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Review

Abeta, oxidative stress in Alzheimer disease: Evidence based on proteomics studies[☆]



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ABSTRACT

The initiation and progression of Alzheimer disease (AD) is a complex process not yet fully understood. While many hypotheses have been provided as to the cause of the disease, the exact mechanisms remain elusive and difficult to verify. Proteomic applications in disease models of AD have provided valuable insights into the molecular basis of this disorder, demonstrating that on a protein level, disease progression impacts numerous cellular processes such as energy production, cellular structure, signal transduction, synaptic function, mitochondrial function, cell cycle progression, and proteasome function. Each of these cellular functions contributes to the overall health of the cell, and the dysregulation of one or more could contribute to the pathology and clinical presentation in AD. In this review, foci reside primarily on the amyloid β -peptide ($A\beta$) induced oxidative stress hypothesis and the proteomic studies that have been conducted by our laboratory and others that contribute to the overall understanding of this devastating neurodegenerative disease. This article is part of a Special Issue entitled: Misfolded Proteins, Mitochondrial Dysfunction, and Neurodegenerative Diseases.

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1. Background

Alzheimer disease (AD), a severe, age-associated neurodegenerative disorder, affects many people aged from 65 years or older, with nearly half of those of age 85 are afflicted with this disorder. As the world population grows and life expectancies increase, the number of AD patients is growing at an ever-increasing rate since the most important contributing factor to AD is age. Current reports estimate that there are about 5.1 million AD patients in the United States, and may increase up to 20 million by the year 2050 [1].

Abbreviations: 3NT, 3-Nitrotyrosine; HNE, 4-Hydroxynonenal; AD, Alzheimer disease; aMCI, amnesic mild cognitive impairment; APP, amyloid precursor protein; AICD, amyloid precursor protein intracellular domain; $A\beta$, amyloid- β ; BACE1, beta-secretase 1; CTF, C-terminal fragment; EAD, early onset Alzheimer disease; LAD, late-stage Alzheimer disease; MCI, mild cognitive impairment; NFTs, neurofibrillary tangles; naMCI, non-amnesic mild cognitive impairment; ONOO⁻, peroxynitrite; PUFA, polyunsaturated fatty acid; PET, positron emission tomography; PCAD, pre-clinical Alzheimer disease; PS-1, presenilin-1; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAMP8, senescence-accelerated prone mouse 8; sAPP α , soluble amyloid precursor protein α ; sAPP β , soluble amyloid precursor protein β ; O₂⁻, superoxide; SOD, superoxide dismutase

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The classic histopathological hallmarks of AD can be summarized as the accumulation of extracellular amyloid β -peptide ($A\beta$)-rich senile plaques (SPs), which are generated from the cleavage of amyloid precursor protein (APP), the accumulation of intracellular neurofibrillary tangles (NFTs), which are largely comprised of the aggregated form of hyperphosphorylated Tau, and synapse loss. Tau, a microtubule stabilizing protein, is hyperphosphorylated in AD neurons and causes microtubular abnormalities with consequent disruption of intraneuronal trafficking. On a molecular basis, cell cycle changes and oxidative stress resulting from increases in ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) have also been shown to play a detrimental role in AD [2,3]. Not all AD cases are sporadic, with a small percentage being the result of hereditary on-set AD.

Classical AD progression can be categorized into four stages: preclinical AD (PCAD), mild cognitive impairment (MCI), early-onset AD (EAD), and late-stage AD (LAD). Many individuals with PCAD have a high amyloid plaque burden, yet function normally. PCAD, however, is difficult to study, as PCAD itself does not show evidence of being fatal and brain samples are only obtained after a death by another means. However, what research has been conducted has been helpful in understanding the earliest of AD progressions.

MCI has been categorized as the transition stage between normal cognition and EAD/AD and can be further sub-divided into amnesic MCI (aMCI) and non-amnesic MCI (naMCI). Pathologically, differences in amyloid load and NFTs vary from patient to patient, yet recent research has shown that a striking difference in aMCI progression to

AD (38%) compared to naMCI (20%). naMCI patients present with higher concentrations of cerebral spinal fluid (CSF) $A\beta(1-42)$ as well as less severe hippocampal atrophy that may play a role in these findings [4].

Patients with LAD present with the highest levels of $A\beta$ peptide and NFTs. Applying imaging techniques such as MRI, various degrees of degeneration are observed for all stages of clinical AD. Positron emission tomography (PET) technology used to probe regional glucose utilization within the brain suggests severe energy deficiency for PCAD and MCI patients, which, considering that glucose is as the main energy resource for brain, demonstrates that the brain is under energy deprivation, consistent with the progression of AD.

2. ROS

The initiation and propagation of ROS and RNS generation have been shown to play a major role in the pathogenesis of AD [5]; thus, it is important to understand the origin of these oxidants, as well as their modes of action. ROS and RNS include, among many others, the superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), nitric oxide ($\cdot NO$), and peroxynitri ($ONOO^-$), most of which are free radicals. ROS/RNS plays necessary and beneficial roles in many biological processes [6]. Oxidative stress is a condition in which there is an imbalance of these reactive species and antioxidant defenses [6–8]. In addition to free radicals produced from environmental exposure, excess production of free radicals or other reactive species and a failure of antioxidant defense systems to adequately handle the ROS/RNS load result in damage to biomolecules including proteins, lipids, and genetic material [3,9–13].

Human mitochondria process oxygen at great potential self-risk due to production and leakage of damaging $O_2^{\cdot-}$ (Fig. 1), which is elevated due to an age-related decrease in the efficiency of electron transport chain reactions, primarily from Complex I [6,14–17]. Reactive $O_2^{\cdot-}$ has been shown to oxidize biomolecules directly through lipid peroxidation and protein oxidation [18–20] and indirectly inducing oxidative/nitrosative stress in the brain through production of other reactive species, as well as through cellular signaling routes such as that of inflammatory cytokine tumor necrosis factor alpha ($TNF-\alpha$) [20–24]. The mitochondrial resident superoxide scavenger, manganese superoxide dismutase (MnSOD) in the matrix, and Cu/ZnSOD in the intermembrane space, reacts with $O_2^{\cdot-}$ producing H_2O_2 . H_2O_2 , though only a weak oxidant, becomes much more damaging in the presence of free copper(I) or iron(II) ions through Fenton chemistry by which H_2O_2 is reduced to the extremely reactive $\cdot OH$ radical [9,20]. The $\cdot OH$ radical is responsible for much of the downstream indirect damage from superoxide. Hard nucleophiles such as the hydroxyl radical may attack biomolecules at carbonyl moieties resulting in structural and functional changes.

Free radicals in close proximity to allylic hydrogen atoms on biomolecules abstract such hydrogens leaving a carbon centered radical. When this process affects poly-unsaturated fatty acids (PUFAs) in the lipid bilayer, which are particularly rich in neurons, a subsequent reaction of this radical with molecular oxygen forms a peroxy radical leading to further abstraction of other allylic hydrogens from nearby biomolecules and consequent chain reaction propagation. Once initiated, lipid peroxidation can lead to increased production of reactive alkenals in the bilayer, loss of lipid asymmetry, and apoptosis [25–28]. A major reactive alkenal produced is 4-hydroxynonenal (HNE). HNE is primarily

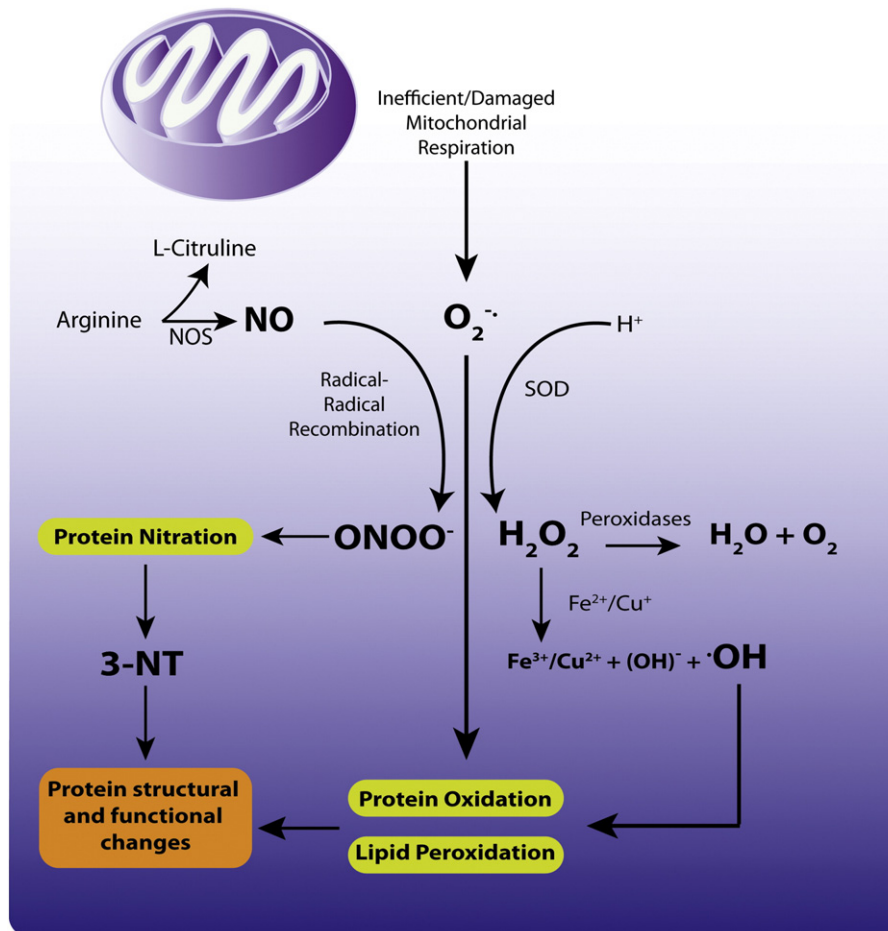


Fig. 1. ROS formation involving mitochondria-derived superoxide free radicals and subsequent processing by MnSOD and Fenton chemistry and RNS formation following NO production via i-NOS and reaction of NO with superoxide free radicals. See text for further details.

produced in the brain via lipid peroxidation of arachidonic acid, an abundant omega-6 PUFA found in the lipid bilayer in brain [27]. Arachidonic acid is released through cleavage of a phospholipid by phospholipase A2 (PLA2) and serves as a second messenger involved in cellular signaling and the regulation of signaling enzymes [29–31]. HNE binds proteins by Michael addition to certain amino acids resulting in protein dysfunction [2]. A β itself has been shown to be a source of ROS and an initiator of free radical damage to biomolecules in brain including lipid peroxidation [32–39].

A free radical in its natural form, \bullet NO is synthesized from L-arginine by endothelial, neuronal, or inducible nitric oxide synthases (NOS). \bullet NO has beneficial effects such as a biological mediator in several processes including neurotransmission, vascular smooth muscle relaxation as well as having antitumor, and antimicrobial activities [40,41] but becomes toxic at high concentrations [42]. \bullet NO and $O_2\bullet$ exert more damaging effects when they react to form ONOO $^-$ [13]. Although the reaction of \bullet NO and molecular oxygen or $O_2\bullet$ may proceed relatively slow in the cytoplasm, the ability of these radical molecules to diffuse through lipid membranes, such as the mitochondrial membranes where large amounts of oxygen reside, has been shown to accelerate the rates \bullet NO autoxidation many times over [43,44]. This increase in autoxidation may be attributed to the 'lens effect' that focuses \bullet NO and $O_2\bullet$ within hydrophobic compartments, not only decreasing the distance they must travel to react with each other and their target proteins, but also increasing the rate of autoxidation [43].

Nitration of tyrosine occurs from the reaction \bullet NO with $O_2\bullet$ in the presence of CO $_2$ by radical–radical recombination producing the reactive intermediate peroxyxynitrite, leading to tyrosine phosphorylation site at the 4-position thereby altering regulation of protein activity by tyrosine kinases and leading to changes in protein function [45–49]. In addition to \bullet NO $_2$, \bullet OH is another potential breakdown product of ONOO $^-$ [49].

MnSOD itself has been shown to be susceptible to tyrosine nitration in the active site of the enzyme [22,46,50], potentially altering the affinity of MnSOD for the $O_2\bullet$ substrate by changing the redox potential of the active site [45]. Tyrosine nitration leads to damaged MnSOD and subsequent mitochondrial dysfunction [22,50].

Increases in 3-nitrotyrosine (3NT) and protein-bound HNE have been found even in the brain of early AD subjects [51,52]. The brain is rich in PUFA and has areas rich in iron. These facts coupled with high oxygen usage and a low antioxidant capacity make the brain particularly susceptible to oxidative damage.

3. APP processing

Central to the A β -induced oxidative stress hypothesis is the generation of A β from its precursor protein, APP, a type I transmembrane protein that is conserved and expressed in many tissues. The isoform of APP preferentially expressed in the CNS is 695 amino acids in length and is heavily concentrated at the synaptic cleft [53]. The exact physiological role of APP remains undetermined, but this protein is thought to play a role in cell growth, neurite outgrowth, cell adhesion, cell signaling and cell survival [53–55]. The proteolytic processing of APP primarily occurs during its anterograde transport along neuronal axons by one of two pathways: non-amyloidogenic and amyloidogenic, the latter producing the neurotoxic A β fragment implicated in AD [56]. Amyloidogenic and non-amyloidogenic pathways are mediated by the actions of β - and γ - and α -secretases, respectively (Fig. 2).

Proteolytic cleavage of APP at position 17 by α -secretase initiates the non-amyloidogenic pathway, producing a membrane-bound C-terminal fragment (CTF α) and the large, soluble fragment, APPs α , from the N-terminal domain of APP [57]. APPs α has been demonstrated to have neurotropic and neuroprotective effects [58,59]. The primary α -secretase in the CNS is a disintegrin and metalloproteinase 10 (ADAM10), a membrane anchored and zinc dependent protease that has been revealed to be regulated by synaptic activity [60].

Further, synapse-associated protein-97 (SAP97) is a necessary protein mediating the trafficking of ADAM10, thereby promoting the non-amyloidogenic pathway by binding to α -secretase and directing it to the post-synaptic membrane where it cleaves APP inside the A β domain of APP. This process negates the amyloidogenic pathway [61]. Moreover, SAP97 expression and activity have been shown to be altered in AD [62,63]. Additionally, clathrin-mediated endocytosis (CME) of ADAM10 is a regulatory process that has been shown to diminish the non-amyloidogenic processing of APP by removing it from its cellular proximity — the plasma membrane [60].

The amyloidogenic pathway is initiated by the proteolytic action of β -secretase at position 671, thereby producing, the large, soluble APPs β (β -secretase-cleaved soluble APP) and the C-terminal fragment (CTF β), C99 [64]. The APPs β fragment may have multiple detrimental effects, such as impairing anterograde axonal transport leading to axonal dystrophy and neuronal cell death, as well as binding to death receptor 6 to recruit caspase 6 to initiate the extrinsic pathway of apoptosis [65,66]. The predominant neuronal β -secretase is β -site APP cleaving enzyme (BACE1), a type I transmembrane aspartyl protease — an enzyme that has been shown to be elevated in AD [67,68]. Further, hydrogen peroxide stimulated oxidative stress increases the activity of the enzyme; and, the presence of 4-hydroxynonenal has been reported to increase the expression of BACE1 [64]. BACE1 has been associated with lipid rafts. Moreover, an increase of membrane cholesterol reportedly recruits APP to lipid rafts, which increases APP-BACE1 proximity, promoting the amyloidogenic processing of APP [69]. In contrast, non-amyloidogenic proteolysis occurs outside of lipid rafts.

The next step involved in both the non-amyloidogenic pathway and amyloidogenic pathway in the proteolytic processing of APP is the cleavage of the CTF α and CTF β , fragments, respectively, by γ -secretase. Gamma secretase is an intra-membrane protease complex consisting of a quartet of proteins: presenilin, nicastrin, anterior pharynx-defective 1 (APH1) and presenilin enhancer 2 (PEN2) [70]. Further, γ -secretase is hypothesized to proteolytically cleave the remaining APP fragment at multiple sites in a step-wise manner within the transmembrane domain (TMD) of CTF α and CTF β , thereby releasing p3 (non-amyloidogenic pathway), A β (amyloidogenic pathway) and APP intracellular domain (AICD) fragments [71]. In the amyloidogenic pathway in particular, this begins with the cleavage at the ϵ -site at either position 48 or 49 on C99, which releases the AICD fragment while the A β remains membrane-bound [70]. Next, it is thought that γ -secretase further cleaves the A β fragment every other 3 or 4 amino acids from the ζ -site to the γ -site until the A β fragment is freed from the membrane [70]. This leads to the production of A β fragments of varying sizes, from 37 to 46 amino acids in length (with heterogeneous C-termini), including neurotoxic A β (1–42), which can easily oligomerize and become more prone to aggregation [72]. Furthermore, mutations in APP and the PS1 protein in γ -secretase can lead to changes in this cleavage pattern [71].

4. A β -induced oxidative stress model

It has long been established that the role of oxidative stress in AD is a critical one that leads to the damage of vital cellular components such as proteins, lipids, and nucleic acids [20,27,73,74]. This damage if left unchecked is a primary reason for the eventual degeneration of neurons, possibly through apoptotic means [75]. The A β -induced oxidative stress hypothesis proposed by our laboratory and others [76,77], states that A β and the damage it initiates are the principal means underlying this injurious increase in oxidative stress observed in AD brain.

As discussed above, APP may be processed into two major isoforms of A β by way of β - and γ -secretases; A β (1–40) and A β (1–42). Studies have demonstrated that as AD progresses, the levels of A β (1–40) in CSF remains relatively constant, while in contrast, levels of A β (1–42) in CSF decrease but levels of A β (1–42) increase in senile plaques [78]. This distribution has been attributed to the possibility of an efflux

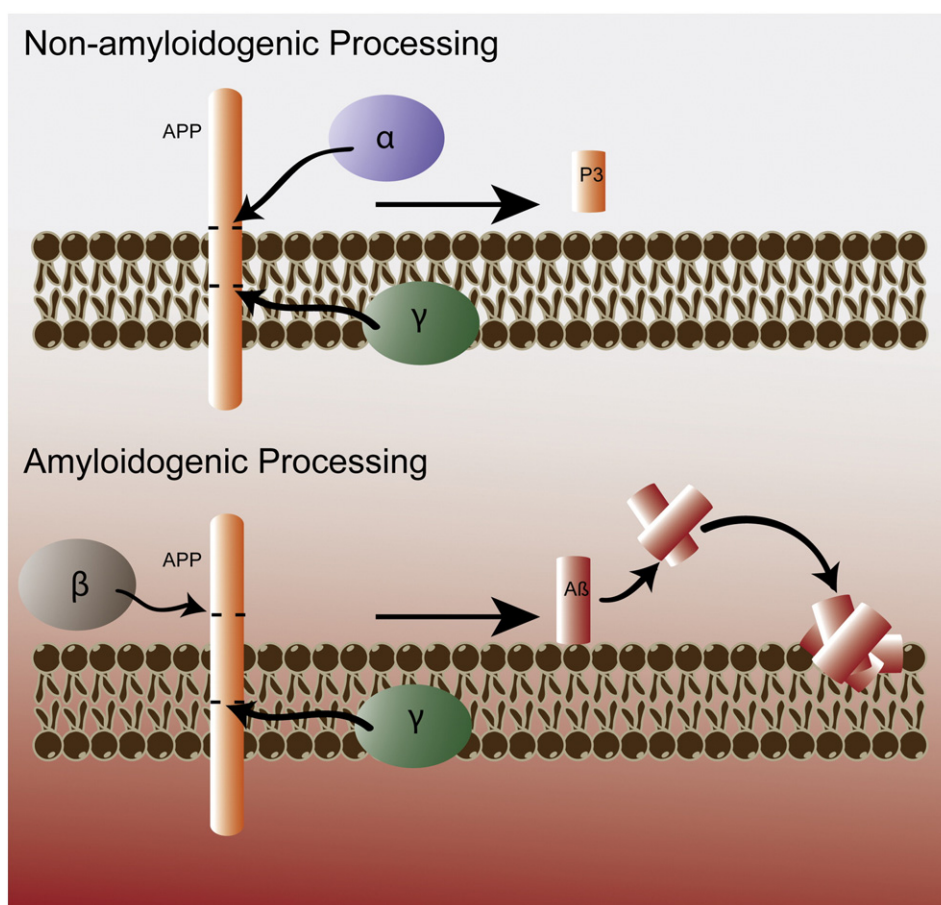


Fig. 2. Proteolytic processing of APP via the non-amyloidogenic and amyloidogenic pathways. The latter produces neurotoxic and oxidative stress-producing A β -peptide. See text for further details.

deficiency for A β (1–42) which may play a role in plaque formation [79]. Previously, plaques were perceived to be the primary pathogenic element of AD, yet more recently evidence provided insights into the notion that plaques may be an extra-cellular storage site for cells to deposit excess A β , suggesting that the real damaging agent, may be a much smaller aggregate form of A β (1–42) oligomers [80,81]. Research has shown that while plaques do not correlate with cognitive dysfunction in AD, soluble oligomers do [82]. More recently, researchers have discovered in AD that distinct assemblies of A β oligomers impair cholinergic neurotransmission [83].

The A β -induced oxidative stress hypothesis places the majority of the causative effect of increased cellular oxidative stress upon oligomeric A β (1–42), as it is believed that only the oligomers are viable to insert into the lipid bilayer wherein they may form alpha-helices to begin the proposed catalytic ROS production that may lead to the lipid peroxidation and protein oxidation found in AD [84].

4.1. Met-35 and A β -induced oxidative stress

While studies have found that oligomeric A β (1–42) correlates with increased oxidative stress, the exact method by which this occurs is still under debate [82]. Strong evidence has been put forth by our lab and others that implicates the methionine-35 (Met-35) residue of A β (1–42) in the process of ROS generation. Since A β (1–42) is cleaved from APP, a transmembrane protein, our lab proposes that A β (1–42) oligomers are capable of re-entering the lipid bilayer where they may adopt an alpha-helical structure. In doing so, Met-35 would interact with the carbonyl of Ile-31 according to the $i + 4$ rule of alpha-helices [85]. It is hypothesized that because the oxygen of the

carbonyl group is more electronegative than the sulfur of Met-35, the electron lone pairs on Met-35 are primed for oxidation by an extrinsic factor.

Once the A β oligomers insert into the bilayer, the hydrophobic environment lends itself towards the stabilization of a one-electron oxidation of an already primed Met to the sulfuranyl free radical [MetS⁺] [86]. It is this sulfuranyl free radical that is hypothesized as being the initiator in a series of free radical chain reactions that take place within the lipid bilayer that generates lipid peroxidation products and oxidatively modified membrane proteins [86,87] (Fig. 3). Substitution of amino acids in critical positions within the A β (1–42) peptide negate the injurious effects of A β (1–42). For example, the substitution of Gly with Asp at residue 37 of A β (1–42) imparts a negative charge to the A β (1–42) peptide that excludes the peptide from the lipid bilayer [88]. Another substitution that replaces Ile-31 with the known α -helix breaker, proline, and again the oxidative stress of the peptide is prevented, consistent with our hypothesis that the α -helical structure of A β (1–42) in the lipid bilayer is important for the production of oxidative stress [85].

Transgenic *Caenorhabditis elegans* that express human A β (1–42) demonstrated increased oxidative stress that was nullified by the substitution of Met-35 with another sulfur containing amino acid, Cys, in an attempt to demonstrate the differences in chemistry of the two sulfur atoms and their associated residencies (thioether vs. thiol) [89]. In an in vitro study, Met-35 in A β (1–42) was substituted by norleucine, i.e., a methylene moiety for the S-atom of Met, to produce [A β (1–42) M35NLE]. This substitution produced a mutant peptide with an amino acid of similar length and hydrophobicity as the original Met-35. A β (1–42)M35NLE was unable to induce toxicity through oxidative stress by way of free radical generation [89–92].

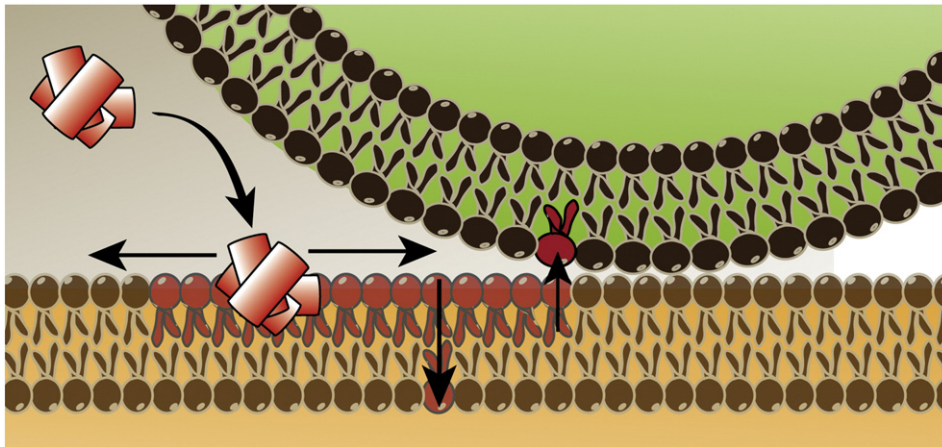


Fig. 3. Insertion of oligomeric A β (1–42), a peptide rich in hydrophobic amino acids, into lipid bilayers leads to lipid peroxidation reactions with subsequent protein modification from lipid peroxidation products. See text for further details.

The J20 mouse, which is a transgenic mouse with human APP containing Swedish (KM670/671NL) and Indiana (V717F) mutations, showed elevated A β (1–40/42) deposition and increased oxidative stress in the brain [93]. Introduction of a third mutation to APP, Met631Leu, corresponding to the Met-35 residue of A β (1–42), resulted in no oxidative stress in brain of these mice at 9 months of age [94]. This results demonstrated *in vivo* in a mammalian model what had been seen earlier in a worm model: Met-35 of A β (1–42) is essential for oxidative stress *in vivo* in AD models, and presumably in AD brain as well.

Important to note are other findings that provide evidence contrary to the Met-35 centric hypothesis such as research conducted that used A β (25–35) instead of A β (1–42) with a substitution of Met-35 with norleucine at the C-terminal position that did not abrogate the oxidative induced by the peptide [95]. These data, however, should be read with the understanding that a C-terminal Met displays altered chemistry from a Met within the α -helix [96].

5. Proteomics applications in AD and models thereof

Proteomics is the study of the proteome, meaning that proteomics studies view the entirety of all proteins present in a given system at any given point in time. Proteomics is far more complex than genomics as it includes all isoforms of a protein, their structure and post-translational modifications as well as protein–protein interactions [97]. In addition, the proteome is not static; it is subject to change during development and in response to various events such as oxidative stress, disease or drug administration. Therefore, proteomics can be applied to compare the proteome of control vs. treated samples or healthy controls vs. a disease state. Knowledge of the affected proteins can help in gathering insights into pathways and cellular mechanisms of a disease and also can help in developing interventions or therapeutic strategies. In addition to providing information on up- or down-regulated proteins (expression proteomics), proteomics techniques can be applied to look at changes in post-translational modifications (e.g., phosphoproteomics). Furthermore, our laboratory pioneered a proteomics technique, redox proteomics (Fig. 4), that can specifically identify differentially oxidized proteins in a given sample [98–100].

Gel-based proteomic studies generally consist of two main steps: In the first step, the sample is separated, e.g., by two-dimensional gel electrophoresis by which the proteins are separated based on their net charge, or isoelectric point, and subsequently by their migration rate in a polyacrylamide gel. The second step consists of identifying the proteins identified by mass spectrometry and data base inquiry. For redox proteomics, an additional step is used in which gel

electrophoresis is followed by Western blot analysis with oxidation marker-specific antibodies (for comprehensive reviews see [98,100]).

Proteomics has been used extensively by our laboratory and others in the field to analyze the effects of A β -mediated oxidative stress in AD models as well as brains from subjects of different stages of AD. Some of these studies and their findings are summarized below.

5.1. A β in cell culture

Early studies have shown that A β (25–35) can produce free radicals in solution [101] or synaptic membranes [102] and that the addition of (pre-incubated/aggregated) A β (1–40) to neurons increases intracellular ROS formation and increases the levels of protein carbonyls [33,37,89]. Many proteomic studies have used this approach, the addition of A β -peptides to a system, to identify proteins that are affected by A β -induced oxidative stress. These studies have shown that A β -peptides change protein levels and lead to the oxidation of specific enzymes.

Treatment of neurons with oligomeric A β (1–42) decreased the levels metabolic enzymes involved in energy production (e.g., citrate synthase) and increased molecular chaperones such as Hsp70 [103]. In primary neurons A β (1–42) treatment also lead to the oxidation (measured as protein carbonyls) of proteins associated with energy metabolism (e.g., GAPDH) as well as regulatory and structural proteins [104–106]. Interestingly, increasing the cells' antioxidant defense systems by pre-incubation of cells with a glutathione precursor or mimetic significantly reduced overall A β -induced protein oxidation (measured as protein carbonyls) as well as specifically inhibited the oxidation of four enzymes: GAPDH, 14-3-3 zeta, malate dehydrogenase and pyruvate kinase [104,105]. In a different study, pretreatment with Vitamin E, a lipid-soluble free radical scavenger also inhibited protein oxidation and lipid peroxidation caused by A β (1–42) incubation [33,107].

Since inflammation is observed in AD brain, a recent study analyzed the effects of A β (25–35) on microglia and found that A β (25–35) activated immortalized microglia and induced down-regulation of metabolic enzymes, redox proteins (e.g., peroxiredoxin 3 and 4) and chaperones [108]. A caveat of these and other studies that used A β (25–35) is that the C-terminal Met-35 of Ab(25–35) has a different chemistry than intrachain Met-35 in AD-relevant A β (1–42) [96]. It is our view that only the AD-relevant peptides should be used in studies of importance to AD, since (a) such peptides are known in AD brain, but (b) A β (25–35) has not been detected in AD and is not relevant to the disease per se but only of academic interest [96].

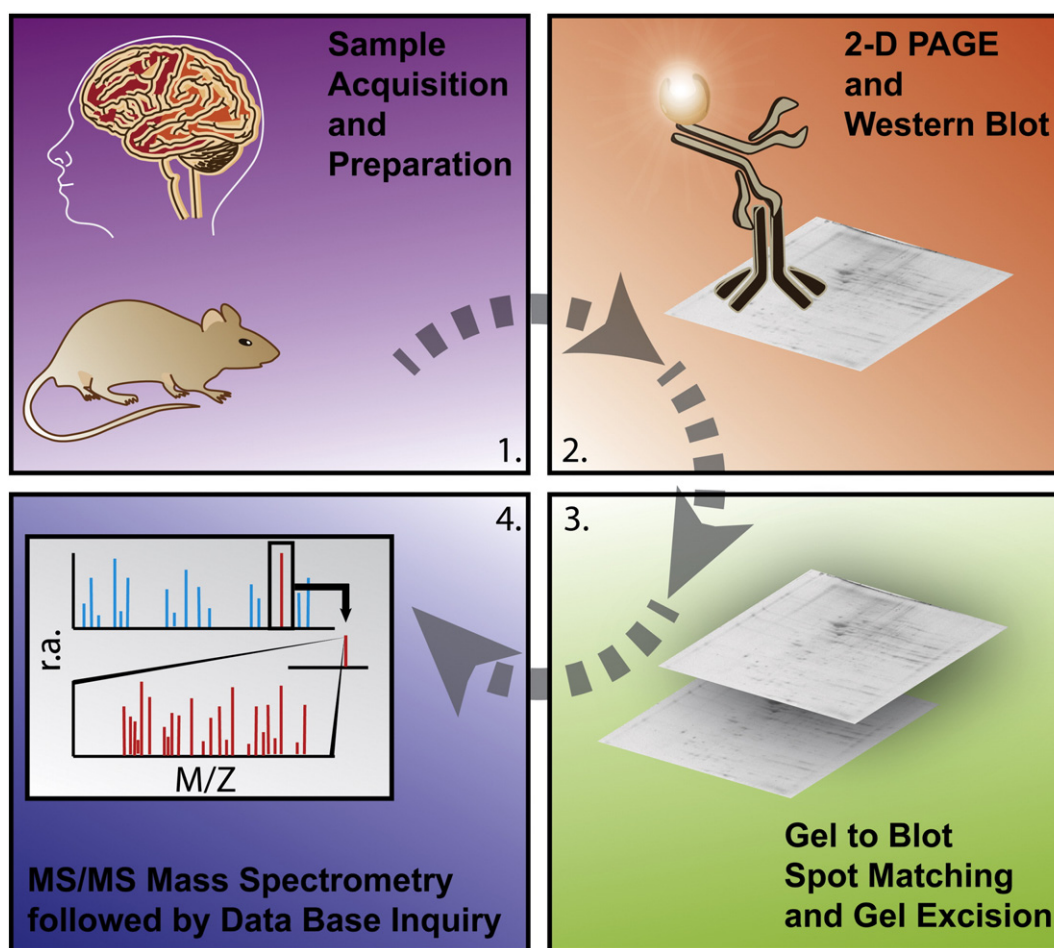


Fig. 4. Schematic illustration of the principal steps involved in redox proteomics used to identify oxidatively modified proteins. See text for further details.

5.2. *C. elegans* AD model

Transgenic *C. elegans* expressing human A β (1–42) have been previously shown to present with increased oxidative stress prior to A β fibrillar deposition [109]. Using this information, another study of transgenic *C. elegans* with the application of redox proteomics was successful in identifying a number of specifically oxidized proteins [110]. Of the oxidized proteins found, many are important in energetic metabolism (i.e., malate dehydrogenase and Acyl-CoA dehydrogenase), antioxidant defense (i.e., glutathione S-transferase), and proteasome function (i.e., proteasome beta subunit).

5.3. AD mouse models

Since the discovery of mutations causing familial AD, different transgenic mouse models have been established to study cellular mechanisms relevant to AD. The human double-mutant APP/PS-1 knock-in mice carry a PS-1 mutation found in familial AD as well as a mutation in the APP gene to humanize the mouse A β sequence. Gene expression is controlled by the endogenous mouse promoters for APP and PS-1 [111]. These mice show increased levels of protein oxidation and lipid peroxidation [112] as well as changes in protein expression when compared to wild-type mice. In particular, proteins involved in energy metabolism such as GAPDH and enolase had decreased levels in APP/PS-1 mice [112–114]. Additional redox proteomics studies comparing APP/PS-1 to wild-type mice showed an age-related significant oxidation of enolase and other proteins (e.g., 14-3-3, actin and Pin-1) [115]. Oral administration of the glutathione precursor N-acetyl cysteine decreased brain protein oxidation and lead to increased levels

of enolase indicating that antioxidants, as shown in cell culture studies, could ameliorate the deleterious effects of A β -induced oxidation in the brain in vivo [113–115].

The SAMP8 mouse, or senescence-accelerated prone mouse, is a mouse model of AD used that shows age-dependent learning and memory deficits and A β accumulation [116], as well as increased oxidative stress [117]. Redox proteomics of 12 month old SAMP8 mice has been used to identify many proteins that are specifically carbonylated in this mouse model for oxidation which include: α -enolase, collapsin response mediator protein-2 (CRMP-2/DRP-2), lactate dehydrogenase (LDH-2), α -spectrin, and creatine kinase (CK). Expression proteomics was also used to determine a decreased expression of critical proteins such as LDH-2, triphosphate isomerase (TPI), α -spectrin, and neurofilament (NF-L) [118]. Many of these proteins affected are vital to both the production of energy as well as the structural organization of the cell, both of which are vital to the neuron, a cell with high energy demand that must maintain the ability to transport vesicles and mitochondrion the length of the cell and to reorganize itself when constructing and deconstructing synapses.

5.4. A β injection in animal models

Intracerebral injection of A β (1–40) leads to behavioral deficits in rats and decreased levels of different proteins, among others proteins associated with ATP production and cytoskeletal structure [119]. Another study used A β (1–42) injections into the nucleus basalis magnocellularis (NBM) of rats followed by a proteomic analysis. The cortex, NBM, and hippocampus were all shown to exhibit significant protein oxidation, affecting proteins such as glutamine

synthetase, 14-3-3 zeta, beta-synuclein, pyruvate dehydrogenase, phosphoglycerate mutase 1 and glyceraldehyde-3-phosphate dehydrogenase [38]. While other groups have reported results from similar experiments, such as intrahippocampal injections of A β [120], the results of Boyd–Kimball et al. are significant on their own as they highlight that injection of A β (1–42) into one brain region has consequences for other regions of the brain [38].

5.5. Human studies

While animal models are adequate and convenient substitutions for human disease, the results have to be interpreted with respect to the facts that the cell biology of humans and the majority of laboratory animal specimens have differences that can make a direct comparison difficult. This inherent difficulty is what makes human proteomic studies so valuable. Proteomic studies in brains of subjects with various stages of AD have identified proteins involved in a variety of cellular pathways to be expressed at differential levels in AD brain when compared to controls. At the University of Kentucky, we are fortunate to have autopsy-derived specimens obtained at a short post-mortem interval (PMI), typically 2–4 h, making comparison to living brain as feasible as possible. In contrast, long PMIs have for some time been reported in the literature, and one wonders what can be realistically and meaningfully determined with such samples?

Proteomics studies from our laboratory have shown that some proteins, like Pin-1 and β -tubulin, were significantly decreased while others, like enolase and heat shock protein 70, were significantly increased in AD [121]. A proteomics study focusing only the detergent-insoluble proteins also found significant changes in samples from AD subjects when compared to controls. Among others, protein levels of 14-3-3 and GAPDH were altered in the detergent-insoluble fractions [122]. Supporting the notion that oxidation of proteins may render them less soluble and thus more prone to aggregation, GAPDH (among others) was shown to be oxidized in AD [123]. A number of redox proteomics studies from our laboratory and collaborators have identified a variety of proteins that are extensively oxidized in AD brain, including, among others, carbonylation of ubiquitin carboxy-terminal hydrolase L-1 (UCHL-1) [124], nitration of enolase [123] and carbonylation and loss of CRMP-2 [125], a protein found to be intricately involved in axonal growth and plasticity, a process heavily impaired in AD, while also being associated with neurofibrillary tangles [126]. In a similar fashion to protein Tau in AD brain, CRMP-2 has been shown to not only be phosphorylated by GSK3 β and CDK5 [127], but is also localized to NFTs [126,128]. A systematic depletion of CRMP-2 via insoluble tangle formation would likely have detrimental effects on the ability of an AD affected neuron to maintain or repair the synaptic connections lost during the disease progression.

5.6. Summary of proteomic data

In summary, proteomic results from cell culture to animal models to human studies identified a number of proteins to be affected by the presence of A β , supporting the notion that A β -mediated oxidative stress contributes to the perturbations seen in a variety of cellular mechanisms in AD. Since some proteomics studies identified a number of afflicted proteins, rather than discussing an exhaustive list of proteins, only a few commonly recurring ones have been mentioned in this review. Throughout the studies of the various animal models and AD brain, a common theme emerges in the form of affected cellular functions. The proteins that show either altered levels or oxidation or both can be summarized into the following functional groups:

- Energy metabolism (i.e., GAPDH, enolase, ATP synthase)
- Signal transduction (i.e., 14-3-3)
- Synaptic function (i.e., SNAPs)
- Structural proteins (i.e., actin, tubulin, DRP2/CRMP2)

- Proteasome function and protein clearance (i.e., UCHL-1)
- Stress response/chaperones (i.e., HSPs and peroxiredoxins)
- Cell cycle, Ab production and tau hyperphosphorylation (i.e., Pin-1)
- Mitochondrial function (i.e., ATP synthase, VDAC).

This overview provides a glimpse into the AD-affected proteome, information that if not for the use of proteomics would have been difficult or nearly impossible to collect. Many of the proteins affected by A β presented herein contribute to vital cellular functions, and the dysfunction or loss of these proteins are consistent with the pathology and clinical presentation in AD. For instance, the fact that GAPDH and enolase, both glycolytic enzymes with multiple additional functions such as cell signaling [129,130], are affected by A β , supports a role of A β in the observed energy impairment in AD [131] while other proteins such as CRMP2 play important roles in cellular organization and structure.

6. Dietary antioxidants and AD biomarkers

Proteomic changes due to A β -mediated oxidative stress can be significantly modulated by pretreatment of cells with different antioxidants. In agreement with cell culture studies, treatment with antioxidants ameliorates negative effects associated with A β in different animal models of AD. Unfortunately, clinical trials and epidemiological studies in AD have produced inconsistent results. While in the Rotterdam study high dietary intake of Vitamins C and E was associated with a lower risk for AD [132], a study in elderly subjects found no association between carotenes, vitamins C or E and a decreased risk of AD [133]. Vitamin E supplementation in MCI patients proved to have no benefit [134]. While it has been shown that antioxidant based treatment in synaptosomes and rodents appears to have ameliorating effects in regard to A β induced oxidative stress production [33,135,136], the lack of efficacy in human studies indicates a lack of a complete understanding in either the mechanisms of A β induced oxidative stress or of the inability of current antioxidant therapies to be effective where they are needed. Regardless, this avenue of research must be thoroughly investigated.

As seen in animal studies (APP/PS-1 mice) [113] the starting point of the intervention influences the outcome, and since A β -mediated oxidative stress is present long before any clinical and pathological hallmarks of AD are detectable, it may prove to be necessary to start therapies years if not decades before symptoms are evident. The availability of reliable AD biomarkers would improve AD diagnostics dramatically; not only could AD be diagnosed unequivocally ante mortem, but it may enable an earlier diagnosis and therefore allow for earlier treatment of people at risk of developing AD. Additionally, the effects of interventional strategies and drug efficacy could be monitored. Unfortunately, reliable biomarkers for AD diagnostics are yet unavailable. Due to its proximity to the brain, much research has focused on cerebrospinal fluid for biomarker identification (e.g., [137]) but the caveat with this strategy is the necessity of a lumbar puncture to obtain the sample. An easily accessible source for potential biomarkers is blood. Given that 80–85% of the plasma proteome consists of albumin and IgGs, AD biomarkers in blood likely would be far less concentrated than in cerebrospinal fluid. To overcome this challenge, a recent study depleted plasma samples of highly abundant proteins and applied a series of fractionation steps before proteomic identification of potential plasma biomarkers [138].

One other approach may be to look at other blood elements. For example, our laboratory recently demonstrated elevated oxidative stress in mitochondria isolated from peripheral lymphocytes from patients with AD [139] and aMCI [140]. The latter study also demonstrated, using proteomics, differential levels of mitochondrial proteins in both AD and aMCI that are consistent with the known mitochondrial alterations in these disorders. Consonant with these findings, and noting that oxidation plays an important role AD coupled with the need for reliable biomarkers for AD, including differentially oxidized

proteins, redox proteomics may be a promising approach (for further comprehensive reviews see [141,142]).

7. Conclusion

Redox proteomics has become an invaluable tool in not only the study of AD, but in many other areas of study in which oxidative stress is believed to be central to disease onset [98]. While the ability to treat a disease is of grave importance, the understanding of how a disease is initiated and progresses may have implications into many different diseases with similar pathology. Data gathered from proteomics studies play a role in this understanding of disease pathology, providing valuable information into the molecular basis of the cell that might otherwise go unnoticed. In its application to AD, proteomics has shown that energy metabolism and structural proteins, among others, are primarily affected by the increase in oxidative stress observed in AD. These cellular processes are important in their maintenance of a healthy cellular environment, while their absence has been demonstrated to be deleterious. Further proteomics studies into the efficacy of antioxidant treatment prior to disease onset should be conducted in order to fully test the oxidative stress hypothesis of AD. Moreover, while biomarkers and antioxidant treatment may possibly contribute to a better disease outcome, general awareness of preventative lifestyle changes by the general populace may also play its role in disease prevention, as many lifestyle factors have been implicated in cognitive performance, which may delay or possibly prevent disease onset [143–145].

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