JGG 2017;65:101-109

Review

From redox proteomics to clinical practice: search for therapeutic targets

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Alzheimer disease (AD) is the most common form of dementia in the elderly population, characterized by a gradual deterioration of memory and other cognitive functions. The major pathological characteristics of AD brains are the presence of senile plaques, made of amyloid β-peptide (Aβ), neurofibrillary tangles, composed of hyperphosphorylated tau protein, and neuronal loss. Among putative mechanisms responsible of neurodegeneration, several studies demonstrated the role of oxidative stress as an important factor contributing to the initiation and progression of AD. If from one side disruption of redox balance and increased production of free radicals are likely to be related to mitochondria dysfunction and/or aberrant accumulation of misfolded proteins, on the other side the abnormal accumulation of Aβ and tau proteins appears to promote the redox imbalance. In addition, evidence has suggested that oxidative stress may augment the production and aggregation of Aβ and facilitate the phosphorylation and polymerization of tau, thus forming a vicious cycle that promotes the initiation and progression of AD.
Taken together, these findings suggest that therapeutic strategies aimed at preventing/reducing oxidative stress-mediated damage may be effective for the treatment of AD and other neurodegenerative disorders.

Key words: Alzheimer disease, Protein oxidation, Antioxidant, Protein aggregation, Chaperones

ALZHEIMER DISEASE AND OXIDATIVE STRESS HYPOTHESIS OF NEURODEGENERATION

Alzheimer disease (AD) is the most common neurodegenerative disorders that affect middle- to old-aged individuals, with an incidence rate that increases almost exponentially with increasing age until 85 years of age. Sporadic AD accounts for approximately 95% of all AD cases and is caused by multiple etiological factors, such as gender, brain injury, education, vascular disease, the presence of the apoE4 gene, among others. The majority of AD cases are sporadic and present considerable heterogeneity in terms of risk factor profiles and neuropathological features. AD may be classified into mainly three stages of progression characterized by gradual increase of AD hallmarks starting from preclinical AD (PCAD), to amnestic mild cognitive impairment (MCI) and early AD (EAD). The core clinical features of AD include gradual and progressive decline in memory, executive function, and ability to perform daily activities. However, there is variability among individuals in age of onset, family history, and the appearance of noncognitive symptoms such as behavioral or motor abnormalities. Rates of disease progression and survival also vary considerably among different individuals.
Pathologically AD is characterized by the deposition of senile plaques (SPs), neurofibrillary tangles (NFTs), decreased synaptic density and brain atrophy particularly in the hippocampus, amygdala and frontal cortex, that correlate with cognitive and memory deficits [1]. SPs are composed by amyloid β-peptide (Aβ), comprising 39-43 amino acids formed by proteolytic cleavage of amyloid precursor protein (APP), a type I trans-membrane protein, by β-secretase and γ-secretase. Though for a long

Received: June 22, 2016 - Accepted: September 9, 2016
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time senile plaques have been considered as the primary pathogenic element of AD; recent evidence supports the notion that plaques may be an extra-cellular storage site for cells to deposit excess Aβ, and suggests that smaller aggregate form of Aβ(1-42) oligomers are the main neurotoxic species [2 3]. Accordingly, experimental data have shown that plaques do not correlate with cognitive dysfunction in AD, but soluble oligomers do [4]. NFTs are composed by tau, a microtubule-associated protein, that once hyperphosphorylated, is increasingly prone to form insoluble aggregates thus loosing its affinity for microtubules [1]. These pathological lesions have been proposed to be the causative features, and mechanism-based therapies have been developed to target both SP and NFTs [2]. However, with a more comprehensive understanding of the molecular mechanisms involved in the neurodegenerative process, several other pathogenic factors have emerged, including excitotoxicity, calcium impairment, mitochondrial dysfunction, neuroinflammation and oxidative stress. All these mechanisms coexist and likely act in concert affecting each other at multiple levels.

Several data indicate that a dysregulation of redox homeostasis strongly participates in the early stage of AD, activating diverse cellular signaling pathways that trigger the initial toxic events [5]. Among these, increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is observed in AD brain. As well, increased levels of oxidative stress (OS) markers of proteins, lipids, carbohydrates and nucleic acids have been detected in a large number of studies from AD brain and peripheral systems. In parallel, the levels of antioxidant enzymes were found to be decreased in different brain regions from AD patients [6 7]. Accordingly, age-related memory impairment correlates with a decrease in brain and plasma antioxidants defense mechanisms. One of the most powerful antioxidant is glutathione (GSH), which is responsible for the endogenous redox potential in cells (GSH/GSSG) [8]. The role of OS in AD has been extensively investigated and several studies showed elevated levels of OS markers in post-mortem brain from AD patients and its early phases, MCI and early AD [9 10].

Moreover, in AD and MCI brains, the increased oxidative damage to lipids and proteins coupled with reduced GSH and antioxidant enzyme activities have been shown to be localized to the synapses and correlate with the severity of the disease, suggesting an involvement of OS in synaptic loss [11]. Importantly, many studies show elevation of OS already in MCI [12-14], which is proposed as an intermediate state between normal ageing and dementia, indicating that the oxidative damage in AD precedes the onset of the disease. These results suggest that OS may be one of the earliest alterations that occur during the initiation and development of AD.

Since the development of sophisticated proteomics platforms, large-scale studies of protein composition have been performed to study the mechanisms of disease pathogenesis, to characterize novel drug targets and to discover potential diagnostic and prognostic biomarkers [15]. With relevance to the OS hypothesis of neurodegeneration, growing attention is given to analyse oxidative post-translational modifications (PTMs) as it has been demonstrated to alter protein function and to be linked to disease pathology [16]. Investigations of oxidative PTMs, that occur in AD and other neurodegenerative disorders, have been performed with success using focused redox proteomics techniques [17 18] supporting the potential impact of this approach on the study of neurological disorder and for the identification of therapeutic targets [19]. This review discusses the most relevant redox-proteomics findings obtained in brain and blood from AD and mild cognitive impairment (MCI) patients with the aim to identify putative therapeutic targets. In addition, we discuss therapeutic strategies involving antioxidants and anti-aggregating compounds that may have the potential to prevent/slow the onset and progression of AD.

OXIDIZED PROTEINS SIGNATURE IN BRAIN AND PERIPHERY: POSSIBLE THERAPEUTIC TARGETS

OS is a common feature of several neurodegenerative diseases and redox proteomics approach has the power to identify pathology-specific alterations in different biological samples.

A comprehensive redox proteomics analysis of different brain regions from AD, MCI subjects and, with some limitations, from subjects with early AD and preclinical AD is currently available. In parallel, the redox proteomics analysis of blood-based biofluids from AD and MCI has been performed. From these studies, it emerges that protein oxidation highly correlates with the clinical features, pathology and biochemistry of AD [20 21]. Among the most relevant findings coming from brain tissue studies, it is important to underlie that oxidative damage targets proteins involved in energy metabolism, antioxidant response, protein degradation, excitotoxicity, neuronal structure and mitochondrial functions. Oxidative-mediated dysfunctions of these proteins are likely involved in neurodegeneration at various stages of the disorder [22]. By comparing the results obtained by different subjects, from early to moderate and to severe AD, it has been possible to identify some common targets of protein oxidative modification among different

phases of the disease. This approach allows identifying molecular events involved in the prodromal phase of AD, which will eventually participate to the chronic accumulation of cellular deficits ultimately culminating in the loss of cognitive functions.

Among others, redox proteomics data on proteins related to energy metabolism suggest that the impairment of ATP synthesis is a crucial event driving the neurodegenerative process [23]. Indeed, ATP, the cell's energy currency, is extremely important at nerve terminals for normal neurotransmission [24 25] and decreased ATP levels may lead to loss of synapses and synaptic function, both of which can affect propagation of action potentials and contribute to memory loss.

Interestingly, chaperones such as heat shock proteins (HSPs) use energy from ATP to help misfolded proteins to refold properly. Several HSPs have been found to be oxidatively-modified in AD including HSP90, HSP60 and HSP27 that were also found to be aberrantly induced in MCI compared with age-matched controls [26 27]. Defective repairing systems may exacerbate protein misfolding and aggregation processes overloading proteasome removal, a condition known to occur in AD [28].

Though the study of post-mortem brain allowed to identify the molecular mechanisms of neurodegeneration, it should be taken into consideration that i) collection of *post-mortem* brain is difficult and allows to analyse limited sample size; ii) brain tissue cannot be used for early diagnosis of cognitive decline; iii) the analysis of early asymptomatic disease stage is needed to fully understand all the intrinsic and extrinsic factors involved in AD onset and progression. Thus, in recent years growing studies have been focused to establish a direct link between tissue specific damage and systemic alteration, as well as to identify biochemical markers of brain dysfunction that can be measured in body fluids such as cerebrospinal fluid (CSF), plasma and urine [29].

Diagnostic criteria for AD and MCI are actually based on clinical features allowing only a "probabilistic" diagnosis and exclusion of other types of dementia. This low specificity represents a major limit for the therapeutic management of AD patients and for testing the efficacy of disease-modifying drugs. So far, CSF biomarkers have been focused on the amyloid cascade hypothesis and cytoskeletal degeneration, measuring the levels of total tau and phosphorylated-tau. The increase in total tau and p-tau, and a decrease in Aβ42 level and Aβ42/Aβ40 ratio have been documented in CSF from AD patients and from MCI subjects [30-32]. However, the sensitivity is still unsatisfactory making this method not useful for monitoring the progression of the disease. In addition to Aβ and tau hallmarks, increased oxidative stress and subsequent damage to protein represent a further potential marker of AD development/progression [33].

Only few clinical two-dimensional electrophoresis-based studies focusing on protein oxidation in AD blood and CSF are currently available. The lack of data is probably due to the difficulty of analysing complex samples, such as biological fluid, with a wide variability among patients.

Proteomics approaches and targeted multi-analyte studies of CSF have been performed and lead to the identification of many proteins that are elevated or reduced in AD compared to cognitively normal controls [34 35]. A recent study from our group applied targeted proteomics approach to discover putative CSF biomarkers that can improve the diagnostic and prognostic accuracy of current leading CSF biomarkers. CSF samples from aMCI, AD and control individuals were analyzed using redox proteomics to identify the specific oxidatively modified proteins in AD and MCI compared with controls. We found that the majority of carbonylated proteins identified by mass spectrometry are present early in the progression of AD, i.e., oxidatively modified CSF proteins were already present in MCI compared with controls and remain oxidized in AD, thus suggesting that dysfunction of selected proteins initiate many years before severe dementia is diagnosed [36]. In parallel, we also investigated the involvement of immune system in AD. Our method allows recognition of natural occurring antibodies by the identification of brain antigen targeted by human IgGs. Collected findings reveal that the alterations of autoantibodies profile both in CSF and serum correlate with disease staging and progression. However, we did not find a strong overlap between CSF and serum suggesting the existence of different immunogenic events. Interestingly, CSF autoantibodies recognized, among others, key players of energy metabolic pathway, including glycolysis and TCA cycle, found oxidatively modified in AD brain studies. These data suggest a potential casual sequence between oxidative damage at brain level, autoantibodies presence in CSF and reduced energy metabolism of AD patients [37].

Compared with CSF, blood samples were the object of several studies [38]. Thereafter, as expected, most of the proteomics studies on plasma/serum samples show the oxidation of protein involved in the inflammatory response [39 40]. However, taking into account the technical and practical limitations in performing proteomics analysis in blood samples, it has been recently demonstrated that haptoglobin (Hp) β chain, among other proteins, was both down regulated and increasingly oxidized in a disease-dependent way [41]. The main physiological role of Hp is represented by its binding activity to hemoglobin, which functions as a way to prevent hemoglobin-mediated production of reactive oxygen species, as it occurs during inflammatory

events. Interestingly, in addition to its well-established inflammatory role, Hp has a specific capacity to inhibit aggregation/precipitation of a wide variety of proteins induced by different stress conditions. Indeed, when incubated with Aβ peptide Hp inhibits the formation of fibrils. The same considerations can also be applied to another chaperone protein found to be oxidized in human plasma from AD patients, alpha-2 macroglobulin. Redox proteomics data on plasma, when compared to studies on AD brain samples, show a parallelism for the oxidation of protein known as molecular chaperones, extracellular in one case and intracellular in the other. The oxidative modification of proteins with similar function but different compartmentalization suggests that the alteration of chaperone proteins may represent a common feature of both central and peripheral damage in AD. In addition, it was demonstrated that the impairment of the heme degradation pathways, due to oxidative modifications of the main components, heme-oxygenase and biliverdin reductase-A (HO-1/BVR-A), occurs in post-mortem brain from MCI and AD patients as well as in plasma samples [42]. Therefore, HO-1/BVR-A system status in plasma could reproduce the on-going pathology at brain level, suggesting that the analysis of HO-1/BVR-A system in blood-related biofluids may reflect the "oxidative index" of the brain.

PHARMACOLOGICAL PROSPECTIVE: ANTIOXIDANTS AND ANTI-AGGREGATING COMPOUNDS

Collectively, results obtained by redox proteomics studies in the brain, blood and CSF from AD patients suggest therapeutic strategies based on antioxidant supplementation and compounds acting on aggregation mechanisms of Aβ and Tau may have the potential to prevent/slow AD neuropathology (Fig. 1). Intriguingly, OS itself seems to influence aggregation processes and "amyloidosis".

Antioxidant therapy, as one of the promising therapeutic strategies for AD, has been studied for years. Antioxidants comprise both exogenous (natural or synthetic) and endogenous compounds acting in different ways. The natural antioxidant system can be classified into two major groups: enzymatic (e.g., superoxide dismutase, catalase) and non-enzymatic or low-molecular-weight antioxidants (LMWAs). As a whole, antioxidants are able to block pro-oxidant enzymes, neutralize radicals, or chelate transition-metal ions that catalyze radical generation. In addition, some antioxidants exert their effects by inducing endogenous antioxidant defenses, up-regulating the expression of redox-sensitive transcriptional factors. Hence, redox homeostasis in cells is derived from a fine-tuning of numerous factors. Considering that OS mediates multiple cellular processes, a therapeutic strategy aimed to prevent/slow OS-induced modifications require molecule/s able to target not only a single mechanism, such as the case of ligand/receptor interaction, but able to act at the crossroad of multiple pathways. Further, the variety of sources and sites of production of free radicals implicates an even higher heterogeneity in the antioxidant response. Moreover, it is important to underlie that there is an extensive crosstalk between OS and other key toxic AD events, which amplify the complexity of these phenomena [43]. Nevertheless, OS has recently been proposed to be a common, key element capable of articulating the divergent nature of different pathogenic mechanisms of AD. So far, several antioxidant compounds have been tested as neuroprotectants. Among the most well characterized exogenous compounds, vitamin E (α-tocopherol), vitamin C, and β-carotene are chain-breaking antioxidants that decrease free radical-mediated damage in neuronal cells. For example, vitamin E has been shown to attenuate Aβ-induced toxicity and improve cognitive performance in rodents [44]. Sano and colleagues showed that treatment with α-tocopherol in patients with moderate AD was able to reduce neuronal damage and slow the progression of AD [45]. Further, vitamin E was shown to suppress brain lipid peroxidation and reduce Aβ levels and senile plaque deposition in Tg2576 mice, if administered early prior to the appearance of the pathological hallmarks of AD [46]. However, if vitamin E supplementation was started at a later time point when amyloid plaques deposition is already occurred, no protective effect on the amyloidotic phenotype of these animals despite a reduction in brain oxidative stress was observed [46]. As well, the levels of carbonyls and 8-OHdG were reduced after α-tocopherol administration in transgenic mice overexpressing human tau protein [47].

Vitamin C is a water-soluble antioxidant that inhibits lipid peroxidation and is a major defence against free radicals in the blood. Bagi et al. have shown that chronic vitamin C treatment is able to decrease high levels of isoprostanes, common markers of oxidative damage to cellular lipids, enhance NO bioavailability, restore the regulation of shear stress in arterioles, and normalize systemic blood pressure in methionine diet-induced hyperhomocysteinemia rats [48]. Further, vitamin C reduces α-tocopheroxyl radicals in membranes and LDL to regenerate α-tocopherol and possibly inhibits α-tocopheroxyl radical-mediated propagation [43].

Carotenoid is another lipid-soluble antioxidant that may reduce lipid peroxidation and improve antioxidant status. The most known and studied carotenoid is the β-carotene that is a potent antioxidant able to quench

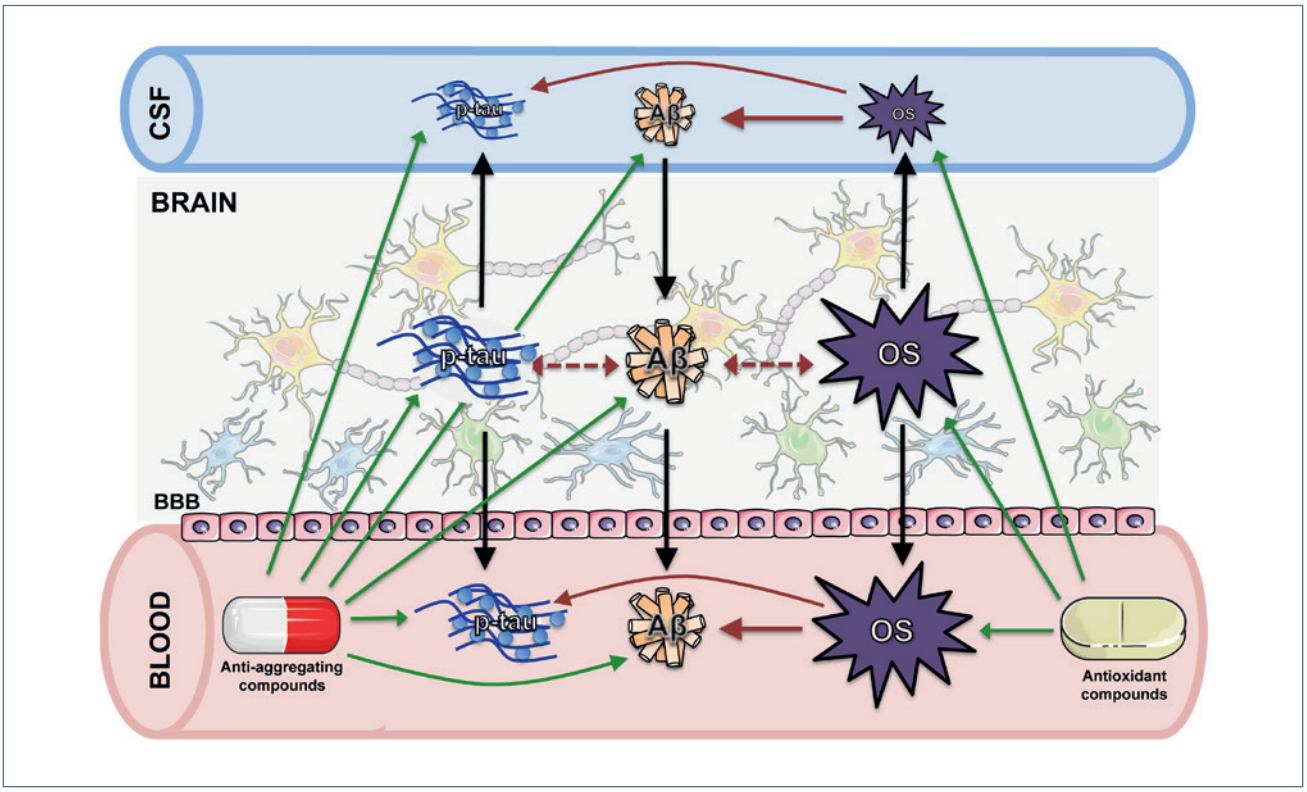

Figure 1. Putative therapeutic strategies to prevent/slow AD neuropathology. Antioxidant supplementation may reduce OS-induced damage in the brain and in the periphery. As well, targeting aggregation mechanism of pathological proteins, Aβ and Tau, has been shown to be a promising approach for the treatment of AD and also for other neurodegenerative diseases caused by protein misfolding.

singlet oxygen rapidly [49]. Taken together, vitamin C, vitamin E, and carotenoids have shown to synergistically interact against lipid peroxidation [43].

Other promising antioxidants that have some potential therapeutic value in the treatment of neurodegenerative diseases are the mitochondrial-targeted antioxidants such as α-lipoic acid (LA), coenzyme Q10, NADH, Mito Q, Szeto Schiller (SS) peptide, and GSH. Mitochondrial dysfunction has been well demonstrated in many neurodegenerative diseases and mitochondrial fragmentation, altered mitochondrial distribution and also structurally and functional damage of mitochondria has been shown to be involved in the pathogenesis of AD [50].

LA is the coenzyme of mitochondrial pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, and is able to recycle other antioxidants such as vitamins C and E and glutathione and increase the production of acetylcholine or to act as a chelator of redox-active metals [51]. It has been reported that chronic administration of the LA decreased lipid peroxidation but not Aβ load within the brains of both control and AD mice models, and improved Morris water maze performance in the Tg2576 mouse model but was ineffective at altering cognition in the Y-maze test [52]. Similarly, Farr Sa et al. showed that chronic administration of LA to SAMP8 mice (a model of accelerated ageing) was able to reverse memory impairment and brain OS [53].

Although promising results have been obtained both by *in vitro* studies and animal models, no significant neuroprotective effects with antioxidant supplementation have been observed in clinical trials [54 55]. So far, it is not completely clear whether nutrient therapy is an effective treatment of AD [56]. Lloret et al. showed that vitamin E supplementation was not able to lower plasma OS for half of the AD patients [57] and results from others have suggested that increased intake, either by diet or supplementation, of carotenes or vitamins C and E did not decrease the risk of developing AD (reviewed in [56]). Taken together, these results indicate that antioxidant therapies have been successful in preclinical studies in animal models of AD but little benefit in clinical trials can be achieved [58]. Moreover, Morris et al. reported that higher intake of foods rich in vitamin E may modestly reduce long-term risk of dementia and AD only among individuals without the APOEε4 allele [59], while dietary intakes of vitamin C, β-carotene, and flavonoids were not associated with dementia risk.

In the effort to understand the reasons of such failure, the first aspect to be considered is the design of trials that set up the treatment when clinical diagnosis of dementia is already significant [60 61]. Another major limit when administrating antioxidants is their poor bioavailability and low permeability across the blood-brain barrier, that needs the development of new delivery systems, such as those based on nanoparticles [62]. Future studies should be re-directed to consider such critical issues.

As discussed above, findings from AD brain and blood suggest that impairment of protein quality control, including chaperones, may significantly contribute to the onset and progression of AD and it may be considered a promising therapeutic target. Accordingly, molecular chaperones and chemical and pharmacological chaperones have been found to be effective in preventing misfolding of different disease-causing proteins. Chaperones are highly specific and can distinguish between the native and non-native states of targeted proteins. However, how they discriminate between correctly and incorrectly folded proteins and how they selectively retain and target the latter for degradation has not been clarified [63]. Proteins that fail to achieve their native state, either as a consequence of amino acid mutation or because of an error in the folding process, are recognized as misfolded and therefore targeted to degradation – protein 'quality control' (QC) system. For this reason, the QC system, including molecular chaperones, the ubiquitin proteasome system and autophagy, plays a critical role in cell function and survival.

In almost all protein-misfolding disorders, an error in folding occurs because of a mutation in the polypeptide or, in a few cases, unknown reasons. The formation of oligomers and aggregates occurs in the cell when a critical concentration of misfolded protein is reached. Protein aggregation involves the self-assembly of proteins into large β-sheet rich complexes. This process could result from aberrant protein folding and lead to "amyloidosis", a condition characterized by deposits of protein aggregates known as amyloids in different tissues. Intriguingly, OS contributes to protein misfolding trough a double mechanism: oxidants induce PTMs on protein structure that increased the propensity to form aggregates; further free radicals damage directly members of the QC thus causing the reduced ability to remove misfiled proteins [64-66]. These effects result in impairment the entire catabolic system of pathogenic proteins such as Aβ, tau and alpha-synuclein among others.

Thus, disease-modifying strategies currently being pursued for AD mainly focus on amyloid β and Tau, especially in the aggregated state. In particular, much effort has been expended in the last decade on developing small molecules that have the ability to inhibiting Aβ aggregation. However, to date, no compounds have been successful and entered into clinical use. This failure has been explained because the inhibition of Aβ aggregation requires blocking interactions between Aβ monomers. Indeed, Aβ42 is an intrinsically disordered peptide [67] that self-assembles into fibrillar aggregates as observed in the brain from AD subjects [68]. The failure of therapeutic strategies based on small compounds that can interfere with self-aggregation is caused by the incomplete knowledge of the mechanisms generating toxic species and how potential drugs are able to interfere with the aggregation pathway of Aβ42. In addition, it is increasingly evident that prefibrillar oligomeric species, rather than mature amyloid fibrils and plaques, represent the main pathogenic agents in AD and other neurodegenerative conditions [69].

As the strategy of inhibiting Aβ aggregation has increasingly gained success, a number of inhibitors have been developed and the structure-activity relationships of potent inhibitors have been described [70 71]. These studies revealed that typical Aβ aggregation inhibitors such as Congo red (CR), chrysamine G (CG) and curcumin share a similar chemical scaffold. These molecules contain two aromatic groups or inositol groups (with a suitable substituted group) separated by a backbone of the appropriate length [71]. It is likely that the two terminal groups interact with Aβ protein residues to provide the binding affinity, whereas the linker facilitates binding of inhibitors to specific subregions.

A significant step-forward has been recently achieved by Vendruscolo's group which demonstrated that bexarotene, an anticancer drug approved, selectively targets the primary nucleation step in Aβ42 aggregation, delays the formation of toxic species in neuroblastoma cells, and completely suppresses Ab42 deposition in a C. elegans model of Aβ42-mediated toxicity [69].

CONCLUDING REMARKS

In summary, evidence has demonstrated that OS is inseparably linked with several major pathological processes in AD including Aβ-induced neurotoxicity, tau pathology, mitochondria dysfunction, and metal dyshomeostasis. Redox proteomics studies performed by our group and others demonstrated that oxidative damage to selected proteins contributes to the onset and progression of AD by impairing the function of proteins involved in energy metabolism, QC, synaptic function, antioxidant response among others.

However, if from one side free radical-mediated damage is a well-established marker of neurodegeneration, it is not the only toxic mechanism. If antioxidant efficacy

exist other mechanisms have to be targeted to modify AD progression. The search for antioxidants compound that present additional pharmacological effects are thought to offer a good possibility for prevention of AD. Removal of ROS or prevention of their formation may delay the onset or slow down the progression of AD through multiple mechanisms. The rationale is that single molecules, endowed with antioxidant properties and able to act at different steps in the neurodegenerative process, can produce additional neuroprotective effects against AD.

Another promising strategy is based on the development of small compounds able to inhibit Aβ and Tau aggregation. A large number of neurodegenerative diseases in humans result from protein misfolding and aggregation. A nascent polypeptide chain can become misfolded due to a specific gene mutation, as it occurs in almost all familial form of neurodegenerative diseases, or a matured native protein can also achieve a misfolded conformation inside the cell. These aggregated/misfolded proteins become neurotoxic because of the impairment of the protein QC. Thus, therapy should be directed to inhibit and/or reverse conformational changes in the protein molecules responsible. To achieve this goal, it is mandatory to understand the molecular details of the inhibition processes. Vey recent and enthusiastic findings have demonstrated the beneficial effects of bexarotene on Aβ aggregation. This strategy can also be translated to other neurodegenerative diseases.

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