Reactive Oxygen Species as Causal Agents in the Neurotoxicity of the Alzheimer's Disease-Associated Amyloid Beta Peptide[a]

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The past decade has seen an explosion of knowledge regarding mechanisms by which reactive free radicals, particularly reactive oxygen species (ROS), damage sensitive proteins, lipids, and nucleic acids under physiologic or toxic stress.[1] It has become clear that oxidation is a cumulative process which progressively damages the brain during aging. Protein oxidation products accumulate in the brain exponentially as a function of age in humans, and biomarkers of protein oxidation (*e.g.*, protein carbonyl content) are elevated in the AD brain relative to control, nondemented brain.[2-5]

Cognitive dysfunction seems to be a general consequence of aging, although there is great variability among individuals with respect to the rate of cognitive decline. Extrapolating from the assumption that tissue oxidation impairs tissue performance, numerous authors have suggested that normal cognitive decline as well as age–specific neurodegenerative conditions (*e.g.*, Alzheimer's disease (AD)) are caused or exacerbated by processes which increase ROS or impair antioxidant systems in the brain.[6-8] Experimental support for this assumption includes the observation that biomarkers of tissue oxidation (protein carbonyl levels, glutamine synthetase (GS)

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activity) and indices of cognitive function (radial arm maze, Morris water maze performance tests) in aged rodents return to levels comparable to young animals during chronic treatment with certain antioxidants.[9,10] The mechanistic relationship (if any) among oxyradical production, brain tissue oxidation, normal human aging, and AD pathology is controversial.

One nascent theory of Alzheimer's disease etiology holds that senile plaques, the histopathologically discriminating feature of the AD brain, are microenvironments of elevated oxidative stress.[2] Specific sources of this putative oxidative stress have been suggested to include redox-active metals,[11,12] defective mitochondrial electron transport systems;[13,14] protein glycation products;[15] reactive glia;[16] and beta amyloid peptide (Aβs).[2,17-34] This paper specifically reviews the potential role of Aβ as a prooxidant. Detailed review of the molecular biology of amyloid, along with a discussion of other risk factors relevant to Alzheimer's disease, are thoroughly treated elsewhere.[35-36]

There are several reasons that Aβ might be a principal factor in AD. Amyloid peptides (mostly 39-43 amino acid variants) are the primary component of senile plaques, which correlate spatially with neuronal damage in the AD brain.[35-37] Mutations affecting the amyloid precursor protein correlate with some familial forms of AD,[38] while transgenic mice overexpressing the precursor exhibit brain pathology similar to that found in AD.[39] Finally, synthetic Aβ peptides are potent inducers of neurotoxicity *in vitro*. For these reasons, research has come to focus largely on Aβ peptides as mediators of cell damage in AD, and recently as progenitors of oxyradical stress.

An emerging body of data indicates that the proximate cytotoxic effects of Aβ peptides are mediated by oxyradicals, while the ultimate result of amyloid treatment is a disruption in cellular ionic homeostasis (particularly Ca^{2+} balance; see FIG. 1). Aβ neurotoxicity can be abrogated by free radical scavengers or enhanced by redox-cycling metals, which catalyze oxyradical production. The solution structure of synthetic amyloid peptides demonstrates an oxygen dependency, while solution incubates of synthetic Aβ generate ROS as detected by salicylate hydroxylation assays and electron paramagnetic resonance spin trapping studies. Reaction of amyloid with oxygen is further indicated by rapid oxidation of toxic amyloid peptide to yield a sulfoxide product, a reaction which is inhibited by antioxidant inclusion. Investigation of the prooxidant properties of Aβ may lead to therapies that slow the clinical progression of Alzheimer's disease.

Amyloid Peptides Promote Free Radical Reactions in Oxygenated Buffer: Genesis for the Hypothesis that Toxic Aβ Is a Prooxidant

Beta amyloid research largely began in the latter half of the 1980s, following the identification and characterization of Aβ peptide in senile plaques. The most common form of amyloid in these plaques was found to be a 40-mer, consequently termed Aβ(1-40).[35-37] Other significant amyloid peptides found in plaques are 39-43 residues in length, while other variants may exist.[40] Synthetic versions of Aβ(1-40) and longer oligomers were synthesized and made commercially available; however, the question of whether this peptide could promote cell death was debated for some time owing to difficulties in replicating observations of amyloid-mediated

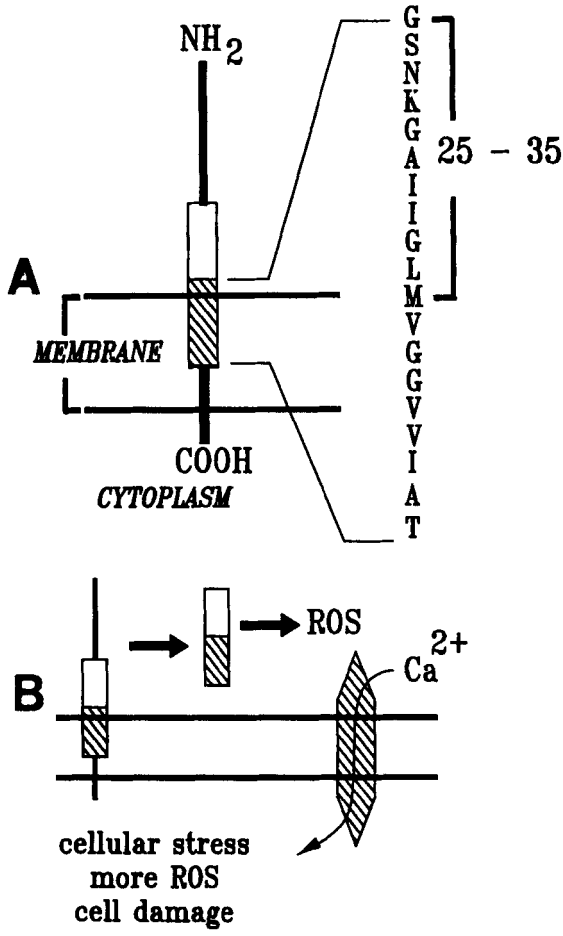

FIGURE 1. (**A**) Schematic diagram illustrating the relationship between amyloid precursor protein (APP) and derived Aβ fragments and the cell membrane. (**B**) Proposed model for release of toxic amyloid peptide fragments (via aberrant proteolytic processing) followed by ROS production, membrane damage, and consequent Ca^{2+} influx.

neurotoxicity.[41] Synthetic amyloid may be either neurotoxic, neurotrophic, or unreactive when tested in cell culture systems, depending upon the experimental paradigm employed.[41]

Eventually, a consensus emerged that synthetic amyloid is in fact neurotoxic when applied to hippocampal neuronal cultures at concentrations of 10-100 μM.[41-44] Other cell types besides hippocampal neurons are more or less susceptible to amyloid toxicity. Full realization of amyloid toxicity may require "preaging" of the peptide by incubation in buffer for some number of days before application to the cell culture system.[43] The amount of time required to preage a given batch of

synthetic amyloid varies from lot to lot.[41] During the preincubation period, the peptide adopts a beta sheet conformation and assembles into fibrillar aggregates.[44] Nontoxic amyloid analogs, *e.g.*, the reverse sequence Aβ(40-1), form amorphous aggregates in solution and demonstrate no beta sheet conversion.[44] Pike and co-workers have localized Aβ toxicity to the a fragment spanning residues 25-35, and these results have subsequently been well replicated.[42] At least one author reports that the short fragment Aβ(31-35) is comparably neurotoxic to Aβ(25-35);[45] however this observation has not yet been thoroughly replicated.

The first indication of a possible free radical component to amyloid toxicity was the finding by Behl and co-workers that cotreatment of clonal PC12 cells (a cell type extremely sensitive to amyloid toxicity) with the antioxidants vitamin E or propyl gallate in addition to Aβ(1-40) or Aβ(25-35) protected the cells against peptide-mediated toxicity.[17] Shortly thereafter, Dyrks and Beyreuther showed that bacterially expressed amyloid analogs corresponding to the carboxyl-terminal 100 residues of the amyloid precursor protein were not competent to aggregate in the same fashion as synthetic Aβ(1-40), but could be induced to do so by treatment with metal-catalyzed oxidation systems.[18] Other, contemporary studies demonstrated convincingly that Aβ(1-40) caused Ca^{2+} influx and rendered cells sensitive to excitotoxic stimuli and ionophores,[36] but at the time these experiments did not address the issue of cellular oxidation.

Given the apparent involvement of oxidative processes in Aβ-mediated cytotoxicity, our group decided to systematically probe the free radical reactivity of synthetic amyloid peptide incubates. Since free radicals are fundamentally unstable compounds, their presence must be inferred indirectly. Detection of free radicals typically involves reaction with a probe to generate a characteristic stable product. The probe may be a spin trap, in which case the product is a nitroxide which is detected by electron paramagnetic resonance (EPR) spectroscopy. Alternatively, the probe may be an aromatic compound that undergoes electrophilic addition reaction with oxyradicals to yield a hydroxylation product. Other probes, such as nitroxide spin probes, redox-activated dies, and colorimetric reagents have recently been used to quantify oxyradical production in various systems. Finally, elevation of ROS levels may be inferred from reaction with a resident biomolecule, such as an oxidatively sensitive enzyme, which loses activity as a consequence of free radical lesioning.

Incubation of synthetic Aβ(1-40) or its toxic fragment, Aβ(25-35), with the spin trap phenyl-*tert*-butyl nitrone (PBN) generates EPR-detectable levels of nitroxide.[19-21] The EPR spectrum of such samples is either a three- or a four-line signature, depending upon the source and history of the peptide sample.[20] Numerous laboratories have observed lot-to-lot variability in amyloid peptide toxicity, though attempts to correlate this variability with some impurity have failed.[41,43] There is a clear relationship between amyloid neurotoxicity and PBN reactivity, with three line-generating variants being much more toxic than those which react with PBN to yield four-line EPR spectra. Similar EPR spectra are obtained when the spin trap is dimethyl pyrolline-N-oxyl (DMPO), though the reaction is less vigorous and less product is formed.[20-21]

These EPR spectra are unusual in that most products of PBN reaction with free radicals are characterized by spectra having 6 or more lines. By using ^{13}C-substituted PBN to magnetically couple the carbon and nitrogen of the PBN nitrone backbone, we demonstrated that the nitroxide derived from amyloid reaction with PBN represent

fragmentation of the trap between the phenyl carbon and the nitrogen centers.[21] The nontoxic reverse sequence amyloid peptide analog, Aβ(40-1), is also reactive towards PBN, but yields a characteristic six-line EPR spectrum indicative of a stable spin adduct rather than a three-line signal indicative of a decomposition product.[21] The levels of nitroxide products resulting from peptide incubation with PBN represent only 1-2% of the total peptide content of the incubate, possibly indicating that the amyloid spin trap reactivity is due to small amounts of radicalized peptide present in the incubate.[19] Attempts to inhibit the amyloid/PBN reaction by inclusion of deferoxamine, EDTA, EGTA, or chelex-100 resin, were unsuccessful.[19] The reaction is effectively prevented by sparging buffer with N_2 prior to peptide addition.[19] These last results imply that the amyloid reaction with synthetic Aβ is oxygen dependent, but that metal catalyzed reactions are not operative. This does not rule out the possibility that metal-catalyzed processes occur during synthesis and lyophilization of the peptide, before the actual addition of buffer, spin trap, and chelator.

Preincubation of synthetic Aβ(1-40) for five days prior to PBN addition results in no PBN reaction, as monitored by EPR spectroscopy. Preincubation of the same peptide for shorter time periods (up to 24 hours) does not affect the ability of the peptide to generate paramagnetic reaction products upon PBN addition. Similarly, Aβ(25-35) remains competent to react with PBN after several hours of preincubation, but becomes incompetent after days in solution.

Amyloid peptide incubates generate detectable hydroxylation products when incubated with sodium salicylate. In particular, significant quantities of dihydroxy benzoic acid (DHBA) are formed within minutes of buffer addition to synthetic Aβ(25-35). Preincubation of the peptide prior to salicylate addition results in no DHBA production.[19]

Biomolecules are also affected by contact with amyloid peptides. Coincubation of either Aβ(1-40) or Aβ(25-35) with the cytosolic fraction of gerbil brain homogenate promotes inactivation of both glutamine synthetase and creatine kinase (CK) enzymes.[19] These two enzymes are known to be sensitive indices of oxidative stress, and are both diminished during normal aging and in the AD brain.[2,3-4] The sensitivity of GS to amyloid is not a specific feature of rodent brain homogenate; we observe similar amyloid sensitivity in samples of commercially available, partially purified sheep-brain GS and in astrocytic cell culture (discussed further below).

Amyloid Peptide Solution Structure and Binding Properties Are Oxygen-Dependent

The chemical mechanism by which amyloid peptides seem to generate ROS in oxygenated buffers is unclear. The most obvious chemical change that occurs to Aβ during solution incubation is the conversion of methionine (residue 35) to the corresponding sulfoxide (FIG. 2).[20] This reaction occurs within three hours at 37°C, in the apparent absence of redox-active metals, and is completely prevented by millimolar levels of PBN.[20] Alkyl sulfides are known to participate in unusual free radical reaction mechanisms, and are particularly prone to metal-catalyzed oxidation. The remarkable feature of methionine oxidation in amyloid is that the reaction occurs rapidly under mild solution conditions in the apparent absence of buffer-carried metals.[20]

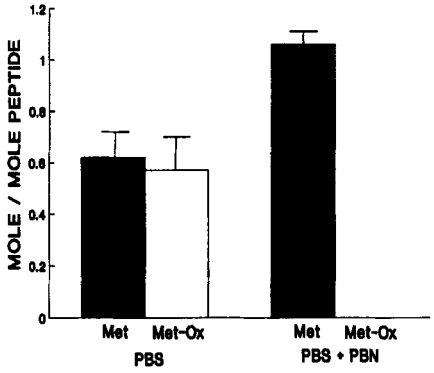

FIGURE 2. Conversion of methionine (Met) to the sulfoxide (Met-Ox) in synthetic amyloid Aβ(25–35) upon incubation in chelated phosphate buffered saline (PBN) for 12 hours with or without 50 mM PBN.

Methionine oxidation has been shown to drastically affect secondary protein structure by stabilizing beta sheet conformation in some model oligopeptides.[46] Model Aβ(1-40) synthesized with methionine sulfoxide in the residue 35 position reportedly aggregates more than twice as rapidly as unmodified Aβ, as indicated by solution hydrodynamics measurements.[23] It is therefore plausible that peptide oxidation is related to aggregation and toxicity. Since Aβ(1-40) beta sheet content is correlated with toxicity and requires "preaging" to develop, we undertook a study to monitor the solution structure of Aβ(1-40) incubated in oxygenated or nitrogen-sparged saline. Circular dichroism spectroscopy indicated a pronounced oxygen dependency on the solution structure of synthetic amyloid, although a clear beta sheet conformation was not detectable under these solution conditions.[25] Our lab was not the first to notice an oxygen dependency to amyloid reactivity. Roses and co-workers, in the course of investigating amyloid binding to apolipoprotein E (ApoE), observed a dramatic increase in the rate of binding between Aβ(1-40) and both ApoE3 and ApoE4 isoforms in oxygenated buffer relative to nitrogen-sparged buffer.[47] Moreover, inclusion of reducing agents prevented Aβ/ApoE binding. These authors also observed that only a fraction of the amyloid peptide was capable of binding to ApoE.

These studies demonstrate that the properties of amyloid peptide can be moderated by oxygen tension and by the presence of oxyradicals and reducing agents, suggesting that redox chemistry may be significant to the role played by Aβ in the Alzheimer's brain.

Amyloid Peptides Inactivate Oxidation-Sensitive Enzymes in Cell Culture and Elevate Intracellular Levels of Reactive Oxygen

As discussed above, amyloid beta peptides inactivate glutamine synthetase and creatine kinase enzymes in brain cytosolic extract and cell free incubates.[19] Aβ is also capable of inactivating neuronal enzymes in cell culture and in human synaptosomes. Enzyme impairment correlates with an elevation of intracellular ROS levels and concomitantly with membrane protein oxidation. Studies performed by our group suggest that the proximate mode of toxicity of amyloid peptides is membrane destabili-

zation due to oxyradical stress, which results in damage to ion motive ATPases, and subsequent loss of Ca^{2+} homeostasis.

In order to investigate the ability of amyloid to induce intracellular ROS, our group employed the redox-sensitive fluorescent dye 2′,7′-dichlorofluorescin diacetate (DCF).[24,27-29] The diacetate compound is taken into cells where it is hydrolyzed to dichlorofluorescin. Oxidation of the dichlorofluorescin converts it to dichlorofluorescein, which is detectable spectrofluorometrically. Use of a confocal scanning laser microscope allows direct observation of individual cells. Neurons probed with DCF and treated with Aβ(1-40) or Aβ(25-35) fluoresce strongly, as do neurons treated with hydrogen peroxide/iron (a paradigm known to generate *OH radicals).

Our DCF findings were published coincidently with those of Behl and co-workers, who also utilized the DCF technique to quantify Aβ-generated oxyradicals.[30-31] These researchers employed clonal PC12 cells rather than primary neuronal culture, and further probed the intracellular redox state with the redox-sensitive dye 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT). MTT is reduced to a colored formazan product, apparently by contact with components of the mitochondrial cytochrome chain.[30-31]

Treatment of cells with H_2O_2 or Aβ diminishes the MTT → formazan conversion, indicating a less reducing (more oxidizing) intracellular milieu. Toxicity in these studies was assessed by lactic dehydrogenase (LDH) release, indicating a membrane compromise and cellular lysis.[30] It is thought that most intracellular MTT reduction occurs at mitochondrial locations; inhibition of MTT reduction by amyloid may suggest a role for mitochondrial processes, and particularly flavin oxidases, in exacerbation of peptide-mediated oxyradical generation.

Intracellular enzymes and DNA are also susceptible to amyloid. Rat hippocampal cultures exposed to 50 μM Aβ(1-40) for 6 hours demonstrate a 30% reduction in GS activity relative to control cells,[24] indicating that GS toxicity data derived from brain cytosolic extract accurately reflects at least one aspect of cell physiology. In addition to the deleterious effects exerted by amyloid upon cellular proteins and lipids, it has been reported that chronic low-dose exposure of rodent cortical neurons to amyloid induces DNA fragmentation similar to that seen following brief exposure to micromolar levels of H_2O_2.[32]

These data demonstrate a striking similarity between the constellation of physiologic perturbations caused by amyloid and that caused by known oxyradical generating systems, *e.g.*, hydrogen peroxide.

Amyloid Causes Membrane Oxidation and Inactivates Ion-Motive ATPases

Synthetic Aβ applied to cortical neurons immunolocalizes to the plasma membrane, which undergoes blebbing as the cells die.[36] These microscopic observations further suggest the membrane as a possible target of amyloid action. As a means of detecting membrane oxidation products, we modified the established dinitrophenyl hydrazide (DNPH) assay for protein carbonyls by coupling 4-aminobenzoic hydrazide to biotin.[24] This probe covalently labels protein carbonyls, and provides a substrate for attachment of fluorescein-conjugated streptavidin. Tight binding of streptavidin

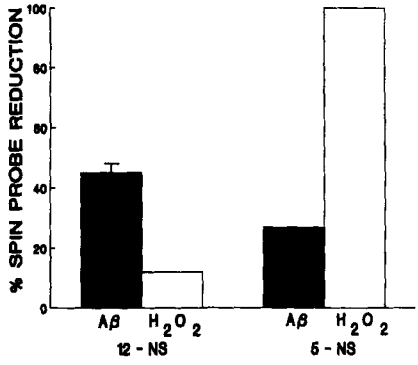

FIGURE 3. Relative effects of synthetic Aβ(25–35) and H_2O_2 on 5-NS and 12-NS spin probes localized to gerbil synaptosomal membrane bilayers.

to biotin results in localization of the fluorescent probe to oxidatively lesioned proteins. As expected, coincubation of cells with 20 μM Aβ(1-40) caused a 3-fold increase in membrane protein carbonyls as measured with biotin-4-aminobenzoic hydrazide staining and confocal microscopic analysis.[24]

In further support of the contention that amyloid reacts aggressively with plasma membrane components, we have shown that amyloid can reduce spin probes localized within synaptosomal plasma membranes,[22] indicating a possible role for Aβ as a lipoperoxidant.[23] Synthetic Aβ is readily able to reduce the paramagnetism of spin probe 12-nitroxyl (doxyl) stearic acid, when the probe is localized to rodent synaptosomal membranes (FIG. 3).[22] Contrastingly, the peptide is less capable of reducing the paramagnetism of the 5-NS isomer (FIG. 3).[22] Hydrogen peroxide also reduces these spin probes; however, H_2O_2 is much more reactive toward 5-NS than 12-NS (FIG. 3). The 5- and 12-NS isomers differ in the location of the nitroxide moiety. The 12-NS nitroxide is located near the midpoint of the stearic acid chain, and consequently probes plasma membranes deep with the hydrophobic interior. 5-NS contains a nitroxide near the acid group, and consequently probes the lipid/water interface.

The selective reduction of the 12-NS signal by amyloid was unexpected. It is interesting to speculate that the 12-NS reactivity of Aβ(25-35) may be due to free radical production deep inside the lipid bilayer of the plasma membrane, where the portion of APP normally resides (FIG. 1). Thus, the hydrophobic segment of Aβ(25-35) may direct the peptide to reinsert into the plasma membrane, where the potentially redox active methionine 35 could facilitate ROS production.

It is plausible that such membrane protein oxidation can lead to inactivation of membrane enzymes. Since intracellular Ca^{2+} levels increase following Aβ administration, we investigated the activity of several ion-motive ATPases in cultured neurons exposed to amyloid.[26] Neurons exposed to 20-50 μM Aβ(25-35) or Aβ(1-40) rapidly lose Na^+/K^+ ATPase (less than 50% activity remaining after 3 hours).[26] In these same experiments, amyloid did not affect the Na^+/Ca^{2+} exchanger and significantly reduced Mg^{2+} ATPase only after 10 hours exposure at 50 μM peptide concentration.[26] Impairment of the Na^+/K^+ ATPase is sufficient to elevate intracellular Ca^{2+} levels, as demonstrated by exposing neurons to 200 μM ouabain (a specific blocker of the Na^+/K^+ ATPase) after loading with the fluorescent Ca^{2+} indicator fura-2.[26] As further indication

that Ca^{2+} influx occurs secondarily to Na^+ influx, we observed that Aβ-mediated Ca^{2+} influx was significantly reduced and cell survival was significantly increased when cells were exposed to the peptide in Na^+-deficient medium or in the presence of 1 μM tetrodotoxin (a specific blocker of voltage-dependent Na^+ channels). Amyloid-mediated impairment of the Na^+/K^+ ATPase, Ca^{2+} influx, and cell death were all abrogated by cotreating cell cultures with vitamin E, propyl gallate, or PBN (discussed below).[26]

Human neuronal Na^+/K^+ ATPase and Ca^{2+} ATPase are also impaired by synthetic amyloid. Human hippocampal synaptosomes prepared from brain tissue and subsequently exposed to 50 μM Aβ(25-35) for 1 hour demonstrate 70% and 30% loss of Na^+/K^+ ATPase and Ca^{2+} ATPase, respectively.[26] Similar effects were observed in synaptosomes treated with Fe^{2+} instead of amyloid.[26]

Amyloid-Stimulated Oxyradical Production Inhibits Na^+-Dependent Glutamate Uptake and Sensitizes Cells to Subsequent Excitotoxic Insult

Amyloid peptides, when administered to mixed astrocytic/neuronal cell culture in low doses, predispose the cells to subsequent glutamate-induced excitotoxic trauma. Astrocytes located in the perisynaptic space provide the major mechanism for removal of glutamate following synaptic transmission; the primary driving force for glutamate absorption is the action of the astrocytic Na^+-dependent glutamate transport system. Volterra and co-workers recently demonstrated that Na^+-dependent glutamate uptake is sensitive to oxyradical stress caused by Fe^{2+}/H_2O_2.[48] Once inside the astrocyte, glutamate is converted to glutamine by GS, so that in effect, the astrocytic glutamate uptake system represents the coupling of two distinct oxidatively sensitive biochemistries. Given the apparent role of amyloid-derived oxyradical stress in disruption of membrane ionic control mechanisms, we hypothesized that similar oxidative impairment to the astrocytic glutamate uptake system by amyloid-induced ROS might explain the synergism between Aβ and excitotoxic amino acids.

Experimental treatment of mixed neuronal/astrocytic hippocampal cultures with 100 μM Aβ(25-35) or to 1 μM Fe^{2+}/100 μM H_2O_2 for 1 hour both significantly diminished Na^+-dependent glutamate uptake and glutamine synthetase activity.[25,49] Treatment with ouabain confirmed that this decrement in glutamate uptake was not simply an effect of Na^+/K^+ ATPase inhibition.[25,49] Concomitant with both amyloid and H_2O_2 treatments, we observed significant elevations in intraastrocytic ROS according to DCF fluorescence and a 3-fold increase in protein carbonyl according to the biotin hydrazide staining protocol.[49] The deleterious effects of both Aβ and H_2O_2 on glutamate uptake were prohibited by pretreatment of cultures with 100 μM of the antioxidant trolox, or by temporarily blocking the glutamate transporter with the specific antagonist pyrollidine-2,4-dicarboxylate (PDC) during amyloid coincubation, followed by washout.[49]

Modulation of Amyloid Peptide Toxicity by Antioxidants

There are two reasons to include antioxidants as experimental variables in amyloid studies. One reason is to differentiate oxyradical-mediated effects from nonoxidative

effects; another reason is to gauge the potential therapeutic benefit of various antioxidants. Several studies have now shown that antioxidant treatments partially abrogate the toxic effects of amyloid in cell-free incubates, in cell culture and in brain homogenates.

The inclusion of PBN in coincubates of gerbil brain cytosolic extract and Aβ(1-40) partially protects GS from peptide-mediated inactivation (mean % change in GS activity in a coincubate consisting of 0.5 mg/ml brain cytosol extract and 1 mg/ml Aβ(1-40) after 3 hours in saline, pH 7.4, at 37°C = –67% without PBN (n = 3) and +5% in the presence of 50 mM PBN (n = 2), relative to controls incubated without Aβ or PBN; no effect was observed from PBN alone). The iron chelator deferoxamine and the nonspecific metal chelating resin chelex-100 do not protect GS against amyloid-stimulated inactivation (concentrations of deferoxamine were limited to <100 μM in these experiments, however, as higher concentrations interfere with the assay). Similarly, PBN was found to protect gerbil brain homogenate against Aβ(25-35)-induced GS inactivation (mean % change with Aβ, without PBN = –48% (n = 3), with 50 mM PBN = –30% (n = 1), with deferoxamine = –70% (n = 1); samples incubated 24 hours at 37°C in saline, pH 7.4; there was no observable effect of PBN or deferoxamine alone).

Behl and co-workers have compared the relative efficacies of numerous antioxidants in protecting PC12 cells against amyloid toxicity, and report significant protection by 5-200 μM concentrations of the organic antioxidants PBN, propyl gallate, vitamin E, mercaptoethanol, acetylcamitine, and ascorbate.[30] These researchers also report that the enzymatic antioxidant catalase and the flavin oxidase inhibitor diphenylene iodonium (DPI) protect PC12 cells from amyloid-mediated death.[30] Meanwhile, Tomiyama and colleagues report that low doses (10 μM) of the tuberculosis drug and potential antioxidant rifampicin protects PC12 cells against Aβ toxocity while inhibiting amyloid aggregation and fibril formation *in vitro*.[33]

Experiments conducted in our lab have confirmed the ability of PBN, propyl gallate, and vitamin E to protect cultured rodent hippocampal neurons against amyloid toxicity.[26] Concentrations of 50 μM PBN, 50 μg/ml vitamin E, or 5 μM propyl gallate significantly protect cultured neurons against Aβ-stimulated Na^+/K^+ ATPase impairment, Ca^{2+} influx, and cell death.[26] Propyl gallate significantly attenuates Aβ-induced elevation of biotin-hydrazide reactive membrane protein carbonyls.[24] In addition to these chain-breaking antioxidants, the lipoxygenase inhibitor nordihydroguaritic acid was found to significantly attenuate amyloid-stimulated neuronal calcium influx and cytotoxicity,[28] indicating a possible contribution of enzymatic oxidation to amyloid-stimulated ROS generation. The list of antioxidants which protect hippocampal neurons against both Aβ stress and H_2O_2 treatment also includes the bacterial alkaloids staurosporine and K-252 (protective at concentrations of 5-100 picomolar),[29] as well as the secreted N-terminal portion of APP (APPs).[27] It is unlikely that all these compounds operate simply as free radical scavengers and chain-breaking antioxidants; in no case is the exact mechanism of antioxidant activity positively known.

Some attention has recently fallen on the lazeroid class of antioxidant compounds as potential therapeutics for pathologies thought to have an oxidative component. These drugs have been observed to specifically diminish glutamate-mediated toxicity, perhaps by scavenging oxyradicals produced during excitotoxic stimulation. Accord-

ingly, Kumar has reported that a 10-μM dose of the lazeroid U-78517F was efficacious in protecting hippocampal neurons against Aβ.[34]

As discussed above, amyloid-stimulated ROS stress on perisynaptic astrocytes may exacerbate glutamate excitotoxicity by damaging components of the astrocytic glutamate uptake system. Certain antioxidants, particularly the vitamin E analog trolox, are effective at protecting astrocytes and particularly the astrocytic Na^+-dependent glutamate transport system against the damaging effects of both amyloid and Fe^{2+}/H_2O_2.[49] The effective concentration of trolox in these studies was 100 μM.

Taken together, these results indicate that antioxidant therapy may be a promising approach to treatment of Alzheimer's disease.

Evidence for Oxidative Damage to the Alzheimer's Diseases Brain: The Senile Plaque as a Microenvironment of Oxidative Stress

Despite growing evidence that the *in vitro* behavior of synthetic amyloid peptides is, at least partially, mediated by oxyradicals, questions remain regarding the significance of oxidative trauma to Alzheimer's disease pathology. Previous studies have indicted an elevation of protein oxidation products in AD, and recent publications have found evidence of an oxidative challenge in the AD brain.[3-7] We have undertaken a study to extend upon these findings by examining AD and age-matched control brain tissue for biomarkers of protein oxidation similar to those documented to be sensitive to amyloid treatment *in vitro*.[2]

We assayed protein carbonyl content, MAL-6 spin labeled synaptosomal W/S ratio, and cytosolic GS and CK activities of AD and age-matched control subjects. For each of these endpoints we compared tissue taken from the cerebellum, which is virtually spared from accumulation of senile plaques and other histopathological correlates of AD, with tissue taken from the hippocampus and inferior parietal lobule, which are severely affected in AD.[2] Protein carbonyl level was elevated in AD subjects relative to control.[2] Carbonyl levels in the AD hippocampus and inferior parietal lobule were elevated 25% and 37% above cerebellar levels, respectively, whereas in control brains cortical and cerebellar carbonyl levels were comparable.[2]

GS and CK activities were significantly lowered in AD relative to age-matched controls, though regional correlations were less well defined than was the case with carbonyl content.[2] The EPR spin labeling experiments revealed no significant difference between W/S ratio of MAL-6 labeled cerebellar synaptosomes taken from control and AD subjects. The W/S ratios of inferior parietal and hippocampal synaptosomes taken from AD subjects, however, were decreased approximately 40% relative to age matched controls.[2] We also observed a striking regional effect, with hippocampal W/S ratio being less than that of the inferior parietal lobule and both less than the cerebellum.[2]

Unfortunately, fluorescence techniques for quantifying oxidation and ROS are limited with their utility as a probe of human autopsy brain tissue due to fluorescence quenching and the endogenous presence of fluorescent components (*e.g.*, lipofuscin).

The regional and disease-related differences in W/S ratio, GS and CK activities, and protein carbonyl content correspond reasonably well with senile plaque counts and neurofibrillary tangle distribution with the AD brain. Unfortunately, it is impossible to

positively separate regional amyloid burden from other factors. For instance, it has been suggested that reactive microglia are recruited to nascent senile plaques, where they may become activated to generate superoxide radical. A further complication with the involvement of microglia is the apparent ability of Aβ to activate previously quiescent glia.[16] We found some indication of elevated HLA-DR immunoreactive microglia in the AD hippocampus and inferior parietal regions, which could synergistically modify amyloid-mediated oxidative processes in unforetold ways.[2]

The conclusion from these autopsy studies is that regions of the Alzheimer's brain that are rich in amyloid possess a constellation of biochemical and biophysical alterations similar to those observed with *in vitro* models for amyloid-stimulated or metal-catalyzed oxyradical insult.

Conclusions: Towards an Integrated Model of Free Radical Involvement in the Progression of Alzheimer's Disease Neuropathology

Increasing evidence implies that AD is caused or exacerbated by oxidative stress. Based on the data outlined in this review, a plausible (if speculative) scenario can be constructed to rationalize the role of oxyradicals in AD pathology.

In this scenario, events leading to a marginal increase in brain steady-state ROS levels or a decrease in competency of antioxidant systems facilitates damage to membrane lipids and enzymes, intracellular and extracellular matrix proteases, and nuclear components. Consequent marginal perturbations of second messenger systems and inefficiencies in protein synthesis or turnover contribute further to metabolic compromise and render cells more susceptible to oxidation. Eventually, physiological perturbations effect the amyloid processing pathway. Abnormal proteolysis releases toxic Aβ into the extracellular space, where it aggregates and may react with redox-catalytic metals, protein glycation products, and other biomolecules. Amyloid is activated to an ROS-inducing form, which interacts with sensitized neuronal or astrocytic membranes, engendering further oxyradical production. Recruitment of microglia to the nascent senile plaque followed by microglial activation superoxide production may further accelerate oxidative tissue degradation. Oxidative damage to glutamine synthetase or other components of the astrocytic glutamate uptake system render cells vulnerable to excitotoxicity and glutamate-enhanced oxyradical stress. The net result of a lifetime exposure to oxyradicals becomes a vicious cycle of neural degeneration, tissue oxidation, and amyloidogenesis.

Confirmation of various aspects of this model require more detailed study of basic amyloid chemistry in addition to basic research into the mechanisms of brain oxidation. Data gained from recently engineered mouse models of Alzheimer's disease may facilitate understanding of these processes, and lead the way to development of therapeutic compounds to slow the clinical progression of Alzheimer's disease.

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