Nitrosative Proteins in the Progression of Alzheimer’s Disease: A Proteomics Comparison of Mild Cognitive Impairment and Alzheimer’s Disease Brain

Rukhsana Sultana, Renë A. Sowell, and D. Allan Butterfield

Abstract Oxidative stress and nitrosative stress have been reported to play important roles in the pathogenesis of a number of diseases including neurodegenerative diseases, cancer, ischemia, etc. Reactive nitrogen species are highly reactive and unstable. One of the best ways to quantify the amount of nitrosative stress is to measure the levels of 3-nitrotyrosine level. In addition, by using proteomics selective targets of protein nitration can be identified. In this chapter we discuss the roles of proteomics-identified nitrated brain proteins to the pathology of both mild cognitive impairment and Alzheimer’s disease. The identity of these nitrated proteins improves understanding of the role of nitrosative stress in the pathogenesis and progression of disease from MCI to AD. Such studies could also help in early detection and may provide therapeutic targets for early treatment that may slow disease progression.

Keywords Nitrosative stress · 3-nitrotyrosine · Proteomics · Alzheimer’s disease · Mild cognitive impairment

1 Nitrosative Stress

Oxidative stress and nitrosative stress have been reported to play important roles in the pathogenesis of a number of diseases including neurodegenerative disease, cancer, ischemia, etc. [1–5]. Nitrosative stress is caused by increased levels of reactive nitrogen species (RNS). RNS are highly reactive and toxic. RNS include nitric oxide (NO), peroxynitrite, nitrogen dioxide, etc. [6, 7]. Nitric oxide synthase (NOS) produces NO, a free radical. Under physiological

This paper is dedicated to the life of Dr. Earl R. Stadtman (1919–2008), a good friend and accomplished scientist and mentor.

D.A. Butterfield (✉)
Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA
e-mail: dabcsn@uky.edu

conditions NO is produced for relatively specific cellular targets [8]. However, under certain cellular conditions NO is produced by inducible NOS (iNOS) in higher amounts, which can then react with superoxide to form peroxynitrite [9]. Peroxynitrite has a half-life of less than one second and is highly reactive. NO or peroxynitrite can react with thiols to form nitrosothiols, or peroxynitrite also can react with tyrosine residues, one of the preferential sites of phosphorylation, at the meta position to form 3-nitrotyrosine (3NT) [10]. These modifications of proteins could affect the structure and thereby function of the proteins including alterations of cell signaling, catalytic activity, cytoskeletal organization, and inflammatory response [11-15]. Hence, increased levels of protein nitration can have detrimental effects on cell viability and function [16].

A number of previous studies showed that nitration of proteins may lead to inactivation of several important mammalian proteins such as antioxidant enzymes [17], structural proteins, energy-related mitochondrial proteins, and neurotransmitter-related proteins [12, 18, 19]. Several studies suggested that protein nitration is a reversible process analogous to phosphorylation and this may serve as a cellular signal [20, 21]. For example, proteins that are nitrated were reported to be more prone to proteosomal degradation than their counterparts [18]. Since RNS themselves are unstable, one of the best ways to quantify the amount of nitrosative stress is to measure the products of RNS, e.g., levels of 3-NT [11].

2 Alzheimer’s Disease and Protein Nitration

Alzheimer’s disease (AD) is an age-related neurodegenerative disorder that is pathologically characterized by the presence of extracellular amyloid plaques, intracellular neurofibrillary tangles (NFT), and loss of synaptic connections within selective brain regions. Hyperphosphorylated tau protein is the main component of NFT that forms paired helical filaments and related straight filaments. Amyloid plaque has amyloid beta-peptide (Aβ) as the main component and is considered to play a causal role in the development and progression of AD [22]. Aβ peptides are generated from Aβ peptide precursor protein (APP) by the action of β- and γ-secretases. Aβ exists in many forms, such as soluble form, aggregated form, oligomeric form, protofibrils (PF), and fibrils [23, 24]. Considerable research has shown that Aβ toxicity is associated with oligomers, PF, and amyloid-derived diffusible ligands (ADDLs) [25-27].

Although the exact mechanism of AD pathogenesis is not clearly understood, mutation of presenilin-1 (PS-1), presenilin-2 (PS-2), and APP genes has been found to be associated with inherited AD [28, 29]. In addition, other genes like allele 4 of the apolipoprotein E (APOE) gene, endothelial nitric oxide synthase –3 gene, and the alpha-2-macroglobulin gene have been associated with AD [30, 31]. Further, the amyloid cascade, excitotoxicity, oxidative stress, and inflammation hypothesis have been proposed for AD mechanisms, and all
Nitrate Proteins in the Progression of Alzheimer’s Disease

these are based on the role of Aβ [32–34]. There are large number of evidences that suggest a role of oxidative stress in the pathophysiology of AD [33, 35, 36]. Oxidative stress in AD brain is manifested by decreased levels of antioxidant enzymes and also by increased protein oxidation (including protein carbonyls and 3-NT formation), lipid peroxidation, DNA oxidation, advanced glycation end products, and reactive oxygen species (ROS) formation, among other indices. The role of oxidative stress is supported by the use of vitamin E in cell culture that diminishes Aβ (1–42)-induced toxicity [37].

A number of previous studies support the role of nitrosative and oxidative injury in the pathogenesis of AD [22, 36, 38, 39]. The early markers of oxidative stress and nitrosative stress in a cell include the formation of protein carbonyls, the lipid peroxidation product, 4-hydroxy-2-nonenal (HNE), and 3-NT [12, 19, 38, 40–45]. The role of RNS in AD pathology is based on the elevated levels of nitrated proteins that were found in AD brain and cerebrospinal fluid (CSF) [12, 19, 44]. In AD brain and ventricular cerebrospinal fluid (VF) increased levels of DiTyr and 3-NT were reported [46] that probably reflect increased leakage of mitochondrial electron equivalents that may nitrate proteins. Further, immunohistochemistry showed the presence of nitrated tau in pretangles, tangles, and tau inclusions in AD brain. However, the levels of 3-NT were found to be more in pretangles of early AD brain compared to that of more advanced brain that suggest the involvement of tau nitration as an early event in AD pathogenesis [47, 48].

3 Mild Cognitive Impairment and Protein Nitration

Mild Cognitive Impairment (MCI) is characterized by loss of recent memory without dementia or significant impairment of other cognitive functions and with no loss of activities of daily living [49]. MCI is divided into two broad subtypes based on memory impairment, i.e., amnestic MCI and non-amnestic MCI. Many MCI subjects show some of the neuropathological features of AD at autopsy such as significant medical temporal lobe atrophy, while others demonstrate low CSF-β amyloid (1–42) concentrations, factors that are associated with the senile plaques common to AD. In addition, there are also genetic similarities such as mutations in allele 4 of APOE, PS1, and the APP [50, 51]

Studies from our laboratory and others have proposed the role of oxidative stress and nitrosative stress in the progression of MCI to AD [52, 53]. Like AD, in MCI patients, plasma mean levels of non-enzymatic antioxidants and activity of antioxidant enzymes appeared to be lower than in controls [53–55]. Further, studies showed increased oxidative damage in nuclear and mitochondrial DNA, as indexed by increased levels of 8-hydroxyguanosine (8-OhdG), 2,6-diamo-4-hydroxy-5-formamidopyrimidine (fapyguanine), 8-hydroxyadenine, 4,6-diamo-5-formamidopyrimidine (fapyadenine), and 5-hydroxycytosine in MCI brain [56, 57]. In addition, other markers of
oxidative stress such as lipid peroxidation products isoprostane were found to be elevated in MCI plasma, urine, and CSF [58]. Our laboratory and others have shown increased levels of protein-bound and protein-free HNE [59–63] in MCI hippocampus and inferior parietal lobules compared to those of control brain [64]. Further, from our laboratory we also showed an increased level of protein-bound 3-NT levels that suggest the involvement of nitrosative stress at MCI stage [15].

4 The Proteomics Approach

4.1 Redox Proteomics

Redox proteomics focuses on the identification and quantification of oxidatively modified proteins. Several oxidative posttranslational markers have been studied by redox proteomics, e.g., protein carbonyls, HNE-bound protein, protein glutathionylation, etc. [65]. In this chapter, we focus on the application of redox proteomics to determine posttranslationally modified proteins via nitration of tyrosine residues. Redox proteomics methods most often involve the coupling of two-dimensional (2D) gel electrophoresis techniques with mass spectrometry (MS) and 2D-Western blotting analysis.

Figure 1 shows an experimental scheme of the overall approach. A detailed description is provided elsewhere [66, 67]. Briefly, the experiment is carried out as follows. Samples are split into two equal aliquots and each separated by isoelectric focusing (IEF) coupled to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The IEF SDS PAGE separation is based on the physiochemical properties of isoelectric point and migration rate (proportional to molecular weight). One gel is stained with Sypro Ruby and scanned at the appropriate wavelengths for total protein detection. The other gel is used to transfer proteins onto a nitrocellulose membrane for 2D-Western blot analysis. After transfer, the 2D-Western blot is probed with anti-3NT primary antibody and nitrated proteins are visualized with a colorimetric alkaline phosphatase assay. Protein spots in gels and biots are aligned and matched utilizing powerful image analysis software (i.e., PDQuest). Nitration levels for individual proteins are calculated by normalization to total protein levels in the gel (i.e., the ratio of the spot intensity on the 2D blot to the spot intensity on the gel). This takes into account changes in protein expression levels that may influence protein nitration levels.

Statistical analysis with ANOVA or Student’s t-tests is carried out to determine the significance of changes in nitration between age-matched control and MCI or age-matched control and AD samples, respectively. Protein spots of interest are excised, digested with trypsin, and analyzed by matrix-assisted laser desorption ionization (MALDI)-MS analysis. Mass spectra are submitted to the MASCOT database search engine for final protein identification.
Goodness of fit of the proteins is embodied in the associated MOWSE score \([-10\log_{10} p]\), where \(p\) represents the probability of a random identification of the protein of intent. Only proteins with MOWSE scores at or above a 95% confidence level (i.e., a score of 65 in these studies) are considered for further analysis. We note that a few proteins having a significant MOWSE score had nonsignificant increases (i.e., \(p > 0.05\)) in protein nitration in MCI [i.e., malate dehydrogenase, \(p < 0.06\] [15]] and AD [i.e., \(\beta\)-actin, \(p = 0.08\); lactate dehydrogenase, \(p = 0.16\); \(\gamma\)-enolase, \(p > 0.20\] [12]]. However, based on the biological functionalities of these proteins and their relationships with MCI and AD pathology, they have been included in the discussions below.
4.2 Identification of Nitrated Proteins in the MCI Brain

As mentioned above, elevated levels of nitration have been demonstrated previously in MCI inferior parietal lobule (IPL) and hippocampal brain regions [15, 52]. A comprehensive list of nitrated proteins in MCI brain, including functional categorizations, is provided in Table 1. This list includes the following proteins: α-enolase, glucose-regulated protein precursor, aldolase, malate dehydrogenase, glutathione-S-transferase Mu (GSTM3), multidrug-resistant protein (MRP3), 14-3-3-γ, peroxiredoxin 6 (PR VI), dihydropyriminidase-like protein-2 (DRP2), fascin 1, and heat shock protein 70 (HSPA8). Nitrated proteins in MCI can be grouped into several distinct biological functions and are discussed below with regard to MCI and AD pathology.

<table>
<thead>
<tr>
<th>Protein function</th>
<th>MCI</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy dysfunction</td>
<td>α-Enolase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>α-Enolase&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Aldolase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>γ-Enolase&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>Triosephosphate isomerase&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Dehydrogenase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Glyceraldehyde-3-phosphate Dehydrogenase&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactate dehydrogenase&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mitochondrial dysfunction</td>
<td>–</td>
<td>ATP synthase α-chain&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Voltage-dependent anion channel protein 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lipid abnormalities and cholinergic dysfunction</td>
<td>–</td>
<td>Neuropolyptide h3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH buffering and CO₂ transport</td>
<td>–</td>
<td>Carbonic anhydrase II&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Neuritic abnormalities and structural dysfunction</td>
<td>DRP2</td>
<td>β-Actin&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Fascin 1</td>
<td></td>
</tr>
<tr>
<td>Antioxidant defense/detoxification system dysfunction</td>
<td>GSTM3</td>
<td>DRP2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MRP3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peroxiredoxin 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose-regulated protein precursor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HSPA8</td>
<td></td>
</tr>
<tr>
<td>Cell signaling dysfunction</td>
<td>14-3-3-γ</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> For nitrated proteins identified in MCI, please see reference [15].

<sup>b</sup> For these nitrated proteins identified in AD, please see reference [12].

<sup>c</sup> For these nitrated proteins identified in AD, please see reference [19].

Nitrated proteins in bold are in common in both MCI and AD.
4.3 Energy Dysfunction

Proteins with elevated levels of nitrination in MCI brain that are involved in energy metabolism are α-enolase, aldolase, and malate dehydrogenase [15]. Aldolase and α-enolase are enzymes directly involved in glycolysis. For example, aldolase catalyzes the conversion of fructose 1, 6-biphosphate into dihydroyacetone phosphate and glyceraldehyde-3-phosphate in the second stages of glycolysis. α-Enolase is one of the subunits of the enolase enzyme that catalyzes the formation of phosphoenol pyruvate from the dehydration of 2-phosphoglycerate in the final stages of glycolysis. Malate dehydrogenase, on the other hand, catalyzes the conversion of malate to oxaloacetate in gluconeogenesis by transporting nicotinamide adenine dinucleotide (NADH) from the mitochondrion to the cytosol.

Because energy metabolism in the brain is heavily dependent on ATP generated from glycolysis any disruptions to the activities of glycolytic enzymes are detrimental to normal brain functions. Our laboratory and others have shown that oxidative modification of α-enolase in MCI and AD results in a loss of enzyme function and hence reduced amounts of ATP [12, 42, 45, 68–70]. Moreover, proper neural communication relies on the stores of ATP present in nerve terminals. Thus decreases in ATP, as a result of loss in glycolytic enzyme function, may contribute to synapse loss and dysfunction that lead to memory impairment and cognitive decline [71] as observed in amnestic MCI and AD. Other consequences of reduced ATP levels may include the following: impaired ion-motive ATPase activity with subsequent altered cell potential, loss of membrane lipid asymmetry and intercellular communication, and the induction of hypothermia which induces abnormal tau hyperphosphorylation [64, 72].

4.4 Neuritic Abnormalities and Structural Dysfunction

Maintenance of cytoskeletal structural integrity is crucial for proper neuronal transmission, especially with regard to the brain’s ability to perform normal memory processes. DRP2 is involved in neuronal repair and in axonal outgrowth [73, 74] by regulating collapsin activity. Collapsin is a protein involved in dendritic elongation and axonal outgrowth. Thus, nitration of DRP2 may lead to a loss of collapsin activity and may be responsible for shortened dendritic length, which has been previously observed in AD brain [75]. Our lab has previously observed DRP2 to have increased oxidation (i.e., protein carbonyls) and decreased expression in AD brain [42, 45]. Ultimately, oxidative and nitrosative modification of DRP2 in MCI may be responsible for or enhance neuritic degeneration and synapse loss, pathological hallmarks of AD [75], resulting in cognitive deficits.

Fascin 1 is a structural protein involved in cell adhesion [76] and cell motility [77] and is a marker for dendritic functionality [78]. Nitrination of fascin 1 could
lead to a loss of protein function. Because fascin 1 has been shown to provide protection against oxidative insult [79], nitration and subsequently loss of function could lessen the cell's natural defenses against oxidative stress. In addition, damage to fascin 1 may lead to poor neurotransmission from dendritic projections based on its role in cell adhesion. To date, this is the first association of fascin 1 with a neurodegenerative-related disorder.

4.5 Antioxidant Defense/Detoxification System Dysfunction

GSTM3, MRP3, PR VI, glucose-regulated protein precursor, and HSPA8 are involved in antioxidant defenses or detoxification processes within the cell. GSTM3 is an enzyme that functions in the detoxification of carcinogens, environmental toxins, therapeutic agents, and byproducts of oxidative stress [80, 81]. The mechanism of action of GSTM3 involves the conjugation of these toxins (e.g., xenobiotics or HNE) with glutathione. MRP3 then removes glutathione-conjugated toxic products out of the cell [82-84]. Thus, impairment of GSTM3 and MRP3 by nitration may result in impairments to the detoxification system crucial for removing ROS and toxic byproducts and maintaining low levels of oxidative stress. In addition to nitration, our lab has reported oxidative modification of GST and MRP1 by HNE in AD brain [84]. The proteomics results are consistent with reports of increased HNE levels in MCI IPL and hippocampal brain regions [66].

Peroxiredoxin VI is an efficient antioxidant enzyme that catalyzes the reduction of peroxynitrite [85] and is also involved in cell differentiation and apoptosis. Interestingly, PR VI forms a complex with GST [86], suggesting that alteration of either of these proteins can have detrimental effects on the detoxification and antioxidant systems of the brain. Changes in PR VI activity may also influence phospholipase A2 activity, a protein regulated by peptidyl prolyl cis/trans isomerase (Pin 1). Pin 1 is downregulated and has reduced activity in AD brain and may contribute to abnormal tau hyperphosphorylation which results in NFT formation [87, 88]. We have previously identified Pin 1 to be oxidatively modified through protein carbonylation in both MCI and AD brain [69, 89]. Nitration of GSTM3, MRP3, and PR VI may ultimately result in increased oxidative and nitrosative stress in MCI brain that contributes to disease pathology.

Glucose-regulated protein precursor is indirectly involved in energy production by regulation of glucose levels. It also belongs to the family of proteins that are molecular chaperones for the endoplasmic reticulum (ER) and thereby regulate proper protein folding of ER-associated proteins [90]. In situations where ER undergoes stress, glucose-regulated proteins (GRPs) help to protect against cell death [90]. Interestingly, inhibition of basal levels of GRP78 has been shown to increase Aβ(1–40) and Aβ(1–42) in cells [91]. Nitration of glucose-regulated protein precursor may influence the normal expression of GRPs under conditions of ER stress (e.g., oxidative damage). This may hinder
protective mechanisms in cells, increase cell death, and lead to increased level of Aβ found in senile plaques (SP) of MCI patients.

Finally, HSPA8 is a chaperone protein belonging to the heat shock family of proteins. HSPs function by repairing misfolded proteins in response to cellular stress (i.e., oxidative damage, elevated temperature, etc.). Nitration of HSPA8 in MCI brain may result in functional impairment that may lead to elevated levels of misfolded proteins and thus increased amounts of protein aggregates [15]. Protein aggregates can cause inefficiencies in the proteasome due to "clogging," and hence these aggregates accumulate in the cells and contribute to disease pathology (e.g., Aβ peptide aggregates result in SP in AD brain). Other HSPs have been identified as oxidatively modified in AD [42] demonstrating the role of normal molecular chaperoning ability as an important pathway to reduce cellular and pathological defects observed in MCI brain.

4.6 Cell Signaling Dysfunction

14-3-3 Proteins are scaffolding proteins involved in signal transduction, protein trafficking, and metabolic processes [92, 93]. Several studies have reported 14-3-3 proteins to be increased in AD brain [94], and CSF [95] as well as in models of AD [45, 96, 97]. Nitration of 14-3-3-γ in MCI has important consequences, primarily due to the binding relationship of 14-3-3 to the proteins glycogen synthase kinase 3β (GSK 3β) and tau [98]. Conformational changes to the structure of 14-3-3 due to nitration could affect normal binding of these proteins and may enhance tau hyperphosphorylation [99]. Thus, nitration of 14-3-3-γ may indirectly contribute to NFT found in MCI and in AD brains [15].

5 Identification of Nitrated Proteins in the AD Brain

In order to fully understand the role of nitration in the progression from MCI to AD, a brief discussion of nitrated proteins that our laboratory has identified with redox proteomics techniques in AD brain is presented. Proteins with increased levels of nitration in AD brain, as listed in Table 1, are as follows: α- and γ-enolase, triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), ATP synthase α-chain, voltage-dependent anion channel 1 (VDAC), neuropolypeptide h3, carbonic anhydrase II, DRP2, and β-actin [12, 19]. In comparing the functional categories associated with these proteins to those observed in MCI, there is overlap in dysfunction of energy-related proteins and those involved in neuritic abnormalities and structural dysfunction. Because these proteins may be involved in recurring pathways in the progression of MCI to AD, the implications of nitration to proteins in these pathways as well as mitochondrial dysfunction, lipid abnormalities and cholinergic dysfunction and pH buffering and CO2 transport are discussed.
5.1 Energy Dysfunction

Energy-related enzymes that are nitrated in AD brain are α- and γ-enolase, TPI, GAPDH, and LDH. As described above in MCI, dysfunction in energy-related enzymes can lead to decreased levels of ATP that are absolutely necessary for normal glucose metabolism in the brain and proper neuronal function. Positron emission tomography (PET) scans have shown that glucose metabolism is altered in the AD brain [100–102] and other studies have reported glucose intolerance in AD [103, 104]. These studies correlate with findings reported from our laboratory using redox proteomics of disturbances in energy-related pathways. Alterations in glucose metabolism are a pathological hallmark of AD, and as demonstrated above in these redox proteomics analyses, also in earlier disease stages such as MCI. These five enzymes are all directly or indirectly involved in the glycolysis pathway and thus are important to maintenance of proper glucose metabolism and ATP levels in the brain.

γ-Enolase is the second subunit, along with α-enolase, of the heterodimer enzyme enolase that is directly involved in glycolysis. Nitration of both enolase subunits, which are the predominant forms in the brain, can cause inefficiencies in the catalysis of 2-phosphoglycerate to phosphoenol pyruvate in glycolysis. These inefficiencies may then lead to a reduction in cellular ATP. α-Enolase has been reported as having increased levels in AD brain, oxidative modification through protein carbonylation, and a decreased activity [12, 42, 45, 70], thus providing direct evidence of its role in glucose metabolism impairment. TPI is an enzyme that provides high catalytic conversion of dihydroxyacetone phosphate into glyceraldehyde-3-phosphate in the second stages of glycolysis. In addition to nitration, our laboratory has found TPI to be oxidatively modified in AD brain [45, 70].

GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate, generated from the aforementioned TPI, into 1,3-biphosphoglycerate utilizing NAD⁺ as a cofactor in addition to inorganic phosphate. GAPDH has been reported to be oxidatively modified in AD brain with decreased activity [105, 106], and in other AD models [96]. Nitrination and oxidative modification of GAPDH can affect its ability to serve as a NO⁻ trap [107], resulting in increased 3NT formation in tyrosine residues of other proteins. The NADH that is generated from the GAPDH glycolytic reaction is used by LDH in its catalysis of pyruvate to lactate. Consequently, in the conversion of pyruvate to lactate, LDH also generates NAD⁺ that is used by GAPDH, as previously mentioned. Thus, while nitration of LDH can influence its own activity it may also have detrimental effects on GAPDH activity (and vice versa) in AD brain.

Disturbances to enzymes in the glycolytic pathway have major consequences in MCI and in AD brain. The primary insult is a reduction in cellular ATP that is necessary for the brain to perform normal functions including neuronal communication and synapse transmission, events that are key to proper memory retrieval and storage functions [71]. ATP is also required by many cellular processes such as ATPases for maintenance of ion pumps and potential
gradients and in lipid asymmetry. It is apparent that our proteomic findings of increased nitration to α- and γ-enolase, TPI, GAPDH, and LDH may contribute to glycolytic dysfunction reduced cellular ATP levels, and ultimately cognitive decline that is prevalent in AD patients.

5.2 Mitochondrial Dysfunction

ATP synthase, α-chain and VDAC are two mitochondrial-related proteins found in the inner and outer mitochondrial membranes, respectively, that we identified to have increased nitration in AD brain. Several studies have reported mitochondrial dysfunction to be an underlying cause to AD pathogenesis and neurodegeneration [108, 109]. In addition, other mitochondrial enzymes have been reported as reduced in AD brain [110, 111]. ATP synthase, α-chain is a subunit of the ATP synthesizing enzyme that is housed in the mitochondrion and generates ATP from the coupling of ADP and inorganic phosphate. ATP generation from ATP synthases is highly dependent on structural conformation such that ADP and inorganic phosphate must be close enough to each other to have a high affinity to bind. Nitration of ATP synthase, α-chain could affect the structure of the ATP synthase machinery and results in insufficient binding of ADP and inorganic phosphate. Thus, lower levels of ATP would be generated in mitochondria resulting in impairments to oxidative phosphorylation and subsequently reduced energy production. Other studies have shown ATP synthase, α-chain to be decreased in AD brain [112] and to accumulate in NFT [113]. Improper ATP synthesis can induce ROS production and leads to oxidative stress and neuronal death [45].

VDAC, also known as mitochondrial porin, is located in the mitochondrial permeability transition pore (MPTP) and is involved in the flux of metabolites, such as ATP, into and out of the mitochondrion. VDAC also plays roles in synaptic communication and apoptosis [114, 115]. Decreased expression of VDAC has been observed in various regions of AD brains [116] and deficits in learning behavior and synaptic plasticity have been reported in VDAC1-deficient mice [114]. Nitration of VDAC may alter MPTP function resulting in mitochondrial depolarization and disrupted signal transduction pathways, key elements for normal synaptic transmission and plasticity [45]. In addition, because VDAC contributes to the release of apoptotic factors such as cytochrome c [117], caspases [118], smac [119], and apoptosis-inducing factors [120], from the mitochondria, nitration of VDAC may induce apoptosis which results in cell death. Both neuron loss and synapse loss are known pathological hallmarks of AD, and apoptosis markers are elevated in AD and MCI [121].

5.3 Lipid Abnormalities and Cholinergic Dysfunction

The redox proteomics identified nitrated protein in AD that potentially is involved in lipid abnormalities and cholinergic dysfunction is neuropolypeptide
h3, also known as phosphatidylethanolamine-binding protein (PEBP), hippocampal cholinergic neurostimulating peptide (HCNP), and Raf-kinase inhibitor protein (RKIP). As PEBP, nitration of neuropolypeptide h3 may influence lipid bilayer integrity. Our laboratory has shown that HNE and Aβ disrupt lipid asymmetry [122, 123] resulting in the exposure of phosphatidylinerine to the outer leaflet, which induces apoptosis. Moreover, loss of phospholipid asymmetry is observed in MCI and AD brain [121] potentially coupling AD-induced ROS and RNS to cell death in MCI and AD brain.

PEBP is also the precursor of HCNP, a peptide that helps in the regulation of choline acetyltransferase and thus plays roles in signal transduction. It has been shown that a loss of choline acetyl transferase results in low levels of acetylcholine, a neurotransmitter important in maintaining normal neurotransmission [124]. Cholinergic neuronal loss is associated with AD [125–127], and thus drug treatments with cholinesterase inhibitors (e.g., Aricept®) have been used to treat the symptoms in AD patients. Moreover, HCNP has been reported as decreased in AD hippocampus [128]. Nitration of HCNP could disturb choline acetyltransferase activity which has been shown to have decreased activity in AD brain [129]. This could lead to poor neurotransmission in AD which is relevant to the already known cholinergic deficits observed in AD patients.

5.4 pH Buffering and CO₂ Transport

Carbonic anhydrase II is an enzyme that catalyzes the reversible hydration of CO₂ to HCO₃⁻ and was identified as nitrated in AD brain. This enzyme also aids in the transport of CO₂ and HCO₃⁻ and in maintenance of cellular pH, electrolytic, and water balance [130]. Proper pH balance in the cell is crucial for pathways such as glycolysis and ATP synthesis and for optimal efficiency of other mitochondrial enzymes. Decreased carbonic anhydrase II activity has been previously reported in AD hippocampus [70]. Nitration of carbonic anhydrase II may lead to reduced enzymatic activity and improper pH status which would have consequences in all of the above discussed processes in AD brain. Moreover, altered pH could influence protein aggregation seen in AD.

5.5 Neuritic Abnormalities and Structural Dysfunction

As discussed above, DRP2 and β-actin are structural-related proteins. Each was identified as nitrated in AD brain. DRP2 was also identified as nitrated in MCI brain (see discussion above). Nitration of DRP2 may lead to diminished enzymatic activity and shortened dendritic length, which has been previously observed in AD brain [75]. Oxidative modification of DRP2 through protein carbonylation has been previously reported in AD brain and in AD models.
42, 45, 96]. In addition, decreased expression of DRP2 has been found both in
AD brain and in Down’s syndrome patients [131]. Nitrosative modification of
DRP2 may be responsible for neuritic degeneration and synapse loss in AD [75],
with consequent decreased inter-neuronal connection as discussed above.

β-Actin is a component of actin microfilaments found in neurons, glia,
presynaptic terminals, dendritic spines, and in growth cones and plays roles in
maintaining cytoskeletal structural integrity. Actin is primarily concentrated in
dendritic spines and in growth cones in the brain [132] and helps in stabilizing
the shape of the Golgi complex and actin filaments [133, 134]. β-Actin was
found to be carbonylated in AD brain [135]. Oxidation and nitration of β-actin
may influence the shape of dendrites, growth cones, and microfilaments resulting
in poor neuron to neuron signal transmission, neuronal death, and ultimately,
improper memory function. Both DRP2 and β-actin are important in the struc-
tural network of neurons that are necessary for normal neural communication in
the brain.

6 Implication of Nitration in the Progression from MCI to AD

This chapter has discussed in some detail the effects that nitration of proteins can
have in the progression of MCI to AD. Among the most profound effects are the
disruptions that occur in energy-related enzymes that results in impaired glucose
metabolism and reduced energy supply to the brain (via ATP). The ultimate
consequences of these disturbances are neuronal death, synapse loss, poor neuro-
transmission, and subsequent memory loss; all of these events are characteristic of
both amnestic MCI and AD. Nitration of proteins was also found to disturb
neuritic and cytoskeletal structural integrity, antioxidant defenses/detoxification
systems and cell signaling in MCI brain, and also mitochondrial function, cell
buffering ability, lipid and cholinergic functions, as well as neuritic and cytoskeletal
structural integrity in AD brain. Overall, we hypothesize that nitration of proteins
can influence enzymatic activity in a manner that directly alters protein function,
leading to MCI and AD pathology.

Early degradation of antioxidant and detoxification defenses in MCI can
promote the induction of damaging ROS and contribute to further established
AD pathology. In addition to augmented nitration of proteins in MCI, our
laboratory has also identified increased carbonylation of proteins and lipid
peroxidation in MCI brain [60, 62, 63, 69]. Thereby, oxidative and nitrosative
stress are key elements in the earliest stages of AD (i.e., MCI) that result in
worsened damage found in late-stage AD brain.

Of the many proteins that were nitrated in MCI and AD, only α-enolase and
DRP2 were commonly identified as nitrated in both disease stages. Thus, these
proteins may be important in the progression of MCI to AD. This implies that
altered glucose metabolism, occurring from α-enolase nitration, and neuritic
degeneration, resulting from DRP2 nitration, may be key components in the
conversion of amnestic MCI to AD. It should be noted that α-enolase has been identified as both carbonylated and nitrated in MCI and AD in the IPL and hippocampus and as having enzymatic dysfunction [64]. Because not all individuals with MCI convert to AD and sometimes resume normal cognitive activity [136], therapeutic targets revolving around α-enolase and DRP2 pathways potentially conceivably may delay the onset of or prevent AD.

Redox proteomics studies that target MCI, an early stage in AD, are important for developing insights into the underlying causes of AD. Brain protein pathways that are revealed in common by redox proteomics analyses of MCI and AD show that energy-related pathways and structural pathways likely are involved in, and perhaps key to, disease pathogenesis. The findings presented in this chapter provide many insights into potential mechanisms of AD pathogenesis and into targets for therapeutic intervention at one of the earliest disease stage, MCI.

Acknowledgments This work was supported in part by NIH grants to D.A.B. [AG-10836 and AG-05119].

References


82. Joshi G, Hardas S, Sultana R, St Clair DK, Vore M, Butterfield DA. Glutathione elevation by gamma-glutamyl cysteine ethyl ester as a potential therapeutic strategy for


