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Review Article

Lipid peroxidation triggers neurodegeneration: A redox proteomics view into the Alzheimer disease brain

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ABSTRACT

Lipid peroxidation involves a cascade of reactions in which production of free radicals occurs selectively in the lipid components of cellular membranes. Polyunsaturated fatty acids easily undergo lipid peroxidation chain reactions, which, in turn, lead to the formation of highly reactive electrophilic aldehydes. Among these, the most abundant aldehydes are 4-hydroxy-2-nonenal (HNE) and malondialdehyde, while acrolein is the most reactive. Proteins are susceptible to posttranslational modifications caused by aldehydes binding covalently to specific amino acid residues, in a process called Michael adduction, and these types of protein adducts, if not efficiently removed, may be, and generally are, dangerous for cellular homeostasis. In the present review, we focused the discussion on the selective proteins that are identified, by redox proteomics, as selective targets of HNE modification during the progression and pathogenesis of Alzheimer disease (AD). By comparing results obtained at different stages of the AD, it may be possible to identify key biochemical pathways involved and ideally identify therapeutic targets to prevent, delay, or treat AD.

Contents

Lipid peroxidation and neurodegeneration

One of the major targets of the lipid peroxidation process is the central nervous system (CNS). Indeed, the brain is highly sensitive to oxidative stress because this 1300-g organ consumes about 20–30% of inspired oxygen, contains high levels of polyunsaturated fatty acids (PUFAs), is an ideal target for free radical attack, and high levels of redox transition metals. The latter play a crucial role in initiation/propagation of the cascade of reactions that start with the abstraction of an electron from the conjugate double bond system of fatty acid acyl chain. This process leads to the formation of a variety of free radical species, commonly

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grouped as reactive oxygen species (ROS), with slightly different reactivity. Altogether, ROS are highly unstable and easily react with all macromolecules such as proteins, nucleic acids, and lipids. These events are further exacerbated in the brain by the relative inability of neuronal cells to neutralize free radicals due to the paucity of both enzymatic and nonantioxidants.

Lipid peroxidation is one of the major sources of free radical-mediated injury that directly damages neuronal membranes and yields a number of secondary products responsible for extensive cellular damage. Free radical attack to PUFAs leads to the formation of highly reactive electrophilic aldehydes, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), the most abundant products, and acrolein, the most reactive (Fig. 1) [1–3]. In addition to aldehyde formation, lipid hydroperoxyl radicals undergo endocyclization to produce fatty acid esters; two classes of these cyclized fatty acids are ispoprostanes and neuroprostanes (Fig. 1) [4,5].

Peroxidation of arachidonic acid (AA) leads to the formation F_2-isoprostanes (F_2-IsoPs), while F_4-neuroprostanes (F_4-NPs) are the stable product of free radical damage to docosahexanoic acid (DHA). Once formed, F_2-NPs and F_4-NPs can undergo hydrolysis to free iso- and neuroprostanes that can be measured in body fluids [6], and F_2-NPs and F_4-NPs can undergo nonenzymatically additional conversions to form isochetals and neurochetals both of which are dangerous to cells [7,8]

However, cells also are endowed with lipid antioxidants, especially lipid-soluble vitamins and glutathione, glutathione *S*-transferases, one isoform of glutathione peroxidase, and beta-alanyl-L-histidine, which can quench lipid oxidants including HNE. In addition, albumin and apolipoproteins in plasma can bind and buffer HNE. However, a specific repair process of lipid peroxidation does not exist as it does for proteins and DNA and this may explain why moderate levels of lipid peroxidation could have physiological significance for cell signaling and membrane remodeling [9].

Peroxidation of membrane lipids affects a variety of functions resulting in increased membrane rigidity, decreased activity of membrane-bound enzymes (e.g., sodium pump), impairment of membrane receptors, and altered permeability [10,11]. In addition to damage to phospholipids, radicals also can directly attack membrane proteins and induce lipid–protein and protein–protein crosslinking, all of which contribute to altered membrane integrity [12]. It is reasonable to hypothesize that perturbation of all the above-noted functions displayed by PUFAs and its metabolites, together with modification of proteins, affects neuronal homeostasis, thus contributing to brain dysfunction.

The role of free radical-mediated oxidative damage in the pathogenesis of neurodegenerative disorders has been firmly established [13–17]. In particular, markers of lipid peroxidation have been found to be elevated in brain tissues and body fluids in several neurodegenerative diseases, including Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), Huntington disease (HD), and Down syndrome (DS) [17–21]. In agreement with these findings, several reports have documented increased levels of reactive products of lipid peroxidation in diseased regions of brain [17,22,23], but generally not in regions uninvolved in the disease [17]. This review focuses on the role of lipid peroxidation in brain of subjects at various stages of AD.

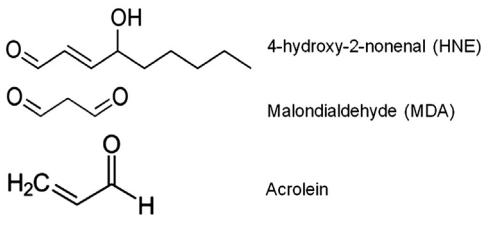

Fig. 1. Chemical structure of 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA), and acrolein.

The chemistry of lipid peroxidation: Focus on HNE

Lipid peroxidation involves a cascade of reactions which cause the degradation of lipids commonly described as a 5-step sequence (Fig. 2).

Step 1: **Initiation**, in which the free radical (hydroxyl $HO^{\bullet}$, alkoxyl $RO^{\bullet}$, peroxyl $ROO^{\bullet}$, and possibly $HO_2^{\bullet}$ but not H_2O_2 or O_2^{-}) abstracts an allylic H from a methylene group in the acyl chain of phospholipids, followed by rearrangement of the double bonds to the conjugated diene form, and simultaneously producing a carbon-centered alkyl radical.
Step 2: A peroxyl radical is produced when the alkyl radical reacts with paramagnetic molecular oxygen.
Step 3: **Propagation**, in which the peroxyl radical abstracts another allylic H atom to initiate a self-perpetuating chain reaction that ultimately leads to a variety of cyclic peroxides and hydroperoxides. These latter can be further degraded to hydrocarbons, alcohols, ether, epoxides, and aldehydes. Among these by-products, MDA, HNE, and acrolein can cause irreversible modification of phospholipids, proteins, and DNA [24].
Step 4: **Termination**, by which different types of radicals react with each other leading to formation of stable products; or
Step 5: Termination, by which reactions between the radicals and antioxidants give rise to nonradical products or unreactive radicals. Both exogenous and endogenous antioxidants such as vitamin E and vitamin C prevent the propagation of lipid peroxidation at the early stages of free radical attack [25,26]. Vitamin E (α-tocopherol) is a "chain-breaking" antioxidant; when the allylic hydrogen is abstracted in step 1, and α-tocopherol radical forms (step 5), the tocopherol radical can be reverted back to vitamin E by the vitamin C (ascorbic acid) and glutathione. The protective effects exerted by antioxidant toward HNE and other toxic aldehydes have been investigated by many groups to test the possibility of a therapeutic use of free radical scavengers and antioxidants against lipid peroxidation-mediated toxicity. In addition to small molecules, antioxidant enzymes such as heme oxygenase-1 (HO-1), catalase, superoxide dismutase, peroxiredoxin, and

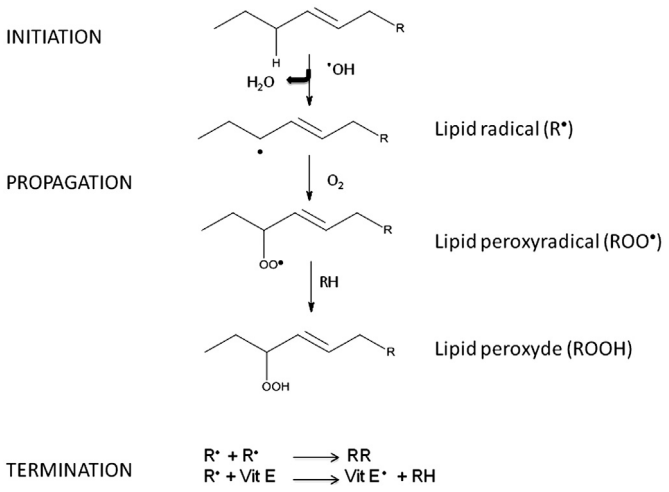

Fig. 2. Lipid peroxidation process.

glutathione reductase have been shown to lead to a significant decrease in lipid peroxidation products [27,28].

4-Hydroxy–2-trans nonenal

Among different types of lipid peroxidation by-products, the best characterized, mostly for its toxic role, is HNE. This alkenal is an α,β-unsaturated aldehyde that is formed by peroxidation of ω-6 polyunsaturated fatty acids such as linoleic acid, linolenic acid, and mostly arachidonic acid (AA). Although HNE is produced by nonenzymatic processes [29], Esterbauer's group demonstrated that the formation of HNE from AA is greater in the presence of NADPH-dependent microsomal enzymes [30]. Degradation of PUFAs to HNE is further accelerated in the presence of iron ions. The cascade of reactions initiates with formation of a lipid peroxyl radical [31], which is further oxidized to a lipid peroxide. The C–O bond is broken by a hydration reaction, resulting in a 9-carbon alkenal, namely 4-hydroxy-2-nonenal. Once formed, HNE is highly reactive and can easily attach to proteins by Michael addition to Cys, His, and Lys residues [32–34]. HNE is not only a potent electrophile reacting with a variety of nucleophilic compounds, but also may act as a stress signaling molecule [1]. The concentration of HNE within cells may vary from 10 μM to 5 mM and causes a wide range of biological activities, including the suppression of basal and inducible NFkB activity [35], disruption of ion homeostasis such as Ca^{2+}, impairment of Na^+/K^+ ATPase activity, and activation of caspase pathways [1,36,37]. HNE can cause an impairment of glucose transport in cultured rat hippocampal neurons and an alteration of the glutamate transport in rat neocortical synaptosomes [37]. Therefore, uncontrolled and/or excessive production of HNE could interfere with normal cellular signaling and lead to the development of pathological conditions as occur in several neurodegenerative diseases [17,32,36].

HNE–protein adducts

HNE is an amphiphilic compound, with both water-soluble and lipophilic properties. Since it has a stronger hydrophobic nature, it is mostly associated with the membranes where it is produced but it can also diffuse to different cellular compartments and interact with many different substrates [38]. The high reactivity of HNE is due to its chemical structure, where the presence of three functional groups potentiates its electrophilic properties [39]. Indeed, a conjugated system of a C=C double bond and a CO carbonyl group provides a partial positive charge to carbon 3. This positive charge is further increased by the inductive effect of the hydroxy group at carbon 4. Therefore, nucleophilic attack, for example, by thiol or amino groups, occurs primarily at carbon 3 and secondly at the carbonyl carbon 1 [38,40].

It is not surprising that proteins are particularly vulnerable to HNE-induced modification [41,42]. HNE forms adducts with three different side-chain amino acids, namely Cys, His, and Lys via Michael addition (Fig. 3), either with thiol (–SH) and amino (–NH_2) groups of these amino acids. Cys residues displayed the highest reactivity in the following order: Cys > His > Lys. However, Cys residues are not always the preferential targets of HNE in proteins. The degree of reactivity mostly depends on the tertiary structure of the protein, that is, the accessibility, and therefore reactivity, of amino acid residues toward exogenous chemicals. No reaction of HNE was detected with Glu [43]. Several proteins have been reported to be modified by HNE including plasma membrane ion and nutrient transporters; receptors for growth factors and neurotransmitters; mitochondrial electron transport chain proteins; protein chaperones; proteasomal proteins; and cytoskeletal proteins [40,44].

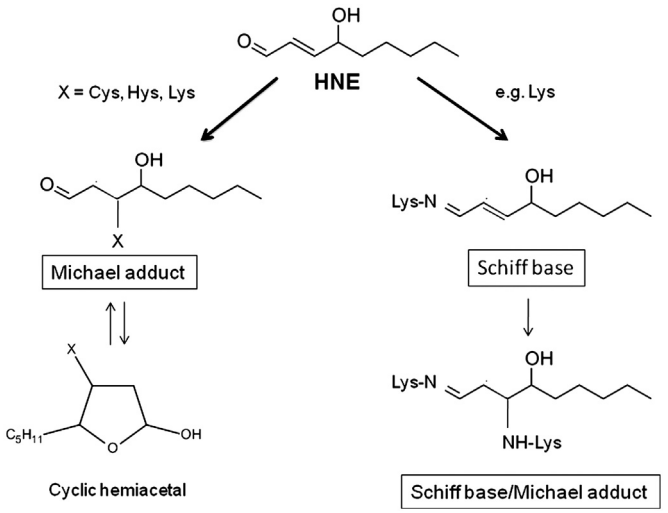

Fig. 3. HNE reacts with proteins either via Michael adduct, Schiff base formation, or both.

Low levels of HNE modification are sufficient to increase the susceptibility of proteins to proteolysis and removal by the proteasomal system. But, while under normal conditions the proteasomal system is able to remove the majority of oxidatively damaged and modified proteins, under severe oxidative stress conditions accumulation of modified proteins occurs. This could take place because of either protein cross-linking or impairment of the aldehyde reductase, aldehyde oxidase, and/or proteolytic machinery of the cell.

Interestingly, HNE can also react directly with amyloid beta-peptide (Aβ), the major component of senile plaques, a pathological hallmark of AD. This process is reported to affect formation of oligomers and to exacerbate Aβ aggregation and toxicity, which in turn causes oxidative stress, even more lipid peroxidation products, such as HNE, and more toxic Aβ oligomers [45]. Interestingly, in AD brain a protein involved in removing Aβ, LRP-1, is also covalently modified by HNE, and the consequent dysfunction of this efflux protein likely contributes to parenchymal accumulation of this neurotoxic peptide [46].

Cells possess different mechanisms for detoxifying HNE and thereby prevent its damaging toxic actions. Glutathione (GSH) is the most powerful and it has the ability to rapidly bind HNE through its Cys residue. In addition, glutathione *S*-transferases, together with the multidrug resistant protein (MRP1), contribute to regulate the intracellular level of HNE. The latter catalyzes the export of the GSH conjugate of HNE out of neurons [47]. The dipeptide carnosine (β-alanyl-L-histidine) also can quench HNE via intramolecular Michael addition [48].

Another major metabolic pathway for aldehyde detoxification is oxidation or reduction of the aldehyde to its corresponding acid or alcohol, respectively, by aldo-keto oxidoreductases [49]. Aldehyde oxidation to the corresponding acid is catalyzed by aldehyde dehydrogenases (ALDH; EC 1.2.1.3). Class 1 and 2 ALDH are NAD+ requiring enzymes that metabolize HNE to its corresponding acid, 4-hydroxy-(2)-nonenoic acid, while class 3 ALDH utilizes either NAD^+ or $NADP^+$ as cofactors in vitro and does not metabolize HNE [50]. A decreased activity of ALDH2 has been reported in AD brain [51], and it may contribute to increased accumulation of toxic aldehydes.

Aldehyde reduction to the corresponding alcohol is catalyzed by members of the aldo-keto reductase superfamily (AKR), aldehyde reductase (AKR1), and aldose reductase (AKR1B1), as well as by 3 classes of alcohol dehydrogenases (AD). Aldehyde reductase is approximately 3-fold more active toward HNE than

aldose reductase, although both metabolize acrolein at the same rate [52]. Carbonyl reductase is a member of this enzyme family and it can reduce HNE [53]. Interestingly, carbonyl reductase is one of the proteins found to be oxidatively modified in amnestic MCI brain by redox proteomics studies from our laboratory [54].

Other proteins expressed at high levels within or outside of cells, such as albumin [55] and apolipoprotein, may also play important roles in binding and thereby quenching HNE.

Redox proteomics: identification of HNE-modified proteins

In the last decade, development of new proteomics platforms has been a powerful tool for investigating the alteration of the proteome profile associated with a disease state. Thus, expression of specific proteins is often altered in disease conditions, and proteomics analysis is essential to help decipher biological processes and phenotyes of both normal and diseased cells. These differences become particularly intriguing when they are associated with a disease process. In addition to variation of protein expression levels, the activity of proteins is crucially regulated by posttranslational modifications, including acetylation, phosphorylation, methylation, glutathionylation, among others. These modifications are reversible and are fundamental for regulating normal cellular functions. However, this tight regulation may be perturbed by a different set of posttranslational modifications which often lead to irreversible protein modifications, mostly with dangerous effects. Among these, oxidative modifications have been extensively investigated mostly in neurodegenerative diseases, a condition where increased oxidative stress is a constant treat for protein homeostasis. Oxidation results in impaired protein function and accumulation of oxidized proteins is a characteristic hallmark of AD [15,16,56] and other neurodegenerative diseases [9].

Redox proteomics, arguably pioneered in the Butterfield laboratory [57–60], is the branch of proteomics that leads to the identification of oxidatively modified proteins, most often by coupling two-dimensional polyacrylamide gel electrophoresis (2D –PAGE), Western blot analyses, and mass spectrometry (MS) [58]. Despite the intrinsic limitations of 2D –PAGE, for example, the challenges involved in studying membrane proteins, it is still the most performed separation tool when dealing with a huge number of proteins. Oxidative stress response activates different signaling pathways which are responsible for different footprints in the cell, including oxidative modifications of proteins. Furthermore, the resolving power of proteomics allows identifying even the target of these oxidatively modified proteins. The study of the "redox proteome" is crucial for deciphering such modifications occurring in "stressed cells" and relating oxidatively modified proteins to the clinical presentation and to the pathology of disease states.

The most common and abundant types of oxidative modifications to proteins are protein carbonylation, 3-nitrotyrosine formation, binding of HNE, and glutathionylation. Redox proteomics can be applied to study all the above-noted modifications and many studies have been performed in our laboratory by following this approach. However, other non-gel-based approaches that utilize liquid or affinity chromatography in combination with MS have also been developed for these modifications by other groups [58].

Alzheimer disease

AD is the most common form of dementia in the elderly, characterized by neuronal degeneration in selective brain regions involved in cognition (hippocampus, entorhinal, and frontal cortex) and emotional behaviors (amygdala, prefrontal cortex, hypothalamus). The major pathological hallmarks of the disease are deposition of extracellular senile plaques (SP) and intracellular neurofibrillary tangles (NFTs) and loss of synapses [61,62]. The core of SP contains mostly Aβ, a 40–42 amino acid peptide that derives from the proteolysis (beta- and gamma-secretases) of the amyloid precursor protein (APP), surrounded by degenerating neuritis; NFTs are composed of aggregated hyperphosphorylated tau protein [63,64].

Due to the progressive nature of AD, it is possible to define at least three stages: mild cognitive impairment (MCI), early-stage Alzheimer disease (EAD), and late-stage Alzheimer disease (LAD). Patients are usually diagnosed on the basis of the severity of symptoms during the transition into each progressing stage. Braak staging (score) characterizes the severity of the disease by assessing the distribution of NFTs and neurophil threads; there are six levels of Braak staging (the higher the stage, the greater the severity of AD).

Some patients showed a presymptomatic phase before MCI, namely preclinical Alzheimer disease (PCAD), characterized by the absence of memory deficits and normal activities of daily living but pronounced AD neuropathology (Braak scores are III or higher) [65,66]. It is difficult to study PCAD brain due to limits in collecting samples; thus experimental data on these subjects are lacking and will likely be available in the near future. However, studies from our laboratory showed proteomics changes in brain from subjects with PCAD [67], and in the transition from PCAD to amnestic MCI, including identification of carbonylated proteins that conceivably may be involved in memory loss of amnestic MCI compared to PCAD individuals [68].

More results have been obtained from subjects with amnestic MCI, which is considered a prodromal phase of AD, though not all AD patients have been previously diagnosed with MCI. MCI patients can be classified as having amnestic (memory-affecting) MCI or nonamnestic MCI [69,70]. Braak scores for MCI are typically III or IV, with deposition of NFTs detectable in the hippocampus and neocortex. Pathologically, amnestic MCI subjects show mild degeneration of the hippocampus, entorhinal cortex, sulci, and gyri as evaluated by using magnetic resonance imaging technology (MRI) [71], while early- and late-stage AD patients show considerably greater loss in these areas [72]. It is estimated that the rate of conversion of amnestic MCI to AD is roughly 10–15% per year [73]; however, in some cases MCI individuals can revert to normal [70].

EAD, an intermediate stage between MCI and LAD, is characterized by increased frontal lobe atrophy, ventricular widening with progressive brain deterioration (Braak scores are between IV and V). Accordingly, these patients show decreased hippocampal volume which correlates with memory decline. There is a significant increase in the neurofibrillary tangle load in EAD subjects compared to MCI subjects in both the frontal and the temporal lobes [74], which correlates with impairments in verbal abilities, visuo-spatial functions, attention, and executive functions. Similar to PCAD, experimental evidence on EAD is still limited because sample availability is quite rare.

LAD is the final stage of the disease: memory loss, dementia, and behavioral changes are evident and significantly compromise all the activities of daily living. Braak score for these subjects is approximately IV–VI, as NFTs already caused substantial neuronal death in the hippocampus and neocortex. Accordingly, synapse loss, Aβ accumulation, and SP are profound [75]. Markers of oxidative stress including DNA oxidation, protein oxidation, and lipid peroxidation are significantly higher in these patients [76–80]. In contrast, brain levels of the antioxidant enzymes such as catalase, superoxide dismutase, glutathione *S*-transferase, and glutathione reductase are significantly decreased in LAD subjects.

Lipid peroxidation in AD

In AD brain increased lipid peroxidation has been identified by measuring the levels of free and protein-bound HNE, F_2-IsoP, F_4-NP, isoprostane 8,12-iso-iPF$_2\alpha$–VI, malondialdehyde, and acrolein [81–83]. Further, studies from our laboratory and others showed increased levels of lipid peroxidation markers such as thiobarbituric acid reactive substances, MDA, F_2-IsoP, F_4-NP, and protein-bound HNE also in subjects with MCI [82,84,85], suggesting that lipid peroxidation is an early event in the progression of AD. Pratico et al. showed increased levels of the isoprostane 8,12-iso-iPF $_2$ α–VI in CSF in AD [86] and MCI [87] and suggested that this lipid peroxidation product conceivably could be used as a marker to identify MCI patients who are at increased risk to progress to symptomatic AD.

Lipid peroxidation products as noted above react with biomolecules resulting in the direct oxidation of amino acids, glycooxidation, and lipoxidation in AD brain as indexed by the presence and concentrations of glutamic and aminoadipic semialdehydes, N_ε-(carboxymethyl)-lysine, N_ε(carboxyethyl)-lysine, and N_ε-(malondialdehyde)-lysine [88]. Pamplona et al. [88] using a redox proteomics approach found neurofilament L, alpha-tubulin, glial fibrillary acidic protein, ubiquinol-cytochrome *c* reductase complex protein I, and the beta-chain of ATP synthase as targets of N_ε-(malondialdehyde)-lysine formation that correlated with increased levels of docosahexaenoic acid. Further, acrolein has been reported to react with DNA bases such as guanine to lead to increased formation of acrolein-deoxyguanosine in AD brain [89]. Quinn et al. showed elevated levels of F_2-IsoPs in CSF from AD patients [90]; further, these researchers showed that the patients receiving alpha-tocopherol and vitamin C had lower levels of CSF F_2-IsoPs, suggesting that antioxidants may be a promising therapeutic strategy to treat AD [90].

HNE-modified common proteins at different stages of AD

The presence of increased lipid peroxidation at different stages of this dementing disorder underscores the crucial role of this process in AD pathogenesis and progression. Redox proteomic studies led to the identification of a number of oxidatively modified proteins in AD and MCI brains that play important roles in regulating energy metabolism, cellular signaling, pH regulation, neuronal communication, antioxidant and detoxification, neurotransmitter regulation, tau hyperphosphorylation, and APP processing. These redox proteomics-identified brain proteins are consistent with the biochemical, histopathological, and cognitive dysfunctions reported in AD [131,132]. Table 1 shows the listing of proteins that have elevated HNE modification in AD, EAD, and MCI, compared to age-matched controls. In this review, we discuss enolase, hemeoxygenase 1, collapsin response mediator protein 2 regulatory protein-2 (CRMP2), and ATP synthase alpha, which are selectively modified during AD progression and contribute to illumination of the molecular events that drive neurodegenerative phenomena. These proteins are discussed in light of their role in AD pathogenesis and progression. It is reasonable to hypothesize that HNE modification may not be a random event but occurs on specific proteins, which, in turn, display altered functions. The biological effects of such modifications are also discussed.

Enolase

Our redox proteomics studies performed on postmortem brain from annestic MCI, EAD, and LAD subjects identified HNE-modified enolase in all the three stages of the disease. We analyzed different brain regions, including the inferior parietal lobule, hippocampus, and frontal cortex, and also cerebellum, a brain region essentially devoid of pathology in AD. Compared with controls, the isoforms, α- and γ-enolase, were found to be

Table 1
HNE-modified brain proteins identified in amnestic MCI, Early AD, and late-stage AD.

AD stage	HNE-modified proteins	Function	References
MCI	Neuropolypeptide h3	Neuronal communication	[76,109]
	Collapsin response mediated protein 2	Neuronal communication	
	Lactate dehydrogenase B	Energy metabolism	
	Phosphoglycerate kinase	Energy metabolism	
	Heat shock protein 70	Stress response	
	carbonyl reductase	Antioxidant	
	ATP synthase alpha	Energy metabolism/mitochondrial function	
	Beta-actin	Cytoskeletal integrity	
	Alpha enolase	Energy metabolism	
	Pyruvate kinase	Energy metabolism	
	Eukaryotic Initiation factor alpha	Protein synthesis	
	Elongation factor-Tu	Protein synthesis	
	Hemeoxygenase 1	Antioxidant	
EAD	Manganese superoxide dismutase	antioxidant defense	[75]
	Collapsin regulatory protein 2	Neuronal communication and neurite outgrowth	
	Alpha-enolase	Energy metabolism	
	Malate dehydrogenase	Energy metabolism	
	Triosephosphate isomerase	Energy metabolism	
	F1 ATPase, alpha subunit	Energy metabolism	
LAD	Aconitase	Energy metabolism	[77,109]
	Aldolase	Energy metabolism	
	Peroxiredoxin VI	Antioxidant defense	
	Alpha-tubulin	Cytoskeletal integrity	
	Alpha-enolase	Energy metabolism	
	ATP synthase alpha	Energy metabolism/mitochondrial function	
	Glutamine synthase	Excitotoxicity	
	Superoxide dismutase 1	Antioxidant	
	Collapsin regulatory protein 2	Neuronal communication	
	Hemeoxygenase 1	Antioxidant	

Proteins highlighted in red are common targets of HNE modifications at different stages of AD.

excessively carbonylated [74,91,92], nitrated [93–95], HNE-modified [54,96], and S-glutathionylated [97]. In addition to our findings, other studies reported α-enolase as one of the most frequently identified differentially expressed proteins in the brain from both human and animal studies [98].

Our results may raise the question whether or not oxidative modification is a specific modification involved in AD or simply a result of its structural susceptibility to oxidation. Further, enolase can be found in different regions of the cell in close proximity with redox reaction centers where its many active -Lys and -His residues can undergo oxidative modifications. To address this question, it is important to recall that one of the striking metabolic features of AD is the drastic reduction of ATP synthesis and glucose metabolism [99]. Typical findings in AD-like dementia include a significant, bilateral reduction in temporal and parietal glucose metabolism, as shown by positron emission tomography (PET) studies. PET imaging with 2-[^{18}F] fluoro-2-deoxy-D-glucose (FDG) as the tracer has long been used to track AD-related brain changes by providing qualitative and quantitative estimates of the cerebral metabolic rate of glucose. Recent evidence suggests that altered glucose metabolism is a very early change in AD and is an excellent correlate of the clinical disabilities in dementia [100]. At the molecular level, metabolic changes are intimately linked to glucose consumption and oxidative phosphorylation. In response to hypometabolism, upregulation of glycolytic enzymes occurs to combat the mounting energy deficit and hypoxic environment [99]. Interestingly, in all studies of MCI, EAD, and LAD brain from our laboratory, enolase levels were increased [101]. In addition to upregulation, we also showed that enolase was among the few proteins, glycolytic or otherwise, consistently oxidatively modified in the progression from MCI to LAD.

We suggest that oxidative modification and dysfunction of α-enolase disrupts neuronal energy metabolism and ATP-dependent ion homeostasis including functions of membrane ion-motive ATPases, glucose and glutamate transporters, loss of membrane asymmetry, and signal transduction. Such oxidative and metabolic compromise may thereby render neurons vulnerable to excitotoxicity and apoptosis.

Recent studies demonstrated that enolase is not simply a glycolytic enzyme but also possesses a variety of different regulatory properties [102,103]. In particular, enolase has been reported to be a neurotrophic factor, 14-3-2 [104,105], a hypoxic stress protein [106], c-Myc-binding protein and transcription factor [107], and a strong plasminogen (PGn)-binding protein [108,109] among others (Table 2). This wide array of functions can be attributed to different DNA base sequences within enolase genes.

Enolase, the plasminogen system, and amyloid β

The plasminogen system is essential for maintenance of vascular potency and thrombolysis, by dissolving fibrin [110]. In order to exert its function, the glycoprotein PGn binds cell surface receptors via domains that recognize exposed C-terminal lysine residues. Therefore, virtually any surface protein exposing C-terminal lysines has the potential to bind and activate PGn processes. Enolase has frequently been reported as a strong PGn-binding protein within the brain and is able to integrate into the cell membrane, although without possessing a specific signal sequence [109]. However, binding α-enolase alone cannot activate PGn conversion to plasmin; the PGn proteolytic cascade must begin with cleavage by either tissue-PGn activator (tPA) or urokinase-PGn activator (uPA) [111], both of which can be found in human brain [112]. For example, when microglial and/or neuronal PGn binds membrane-integrated enolase, PGn is rapidly activated through tPA proteolytic cleavage. Consequent production of plasmin activates a number of proenzymes, prohormones, progrowth factors, and procytokines as a result of the catalytic amplification of tPA/PGn signaling [113]. Moreover, binding enolase protects plasmin from inactivation from inhibitors, like α2-antiplasmin [111]. Therefore, it can be speculated that during AD progression, when excitotoxic events occur, the upregulation of enolase may initially be an attempt to propagate neuronal-preservation pathways that ultimately go amiss.

As well known, Aβ(1–42) has the ability to aggregate into fibrils in a β-sheet conformation, similar to the cross-beta-structure that fibrin peptides adopt during fibrinolysis [114]. Due to these structural similarities, which do not reflect sequence similarity, Aβ(1–42) can bind and activate tPA through its aggregated β-sheet structure, thereby substituting for fibrin in PGn activation by tPA, in the brain, where fibrin is not present [115]. However, through tPA cleavage of PGn, activated plasmin can degrade oligomeric and fibrillar Aβ, effectively blocking Aβ neuronal toxicity. Van Nostrand and Porter [116] further demonstrated that plasmin cleavage yields an N-terminal truncated form of Aβ with altered β-sheet properties that enhanced stimulation of tPA activity in a positive feedback-loop manner.

We suggest that the multifaceted roles of enolase in AD can be described in the following model, in which concomitant upregulation and oxidative modification of enolase occur. From one side, the upregulation and membrane integration of α-enolase promote surface binding of the tPA/PGn complex, which results in the production of the protease plasmin. Plasmin, in turn, has the ability to degrade Aβ peptides associated with the bilayer and can also activate the MAPK/MEK/ERK1/2 pathway, promoting upregulation of enolase transcription. This pathway would lead to increased production of enolase and would catalytically amplify an internal signal for cell survival during AD progression. However, in contradiction, enolase, once oxidized, becomes unable to facilitate the initiation of survival pathways, which would lead to the neuronal death in brain of subjects with MCI, EAD, and LAD versus normal aged brain.

Heme oxygenase-1

Among the proteins we found to be HNE modified in brains of subjects with amnestic MCI and AD compared with control brain was heme oxygenase-1. Under conditions of oxidative stress the

Table 2
List of different functions of enolase and their possible implications in disease.

Enolase functional diversity	Disease
GLYCOLYSIS/GLUCONEOGENESIS	Various human diseases (cancer,neurological disease, autoimmunity,etc);
C-MYC BINDING PROTIEN	Tumor formation/Tumor maker;
PLASMINOGEN BINDING PROTEIN	Various human diseases (cances,neurological disease, autoimmunity, etc);
HEAT-SHOCK PROTEIN	Saccharomyces cervesiave
IMMUNODOMINANT ANTIGEN	Candida infection
NEUROTROPHIC FACTOR	Activation of pro-survival ERK1/2

– =unknown function.

brain reacts by upregulating genes involved in cell stress response to counteract neuronal damage [117]. Indeed, activation of HO-1 is one of the earlier events in AD and plays a crucial role in the adaptive response to stress [118].

HO is a microsomal enzyme that exists in two isoforms: the inducible HO-1 and the constitutive HO-2 [119]. HO-1, also known as heat shock protein-32 (HSP32), is induced by various stimuli, including ROS/RNS, ischemia, heat shock, and bacterial lipopolysaccharide and is primarily involved in the cell stress response. Conversely, HO-2 is involved in the physiological turnover of heme and is responsive to developmental factors and adrenal glucocorticoids [119,120]. HO-1 is the rate-limiting enzyme in the production of bilirubin and catalyzes the degradation of heme in a multistep, energy-dependent way, resulting in equimolar amounts of carbon monoxide, ferrous iron, and biliverdin-IXα. Biliverdin-IXα is further reduced by the cytosolic enzyme biliverdin reductase-A (BVR-A) to bilirubin-IXα, the final product of heme catabolism and a potent antioxidant [121]. The activities of both HO-1 and BVR-A are regulated by the phosphorylation of serine/threonine/tyrosine residues [122]. In the CNS, HO-2 is ubiquitous in almost all brain areas [119], whereas HO-1 is present at low levels in scattered groups of neurons [120] and also found in glial cells, where its expression can be induced by oxidative stress [123].

Increasing evidence suggests that the HO-1 gene is redox-regulated and contains in its promoter region the antioxidant responsive element (ARE), similar to other antioxidant enzymes. Since the expression of HSPs is closely related to that of amyloid precursor protein (APP), this family has been studied in brains of patients with AD [124]. Increased levels of HO-1 have been observed in association with neurofibrillary tangles and colocalized with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains [124,125]. It is conceivable that the high increase in HO-1 in AD may be a direct response to increased free heme associated with neurodegeneration and an attempt to convert the highly damaging heme into the antioxidants biliverdin and bilirubin.

Recent studies raised questions about the activation of the HO-1/BVR-A system in neurodegenerative disorders, opening a debate on its pathophysiological and clinical significance. Despite an upregulation of the HO-1/BVR-A system, a substantial protection against oxidative and nitrosative stress is not observed in AD brain. Furthermore, Hui et al. have recently proposed that excessive iron production mediated by HO-1 overexpression may be responsible for increased tau aggregation [126]. In addition, Schipper et al. showed that suppression of glial HO-1 hyperactivity may be an effective neurotherapeutic intervention in AD [127].

In this complex scenario, we have analyzed, in addition to expression levels, posttranslational modification of both HO-1 and BVR-A, which are crucial for regulating protein function, either neuroprotection or metabolic activity. In agreement with previous findings [128], we found increased levels of HO-1 in the hippocampus of AD subjects, whereas HO-2 protein levels were significantly decreased in both AD and MCI hippocampus. In addition, significant increases in Ser-residue phosphorylation together with increased HNE modification of HO-1 were found in the hippocampus of AD subjects [129]. Because HO-1 is a stress-inducible protein, and phosphorylation on Ser residues seems to be important for its activation, the increase in oxidative stress levels in the hippocampus of AD subjects could lead to an increase in HO-1 protein levels and phosphorylation to promote its activity and its interaction with BVR [130]. At the same time, the increased oxidative stress results in HNE/HO-1 adduct formation, leading to altered protein structure and function impairment. With regard to amnestic MCI, levels of HO-1 protein did not show significant differences [131], while the formation of HNE adducts on HO-1 was already evident in the hippocampus of subjects with amnestic MCI compared with controls.

Based on our findings we propose the following scenario: (1) Increased oxidative stress conditions in the hippocampus of AD subjects promote the increase in HO-1 oxidative damage (HNE adducts on its structure). Consequently, the cell tries to restore the functionality of HO-1 by increasing Ser-residue phosphorylation. (2) Increased oxidative stress results in increased Ser phosphorylation to activate protein function, then HO-1 quickly becomes a target of oxidative posttranslational modifications, which in turn could impair its function [129].

Considering the importance of this defense mechanism in neuronal homeostasis, the impairment of HO-1 function as a consequence of oxidative modification may leave neurons more susceptible to toxic stimuli and eventually to cell death. In addition, since oxidative modification of HO-1 occurs already in AD pathogenesis, i.e., amnestic MCI subjects, and continue to be robust in AD patients, oxidative damage to HO-1 may represent a putative marker of disease progression.

Collapsin response mediator protein 2

CRMP2, also known as dihydropyrimidinase-related protein 2 (DRP2), is a ~62- to 75-kDa protein that plays an important role in cytoskeletal organization, membrane trafficking, axonogenesis, axon outgrowth, and neuronal polarity [132–134]. CRMP2 was found to be HNE modified in both MCI and AD brain [54,96]. Hence, the oxidative modification of CRMP2 might play an important role in shortening of axons, thereby impairing neuronal communication and as discussed below might also be a key contributor in the development of neuropathological hallmarks of AD, such as NFTs, and loss of synapses. Recent research suggests that loss of synapses is an early feature of AD [61,135]. Therefore, the oxidative modification of CRMP2 might be a driving force in the progression of AD.

CRMP2 is phosphorylated by kinases such as glycogen synthase kinase-3β (GSK-3β) [136], cyclin-dependent kinase 5 (cdk5) [136], Rho kinase [137], calmodulin-dependent protein kinase II (CaMKII) [138], and the src family kinase Fyn [139]. Both CDK5 GSK-3β levels and activity were found to be altered in AD and MCI brain [140–142]. Phosphorylation of CRMP2 at Ser-522 and Thr-509/514 by CDK5 and GSK3β, respectively, reduces its ability to interact with tubulin and consequently reduced the stability of microtubule and axonal retraction [143,144]. Hyperphosphorylated CRMP2 proteins coexist with NFT, though the reasons for this are unclear [145,146]. We hypothesized that amyloid beta-peptide-induced oxidation of the regulatory protein, Pin1 [74,92,147] leads to dysregulation of the activities of CDK5, and GSK3β and protein phosphotase 2 A, leading to increased phosphorylation of tau protein, thereby inhibiting tau function. Such changes could lead to impaired axonal transport. Inhibition of axonal anterograde and retrograde transport deprives synapses of energy-producing mitochondria, which likely contribute to synapse loss. This, in turn, may act as signal to recruit CRMP2 to axonal sites to promote neuronal sprouting and synapse formation. That CDK5 and GSK3 β are already recruited to this site might lead to hyperphosphorylation of CRMP2 protein consequent to the loss of synapses, an early feature in AD pathogenesis.

Apart from tubulin, CRMP2 protein also interacts with various other proteins including cytoskeletal proteins actin, vimentin, the Ca^{+}-binding protein calmodulin (CaM) [148], *N*-methyl-*D*-aspartate receptors (NMDARs) subunits NR2A/2B [149], and N-type voltage-gated calcium channel (CaV2.2) [150], thereby playing an important role in endocytosis, vesicle recycling, synaptic assembly, calcium channel regulation, Ca^{2+} homeostasis, and neurotransmitter release [151]. Hence, oxidative modification of CRMP2 is consistent with altered neuronal functions as reported in MCI and AD [54,96].

A recent study showed that in addition to the phospho-CRMP2 colocalization with neurofibrillary tangles (NFTs), another protein, the Wiskott-Aldrich syndrome protein family verprolin-homologous protein 1 (WAVE1), is also found at this site. WAVE1 is important for actin assembly at the distal end and thereby important in neurite outgrowth. Hence, the oxidative modification of CRMP2 alters the functions of protein that are downstream to it such as WAVE1 consequently leading to growth cone collapse. Knock out and RNA interference studies supported this observation [152,153].

Studies conducted so far on CRMP2 suggest that posttranslational modification of CRMP2 (oxidation and phosphorylation) might impact neurons by impairing axonal transport, affecting pathways involving CRMP2 and consequently leading to synapse loss [139]. Since the oxidation of CRMP2 was observed at the amnestic MCI stage, arguably the earliest stage of AD, targeting CRMP2 conceivably could prevent or delay the progression of this devastating disorder.

ATP synthase alpha

Mitochondrial dysfunction and energy metabolism deficiencies have been recognized as earliest events in AD and have been correlated with impairments of cognitive abilities in this disorder [154,155]. The oligomeric form of Aβ species interferes in molecular and biochemical alterations in AD more so than the extracellular, insoluble amyloid deposits [156–158], suggesting that Aβ-induced mitochondrial dysfunction can play a major role in AD progression and pathogenesis [159–161].

ATP synthase subunit α is a part of complex V, responsible for mitochondrial-resident ATP synthesis. As well known, ATP synthase consists of a membrane spanning component called Fo and another part that projects out from the membrane into the mitochondrial matrix called F1. The F1 component binds to ADP and inorganic phosphate and synthesizes ATP on its surface. Subunits alpha along with subunit beta form the catalytic core in F_1, and hence it is critical for the production of ATP. Further, studies showed that the F_1F_O ATP synthase also plays an important role in cellular response to antiangiogenic agents, intracellular pH, and cholesterol homeostasis [162,163].

In AD brain the activity of ATP synthase activity was reported to be decreased [164]. Further, low levels of complex V have been reported in the isolated AD mitochondria [164]. Studies from our laboratory showed that ATP synthase alpha undergoes HNE modification in MCI and AD brain [54,96], likely explaining the reduced activity of ATP synthase and reduced ATP levels in AD brain compared to age-matched controls. Lu et al. showed oxidative damage of the α subunit of the mitochondrial ATP-synthase gene promoter, resulting in decreased levels of the ATP synthase [165]. Such changes would lead to reduced ATP synthesis and consequently to nuclear DNA damage of vulnerable genes [165]. In the hippocampus and parietal cortex of individuals with amnestic MCI, ATP-synthase lipoxidation has been reported, supporting the finding of oxidative damage of this protein reported from our laboratory and further supporting the fact that oxidative modification of key proteins plays an important role in the progression and pathogenesis of AD [54,166]. The oxidation of ATP synthase alpha would not only compromise brain ATP synthesis but would also lead to increased production of ROS, further exacerbating the affect of its oxidative damage and mitochondrial dysfunction, with consequent neuronal damage.

Studies showed that the oligomeric form of the amyloid beta-peptide is present in mitochondria that can induce oxidative damage in mitochondria [159–161]. Further, mitochondria have been reported to alter APP metabolism, enhancing the intraneuronal accumulation of amyloid β-peptides and enhancing the neuronal vulnerability [167]. Like CRMP2, ATP synthase alpha was also found to be colocalized with the NFT, suggesting that the oxidation of these proteins plays an important role in the progression and pathogenesis of AD [168]. A recent study by Vacirca et al. [169,170] reported the presence of autoantibodies to ecto-F1-ATPase (ASabs) in sera and cerebrospinal fluids from patients with AD. Further, these researchers also showed that ASabs can increase cellular uptake of high density lipoprotein (HDL) via a mechanism involving the prototypical function of ecto-F1-ATPase. Hence, oxidation of ATP synthase could also lead to increase levels of HDL, one of the risk factors for the development of AD.

Conclusions

These cytotoxic metabolites of lipid peroxidation such as 4-HNE can have severe adverse effects on protein function. The identification of specific HNE-modified proteins in the brain of subjects with AD, EAD, and amnestic MCI provides an overview of the selective cellular functions that are altered and how they possibly relate to pathology and clinical presentations of both disorders. Studies conducted so far from our laboratory suggest that HNE modification of enolase, HO-1, CRMP2, and ATP synthase is critical in the progression of AD (Figs. 4 and 5). Further studies are needed to understand the link of these proteins and other specific pathways that are altered by products of lipid peroxidation at different stages of AD. The current literature suggests that targeting brain proteins in common

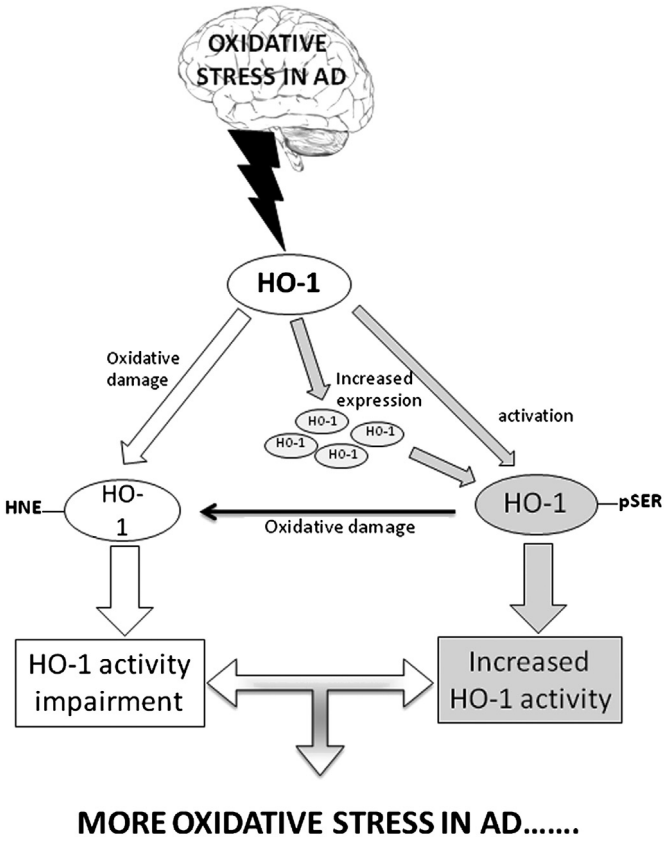

Fig. 4. Putative scenario of the oxidative stress-induced modification of HO-1 in AD hippocampus. (1) White arrows: increased oxidative stress levels in AD lead to oxidative modification of HO-1 (e.g., HNE), thus resulting in impairment of its functions. (2) Gray arrows: in response to oxidative stress, cell activates HO-1 by upregulating protein synthesis or by Ser-phosphorylation. At the same time, "activated" HO-1 is a target of oxidative modifications. Impairment of HO-1 functions may contribute to exacerbate oxidative damage in AD.

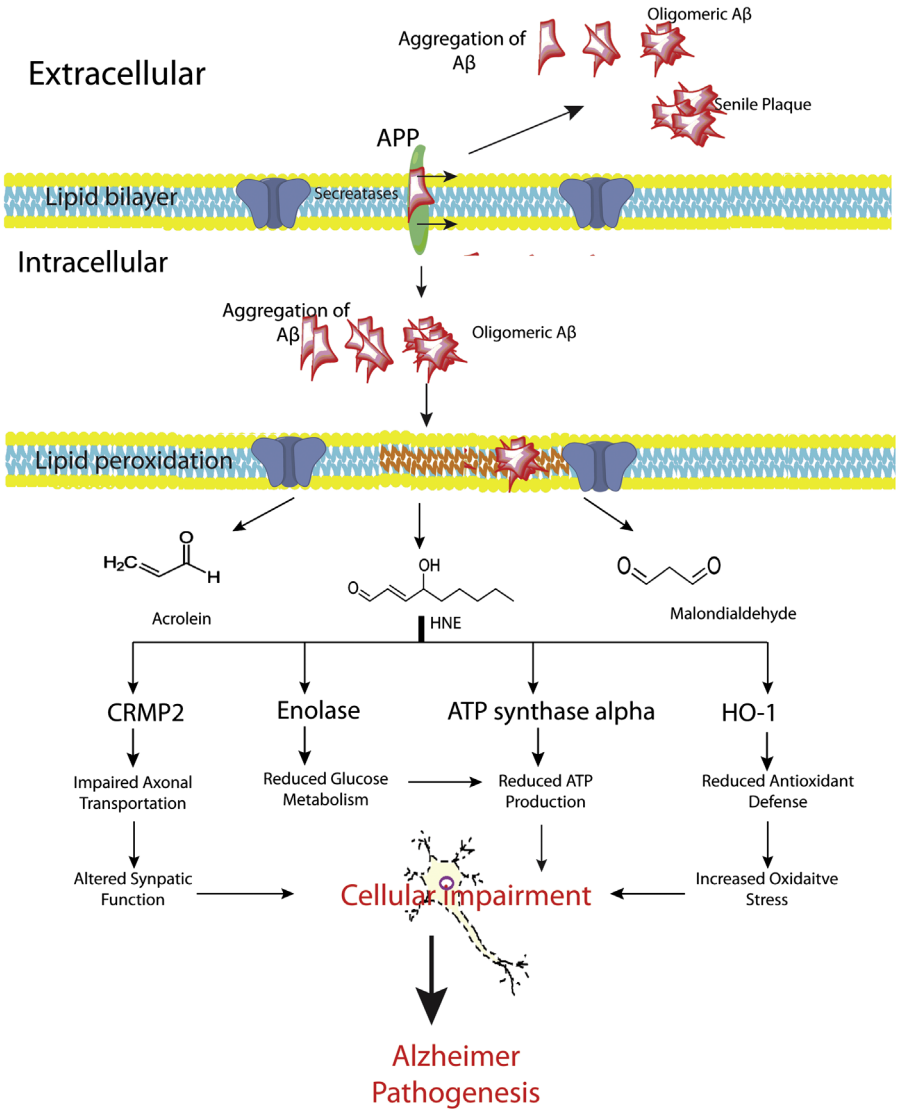

Fig. 5. Amyloid β-peptide (Aβ) is generated by proteolytic cleavage of amyloid precursor protein (APP) by the action of secretases. Once generated Aβ undergoes aggregations and is eventually deposited extracellularly as senile plaques. Oligomeric Aβ can insert itself into the lipid bilayer, subsequently initiating the lipid peroxidation process leading to the formation of highly reactive products such as malondialdehyde (MDA), 4-hydroxy 2-*trans* nonenal (HNE), and acrolein. HNE can react with the proteins forming HNE–protein adducts consequently altering the function of proteins. During the progression of the Alzheimer's disease (AD), enolase, hemeoxygenase-1 (HO-1), collapsin response mediated protein 2 (CRMP2), and ATP synthase alpha are selectively modified by HNE, eventually leading to cellular impairment and AD pathogenesis.

in LAD, EAD, and amnestic MCI with HNE modification could possibly provide a therapeutic strategy to treat or prevent this devastating disorder. The identification of early markers of the AD is still difficult because of numerous factors, including, among others, pathology occurs prior to clinical symptoms and the dynamic range of plasma or CSF protein concentrations. Development of ever-increasing sophisticated experimental approaches together with advances in tissue sampling may help to detect subtle changes of protein oxidative modification, levels, and functions that may relate to a disease state, thereby helping in the treatment, or delay AD progression. Redox proteomics will be one method to achieve this goal.

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