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Review

Proteomics: a new approach to investigate oxidative stress in Alzheimer's disease brain

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Abstract

In Alzheimer's disease (AD) brain oxidative stress is observed indexed by several markers, among which are protein carbonyls and 3-nitrotyrosine, markers for protein oxidation. We hypothesized that identity of these oxidatively modified proteins would lead to greater understanding of some of the potential molecular mechanisms involved in neurodegeneration in this dementing disorder. Proteomics is an emerging method for identification of proteins, and its application to neurodegenerative disorders, especially AD, is just beginning. Posttranslational modification of brain proteins, particularly that due of oxidation of proteins, provides an effective means of screening a subset of proteins within the brain proteome that likely reflects the extensive oxidative stress under which the AD brain exists, and this new methodology provides insights into mechanisms of neurodegeneration in and new therapeutic targets for AD. In this review, the use of proteomics to identify specifically oxidized proteins in AD brain is presented, from which new insights into mechanisms of neurodegeneration and synapse loss in this dementing disorder that is associated with oxidative stress have emerged.

Theme: Disorders of the nervous system
Topic: Degenerative disease: Alzheimer's beta-amyloid

Keywords: Proteomics; Oxidative stress; Alzheimer's disease brain

1. Introduction

Alzheimer's disease (AD), a progressive, age-associated neurodegenerative disorder characterized by loss of memory and cognition [31], affects nearly 5 million persons in the United States currently. Estimates of 22 million persons worldwide with AD in the near future exist [30].

Oxidative stress, indexed by protein carbonyls (protein oxidation), 4-hydroxy-2-nonenal (HNE) and isoprostanes (lipid oxidation) and 8-hydroxy-2-deoxyguanine (DNA oxidation) is extensive in AD brain [9,11,12,35,39]. Amyloid β-peptide (1–42) [Aβ(1–42)] is thought to be central to the pathogenesis of AD [48].

We combined these two notions—the centrality of Aβ(1–42) to the pathogenesis of AD and the oxidative stress under which the AD brain exists—into a comprehensive, Aβ(1–42)-centered model for neurodegeneration in AD brain [9,11,12,56]. In support of this model, we and others demonstrated that Aβ(1–42) induces in neurons protein oxidation, lipid peroxidation, and reactive oxygen species formation and that free radial scavengers could inhibit Aβ(1–42)-induced oxidative stress (reviewed in [Refs. 9,11,12,56]). In vivo, oxidative stress was found in animals that express human Aβ(1–42) [22,58] or express a human mutated gene that leads to familial AD [33,52].

The mechanisms by which Aβ(1–42)-associated oxidative stress occur in neurons and putatively in AD brain are under active investigation. We showed that the single methionine residue of Aβ(1–42) at residue 35 is critical for the oxidative stress and neurotoxic properties of this peptide, both in vitro and in vivo [4,8,27,28,58]. Others invoke the involvement of peptide-bound redox metal ions in these properties [25]. Aggregated, not fibrillar, Aβ(1–42) is likely the toxic species of this peptide [2,22,34,45,57], consistent with the notion of small aggregates of the peptide inserting into the membrane bilayer to induce oxidative stress. Such membrane insertions are less likely with large fibrillar structures.

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As noted, protein oxidation is evident in AD brain that correlates with markers of AD histopathology [23], i.e., protein oxidation occurs in AD brain where Aβ(1–42) is but not in Aβ(1–42)-poor cerebellum. But which proteins are oxidized? And could their identity provide insight into potential mechanisms of neurodegeneration in AD brain?

Our initial attempts at addressing these questions involved immunochemical methods. Brain slices or tissue were double immunoprecipitated, one antibody for protein carbonyls and a second for the protein of interest, and densitometric analysis of bands was compared between control and AD samples [3]. Creatine kinase and β-actin were shown to be selectively oxidized in AD brain by this approach [3]. However, this means of protein identification, one at a time, is impractical for the brain proteome. Moreover, one has to know beforehand the identity of the protein of interest in order to employ a specific antibody. A different approach to identification of large numbers of potentially oxidized proteins was needed. Hence, we used proteomics for the first time to identify specifically oxidized proteins in AD brain [4–7,13–16], and the results are presented in this review. Insights into potential neurodegenerative mechanisms that stem from oxidative stress-induced protein oxidation and that are consistent with the biochemical and pathological alterations in AD brain have emerged.

2. Methods

2.1. Brain tissue sampling

Inferior parietal lobule (IPL) tissue samples used for analyses were taken at autopsy from AD and control subjects, immediately frozen in liquid nitrogen, and stored at −80 °C. The Rapid Autopsy Program of the University of Kentucky Alzheimer's Disease Research Center (UK ADRC) resulted in extremely short postmortem intervals (PMIs). All AD subjects displayed progressive intellectual decline and met NINCDS ADRDA Workgroup criteria for the clinical diagnosis of probable AD [42]. All AD subjects met accepted guidelines for the histopathologic diagnosis of AD [44]. Hematoxylineosin and modified Bielschowsky staining and 10-D-5, ubiquitin, and α-synuclein immunohistochemistry were used on multiple neocortical, hippocampal, entorhinal, amygdala, brainstem, and cerebellum sections for diagnosis. Some AD patients were also diagnosed with dementia with Lewy bodies, but results were no different from AD patients with or without the presence of Lewy bodies. Control subjects underwent annual neuropsychological testing as a part of the UK ADRC normal volunteer longitudinal aging study, and did not have a history of dementia or other neurological disorders. All control subjects had test scores in the normal range. Neuropathologic evaluation of control brains revealed only age-associated gross histopathologic alterations. Brain samples were minced and suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$, and proteinase inhibitors: leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), type IIS soybean trypsin inhibitor (0.5 μg/ml), and PMSF (40 μg/ml). Homogenates were centrifuged at $14{,}000 \times g$ for 10 min to remove debris. Protein concentration in the supernatant was determined by the Pierce BCA method.

2.2. Two-dimensional (2D) gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separates a mixture of proteins into single detectable protein spots. This analytical method has been widely used in the process of examination of complex protein mixtures [46]. In the first step of 2D-PAGE, isoelectric focusing (IEF), that separates proteins according to their isoelectric point is used. A second step, SDS-PAGE, further separates proteins based on their molecular weight. The result is a two-dimensional map, in which each spot most often corresponds to a single protein. Protein profiling is the major information that is obtained from a 2D PAGE map, allowing comparison between different samples, thereby indicative of differences in protein expression [55]. 2D PAGE is used to create maps in order to catalogue proteins and create databases [26]. The power of 2D-PAGE relies in its potential to resolve thousands of proteins simultaneously, providing important clues even for post-translational modifications that involve changes in protein total charge. 2D-PAGE is a reasonably reliable method, but many challenges still exist. The solubilization process for proteins is the main difficulty, since IEF is not compatible with the introduction of charge, i.e., ionic detergents. Chaotropic agents, such a urea and thiourea, coupled with nonionic or zwitterionic detergents, offers a good solubilization and avoids protein precipitation during the IEF and in the process of protein transfer from the IEF gel into the SDS gel. Protein solubilization issues are most severe with regard to membrane proteins [47]. Immobilized pH IEF strips, introduced to replace the previously used gel tubes containing ampholytes, eliminate the typical "cathodic drift" during IEF and increase the map reproducibility between samples [43]. However, the limited pH range (usually 3–10) that the strips provide excludes the identification of highly basic proteins. Improved resolution of map spots representing proteins of close isoelectric points may be possible with use of narrow range pH strips (as low as 1 pH unit). Moreover, low-abundance proteins represent a technical limitation of proteomics. When proteomics is applied to disease, in which low-abundant proteins can often play an important role, the results are usually non-informative.

Non SDS-page methods for protein are also used. HPLC is often used to achieve separation of peptides produced by trypsin digestion of a protein mixture. The technique, 2D-HPLC, utilizes a series of columns that obtain separation of the peptides that then are submitted for mass spectrometry analysis. Evaluation of protein expression can also be

achieved by previously labeling a mixture of two samples with compounds that contain different isotopes and that bind to specific amino acid side chains. The resulting mass spectrometry distribution of masses allows evaluation of the differences in expression of a protein in two samples. This technique is referred as isotopically coded affinity tags (ICAT) [53].

Our proteomics analysis to identify specifically oxidized proteins in AD brain coupled 2D-gel electrophoresis with immunochemical detection of protein carbonyls, followed by mass spectrometry analysis, as shown in Fig. 1. Proteins containing reactive carbonyl groups in AD and control brain samples are detected by 2D Oxyblot analysis using specific antibodies. 2D Oxyblots (Western blots) and the subsequent 2D gel images are matched and the anti-DNP immunoreactivity of individual proteins are normalized to their content, obtained by measuring the intensity of colloidal Coomassie Blue staining. This procedure allows comparison of oxidation levels of brain proteins in AD versus control subjects.

2.3. Mass spectrometry and database searching

The protein of interest, separated from the mixture into a single detectable spot, is eluted from the gel to undergo mass spectrometry analysis. The most common methods to obtain the protein from an excised spot utilize the procedure of in-gel digestion, in which a protease, solubilized in a buffer that optimizes the digestion, is absorbed into the gel surface to cleave the protein into several peptides. In-gel digestion has two advantages: first, the recovery is much higher when the protein is cleaved in smaller particles; more importantly, collection of peptides that result from sequence-specific proteolysis is an important means to identify the protein itself, because their masses constitute mass fingerprints that are characteristic of a particular protein. This peptide mass mapping involves the use of MS and a suitable database (Table 1) containing protein sequences to which the experimental masses are compared.

Table 1
Mass spectrometry search engines for peptide mass fingerprinting

Search engine	URL
Mascot	http://www.matrixscience.com
MOWSE	http://www.hgmp.mrc.ac.uk/Bioinformatics/Webapp/mowse
Profound	http://www.prowl.rockefeller.edu/sgi-bin/Profound
MS-fit	http://prospector.ucsf.edu/ucsfhtlm3.4/msfit.htm
Peptident	http://www.expasy.ch.ch/tools/peptident.html

Peptide masses and amino acid sequence, which are necessary to identify the protein of interest are attained by mass spectrometry. Early MS studies failed to provide an accurate peptide mass due to molecular fragmentation of the sensitive ion. The introduction of "softer" ionization techniques has given mass spectrometry new impetus as a tool in protein identification studies. The two most-often techniques employed are MALDI (matrix assisted laser desorption/ionization) and ESI (electrospray ionization). In MALDI, the peptide sample is mixed to a matrix (usually α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid) and deposited to a plate that is subjected to laser radiation. The chromophores (highly UV absorbents) incorporate high energy that is then released to the peptides. The latter evaporate as detectable MH^+ ions, due to the acidic nature of the matrix, which donates a proton in the gas phase in processes that are not well understood. In ESI, a high potential difference is established between the MS inlet and a microcapillary tube, through which the liquid sample flows. Dispersion of the liquid released by the capillary into fine droplets is caused by the potential difference. These droplets undergo solvent evaporation until droplet fission occurs, due to the high charge to surface tension ratio, ultimately ending in the formation of a single detectable ion. Low salt concentration in the sample of interest is

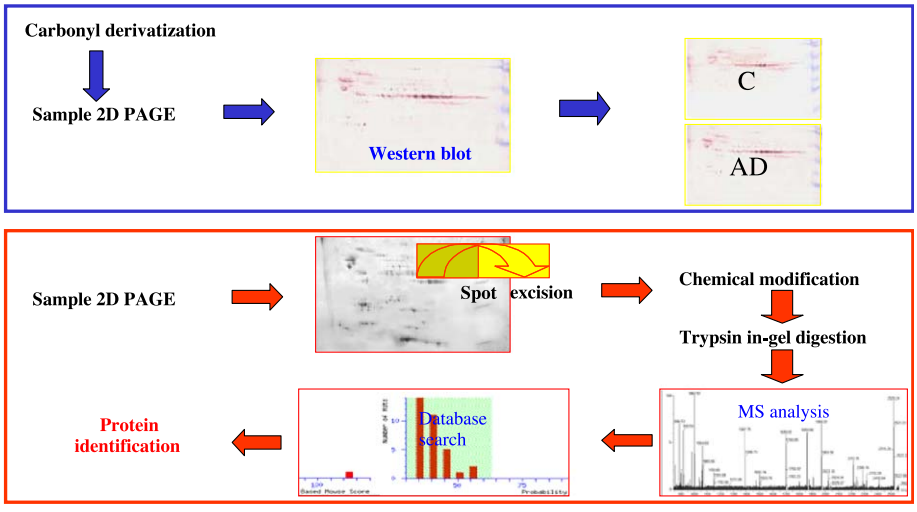

Fig. 1. Parallel analyses used to identify oxidatively modified proteins in AD brain. See Refs. [5–7,13–16].

required and offers the advantage of allowing on-line pre-separation of the peptides with HPLC. Moreover, the flow rate can be reduced to nanoliters per minute, thereby increasing the time available for analysis. Tandem MS provides conformational information by further isolation of a single peptide and fragmentation. This process provides insight into protein identity and sequence [1].

In MS/MS analysis, a single ion is isolated and fragmented. Once all the ions are collected, the ion of interest is isolated, ejected from the trap and fragmented in an excitation step. The isolation occurs by applying a large range of frequencies at once, except the frequency of the desired ion that is retained in the trap. Fragmentation of the isolated ion, which provides additional information necessary for protein identification or for evaluation of possible protein modification, is the final step in MS/MS.

An additional proteomics tool in which mass spectrometry is utilized is surface-enhanced laser desportion/ionization time-of-flight that couples the classical methods of chromatographic sample preparation with mass spectrometry analysis. This method was not used in our studies. For additional information the reader is directed to our recent review [13].

Mass spectrometic analysis is followed by the informatics part of proteomics studies, which involves on-line protein or genomic databases. The protein sequence database SwissProt [24], to which it is possible to submit a protein identification request by using identification tools based on computer algorithms that are freely accessible through the internet, is likely the most widely used protein database. These databases are given in Table 1. These search engines provide a theoretical protease digestion of the proteins contained in the database, and the search engines compare the resulting peptide masses to the experimental masses obtained from the in-gel digested proteins. This process is not a simple comparison of peptide masses, since it accounts for several factors, such as the protein size and the probability of a single peptide to occur in the whole database. The search engine produces a probability score for each entry, which is calculated by a mathematical algorithm that is specific for each search engine. Any hit with a score higher that the one obtained from the search, which is usually set for $p < 0.05$, has a legitimate chance to be the protein cut from a given spot. False identification conceivably could occur. To avoid this possibility, the molecular weight and IP range in the 2D map must be taken into account.

3. Results and discussion

3.1. Proteomic studies on oxidatively modified proteins in AD

In AD brain, protein oxidation is increased, indexed by protein carbonyls [9,11,14,15] and 3-nitrotyrosine [16,51]. Identification of specifically oxidized proteins in AD brain allows one to determine which proteins are more affected by oxidation in AD and, consequently, more prone to inactivation, and thus represents a significant step in linking well-established AD neurodegeneration with oxidative events at a protein level.

The first use of proteomics to identify specifically oxidized proteins in AD brain indicated several such proteins: creatine kinase BB (CK), glutamine synthase (GS), ubiquitin carboxy-terminal hydrolase L-1 (UCH l-1), α-enolase, and dihydropyrimidinase related protein 2 (DRP2) [14,15]. Similarly, proteomics has been used to identify posttranslationally modified proteins in AD brain, e.g., 3-nitrotyrosine: neuropolypeptide h3, triosophosphate isomerase and α-enolase [16].

Plausible and testable mechanisms of neurodegeneration in AD brain, summarized in Table 2 can be posited based on each of these specifically oxidized proteins. Specifically oxidatively modified proteins thus far identified in AD brain can be classified as those dealing with energy metabolism, excitotoxicity, recycling of damaged or aggregated proteins through the proteasome, membrane structure and apoptosis, and neuronal communication. In addition, some of the pathological characteristics of AD brain, such as accumulation of aggregated, damaged proteins, excess ubiquination, and shortened dendritic lengths are consistent with putative dysfunction of some of these specifically oxidized proteins. The assumption involved is that oxidative modification affects protein function, and this assumption has been proved correct for three cases thus far investigated in our laboratory: CK and GS [23] and the glutamate transporter, EAAT2 [35].

3.1.1. Proteins involved in energy metabolism

Creatine kinase (BB isoform), α-enolase, and triosephosphate isomerase are directly or indirectly involved in ATP production, and if, like CK, the enzymatic activity of these other energy-related enzymes is diminished in AD brain, ATP production will be compromised. ATP diminution may be important especially to the synapse, the probable initial site of attack in neurons in AD [41]. Ion pumps, electro-

Table 2
Proteomic identification of specifically oxidatively modified proteins in ad brain

Energy-Related Enzymes: Creatine Kinase[a]; α-Enolase; Triosephosphate Isomerase
Excitotoxicity-Related Proteins: Glutamine Synthetase[a]; Glutamate Transporter, EAAT2[a,b]
Proteasome-Related Clearance Function: Ubiquitin Carboxyl Terminal Hydrolase L-1; Heat Shock Cognate 71
Cholinergic System: Neuropolypeptide h3 (also known as Hippocampal Cholinergic Neurostimulating Peptide; Phosphatidyl Ethanolamine Binding Protein; and Raf Kinase Inhibitor Protein)
Structural Proteins: Dihydropyrimidinase-Related Protein 2; β-Actin[b]

[a] Activity known to be decreased in AD brain.
[b] Determined by immunochemical means.

chemical gradients, the cell potential, voltage-gated ion channels, phospholipid asymmetry, and many other aspects of the neuron would be compromised by diminished ATP levels. Neurodegeneration, following influx of Ca^{2+} and subsequent Ca^{2+}-dependent degradative processes, would ensue.

3.1.2. Proteins involved in glutamate reuptake or conversion

The glutamate transporter EAAT2 in AD brain is oxidatively modified by the lipid peroxidation product, 4-hydroxy-2-trans-nonenal (HNE) [35]. Aβ(1–42) induced the same modification to this glutamate transporter [35], and Aβ(1–42) causes HNE formation [35,38]. HNE modification of synaptosomal membranes leads to altered protein conformation (structure) [54]. Therefore, oxidative modification of EAAT2 in AD brain likely explains the loss of activity of this transporter [40]. This result, coupled with the loss of activity of GS in AD brain [23,50] that is likely explained by the oxidative modification of this enzyme as determined by proteomics [14], would lead to excess glutamate on the outside of neurons. NMDA receptor stimulation by excess glutamate leads to the influx of Ca^{2+}, with significant effects on LTP, organelle structure, and subsequently neuronal death via excitotoxicity.

3.1.3. Proteasomal dysfunction

The sequelae of a dysfunctional proteasomal degradation system, known to be defective in AD brain [29], include accumulation of damaged or aggregated proteins, with subsequent loss of neuronal function [49]. The proteasomal system might be compromised in AD due to the excess HNE-bound proteins present. The increased hydrophobic load associated with this alkenal bound to proteins, or potential crosslinks between protein-bound HNE and the proteasomal proteins [10] could provide steric interference to the pore of the proteasome. Moreover, loss of activity of UCH L-1, a specifically oxidatively modified protein in AD brain [14], is predicted to lead to excess protein ubiquitination (found in AD), loss of activity of the proteasome (found in AD), and accumulation of aggregated or damaged proteins (found in AD). The deleterious effects of proteasome dysfunction on neuronal survival are pronounced.

3.1.4. Lipid abnormalities and cholinergic failure

Neuropolypeptide h3, a specifically oxidatively modified protein in AD brain [16], has a role as a phosphatidylethanolamine binding protein. It is conceivable that loss of activity of this protein activity as a consequence of its oxidative modification could compromise phospholipid asymmetry in AD brain. Phospholipid asymmetry is essential for membrane structure and function [20]. Phosphatidyl serine would become exposed on the outer lamellae of the membrane, a signal to initiate apoptosis, leading to neuronal death. Neuropolypeptide h3 is also the hippocampal cholinergic neurostimulating peptide, one function of which is to stimulate choline acetyltransferase. This latter enzyme was one of the earliest biochemical abnormalities reported in AD brain [21]. HNE is bound to cholineacetyltransferase upon interaction of Aβ(1–42) with synaptosomes [9], thereby possibly explaining the loss of activity of this enzyme in AD brain. Conceivably oxidative modification of neuropolypeptide h3 contributes to the loss of cholineacetyltransferase activity.

3.1.5. Neuritic abnormalities

Shortened dendritic lengths are found in AD neurons [19], which conceivably could decrease interneuronal communication, likely important in a disorder like AD in which memory loss is so characteristic. Dihydropyrimidinase related protein 2 (DRP2), a specifically oxidatively modified protein in AD brain [15] and whose expression is altered in AD [36], performs two important functions in neurons. DRP2 modulates the activity of collapsin, a protein that elongates dendrites and directs them to adjacent neurons. This protein may also be involved in neuronal repair and normally is highly expressed during brain development, but rarely in adult brain. Under oxidative attack and subsequent synaptic loss, neurons require new synapses to be formed. Thus, the altered expression of DRP2 would be explained [36]. However, as soon as it is expressed, it may become oxidatively modified and lose function. Hence, dendritic lengths would be shortened, and memory conceivably would decline, both observed in AD brain.

Thus, our proteomics analysis of posttranslationally modified proteins, i.e., oxidation, in AD brain has identified proteins whose decreased functions are consistent with the pathophysiology of AD.

Others have used proteomics analysis to identify oxidized proteins in AD plasma [18]: isoforms of fibrinogen gamma-chain precursor protein and of alpha-1-antitrypsin precursor, proteins that are already been implicated in the pathology of this neurodegenerative disorder. Potentially, proteomics offers the possibility of using oxidized proteins in plasma as markers for AD. Studies to test this idea are in progress in our laboratory. A recent study on AD brain also identified targets of protein oxidation, although no specific identification of the oxidized proteins was achieved [32].

Other age-related neurodegenerative disorders are associated with brain-resident oxidative stress, e.g., Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), diffuse Lewy body disease (DLBD), among others [8]. A rare form of familial PD is inherited by a defect in the gene for UCH L-1 [37]. Interestingly, the gracile axonal dystrophy (GAD) mouse, which results from a partial expression of UCH L-1, leads to protein oxidation in brain [17]. While a search of the literature has not revealed proteomics analysis of oxidized proteins in brain in PD, ALS, HD, or DLBD, such analyses may provide insight into these disorders as our proteomics studies have for AD. Currently, we are examining animal models of some of these disorders in order to establish a database of potential brain

protein targets of oxidation. This database then will be used in studies of human brain proteins in these disorders.

Proteomics is an emerging technique that, in identifying oxidatively modified proteins, accounts for some biochemical and morphological alterations in AD. The promises of proteomics include the possibility to use peripheral tissue to diagnose AD and monitor therapeutic efficacy, to provide potential new therapeutic targets, and to learn more of the mechanisms of neurodegeneration. Continued proteomics analysis of AD and models thereof are underway in our laboratory.

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