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Hallmarks of protein oxidative damage in neurodegenerative diseases: focus on Alzheimer's disease

Review Article

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Summary. The pathogenesis of several neurodegenerative diseases, including Alzheimer's disease, has been linked to a condition of oxidative and nitrosative stress, arising from the imbalance between increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) production and antioxidant defences or efficiency of repair or removal systems. The effects of free radicals are expressed by the accumulation of oxidative damage to biomolecules: nucleic acids, lipids and proteins. In this review we focused our attention on the large body of evidence of oxidative damage to protein in Alzheimer's disease brain and peripheral cells as well as in their role in signalling pathways. The progress in the understanding of the molecular alterations underlying Alzheimer's disease will be useful in developing successful preventive and therapeutic strategies, since available drugs can only temporarily stabilize the disease, but are not able to block the neurodegenerative process.

Keywords: Alzheimer's disease – Aging – Oxidative stress – Nitrosative stress – Protein

1. Introduction

A large body of experimental research suggests an important pathophysiological role of increased reactive oxygen species (ROS) production leading to oxidative stress (Sies, 1985) as well as of increased reactive nitrogen species (RNS) in aging (Polidori et al., 2001) and in neurodegenerative disorders (Calabrese et al., 2001) including Alzheimer's disease (Beal, 2000). Alzheimer's disease is the most common neurodegenerative disorder worldwide, affecting approximately 4.5 million people in the United States and 4.8 million people in the European Union. Histopathologically, Alzheimer's disease is characterized by synaptic loss, nerve cell loss, extracellular deposition of β-amyloid (Aβ) protein (forming senile plaques) and intracellular precipitation of hyperphosphorylated tau protein (forming neurofibrillary tangles).

2. Brain susceptibility to oxidation

In physiological conditions, most of the damaging effects of free radicals are prevented by the action of enzymatic and non-enzymatic compounds with various degrees of antioxidant capacity (Frei, 1994). These antioxidants, however, might become themselves target of ROS damage associated with aging, thereby reducing their efficiency to counteract free radical hyperproduction (Beckman and Ames, 1998). Several clinical and epidemiological studies have found an inverse relationship between some circulating antioxidants and cognitive performance in healthy adults and in aged subjects (Goodwin et al., 1983; Gale et al., 1996; La Rue et al., 1997; Perrig et al., 1997; Short et al., 1997; Schmidt et al., 1998; Riviere et al., 1998; Berr et al., 2000) suggesting that some dietary antioxidants may protect against cognitive impairment in older people. Unfortunately, despite the convincing scientific evidence supporting a role for oxidative stress in the pathogenesis of Alzheimer's disease, the overall data regarding concentrations of antioxidants in plasma and/or cerebral tissue in Alzheimer's disease remain conflicting. Similarly, the interpretation of few clinical trials conducted to date in order to assess the efficacy of antioxidant therapy in dementia

is still not clear (Sano et al., 1997; Pitchumoni and Doraiswamy, 1998). This is due to a number of reasons, including the lack of homogeneous methods of antioxidant measurement among studies, and the fact that Alzheimer's disease patients and control subjects considered in different studies have also different diet, age, smoking or alcohol habits, drug therapies, comorbidities and social condition.

The brain is particularly vulnerable to oxygen radical damage, through the prevalence of oxidizable polyunsaturated fatty acids in membranes, the presence of redox active metal ions and the high metabolic requirement for oxygen. Furthermore, the endothelium of the small blood vessels in the brain is much less permeable than other vascular endothelia; apart from essential molecules such as glucose and most lipid-soluble small compounds, in normal conditions components such as peripheral phagocytes are excluded from the brain by the blood–brain barrier. Nonetheless, the brain is rich in microglial cells; an important source of oxygen and nitrogen radicals.

Free radicals produced during oxidative stress are thought to play an early pathophysiological role in Alzheimer's disease, and oxidative modification to virtually all classes of biomacromolecules has been described in association with susceptible neurons in Alzheimer's disease. Indeed, high levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) indicating DNA oxidation have been shown in the aging brain (Mecocci et al., 1993) as well as in the post-mortem brain tissue from Alzheimer's disease patients (Mecocci et al., 1994; Wang et al., 2005), and high concentrations of thiobarbituric acid reactive substances, malondialdehyde, 4-hydroxy-2-nonenal and isoprostanes in Alzheimer's disease provide evidence of increased lipid peroxidation in this disorder (Bassett and Montine, 2003).

If DNA oxidation and lipid peroxidation have been consistently shown in a large number of well conducted studies on Alzheimer's disease, protein oxidation does not constitute an exception. Increased brain protein oxidation has been found in Alzheimer's disease as well as in other neurodegenerative disorders (Butterfield and Kansky, 2001). Oxidative damage of proteins in Alzheimer's disease is indicated by high concentrations of several modified amino acids including protein carbonyls and nitrated tyrosine residues. Differently from DNA oxidation products, oxidized amino acids are rarely repaired as mildly oxidized proteins are usually degraded by the 20S proteasome (Grune and Davies, 2003). The exception to this is repair of oxidized methionine. Methionine has been suggested to act as an antioxidant in proteins and peptides, such as amyloid beta, by scavenging oxidizing species and forming methionine sulphoxide (Hou et al., 2002). This oxidized amino acid is specifically reduced in its native form by methionine sulphoxide reductase (Stadtman et al., 2003). However, this enzyme activity has been reported to decline in the superior and middle temporal gyri and hippocampus in the brain of Alzheimer's disease patients resulting in a loss of antioxidant defence and increase in oxidized methionine residues (Gabbita et al., 1999). There has also been a suggestion that nitrotyrosine may be repaired by a denitrase enzyme although this has not been substantiated (Irie et al., 2003).

In this review we will focus on protein oxidation not only because it results in functional disruption, but also because the cross-linking of proteins by oxidative processes may lead to the resistance of the lesions to intracellular and extracellular removal, even though they are extensively ubiquinated. Several studies have shown that the proteasome is impaired in Alzheimer's disease and this has been suggested to reduce the clearance of intracellular protein aggregates (Song and Jung, 2004). Therefore, the accumulation of oxidized proteins in Alzheimer's disease is likely a consequence of imbalance in any one of a number of different systems including free radical generation, antioxidant defences or efficiency of oxidized protein repair or removal. Furthermore, protein oxidation appears not to be a random process but rather to be associated with increased oxidation in specific proteins. This latter aspect is being explored by means of the proteomic approach, an emerging method for identification of proteins possibly allowing the screening of a subset of proteins within the brain proteome that might reflect the extent of oxidative stress within the Alzheimer's disease brain.

3. Protein modification by oxygen and nitrogen free radicals in Alzheimer's disease

In a post-mitotic environment such as neurons, oxidative stress can be used as a marker of age-related deterioration in cellular homeostatic mechanisms. Aging is the major risk factor for Alzheimer's disease, and the accumulation of oxidized proteins in many tissues, particularly the ones formed by cells with low mitotic rate such as brain or muscle (Mecocci et al., 1999) is widely considered a hallmark of aging.

Neurons have a diminished capacity to deal with redox imbalance, so that even minor stresses can lead to irreversible injury. Oxidative deamination of lysine and deguanidination of arginine results in protein-based aldehyde groups that can be detected with 2,4-dinitrophenylhydrazine (DNPH). It appears that most of the DNPH-detectable carbonyls found on proteins result from modification by

bifunctional reactive aldehyde products of lipid and sugar oxidation, thus acting as secondary toxins. These carbonyl species, as described below, have been shown to play a role in the pathophysiology of Alzheimer's disease.

Most of the studies conducted in the aging brain conclude that the amount of oxidized protein does increase with age. In the study from Smith et al. (1991), the amount of protein oxidation was measured by a general 2,4-dinitrophenylhydrazine assay of protein carbonyl groups, formed as result of oxidation of several amino acid residues in proteins. The results showed that there was a general logarithmic increase in protein damage in human cerebral cortex with age, and no significant difference was observed between aged controls and subjects with Alzheimer's disease. In this study not only protein carbonyl levels, but also the activities of creatine kinase and glutamine synthase were measured in the frontal and occipital lobe regions as a function of age. While protein carbonyl content increased with age, the activities of creatine kinase and glutamine synthase decreased, glutamine synthase activity appeared to be selectively lost in Alzheimer's disease brains when the comparisons were made between the latter and age-matched controls (Smith et al., 1991). Other studies conducted in animal models of aging brain showed that indices of protein oxidation such as dityrosine and *ortho*-tyrosine-formed by the addition of two free radicals of tyrosine and by hydroxyl free radical reaction with tyrosine, respectively – had little relationship with age (Leeuwenburgh et al., 1997; Cakatay et al., 2001). Mitochondria, being the major source of ROS, might be expected to have high levels of protein carbonyls; instead no age-related increase in the amount of *ortho*- and *meta*-tyrosine and protein carbonyls was found in the proteins from matrix and membrane fractions of mitochondria isolated from liver, heart and brain of old female rats compared to young animals (Davies et al., 2001). This result probably reflects an efficient protease activity in mitochondria able to remove oxidized proteins.

Hayn et al. (1996) found no difference in *ortho*-tyrosine levels from the frontal cortex between controls and patients with Alzheimer's disease. Peroxynitrite, a powerful oxidant produced as a result of the diffusion-limited reaction of superoxide with nitric oxide, is a source of hydroxyl radical-like reactivity that directly oxidizes proteins and other macromolecules, with resultant carbonyl formation from side-chain and peptide-bond cleavage. Peroxynitrite also causes the nitration of tyrosine residues, which can be used as an index of peroxynitrite action. In two reports, increased 3-nitrotyrosine was found in neurons from Alzheimer's disease patients both containing neurofibrillary tangles and in those in which they were absent (Smith et al., 1997; Su et al., 1997). In these studies, nitrotyrosine immunoreactivity in Alzheimer's disease was increased in the neuronal cytoplasm of the cerebral cortex within regions of neurodegeneration, whereas it was undetectable in the same brain regions of controls. The distribution of nitrotyrosine found by Smith et al. (1996) was essentially identical to the distribution of free carbonyls. In another study, four biomarkers of neuronal protein oxidation including phenylhydrazine-reactive protein carbonyl content were assessed in three brain regions (cerebellum, inferior parietal lobule and hippocampus) of Alzheimer's disease patients and age-matched control subjects (Hensley et al., 1995). Protein carbonyls were significantly increased in both hippocampus and the inferior parietal lobule, but unchanged in the cerebellum, consistent with the regional pattern of histopathology in Alzheimer's disease and indicating that Alzheimer's disease brain protein may be more oxidized than that of control subjects. Others also reported brain regional elevations of modified tyrosines in Alzheimer's disease: both dityrosine and 3-nitrotyrosine levels were elevated 5–8 fold in the hippocampus, neocortex and ventricular CSF of Alzheimer's disease patients when compared with cognitively normal controls (Hensley et al., 1998).

Due to the impossibility to assess protein oxidation in vivo in Alzheimer's disease brains, a recent report has described elevated tyrosine and tryptophan modification products in proteins within the CSF from Alzheimer's disease patients: the concentration of 3-nitrotyrosine, the glycation product CML and the oxidized tryptophan moiety, N-formyl kynurenine were significantly elevated in AD where the mini mental state score showed an inverse correlation with 3-nitrotyrosine levels. Moreover, after removal of the CSF proteins using ultrafiltration techniques, increased levels of free 3NT were recorded indicating that degradation of the nitrated proteins was occurring (Ahmed et al., 2005).

Since it has been shown that oxidative stress in Alzheimer's disease can be also detected in peripheral cells and not only in neuronal cells (Mecocci et al., 1998, 2002; Cecchi et al., 1999; Vina et al., 2004), we decided to evaluate the carbonyl and dityrosine content in immunoglobulins from Alzheimer's disease patients and control subjects (Polidori et al., 2004). The choice of evaluating the carbonyl and dityrosine content in immunoglobulins rather than total oxidized proteins was based upon a number of considerations. First of all, and bearing in mind that targets of protein oxidation are determined by proximity to the source of reactive oxygen species, relative

concentration and size, immunoglobulins are the second most prevalent serum protein; in contrast, not only albumin is smaller, but it also carries lipids, thereby rendering difficult the discrimination between primary and secondary oxidation. Furthermore, immunoglobulins have a half life of 15 days, making them a good temporal indicator of oxidative stress. In our study, the oxidative modification of protein as formation of carbonyl groups was assessed by ELISA using the method of Carty (Carty et al., 2000). The analysis of protein oxidation as dityrosine was performed by reverse-phase HPLC with UV detection (Griffiths et al., 1992). Immunoglobulin levels of dityrosine but not of carbonyls were shown to be significantly higher in demented patients as compared to controls in our study, in disagreement with a previous report of increased total amount of oxidatively modified proteins measured by HPLC in plasma from Alzheimer's disease patients as compared to controls (Conrad et al., 2000).

Oxidative inactivation of enzymes is another index of age-dependent oxidative damage to proteins, and in fact Smith et al. (1991), as mentioned above, showed that glutamine synthase activity is selectively lost in Alzheimer's disease brains. This implies, among others, that the activities of various antioxidant molecules that would normally counteract the injurious effects of ROS are decreased. The antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase display lowered activities in Alzheimer's disease brains (Pappolla et al., 1992). Sohal et al. (1993), who had initially shown that in houseflies protein carbonyl content is associated with life expectancy, also found that in the same model mitochondrial aconitase, an enzyme in the citric acid cycle, is a specific target during aging (Yan et al., 1997). The oxidative damage detected immunochemically was paralleled by a loss of catalytic activity of aconitase, that contains an iron-sulfur cluster rendering it very susceptible to oxidative stress. While the potential serious implications of impaired activity of aconitase have been recently elegantly summarized (Shadel, 2005), we recently observed that the activity of aconitase is decreased in both lymphocytes and purified mitochondria from Alzheimer's disease patients compared to controls (Mecocci et al., unpublished data). Not only this is of interest in light of the possibility to peripherally assess biomarkers of oxidative stress in Alzheimer's disease, but also because the decrease of activity of aconitase in patients with Alzheimer's disease markedly resembles that of aconitase activity in subjects with mild cognitive impairment (MCI), a prodromal stage of Alzheimer's disease (for review on oxidative stress in MCI, see Mecocci, 2004). This suggests that mitochondrial dysfunction and oxidative stress have chronological primacy in Alzheimer's disease. Interestingly, Keller et al. (2005) showed a 25% increase of protein carbonyls in the superior and middle temporal gyri of individuals with MCI and early Alzheimer's disease in comparison to controls.

The first use of proteomics to identify specifically modified proteins in Alzheimer's disease brains indicated compounds such as glutamine synthase or α-enolase, and also posttranslationally modified proteins such as 3-nitrotyrosine (Butterfield and Castegna, 2003). Thus far, several proteins have been identified by proteomics, and these include proteins dealing with energy metabolism, excitotoxicity, and the recycling of damaged proteins through the proteasome (reviewed in Butterfield, 2004). Not only age-associated oxidative changes of proteins (Poon et al., 2005) but some of the hallmarks of Alzheimer's disease, such as the accumulation of aggregated and damaged proteins, excess ubiquitination and shortened dendritic lengths might be related to these oxidized proteins (Choi et al., 2004). Proteomics appears to be an extremely helpful approach to study some of the processes rendering synapses and axons vulnerable in Alzheimer's disease, including their high content of the disease-related proteins APP (Amyloid-Precursor Protein), presenilins and tau (for review see Ross and Poirier, 2004), and their metabolic and oxidative loads. Protein nitration, for instance, seems to be target-specific, as nitration of tyrosine residues within the tau protein has been found in Alzheimer's disease (Horiguchi, 2003); it has been suggested that Alzheimer's disease, like other neurodegenerative disorders, might have special features that contribute to the interaction between oxidative stress and specific protein misfolding (Andersen, 2004). More recently, Reynolds et al. (2005) have shown that tau polymerization is affected differentially by oxidation or nitration, where peroxynitrite mediated cross-linking could facilitate tau aggregation in Alzheimer's disease.

Even if slowly, Alzheimer's disease patients will inexorably experience loss of memory, cognitive, functional and behavioral activities. Although drugs can temporarily stabilize the disease, they cannot stop the neurodegenerative process at the moment. Therefore, improvement in understanding the molecular alterations underlying Alzheimer's disease may help in developing effective preventive and therapeutic strategies. Exercise, cognitive stimulation and dietary control may exert a beneficial effect through similar mechanisms involving increased production of brain-derived neurotrophic factor (BDNF). Recently, for instance a decreased number of oxidised proteins

were observed in SHSY-5Y cells that had been exposed to=hydrogen peroxide after ascorbate treatment, and the induction of BDNF was demonstrated. BDNF was able to protect the cells against oxidative attack which raises the intriguing possibility that antioxidants such as ascorbate might offer not only a mechanism to prevent damage to proteins through radical scavenging but may also afford prolonged benefit through altered gene expression patterns due to alterations in intracellular redox state (Grant et al., 2005).

4. Oxidative stress and signalling pathway in Alzheimer's disease

There is increasing evidence that ROS and RNS are important mediators of signal transduction via several pathways including ras/raf, protein kinase C and MAP kinase cascades (Jackson et al., 2002). The reduction/oxidation (redox) state of the cell is a consequence of the balance between the levels of oxidising equivalents and reducing equivalents. A reducing intracellular environment is often associated with cell survival; however, redox imbalance is necessary since it represents a regulatory sensor for several nuclear transcription factors. In effect, there is a possibility that external interferences with such signalling pathways through antioxidants may contribute to failure to adapt to oxidative stress or even failure to survive. Evidence to support the concept that ''antioxidants'' can attenuate the adaptation to exercise induced stress has been shown recently in a supplementation study using antioxidant vitamins C (ascorbic acid) and E (tocopherol) as measured by the induction of the heat shock protein, HSP72, in the skeletal muscle and in the circulation.

Modified gene expression and enzyme activity induced by cellular oxidative stress are mediated through the interplay of several signalling pathways. The stress-activated protein kinase (SAPK) pathways, for instance, are the central mediators that propagate stress signals from the membrane to the nucleus. In neuronal cells, stimuli-like free radicals and ischemia cause an intracellular stress response that either leads to apoptosis or to defensive-protective adaptation. SAPKs and its downstream effectors are the major molecules involved in this bipartite response, which can accordingly lead to either neurodegeneration or to neuroprotection, depending on the cellular and environmental conditions as well as cooperation with other signalling pathways (Zhu et al., 2004). It has been suggested that oxidative stress, as an earlier event in Alzheimer's disease pathogenesis, may activate JNK/SAPK and elevated levels of β-amyloid, as a later event, contribute to further activation of JNK/SAPK (Zhu et al., 2004).

Many transcription factors (e.g. NFkB, STAT, AP-1, Ets and CREB) are subject to redox/thiol regulation. This suggests that the controlled production of ROS is important in cell activation, proliferation or programmed cell death.

One of the upstream kinases in the JNK and p38 MAPK modules is the apoptosis signal regulating kinase-1 (ASRK-1), that is maintained in an inactive state by binding reduced thioredoxin (Saitoh et al., 1998). Oxidation of Trx by ROS releases ASRK1 permitting its activation and allowing downstream signalling to apoptosis. The subtlety of this effect is exemplified by the observation that caspase 3, a downstream effector of apoptosis, is only active when a critical thiol residue is reduced. Recent data suggests that the cellular redox environment selectively regulates stress signalling through MEKK1 via glutathiolation (Cross and Templeton, 2004). This can be viewed as a simple redox controlled molecular switch in signal transduction. Such switches could be turned off in inflammatory conditions after exposure to a sustained source of ROS or RNS that alters the redox balance (Fig. 1).

So, the influence that ROS/RNS have in modulating many different signalling pathways might explain the scarce efficacy of antioxidant supplementations in preventing or treating Alzheimer's disease although it cannot be excluded that the lack of efficacy is due, instead, to the use of high doses of a single antioxidant. In fact, epidemiological studies on dietary intake of antioxidants showed a higher efficacy than supplementation in preventing Alzheimer's disease, suggesting that the antioxidant mixture contained in food is more effective (Engelhart et al., 2002; Morris et al., 2002) although other studies did not confirm these findings (Laurin et al., 2004).

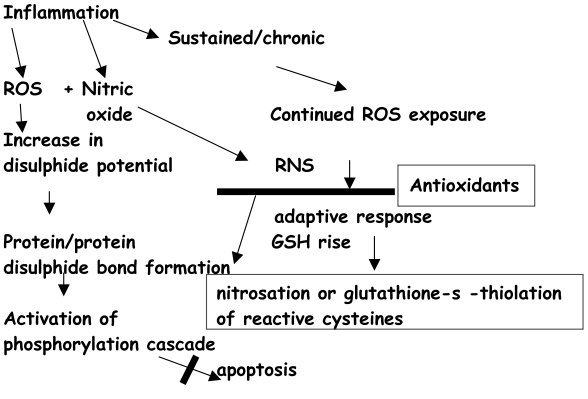

Fig. 1. Redox-controlled molecular switch in signal transduction

In conclusion, oxidative stress, as one of the earliest events in AD pathogenesis, plays a significant role in the development of AD pathology. Although the formation of highly reactive hydroxyl radicals poses a great threat on neuronal cells by damaging important macromolecules, such as proteins, compensatory responses provoked by ROS/RNS via the activation of different signalling pathways and downstream adaptations such as induction of anti-oxidant enzymes, tau phosphorylation and neurofibrillary tangles formation may provide some protective mechanisms to ensure neuronal cells do not succumb to such oxidative insults.

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