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Sources Contributing to the Initiation and Propagation of Oxidative Stress in Alzheimer Disease

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Oxidative damage is a striking feature of neurons at risk of damage and ultimately death in Alzheimer disease. Recent evidence has indicated that oxidative stress is an early event in the progression of Alzheimer disease, proximal to the development of hallmark pathology. In consideration of the temporal relationship between oxidative stress and the pathology of the disease, in this review, we provide a summary of recent work demonstrating key sources of the initiation and propagation of neuronal oxidative damage and the relationship these sources have to the development of Alzheimer disease. For example, interactions between abnormal mitochondria, redox transition metals, and oxidative stress response elements, contribute to the generation of reactive oxygen species in diseased neurons. Interestingly, although oxidative stress is a prominent feature in Alzheimer disease, few vulnerable neurons show established signs of apoptosis, suggesting that levels of oxidative stress do not significantly exceed neuronal oxidative defences. In light of this observation, we propose that neurons in Alzheimer disease are exposed to low, but chronic, levels of oxidative stress whereby neurons elicit adaptive response mechanisms to adjust to chronic oxidative challenge.

Key Words: Alzheimer disease, Heme oxygenase, Mitochondria, Oxidative stress, Transition metals, Stress-activated protein kinase

Introduction

Oxygen radical-mediated damage, an event known as oxidative stress, has been demonstrated to play a prominent role in Alzheimer disease (AD) with marked increase of oxidative damage to lipids, sugars, nucleic acids, and proteins (Smith et al. 1994a, Nunomura et al. 1999, 2000, 2001). Oxidative stress is one of the earliest events of AD (Nunomura et al. 2001), with implications as an important mediator in the onset, progression, and pathogenesis of the disease. The generation of reactive oxygen species (ROS) and its consequent cellular damage contributes to much of the hallmark AD pathology seen in susceptible neurons; namely neurofibrillary tangles (NFT), composed of highly phosphorylated tau (τ), and senile plaques containing amyloid-β (Aβ) (Smith et al. 1997a, Sayre et al. 2000, Wataya et al. 2002). The sources of oxygen radical-mediated damage appear to be multi-faceted in AD, with interactions between abnormal mitochondria, redox transition metals, and oxidative stress response elements [heme oxygenase (HO) and mitogen and

Abbreviations:
AD, Alzheimer disease; *Aβ*, amyloid β; *AβPP*, amyloid β protein precursor; *HO* heme oxygenase; *JNK*, c-Jun N-terminal kinase; *mtDNA*, mitochondrial DNA; *NFT*, neurofibrillary tangles; *ROS*, Reactive oxygen species; *SAPK*, stress-activated protein kinase pathways

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stress-activated protein kinase pathways (SAPK)] as key responders and effectors of oxidative stress. In this review, we describe the roles each of these components have in producing oxidative stress and provide explanations linking these events to AD pathogenesis.

Mitochondrial Abnormalities

As the predominant site of oxidative/energy metabolism within the cell, mitochondria make a significant contribution to oxidative damage and related events. Extensive evidence gathered over the last 50 years indicates that cerebral metabolism is reduced in AD, placing mitochondria at the source of this dilemma (Smith et al. 1999, 2000). It has been reported that activities of specific mitochondrial enzyme complexes are reduced in AD including cytochrome oxidase (COX), the pyruvate dehydrogenase complex, and the α-ketoglutarate dehydrogenase complex (Sorbi et al. 1983). Because COX is the component of the mitochondrial electron transport chain that directly interacts with molecular oxygen, its abnormality in AD (Hirai et al. 2001) could result in the genesis of increased ROS production in mitochondria. Although side-production of superoxide is a normal phenomenon in aerobic organisms, increased superoxide production is a possible phenomenon in AD due to a loss of activity in COX which could back up electrons at the Complex III site causing formation of ROS. Although it is hypothesized that mitochondria are significant producers of ROS, mitochondria in AD do not exhibit striking evidence of oxidative damage (Nunomura et al. 1999). Using 8-hydroxyguanosine as a marker for nucleic acid oxidation, oxidative damage is primarily limited to the cytoplasm of susceptible neurons in AD with no significant increase in AD mitochondria (Nunomura et al. 1999, 2001). It is likely, therefore, that mitochondria in AD supply a key reactant that, once in the cytoplasm, releases free radicals (Sayre et al. 2000). Although increased amounts of the superoxide radical ($O_2^{\cdot}$) may be produced, it diffuses poorly past membranes. However, $O_2^{\cdot}$ once converted, via the mitochondrial specific enzyme Mn superoxide dismutase (MnSOD), to H_2O_2 can freely diffuse across the outer membrane of the mitochondria and into the cytoplasm. Once H_2O_2 is present in the cytoplasm, it can interact with redox-active iron (see below) into produce the highly damaging hydroxyl radicals (•OH) via the Fenton reaction (Sayre et al. 2000). Therefore, it is likely that mitochondrial abnormalities are clearly involved as a source of ROS that culminates in perikaryonal oxidative damage.

In AD, mitochondrial DNA (mtDNA) and protein are increased while mitochondrial number is decreased in vulnerable neurons (pyramidal neurons but not granule cells or glia) (Hirai et al. 2001). This dichotomy of mtDNA/protein increase and mitochondrial decrease indicates that vulnerable neurons in AD have greater amounts of mitochondrial degradation products (Smith et al. 2000). In fact, ultrastructural examination shows that increased mtDNA and protein was found in vacuoles associated with lipofuscin, a lysosome that, in previous studies, has been suggested as the site of mitochondrial degradation by autophagy (Brunk et al. 1992). It is likely that this degradation is either attributed to greater turnover of mitochondria by autophagy or a reduction of proteolytic turnover leading to accumulation of mtDNA and protein. These data thereby support the notion of increased oxidative stress in AD due to an increase in dysfunctional mitochondria.

Redox-Active Metals

The generation of oxygen-free radicals and consequent cellular oxidative stress is thought to be mediated by redox reactions associated with transition metals. Perturbations in metal homeostasis (primarily iron and copper) result in an array of cellular disturbances characterized by free radical production. Recent data has shown that redox-active iron and copper are associated and concentrated within the major pathological markers of AD including senile plaques, NFTs, and neuropil threads (Lovell et al. 1998, Sayre et al. 2000).

Tissue oxidative stress *in vivo* is thought to be mediated by redox transitions associated with "free" iron, more than any other transition metal. Indeed, iron plays a key role in much of the pathophysiology of AD, with an especially significant role in Aβ. The association and interaction of iron with Aβ have shed much light

around the debate of Aβ toxicity and its subsequent relation to oxidative stress. While it is clear that Aβ causes oxidative stress, the precise mechanism by which Aβ leads to increased oxidative stress was unclear. Recent evidence indicates that the pro-oxidant and cytotoxic effects of Aβ are likely mediated by its interaction with redox-active iron (Rottkamp et al. 2000). Pre-treatment with the iron chelator deferoxamine significantly decreases *in vitro* neurotoxicity of pre-aggregated Aβ while subsequent incubation of Aβ with excess redox-active iron restores Aβ neurotoxicity (Rottkamp et al. 2000). Although senile plaques accumulate iron in both Fe(II) and Fe(III) redox states, Fe(II) can be oxidized by H_2O_2 to increase Fe(III) levels at the expense of Fe(II) (Sayre et al. 2000). Importantly, this reaction between Fe(II) and H_2O_2 is the primary source of highly damaging •OH. It can therefore be suggested that redox-active iron is required for Aβ-induced oxidative stress. Furthermore, dysregulation of cellular iron metabolism supports an impaired iron homeostasis in this disease. Iron regulatory protein (IRP)-2, and not IRP-1, is increased in AD and selectively associated with the pathologic hallmarks of AD (Smith et al. 1998). Also, an increase in iron concentration with a concurrent decrease in ferritin is seen in AD brain (Connor et al. 1995, Loeffler et al.1995). IRPs are involved in intracellular regulation of iron homeostasis regulating the iron storage protein ferritin by interacting with a conserved RNA structure termed the iron-responsive element (IRE). Much data supports the theory that alterations in the IRP/IRE interaction are the cause for this observed disruption in iron homeostasis (Smith et al. 1998, Pinero et al. 2000). Such an increase in iron without an appropriate increase in ferritin, to detoxify the iron, would leave the neuron vulnerable to ROS.

Copper has a functional role in many enzymes that require oxidation-reduction reactions. For example, copper is found in the catalytic site of COX, of the mitochondrial electron transport chain, and Cu-Zn superoxide dismutase (SOD). In AD, copper interactions possess potential to yield oxidative damage by at least two pathways: (1) alterations in ceruloplasmin and (2) copper interaction with amyloid β protein precursor (AβPP). The entry of copper to the brain is mainly mediated by ceruloplasmin, a copper binding protein that plays a role into protecting cells against oxidative stress. Specifically, ceruloplasmin is a key protein involved in the regulation of the redox state of iron by converting the ROS catalytic-Fe(II) to a less reactive Fe(III). While ceruloplasmin is increased in brain tissue and cerebrospinal fluid in AD (Loeffler et al. 1996), neuronal levels of ceruloplasmin remain unchanged (Castellani et al. 1999). Thus, while increased ceruloplasmin may indicate a compensatory response to increased oxidative stress in AD, its failure to do so in neurons may play an important role in metal-catalysed damage (Castellani et al. 1999). In fact, studies directed at clarifying the relationship between oxidative stress and tissue metal ion levels indicate that the ratio of copper to zinc and levels of ceruloplasmin are significantly higher in cases with neurodegeneration (Mezzetti et al. 1998). Copper has also been shown to play a role in generating ROS through its binding to AβPP. AβPP can reduce Cu(II) to Cu(I) involving an electron-transfer reaction that could enhance the production of •OH through formation of an AβPP-Cu(II)-hydroxyl radical intermediate. As with iron, copper concentrations are also highly concentrated within Aβ plaques setting up conditions for Fenton-type chemistry through the reduction of Cu(II) by Aβ-H_2O_2 reactions.

Heme Oxygenase

HO is a cellular stress protein expressed in the brain and other tissues in response to oxidative challenge and other noxious stimuli. The main function of HO, as a major enzyme in the cellular oxidative stress response, is to cleave iron from the pro-oxidant heme. This chelation of heme iron is extremely important in regulating oxidative stress due to the potential reaction of heme with H_2O_2 to produce highly damaging •OH. By chelating iron, HO forms biliverdin IX•, water, and carbon monoxide. Subsequently, biliverdin IX• is then reduced by biliverdin reductase to the antioxidant bilirubin and free iron is sequestered by iron storage proteins such as ferritin.

Three distinct HO isozymes have been identified in the brain, an inducible form, HO-1,

and constitutive forms, HO-2, and the related species HO-3 (Maines et al. 1986, McCoubrey et al. 1997). Both HO-1 and HO-2 catalyse the identical biochemical reaction but differ in reaction rates. HO-3 displays a high sequence homology to HO-2, yet a poor heme catalytic activity (McCoubrey et al. 1997). Of the three isoforms, constitutive expression of HO-2 levels are highest in the brain, however, HO-1 is the major catalyst for heme degradation, attracting much research to this isozyme.

In AD, HO-1 has been shown to be associated with degenerating neurons (Smith et al. 1994b, Castellani et al. 1995, 1996) and that specific induction of HO-1, but not HO-2, parallels the regional susceptibility of neuronal degeneration (Premkumar et al. 1995). In AD, HO-1 has been shown to be significantly overexpressed in hippocampal neurons (Schipper et al. 1995) and is associated with the cytoskeletal pathology of AD, specifically NFTs (Takeda et al. 2000a,b). Also, when HO-1 was overexpressed in neuroblastoma cells, physiological expression of τ was inhibited and this suppression was partially alleviated by an HO inhibitor (Takeda et al. 2000b). This observation suggests that HO-1 expression may play a role in the regulation of the τ gene, consequently supporting the view that τ phosphorylation is related to oxidative stress. Also, in the AD brain, the expression of HO-1 is closely associated with pathological changes in the τ protein. *In vitro*, HO-1 induction was found to be completely coincidental with a conformational change in the τ protein that likely appears after τ phosphorylation (Takeda et al. 2000a). This conformational change is recognized by the Alz50 epitope in τ in which its appearance is considered an early pathological change, followed by further pathological changes, such as formation of NFTs that also contain the Alz50 epitope (Benzing et al. 1993). In study of the Alz50 epitope, its appearance in the τ protein is likely related to oxidative damage. This support for an oxidative damage effect is due to the fact that the reactive lipid peroxidation product 4-hydroxy-2-nonenal and other bifunctional carbonyl compounds effectively generate the conformational change defining the Alz50 epitope in τ (Takeda et al. 2000a). Because HO-1 is associated with the Alz50 epitope in τ, HO-1 appears to play a role in the pathogenesis of τ through oxidative stress events. The conformational change of the τ protein leading to cytoskeletal AD pathology can be attributed to oxidative stress events relating to dysregulation of the heme degradation pathway. Since mitochondria are rich in heme molecules and AD mitochondria exhibit increased degradation products, it is plausible that HO-1 expression is induced by excess heme release from damaged mitochondria. However, dysregulation of the heme homeostatic pathway may occur after chelation of heme iron from HO. Although free heme iron is normally sequestered by ferritin, this iron storage protein in AD does not appear to be simultaneously upregulated with increased free iron present (Connor et al. 1995, Loeffler et al. 1995). As previously stated, a decrease in ferritin was associated with an increase in iron concentration. Also, overexpression of HO-1 in neuronal cells did not affect ferritin expression (Takeda et al. 2000b). With no ferritin to store highly reactive free iron, HO-1 potentially serves as a pro-oxidant, catalysing the release of cellular iron to produce ROS via fenton chemistry. Although HO serves as a major oxidative stress response protein, its oxidant properties are highly dependent on the cellular environment, in which HO-1 appears to be a pro-oxidant in AD.

Stress Activated Protein Kinase (SAPK) Pathways

Alterations in gene expression and enzyme activity induced by cellular stresses are mediated through the interplay of multiple signalling pathways. Among these are the SAPK pathways, which are the central mediators that propagate signals from the membrane to the nucleus. SAPK pathways play important roles in cellular processes including gene expression, cell growth, and cell cycle control. Two of the best described SAPK (JNK and p38) pathways are activated primarily in response to oxidative stressors and have been found to be activated in AD (Zhu et al. 2002). The roles of these two SAPKs in AD as oxidative stress response elements are discussed below.

JNK (c-Jun N-terminal kinase) is specifically activated in response to UV irradiation, pro-inflammatory cytokines, as well as environmental oxidative stress. JNK is activated by dual phosphorylation of threonine in which its

immediate upstream activators include JKK1/MKK4 and JKK2/MKK7, respectively. Once phosphorylated, JNK translocates into the nucleus and activates transcription factors such as c-Jun, ATF-2, and Elk-1. In AD, phosphorylated JNK is localized to the same neuronal population that exhibits oxidative damage. Immunocytochemical studies have shown that JNK activation is increased significantly in both sporadic and familial AD cases and is associated with neurofibrillary pathology including a complete overlap with phosphorylated τ (Shoji et al. 2000, Zhu et al. 2001a). Notably, the immediate upstream activator, JKK1, is also activated in AD (Zhu et al. 2003). The capability of JNK to phosphorylate 10 proline-directed sites on τ *in vitro* (Goedert et al. 1997, Reynolds et al. 1997a,b, 2000) and the upregulation of τ-associated active JNK indicates that active JNK may be involved in the phosphorylation of τ *in vivo*. In fact, several groups reported that JNK can phosphorylate tau in neuronal and non-neuronal cells and in two different animal models (reviewed in Zhu et al. 2002). The fact that JNK is activated in control cases with limited pathology suggests that JNK activation is an early event in AD. Interestingly, the active JNK is redistributed from nuclei to cytoplasm and neurofibrillary pathology correlating with the progress of the disease and the development of the neurofibrillary pathology. It is therefore likely that rather than initially being involved in phosphorylating cytoprotective targets, JNK also plays a pathogenic role in AD by phosphorylating τ. Aβ induces a 2-3 fold activation of JNK in different neuronal cell types and that this activation directly contributes to Aβ-induced cell death (Troy et al. 2001). However, the mechanism by which Aβ activates JNK remains unclear. Given that JNK was activated in AβPP transgenic mice (K670N/M671L), which accumulates iron extensively, but not in YAC AβPP transgenic mice (K670N/M671L and V717I), which accumulate little iron, iron and related oxidative stress may play an important role in mediating Aβ-induced JNK activation (Zhu, Perry, and Smith, unpublished data).

The p38 kinase pathway is primarily activated by cellular stresses similar to the activation of JNK which includes UV irradiation, heat shock, pro-inflammatory cytokines, and oxidative stress. The p38 family is activated by dual phosphorylation on threonine and tyrosine within the Thr-Gly-Tyr site located in the activation loop. This phosphorylation is mediated by the upstream dual-specificity kinases MKK3 and MKK6 (Derijard et al. 1995). The role of p38 appears to be cell type and stimuli specific. However, p38 has been widely implicated in cell death regulation. An increase in p38 level and activity in AD brain tissues has been described (Zhu et al. 2000, Atzori et al. 2001). Immunocytochemical studies show that both increased and activated levels of p38, and its immediate upstream activator, MKK6, are associated with neurofibrillary pathology including NFTs, senile plaques, and neuropil threads in the AD brain (Hensley et al. 1999, Zhu et al. 2000, 2001b). In addition, a complete overlap of phospho-p38 immunoreactivity with phosphorylated τ was observed. It is likely that p38 may be involved in the phosphorylation of τ *in vivo* similarly to the phosphorylation of τ by JNK (Hensley et al. 1999). Since p38 is widely implicated as a primary response to oxidative stress, the association of activated p38 with hallmark AD pathology *in vivo* suggests a mechanistic response of neurons to oxidative challenge. The activation of the p38 pathway has been shown to mediate expression of downstream antioxidant proteins including HO-1 (Smith et al. 1994b, Premkumar et al. 1995, Schipper et al. 1995) as well as heat shock proteins in AD (Pappolla et al. 1992, Renkawek et al. 1994). Although the activation of p38 may indicate an effort by neurons to induce protective mechanisms, the ultimate consequence may vary depending on cellular and environmental conditions.

A comparison of JNK and p38 immunocytochemical studies showed a nearly identical immunoreactivity for both activated JNK and p38 kinases in severe AD cases. This finding suggests that these two SAPKs might be activated by the same signal, a signal that likely relates to oxidative stress. Although high levels of acute oxidative stress would inflict neuronal death, this type of oxidative damage is not the case in AD. Such high and acute levels of oxidative stress are seen in cases of trauma and ischemia in which neuronal pathology is significantly different from that in AD. Rather, oxidative challenges in AD cannot

exceed oxidative defences or rapid apoptotic death will result. This is exactly the case because few vulnerable neurons in AD show established signs of apoptosis (Perry et al.1998a,b). Although the extent of neuronal loss in AD can be great in some cases, it is often not greater than that seen in other aged individuals not suffering from AD (Cras et al. 1995). Further, it was shown that although the upstream caspases were activated, the concurrent activation of downstream caspases was virtually absent (Raina et al. 2001). With no significant signs of neuronal apoptosis brought on exclusively by oxidative stress, it is likely that neurons in AD are being chronically subjected to low levels of oxidative stress in which SAPKs serve as part of a neuroprotective adaptive response.

Because much evidence points to oxidative stressors as the primary activators of JNK, the prolonged activation of this pathway in AD suggests a chronic exposure of neurons to low levels of oxidative stress (figure 1). Oxidative damage is shown not to be limited to AD pathology, but rather uniformly involves members of entire populations

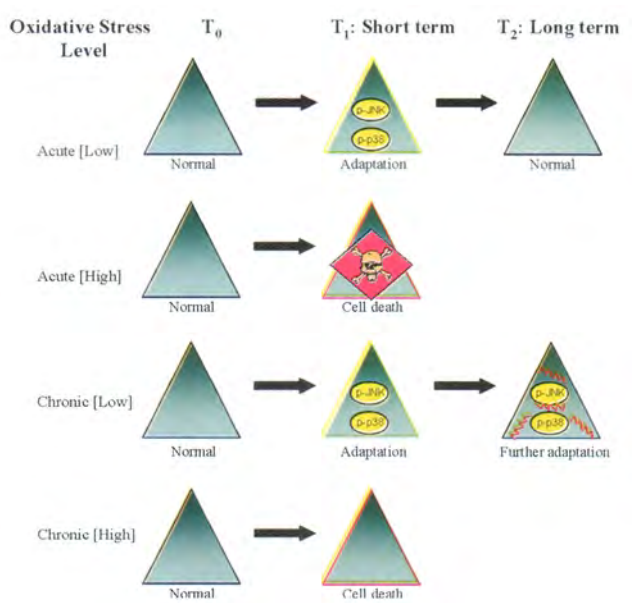

Figure 1 Neuronal responses to varying degrees and periodicities of oxidative stress. Although the neuron can encounter four distinct types of levels of oxidative stress, a chronic [low] stress level in AD leads to primary adaptation followed by secondary adaptive responses. Primary adaptation involves activating the stress response pathways of JNK (p-JNK) and p38 (p-p38) that lead to secondary adaptations involving cytoskeletal alterations including the phosphorylation of t ( ).

of neurons at risk for damage or death in AD (Smith et al. 1997b, Nunomura et al. 1999). This abnormal oxidative damage, as an early event in AD, may activate JNK in these neurons as an oxidative stress response with JNK pathway targets becoming altered as the disease progresses. It has been shown that active JNK is present in the nuclei of neurons in non-AD elderly. Therefore, active JNK may reflect an accumulative stress process during aging that appears to be an extremely early event in AD pathology. In fact, control cases without any pathology (Braak stage 0) showed select neurons with JNK activation exclusively in the nuclear compartment. Controls with limited pathology (Braak stages I and II) showed JNK activated in the cytosol as well as nuclei, while cases deeming a clinical diagnosis of AD (Braak stages III-IV) showed JNK activation exclusively in the cytosol (Zhu et al. 2001c). This redistribution of active JNK from nuclei to cytosol signifies an adaptation of neuronal cell signalling pathways in response to a constant threat of oxidative damage. This abnormal cellular adaptation due to chronic sources of oxidative stress may thus lead to a pathogenic response, with JNK contributing to the phosphorylation of the t protein. The temporal relationship of JNK and p38 activation in AD may further support their role in a neuronal adaptive response to oxidative challenges. Although activated in cases with mild and severe AD pathology, p38 is not activated in non-demented cases with limited pathology (Zhu et al. 2001c). The activation of p38 in post-JNK activation may imply an effort by neurons to adapt to a long term constant oxidative challenge whereby AD pathology is already manifested. Although p38 has been traditionally thought to mediate a stress response that is often associated with subsequent cell death, some reports (Zechner et al. 1997, Nagarkatti & Sha'afi 1998, Rausch & Marshall 1999) have suggested protective roles for p38 kinase. This non-apoptotic protective role is further supported by the immuno-histochemical finding that intact neurons with AD pathology contain high localization of active p38 (Zhu et al. 2000). A low level of chronic oxidative stress attributed to AD may explain the finding that p38 does not appear to induce an apoptotic response in AD susceptible neurons but rather a cytoprotective

response. It can therefore be explained that due to a constant oxidative challenge faced by susceptible neurons in AD, the cell activates JNK, p38, and other adaptive stress responses that ultimately contribute to AD pathology through the phosphorylation of τ.

Conclusions

Oxidative stress, as one of the earliest events in AD pathogenesis, plays a significant role in the formation of AD pathology. The complex nature and genesis of oxidative damage in AD can be partly explained by mitochondrial abnormalities as the loci of oxidative stress. By releasing excess levels of H_2O_2 and heme, dysfunctional mitochondria propagate a series of interactions between redox metals and oxidative stress response elements, providing a pro-oxidant environment through the formation of highly reactive free radicals. This dangerous pro-oxidant environment thus leads to AD pathology marked by Aβ and NFTs. The specific formation of Aβ and NFTs can be explained by interactions with excess free iron and long term induction of SAPKs resulting in the phosphorylation of τ, respectively (figure 1). Although oxidative stress is a pervasive feature in AD, what is striking is that few neurons exhibit signs of apoptosis, a response that is characteristic of acute oxidative stress (Raina et al. 2001). Rather, a uniquely low and chronic exposure of neurons to oxidative stress provides an explanation for the low apoptotic neuronal loss observed in AD as well as for the abnormally sustained activation of select oxidative stress response elements (figure 1). Such a low, but chronic, threat of oxidative stress causes susceptible neurons to enable adaptive mechanisms that are activated continuously due to the chronic nature of AD. It is this chronic adaptation as a result of persistent oxidative stress that may in fact be pathogenic rather than protective, contributing to the onset of AD pathology. Given the proposed persistent nature of oxidative stress in AD and the genesis of such stress preceding AD pathology, therapies involving the timely prevention of oxidative stress and effective removal of ROS appear beneficial.

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