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COMMENTARY

Reactive Oxygen Species and Alzheimer's Disease

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ABSTRACT. Although a consensus that Alzheimer's disease (AD) is a single disease has not been reached yet, the involvement of the amyloid precursor protein (APP) and βA4 (Aβ) in the pathologic changes advances our understanding of the underlying molecular alterations. Increasing evidence implicates oxidative stress in the neurodegenerative process of AD. This hypothesis is based on the toxicity of βA4 in cell cultures, and the findings that aggregation of βA4 can be induced by metal-catalyzed oxidation and that free oxygen radicals may be involved in APP metabolism. Another neurological disorder, familial amyotrophic lateral sclerosis (FALS), supports our view that AD and FALS may be linked through a common mechanism. In FALS, SOD–Cu(I) complexes are affected by hydrogen peroxide and free radicals are produced. In AD, the reduction of Cu(II) to Cu(I) by APP involves an electron-transfer reaction and could also lead to a production of hydroxyl radicals. Thus, copper-mediated toxicity of APP-Cu(II)/(I) complexes may contribute to neurodegeneration in AD. BIOCHEM PHARMACOL **54**;5:533–539, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Alzheimer's disease; familial amyotrophic lateral sclerosis (FALS); βA4 toxicity; free radicals; hydrogen peroxide; copper-mediated toxicity

AD§ is the most prevalent cause of dementia in old age. In all forms of the disease, including FAD and sporadic cases, accumulating evidence supports the hypothesis that cerebral amyloid βA4 (Aβ) protein deposition is an essential pathogenic event [1]. The major component of amyloid βA4 is derived from the transmembrane APP, the gene for which is localized on chromosome 21 (reviewed in Ref. 2). Mutations in the *APP* gene [3–7] and two other genes that cosegregate with affected members of familial AD pedigrees have been identified. Both genes on chromosome 14 (presenilin 1, PS1) and chromosome 1 (presenilin 2, PS2) encode integral membrane proteins. PS1 and 2 share extensive amino acid sequence identity and are predicted to contain either seven or nine transmembrane domains [8, 9].

Although the underlying mechanisms for AD are far from being understood, a significant increase in the extracellular concentration of amyloid βA4 is demonstrable in all groups with a genetic cause of AD. The production of a 42/43-amino acid peptide is increased in favor of a 40-amino acid peptide which is the predominant form in the normal processing of APP to the amyloid βA4 protein [1, 3, 10–12]. Recently, it has been shown that βA4(42/43) is deposited in diffuse plaques of the brain in trisomy 21 patients before βA4(40) deposition starts [13]. Thus, the faster formation of insoluble aggregates of βA4(42/43) in vitro [14–16] and its predominance in classical AD plaques [17, 18] favor a more pathogenetic role for such βA4(42/43) forms over the βA4(40) species.

Amyloid βA4 is normally released from APP through cleavage by proteases referred to as β- and γ-secretases [19–22]. The N-terminal 28 residues of βA4 are encoded by the ectodomain, and the remaining residues encode the first 12–15 residues of the transmembrane domain of APP (Fig. 1). Exogenous βA4 forms aggregates greater than 100 kDa [23], disrupts cellular ion homeostasis, induces cytotoxic cellular oxidant stress, and promotes microglial activation [24–28]. Recently, it has been suggested that RAGE is a specific cell-surface acceptor that mediates the effects of βA4 on neurons and microglia [29]. But since synthetic βA4 alone is able to induce oxidative stress and cytotoxicity [30], the underlying mechanisms at the molecular level need further elucidation.

βA4 Toxicity and Involvement of Free Radicals

Several years after it has been suggested that oxidative stress could be involved in AD [32] and the neurotoxicity of

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§ *Abbreviations:* AD, Alzheimer's disease; APP, amyloid precursor protein; βA4/Aβ, amyloid βA4 protein; A4CT, COOH-terminal 100 residues of APP, FALS, familial amyotrophic lateral sclerosis; SOD, superoxide dismutase, FAD, familial Alzheimer's disease; PS, presenilin; RAGE, receptor for advanced glycation end products; KPI, Kunitz-type inhibitory domain; FXIa, coagulation factor XIa; and DS, Down's syndrome.

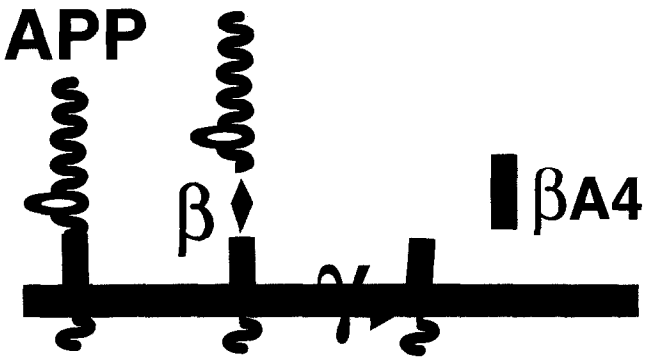

FIG. 1. Processing of APP. APP contains a single membrane-spanning domain. The βA4 fragment includes 28 residues of the ectodomain and 12–15 residues of the transmembrane domain. Proteolytic cleavage of APP770 after residue 671 by an uncharacterized event called β-secretase results in the secretion of the truncated APPβ-sec and the retention of a 99 residue C-terminal fragment. From this 12-kDa fragment, βA4 is released by γ-secretase activity.

βA4 had been demonstrated [32], evidence was provided that βA4 toxicity is related to an increased sensitivity of hippocampal neurons to glutamate-mediated cell death [33]. It was then shown that hydrogen peroxide mediates βA4-induced apoptosis due to oxidative damage [34–37]. A number of antioxidants were shown to protect cells from βA4 toxicity. Amyloid βA4 was found to increase the intracellular peroxides in cells in a time- and concentration-dependent manner. Since catalase could block this effect, it has been suggested that H_2O_2 or one of its metabolites mediates the toxicity of βA4 residues 25–35 in PC12 cell cultures [38]. A βA4-mediated superoxide production was observed with endothelial damage [39]. Based on these observations, amyloid itself should be capable of generating free radicals. EPR spectroscopy and spin trapping represent the important techniques that enabled Hensley *et al.* [40] to study the generation of free radicals by a synthetic fragment of βA4 (residues 25–35) in aqueous solutions. In contrast to a rapid radicalization observed by βA4(25–35), a lag-time period for radical formation by βA4(1–40) was found. Since βA4(1–40) is only neurotoxic after preincubation and βA4(25–35) immediately upon solubilization, and both effects arise within the same time frame, they seem to be linked. The key amino acid was suggested to be Met-35 that would react with oxygen to produce a sulfoxide. Recently, it was suggested that monomeric and dimeric βA4(1–42) purified from brains are toxic to cultured nerve cells through a microglia-dependent mechanism [41]. This does not exclude a radical-mediated reaction being involved in the activation of microglia to neurotoxicity; rather, a cascade might be started by activated microglia when those cells secrete a number of potentially neurotoxic compounds, including free radicals, cytokines, and nitric oxide [42].

Thus, formation of radical species may be a mechanism for the neurotoxicity of βA4. Although a metal-catalyzed redox reaction was excluded to evoke the βA4-associated EPR signal [40], other reports indicate an involvement of transition metal ions in radicalization and aggregation of βA4 [40, 43, 44].

Metal Ions May Induce βA4 Amyloid Formation

There exists some indirect and direct support for free radical involvement in βA4 insolubilization. Concentrations of Zn(II) above 300 nM induce the aggregation of βA4 [45]. A rapid amyloid formation of synthetic human βA4 was found in the presence of physiological salt concentrations. In contrast to the human sequence, the rat/mouse substitutions at positions 5, 10, and 13 appear to change the physiochemical properties of the peptide since rat βA4(1–40) remained stable and soluble in the presence of up to 10 μM Zn(II). Human βA4(1–40) contained two specific binding sites of different affinities for Zn(II), whereas only one such site appears to exist for the corresponding rat sequence [45].

More direct support derives from reports that analyzed βA4 aggregation in the presence of free radical catalysts such as Fe(III) and Cu(II). Mantyh *et al.* [46] showed that Fe(III) and Al(III) markedly accelerate the *in vitro* aggregation of radioiodinated human βA4 into amyloid. Cu(II) may also stabilize dimeric forms of βA4 [45] and can be used to capture the peptide from solution or suspension [45]. Although some observations in these reports are contradictory, it can at least be concluded that βA4 has the propensity to bind metal ions.

Dyrks *et al.* [44] reported that the aggregation of βA4 and A4CT requires metal-catalyzed oxidation. Both proteins formed highly insoluble aggregates if expressed in the rabbit reticulocyte lysate system and analyzed by SDS-PAGE [44]. A transformation of soluble βA4 and A4CT into insoluble and aggregating molecules occurred after hydrogen peroxide was added in conjunction with metal-catalyzed oxidation systems such as hemoglobin and hemin. The aggregation process was prevented in the presence of radical scavengers or free amino acids. Therefore, it was concluded that the aggregation was induced by amino acid oxidation and protein cross-linking. These findings revealed for the first time that not only βA4 but also larger amyloidogenic APP fragments, such as A4CT, could be transformed into stable aggregates. This report was the first to introduce metal-catalyzed oxidation systems as possible pathological events causing Alzheimer's disease.

Evidence for Involvement of Free Oxygen Radicals in APP Metabolism

The following comments about the binding of divalent metal ions to the extracellular domain of APP allow one to postulate that a radical-promoted, protease-independent or protease-dependent cleavage may lead to an initial perturbation of proteolytic processing or other pathways of APP, and finally result in the release of βA4 into the extraneuronal space. The mechanism of βA4 neurotoxicity might

start initially as a cascade of excessive formation of hydrogen peroxide and oxygen-derived free radicals at the APP level. APP may act on βA4 in a feedback reaction, thereby increasing oxidative stress and possibly other age-related pathological factors.

Initial binding studies of metal-ion binding to APP were undertaken to investigate an association between Zn(II) and APP metabolism [43]. A novel Zn(II) binding motif was discovered in the region of APP 181–200, located more than 400 residues distant from the Zn(II) binding sites in the βA4 region [45]. Incubation with Zn(II) increased binding of APP to heparin [47, 48] and has been shown to potentiate the inhibition of FXIa by an APP isoform that contains the KPI [49]. The interaction of Zn(II) with KPI-containing isoforms of APP has been shown to be necessary for optimal inhibition of FXIa [50]. Thus, Zn(II) binding modulates the functional properties of APP by enhancing its macromolecular conformation. An active transport of neuronal Zn(II) along cell processes or from cell to cell has been hypothesized [51].

Whereas Zn(II) is assumed to play a purely structural role, we found that APP binds Cu(II) [52] and reduces bound Cu(II) to Cu(I) [53]. Since Zn(II) exists exclusively in one oxidation state, only APP-Cu(II) complexes are sensitive to redox reactions. Cu(II) binds at a dissociation constant of 10 nM at pH 7.5 in the region of APP 135–155. This APP domain contains a consensus motif for type II copper binding proteins, and is encoded by exon 4. The reduction of Cu(II) to Cu(I) by APP results in a corresponding oxidation of cysteines 144 and 158 in APP that involves an intramolecular reaction leading to a new disulfide bridge. This reaction was found to be very specific, as APP did not bind and reduce other metals such as Fe(III), Ni(II), Co(II), or Mg(II).

Thus, Cu(II) binding leads to oxidative modification of APP, resulting in cystine and Cu(I) formation. Accordingly, *in vitro* APP has a function in electron transfer to Cu(II). Once Cu(I) is formed, it may produce activated oxygen species in the presence of peroxides. In general, when the balance between the production of these oxygen-derived species (such as O_2^-, H_2O_2, and $^{\bullet}OH$) and antioxidant is disturbed, oxidative stress results [54]. Endogenous sources appear to account for most of the free radicals produced in cellular aerobic metabolism [55]. Hydrogen peroxide and peroxynitrite ($ONOO^-$), although not themselves free radicals, are important contributors to the cellular redox state. About 10^{12} O_2 molecules are processed by each rat cell daily. The leakage of partially reduced oxygen molecules from various components of the cellular electron transport chains is about 2%, yielding about 2×10^{10} O_2^- and H_2O_2 molecules per cell per day [56]. For instance, superoxide radicals are formed in almost all aerobic cells by a variety of cytosolic and membrane-bound enzymes, including xanthine oxidase, the cytochrome P450 complex, and phospholipase A_2, and is produced during the respiratory burst of phagocytic cells [57]. It has become well established that O_2^- is produced in human cells and is the precursor of H_2O_2 that is formed by SOD-catalyzed dismutation [58]. Hydrogen peroxide has a limited reactivity, but it can cross biological membranes, while O_2^- species only move very slowly unless there is an anion channel through which they can travel [59, 60]. Since superoxide species and H_2O_2 at physiological levels are poorly reactive [reviewed in Ref. 61], the toxicity of O_2^- and H_2O_2 was proposed to be derived from a conversion into the much more reactive $^{\bullet}OH$. Accordingly, an interaction between O_2^- and nitric oxide ($^{\bullet}NO$) leading to $^{\bullet}OH$ could be demonstrated *in vitro* [55]. But an earlier mechanism that brings us back to the APP of Alzheimer's disease was the metal ion-catalyzed Haber-Weiss reaction [58],

$$\textbf{A:}\quad O_2^- + H_2O_2 \xrightarrow{Fe/Cu} {}^{\bullet}OH + OH^- + O_2$$

or in the involvement of iron by the so-called superoxide-driven Fenton reaction

$$\textbf{B:}\quad Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^-$$

Copper(I) can catalyze the same reaction to form $^{\bullet}OH$. The rate constant for a reaction of Cu(I) with H_2O_2 is magnitudes higher than that for Fe(II) [58].

$$\textbf{C:}\quad Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + OH^-$$

A Cu(III) (cupryl) intermediate has been suggested to be formed as well as or instead of $^{\bullet}OH$, and there is an ongoing controversy over the physiological significance of copper-dependent radical production *in vivo* [reviewed in Ref. 58]. Nevertheless, $^{\bullet}OH$ formation requires the presence of catalyzing metal ions. Also, if one adopts a broad approach and defines a free radical as any species that has one or more unpaired electrons, Cu(I) as a transition metal ion is therefore a radical by itself. Damage might occur when such radicals react with the binding molecule and are not accessible to added scavengers. More generally, radicals are thought to be involved in lipid peroxidation of the cell membrane, leading to increased membrane fluidity and disturbance of divalent ion homeostasis [58].

It is suggested that APP-Cu(I) complexes could react according to the Fenton reaction in a site-specific manner to modify amino acid residues at the metal binding site of APP. In this mechanism, it is assumed that the hydroxyl radical ($^{\bullet}OH$) is formed by the reaction of Cu(I) with H_2O_2. Copper ions *in vivo* are invariably associated with amino groups, to which they are ligated. Nevertheless, this binding does not prevent copper from participating in oxygen-radical reactions, but rather limits radical formation to the site of copper binding [58, 61]. Therefore, the reaction is viewed as a caged process in which the active oxygen species is not released into the surrounding medium but preferentially reacts with functional groups of amino acids at the metal binding site. This site-specific nature of the metal-catalyzed reactions has been confirmed by the

results of studies with incubations of APP and Cu(II), which show that the copper-binding site in APP remains intact even after the redox reaction. The formation of one intramolecular disulfide bridge did not interfere with copper binding [53]. Additionally, the redox reaction was not inhibited by SOD and catalase and, therefore, did not require either superoxide or hydrogen peroxide in free solution. However, a possible inhibitory action of SOD and catalase in the proposed Fenton reaction is under investigation and will clearly show if O_2^- or H_2O_2 are involved in the decomposition of the APP-Cu(I) complex (see equation F).

The generation of reactive oxygen species is a normal byproduct of metabolism. One of the consequences of their production is an increased level of protein oxidation in Alzheimer's disease and normal aging [62]. Moreover, apoptosis and an increased generation of reactive oxygen species were observed in DS neurons *in vitro* [63].

On the molecular level, all amino acids are subject to attack by $^{\bullet}OH$. Pro, Arg, and Lys are preferred targets for oxidation by metal-catalyzed oxidation systems, are converted to carbonyl derivatives [reviewed in Ref. 64], and are found to be increased in hippocampal brain tissue of Alzheimer cases [65]. Many of the known proteases degrade oxidized proteins more rapidly than unoxidized forms [66]. Intracellularly, the degradation of proteins is greatly stimulated by exposure of the cells to oxygen radical generating systems and H_2O_2 [67, 68]. In the light of current studies investigating the catabolism of APP, this seems to be an area to focus on for three reasons. First, hydroxyl radicals denature proteins such that susceptibility to proteolytic enzymes is increased remarkably [68, 69]. Second, APP molecules undergo proteolysis by poorly identified activities at the molecular level, designated either as an α-secretory or amyloidogenic (β- followed by γ-secretase cleavage of APP) pathway. Third, the defect of DS neurons in the metabolism of reactive oxygen species causes neuronal apoptosis and may also predispose to Alzheimer's disease in adults.

Considerations from FALS

Free radical damage to cellular function has been found to be associated with a number of age-related diseases, such as atherosclerosis, arthritis, cataractogenesis, and pulmonary dysfunction, and in various neurological disorders, such as Parkinson's disease and ALS [70]. The most convincing evidence thus far for a link between neurological disorders and oxygen radical formation is the strong association observed between FALS and mutations in the Cu/Zn SOD gene. The human motor neuron disease ALS is characterized by the degeneration of large motor neurons of the spinal cord, brainstem, and motor cortex. ALS occurs in sporadic and familial forms, which are clinically and pathologically similar [71]. Some pedigrees of autosomal dominant FALS have missense point mutations in the gene located on chromosome 21, encoding cytosolic Cu/Zn SOD 1 (SOD1) [72]. Currently, much evidence argues strongly that the disease arises not from loss of SOD1 function but rather from an adverse or novel property of the mutant SOD1 molecule [70, 73]. Transgenic mice that over-express the human *SOD1* gene develop a clinically analogous form of motor neuron disease similar to human FALS [74]. Insight into the mechanism was gained after it had been discovered that SOD is inactivated by H_2O_2 that rapidly reduced Cu(II) at the active site [75, 76] and Cu/Zn-SOD was found to generate free $^{\bullet}OH$ radicals from H_2O_2 [77]. The most important findings were recently published by Wiedau-Pazos and colleagues who reported that in an *in vitro* system, FALS-associated mutant SOD1 enzyme catalyzes the reduction of H_2O_2 thereby acting as a peroxidase. Thus, Cu/Zn-SOD has a peroxidative function that utilizes its own dismutation product, H_2O_2, as a substrate. This was found to occur more rapidly with mutant than wild-type SOD1, with the mutants at least twice as reactive to that of the wild-type enzyme. These findings were confirmed by Yim *et al.* [77] who showed that the same FALS mutant G93A of Cu/Zn-SOD, overexpressed in insect cells, generates more free radicals during the peroxidase reaction. Using the spin-trapping method, it was found that the enhanced free radical generating function of the mutant is due to a decrease in K_m for hydrogen peroxide [77]. A proposed model by Wiedau-Pazos [78] results from the following equations:

$$\textbf{D:}\quad \textbf{SOD-Cu(II)} + \mathbf{H_2O_2} \rightarrow \textbf{SOD-Cu(I)} + \mathbf{O_2^-} + \mathbf{2H^+}$$

$$\textbf{E:}\quad \textbf{SOD-Cu(I)} + \mathbf{H_2O_2} \rightarrow \textbf{SOD-Cu(II)}(\mathbf{^{\bullet}OH}) + \mathbf{OH^-}$$

$$\textbf{F:}\quad \textbf{APP-Cu(I)} + \mathbf{H_2O_2} \xrightarrow{?} \textbf{APP-Cu(II)}(\mathbf{^{\bullet}OH}) + \mathbf{OH^-}$$

This suggests that in the critical step H_2O_2 is reduced and accepts an electron from Cu^+. In this process the copper ion is bound by imidazole groups on neighboring histidine residues within the SOD molecule, as has been described for APP earlier [52]. This suggests that oxygen radicals may be responsible for the selective degeneration of motor neurons occurring in this fatal disease [79]. One specific example of copper-dependent gain of function would be disruption in intracellular copper trafficking. At least three genes have been identified that genetically interact with SOD and influence the intracellular trafficking of copper [for a review see Ref. 73]. Their gene products are clearly involved in metal homeostasis, and SOD mutations adversely affect copper homeostasis, thus leading to dysfunction and death of neurons. In Alzheimer's disease, APP-Cu(I) complexes on the surface of neurons may be particularly vulnerable to peroxides generated by extracellular forms of SOD, according to equations E and F. Such complexes are spontaneously formed since APP itself reduces bound Cu(II) to Cu(I). This Cu(II) ion-mediated redox reaction leads to disulfide formation in APP and to

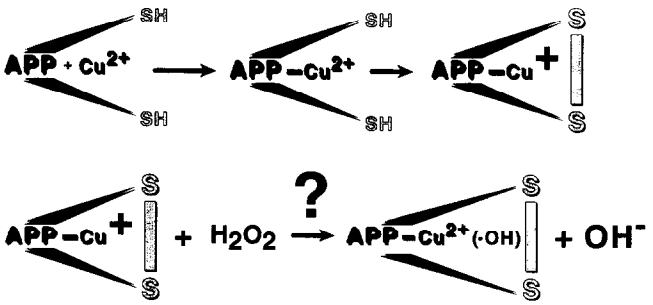

FIG. 2. APP in the reduction of Cu(II) to Cu(I). APP reduces bound Cu(II) to Cu(I) in a redox reaction that leads to disulfide bond formation in APP. Potential formation of hydroxyl radicals from hydrogen peroxide and APP-Cu(I) complexes may contribute to the neurodegeneration of Alzheimer's disease.

the formation of APP-Cu(I) complexes, even in the absence of hydrogen peroxide [53] (Fig. 2).

Both APP and SOD may be involved in Cu(II)/Cu(I) homeostasis. An interplay of APP and SOD could affect the metabolism of Cu(II), as has been suggested for mutant SOD in FALS. In agreement with this, neurons in trisomy 21 exhibit a 3- to 4-fold increase in intracellular reactive oxygen species [63]. Therefore, both SOD and APP genes that are overexpressed in Down's syndrome may be responsible for the premature onset of AD [17, 80].

The body of an average adult contains about 80 mg copper, and copper reaches concentrations in body fluids of about 10 μM. It has been discussed as a possible effector of βA4 aggregation in AD [45, 81, 82]. Also, in cerebrospinal fluid of AD patients, concentrations of copper are increased by a factor of 2.2 [83].

Two major hypotheses have been proposed concerning the mechanism of amyloid deposition in AD. In the first, synaptic and axonal injury leading to the formation of dystrophic neurites with accumulations of APP are postulated to precede the deposition of βA4 amyloid. The alternative hypothesis is that amorphous deposits or soluble forms of βA4 (diffuse plaques) constitute the first abnormality. A mechanism of disease mediated by disruption of vesicular-mediated APP-Cu(I) complex trafficking is consistent with both hypotheses. In the presence of hydrogen peroxide, the key feature of such an APP-Cu(I) accumulation would be the gain of copper-mediated toxicity, and the final common pathway in AD may be oxygen radical-induced cellular damage and radical-mediated aggregation of βA4 monomers, producing toxic forms of βA4 amyloid [84, 85].

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