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REVIEW ARTICLE

# Oxidative stress in Alzheimer disease and mild cognitive impairment: evidence from human data provided by redox proteomics

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**Abstract** Alzheimer disease (AD) is a neurodegenerative disease with many known pathological features, yet there is still much debate into the exact cause and mechanisms for progression of this degenerative disorder. The amyloid-beta (Aβ)-induced oxidative stress hypothesis postulates that it is the oligomeric Aβ that inserts into membrane systems to initiate much of the oxidative stress observed in brain during the progression of the disease. In order to study the effects of oxidative stress on tissue from patients with AD and amnestic mild cognitive impairment (MCI), we have developed a method called redox proteomics that identifies specific brain proteins found to be selectively oxidized. Here, we discuss experimental findings of oxidatively modified proteins involved in three key cellular processes implicated in the pathogenesis of AD progression: energy metabolism, cell signaling and neurotransmission, as well as the proteasomal degradation pathways and antioxidant response systems. These proteomics studies conducted by our laboratory and others in the field shed light on the molecular changes imposed on the cells of AD and MCI brain, through the deregulated increase in oxidative/nitrosative stress inflicted by Aβ and mitochondrial dysfunction.

**Keywords** Alzheimer disease · Mild cognitive impairment · Amyloid-beta · Reactive oxygen species · Redox proteomics

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**Abbreviations**

| | |
|---|---|
| 3NT | 3-Nitrotyrosine |
| AD | Alzheimer disease |
| Aβ | Amyloid-beta |
| CRMP2 | Collapsin response mediator protein-2 |
| EAD | Early-onset Alzheimer disease |
| ESI–MS/MS | Electrospray ionization tandem mass spectrometry |
| FAD | Familial Alzheimer disease |
| GSH | Glutathione |
| HNE | 4-Hydroxynonenal |
| IPL | Inferior parietal lobule |
| LAD | Late-onset Alzheimer disease |
| MCI | Mild cognitive impairment |
| NFT | Neurofibrillary tangle |
| PC | Protein carbonyl |
| PCAD | Preclinical Alzheimer disease |
| PET | Positron emission tomography |
| Pin1 | Peptidyl-prolyl *cis/trans* isomerase NIMA-interacting 1 |
| PMI | Postmortem interval |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SP | Senile plaque |
| UCH L-1 | Ubiquitin carboxy-terminal hydrolase L-1 |

## Introduction

Alzheimer disease (AD) is a devastating neurodegenerative disease and the primary known cause of dementia. While there are many factors that go into the development of AD, the one primary risk factor is aging. It is important, however, to make the distinction between normal aging and AD as most individuals do not develop AD as a part of their

aging process, though as medicine continues to advance in effectiveness and the prolonging of the normal age span, the rate of AD development is expected to continue to increase (Hebert et al. 2003).

Alzheimer disease is clinically characterized by a loss of cognitive ability and short-term memory formation. Patients may be subjected to the Mini-Mental Status Examination (MMSE) if their clinician suspects abnormal cognitive performance. Pathologically, AD is a diverse and complex disease affecting nearly every subcellular system of the affected neurons (Oddo 2008; Tu et al. 2014; Moh et al. 2011; Wilkins et al. 2014). One primary pathological characteristic has been identified as being an accumulation of amyloid-beta 1–40 and 1–42 ($A\beta_{1-40}$ and $A\beta_{1-42}$) though the primary amyloidogenic product implicated in AD has been $A\beta_{1-42}$ (Glabe 2001; Walsh and Selkoe 2004). Aβ has long been known to form extracellular agglomerations termed senile plaques (SPs), but more recently it has been found that the more toxic forms of Aβ are not the insoluble (SPs) but rather more soluble Aβ oligomers (Glabe 2005). It is these oligomers that are proposed to do the most oxidative damage to the cell, perhaps due to their ability to translocate into membrane systems such as the cell membrane and the mitochondrial membrane. Aside from Aβ, hyperphosphorylated tau has been shown to lead to microtubule destabilization and formation of neurofibrillary tangles (NFTs) (Braak and Braak 1997; Andreadis et al. 1992). NFTs are composed primarily of paired helical filaments of hyperphosphorylated tau, and the severity of an AD patient's dementia is correlated closely with NFT density (Braak and Braak 1997). The loss of tau and the subsequent destabilization of microtubules may play a large role in the pathogenesis of AD due to a loss in structural integrity and methods for intracellular trafficking of vesicles and organelles which rely in part on organized microtubule networks to function properly.

While Aβ and NFTs have been the focus of the majority of AD research since its official recognition, there are many other systems, as previously discussed, that go awry in AD such as metabolism, insulin signaling, the proteostasis network, synaptic growth and maintenance, cell cycle control, and the antioxidant response system to name a few. While systems affected may at first seem disparate, there is a common unifying theme in oxidative/nitrosative stress and altered redox state of the cell (Butterfield et al. 2013).

Alzheimer disease can be further categorized into three primary and one alternate stagings: amnestic mild cognitive impairment (MCI), early-onset AD (EAD), late-stage AD (LAD), and preclinical AD, respectively. MCI is often referred to as the first stage or transition stage of AD, and it is the stage in which the patient and those around them begin to first notice signs of memory loss, yet not all patients with MCI transition to AD (Jicha et al. 2006). Aβ load and NFT density increase in number and concentration through the progression of MCI to LAD, with LAD presenting with the highest loads and subsequently the lowest neuron density and highest cognitive impairment and ultimately death. PCAD is an unusual stage in which the patients has significant levels of Aβ and NFT, yet perform normally in cognitive testing and activities of daily living (Bradley et al. 2010). PCAD has of late been the focus of experimentation for some in order to determine the differences in patient outcome, though PCAD is currently difficult to study due to the necessity to determine Aβ and NFT density by PET scanning methods, so subjects are usually identified only after death from another means (Aluise et al. 2011).

## Oxidative stress

The maintenance of the cellular redox state is of utmost importance in regard to not only the normal functioning of the cell, but more importantly its survivability. The basis of the cellular redox state is composed of molecules termed oxidants and reductants, based on their chemical ability to oxidize or reduce substrate molecules. Common oxidants known to participate in the redox state are molecules such as hydrogen peroxide ($H_2O_2$), superoxide ($O_2^-$), the hydroxyl anion ($OH^-$), the hydroxyl radical (OH), nitric oxide (NO), and peroxynitrite ($ONOO^-$) (Dasuri et al. 2013). Many diseases such as AD have been found to have a dysregulation of oxidant levels which have been implicated in the disease pathogenesis (Butterfield and Sultana 2011; Nourazarian et al. 2014; Indo et al. 2015; Tangvarasittichai 2015). This loss of homeostasis can both be attributed to an over-production of oxidants as well as a loss of antioxidants or the antioxidant defense system. Within the brain, the primary means of dealing with an excess of oxidants is the antioxidant glutathione (GSH) and its supporting enzymes, glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-S-transferase (GST). A dysregulation in the redox state of the cell if left unchecked leads to oxidant-induced damage of organelles, membranes, and biomolecules such as proteins, lipids, and nucleic acids (Fig. 1) (Dasuri et al. 2013; Uttara et al. 2009; Halliwell 2012; Valko et al. 2007).

One major source of oxidant generation in AD is from the functioning of the mitochondria during oxidative phosphorylation in which it produces ATP for the cell. Healthy mitochondria have been reported to leak superoxide radical anion as a natural part of its normal functioning due to the imperfect efficiency of electron transfer through the electron transport chain (Dasuri et al. 2013; Adam-Vizi 2005; Calabrese et al. 2005). This superoxide radical leak has also long been attributed to one of the primary theories of

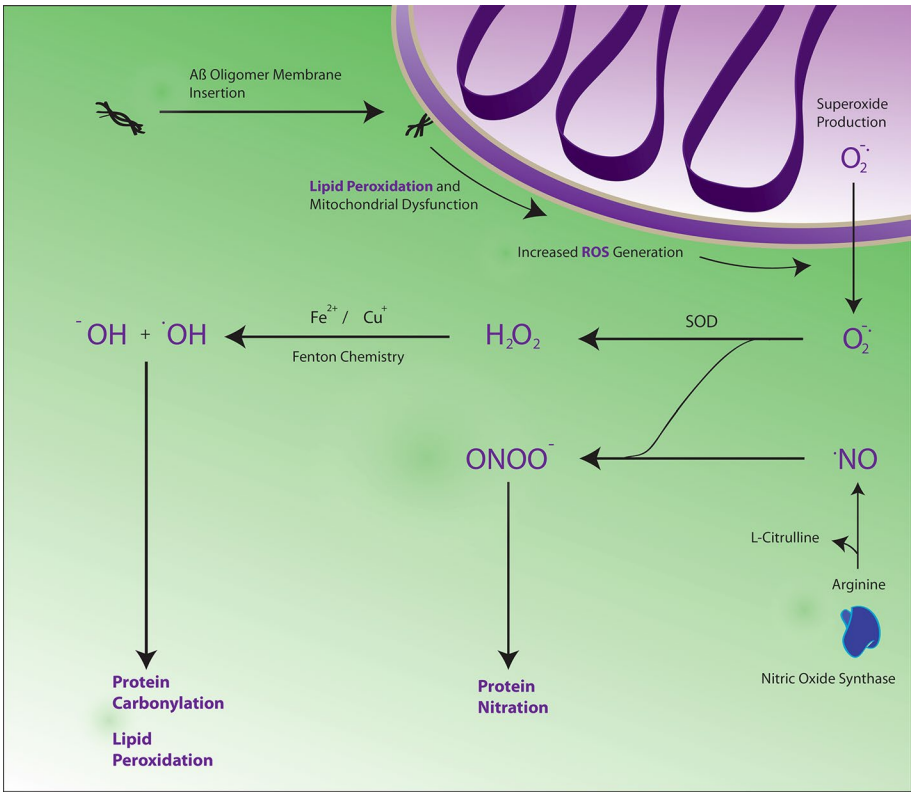


**Fig. 1** Proposed pathways involved in the production of ROS/RNS in Alzheimer disease that lead to protein oxidation/nitration and lipid peroxidation

normal aging. However, as AD progresses and mitochondria are damaged, the normal efficiency of the mitochondria also deteriorates leading to a feed-forward mechanism of superoxide production (Friedland-Leuner et al. 2014). Superoxide in and of itself is a potent oxidant, yet it has its limitations, one being that it cannot easily cross into membrane systems due to its negative charge. However, some suggest that the $H^+$ within the intermembrane space of mitochondrion leads to $HO_2^{\cdot}$,which, being neutral, can cross membranes (Hernandez-Saavedra and McCord 2007). In an attempt to rid the cell of superoxide, enzymes such as the mitochondrial-resident manganese superoxide dismutase (Mn-SOD) and the cytosolic copper/zinc-superoxide dismutase (Cu/Zn-SOD) reduce superoxide to hydrogen peroxide (Halliwell 2012). Hydrogen peroxide is a relatively weak oxidant; however, its lack of a dipole moment allows it to cross into and through membranes to other cellular compartments, whereas superoxide itself is essentially localized upon production. Hydrogen peroxide may also react with either copper (I) or iron (II) in a Fenton chemical reaction to produce the much more reactive hydroxyl radical that is accountable for much of the downstream damage of superoxide production (Halliwell and Gutteridge 1984). The production of hard nucleophiles such as hydroxyl radical leads directly to the damage of proteins and nucleotides.

The proper functioning of proteins is primarily the result of the integrity of its three-dimensional structure. Protein oxidation has implications for protein dysfunction or loss of function, depending on the severity and location of the oxidative damage to the protein (Butterfield 2002). Upon protein oxidation, proteins may lose their secondary and tertiary structural integrities which may lead to the exposure of hydrophobic amino acids to the protein surface causing aggregation and further unfolding and loss of activity (Dunlop et al. 2009; Dean et al. 1997). Direct oxidative insult to the peptide chain backbone may even result in fragmentation (Dean et al. 1997). The most common type of direct protein oxidation is that of protein carbonylation, in which an aldehyde or ketone is formed. Both the side chains of every amino acid along with the peptide backbone are susceptible to this form of oxidation, though the aromatic amino acids such as tryptophan and tyrosine are prone to generate hydroxy derivatives (Stadtman and Levine 2000). Among the more susceptible amino acids to oxidation are methionine and cysteine, with methionine being oxidized to form methionine sulfoxide (MeSOX) while the sulfhydryl group of cysteine may be oxidized to form disulfide bonds either intra or intermolecularly, though these forms of oxidation have their own enzymatic mechanisms of reversal (Dunlop et al. 2009). Moreover, cysteine can be oxidized to sulfenic (–SOH), sulfinic ($–SOH_2$), and sulfonic ($–SOH_3$) acids, the first being often reversible and involved in signaling (Wang et al. 2008; Nakamura et al. 2015).

Indirect protein oxidation may also occur in which the oxidant interacts with another molecule prior to an interaction with the actual protein (Suzuki et al. 2010). One common form of indirect protein oxidation is through the conjugation of reactive aldehydes to the protein. Reactive aldehydes such as acrolein or 4-hydroxynonenal (HNE) are α/β-unsaturated aldehydes which are the products of the lipid peroxidation of unsaturated fatty acids such as arachidonic acid. The brain is specifically prone to secondary carbonylation via lipid peroxidation due to the large concentrations of poly-unsaturated fatty acids (PUFAs) resident in brain tissue (Butterfield et al. 2010a, b). The process of lipid peroxidation is specifically toxic due to allylic hydrogen abstraction and subsequent radical chain reaction and propagation on acyl chains of polyphenols, processes that may spread between membrane systems and even adjacent cells (Butterfield et al. 2010a, b; Bader Lange et al. 2010; Castegna et al. 2004). Reactive aldehydes in the presence of nucleophilic protein side chains undergo Michael addition, resulting in indirect protein carbonylation. This covalent modification results in structural changes which likely results in protein activity and cause dysfunction (Perluigi et al. 2012).

Lipid peroxidation in the AD brain may be measured by the detection of elevated levels of free or protein-bound acrolein, HNE, isoprostane 8,12-iso-iPF$_{2}$α-VI, F$_{2}$-isoprostane (F$_{2}$-IsoP), and F$_{4}$-neuroprostane (F$_{4}$-NP) (Markesbery et al. 2005; Yao et al. 2003). A primary means of defense against lipid peroxidation products for the neuron is the ability of GSH to bind free HNE prior to reaction with a biomolecule of importance such as a protein (Lovell et al. 1998). Not only has it been shown that there was an increase in HNE-bound by GSH (HNE–GSH) in AD brain, but many of the enzymes responsible for the upkeep and maintenance of GSH levels, such as glutathione-S-transferase (GST) and multidrug-resistant protein-1 (MRP-1), were found to be modified by HNE themselves and subsequently a buildup of HNE-bound protein adducts (Lovell et al. 1998; Sultana and Butterfield 2004). This finding suggests that there is a significant increase in free and protein-bound HNE in AD which has overwhelmed the cells defense against reactive aldehydes. This deficiency in HNE clearance likely plays a role in HNE-modified proteins (Tables 1, 2, 3).

The detection of carbonylated proteins have had been simplified with the use of 2,4-dinitrophenylhydrazine (DNPH), which upon contact with a carbonyl may form a hydrazone, detectable through the use of immunochemical assay and antihydrazone adduct antibodies (Suzuki et al. 2010; Levine et al. 1994). Indirect carbonylation via reactive aldehyde addition is also detected immunochemically as well as through the use of spectrophotometry (Sayre et al. 1997; Lovell et al. 2001).

Nitric oxide is an important signaling molecule in regulating vascular smooth muscle relaxation, antixenobiotic actions, and postsynaptic density functions, among other processes (Shinde et al. 2000; Xu et al. 1994). However, nitric oxide itself is also a free radical which may become toxic if its concentrations become too great (McCarty 2006). Nitric oxide when in the presence of superoxide may react to form peroxynitrite ($ONOO^{-}$), a reactive anion (Williamson et al. 2002). The reaction between nitric oxide and superoxide in the cytoplasm is relatively slow in part due to the rarity of two radical molecules coming into contact in a radical–radical recombination step; however, if nitric oxide diffuses into the mitochondria, where large amounts of oxygen and superoxide reside, the rate of nitric oxide autoxidation has been shown to increase dramatically contributing to a "lens effect" wherein nitric oxide and superoxide are focused into hydrophobic compartments further increasing the rate of autoxidation (Moller et al. 2007; Liu et al. 1998). This production of peroxynitrite (in the presence of CO2) induces protein nitration, covalently modified onto tyrosine side chains in the 3′ position resulting in the protein nitration product, 3-nitrotyrosine (3-NT) (Ischiropoulos 2009). Nitration of the 3′ position likely contributes to an interruption in protein signaling due to the ability of tyrosine to be phosphorylated on the 4′ hydroxyl group in specific target proteins (Feeney and Schoneich 2012).

**Table 1** Summary of metabolically involved proteins discussed within the text, modifications identified, and the AD stage and/or brain region analyzed

| Identified protein | Modifications |
|---|---|
| ATP synthase | HNE, 3NT |
| Alpha-enolase | HNE, 3NT, PC |
| Aldolase | HNE, 3NT |
| Aconitase | HNE |
| Creatine kinase BB | PC |
| Phosphoglycerate mutase 1 | PC |
| Carbonic anhydrase II | PC |
| Triose phosphate isomerase | 3 NT, PC |
| Lactate dehydrogenase | HNE, 3NT |
| Phosphoglycerate-3-dehydrogenase | 3NT |
| VDAC | 3NT |
| Pyruvate kinase | HNE, PC |
| Phosphoglycerate kinase | HNE |
| Malate dehydrogenase | 3NT |
| Glucose-regulated protein precursor | 3NT |

## Amyloid-β-induced oxidative stress hypothesis of AD and the importance of Met-35

The amyloid-β-induced oxidative stress hypothesis posits that amyloid-β, specifically A$\beta_{1-42}$, is primarily responsible

**Table 2** Summary of signaling proteins discussed within the text, modifications identified, and the AD stage and/or brain region analyzed

| Identified protein | Modifications |
|---|---|
| Glutamine synthase | PC |
| CRMP2 | HNE, 3NT, PC, SNO |
| Alpha-enolase | HNE, PC, SNO |
| Pin1 | PC |
| Gamma-SNAP | PC |
| Alpha-tubulin | HNE |
| Actin | HNE |
| Transition initiation factor alpha | HNE |
| Elongation factor Tu | HNE |
| Syntaxin-binding protein 1 | PC |
| Carbonic anhydrase II | PC |
| Mitogen-activated kinase 1 | PC |
| Alpha-INTERNEXIN | SNO |
| Glutamate dehydrogenase | SNO |
| Proenkephalin | SNO |
| Proopiomelanocortin | SNO |
| GFAP | SNO |
| Septin | SNO |

**Table 3** Summary of proteins involved in the proteasome or unfolded protein response discussed within the text, modifications identified, and the AD stage and/or brain region analyzed

| Identified protein | Modifications |
|---|---|
| UCH L-1 | PC |
| Cu/Zn-SOD | PC |
| Mn-SOD | HNE |
| HSPA8 | 3 NT, PC |
| HSP70 | HNE, PC |
| Glutathione-S-transferase Mu | 3NT |
| Multidrug-resistant protein-1 | HNE |
| Multidrug-resistant protein-3 | 3NT |
| Peroxiredoxin 6 | HNE, 3NT |
| Glutathione-S-transferase | HNE |
| Proteasome | HNE |

in large part for the oxidative damage observed to take place in AD (Butterfield et al 2001; Markesbery 1997). Oxidative stress has long been associated with AD and MCI and has been found to associate with Aβ. As previously discussed, there are two primary forms of Aβ: $A\beta_{1–40}$ and $A\beta_{1–42}$, with $A\beta_{1–42}$ being found to be more neurotoxic and aggregate more quickly than the 1–40 proteolytic variant (Butterfield and Sultana 2011). The effect of $A\beta_{1–42}$ on the levels of intracellular ROS has been verified in a number of experiments by our laboratory and others using in vivo and in vitro methods. For instance, $A\beta_{1–42}$ was added to 9- to 11-day-old primary hippocampal neuronal cultures which led to an increase in markers of oxidative stress and resulted in neurotoxicity (Yatin et al. 1999; Boyd-Kimball et al. 2004). Other studies have shown that this Aβ-induced oxidative stress may be mitigated through the use of free radical scavengers such as melatonin, estradiol, vitamin E, while more studies demonstrate that the neurotoxic effects of the $A\beta_{1–42}$ peptide were modulated specifically by vitamin E (Boyd-Kimball et al. 2004; Yatin et al. 2000; Quintanilla et al. 2005; Olivieri et al. 2001; Behl and Moosmann 2002). While these experiments demonstrate the neurotoxic effects of $A\beta_{1–42}$, the method of Aβ-induced ROS generation was as of then unclear. We have since provided evidence that suggests a major method of ROS generation by $A\beta_{1–42}$ is through the one-electron oxidation of the methionine-35 residue followed by lipid peroxidation, a process that involves a chain reaction (Fig. 2) (Butterfield and Boyd-Kimball 2005; Butterfield et al. 2007, 2010a, b). This mechanism is a way for a small free radical initiation step on $A\beta_{1–42}$ to be greatly amplified by continuous production of acyl-chain-resident C-centered free radicals that subsequently leads to production of HNE.

## Redox proteomics of human samples

Brain samples from subjects diagnosed with MCI- or AD-related dementia are invaluable because of their absolute physiological relevance. While data from animal models provide valuable insight into disease progression, extrapolating the data for use in humans has proven to be difficult in large part due to the inherent physiological and biochemical differences. Moreover, it is important to maintain sample integrity with a low postmortem interval (PMI), as higher PMIs may result in protein degradation and oxidation before analysis can begin. At the University of Kentucky, autopsy-derived specimens are obtained with a very short PMI, usually between 2 and 4 h. This low PMI allows us to perform our studies, such as redox proteomics, using human samples as close to living brain as feasibly possible.

Proteomics itself is a global term for the study of the proteome, whether in its entirety or a specific portion. The objective of redox proteomics is to narrow this focus onto that of oxidatively/nitrosatively modified proteins with the ultimate goal of protein identification matched with oxidative modification. Our laboratory pioneered the establishment and utilization of redox proteomics in the study of AD and has contributed a great deal to the understanding of the diseased proteome in the various stagings of AD: PCAD, MCI, and AD. There are two primary methods for the employment of redox proteomics: the first being the gel-free enrichment of proteins which present oxidative

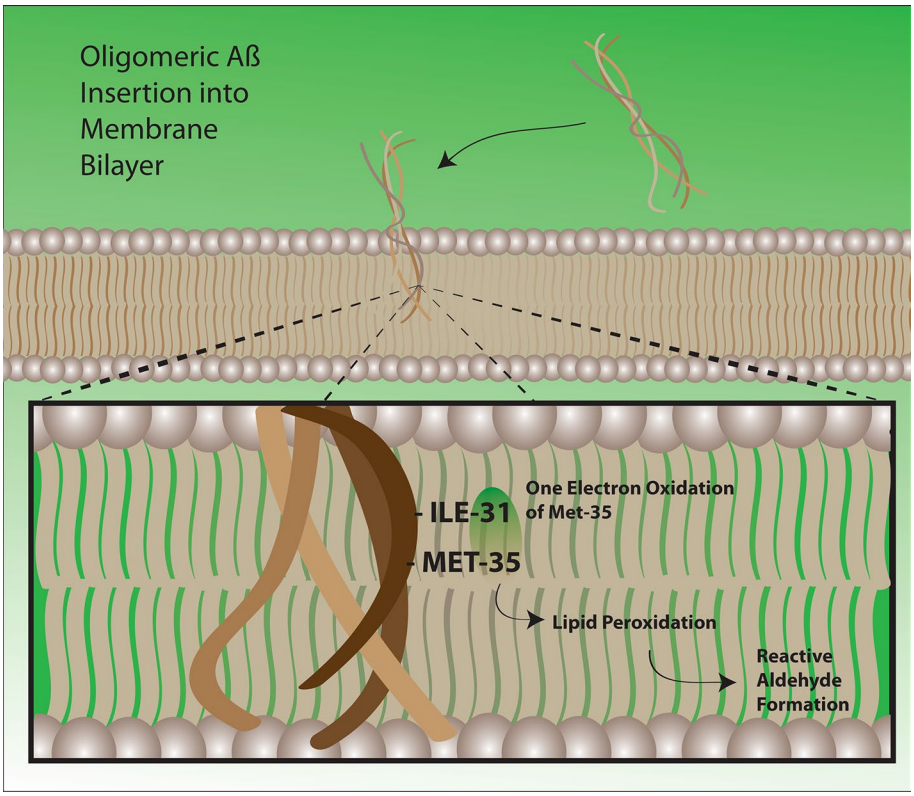


**Fig. 2** Schematic illustration of our proposed schematic for the method by which Aβ oligomers insert into a bilayer and subsequently initiate lipid peroxidation through the one-electron oxidation of Met-35 of the $A\beta_{1-42}$ peptide

modifications, while the second a gel-based global method (Butterfield et al. 2012). A non-gel method for redox proteomic analysis utilizes strong cation exchange and reverse-phase liquid chromatography and both mass spectrometry and tandem mass spectrometry (MS/MS). In the non-gel-based method, the proteins are digested in solution and peptides separated using nanoflow liquid chromatography where the separated peptides are subjected to automated MS identification.

In the gel-based method, samples are prepared accordingly to established protocols and the proteins separated in the first dimension according to their respective isoelectric points. Immediately following isoelectric separation, the proteins are run in the second dimension according to their migration rate. The two-dimensional separation yields distinct and isolated protein localizations within the gel which are ideally suited for comparison and identification. In order to determine the oxidatively modified proteins, the gels are first imaged and then transferred to a nitrocellulose membrane for immunochemical detection of the oxidative modification of interest on a 2-D western blot. The actual spot comparison is made using spot-matching software such as PD-Quest or Dimensional Delta 2D by comparing individual spot density to matched proteins. Once analysis is completed, identification of selected proteins is initiated through the excision of the target spot and in-gel trypsin digestion. The digested spots are then identified based on their peptide mass fingerprint (PMF) through the use of either matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry or sequencing of the tryptic peptides using electrospray ionization tandem mass spectrometry (ESI–MS/MS). ESI–MS/MS is especially useful due to the ability to select parental peptides to send through to an additional fragmentation which allows for an identification of the amino acid sequence and subsequently the tryptic peptide. Further, this technique has been developed to probe possible oxidatively modified sites or sites for other posttranslational modifications. The identification of the tryptic peptide is accomplished through the searching of peptide databases such as MASCOT and SEQUEST.

## Oxidized brain proteins from subjects with AD and MCI gathered using redox proteomics

### Energy metabolism

A prominent hallmark of AD and MCI is a marked decrease in the metabolism of glucose, primarily glycolysis, the tricarboxylic acid cycle (TCA), and the electron transport chain. Evidence of glucose utilization deficiency has been known for some time with data collected from fluorodeoxyglucose positron emission tomography (FDG-PET) scans

(Rapoport 1999). The importance of an impaired metabolic function in the brain is magnified by the fact that the brain consumes 30 % of inspired oxygen and 20 % of ingested glucose. Any impairment in glucose metabolism may have drastic effects to the overall health of the brain, and thus the organism. Redox proteomics has been able to shed some light on some possible reasons for this glucose utilization deficiency with the discovery of many proteins associated with metabolic processes being posttranslationally modified either by lipid conjugation or by reaction with ROS/RNS.

ATP synthase, an enzyme in the final step in the production of ATP from the electron transport chain, was found to be modified by HNE in the AD hippocampus, while α-enolase, aldolase, and aconitase were all found to be HNE-bound in the AD cortex (Perluigi et al. 2009). AD IPL was shown to have increased protein carbonylation of creatine kinase BB and α-enolase compared with age-matched controls, while AD hippocampus yielded carbonylated phosphoglycerate mutase 1, carbonic anhydrase II, triose phosphate isomerase (TPI), and α-enolase (Castegna et al. 2002a, b; Sultana et al. 2006a, b, c). In further studies, nitrated adducts of both α- and γ-enolase, TPI, and lactate dehydrogenase were found in AD IPL, while nitrated α-enolase, phosphoglycerate-3-dehydrogenase (GAPDH), ATP synthase alpha chain, and voltage-dependent anion channel (VDAC) were found in AD hippocampus (Sultana et al. 2006a, b, c; Castegna et al. 2003). Redox proteomics of familial AD (FAD) found specific increased carbonylation of γ-enolase (Butterfield et al. 2006).

Furthermore, redox proteomics of MCI cortex and hippocampus identified HNE-modified α-enolase as well as lactate dehydrogenase B (LDH-B), phosphoglycerate kinase (PGK), pyruvate kinase, and ATP synthase alpha chain (Reed et al. 2008). Nitration was observed of malate dehydrogenase (MDH), α-enolase, glucose-regulated protein precursor (GRP) and aldolase in MCI IPL, while α-enolase and MDH were found to be nitrated in MCI hippocampus (Sultana et al. 2007). Furthermore, protein carbonylation of α-enolase and pyruvate kinase M2 in MCI brain has also been reported (Butterfield et al. 2006). These findings of oxidatively modified proteins in MCI suggest that enzymes crucial to the normal functioning of energy metabolic pathways are affected in the early stages of AD, supplying evidence that corroborates the PET imaging discussed.

Enolase and its isoforms, which were found to be modified in the majority of studies mentioned, is specifically important to note as not only does it play a large role in the second stage of glycolysis in glucose metabolism, but recent research indicates it has a pleiotropic role in many other non-glycolytic functions such as in cellular signaling, activation of survival pathways, a neurotrophic factor, hypoxic stress protein, a transcription factor, and clearance of Aβ (Butterfield and Lange 2009; Takei et al. 1991; Aaronson et al. 1995; Subramanian and Miller 2000). As discussed above, oxidative insult to a protein involved in a large number of systems may have a pronounced negative effect downstream, whether that be energy metabolism or cell signaling.

## Signaling, structure, and neurotransmission

The primary function of the brain is to communicate with itself and all other bodily systems, and consequently proper functioning of cellular signaling and neurotransmission is of the utmost importance for the maintenance of a healthy brain. Likewise, the ability of the neuron to maintain its internal integrity while retaining the ability to guide the growth cone and form new synapses also is important to overall neuronal health. However, redox proteomic investigations demonstrated that many enzymes and proteins involved in cellular subsystems such as growth, axonal guidance and integrity, and neurotransmission have been oxidatively modified in AD and MCI brain.

Protein carbonylation of glutamine synthase, dihydropyrimidinase-related protein-2 also known as collapsin response mediator protein-2 (DRP-2 or CRMP2), and the pleiotropic α-enolase was observed in AD IPL, while carbonylation of the phosphorylation-specific peptidyl-prolyl cis/trans isomerase Pin1, CRMP2, α-enolase, and γ-SNAP was found in AD hippocampus when compared to age-matched controls (Sultana et al. 2006a, b, c; Castegna et al. 2002a, b). Being important in demonstrating that oxidative damage may have a significant impact on the ability of a protein to fulfill its normal function, this study also demonstrated that enzyme activities of Pin1, enolase, and carbonic anhydrase were all decreased (Sultana et al. 2006a, b, c). The oxidation of CRMP2 is notable as it is heavily involved in the control of axonal growth and overall neuroplasticity, so its modification may well contribute to the loss of synaptic integrity observed in AD. Pin1 is a regulatory isomerase of whose loss is implicated in nearly every pathogenic hallmark of AD, and is directly involved in amyloid-β-precursor protein (AβPP) processing and the phosphorylation status of protein-tau (Ma et al. 2012). Pin1 is also intricately involved in many stages of the cell cycle through direct contact with target proteins as well as indirect action through the regulation of important kinases and phosphatases (Keeney et al. 2012; Driver and Lu 2010; Lin et al. 2015). We recently reported that the active site Cys-113 residue is oxidatively modified to sulfonic acid in AD brain, and we posit that this modification may account for the decreased Pin1 activity we have observed in AD brain (Chen et al. 2015; Sultana et al. 2006a, b, c).

Carbonylation was not the only modification relating to signaling and structural proteins found in AD brain.

Secondary carbonylation, demonstrated by the identification of HNE-modified proteins, was observed for CRMP2 in AD hippocampus and α-enolase, and α-tubulin in AD cortex (Perluigi et al. 2009). In MCI hippocampus and cortex, redox proteomic identification of protein-bound HNE was found to affect α-enolase, actin, translation initiation factor-α, and elongation factor Tu, while experiments into the nitration of proteins in MCI hippocampus yielded nitrated CRMP2 (Reed et al. 2008; Sultana et al. 2007). Carbonylation of glutamine synthetase, Pin1, syntaxin-binding protein I, carbonic anhydrase II, and mitogen-activated protein kinase I was likewise identified in MCI brain (Butterfield et al. 2006; Sultana et al. 2009). Interestingly, within the entorhinal cortex of AD brain, S-nitrosylation of cysteine residues was reported to modify CRMP2, alpha-internexin, glutamate dehydrogenase, proenkephalin, proopiomelanocortin, α-enolase, glial fibrillary acidic protein (GFAP), and septin, which may suggest that glial cells around SPs present higher levels of nitrosylation of GFAP and may contribute to the pathogenesis of AD (Riederer et al. 2009).

**Proteasome system and the oxidative response system**

As discussed, oxidative insult to proteins mediated by free radicals is key in both normal aging and age-related diseases, and the clearance of damaged peptides from the cell serves as protection from non-functioning proteins and/or protein aggregation primarily due to the fact that most protein oxidation is non-reversible, aside from the action of carbonyl reductase (Perluigi et al. 2014). Left unchecked, protein oxidation may affect gene regulation and downstream protein expression, general cell signaling, apoptosis and necrosis, and protein turnover due to non- or dysfunctional protein regulators, which subsequently may lead to cell death (Butterfield et al. 2012). Moreover, because of the structural changes that oxidative damage may cause, protein aggregation is a likely end product, and such aggregation has been evidenced in neurodegenerative diseases such as amyotrophic lateral sclerosis with SOD1, Parkinson disease with α-synuclein, and AD with Aβ.

Two proteasome systems found to be important in AD pathology are the 20S proteasome and the 26S proteasome. The 26S proteasome, also known as the ubiquitin–proteasome system (UPS), degrades shortly lived or misfolded proteins (Forster et al. 2013). The target protein is first poly-ubiquitinylated through the concerted effort of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ligases preceding the chaperone-mediated transport to the 26S proteasome complex for degradation. Just prior to entry, the enzyme ubiquitin carboxy-terminal hydrolase-1 (UCH L-1) cleaves the ubiquitin moieties from the target protein one at a time, allowing the target protein to be degraded and preserving an active pool of ubiquitin for cellular usage (Forster et al. 2013; Kleiger and Mayor 2014).

The 20S proteasome is a ubiquitin-independent system and primarily degrades oxidatively modified, mutated, or aged proteins recognized by the 20S system due by the structural disorder inherent in the damaged proteins (Ben-Nissan and Sharon 2014; Hwang et al. 2011; Pickering and Davies 2012; Jung et al. 2013). Additionally, the 20S proteasome may degrade proteins which contain both intrinsically disordered regions (IDRs) and proteins containing intrinsically disordered sequences (IDPs) (Ben-Nissan and Sharon 2014; van der Lee et al. 2014; Dyson and Wright 2005). This function is especially important with concern to IDPs, as many function as regulator and signaling proteins in cell cycle control, growth control, and oncogenesis, and proper control over their protein levels is needed to prevent development of disease (Dyson and Wright 2005; Aiken et al. 2011). Therefore, properly functioning proteasomal systems are vital to the health of a cell, and disturbance of these systems may trigger catastrophic events leading to cell death through loss of control or protein aggregation.

Using redox proteomics, we found that UCH L-1 was carbonylated in AD brain, specifically AD IPL, AD hippocampus, and FAD IPL (Sultana et al. 2006a, b, c; Butterfield et al. 2006; Castegna et al. 2002a, b). These results were corroborated by others who also found oxidatively modified Cu/Zn-superoxide dismutase (Choi et al. 2004). An oxidatively modified and dysfunctional UCH-L1 poses a threat to the cell as ubiquitinylated proteins cannot enter the proteasome without first having their ubiquitin moieties hydrolyzed away from the ubiquitinylated protein. This bottleneck may lead to not only an accumulation of damaged and ubiquitinylated proteins and a loss of cellular signaling control if important short-lived regulators are not kept in check, but also a depletion of the ubiquitin pool since the fixed amount of ubiquitin is not recycled for reuse (Kleiger and Mayor 2014; Castegna et al. 2002a, b). Chaperone proteins and proteins involved in the unfolded protein response were also found to be oxidatively modified. Heat shock cognate 71 (HSPA8) was identified as being both carbonylated and specifically nitrated in AD IPL and MCI hippocampus, respectively (Sultana et al. 2007; Castegna et al. 2002a, b). Heat-shock protein 70 (HSP70) was also reported to be carbonylated and HNE-modified in MCI brain (Reed et al. 2008; Sultana et al. 2009). Glutathione-S-transferase Mu (GST-M) and multidrug-resistant protein 3 (MRP3) were found to be nitrated in MCI IPL, while peroxiredoxin 6 (PR VI) was found nitrated in MCI hippocampus and HNE-bound in AD cortex (Perluigi et al. 2009; Sultana et al. 2007). Mn-SOD was found to be covalently modified by HNE in the AD hippocampus (Perluigi et al. 2009). GST, MRP-1, and GSH were all found to

be HNE-modified, and an increase in HNE protein adducts was reported (Lovell et al. 1998; Sultana and Butterfield 2004). The proteasome itself has also been reported to be the target of oxidative injury as HNE, and neuroprostane conjugation was found in the brain of both MCI and AD tissues (Cecarini et al. 2007). We posit that the oxidation and modification of the aforementioned proteins, which play large roles in the defense against such oxidative insult, protein turnover/degradation, and protein folding, likely contribute to disease pathogenesis in the progression of AD.

## Conclusion

Redox proteomics studies of human brain have provided a wealth of information in regard to the molecular progression of AD and MCI, as well as a number of other detrimental and degenerative diseases. Providing information about the molecular degeneration of the disease enables others more to use that knowledge to not only improve upon existing treatments, but develop new ones by targeting specific proteins key to AD progression, which have been found to be oxidatively modified and dysfunctional. Not only are eventual treatments an end goal, but the identification of potential protein biomarkers for the successful and early detection of AD and MCI are critical in delaying AD onset among the coming tide of patients with AD dementia that are being predicted. Research has shown that by the time AD presents itself, much of the damage has already occured, which makes the need for early biomarker identification an absolute necessity. Here, we have shown evidences from a multitude of studies demonstrating that systems vital to the health of the brain are impaired by way of oxidative insult through ROS/RNS and reactive aldehyde-mediated conjugation. We posit that the damage to these proteins leads to deficiencies in systems important to the brain, such as energy metabolism, cell signaling, neurotransmitter release, and the proteasome, which may lead to the progression and pathogenesis of AD.

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