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REVIEW ARTICLE

Roles of 3-nitrotyrosine- and 4-hydroxynonenal-modified brain proteins in the progression and pathogenesis of Alzheimer's disease

D. ALLAN BUTTERFIELD[1,2,3], TANEA REED[4] & RUKHSANA SULTANA[1,2,3]

[1]*Department of Chemistry, University of Kentucky, Lexington, KY 40506-0055, USA,* [2] *Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506-0059, USA,* [3]*Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40536, USA, and* [4]*Department of Chemistry, Eastern Kentucky, University, Richmond, KY, USA*

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Abstract

Proteins play an important role in normal structure and function of the cells. Oxidative modification of proteins may greatly alter the structure and may subsequently lead to loss of normal physiological cell functions and may lead to abnormal function of cell and eventually to cell death. These modifications may be reversible or irreversible. Reversible protein modifications, such as phosphorylation, can be overcome by specific enzymes that cause a protein to 'revert' back to its original protein structure, while irreversible protein modifications cannot. Several important irreversible protein modifications include protein nitration and HNE modification, both which have been extensively investigated in research on the progression of Alzheimer's disease (AD). From the earliest stage of AD throughout the advancement of the disorder there is evidence of increased protein nitration and HNE modification. These protein modifications lead to decreased enzymatic activity, which correlates directly to protein efficacy and provides support for several common themes in AD pathology, namely altered energy metabolism, mitochondrial dysfunction and reduced cholinergic neurotransmission. The current review summarized some of the findings on protein oxidation related to different stages of Alzheimer's disease (AD) that will be helpful in understanding the role of protein oxidation in the progression and pathogenesis of AD.

Keywords: *Alzheimer's disease, mild cognitive impairment, early Alzheimer's disease, protein nitration, lipid peroxidation, 4-hydroxy 2-trans nonenal, proteomics*

Introduction

Proteins play an important role in normal structure and function of the cells. Oxidative modification of proteins may lead to loss of normal physiological cell functions and may eventually lead to cell death. The current review summarizes some of the findings on protein oxidation related with different stages of Alzheimer's disease (AD) that may relate to the progression and pathogenesis of AD.

AD is an age-related neurodegenerative disease that is pathologically characterized by the presence of extracellular amyloid plaques, intracellular neurofibrillary tangles (NFT) and loss of synaptic connections. The exact mechanism of AD pathogenesis is not clearly understood; however, mutation of *presenilin-1* (PS-1), *presenilin-2* (PS-2) and *amyloid precursor protein* (APP) genes has been found to be associated with inherited AD [1,2]. In addition to the above-mentioned genes, other risk factor genes such as allele 4 of the *apolipoprotein E* (APOE), *endothelial nitric oxide synthase–3* and *alpha-2-macroglobulin* have been associated with AD [3,4]. A number of hypotheses have been proposed to explain AD pathogenesis such as the amyloid cascade, excitoxicity, oxidative stress and inflammation hypothesis and all these are based on the role of amyloid beta-peptide (Aβ) [5–7].

Considerable evidence suggests a role of oxidative stress in the pathophysiology of AD [6,8,9]. For example, oxidative stress in the AD brain is evidenced by decreased levels of antioxidant enzymes and by increased levels of oxidative stress markers such as protein oxidation (indexed by protein carbonyls and 3-nitrotyrosine

Correspondence: Professor D. Allan Butterfield, Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington KY 40506-0055, USA. Tel: +1-859 257-3184. Fax +1-859-257-5876. Email: dabcns@uky.edu

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(3-NT)), lipid peroxidation, DNA oxidation, advanced glycation end-products and reactive nitrogen species (ROS) formation. Further, the role of oxidative stress in AD pathogenesis is supported by studies that showed diminished Aβ (1-42)-induced toxicity in the presence of vitamin E in cell culture [10].

Elevated levels of oxidative stress markers have also been found in the mild cognitive impairment brain (MCI), a condition characterized by loss of recent memory without dementia or significant impairment of other cognitive functions and with no loss of activities of daily living [11]. Many MCI subjects show some of the neuropathological features of AD at autopsy and MCI is considered a transition stage between normal cognition and AD. In addition to histopathological similarities to AD, MCI patients also show genetic similarities such as mutations in allele 4 of *apolipoprotein E*, *presenilin* 1 and *amyloid precursor protein* [12,13]. Moreover, increased levels of oxidative stress and nitrosative stress have also been reported in early AD (EAD). EAD is considered as an intermediate condition between MCI and late-stage AD; however, information involving this disease stage is limited due to lack of autopsy material. A detailed study involving AD, MCI and EAD would add to the understanding of AD pathogenesis and progression and may also lead to identification of important biomarkers.

Nitrosative stress in the brain of subjects with AD, MCI and EAD

Free radicals such as nitric oxide, produced during the nitric oxide synthase (NOS)-catalysed conversion of L-arginine to L-citrulline, play an important role in physiological conditions, such as vasodilation, but also can be harmful [14]. In 1998, Ferid Murad, Robert F. Furchgott and Louis Ignarro were awarded the Nobel Prize in physiology or Medicine for the discovery of the signalling properties of $NO^{\cdot}$. However, an increase level of $NO^{\cdot}$ is harmful to the brain [15]. In addition to $NO^{\cdot}$, other reactive nitrogen species (RNS), such as peroxynitrite, nitrogen dioxide, etc., also are produced in the body [16,17]. Protein modification via RNS usually results from either the reaction of peroxynitrite ($ONOO^{-}$), a highly reactive molecule formed from the reaction of nitric oxide with superoxide anion ($O_2^{\cdot -}$), resulting in protein-resident 3-NT (Figure 1) or from the reaction of $NO^{\cdot}$ (or N_2O_3) with thiols to form S-nitrosothiols (RSNO) [18–20]. Hence, nitration of the proteins could affect the secondary, tertiary and consequently the quaternary structure (if applicable) and thereby alter function of proteins including those involved in cell signalling, catalysis, cytoskeletal organization and inflammatory response. Such altered functions could have detrimental effects on cell viability [21–27]. Protein nitration is a reversible process and has been suggested to serve as a cellular signal [28,29].

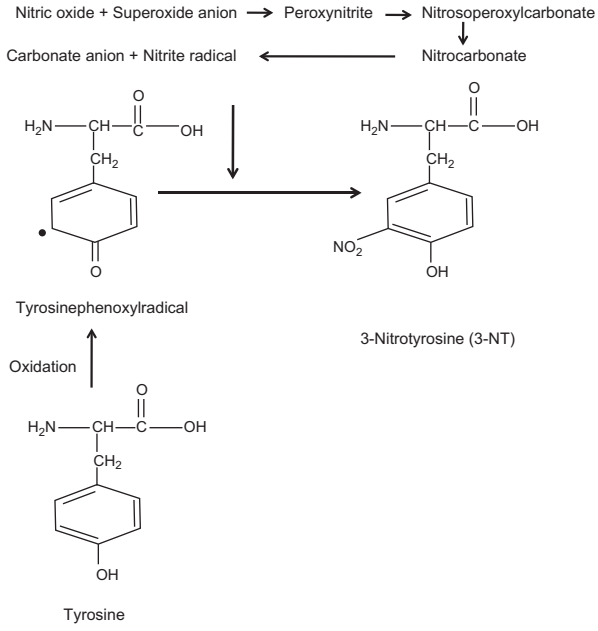

Figure 1. Formation of 3-nitrotyrosine.

Increased levels of RNS lead to increased nitrosative stress that has been reported to play important roles in the pathogenesis of a number of diseases including neurodegenerative disease, ischemia, etc. [30–34]. In AD brain an increase in protein and lipid nitration has been reported [35]. Williamson et al. [36] reported a 2–3-fold increased level of the lipid nitration product, 5-nitro-gamma-tocopherol in AD brain, which correlated with increased NOS levels and thereby suggested a role of nitration in AD pathogenesis [37]. AD brain and ventricular cerebrospinal fluid (VF) showed increased levels of dityrosine (DiTyr) and 3-NT [38] and immunohistochemical analysis showed the presence of nitrated tau in pre-tangles, tangles and tau inclusions in the AD brain. Further, the finding of the increased levels of 3-NT in pre-tangles of early AD brain suggests the involvement of tau nitration as an early event in AD pathogenesis [39,40]. The notion of the protein nitration role in AD was further supported by the fact that the MCI and EAD brain also showed increased levels of protein bound 3-NT [41]. The finding of increased nitration in MCI and EAD brain implies the role of protein nitration in the progression and pathogenesis of AD.

Redox proteomics [42] studies identified a number of proteins to be nitrated in MCI, EAD and AD brains [41]. These proteins were classified based upon their function in various classes (see Table I) and are discussed below with regards to AD progression and pathology.

Energy dysfunction

Of the many proteins that were nitrated in MCI, EAD and AD, only α-enolase is found to be a common target of nitration between these different disease stages. This result suggests that α-enolase nitration may be

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Table I. Functional categorization of nitrated proteins identified in MCI, EAD and AD.

Protein function	MCI[a]	EAD[d]	AD[b,c]
Energy dysfunction	**α-enolase**	**α-enolase**	**α-enolase**
		phosphoglycerate mutase 1	γ-enolase
			lactate dehydrogenase[b]
	aldolase	fructose 1,6-	glyceraldehydes-3-
	malate dehydrogenase	bisphospate aldolase	phosphate
		dehydrogenase	dehydrogenase
		triose phosphate isomerase	**triose phosphate isomerase**
Mitochondrial dysfunction	—	—	ATP synthase
			Voltage dependent anion channel protein 1
Antioxidant defence/detoxification system dysfunction	GSTM3	**peroxiredoxin II**	—
	MRP3		
	peroxiredoxin VI		
	glucose regulated protein precursor		
	HSPA8		
Cell signalling	14-3-3-γ	—	—
Lipid abnormalities and cholinergic dysfunction	—	neuropolypeptide h3	—
Neuritic abnormalities and structural dysfunction	**DRP2**		**DRP2**
	Fascin 1		**β**-actin
Excitotoxicity	—	Glutamate dehydrogenase	—

[a]For nitrated brain proteins identified in MCI see reference [41].
[b,c]For these nitrated brain proteins identified in AD see references [25,27].
[d]For these nitrated brain proteins identified in EAD see reference [58].
Nitrated proteins **in bold** are common targets of 3-NT modification.

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an important event in the progression of AD. Alpha enolase catalyses the conversion of 2-phosphoenolpyruvate to phosphoenolpyruvate in the glycolytic pathway of glucose metabolism and is a frequently oxidatively modified protein in several neurodegenerative diseases including: amyotrophic lateral sclerosis [43], a mouse model of Huntington's disease [44] and a Parkinson's disease mouse model [45,46]. Increased oxidation of alpha-enolase occurs even in models of AD [25,47–51]. In addition to its role in energy metabolism alpha-enolase also perform other functions [52]. Among these are involvements in the regulation of proteolytic clearance of Aβ and induction of pro-survival pathways. Our laboratory and others have shown that oxidative modification of α-enolase in MCI, EAD and AD results in a loss of enzyme function and, hence, in reduced amounts of ATP [53–57]. Elevated chances for neuronal death derived from decreased clearance of Aβ and diminished induction of ERK1/2-mediated survival pathways also are possible. Consequently, nitration of this protein in MCI, EAD and AD brain would promote progression of AD.

Triose phosphate isomerase (TPI) is a common target of protein nitration between the EAD and AD brain. TPI is a glycolytic enzyme that catalyses isomerization of glyceraldehyde-3-phosphate and dihydroxy acetone phosphate (DHAP). This triose isomerization reaction is important for two reasons: first, it provides the substrate for the next step of glycolytic cycle and is crucial for ATP production; and, second, by reducing the levels of DHAP TPI prevents the formation of methyl glyoxal, a highly reactive and toxic product. It is interesting to note that the activity of TPI is not altered in the AD or EAD brain [50,58]. TPI deficiencies are associated with hemolytic anaemia, resulting in mitochondrial myopathy [59–61].

Positron emission tomography (PET) studies showed that MCI, EAD and AD brains exhibit decreased glucose utilization compared to age-matched controls. Decreased glucose utilization could be due to impaired functions of the proteins that are involved in the glucose metabolism pathway, which is detrimental to normal brain activity. The stores of ATP at nerve terminals are key for proper neural communication. The decrease in ATP as a consequence of nitration may contribute to synapse loss and dysfunction and, consequently, to the memory impairment and cognitive decline [62] observed in amnestic MCI, EAD and AD. In addition to synapse loss, reduced ATP levels would impair ion-motive ATPase activity with subsequent altered cell potential, loss of membrane lipid asymmetry, intra- and inter-cellular communication and elevated intracellular Ca^{2+} [63,64].

Not all the proteins belonging to energy metabolism pathways that were found to be excessively nitrated in MCI are nitrated in late-stage AD. It is unclear as to what processes reverse nitration of selected proteins.

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A recent study suggests bilirubin may play such a role [65]. Bilirubin is a final product of the action of heme oxygenase-1 (HO-1) and the levels of HO-1 and its activity are altered in AD and Aβ [34,66]. A better understanding of proteins identified that reduce protein nitration may help in development of eventual treatments to slow progression of AD.

Neuritic abnormalities

Axonal growth and repair is crucial for proper neuronal transmission, a key process for memory formation and retrieval. One of the proteins that is important for these functions is dihydropyrimidine-related protein 2 (DRP2), which has been found to be nitrated in both AD and MCI brains [27,41]. DRP2 is involved in neurite outgrowth, so nitration of DRP2 would be predicted to lead to shortened dendritic length, which has been reported in the AD brain [67]. This protein also undergoes other oxidative post-translational modifications, such as protein carbonylation and decreased levels of DRP2 in the AD brain and its animal models have been reported [53,68,69]. Further, individuals with Down syndrome, having an extra copy of *APP* gene and consequently increased Aβ, also showed decreased levels of DRP2 [70]. Hence, decreased expression and elevated nitration of DRP2 in AD and MCI brains may contribute to shortened neurites with consequent diminution in cognition [67].

Antioxidant defense/detoxification system dysfunction

Peroxiredoxins (Prx) exist in six isoforms, i.e. Prx I, -II, -II, -IV, V and VI, that reduce hydrogen peroxide. Of these isoforms Prx II and Prx VI are found to be significantly nitrated in EAD and MCI, respectively [41,58]. Both these Prx use different electron donors, i.e. thioredoxin and glutathione for II and IV, respectively; however, they catalyse similar functions. Peroxiredoxin II is neuronal-specific and functions also to help regulate the opening of the mitochondrial permeability transition pore [71] and is thereby important in inhibiting apoptosis [71] and promoting neuronal cell survival [72,73]. Prx2 over-expresssion in cell lines prevents oxidant-induced apoptosis [74]. The levels of Prx2 were reported to be increased in Parkinson's disease [75] as well as AD and in Down's syndrome [76,77]. In the AD brain the increased level of Prx VI was found to be associated with SP and NFT [78]. In addition to reduction of hydrogen peroxide, Prx II also reduces peroxynitrite and is, thereby, important for detoxification of RNS [79]. Hence, loss of Prx activity may lead to increased ROS and RNS that has been reported to be elevated in the AD brain. Prx VI is an efficient antioxidant enzyme that catalyses the reduction of peroxynitrite [80] and is also involved in cell differentiation and apoptosis. Further, it has been reported that changes in Prx VI activity may also influence phospholipase A2 activity, a protein regulated by peptidyl prolyl *cis*/*trans* isomerase (Pin 1). In AD and MCI brains Pin 1 has been shown to be down-regulated, oxidatively modified and has reduced activity that could lead to abnormal tau hyperphosphorylation and consequently to NFT formation [81–83]. Further, Prx VI has been shown to form a complex with glutathione-S-transferase [84], so nitration of Prx is an indication that oxidative stress conditions exist in AD and MCI brains and that peroxiredoxin is an important antioxidant enzyme in the human brain defenses system.

HNE-modification of brain proteins in AD, MCI and EAD

Lipid oxidation in the brain readily occurs due to the fact that lipids are particularly vulnerable since polyunsaturated fatty acids are abundant in brain and oxygen is present in membrane bilayers at high levels. Lipid peroxidation is a complex process involving the interaction of oxygen-derived free radicals with polyunsaturated fatty acids, resulting in a variety of highly reactive electrophilic aldehydes that are capable of easily attaching covalently to proteins by forming adducts with cysteine, lysine or histidine residues (Figure 2) through Michael addition [85–88]. Although malondialdehyde and 4-hydroxy-2-nonenal (HNE) represent the major products of lipid peroxidation [85], other carbonyl-containing products are formed, among which are acrolein, neuroprostanes and isoprostanes [89–91].

Lipid peroxidation is highly evident in several neurodegenerative diseases including PD [92], Huntington's disease (HD) [93] and AD [94–97]. Specifically in AD, protein-bound HNE and acrolein have been found to be significantly elevated [98–100]. Evidence

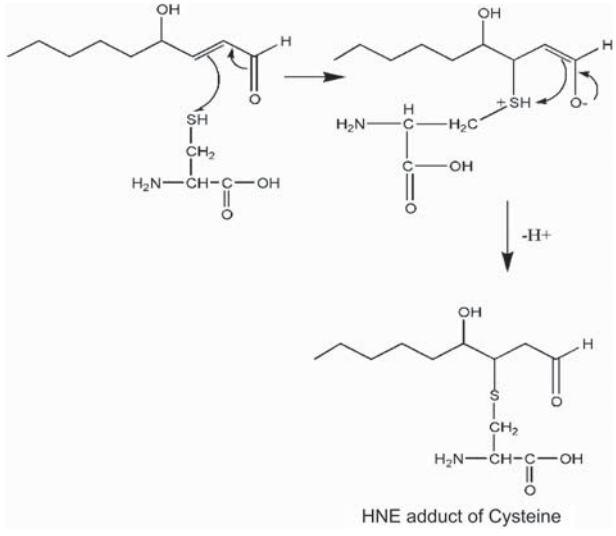

Figure 2. Michael addition of a cysteine residue to HNE.

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indicates that lipid peroxidation is an early event during the progression of AD, as demonstrated by its appearance in MCI [101] as well as early AD [102] and late-stage AD [103].

Analysis of AD brains demonstrates an increase in free HNE in amygdala, hippocampus and parahippocampal gyrus of the AD brain compared with age-matched controls [104]. This increased alkenal concentration corresponds with the regions showing the most striking histopathologic alterations in AD. A significant elevation of free HNE in ventricular CSF and serum provides a potential biomarker for AD [105,106] Likewise, protein-bound HNE, which is an indication of Michael addition of HNE to proteins, is elevated in AD [27,107,108]. Other lipid peroxidation products such as acrolein, malondialdehyde and isoprostanes have been significantly elevated in MCI [102,109,110], EAD [102,110] and late-stage AD [90,91] as well.

Protein-bound HNE alters conformation and function of proteins [107,111–113]. Several important categories of proteins are HNE-modified throughout the course of AD. They include: energy metabolism, mitochondrial dysfunction, cytoskeletal integrity, antioxidant defense, protein synthesis, stress response, neuronal communication and excitoxicity (Table II).

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Energy metabolism

Since altered energy metabolism and reduced cholinergic activity are two well-documented characteristics of AD, it is important to note the HNE modification of several cholinergic, glycolytic and ATP generating proteins, thereby providing insights into these functions at an early time point of the disease. As noted above, α-enolase catalyses the conversion of 2-phosphoenolpyruvate to phosphoenolpyruvate and is a frequently oxidatively modified protein in several neurodegenerative diseases including: amyotrophic lateral sclerosis [43], a mouse model of Huntington's disease [44] and a Parkinson's disease mouse model [44,45]. α-enolase demonstrates increased oxidation in AD and models of AD [49,53,57,68,69,114]. Protein oxidation and reduced enzyme activity has been previously established in MCI [27], EAD [107] and AD [53,115]. Protein modification of α-enolase may disrupt neuronal energy metabolism and ion homeostasis, thereby impairing the function of membrane ion-motive ATPases and glucose and glutamate transporters [113,116], loss of membrane asymmetry [117] and signal transduction. HNE modification of this protein bolsters the concept that altered energy metabolism is a common theme in neurodegenerative disease. ATP, the energy source of the cell, is extremely important at nerve terminals for normal neural communication. Decreased levels of cellular ATP at nerve terminals may lead to loss of synapses and synaptic function and loss of cell potential that can affect propagation of action potentials, all of which may ultimately contribute to memory loss, potentially correlating evidence of protein modification to memory loss throughout the spectrum of AD. Phosphoglycerate kinase catalyses the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate. This reaction undergoes substrate phosphorylation by phosphoryl transfer from 1, 3-bisphosphoglycerate to ADP to produce one molecule

Table II. Functional categorization of HNE-modified proteins identified in MCI, EAD and AD.

Protein function	MCI[a]	EAD[b]	AD[c]
Energy dysfunction	***α*-enolase**	***α*-enolase**	***α*-enolase**
	phosphoglycerate kinase	aldolase triose phosphate isomerase	
	pyruvate kinase	aconitase	
	lactate dehydrogenase B		
Mitochondrial dysfunction	**ATP synthase**	**ATP synthase manganese superoxide dismutase malate dehydrogenase**	**ATP synthase manganese superoxide dismutase**
Antioxidant defence/ detoxification system dysfunction	carbonyl reductase 1	—	peroxiredoxin VI
Protein synthesis	elongation factor Tu; initiation factor alpha	—	
Stress response	heat shock protein 70		
Lipid abnormalities and cholinergic dysfunction	neuropolypeptide h3		
Neuritic abnormalities and structural dysfunction		**DRP2**	**DRP2**
	β-actin		*α*-tubulin
Excitotoxicity			glutamine synthetase

[a]For HNE-modified proteins identified in MCI, see reference [97].
[b]For these HNE-modified proteins identified in EAD, see reference [107].
[c]For these HNE-modified proteins identified in AD, see reference [108].
HNE-modified proteins **in bold** are a common target of modifications.

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of ATP. Impairment of this glycolytic enzyme would result in decreased energy production and irreversible downstream effects, such as multi-drug resistance [118]. Pyruvate kinase catalyses the final step in glycolysis, the conversion of phosphoenolpyruvate to pyruvate, with the concomitant transfer of the high-energy phosphate group from phosphoenolpyruvate to ADP, thereby generating ATP. Under aerobic conditions, pyruvate can be transported to the mitochondria, where it enters the TCA cycle and is further metabolized to produce considerably more ATP through oxidative phosphorylation and substrate for brain-requiring glutamate. Additionally, enzymatic activity is reduced, thus suggesting that oxidative modification leads to impairment of protein function [97]. Lactate dehydrogenase B (LDH) reduces pyruvate to lactate by NADH in glycolysis. The generation of NAD^+ to oxidize glyceraldehyde-3-phosphate in glycolysis is one pertinent reason for the reduction of pyruvate to lactate. Lactate is a substrate for gluconeogenesis and, given that glucose is the major supplier of energy to the brain, proper lactate production is crucial [119]. The enzyme activity of lactate dehydrogenase was significantly reduced in the MCI hippocampus, which further correlates protein dysfunction and enzyme activity impairment. Impairment of this enzyme could initiate reduction of glucose production and creation of excess pyruvate. Aldolase (ALDO1) cleaves fructose 1,6-bisphosphate into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate in glycolysis. This is a critical step as it generates two substrates that will further undergo reactions to produce two molecules of ATP. Consequently, HNE modification results in decreased energy metabolism. Levels of ALDO1 are significantly decreased in the AD hippocampus [120] and PD [121]. Enzyme activity is reduced [122] and impairment can cause increased levels of fructose 1,6- bisphosphate, inhibition of complete glycolysis and ATP depletion. TPI easily isomerizes dihydroxyacetonephosphate (DHAP) to glyceraldehyde-3-phosphate (G3P) in glycolysis. Transformation of DHAP to G3P is imperative to continue glycolysis and for ATP production. ATP is essential in maintaining ATPases, ion motive pumps and potential gradients. In the AD brain, TPI is oxidatively modified as shown by our group in late stage AD [57,123]. These results support the notion that energy metabolism is a critical component in the progression of AD pathogenesis from MCI to EAD and terminal late-stage AD.

Energy metabolism/mitochondrial dysfunction

Aconitase is a TCA cycle enzyme involved in the conversion of citrate to isocitrate via a dehydration–hydration reaction. The TCA cycle generates a higher level of ATP than glycolysis by energetically equivalent molecules (i.e. GTP, FAD and NAD). Enzymatic activity of aconitase is significantly reduced in the AD brain [108]. Since aconitase is a mitochondrial protein, impairment can lead to mitochondrial dysfunction as observed in several disorders including PD, Friedrich's ataxia and Huntington's disease [124]. Decreased ATP production can lead to voltage-gated channel and ion-motive pump disruption as well as synapse loss, an early event in AD pathology [125]. ATP synthase is a mitochondrial regulating sub-unit of complex V that plays a key role in energy production. This enzyme complex goes through a sequence of coordinated conformational changes of its major subunits (α, β) to produce ATP. ATP synthase has been previously shown to be HNE-modified in MCI [97], EAD [107] and late-stage AD [108]. Activity for this enzyme is significantly reduced in the aforementioned stages of AD. The oxidation of ATP synthase leads to the inactivation of this mitochondrial complex.

Altered expression and elevated oxidative modification of mitochondrial proteins, functional deficits and lowered activity in different complexes of the ETC are seen in AD [126–130]. These changes, coupled with the changes in complexes I, III and IV, may cause electron leakage from the mitochondria to produce ROS. This action may also affect the mitochondrial proton gradient and its ROS generation by reason of mitochondrial dysfunction suggests a different hypothesis [131,132] for the acknowledged existence of oxidative stress in AD [9,101,133–135].

Additional mitochondrial dysfunction

Superoxide dismutase (SOD) catalyses the conversion of two superoxide anions to hydrogen peroxide and oxygen (Figure 3). Maintenance of this enzyme is critical to achieving oxidative balance; otherwise the cell would be in a continual state of oxidative stress. There are several distinct forms of SOD including Cu/ZnSOD (SOD1), MnSOD (SOD2), NiSOD and FeSOD. MnSOD is located in the mitochondria and thus impairment of this protein can greatly affect the proteosome causing an oxidized protein 'overload' with the inability to correctly ubiquitinate and degrade oxidized proteins. This notion is further bolstered by research stating that oxidative modification of manganese superoxide dismutase inactivates the enzyme [136]. Activity of MnSOD is significantly reduced in EAD compared to the age-matched control brain [107], consistent with this hypothesis.

Malate dehydrogenase (MDH) catalyses the reversible oxidation of malate to oxaloacetate by NAD^+ in the TCA cycle. MDH links glycolysis to the ETC by transferring NADH to complex I through the

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Figure 3. Disproportionation of superoxide catalysed by the enzyme, superoxide dismutase.

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malate-aspartate shuttle, thus stimulating ATP production. Activity of MDH increases during ageing [137,138], which can further bolster the hypothesis of mitochondrial dysfunction in AD.

Cytoskeletal integrity

Actin plays a central role in maintaining cellular integrity, morphology and the structure of the plasma membrane. Actin microfilaments stabilize the neuronal membrane cytoskeleton by maintaining the distribution of membrane proteins and segregating axonal and dendritic proteins [139]. In the CNS, actin is distributed widely in neurons, astrocytes and blood vessels [140] and is particularly concentrated in presynaptic terminals, dendritic spines and growth cones. Oxidative modification of actin, by HNE, can lead to loss of membrane cytoskeletal structure, decreased membrane fluidity and trafficking of synaptic proteins and mitochondria [141]. Moreover, actin is involved in the elongation of the growth cone and loss of function of actin could play a role in the loss of synapse and neuronal communication documented in AD [142]. α-tubulin is an isoform of tubulin that alternates with β-tubulin to form a prominent cytoskeletal structure, the microtubule. Microtubules, used to transport cargo from the cell centre to the periphery and vice versa are stabilized by tau, which can become hyperphosphorylated and produce neurofibrillary tangles, a characteristic hallmark of AD. Upon HNE conjugation, α-tubulin is structurally modified and microtubules begin to depolymerize [143]. Therefore, cargo, including mitochondria delivery to and from the pre-synaptic terminal, cannot reach their destination comprising the survival of the neuron [144,145].

Antioxidant defence

Carbonyl reductase is an important enzyme that can reduce carbonyl-containing compounds to their resultant alcohols, thereby diminishing protein carbonyl levels (Figure 4). Subsequent malfunction or down-regulation of this enzyme could contribute to the increased protein carbonyls of AD, which because of the polarity of the carbonyl moiety could expose ordinarily buried hydrophobic amino acids to the solvent (i.e. disrupt conformation). Carbonyl reductase has been shown to reduce the lipid peroxidation product, HNE [146,147]. Expression of carbonyl reductase is altered in Down's syndrome and AD subjects [148]. The gene for carbonyl reductase is located in close proximity to the gene for Cu/Zn superoxide dismutase (SOD1) [149] and the genes for SOD1, carbonyl reductase and APP are located on chromosome 21, which is a trisomy in Down's syndrome patients [150,151]. A potential association between Down's syndrome and Alzheimer's disease by irregular meiotic recombination in chromosome 21 has been postulated [152]. Our proteomics studies lead us to posit a possible intriguing relationship among Aβ, Down syndrome and carbonyl reductase in neurodegeneration [97,107,108].

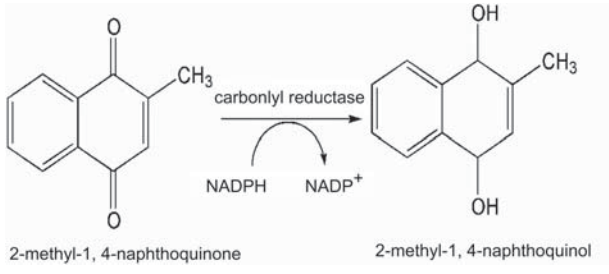

Figure 4. Illustration of one example of a Carbonyl reductase-catalysed reaction.

Peroxiredoxin VI, as stated above, is a nitrated protein and is also found as an HNE-modified protein [108,153]. Prx VI also plays important roles in cell differentiation and apoptosis, so HNE modification may lead to tau hyperphosphorylation and neurofibrillary tangles formation, in addition to development of oxidative stress.

Protein synthesis

Elongation factor Tu and initation factor α (eiF-α) are intimately involved in protein synthesis machinery. Human mitochondrial EF-Tu (EF-Tu) is a nuclear-encoded protein and functions in the translational apparatus of mitochondria [154]. Mammalian EF-Tu acts as a GTPase and hydrolyses a molecule of GTP each time an amino-acylated tRNA is accommodated on the A site of the ribosome and its recycling depends on the exchange factor EF-Ts. Nuclear genes encode most respiratory chain sub-units and all protein components necessary for maintenance and expression of mtDNA. Mitochondrial protein synthesis inhibition is associated with the impairment of differentiation in different cell types, including neurons [155]. The co-ordination of mitochondrial and nuclear genetic systems in the cell is necessary for proper mitochondrial biogenesis and cellular functioning. eIF-α is an abundant protein required to bind aminoacyl-tRNA to acceptor sites of ribosomes in a GTP-dependent manner during protein synthesis [156]. EiF-α has been shown to be involved in cytoskeletal organization by binding and bundling actin filaments and microtubules. Inhibition of eIF-α induces apoptosis [157], indicating that eIF-α activity is critical to normal cell function. Increased levels of HNE-bound eIF-α and EF-Tu suggest an impairment of the protein synthesis machinery, either in mitochondria or cytosol, associated with an impairment of the rate and specificity of ribosome functions [97]. Numerous studies have provided indirect evidence that suggests alterations in protein synthesis may occur throughout the progression of AD [158–163]. The dysfunction of the protein synthesis apparatus, mediated in part by oxidative stress, could compromise the ability

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of cells to generate the various factors needed to regulate cell homeostasis, thus contributing to impaired neuronal function and to the development of neuropathology in AD and pathogenesis of this disorder.

Stress response

Heat shock proteins act as chaperone proteins and aid in protein misfolding, protein aggregation and directing misfolded proteins to the proteosome. Heat shock proteins are involved in combating stress by protecting proteins from denaturation [164]. Heat shock 70 is a member of the heat shock protein family. Several other heat shock proteins have been found to be oxidatively modified in AD [53] and Huntington's disease [44] including Hsp90 and Hsp60 [66]. In the MCI brain, several additional heat shock proteins, including Hsp27, Hsp32 and Hsp70, showed an increase in protein level [66]. Impairment of HSPs may exacerbate protein misfolding and aggregation and eventual proteosomal overload and dysfunction known to occur in AD [165,166]. Aβ peptide aggregates are the major components of senile plaques, which are a hallmark of AD. Aβ-treated synaptosomes show that heat shock proteins are oxidatively modified [69], further illustrating the importance of functioning heat shock proteins in the cell.

Neuronal communication

Neuropolypeptide h3 is critical for modulation of the enzyme choline acetyltransferase, which is vital for synthesis of acetylcholine, a neurotransmitter important in signal transduction and cell communication. The loss of choline acetyltransferase leads to reduced levels of acetylcholine causing poor neurotransmission [167]. NMDA receptors activate the production of this enzyme and alteration of the NMDA receptor mediates cholinergic deficits [168]. AD has cholinergic deficits, consistent with dysregulation in acetylcholine levels and loss of cholinergic neurons [169–172]. The HNE modification of neuropolypeptide h3 further supports the involvement of cholinergic neurons, the dysfunction of which is a major neuroclinical deficit in AD [173]. Moreover, cholinergic neurons in the basal forebrain project to the outer molecular layer of the hippocampus, providing a potential link between cholinergic deficits and memory loss in amnestic MCI and AD.

Neuropolypeptide h3 is also known as phosphatidylethanolamine binding protein (PEBP). As a phosphatidylethanolamine binding protein, PEBP could be important in phospholipid asymmetry. A signal for apoptosis to commence is phophatidylserine becoming exposed to the outer leaflet of the membrane. Loss of function and changes in conformation of PEBP conceivably could lead to loss of phospholipid asymmetry as observed in AD [117,174,175], which can disrupt cellular homeostasis.

As described above, DRP-2 is a member of the dihydropyriminidase-related protein family that is involved in axonal outgrowth and pathfinding through transmission and modulation of extracellular signals [176,177]. Previous studies reported that DRP-2 can induce growth cone collapse [178,179] by rho-kinase phosphorylation [180] and binding to tubulin heterodimers and bundled microtubule as carriers to promote microtubule assembly and dynamics [181,182]. DRP-2 has been reported to be associated with neurofibrillary tangles, which may lead to decreased levels of cytosolic DRP-2. This, in turn, would eventually lead to abnormal neuritic and axonal growth, thus accelerating neuronal degeneration in AD [183], which is a classic hallmark of AD pathology. Since memory and learning are associated with synaptic remodelling, HNE conjugation and subsequent loss of function of this protein could conceivably be involved in the observed cognitive impairments in this disease. Moreover, the decreased function of DRP-2 could be responsible of shortened dendritic length and synapse loss observed in AD [184]. Shortened dendritic length likely would lead to less neuronal communication with adjacent neurons that could contribute to memory loss and cognitive decline associated with AD.

Excitotoxicity

Glutamine synthetase (GS), an important enzyme in maintaining the glutamate–glutamine cycle, catalyses the conversion of the acidic amino acid, glutamate, to the basic amino acid, glutamine. Glutamate is taken up from extracellular fluid from neuronal tissues via glutamate transporters, particularly EAAT2 (or GLT-1). Once GS is conjugated with HNE, it becomes structurally altered [185] and can no longer preserve glutamate levels, resulting in possible excitotoxicity and neurodegeneration. Glutamate synthetase levels are significantly decreased in the AD brain [186,187], which leads to protein dysfunction and neurodegeneration.

Conclusions

Early diagnosis of AD is essential in delaying the progression of this dementing disease. Hippocampus and IPL are ideal models to use, because they are both damaged in the AD brain. As described above, MCI and EAD are two stages preceding late-stage AD; therefore, especially MCI is a better phase of AD to study in order to gain insights into progression of AD and to potentially identify effective therapeutic agents to slow or halt progression. By using a proteomics-based approach to investigate the oxidative modifications of protein nitration and excessive protein-bound HNE in MCI, EAD and the AD brain, better understanding of the proteins altered during the progression of AD arise. For example, multi-functional α-enolase [52] is a common target protein for both HNE and 3-NT modification, providing a

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potential key element in the observed altered energy metabolism, elevated Aβ and decreased neuronal survival of MCI, EAD and AD. The results of these redox proteomics studies suggest that oxidation of α-enolase might be crucial in the progression of the disease [188–190]. Further, the use of antioxidants or agents that elevate the endogenous levels of cellular defence may help in delaying or preventing this devastating, dementing disorder.

Declaration of Interest

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