Butterfield and Lauderback, 2002; Butterfield et al., 2002). Such Aβ(1-42)-induced oxidative stress is inhibited by antioxidants (Yatin et al., 2000). Taken together, these findings suggest that Aβ(1-42) plays a primary role in the oxidative damage evident in AD.

Consistent with this notion, protein oxidation in AD occurs in Aβ-rich brain regions such as the hippocampus and cortex, but not in Aβ-poor regions such as the cerebellum (Hensley et al., 1995). Until recently the ability to determine which proteins were specifically oxidized has been limited to immunoprecipitation techniques, which requires an educated guess as to the identity of the protein and readily available antibodies. These procedures are lengthy and laborious. Fortunately the coupling of two-dimensional gel electrophoresis and improvement of mass spectrometry (MS) techniques has allowed for the rapid screening and identification of proteins.

In this review we discuss the tools that have made proteomics possible, as well as their application to AD. Results thus far have offered invaluable insight into the pathogenesis of the disease. The implications of these results with respect to the pathological and biochemical abnormalities that have been reported for AD will be discussed, as well as our opinion of the future direction of proteomics studies in AD.

II. Proteomics Tools

A. Two-Dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of biological samples involves the separation of proteins based on their properties of isoelectric point and size. The first step in the technique is isoelectric focusing (IEF), in which the proteins are focused within a pH gradient by an applied electric field to their isoelectric point, or the point at which the net charge on the protein is zero. The second step is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in which the proteins are further purified based on their migration within an applied electric field according to molecular size. The result is a two-dimensional map in which there exists a high probability that each individual spot represents an individual protein. Ultimately, the two-dimensional map provides
an expression profile of the proteins present in a given sample. Computer-assisted
collection of such profiles, or maps, allows for the determination of differences in
the expression of proteins between different states (i.e., diseased versus control).
Additionally, 2D-PAGE maps have enabled development of databases, which
catalog the proteins (Gauss et al., 1999). One of the advantages of 2D-PAGE is the
ability to separate a large number of proteins from a given sample at one time.
This allows for the screening of thousands of proteins at once and provides
information on post-translational modifications, which result in changes in total
protein charge (i.e., shift in the position of the protein spot on the gel).

Several techniques have been developed to improve and expand on the
capabilities of 2D-PAGE, resulting in the evolution of a reliable and reproducible
method. The introduction of immobilized pH gradient (IPG) strips to replace
the tube gels with ampholytes has eliminated “cathodic drift” during IEF, resulting
in an increase in reproducibility between samples (Molloy, 2000). Additionally,
the development of narrow pH ranges (e.g., 4–7, 5–8) within the IPG strips has
allowed for the separation of proteins that are close in isoelectric point and may
not have resolved well on the more traditional 3–10 broad pH range strips.
Solubilization of proteins has been the so-called stumbling block of proteomics.
Because IEF does not allow induction of charge, which can interfere with the
focusing process, ionic detergents such as SDS cannot be used to solubilize
lipidated proteins (e.g., transmembrane proteins). The use of SDS has been
reported, but the samples must be dialyzed before IEF. This produces a further
limitation when working with precious biological samples that can potentially be
lost in the process. As a result, chaotropic agents such as urea and thiourea
(Rabilloud, 1998), coupled with nonionic or zwitterionic detergents such as
CHAPS, have been used to prevent the precipitation of proteins during IEF
and SDS-PAGE (Herbert, 1999). Tributyl phosphine also has been used as a
reducing agent in place of dithiothreitol (Herbert et al., 1998). Regardless of the
improvements that have been made in the methods of 2D-PAGE, several
obstacles and limitations remain. Solubilization techniques are still mainly limited to
cytoplasmic proteins, resulting in difficulties in obtaining gel maps of membrane
proteins and lipidated proteins. Maps of membrane proteins have been reported
(Friso and Wikstrom, 1999; Molloy et al., 1998; Pasquali et al., 1997; Santoni et al.,
2000); however, further investigation is necessary in order to develop a method
that is reliable and reproducible. Additional limitations include the resolution of
very basic proteins, although IPG strips have recently been developed to address
this issue (Hoving et al., 2002). Finally, low-abundance proteins are typically
undetectable on a gel map and thus remain elusive. This is particularly important
when low-abundance proteins may play a role in the pathogenesis of a disease
but are virtually silent.

The proteomics techniques of our laboratory have revolved around the
identification of specifically, oxidatively modified proteins (Butterfield, 2004;
Butterfield and Castegna, 2003a,b,c; Castegna et al., 2002a,b, 2003, 2004). We used a parallel analysis in which the 2D gel map is coupled with immunochemical detection of protein carbonyls derivatized by 2,4-dinitrophenylhydrazine (DNPH), followed by MS analysis to identify proteins of particular interest. Immunochemical detection of protein carbonyls is carried out by transfer of DNPH-derivatized protein from the gel to a membrane, which is developed with specific antibodies to create a 2D oxyblot (Fig. 1). The 2D oxyblots and 2D gel maps are matched, and the immunoreactivity is normalized to the actual protein content as measured by the intensity of a protein stain such as colloidal Coomassie blue or SYPRO ruby. Such parallel analysis allows the comparison of oxidation levels of individual brain proteins in AD versus control.

B. NEW TECHNIQUES/NONTRADITIONAL PROTEOMICS

Alternative techniques that do not use 2D-PAGE have also been developed (Ferguson and Smith, 2003). These methods reflect the growing technology that addresses and circumvents the disadvantages and obstacles present in the traditional 2D-PAGE method associated with proteomics analyses. It should be noted, however, that no single technique is ideal and the methodology of proteomics is constantly expanding and evolving.
1. **2D-HPLC**

High-performance liquid chromatography (HPLC) has been used to separate the peptides produced from the tryptic digestion of a protein solution. To accomplish separation of complex peptide mixtures for mass spectrometry analysis, a series of columns that separate the peptides based on different chemical and physical properties is used (Wang and Hanash, 2003). This technique is referred to as 2D-HPLC and commonly utilizes a strong cation-exchange (SCX) column, coupled with a reverse-phase (RP) column (Stevens et al., 2003; Wagner et al., 2003). HPLC is typically tied to a nanoelectrospray inlet mass spectrometer. This technique has also been referred to as multidimensional protein identification technology (MudPIT) (Washburn et al., 2001). An ion exchange column and a size-exclusion column can be used to separate peptides based on the same principles as 2D-PAGE (Issaq et al., 2002).

2. **Isotope-Coded Affinity Tags**

The technique referred to as isotope-coded affinity tags (ICAT) is one that circumvents the challenges and shortcomings of 2D-PAGE, such as problems with solubility of membrane proteins and proteins that fall outside of the \( pI \) and molecular weight range commonly used. ICAT uses tags that consist of three functional moieties, including a cysteine reactive site, a linker containing either 8 hydrogen atoms or 8 deuterium atoms, and a biotin affinity tag. The cysteine reactive moiety forms a chemical bond to the reduced cysteine residue of the protein, while the biotin label allows for the isolation of the isotopically tagged peptide fragment following separation on an avidin affinity column. In this manner the isotopic label allows for quantitation of the spectra generated by MS analysis between two different cellular states by a difference of 8 Da (Gygi et al., 1999). Although ICAT provides a variety of advantages over the traditional methods of protein separation and mapping based on 2D-PAGE, these advantages are accompanied by a variety of disadvantages, including the fact that the method only tags proteins with cysteine residues that are available for reaction with the isotope-coded affinity tag (Moseley, 2001).

3. **Protein Phosphorylation**

Protein phosphorylation is an important post-translational modification involved in cellular signaling and regulation (Butterfield and Stadtman, 1997). Consequently, the phosphorylation state of proteins under specific cellular conditions is of great interest; however, such studies typically involve immunoprecipitation and probing with specific phosphoprotein antibodies. Proteomics has provided the basic tools for high-throughput screening of phosphoproteins. In spite of this advance, screening of 2D gel maps transferred to membranes with antibodies has proved unsuccessful because of the lack of specific
phosphoantibodies (e.g., antibodies to phosphotyrosine, phosphothreonine, and phosphoserine). As a result, a number of methods have been used to detect differences in phosphorylated proteins. In cell culture $^{32}$P or $^{33}$P, radiolabeling can be used to visualize phosphorylated protein separated in a 2D gel. This method has been utilized in conjunction with desalting to concentrate and purify the peptides generated from in-gel digestion of phosphoproteins with various proteases, resulting in an increase in identification of phosphorylated proteins (Larsen et al., 2001). Phosphospecific gel stains (Steinberg et al., 2003) have also been developed for 2D-PAGE to be used in conjunction with SYPRO ruby protein stain (Berggren et al., 2002), allowing detection of phosphoproteins and total expression in the same gel. Additionally, enzymatic dephosphorylation of the phosphopeptides with alkaline phosphatase was coupled with peptide mass mapping to determine the amino acid site of phosphorylation (Larsen et al., 2001).

An alternative method was developed independently by two different groups (Goshe et al., 2001; Oda et al., 2001), which involved the $\beta$-elimination of the phosphate moiety from phosphothreonine and phosphoserine residues, followed by Michael addition of 1,2-ethanediol (EDT), biotinylation, and separation on an avidin affinity column. This method did not prove ideal because of problems with isolating the peptides based on the biotin/avidin affinity. A modification of the Goshe et al. (2001) study was proposed by Qian et al. (2003). The method, referred to as phosphoprotein isotope-coded solid-phase tag (PhIST), varies from the previously reported phosphoprotein isotope-coded affinity tag (PhIAT) by reaction of the EDT thiolate moiety with either light (six $^{12}$C and one $^{13}$N) or heavy (six $^{13}$C and one $^{15}$N) leucine isotope-coded beads rather than a biotin affinity tag. The captured peptides are released from the beads by photocleavage of the linker from the solid support, resulting in transfer of the leucine isotope-coded linker to the EDT-modified peptides, thereby generating the PhIST peptides. The mass of serine and threonine residues is increased by 246.08 Da or 253.10 Da for the light and heavy tags, respectively. The improved method boasts approximately 80% recovery and requires considerably less protein ($<100$ $\mu$g) (Qian et al., 2003).

Another method for the detection and mapping of protein phosphorylation sites was recently reported (Knight et al., 2003). This method uses chemical modification of the site of phosphorylation to specifically target the site for proteolytic cleavage. Such a technique would result in hydrolysis of the amide bond adjacent to the site of phosphorylation, allowing detection of the site based on the cleavage of the peptide rather than the sequence itself, because the phosphoaminoacid would always be the carboxy-terminal amino acid. The method is specific for phosphoserine and phosphothreonine and relies on $\beta$-elimination of the phosphate group to produce dehydroalanine and $\beta$-methyl-dehydroalanine, respectively, followed by reaction via Michael addition with cysteamine to generate aminoethylcysteine and $\beta$-methylaminoethylcysteine.
Aminoethylycysteine is isosteric to lysine; therefore proteases that cleave with respect to lysine residues will cleave proteins at the aminoethylycysteine residue and, consequently, at the site of phosphorylation. Trypsin was found to cleave at the β-methylaminoethylycysteine residue less efficiently than at the aminoethylcysteine; however, the protease Lys-C was found to cleave both aminoethylycysteine and β-methylaminoethylycysteine efficiently (Knight et al., 2003). This method provides an efficient and effective means of identifying the specific site of protein phosphorylation that is often a limitation of current techniques; nevertheless, this method does not provide quantitative information regarding differences in the extent of phosphorylation of a protein, with the exception of the number of phosphorylated residues, and would need to be coupled to techniques such as 2D-PAGE and autoradiography.

4. Membrane Proteins: High pH and Proteinase K

Membrane proteins have been difficult to study because of their low solubility in the buffers typically used in 2D-PAGE. A method using high pH and proteinase K (hpPK) was recently described for the study of both membrane and soluble proteins (Wu et al., 2003). The high pH disrupts the membrane structures and favors the formation of membrane sheets, allowing for the cleavage of the hydrophilic domains of membrane proteins that are exposed by proteinase K without disrupting or solubilizing the lipid bilayer. These experimental conditions coupled with MudPIT allow for the cleavage and identification of integral membrane proteins (Wu et al., 2003).

C. Mass Spectrometry

1. MALDI and ESI LC-MS

Once protein spots of particular interest have been detected, the protein must be eluted from the gel to undergo MS analysis. Most often the spot(s) corresponding to the protein(s) of particular interest are excised from the gel and undergo a series of manipulations and chemical modifications of the protein in order to facilitate the cleavage of the protein by a protease into several peptides. This procedure, more commonly referred to as in-gel digestion, has several advantages, including higher recovery, because the protein is cleaved into many smaller peptides that result from sequence-specific proteolysis, which is an important means of identifying the protein of interest. These smaller peptides constitute mass fingerprints that are characteristic of a particular protein, and the molecular weight of each peptide is determined by MS. Peptide mass fingerprinting, as the process is known, requires the use of MS analysis to determine the experimental masses. Database searching, in which the experimental masses are compared and matched to protein-specific mass fingerprints generated by the
"in silico" "digestion" of proteins, is used to identify the protein of interest based on the quality of peptide matches (Aebersold and Goodlet, 2001).

Mass spectrometry is an invaluable tool for the identification and study of proteins without which proteomic analysis would be relatively unknown. Before the evolution of "softer" ionization techniques that prevent or lessen the extent of fragmentation of ions generated by polypeptides, proteins were identified by the use of specific antibodies, which requires a well-educated guess based on the knowledge of the molecular weight and pI of the protein of interest, or by Edman degradation and protein sequencing. These are both long and laborious processes, and Edman degradation also requires the use of database searching.

The two techniques most commonly used for MS analysis of proteins are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). MALDI requires the mixing of the analyte with a matrix (commonly α-cyano-4-hydroxycinnamic acid) and crystallization of the mixture on a plate that is subjected to a laser radiation. The matrix absorbs the energy from the laser and transfers the energy in the condensed phase to the analyte, generating a gas mixture in which the matrix carries the analyte. Ionization occurs in the gas phase between the matrix and the analyte, where bimolecular proton transfer, due to the acidic nature of the matrix, may occur by a mechanism that is not well described to transform peptides into detectable MH+ ions. ESI, on the other hand, involves the transport of ions from solution to the gas phase rather than ionization. Typically ESI is interfaced with HPLC, in which the sample moves through the capillary inlet into a vacuum and the MS. This requires the application of a large potential difference applied between the capillary and the MS instrument inlet. The high voltage applied to the capillary overcomes surface tension and results in the formation of a Taylor cone, a hallmark of the ESI process, due to a charged pair deconvolution, in which cations get pushed away and anions are held back within the capillary. This process generates droplets with an electrical double layer and a net positive charge. In this state the solvent continues to evaporate from the droplet until the droplet reaches the Rayleigh limit, at which point the coulombic repulsion exceeds the surface tension of the droplet. This situation results in droplet fission and, ultimately, one ion per droplet. This process requires low salt concentration and can often generate multiply charged ions.

Additional information can be obtained about a particular peptide by the use of tandem MS/MS, which requires the use of ESI technology. Tandem MS/MS allows the isolation and fragmentation of a specific ion. The fragments undergo further MS analysis to generate information about the sequence of the peptide. The isolation of a single ion is accomplished by scanning all of the ions that were generated from a sample, followed by the application of a wide range of frequencies, except for the resonating frequency of the ion of interest. This allows for the ion of interest to be retained in the trap. The ion is then fragmented and
analyzed to generate information about the sequence of the peptide aiding in identification and, possibly, information about modifications of the specific amino acids in the peptide (de Hoffmann, 1996; March, 1997).

2. **SELDI-TOF**

Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) is a novel approach to proteomics that couples the classical methods of chromatographic sample preparation with MS analysis. The SELDI technique consists of a chip, which has been modified on the surface, either chemically or biochemically, in such a way as to optimize the isolation of a particular group of proteins. Chemical modifications include making the surface hydrophobic, hydrophilic, anionic, cationic, or metallic, while biochemical modifications take advantage of antibody-antigen, receptor-ligand, and DNA-protein interactions (Merchant and Weinberger, 2000). Advantages of SELDI-TOF technology include the potential to screen biomarkers from a variety of complex samples, including blood, urine, serum, plasma, etc., which are typically difficult to work with under 2D-PAGE conditions because of the high abundance of albumin and immunoglobulins. Additionally, SELDI has detection limits at the attomolar level and thus requires a small sample volume. SELDI can also be used to detect post-translational modifications such as glycosylation and phosphorylation by screening for particular mass shifts in the protein peaks; however, the technology depends on a change in the expression profiles between samples and is limited because the technique cannot be used to specifically identify a protein that is differentially expressed. Consequently, isolation of the protein itself is required (Issaq et al., 2002). Moreover, SELDI is limited to detection of proteins of relatively low molecular mass (Issaq et al., 2002).

**D. Database Searching**

Informatics is the final, and perhaps, most critical stage of the protein identification process. Once the list of peptide masses has been generated by MS analysis, it is necessary to utilize various search algorithms of various available databases to compare and match the peptide masses with those of proteins in the database (Butterfield et al., 2003). The search engines provide a theoretical digestion of the proteins contained within the database with which the peptide masses generated by MS can be compared. The search also takes into account a variety of factors, including protein size and the probability of a single peptide to occur within the database. The search engine provides a probability score for each entry based on the mathematical algorithms that are specific to the individual search engine used. The score corresponds to the probability the peptides match those of the theoretical digest of a particular protein contained in the database, and therefore the
TABLE I
Table of Mass Spectrometry Databases and Search Engines

<table>
<thead>
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<th>Address</th>
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</tbody>
</table>

correct identification of the protein. Any hit with a probability score corresponding to \( p<0.05 \), which can be set by the database, is generally considered to have a legitimate chance of being the protein cut from the gel, thereby allowing for the unambiguous identification of the protein of interest. Table I outlines some of the databases and search engines available online.

III. Proteomic Studies in AD

A. Protein Expression Alterations

Proteomics is an invaluable resource for the investigation of neurodegenerative disease and offers the potential to identify protein and modifications of proteins involved in disease processes. As a result, proteomics offers the capabilities to identify biomarkers of disease and potential therapeutic targets. Expression proteomics, in particular, has enabled researchers to compare and contrast various conditions due to its ability to quickly and reproducibly map, screen, quantitate, and identify a vast number of proteins at once. Such principles have been used to detect specific alterations in the protein expression levels of various regions of the AD brain compared to control brain, which may in turn facilitate an explanation of the mechanisms by which the disease progresses.

Extensive protein expression analysis has been reported by Lubec and coworkers. Table II summarizes the results. This group and others have contributed to the identification of a number of protein expression alterations in the AD brain, which offer substantial speculation into the possible mechanisms underlying the pathogenesis of the disease.

In addition to the presence of neurofibrillary tangles and senile plaques, AD is associated with synapse loss, oxidative stress, altered glucose metabolism,
<table>
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<tr>
<th>Protein</th>
<th>Tissue</th>
<th>Expression alteration</th>
<th>Reference</th>
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mitochondrial deficit, increased protein misfolding, and decreased protein turnover (Butterfield and Lauderback, 2002). The expression alterations observed in many proteins involved in a number of pathways help to develop testable hypotheses of neurodegenerative mechanisms in AD brain, as discussed in the following sections of this chapter.

1. Altered Energy Metabolism

The accumulation of glycolytic enzymes such as α- and γ-enolase and glyceraldehyde 3-phosphate dehydrogenase confirms the indication that glucose metabolism is affected in AD (Schonberger et al., 2001). Additionally, the decrease in the expression of voltage-dependent anion-selective channel protein-1 (VDAC-1), which is responsible for the regulation of mitochondrial metabolism by manipulating the ion flux of metabolites such as adenosine triphosphate (ATP), supports this hypothesis (Yoo et al., 2001a). Decreased expression of β-enolase, one of the subunits that compose the enzyme enolase, which catalyzes the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis, has also been reported. Moreover, a decrease in the precursor α- and β-subunits of mitochondrial ATP synthase has been reported (Tsuji et al., 2002).

Decreased glucose metabolism is also associated with the decrease in expression of the 24- and 75-kDa subunits of the multiprotein enzyme complex NADH:ubiquinone oxidoreductase, more commonly known as complex I of the mitochondrial electron transport chain. This complex is located in the mitochondrial membrane and is responsible for the flow of electrons from NADH to ubiquinone in the initiating steps of oxidative phosphorylation to produce ATP (Kim et al., 2001d). A decrease in complex I expression, consistent with altered message (Aksenov et al., 1999), may result in impaired ATP production, as well as a decrease in the activity of the entire electron transport chain. This in turn may result in the departure of electrons from their carrier molecules to generate reactive oxygen species (ROS). ROS production caused by the decreased expression of complex I suggests an alternative rationalization for the well-documented existence of oxidative stress in AD (Butterfield and Lauderback, 2002; Kim et al., 2001d). Additionally, a decrease in core protein 1 of ubiquinolcytochrome c oxidoreductase (complex III) has been reported. Complex III consists of nine polypeptides and catalyzes the transfer of electrons from ubiquinol to cytochrome c. A decrease in core protein 1 of complex III supports the proposed model of oxidative stress and, consequently, altered energy metabolism in AD (Kim et al., 2000).

2. Altered Antioxidant Expression

An increase in the expression of several antioxidant proteins was also observed in AD brain, which agrees with the premise of oxidative stress in the pathogenesis of the disorder (Butterfield, 2002; Butterfield and Lauderback,
An increase in carbonyl reductase (CBR), alcohol dehydrogenase (ADH), peroxiredoxin-I (Prx-I), peroxiredoxin-II (Prx-II), antioxidant protein 2, and Cu/Zn superoxide dismutase was detected (Balcz et al., 2001; Kim et al., 2001b; Krapfenbauer et al., 2003; Schonberger et al., 2001). Moreover, a decrease in peroxiredoxin-III (Prx-III), metallothionein-1 (MT-1), and metallothionein-3 (MT-3) expression was found (Kim et al., 2001b; Prange et al., 2001). CBR and ADH are cytosolic enzymes that catalyze the reduction of carbonyls to their corresponding alcohols. Carbonyls are generally toxic metabolic intermediates that serve as a marker of oxidative stress (Butterfield and Stadtman, 1997). An elevation in the expression of the enzymes involved in the removal of these cytotoxic intermediates suggests an adaptive mechanism of up-regulation to counteract the accumulation of these species (Balcz et al., 2001). Prx-I and Prx-II play an important antioxidant role in the protection of neurons from damage induced by hydrogen peroxide. The up-regulation of these enzymes is consistent with the role of oxidative stress in AD. However, Prx-III, found only in mitochondria, was decreased in AD brain. This could be a result of damage to the enzyme caused by ROS produced within the mitochondria (Kim et al., 2001b). MT-1 and MT-3 are proteins with antioxidant function, with respect to the clearing of heavy metals. The reduced expression of these proteins is consistent with the notion that metals in AD brain may play a role in this disorder (Bush, 2003; Lovell et al., 1998; Prange et al., 2001).

3. **Altered Synaptic Function**

Synaptic dysfunction and alterations in neuronal growth in AD can be related to the decrease in nucleoside diphosphate kinase-A (NDPK-A), 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase), synaptosomal associated protein 25-kDa (SNAP-25), vesicular-fusion protein N-ethylmaleimide-sensitive factor (NSF), t-complex polypeptide 1 (TCP-1), stathmin, neurofilament protein-L (NF-L), drebrin, dihydropyrimidinase related protein-2 (DRP-2), and α-endosulfine. NDPK-A has been reported to be colocalized with microtubules and is believed to be involved in neuronal cell proliferation and neurite outgrowth (Kim et al., 2002). CNPase is associated with oligodendrocytes and therefore myelination (Vlkolinsky et al., 2001). SNAP-25 is believed to play a role in neurotransmission via the exocytosis and docking of synaptic vesicles (Greber et al., 1999). Vesicular-fusion protein NSF is associated with synaptic transmission and neuronal outgrowth (Schonberger et al., 2001). TCP-1 is a protein complex involved in the folding of actin and various isoforms of tubulin (Schuller et al., 2001), whereas stathmin is responsible for the integration of multiple signal-transduction cascades and the destabilization of microtubules (Cheon et al., 2001). NF-L is a protein implicated in maintaining the integrity of the neuronal cytoskeleton (Bajo et al., 2001). Drebrin is a dendritic spine protein that modulates synaptic plasticity (Kim and Lubec, 2001). DRP-2 is a pathfinding protein associated with axonal outgrowth and guidance (Castegna
et al., 2002b; Lubec et al., 1999; Schonberger et al., 2001; Tsuji et al., 2002). α-Endosulfine is an endogenous regulator of ATP-sensitive potassium channels (Kim and Lubec, 2001). All of the aforementioned differences in expression alterations reported in AD could result in the loss of synaptic function and neuronal communication, cognitive decline, and neuronal death.

4. Neurotransmitter and Receptor Dysfunction

Cognitive decline is associated with neurotransmitter imbalance and neuronal death, both of which can be conceivably explained by changes in protein expression levels. Histamine-releasing factor (HRF), which regulates the release of the neurotransmitter histamine, is expressed ubiquitously throughout the brain. The decrease in HRF expression may be associated with cognitive decline, indicating that histamine plays a role in whole-brain function (Kim et al., 2001a). The α7 and 26-kDa truncated isoform of the α3 subunit of nicotinic acetylcholine receptors (nAChRs) were significantly reduced in AD brain, suggesting the decreased expression may influence the function of cholinergic neurons, which are the most affected neurons in AD (Engidawork et al., 2001a). Interaction of Aβ(1-42) with the α7 nAChR, which regulates acetylcholine release and calcium homeostasis, is reported. Aβ(1-42) binds to the α7 nAChR with high affinity, and the complex is endocytosed, resulting in intraneuronal accumulation of Aβ (Nagele et al., 2002). Consequently, Aβ exhibits an inhibitory effect on α7 nAChR, resulting in altered release of acetylcholine and therefore memory and learning deficits (Tozaki et al., 2002). Alternatively, the 45-kDa α3 subunit was increased. The expression of the α3 nAChR subunit is normally down-regulated by cyclic adenosine monophosphate (cAMP) via protein kinase A (PKA). The decreased basal level of cAMP and PKA (Kim et al., 2001c) in AD brain likely results in dysregulation in which the expression of the α3 subunit goes unchecked and therefore increases (Engidawork et al., 2001a).

5. Apoptotic and Antiapoptotic Regulation

Apoptosis may play a role in the neuronal loss exhibited in AD. The pro-apoptotic proteins zipper-interacting protein kinase (ZIPK), Bcl-2-interacting mediator of cell death/Bcl-2 related ovarian death gene (Bim/BOD), and the receptor interacting protein (RIP)-like interacting CLARP kinase (RICK) were found to be increased in AD brain, whereas the antiapoptotic protein p21 is up-regulated in response to oxidative stress (Engidawork et al., 2001c). Protein p21 is believed to involve the inhibition of the MAPK/JNK pathway, which has been shown to be activated in AD brain and by Aβ peptide in vitro (Morishima et al., 2001). The antiapoptotic protein Bcl-2 is up-regulated only in the cerebellum of AD brain, suggesting a possible mechanism by which this area of the brain is less affected in AD (Engidawork et al., 2001c; Hensley et al., 1995). Likewise the apoptosis repressor with caspase recruitment domain (ARC) was also
up-regulated as an antiapoptotic mechanism in response to apoptotic signals such as Aβ-peptide (Engidawork et al., 2001b). Conversely, the Fas-associated death domain (FADD)-like interleukin-1β-converting enzyme inhibitory protein (FLIP) was decreased because it is a substrate for caspases and forms an inactivating complex with caspase-8. Additionally, the antiapoptotic protein DNA fragmentation factor 45 is decreased due to degradation by caspase-3. The down-regulation of FLIP and DFF45 also provides supporting evidence for the activation of caspases as well as the down-regulation of procaspase-3, procaspase-8, and procaspase-9 (Engidawork et al., 2001b). Additionally, the activation of caspase-3 in the limbic cortex has been shown to be an early event in the pathogenesis of AD (Gastard et al., 2003). Finally, glial fibrillary acidic protein (GFAP), a marker of glia, was elevated in AD brain, supporting the hypothesis of neuronal cell death (Greber et al., 1999; Tsuji et al., 1999, 2002).

6. Chaperone Proteins

AD is associated with protein misfolding and decreased protein turnover. Stress or chaperone proteins are thought to play an important role in protecting cells by accelerating protein degradation and preventing the binding of aggregated proteins to hydrophobic surfaces (Anthony et al., 2003). The protein misfolding and decreased protein turnover associated with AD can be related to the dysregulation, and possibly dysfunction, of these molecular chaperone, or stress, proteins in AD brain. Heat shock protein 60 (HSP60), heat shock conjugate 71 (HSC71), glucose-regulated protein 75 (GRP75), and alpha crystalline B have all been reported to be down-regulated in AD. Conversely, heat shock protein 70 RY (HSP70 RY) and glucose regulated protein 94 (GRP94) are up-regulated (Yoo et al., 2001b). Down-regulation of HSP60 (resident in mitochondria) conceivably could be related to elevated oxidative stress, as already noted.

7. Plasma and CSF Studies

For AD research using proteomics, brain is not the only tissue that has been examined to elicit protein expression changes. Blood plasma samples have been examined for apolipoprotein E (ApoE), tau, and presenilin-2 in order to develop a less invasive screening process for AD using immunodetection (Ueno et al., 2000). Cerebrospinal fluid (CSF) has also been used to determine differences between control and AD patients (Davidsson et al., 2002). Several significant protein expression changes were determined in this study, including a decrease in the isoforms of ApoE and proapolipoprotein, suggesting that there may be a decrease in these levels in the brain as well (Davidsson et al., 2002). An additional study detected nine proteins that were differentially expressed in AD CSF versus control, although these biomarkers have yet to be identified (Choe et al., 2002).
8. SELDI and AD Studies

SELDI has been used as a nontraditional method of proteomics study in AD. For example, SELDI has been used to demonstrate that PS1 plays a role, either directly or indirectly, in the production of Aβ as either γ-secretase itself or in the function of γ-secretase, respectively (Chen et al., 2000). Also, SELDI studies have shown that BACE1 is the primary neuronal β-secretase (Cai et al., 2001), and that an increase in intracellular cholesterol in cells transfected with APP increased the production of Aβ(1-42) (Austen et al., 2000). Likewise, SELDI technology has shown that in the presence of γ-secretase inhibitors, truncated amyloid β-peptides are not produced for the C99 fragment that results from the cleavage of APP by β-secretase. Additionally, increased cleavage of the membrane-bound C99 fragment by α-secretase was detected (Beher et al., 2002). Additionally, it has been shown that Aβ(1-40) and Aβ(1-42) are deposited in the lens in AD, resulting in increased protein aggregation and supranuclear cataract formation (Goldstein et al., 2003). A SELDI analysis of CSF detected five proteins that were differentially expressed in AD, including cystatin c, two β-2-microglobulin isoforms, a nerve growth factor (VGF polypeptide), and a 7.69 kDa polypeptide, which was not identified (Carrette et al., 2003). SELDI has also been used to study the variants of Aβ-peptide present in CSF and brain homogenates of AD versus control. A peptide with a molecular mass corresponding to Aβ(1-45) or Aβ(2-46) was observed only in AD CSF. Additionally, in the AD CSF the ratio of Aβ(1-38) to total Aβ was increased. Several variants of Aβ peptide were present in AD brain but not in control, including Aβ(1-29), Aβ(2-33), Aβ(1-40), Aβ(1-42), Aβ(2-46) or Aβ(1-45), Aβ(8-42), Aβ(7-42), Aβ(5-42), Aβ(4-42), and Aβ(2-42). The molecular mass of Aβ(3-42) is very similar to that of Aβ(1-40) and therefore could not be differentiated (Lewczuk et al., 2003).

It is important to note that the expression changes detected in AD do not provide information regarding the integrity of the function of the proteins themselves, and that these changes are not global but correspond to specific brain regions. Consequently, protein expression changes provide only a clue as to the altered state of the protein that the disease causes. More research is required to determine if these proteins are modified in some way, which would result in loss of function (i.e., oxidation). For example, up-regulation of antioxidant proteins such as CBR, ADH, Prx-I, Prx-II, antioxidant protein 2, and Cu/Zn superoxide dismutase, may be a compensatory action; however, if the proteins do not function properly, the up-regulation is all for naught. Additionally, increased expression may be caused by decreased protein turnover, which has been implicated in AD. Nevertheless, this proteomics work has validated the association of altered energy metabolism, decreased synaptic function, and oxidative stress in AD; however, this work has provided only the beginning from
which researchers can, in future studies, determine how these individual alterations affect the brain and lead to cognitive decline in AD.

B. OXIDATIVELY MODIFIED PROTEINS IN AD

Extensive protein oxidation is evident in AD, as indexed by protein carbonyls and 3-nitrotyrosine (Butterfield and Lauderback, 2002; Butterfield et al., 2001, 2002; Castegna et al., 2003; Good et al., 1996; Smith et al., 1997). However, the detection of markers of oxidative stress offers little insight into the proteins that are specifically oxidatively modified and, subsequently, the cellular mechanisms and pathways that are affected. Identification of proteins specifically oxidized in AD brain allows for the determination of proteins that are more susceptible to oxidation and, consequently, more prone to inactivation and loss of function (Table III). Our initial attempts along this area of research identified creatine kinase BB and β-actin as oxidized proteins in AD by coupling immunochemical quantitation of carbonyl reactivity with immunochemical detection on the protein on 2D gels (Aksenov et al., 2000, 2001). This process is time-consuming, laborious, and requires a good educated guess as to the identity of the protein based on pI and molecular weight. This method also ultimately depends on the availability of specific antibodies. Consequently, our laboratory has turned to the techniques of proteomics to provide a method of screening oxidized proteins in AD brain (Butterfield, 2004; Butterfield and Castegna, 2003a,b,c; Butterfield et al., 2003; Castegna et al., 2002a,b, 2003).

The first proteomics analysis to detect specifically oxidized proteins in AD brain (short PMI of <4 hr) led to the identification of five proteins, including: creatine kinase BB (CK), glutamine synthase (GS), ubiquitin carboxy-terminal hydrolyze L-1 (UCH L-1), α-enolase, and DRP-2 (Castegna et al., 2002a,b). These techniques have recently been modified to detect proteins that are

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specifically modified by 3-NT. Three proteins were found to be specifically nitrated in AD—neureguline H3, triosephosphate isomerase, and α-enolase (Castegna et al., 2003). These oxidatively modified proteins vary across a wide range of classes of proteins, including those dealing with energy metabolism, excitotoxicity, proteosomal function, membrane structure, apoptosis, and neuronal communication (Butterfield, 2004; Butterfield et al., 2003). Based on the principle that oxidative modification affects protein function, as has been demonstrated with respect to the glutamate transporter EAAT2 (Lauderback et al., 2001), CK, and GS (Hensley et al., 1995), a number of plausible mechanisms of neurodegeneration can be proposed based on each of the oxidized proteins.

1. Energy Metabolism

Either directly or indirectly, creatine kinase BB, α-enolase, and triosephosphate isomerase are all involved in the synthesis of ATP. CK activity is severely compromised in AD brain (Hensley et al., 1995). Thus oxidation of CK leads to loss of its function, which would suggest that ATP synthesis is severely affected in AD. Such alterations in energy metabolism and ATP production are consistent with positron-emission tomographic (PET) scanning studies of AD patients (Blass and Gibson, 1991; Scheltens and Korf, 2000) and with the altered accumulation of glycolytic enzymes in AD, such as α-enolase, γ-enolase, and glyceraldehyde 3-phosphate dehydrogenase (Schonberger et al., 2001). Likewise, the expression of CK was also found to be altered in AD brain (Schonberger et al., 2001). Lack of ATP would consequently lead to dysfunction in ion pumps, electrochemical gradients, voltage-gated ion channels, and cell potential.

2. Glutamate Uptake and Excitotoxicity

It has been previously reported that Aβ(1-42) leads to the production of the lipid peroxidation product, 4-hydroxy-2-nonenal (HNE) (Mark et al., 1997). Additionally, Aβ(1-42) has been shown to induce oxidative modification of the glutamate transporter EAAT2 by HNE in synaptosomal preparations (Lauderback et al., 2001). Likewise, EAAT2 is oxidatively modified by HNE in AD brain (Lauderback et al., 2001). HNE has been shown to induce protein conformational changes that could lead to loss of protein function (Subramanian et al., 1997). Therefore it has been proposed that the loss of activity of EAAT2 in AD (Masliah et al., 1995) is due to oxidative modification by HNE (Lauderback et al., 2001). Likewise, it is likely that oxidative modification of GS explains the loss of activity of GS reported in AD (Hensley et al., 1995). Loss of function of these proteins would result in a decreased conversion or uptake of glutamate, resulting in accumulation of extracellular glutamate. The excess glutamate would stimulate N-methyl-D-aspartate (NMDA) receptors, leading to an increase in Ca^{2+} influx. Altered calcium homeostasis would lead to alteration in long-term potentiation (LTP) and, consequently, learning and memory, mitochondrial
swelling with consequent ROS leakage and release of proapoptotic cytochrome c, stress in the endoplasmic reticulum, as well as activation of calcium-sensitive proteases such as calpain. Clearly such changes would lead to neuronal death and may be important in AD.

3. Proteasomal Dysfunction

Proteasomal dysfunction has been reported in AD (Keller et al., 2000) and leads to a buildup of damaged, misfolded, and aggregated proteins (Shringarpure et al., 2001). Protein oxidation typically results in protein cross-linking and aggregation (Butterfield and Stadtman, 1997). Such aggregated proteins could "clog" the pore of the proteasome, leading to a compromise in proteasomal function. Loss of activity of UCH L-1, which was found to be oxidized in AD (Castegna et al., 2002a), would lead to excess protein ubiquitination, loss of activity of the proteasome, and accumulation of damaged or aggregated proteins, all of which are found in AD.

4. Membrane Structure and Cholinergic Dysfunction

Neuropolyepptide h3 has been identified as specifically nitrated in AD (Castegna et al., 2003). Neuropolyepptide h3 has a variety of names and functions, two of which are phosphatidylyethanolamine-binding protein (PEBP) and hippocampal cholinergic neurostimulating peptide (HCNP). It is possible that loss of function of PEBP could lead to loss of phospholipid asymmetry, resulting in the exposure of phosphatidylyserine on the outer leaflet of the lipid bilayer, a signal of apoptosis. Among the functions of HCNP is in vitro up-regulation of the production of choline acetyltransferase in cholinergic neurons following NMDA receptor activation (Ojika et al., 1998). Choline acetyltransferase activity is known to be decreased in AD (Rossor et al., 1982), and cholinergic deficits are prominent in AD brain (Giacobini, 2003; Katzman and Saitoh, 1991). Nitration of neuropolyepptide h3 may help to explain the decline in cognitive function caused by lack of neurotrophic action on cholinergic neurons of the hippocampus and basal forebrain.

5. Neuritic Abnormalities

DRP-2 has been shown to be decreased (Lubec et al., 1999; Schonberger et al., 2001; Tsuji et al., 2002) and is oxidatively modified in AD brain (Castegna et al., 2002b). DRP-2 is a pathfinding and guidance protein for axonal outgrowth. Additionally, DRP-2 interacts with and modulates collapsin, a protein responsible for the elongation and guidance of dendrites. Consequently, DRP-2 plays an important role in forming neuronal connections and maintaining neuronal communication. The oxidation and impaired activity of DRP-2 could result in the known shortened dendritic lengths in AD brain (Coleman and Flood, 1987). Neurons with shortened neurites would not be expected to communicate well
with adjacent neurons, a process that could conceivably contribute to memory and cognitive loss in AD.

6. Oxidized Proteins in AD Plasma

Recently, similar proteomics techniques have been applied to identify specifically oxidized proteins in AD plasma. Isoforms of γ-chain precursor protein and α-1-antitrypsin precursor were identified, both of which have previously been implicated in the disease process (Choi et al., 2003).

IV. Proteomics Analysis of Transgenic Models of AD

Application of proteomics to transgenic models of AD is an increasing field of research in AD. Such approaches allow methods of testing the effects of genetic mutations associated with AD on specific proteins at both the expression and post-translational modification levels. Additionally, transgenic knock-in and knock-out mice allow for the study of the effect of inactivation of specific proteins, which have been found to be oxidized in AD brain. For example, the gracile axonal dystrophy (GAD) mouse model allows for the study of the consequences of the inactivation of UCH L-1 in the brain, which was found to be oxidized in AD brain (Castegna et al., 2002a, 2004). Loss of function of UCH L-1 led to specifically oxidized proteins in brain as identified by proteomics (Castegna et al., 2004).

MudPIT techniques were combined with a hydrazide biotin-streptavidin isolation of carbonylated proteins to identify oxidized proteins in aged mice (Soreghan et al., 2003a). These techniques have recently been applied to the study of the PS1/APP transgenic mouse model, which develops both the amyloid and tau pathologies of AD (Soreghan et al., 2003b). Several membrane proteins were identified in both models; however, this technique is not quantitative. Therefore no information was generated with regard to the extent of carbonylation of the individual proteins.

Studies by Tilleman et al. (2002a,b) have led to the identification of 51 proteins differentially expressed in glycogen synthase-3β (GSK-3β) mutant mice compared to control. GSK-3β is believed to play a role as a tau kinase, resulting in hyperphosphorylation of tau and, consequently, the formation of paired helical filaments and, ultimately, neurofibrillary tangles. Cytoskeletal proteins were identified as well as those involved in energy metabolism, vesicle transport, protein folding, signal transduction, amino acid synthesis, and detoxification (Tilleman, 2002a). Likewise, studies of a mutant mouse model expressing human tau have resulted in the identification of 34 differentially expressed proteins (Tilleman, 2002b). The proteins implicated were involved in similar processes
as those described in the GSK-3β study. Taken together, four proteins were differentially regulated between the two models. These include α-enolase, D-3-phosphoglycerate dehydrogenase, NADH:ubiquinone oxidoreductase, and nucleoside diphosphate kinase, almost all of which have been described as having altered expression in AD (Kim et al., 2001d, 2002; Schonberger et al., 2001).

V. Future of Proteomics in AD

In summary, a number of proteins are oxidatively modified in AD brain. Likewise, the expression of many proteins is altered in the AD brain, resulting in a cascade of potential alterations of multiple pathways within the brain. Perhaps it is the relationship between these pathways that maintains the careful balance between neuronal survival and neuronal death. To date, proteomics has shed light on only a small portion of the puzzle; however, with improved techniques that allow for increased solubilization and loading of membrane-bound proteins and very basic proteins, along with better detection limits, more information will become available on the pathogenesis of AD.

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References


Sears et al., Fig. 5. The secretogranin family. A. Region of gel showing the seven secretogranin proteins identified in CSF proteome. B. Expression of one of the secretogranin proteins in AD versus control patients. AD subjects were subdivided by disease duration. D < 3: disease duration of less than 3 years. D = 3-6: disease duration between 3-6 years. D > 6: disease duration of greater than 6 years. NCO: normal controls, NCI: normal controls with first degree AD relative. Note that levels of the secretogranin protein are decreased in AD versus normal controls.
**Butterfield and Boyd-Kimball, Fig. 1.** Summary of procedure for parallel analysis of 2D gel maps and 2D oxyblots.

**Witzmann and Strother, Fig. 3.** This diagram illustrates a strategy for reducing brain protein sample complexity and increasing analytical depth of field for 2DE gel-based proteomics. This approach is designed to increase the number of proteins that can be detected, comparatively quantified, and identified by at least one order of magnitude over current methods.