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Molecular Targets in Alzheimer's Disease

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Abstract
Alzheimer's disease (AD) is known as a devastating neurodegenerative disorder in aged subjects, which is related to multiple heterogeneous genetic factors. The two basic pathological aspects of AD are related to amyloid beta (Aβ) peptides and *tau* proteins. Some researchers have demonstrated plaques and tangles as apparently primary lesions. Also, experimental data propose that these two lesions are intimately related. In the present review, we highlight some molecular mechanisms linking tau and Aβ toxicities involving oxidative stress, aging, Aβ turnover, the contribution of thiol groups, and the role mitochondrial activities in the AD pathogenesis. Understanding the interplay of these mechanisms as parts of common pathophysiological pathways could reveal molecular targets to control or even treat AD.

Keywords Alzheimer's disease · Amyloid beta · Tau protein · Oxidative stress

Introduction

Alzheimer's disease (AD) is known as a degenerative, progressive brain disorder of the elderly, which is reported as the sixth agent of death in US citizens [1]. The worldwide impact of AD in neurodegeneration and human dementia is particularly important, due to the increasing rate in lifespan and elderly people. Despite this, medical research on dementia accounts for only 1 to 12 research papers respect to cancer, with a global societal cost in 2018 that did not exceed 1000 billion US dollars [2, 3]. Yet, costs to face at AD pathogenesis and development are much higher. The Alzheimer's Disease International estimated, only 3–4 years ago, an amount of about 46.8 millions of AD-dementia cases in the world, calculating total costs for healthcare of about 818 billion US dollars for 2015 [4, 5]. Therefore, global concern about dementia-related neurodegeneration, such as AD, is a major compulsory issue [6]. This should encourage researchers to improve our current knowledge and experimental research about AD diagnosis and prevention. Recently, two forms of the AD were identified, a hereditary form and a sporadic form [7]. A genetic classification in familial and sporadic forms of AD has been recently reviewed [8, 9]. In a more general way, AD is a complex multi-factorial neurodegenerative disease, where the genetic component accounts for about 70% respect to the epigenetic and environmental etiopathogenetic determinants [9]. The apolipoprotein E gene (APOE gene) seems to be major responsible for sporadic cases [9], while the genes APP (amyloid precursor protein), PSEN1 (presenilin 1), and PSEN2 (presenilin 2), together with other co-morbidity factors, such as TREM2, the gene involved in the disorder of the lipid ABCA1 and ABCA7, or for biothiol metabolism MTHFD1 and of the transport of metabolites (BIN1) and gamma-secretase, were all associated with the familial form of AD [9, 10]. However, both forms, i.e., familial and sporadic AD, show similar kinds of molecular and neuropathological manifestations such as impairment of N-methyl-D-aspartate receptor-related signaling pathways, intracellular accumulation of hyperphosphorylated tau protein, extracellular deposition of amyloid-beta, oxidative stress, metal ion metabolism disorders, abnormalities of lipid metabolism, and disturbances

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in both the structure and functions of mitochondria [7, 11]. AD is characterized by selective neuronal cell death, preceded by the intracellular production of neurofibrillary tangles and intercellular precipitation of amyloid beta (Aβ) peptides in the brain of the patients [12–14].

Furthermore, increases in β-amyloid induce neurodegeneration in memory systems before overt cognitive impairment can manifest [15]. AD is associated with a significant loss of the presynaptic cholinergic markers in the brain's cerebral cortex in aged subjects. This occurs particularly in the areas related to learning and memory [16, 17]. This investigation led to the formulation of the "cholinergic hypothesis of AD" as well as the improvement of cholinesterase inhibitor therapies [16]. AD is believed to originate from a heterogeneous and complex etiology, in some cases induced by gene mutations, for example in the codes of β-amyloid precursor protein (APP) [18, 19] and presenilins (PSEN1-L435F and PSEN1-C410Y) [20]. The accumulation of extracellular Aβ in the brain of patients with AD is an important factor in the present of senile plaques of brains [21]. This induces production of significant amounts of free radicals and also the elevation of intracellular levels of calcium ions, followed by continued loss of neurons in the hippocampal area of the brain [22]. The destructive cascade in the AD brain is comprised of neurochemical loss that disturbs cell-to-cell interactions, abnormal structuring of cytoskeletal proteins, pruning of dendrites, loss of synapses, damage induced by oxidative metabolism, and ultimately cell death [12]. Clinical and experimental examinations have reported several mediators of inflammatory responses involved in neurological disorders [23]. Histological studies of brain tissue in the AD patients revealed chronic inflammatory responses in brain tissue leading to neuronal loss. Other studies demonstrated the relevance of hyperhomocysteinemia, oxidative stress, and changed cerebrovascular remodeling as factors accompanying the neuroinflammation state in AD patients [24]. Furthermore, homocysteine over long periods may contribute to dysfunctional clearance of Aβ peptides and blood-brain barrier impairment which may cause inflammatory processes or cerebrovascular dysfunction resulting in the AD development [25]. Another study also revealed that stimulated microglial cells and reactive oxygen species (ROS) play an important role in the neuron loss of brain, and the apolipoprotein E allele epsilon 4, as well as nuclear factor κB (NF-κB), contributes in the inflammatory responses of patients with AD [26]. Moreover, dysfunction of mitochondria and oxidative damage of DNA are still contributing to the many molecular aspects of the AD brain [27]. It has also been demonstrated that perturbed mitochondria-associated endoplasmic reticulum (ER) is involved in the AD pathogenesis [28]. Recent advances in our knowledge about the pathogenic process of AD brain provide fundamental knowledge for early detection and also for the improvement of therapeutic approaches to arrest the AD progression. The objective of this review is to highlight some molecular mechanisms linking tau pathology and amyloid β toxicities to oxidative stress, which could be increased by the involvement of thiol groups, and mitochondrial dysfunction in the AD pathogenesis. This review work should suggest therefore novel approaches to better diagnose AD and gather the many reports on AD pathogenesis and development to highlight which biomarkers can be useful in AD diagnosis.

Amyloid-β Protein (*Abeta*), Oxidative Stress, Aging, and Aβ Turnover

Amyloid beta (Aβ) peptides are key molecules in the pathogenesis of AD [29]. The subsequent cleavage of amyloid precursor protein (APP) by the γ- and β-secretases generates Aβ peptides; in contrast to the physiological cleavage of APP by the α- and γ-secretases, which decreases Aβ production [14, 29]. Self-assembly of Aβ protein into toxic oligomers, polymers, and precipitates is believed to cause or contribute to AD [30]. The occurrence of Aβ in mitochondria induces mitochondrial dysfunction and free radical production [31–33].

It has been suggested that the endoplasmic reticulum (ER) is the original source for much of the Aβ precipitation [34]. Misfolded membranes and secretory proteins, which are degraded by proteasomes, are moved from the ER to the cytosol. Proteins concentrate as pericentriolar aggregates (aggresomes) when the amount of moved misfolded proteins exceeds the capacity of the degradation mechanism [34]. However, it has been suggested that A*beta* generates cytosolic aggregates after its transport from the endoplasmic reticulum [35]. After that, several constituents of aggresomes are shared by the same aggregates. Therefore, A*beta* aggregates are derived from aggresomes after their accumulation randomly around the nucleus. Thus, A*beta* can form cytosolic aggregates and also intranuclear aggregates [34].

It is conceivable that at least part of the A*beta* might be formed in the cytosol. This occurs by an abnormal degradation of something that originally has entered the cytosol in the form of a normal protein molecule and modified the amyloid precursor protein (APP) in its physiological form. Then, APP is degraded, as already mentioned, by successive cleavage by different peptidases, which are called α-, β-, and γ-secretases [29, 36]. In the subsequent cleavage of APP itself by β-secretase, a protein fragment called the APP C-terminal fragment (APP-βCTF or C99) is formed [36]. This occurs next to the fragmentation of C99 by γ-secretase leading to A*beta* production. When C99 is cleaved by γ-secretase, another protein fragment is also formed, which is called the APP intracellular domain (AICD) [37]. AICD is also harmful. AICD and C99 can both be degraded through the ubiquitin-proteasome system, with this degradation being regulated by ligand-activated EphA4 signaling [38].

This might mean that the restriction of the ubiquitin-proteasome system may increase the accumulation of C99 at a given rate of the APP synthesis in the cell. Furthermore, to increase the production of A*beta* and AICD, A*beta* excesses are expelled to the extracellular space.

In addition, it has been showed that ligand-stimulated EphA4 signaling rules the proteostasis of AICD, Aβ, and C99, without notably affecting the activity of γ-secretase [36]. EphA4 was found to induce aggregation of AICD and C99 by a Lyn-dependent process. The induction of this pathway causes EphA4 phosphorylation, leading to the positive feedback of AICD and C99 proteostasis. Restriction of EphA4 through the dasatinib, a receptor tyrosine kinase inhibitor, was found to suppress the accumulation of AICD and C99 effectively [36].

Lyn is a protein tyrosyl kinase (belonging to a family member of Src protein tyrosyl kinases), which can be activated by various pathways. It is not only specifically triggered by other proteins that are found upstream as part of signal cascades passing via Lyn, but also in a more unspecific way by oxidative stress induced by H_2O_2, peroxynitrite [38–41], or A*beta* [42]. It is very common that tyrosyl protein phosphatases are inhibited by oxidative stress, which also occurs in the case with phosphatases reversing the action of Src kinases [43–46] It has been demonstrated that peroxynitrite inhibits tyrosyl protein phosphatases in the synapses [47]. Protein tyrosyl phosphatases can also be inhibited by reaction with protein hydroperoxides [48]. In this perspective, oxidative stress in the cytosol will lead to simultaneous stimulation of Lyn activity and inhibition of phosphatases that have an opposite regulatory effect. This should lead to C99 accumulation, causing in turn improvement of the rate of A*beta* production [40].

Inhibition of the activity of proteasome by an excessive burden of oxidative and nitrative stress has been reported recently [49]. The ubiquitin-proteasome process is the primary machinery of a cytosolic proteolytic mechanism for selective degradation of different forms of damaged proteins [50]. In healthy and young subjects, proteasome activity could rapidly and selectively degrade moderately oxidized soluble cell proteins [51]. However, proteasome could be inhibited by severely oxidized, cross-linked proteins because they are poor substrates for degradation [51]. Recently, it has been reported that the activity of proteasome is reduced during aging since the protease is constantly inhibited by binding to elevated contents of oxidized and cross-linked protein aggregates. For this reason, cellular aging likely induces a constant decrease in the proteasome activity, indicating the growing accumulation of oxidatively damaged protein aggregates that eventually are involved in the cellular senescence and dysfunction [51].

An age-related loss in function of proteasome has been documented in many tissues such as the retina [52, 53], which is a tissue embryologically closely related to the brain with a histological structure strongly overlapping with the latter. However, this occurs in a far more amenable way than any further non-invasive study on the brain. In a research study of old and young adult F344BN rats, the protein oxidation and function of proteasome in retinal homogenates have been studied and compared [52]. In aged rats, in the rate of casein degradation, a decrease of 80% in the retinal proteasome and a loss of 75% in chymotrypsin-like function were reported. This activity loss could be partly attributed to a 50% decrease in the 20S proteasome expression. Furthermore, the immunochemical study with antibodies that identify these specific modifications of proteins reported that aged rat retinal proteins revealed considerable immunoreactivity [52]. These observations proposed that the age-related decrease in the activity of proteasome is contributed in the oxidized retinal protein accumulation. Thus, a mixed effect of an inactivated protease responsible for the increase in oxidized proteins (so ridding the cell of oxidized proteins) identified in the aged retina involves an important risk for permanent damage induced by oxidative stress [52]. It is a plausible working hypothesis that what happens in AD may be a similar age-related loss of proteasome activity that does not only lead to an accumulation of oxidized proteins inside the nerve cells but also of A*beta* extracellularly [54].

In a study on the molecular pathways in the age-associated loss of proteasome activity, it was detected that the cysteine residues in proteasomes of young rats were modified with the sulfhydryl-reactive chemical *N*-ethylmaleimide [53, 55]. Similar observations as reported in aged retinas, such as the inhibition of the chymotrypsin-like function and declined degradation of casein, were also observed [53]. Therefore, chemical changes of cysteine as proposed from in vitro assays may partly represent changes in the aging proteasome, suggesting that cysteinyl group oxidation may be one of the important mechanisms explaining the loss of proteasome activity in aged animals [53].

Tau Protein

Tau (tubulin-associated unit) protein is known as a heat-stable highly native and soluble unfolded protein which in its physiological form is bound to microtubules of neurons [56]. Together with other MAPs (microtubule-associated proteins), tau exerts important effects to create the network of neuronal microtubules in the cytoskeleton [57]. Dysfunction and intraneuronal accumulation of the microtubule-related protein tau in the form of insoluble paired helical filaments appear to be involved in early stages of the AD pathogenesis [58]. It has been suggested that oligomerization, hyperphosphorylation, and propagation of tau have important roles in the pathological pathways leading to cellular disintegration and the consequent

neurodegeneration [59]. Tau phosphorylation has been studied at AD-associated sites via recombinant human tau phosphorylated by in vitro DNA damage-stimulated checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) [60] and revealed 27 residues of Ser/Thr as target sites of Chk1 or Chk2. It has been shown that 13 of these sites could be phosphorylated in the brains of AD subjects [61]. In addition, various protein kinases such as (1) proline-directed protein kinases (PDPK) including mitogen-activated protein kinases (MAPK) (such as Erk1/2, JNK1/2/3, and p38), cyclin-dependent protein kinase-5 (CDK5), glycogen synthase kinase-3 (GSK3), and dual specificity tyrosine-phosphorylation-regulated kinase 1A/B (Dyrk1A/B); (2) non-PDPK, containing cAMP-dependent protein kinase A (PKA), tau-tubulin kinase 1/2 (e.g., casein kinase 1α/1δ/1ε/2), PKB/AKT, phosphorylase kinase, protein kinase N, microtubule affinity-regulating kinases, protein kinase C, and Ca2+/calmodulin-dependent protein kinase II (CaM kinase II); (3) tyrosine protein kinases, such as Src family kinase (SFK)members (e.g., Syk, Src, Lck, and Fyn) and Abelson family kinase members, ABL1 and ABL2 (ARG) could phosphorylate tau sites [58, 62]. Tau hyperphosphorylation negatively influences its physiological ability to stimulate the physiological microtubules assembly. Among the kinases listed above, GSK3 appears to be directly involved in the AD pathogenesis, also contributing to the production of Aβ and Aβ-associated neuronal death. The sequence of these events is initiated through tau phosphorylation in most threonine and serine residues and activation of hyperphosphorylation in paired helical filaments [63]. It has also been reported that casein kinase1 (CK1) and CDKs play important roles in the tau hyperphosphorylation and formation of intracellular tangles, and secondarily Aβ peptides aggregation [64]. An immunohistochemical analysis on human postmortem brain tissue using tau-1 antibody, tau mRNA expression analysis in the mouse brain and electron microscopy, revealed that tau protein was primarily found in the neuronal axons and in very small amounts in oligodendrocytes and astrocytes [65–67]. It has been reported that tau dysregulation or malfunction alone can induce neurodegeneration with mutations in the MAPT gene on chromosome 17, causing cytoskeletal abnormalities in AD patients [68, 69]. More particularly, abnormal accumulation, phosphorylation, and proteolysis of the tau protein in a "pre-tangle" stage of neurofibrillary degeneration have been demonstrated to be a primary step in the AD pathogenesis [58]. Thus, a study reported that tau toxicities in AD individuals are associated with free radicals signaling and oxidative stress-induced p38 activation [70]. Moreover, some molecular pathways related to tau and amyloid β toxicities are linked with p38, glycogen synthase kinase 3β, cyclin-dependent kinase 5, Pin1, and regulation of calcineurin 1 [71].

Involvement of Thiol Groups and Oxidative Stress

Thiol groups can be oxidized by reaction with reactive oxygen species as well as with peroxynitrite [72–74], the latter reaction proceeding much faster than the first one, e.g., the reaction between glutathione (GSH) and H_2O_2 [72]. Kinetics of thiol group oxidation by H_2O_2 and peroxynitrite has been studied and compared already in early reports [72]. Apparent second-order rate constants were found to be 2600–2800 M s^{-1} and 5900 M s^{-1} for the single thiol of albumin and the reaction of peroxynitrite anion with free cysteine, respectively, at pH 7.4 and 37 °C. These rate constants are three times higher than the corresponding rate constants for the H_2O_2 reaction with sulfhydryls at pH 7.4.

Moreover, unlike H_2O_2, which can oxidize thiolate anion, peroxynitrite anion can interact directly with the non-dissociated thiol (-SH) group. Repair of protein molecules that have been damaged because of thiol group oxidation to form disulfide groups will normally be carried out by collaboration between reduced glutaredoxin and reduced thioredoxin, with glutaredoxin reducing the mixed protein-glutathione disulfides, while thioredoxin reduces abnormal internal protein disulfide groups. Glutaredoxin uses GSH as a reducing cofactor for regeneration of its active reduced form after it has been oxidized, and therefore the function of glutaredoxin as a protein repair enzyme might be highly vulnerable to intracellular GSH depletion. And furthermore, the regeneration of thioredoxin to its active reduced form depends on the activity of the selenoprotein thioredoxin reductase. Adequate intakes of essential sulfur amino acids and selenium to fortify GSH and the selenoenzymes have been recommended [75, 76].

As the observed age-related decline in proteasome function in the retina of rats most likely can be explained mainly as a consequence of increased production of ROS in the mitochondria because of mitochondrial DNA aging [77], it will certainly not ameliorate the situation if the function of glutaredoxin is compromised because of intracellular GSH depletion or if the function of thioredoxin as a protein-repairing enzyme is compromised due to low dietary intake of selenium (Se) [76]. GSH-dependent and Se-dependent enzyme systems are, moreover, critically important for scavenging the thiol-oxidizing species H_2O_2 and peroxynitrite, with thioredoxin reductase, as well as the glutathione peroxidases, being very important. GSH depletion and inhibition of the protective enzymes can result from exposure to toxic metals, involving the

fact that the repair of the oxidized protein molecules should be inhibited.

Altered proteasome function as a result of aging has also been observed in other organs, such as the muscle [78, 79], liver [80–82], heart [83], epidermis [84, 85], and human lymphocytes [86]. But, in skeletal muscle, the loss of activity of individual proteins seems to be compensated for by enhanced synthesis [78]. However, it is reasonable that this form of compensation might be compromised in aged individuals who have poor diets with protein malnutrition accompanied by catabolism, which might also influence cerebral functions.

A study on overexpressed scavenger enzyme selenoglutathione peroxidase-1 (GPx-1) on the interaction with the chymotrypsin-like function of the 20S proteasome in the T47D human cells was reported that GPx-1 overexpression paradoxically led to 30% declined activity of the chymotrypsin-like function of 20S proteasomes [87]. The most plausible explanation for this observation is some form of redox control of the expression of 20S proteasomes (since both the 20S and 26S proteasomes are multiprotein complexes), so that oxidative inactivation of individual proteins can be balanced by increased synthesis of the same protein—in accordance with the observations on rat skeletal muscle mentioned above [78]. However, with too much oxidative inactivation of proteasome proteins, there might be an upper limit for the possibility of full compensation through the enhanced synthesis of the same proteins. However, it is likely that such limits might be greatly exceeded in Alzheimer's brains. In another study, it was reported that the activity of aged 20S could be by partially rescued by the antioxidant dithiothreitol (DTT). The latter observation is compatible with the hypothesis that oxidation of functionally significant cysteines is an important mechanism of protein inhibition [79].

Studies on the activity of peptidylglutamyl peptide hydrolyzing have revealed a gradual decline with age, indicating about 60% lower contents in the aged animals respect to the younger rats. These changes were evaluated as the outcomes of the age-associated extension of the half-life of proteins [82]. If a similar age-related decline in proteasome function also occurs in the brain, it must be thought to lead to more accumulation of the A*beta* precursor C99 and hence more A*beta* formation. However, it has also been observed that while an age-related decline in liver 20S proteasome peptidylglutamyl-peptide hydrolase activity could be observed in rats fed with a standard diet, proteasome activity was restored to the normal values in old rats eating a self-selected diet, leading to a reduction of the protein intake. The animals must evidently have some primordial instinct permitting them to adjust their diet, to compensate for the harmful effects of the aging process. Surprisingly, a reduction of protein intake thus acts as protective. But this is in accordance with the observations of Barja and coworkers, who found that a high intake of methionine enhances mitochondrial ROS production and shortens the life span of the animals [88–90].

Furthermore, in a study of age-associated modifications in the activity of 26S proteasome in human lymphocytes from 20 to 63-year-old blood donors, it was found that the identified age-associated decline of 26S proteasome-specific functions was related to an elevated yield of post-translational modifications of proteasome subunits, although the proteasome content and subunit composition remained unaltered [86]. Principally, some assembly and catalytic subunits of the 26S proteasome must have been selectively changed with age [86, 91]. Moreover, based on these observations, it was proposed that structural changed of proteasome subunits may result in the reported decline of proteasome functions with age and could explain components of the immune senescence [91]. These observations on peripheral lymphocytes are obviously also relevant for obtaining a better understanding of sequences of events in Alzheimer's brains but need to be repeated with direct studies on brain tissue from Alzheimer patients.

In the canonical ubiquitin-proteasome pathway, ubiquitin, as well as the 26S proteasome, is an important contributor [50]. Adequate sensitivity of 26S proteasome and the ubiquitin-conjugating enzymes to oxidative damage appears to afford an important response to mild oxidative stress [50]. Once A*beta* has been formed, it can be degraded in the mitochondria by a proteolytic enzyme or peptidase which because of its special structure is called a peptidasome.

Pre-proteins and Peptides

The mitochondrial peptidase [92, 93] named presequence protease (PreP) could degrade unstructured peptides such as Aβ, whose progression may have harmful effects on mitochondrial activity [92, 94, 95]. Unstructured peptides and presequences are formed by cleavage inside the mitochondria of preproteins that are coded by the nuclear DNA and contain a target sequence making them inactive outside the mitochondria while targeting them for transport into the mitochondria by a special membrane transport system called the presequence translocase [96]. The preproteins (PreP) contain amino-terminal targeting sequences that are deleted through the mitochondrial processing peptidase (MPP). Some of the PrePs contain bipartite presequences that are divided twice, by the inner membrane protease (IMP) and MPP, while PreP finishes the job by removing the degradation products formed by MPP and IMP [96].

PreP was mainly recognized and described in *Arabidopsis thaliana* as a localized metalloprotease in the chloroplast stroma [92]. PreP shows an effective role as a peptide-clearing protease with a function in the degeneration of unstructured peptides (up to 65 amino acid residues) that may be toxic to chloroplasts or mitochondria [92, 93]. In the *Arabidopsis*

thaliana, it has been reported a particular enclosed large cavity of 10,000 Å^3 indicating a new catalytic pathway for proteolysis in which a hinge region encloses a large catalytic chamber opening by two halves of the enzyme-linked and closing in peptide binding response. Furthermore, in the human mitochondria, PreP homologs have been detected [92, 93, 95]. Molecular form of human PreP according to the crystal structure at 2.1A resolution of PreP in the *Arabidopsis thaliana* has revealed two cysteyl groups, named Cys(90) and Cys(527), which produce disulfide bridges in the oxidizing environments and could be a target in redox modulation of the enzyme [94]. Recent investigation has reported that PreP function is decreased in AD patients and mouse models of AD compared to controls, which is related to an increased ROS production in the mitochondria [94, 95].

The characteristic pathological changes related to AD are reported in some sections of the brain characterized by large mitochondrial ROS production, and a reduction of PreP has been reported in those parts of the brain [32]. By contrast, no significant difference in the activity of hPreP could be found in the cerebellum, which is a brain region typically spared from Aβ aggregation, in compared AD samples to non-AD controls [32]. Mitochondrial fractions isolated from brains of Alzheimer disease and the brains of a transgenic mouse Alzheimer model were also found to have higher contents of 4-hydroxynonenal, as compared with those from non-Alzheimer humans and non-transgenic, normal mice. Cytochrome *c* oxidase activity was also demonstrated to be significantly decreased in the mitochondria of AD subjects. These findings were thought to propose that declined PreP proteolytic function, possibly because of increased production of ROS, contributes to the accumulation of Aβ in mitochondria inducing to the neuronal death and mitochondrial toxicity that is exacerbated in patients with AD [32, 33]. The H_2O_2 effect, which is a relevant biological oxidant, on the recombinant human PreP (hPreP) activity has been studied [95]. It was found that H_2O_2 inhibited the activity of hPreP in a concentration-related manner, oxidized amino acid residues (identified via carbonylation), and decreased protein stability. The replacement of the evolutionarily preserved methionine 206 for leucine was found to result in elevated hPreP susceptibility to oxidation, suggesting a likely protective effect of methionine 206 as an internal antioxidant [95]. It was also reported that the hPreP-oxidized activity at low contents of H_2O_2 could be repaired through methionine sulfoxide reductase A (MsrA) (which is an enzyme that, while being found in various parts of the cell, is also found in the mitochondrial matrix), indicating that hPreP could establish a substrate for MsrA [95, 97]. These in vitro observations proposed an effect of hPreP redox control in the mitochondrial matrix and revealed the protective effects of the preserved methionine 206 residues as an internal antioxidant [95].

The observations quoted here concerning the role of PreP in the normal degradation of Aβ and how PreP is inhibited by too much oxidative stress inside the mitochondria are critical to better understanding the pathogenesis and also the etiology for AD [98]. The disease clearly must start when the ROS plus peroxynitrite in the mitochondria in vulnerable parts of the brain has reached a certain critical threshold, causing so much inhibition of PreP that Aβ will start to accumulate in the form of a precipitate. Once this has occurred, there will be a cascade effect because an Aβ precipitate will itself function as a strong oxidant stressor [99–101] and contributes to the further inhibition of PreP. Moreover, Aβ fibrils have also been found to enhance nitrosative stress [100], because they function as redox cycling agents [102, 103], generating superoxide anion radicals that can react with NO to produce peroxynitrite. The NO reaction with superoxide anion radical within the mitochondria might be produced inside the mitochondria themselves by mitochondrial NO synthase [104–106].

Aβ Fibrils and the Hypothesis of Mitochondrial Dysfunction

It has been reported that Aβ fibrils cause not only oxidation, and nitrotyrosination, but also glycation of cell proteins in yeast cells [100]. It was also found that Fe and Cu chelators and the antioxidants GSH and Trolox®, were neuroprotective on the mouse hippocampal neurons and neuroblastoma cells treated with Aβ fibrils [100]. GSH was reported to prevent the glycation, nitrotyrosination, and oxidation of cell proteins stimulated by Aβ [107].

These observations might indicate that the mitochondria are the initiators of the Aβ generation. Once formed, Aβ fibrils are resistant to degradation, and they leave the place where they were first produced and accumulate in other locations—where they will continue to harm because of their toxic properties. This occurs when the whole cell where the fibril was produced dies by apoptosis [108, 109] or by necrosis. The Aβ fibrils will then be released to the extracellular environment, but may be available for re-uptake, e.g., in phagocyting cells. However, while many substances will be degraded completely by macrophages, there are also well-known examples of substances, such as cholesterol from LDL (in atheromas), asbestos crystals (in asbestosis), and quartz crystals (in silicosis) that the macrophages cannot degrade, and that will continue to be harmful after endocytosis by macrophages.

We hypothesize that Aβ fibrils may leave their mitochondrial site of generation and be released into the cytosol of a cell that is still alive, either by the regulated process called mitoptosis, which has been reported to take place also in

mitochondria in the synaptic region of nerve cells [108]. There is no doubt that Aβ fibrils may cause much disturbance and damage to mitochondrial membranes [110–113].

Aβ fibrils are found to accumulate in the endoplasmic reticulum of neurons and extracellularly as plaques [112, 114, 115]. If they are formed mainly in the mitochondria, and cannot be formed in the endoplasmic reticulum, one needs to postulate some transport process, whereby the Aβ fibrils, following release from those mitochondria can also be transferred to the endoplasmic reticulum.

Mitochondria and Their Role in AD

Normal apoptosis in other cell types than neurons or astroglia depends on signal cascades that can start in different parts of the cell, in either the plasma membrane or the mitochondria, leading to some form of digestion of all parts of the cell by autophagia involving DNA and RNA molecules. Neurons are much larger than most other non-syncytial cells (i.e., cells that have only one nucleus), and the distance is often large from a synapse to the nucleus. This means that it may be kinetically difficult for pro-apoptotic signals arising in a remote synaptic region to travel to the nucleus, which is necessary if the chromosomes in the nucleus shall be degraded, as occurring during apoptosis in other cell types.

A working hypothesis is that the death of only part of the cell without the death of the entire cell by apoptosis may be common both in the central and peripheral nervous system. This is documented by clinical observations during and following stroke and mechanical brain trauma. During a stroke, there will normally be several nerve cells that die completely, but there will also be a large number of nerve cells that have been only partially damaged, and that have a potential for regeneration afterward. Something similar might happen in AD, although it is also differenced as regards the pattern of localization of the damage in individual neurons. However, in the Alzheimer process, it may be possible that the synapses are most strongly affected, while the rest of the dendrite or axon and also the perikaryon will survive until the disease has reached an advanced stage of progression. The reason for this can be explained as follows: The magnitude of oxidative and nitrative stress inside the mitochondria is determined by the ratio between rates of ROS and NO production on one side and the total scavenging capacity of enzymes and small organic scavengers on the other. NO can be scavenged by reaction with glutathione [116], and several among the harmful substances can be scavenged by reaction with melatonin [117], but most of the scavenging is carried out by intramitochondrial enzymes, including the mitochondrial Mn-dependent superoxide dismutase-2 [118–121].

Se-dependent GPx-1 [122–124], 2-Cys peroxiredoxins, such as peroxiredoxin-3 [125–129], and peroxiredoxin-5 [128, 130] are major factors involved in AD pathogenesis. Furthermore, also thioredoxin-2 as reducing cofactor for the 2-Cys peroxiredoxins [120, 124, 126, 131, 132], and thioredoxin reductase-2 for regenerating thioredoxin-2 in its reduced form [124, 133, 134], are important factors if present at all, inside the mitochondria, although they are better related to the mitochondrial outer membrane [122]. The thioredoxin reductase/thioredoxin/peroxiredoxin system is much more important than GPx-1 for the removal of H_2O_2 from brain mitochondria [124, 135]. Levels of GPx-1 and glutathione reductase in brain mitochondria are low [136]. Resveratrol, which is found in red wine, has been reported to increase the expression of Mn-dependent superoxide dismutase and thioredoxin-2, as well as of X chromosome-linked inhibitor of apoptosis protein (XIAP) in PC6.3 cells [120]. XIAP has also been found to enhance the expression of Mn-dependent superoxide dismutase and thioredoxin-2 [131].

The rate of ROS production in the mitochondria is associated with numerous factors. Most of it is due to the reaction between molecular O_2 and electrons in enzymes in the respiratory chain, especially at the top of the chain in complex I, but also in complex 3 and perhaps other respiratory chain enzymes [137]. It is likely that the selenide/sulfide contents ratio in iron-sulfur groups in respiratory chain enzymes may be one of the key elements influencing the ROS production rate in the mitochondria, with the ROS production rate being enhanced if the selenide/sulfide ratio in the iron-sulfur groups is low [137]. The taurine concentration inside the mitochondria may be another major factor since it has been showed that taurine can very significantly decline the ROS production rate in the mitochondria [138]. The reason for this is however not completely understood. It is possible that it may occur mainly because taurine depletion can lead to reduced synthesis of mitochondrial tRNAs, which contain bases that have been modified by containing taurine [139–142]. But it is also arguable that the sulfonic acid group of taurine might participate in mixed complex formation with Fe atoms in iron-sulfur groups, shielding the Fe atom against reaction with molecular O_2.

The density of electrons that can react with O_2 in complex I depends partly on the rate of electron feeding from the tricarboxylic acid cycle into the respiratory chain and partly on the Ohmian resistance against passage of electrons from complex I to cytochrome *c* inside the chain. The rate of electron feeding into the respiratory chain is in large measure controlled by the Ca^{++} concentration inside the mitochondrial matrix since Ca^{++} regulates the functions of pyruvate dehydrogenase, NAD-isocitrate dehydrogenase, and oxoglutarate dehydrogenase [143]. The Ohmian resistance against the passage of electrons from complex I to cytochrome *c* also related to several factors that probably may include the composition of fatty acid in the membrane lipids [137], the concentration in the membrane of the electron shuttle ubiquinone, and the concentration of ceramide acting as a blocking factor to electron transport by the

respiratory chain [144]. But the most important factor is the synthesis rate for proteins being part of various respiratory chain enzymes, which are multiprotein complexes, where some of the component proteins are coded for by DNA in the cell nucleus, and others are coded for by mitochondrial DNA.

The mutation rate is much higher in mitochondrial DNA than in the nucleus, and as we become older, more and more mitochondrial mutations will accumulate [77], with many of the mutations being in the form of deletions, which sooner or later will lead to decrease of the number of copies of normal mitochondrial genes per cell. This must, in turn, sooner or later lead to a decrease of the production of mitochondrial RNA, both rRNA, tRNA, and mRNA, to the extent that this will limit the rate of mitochondrial production [77, 145].

It must be expected for theoretical reasons that there must be a synergistic interaction between protein malnutrition leading to deficient supply of some of the amino acids and age-related reduction of the production of mitochondrial tRNAs as causes of impaired mitochondrial synthesis of aminoacyl-tRNAs for which reason protein malnutrition may be even more dangerous for old patients than it is for young adults [145]. It is not well known, however, to what extent this might also be true for mitochondrial protein synthesis in the brain since it is conceivable that the brain might have better homeostatic capacity than most other organs for regulating its own supply of amino acids.

It is a reasonable hypothesis that the rate of mitochondrial DNA aging in the nerve cells is highest at the synaptic site. This is because excitatory transmission is always attended by enhancement of the cytosolic Ca^{++} concentration in the synaptic region of the post-synaptic neuron, and enhancement of the cytosolic Ca^{++} concentration will, as earlier described, lead to enhanced activation of the neuronal NO synthase and uptake of Ca^{++} by the local mitochondria, which will lead to elevated production of intramitochondrial ROS.

Thus, the formation of Aβ fibrils may start sooner in mitochondria in the synaptic regions than in other parts of the nerve cells. It is partly not only because the rate of mitochondrial DNA aging is highest there, but also because there is much more stimulation of mitochondrial ROS production than in the mitochondria of other parts of the same nerve cells.

The reason why the hippocampus is especially vulnerable in AD might perhaps very simply be that the normal synaptic traffic associated with learning processes is especially large there, compared to other parts of the brain.

Also, if the disease process starts in the synapses, it gives a fairly straightforward explanation of why the possibility of new learning is blocked. At the same time, this hypothesis involves a possibility of repair, as long as it is only parts of the nerve cells affected that are deteriorated, while the perikaryon of the same cells is still alive. First, it is necessary to stop the formation of new Aβ fibrils by improving as much as possible the antioxidative and antinitrative defense capacity in the synapses. Next, one may try to remove, if possible, Aβ fibrils that already have been formed. The last step of the therapy is to wait for regeneration of the synapses to occur, depending on the migration of "young and healthy" mitochondria from the "stem mitochondria" reservoir to the synaptic regions. This final step of therapy is in principle not much different from repair and rehabilitation following a stroke or severe head trauma because of traffic injuries. It will be facilitated by intense training programs accompanied by an optimal "brain food" diet, presumably with nutrients that are found at high concentrations in human milk because they are needed for normal development of the neonatal brain.

What has been said above about the start of the AD process makes it more easy to understand the pathogenesis. Everything that may involve in the elevated rate of mutations in the mitochondrial DNA in some parts of the brain that is more directly affected in AD must be thought to lead to higher risk for AD, and also everything that may involve the reduction of the ability of the antioxidative/antinutritive defense system of brain mitochondria. This means that the AD etiology must be very multifactorial. It is an example of the rule of many small brooklets making a big river, and where the total number of brooklets must be large, although it is not every brooklet that is equally small. Some of them should perhaps better be considered rivers in their own right.

Concluding Remarks

The possibility to be fully endowed of a wide panoply of methodological tools and biomarkers to enlight our knowledge about AD pathogenesis and development is a keystone in current neurobiology of age-associated neurodegenerative disorders. Many steps ahead must be walked down. However, in this review, we attempted to describe some recent molecular markers on AD, which are useful in this pathway. The destruction of tubulin in the cytoskeleton accompanied by the release of associated tau protein is central in the early stages of the AD pathogenesis. It has been proposed that propagation oligomerization, hyperphosphorylation, and fibrillization of tau show effective roles in the pathological pathways to cellular disintegration and the consequent neurodegeneration. Tau hyperphosphorylation also negatively influences its physiological ability to activate microtubules assembly in the AD pathogenesis, contributing to the Aβ production and Aβ-activated neuronal death via the activation of hyperphosphorylation in paired helical filaments and tau phosphorylation in most threonine and serine residues. Furthermore, a role of age-related mitochondrial dysfunction may explain the connections between all the AD symptoms and aberrant cell cycle re-entry, metal metabolism disorders, and autophagy in neurons, indicating a complex process

involving aging effects. If the Alzheimer disease process starts with mitochondrial production of Aβ fibrils, especially in the synaptic regions, this is just the top of a vast cascade of pathophysiological processes also including the hyperphosphorylation and accumulation of *tau* proteins and secondary inflammatory reactions, both cascades contributing to the enhancement of the anatomical lesions and loss of normal function. All the secondary processes may occur as a result of Aβ not being normally degraded in the mitochondria. Moreover, localized functions of mitochondria-associated ER membranes are markedly changed in cells from patients with AD and in cellular and animal models of AD. The mitochondrial cascade hypothesis indicates the Aβ is only a biomarker of brain aging, and not a cause of AD. The association of the many molecular pattern and markers in AD with oxidative stress may be particularly strategic for AD prevention. Deepening such factors leading to AD-caused dementia by the many molecular markers involved in the mitochondria-ER stress relationship and ROS scavenging, is fundamental to attempt a reliable prevention activity before senescence, to warrant elderly population in avoiding sporadic AD, or to reduce the effects of a familial AD. This can occur by acting also on diet, lifestyle, and environmental pollution. The present review has highlighted basal mechanisms for the AD development beyond the prominent amyloid-beta hypothesis, tracing an opportunity to shed light on interesting novel biomarkers for AD diagnosis and prevention.

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