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The Aβ Peptide of Alzheimer's Disease Directly Produces Hydrogen Peroxide through Metal Ion Reduction†

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ABSTRACT: Oxidative stress markers characterize the neuropathology both of Alzheimer's disease and of amyloid-bearing transgenic mice. The neurotoxicity of amyloid Aβ peptides has been linked to peroxide generation in cell cultures by an unknown mechanism. We now show that human Aβ directly produces hydrogen peroxide (H_2O_2) by a mechanism that involves the reduction of metal ions, Fe(III) or Cu(II), setting up conditions for Fenton-type chemistry. Spectrophotometric experiments establish that the Aβ peptide reduces Fe(III) and Cu(II) to Fe(II) and Cu(I), respectively. Spectrochemical techniques are used to show that molecular oxygen is then trapped by Aβ and reduced to H_2O_2 in a reaction that is driven by substoichiometric amounts of Fe(II) or Cu(I). In the presence of Cu(II) or Fe(III), Aβ produces a positive thiobarbituric-reactive substance (TBARS) assay, compatible with the generation of the hydroxyl radical (OH·). The amounts of both reduced metal and TBARS reactivity are greatest when generated by Aβ1−42 ≫ Aβ1−40 > rat Aβ1−40, a chemical relationship that correlates with the participation of the native peptides in amyloid pathology. These findings indicate that the accumulation of Aβ could be a direct source of oxidative stress in Alzheimer's disease.

Increasing evidence has implicated oxidative stress in the pathogenesis of Alzheimer's disease (AD). Metabolic signs of oxidative stress in AD-affected neocortex include increased glucose-6-phosphate dehydrogenase activity (*1*) and increased heme oxygenase-1 levels (*2*). Signs of oxygen radical-mediated chemical attack include increased free protein carbonyls (*3*−*5*), lipid peroxidation adducts (*6*, *7*), protein nitration (*8*), and mitochondrial and nuclear DNA oxidation adducts (*9*). Prevention of such oxidation injury may be the basis for treatment with the antioxidant vitamin E, reported to delay the progression of clinical AD (*10*).

Aβ is a 39−43 amino acid mixture of polypeptides that characteristically collects in the cortical interstitium and cerebrovasculature in AD. Aβ1−40 is the major soluble Aβ species in biological fluids (*11*), and Aβ1−42 is a minor soluble species, but is more fibrillogenic, and is enriched in

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interstitial plaque amyloid (*12*). Familial AD-linked mutations of APP, presenilin-1 and presenilin-2, correlate with increased amyloid burden and induce an increase either in the total concentrations of all Aβ forms or in the relative concentration of Aβ1−42 (*13*, *14*). However, the mechanism by which Aβ1−42 exerts more neurotoxicity than Aβ1−40 (*15*) remains unclear. An important clue to the biochemistry of Aβ neurotoxicity comes from the observation that amyloid deposits are scarce in the brains of aged rats and mice (*16*). The absence of Aβ deposition in these animals may be related to undetermined properties of these species' homologue of Aβ ("rat Aβ") conferred by its three amino acid substitutions (R_5→G, Y_{10}→F, and H_{13}→R) (*17*), since the solubility of rat Aβ is not greater than the human peptide (*18*, *19*).

A relationship exists between signs of oxidative stress and Aβ cortical deposits. The brain regional variation of oxidation biomarkers indicates that Aβ amyloid plaques represent environments of elevated oxidative stress (*4*), and transgenic mice expressing the cerebral amyloid phenotype exhibit cerebral oxidation markers that match the human pathology (*20*). Synthetic Aβ peptides exert toxicity correlating with the generation of cellular hydrogen peroxide (H_2O_2) (*21*), and abolished by SOD (*22*), O_2^-/H_2O_2 scavengers (*23*), and vitamin E (*24*). Aβ1−40 has also been reported to generate the hydroxyl radical by mechanisms that are unclear (*25*).

We have characterized Aβ as a metalloprotein that binds Zn(II) (*19*, *26*, *27*), Cu(II), and Fe(III) (*28*, *29*). A histidine-mediated zinc binding site was mapped to a contiguous sequence between positions 6 and 28 of the Aβ sequence (*26*). Rat Aβ, which has two amino acid substitutions in this region, binds zinc less avidly and, unlike the human peptide, is not precipitated by Zn(II) or Cu(II) at concentrations ≤25 μM (*19*, *29*).

The brain levels of Zn, Cu, and Fe, and their binding proteins, are dysregulated in AD (*30*−*32*). Zn, Cu, and Fe are concentrated in the neocortex, but are highly enriched (Cu ≈ 400 μM, Zn and Fe ≈ 1 mM) in cerebral amyloid deposits in AD (*33*, *34*). Enrichment of Cu and Fe in Aβ collections may not only serve to assemble the Aβ deposits, but since these metal ions are redox-active, we hypothesized that interactions of Aβ with Fe and Cu may contribute to the oxidation insults that are observed in the AD-affected brain.

We have recently discovered that Aβ possesses a strongly positive formal reduction potential, rapidly reduces Cu(II), and then traps molecular oxygen to generate H_2O_2 (*35*, *36*). These effects are greatest for Aβ1−42 > Aβ1−40 ≫ rat Aβ1−40, a chemical relationship that correlates with the participation of these peptides in amyloid pathology (Huang et al., submitted). We now report that Aβ is also able to reduce Fe(III) and generate H_2O_2, although to a lesser extent. We also report that Aβ−Fe(III)/Cu(II) interaction generates positive thiobarbituric acid-reactive substance (TBARS)[1] assays which may indicate the presence of the hydroxyl radical (OH•) being generated by the reaction of reduced metals with H_2O_2. Together, these data indicate that high concentrations of Aβ may directly contribute to oxidative stress in the brain in AD.

MATERIALS AND METHODS

Reagents. Aβ peptides 1−40 and 1−42 were synthesized by the W. Keck Laboratory, Yale University, New Haven, CT. Confirmatory data were obtained by reproducing experiments with Aβ peptides synthesized and obtained from other sources: Multhaup Laboratory, University of Heidelberg; U. S. Peptides, Bachem (Torrance, CA); and Sigma. Rat Aβ1−40 was synthesized and purified by the Multhaup Laboratory. Aβ1−28 and Aβ25−35 were purchased from U. S. Peptides, Bachem, and Sigma. Aβ40−1 was purchased from Bachem, and also synthesized by the Multhaup Laboratory (giving corroborating results). Aβ peptide stock solutions were prepared on the day of the experiment. Lyophilized peptide was first solubilized in Chelex-100 resin (BioRad, CA) treated water to 200 μM and then indirectly sonicated for 3 min (30 s on, 10 s off) through a water bath to avoid frothing. The peptide preparation was then filtered through a water-washed Spin-X cellulose acetate filter unit (0.22 μm; Corning Costar Corp.). Concentrations of Aβ were determined by MicroBCA assay (Pierce), which we have previously validated as an assay for Aβ concentration. (*27*). Other reagents are from Sigma, St Louis, MO, unless otherwise mentioned. To prevent formation of metal−hydroxy and metal−oxy polyhydrates in neutral metal ion solutions due to their hydrolyses, metal stock solutions [Fe(III)-citrate, Cu(II)-glycine, or Zn(II)-histidine] of 1 mM used in the experiments were prepared by mixing National Institute of Standards and Technology (NIST) standards with ligands at a metal:ligand molar ratio of 1:6.

Metal Reduction Assays. Assays were performed using a 96-well microtiter plate (Costar, MA), based upon a modification of established protocols (*37*−*39*). Polypeptides (10 μM) or vitamin C (10 μM); Fe(III), Cu(II), or Zn(II) (25 μM); and reduced metal ion indicators (250 μM) [Fe(II): BP or PDT; Cu(I): BC or BCA] were coincubated in Dulbecco's phosphate buffered saline (PBS: $CaCl_2$ 1.19 mM, $MgCl_2$ 0.6 mM, KCl 2.7 mM, KH_2PO_4 1.4 mM, NaCl 137 mM, Na_2HPO_4 7.68 mM, pH 7.4), at 37 °C. Absorbances were then measured using a plate reader (SPECTRAmax Plus, Molecular Devices, CA). In control samples, both metal ion and indicator were present to determine the background buffer signal. The absorbances of metal ion and peptide present in the absence of indicator were taken to estimate the contribution of light scattering due to turbidity. The net absorbances (ΔA) were obtained by deducting the absorbances from these controls from the absorbances generated by the peptide and metal in the presence of the indicator. Fe(II) or Cu(I) concentrations (μM) were calculated as: $\Delta A \times 10^6/ML$, where M is the known molar absorption coefficient (M^{-1} cm^{-1}) and L = the vertical path length which is corrected automatically by the platereader to 1 cm. For Fe(II)-BP, M = 22 140 at 535 nm; for Fe(II)-PDT, M = 27 900 at 562 nm; for Cu(I)-BC, M = 12 250 at 483 nm; and for Cu(I)-BCA, M = 7700 at 562 nm.

Hydrogen Peroxide Assays. The colorimetric H_2O_2 assay was performed in a 96-well microtiter plate (SpectraMax

[1] Abbreviations: BC, bathocuproinedisulfonic acid; BCA, bicinchoninic acid; BP, bathophenanthrolinedisulfonic acid; DCF, 2′,7′-dichlorofluorescin diacetate; DFO, desferrioxamine; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTB, 2-nitro-5-thiobenzoate; BSA, bovine serum albumin; PBS, Dulbecco's phosphate-buffered saline; PDT, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine; TBARS, thiobarbituric acid-reactive substance; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TETA, triethylenetetramine dihydrochloride.

Plus, Molecular Devices, CA) according to a modification of an existing protocol (40). Polypeptides (10 μM) or vitamin C (10 μM); Fe(III) or Cu(II) (1 μM); and a H_2O_2 trapping agent, TCEP (Pierce, 50 μM), were coincubated in PBS buffer (300 μL), pH 7.4, for 1 h at 37 °C. Following incubation, the excess TCEP was allowed to react with DTNB (50 μM) stoichiometrically and was measured by monitoring their reaction product: NTB, which has a strong absorption at 412 nm. The amount of H_2O_2 produced was quantified based on the formula: H_2O_2 (μM) = $\Delta A \times 10^6/2LM$, where ΔA is the absolute absorbance difference between a sample and catalase-only (100 units/mL) control at 412 nm and M is the molecular absorbance for NTB (14 150 M^{-1} cm^{-1} at 412 nm).

For the fluorometric DCF assay, 5 mM 2′,7′-dichlorofluorescin diacetate (DCF-DA, Molecular Probes) in 100% ethanol was de-acetated by 0.01 M NaOH for 0.5 h, 200 units/mL horseradish peroxidase was then added, and the DCF solution was neutralized and diluted to 200 μM by PBS before use. Then 20 μM DCF, 10 μM Aβ1−42, and 1 μM Fe(III) were coincubated at 37 °C for 20 min in PBS. Catalase (1000 units/mL) with or without heat inactivation (100 °C for 30 min) was used to validate the signal. The fluorescent readings were recorded by a Packard 96-well fluorocounter (485 nm excitation; 530 nm emission).

Where the O_2 tension of the buffers was manipulated, the buffer vehicle was continuously bubbled for 2 h at 20 °C with 100% O_2 to create conditions of increased O_2 tension, or purged with argon (Ar) to create anaerobic conditions, prior to the addition of vitamin C or polypeptides.

Thiobarbituric Acid-Reactive Substance Assay. The thiobarbituric acid-reactive substance (TBARS) assay for incubation mixtures with Fe(III)-citrate or Cu(II)-glycine was performed in a 96-well microtiter format modified from established protocols (*41*). Vitamin C was used as a positive control for the generation of OH· by Fenton chemistry (*42*). Aβ peptide species (10 μM) or vitamin C (10 μM) was incubated with Fe(III) or Cu(II) (1 μM) and deoxyribose (0.9375 mM, Sigma) in PBS, pH 7.4 (500 μL). Following incubation (37 °C, 1 h), trichloroacetic acid (250 μL × 17 M in double-distilled H_2O) and 2-thiobarbituric acid (250 μL × 1%, w/v, in 0.05 M NaOH) were added, and the sample was heated (100 °C, 10 min). The final mixtures were placed on ice for 1−3 min before the sample was distributed in 3 × 300 μL samples and absorbances at 532 nm were measured and averaged. The net absorbance change for each sample was obtained by deducting the mean absorbance from a control sample consisting of identical chemical components except for the vitamin C or Aβ peptides. To determine the effects of OH· scavengers on the generation of TBARS reactivity, mannitol (5 mM) or dimethyl sulfoxide (DMSO, 5 mM) was coincubated with vitamin C/peroxide (10 μM + 500 μM H_2O_2) or Aβ1−42/Fe(III) (10 μM + 1 μM metal ion).

RESULTS

Human Aβ, but Not Rat Aβ, Reduces Copper and Iron. We first measured the ability of Aβ peptides to reduce Fe(III) to Fe(II), as compared to vitamin C (as a positive control) and other polypeptides (Figure 1A). Vitamin C reduced Fe(III) with 36% efficiency during the 1 h incubation period. Aβ1−42 was the only peptide tested that induced

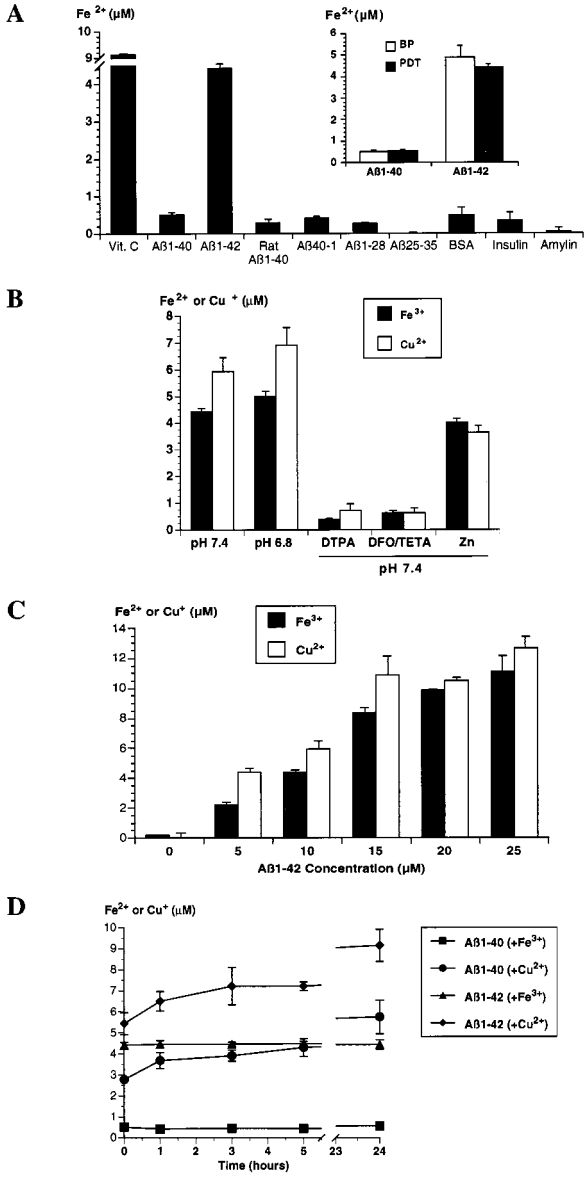

FIGURE 1: Fe(III) or Cu(II) reduction by Aβ peptides. Aβ and control peptides were incubated with Fe(III) or Cu(II), and the concentration of reduced metal ions generated was measured by PDT [for Fe(II)] and BCA [for Cu(I)] unless otherwise mentioned. Data points for panels A−D are means ± SD, $n = 3$ replicate wells, from a single microplate, and are typical of three experiments. (A) Reducing capacity of Aβ species, compared to vitamin C, insulin, and human amylin (Bachem, CA) (all 10 μM), toward Fe(III)-citrate (25 μM) in PBS, pH 7.4, after 1 h coincubation, 37 °C. Inset: Fe(II) assay validation. The amounts of Fe(II) generated by Aβ1−40 and Aβ1−42, under the same conditions, were compared by two different Fe(II) detection assays, BP with PDT. (B) Effect of pH, chelation, and Zn(II) upon Aβ1−42 metal reduction: comparison of Fe(II) to Cu(I) generation. Data show the concentrations of Fe(II) and Cu(I) generated by incubation of Aβ1−42 (10 μM) with Fe(III)-citrate or Cu(II)-glycine (25 μM). The reaction was performed in PBS, pH 7.4, except the pH was adjusted to 6.8, and additional reagents were coincubated: DTPA (200 μM), DFO (200 μM in the presence of Fe), TETA (200 μM in the presence of Cu), or Zn(II) (25 μM), as indicated. (C) Relationship between Aβ1−42 concentration and amount of metal ion reduction. Incubation conditions and assays were as in panel B, except that the amount of reduced metal ion was titrated against the peptide concentrations indicated. (D) Time dependence of Aβ1−42-mediated metal ion reduction. Incubation conditions and assay were as in panel B, but readings were taken at the time intervals indicated.

marked reduction of Fe(III) (18%) in the incubation period, and significantly more than Aβ1−40 (2%). Rat Aβ1−40, Aβ40−1, Aβ1−28, Aβ25−35, BSA, insulin, and amylin each produced significantly ($p < 0.05$, two-tailed *t*-test) less Fe(II) than human Aβ1−40. In a specific comparison of the native peptides, human Aβ1−40 produced 0.51 ± 0.06 μM Fe(II), whereas rat Aβ1−40 produced significantly less Fe(II) (0.30 ± 0.08 μM, $p < 0.001$). We corroborated the relative iron-reducing activities of Aβ1−42 and Aβ1−40 by comparing results obtained using two different assays (BP and PDT) to estimate the Fe(II) generated, and found that the results were in excellent agreement (Figure 1A, insert). The same peptides reduce comparable amounts of Cu(II) (*35*, *36*), except that the corresponding amounts of Cu(I) generated (24% for Aβ1−42; 12% for Aβ1−40) are higher than Fe(II) generated under the same experimental conditions, but the other peptides tested (rat Aβ1−40, Aβ40−1, Aβ1−28, Aβ25−35, BSA, insulin, and amylin) also did not reduce Cu(II) (Huang et al., manuscript submitted).

We next tested the nature of the redox-active binding site on Aβ1−42 by examining the effects of pH, complexation of metal ions with chelators, and competition with Zn(II) (Figure 1B). Precipitation of Aβ by Cu(II) and Fe(III) is dramatically exaggerated at pH 6.8 compared to pH 7.4 (*29*); however, compared to pH 7.4, incubation at pH 6.8 only slightly increased the amounts of Cu(I) and Fe(II) generated. This finding suggests that metal-induced aggregation of Aβ and metal reduction by Aβ are not chemically linked reactions at pH 6.8. Since incubation at pH 6.8 promotes precipitation of Aβ in the presence of Fe(III) or Cu(II) (*29*) but did not inhibit metal reduction, the peptide appears to reduce Fe and Cu even when aggregated.

Chelators with high affinity for Fe(III) (DTPA, DFO) and Cu(II) (DTPA, TETA) inhibited the reduction of the respective metal ions (Figure 1B), indicating that specific coordination of the metal ion with an active site on the peptide is required for electron transfer to metals, and subsequently to oxygen to form peroxide (Figure 2D). This coordination site appears to be selective for Cu(II) and Fe(III) since the presence of Zn(II), at equimolar concentrations, does not decrease the amount of Fe(II) generation and only partially attenuates the reduction of Cu(II). Hence, despite possessing two binding sites on Aβ that may be occupied in this concentration range (*26*), Zn(II) does not appear to compete efficiently for the redox-active Cu/Fe interaction sites on Aβ.

To characterize the reaction further, we titrated the amount of Fe(II) or Cu(I) generated from 25 μM Fe(III) or Cu(II) against Aβ1−42 concentration (Figure 1C). We found a linear relationship where the amount of reduced metal ion was consistently half the concentration of peptide. Since Aβ exists in solution as a dimer (*27*), it may be possible that one electron is donated from each dimeric subunit:

$$(A\beta)_2 + M^{(n+1)+} \rightarrow A\beta{:}A\beta^{+\bullet} + M^{n+} \qquad (1)$$

In this case, electron donation from Aβ creates a putative radical (Aβ:A$\beta^{+\bullet}$) in a metal ion dependent manner. In the absence of the detection reagents used to assay the reduced metal ions, the metal ion and Aβ may be in equilibrium between oxidized and reduced states. The basis for electron donation from only 50% of the available peptide, even in the presence of up to 5-fold excess metal ion, is not clear.

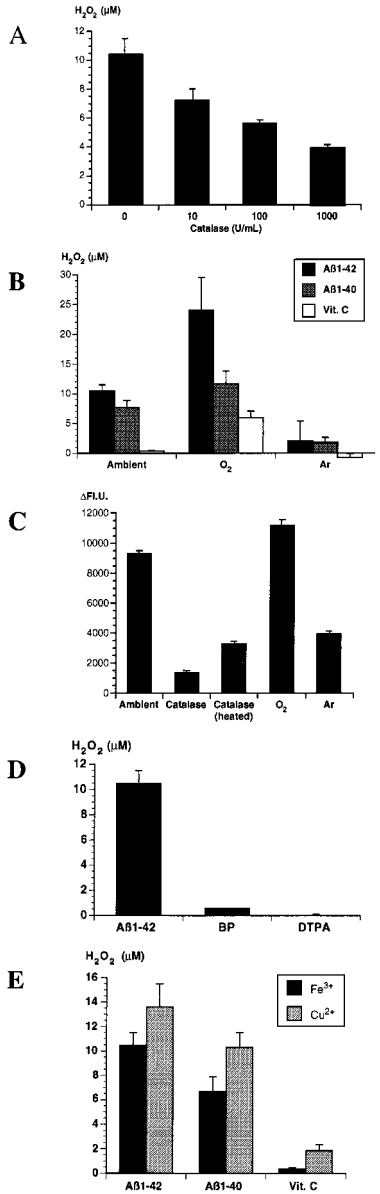

FIGURE 2: Production of H_2O_2 from the incubation of Aβ in the presence of substoichiometric amounts of Fe(III) or Cu(II). All data points are means ± SD, $n = 3$ replicate wells, from a single microplate, and are typical of three experiments. (A) H_2O_2 produced by Aβ1−42 (in PBS, pH 7.4, under ambient gas conditions, 1 h, 37 °C) following coincubation with various concentrations of catalase in the presence of 1 μM Fe(III). (B) H_2O_2 produced by Aβ1−42, Aβ1−40, and vitamin C in the presence of Fe(III) (1 μM) (in PBS, pH 7.4, buffer, 1 h, 37 °C) under various buffer gas conditions. "Ambient" = no efforts were made to adjust the gas tension in the bench preparations of the buffer vehicle; "O_2" = 100% O_2 was continuously bubbled through the PBS vehicle for 2 h (at 20 °C), before the remainder of the incubation components was added; "Ar" = 100% Ar was continuously bubbled through the PBS vehicle for 2 h (at 20 °C), before the remainder of the incubation components was added. (C) Corroboration of H_2O_2 generated by Aβ1−42 using DCF assay. H_2O_2 signal generated by Aβ1−42 (10 μM) in the presence of Fe(III) (1 μM) (in PBS, pH 7.4, buffer, 1 h, 37 °C) under various dissolved gas conditions (as in Figure 2B). The effect of catalase (1000 units/mL), with or without heat-inactivation, to abolish the H_2O_2 signal was used to validate the signal. (D) Metal ion dependency of H_2O_2 production by Aβ1−42. The effects of chelators (200 μM) upon the Aβ1−42-mediated generation of H_2O_2 in the presence of Fe(III) (1 μM) were measured by the TCEP assay (vehicle conditions as in Figure 2A). (E) Comparison of H_2O_2 production by Aβ1−42, Aβ1−40, and vitamin C (conditions as in Figure 2A) in the presence of either 1 μM Fe(III) or 1 μM Cu(II) as indicated.

Perhaps after donating the first electron, there is a conformational change in the peptide that inhibits further electron transfer. Or perhaps each dimeric Aβ unit can coordinate only one Fe(III) in a redox-competent manner.

The rates of Fe(III) reduction by Aβ1−40 and Aβ1−42 differ from the rates of Cu(II) reduction (Figure 1D). The reduction of Fe(III) was completed instantaneously with no further reduction by either peptide over 24 h. However, the reduction of Cu(II) had two phases with similar rates for both peptides: an instantaneous phase, followed by the slow doubling of the concentration of Cu(I) over 24 h. Under these conditions, the amount of Cu(I) equaled the amount of Aβ1−42 present, suggesting that the Aβ1−42 eventually reacted with Cu(II) at a 1:1 ratio. The putative second step reaction may be

$$A\beta{:}A\beta^{+\bullet} + Cu^{2+} \rightarrow A\beta{-}A\beta + Cu^{+} \qquad (2)$$

where Aβ−Aβ is a covalently modified, SDS-resistant Aβ dimer, which we have found is produced over 24 h upon incubation with Cu(II) but not Fe(III) at these concentrations (*43*), in agreement with the slow-phase production of Cu(I) described in Figure 1D and reaction 2. Since reaction 2 continues over 24 h, Aβ:A$\beta^{+\bullet}$ might be a stable radical. Aβ is negatively charged at neutral pH (pI = 5.5); therefore, the donation of one electron (generating Aβ:A$\beta^{+\bullet}$) is unlikely to create electrostatic conditions that obviate further metal ion interactions.

Human Aβ, but Not Rat Aβ, Directly Produces Hydrogen Peroxide. We tested Aβ and control peptides for the direct production of H_2O_2 since the reduced metal ions produced by Aβ could react with molecular oxygen (O_2) to generate O_2^- or O_2^{2-} (*44*). We detected the generation of ≈10 μM H_2O_2 by Aβ1−42 in the presence of 1 μM Fe(III) under ambient O_2 conditions (Figure 2A). Validation of the assay was achieved by coincubating Aβ with catalase, which abolished the H_2O_2 signal in a dose-dependent manner.

To determine whether the formation of H_2O_2 by Aβ is due to the reduction of O_2, we studied the generation of H_2O_2 by Aβ1−42, Aβ1−40, and vitamin C under different O_2 tensions in the presence of 1 μM Fe(III) (Figure 2B), using a colorimetric assay. The presence of vitamin C was used as a control measure to estimate the maximum amount of H_2O_2 that could be detected in the buffer vehicle by the nonprotein generation of reduced metals (*42*). There was a significant increase in the amount of H_2O_2 produced under higher O_2 tensions. The presence of either Aβ1−42 or Aβ1−40 generated significantly more H_2O_2 (Aβ1−42 > Aβ1−40) than vitamin C under any O_2 tension studied. Under ambient and argon-purged conditions in this system, the generation of reduced metal ions alone by the positive control, vitamin C, is not enough to produce detectable H_2O_2. Therefore, Aβ is shown in this experiment to have facilitated the reduction of O_2 more than would be expected by the interaction of the metals reduced by Aβ with passively dissolved O_2. Hence, Aβ acts not only to reduce metal ions, but also to trap molecular O_2 to react to form H_2O_2. The O_2-dependent generation of H_2O_2 by Aβ1−42 was confirmed using a fluorescent dichlorofluorescein (DCF) assay (Figure 2C).

To prove that Aβ-mediated H_2O_2 formation is metal ion dependent, H_2O_2 production by Aβ1−42 in the presence of

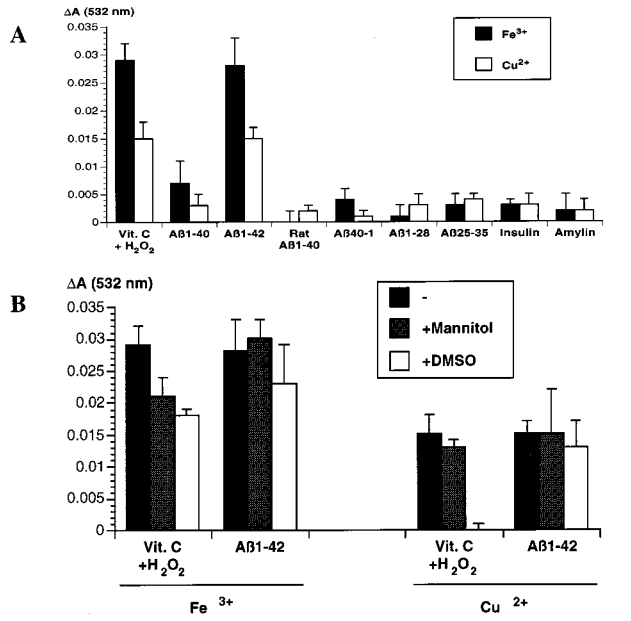

FIGURE 3: Production of TBARS reactivity from the incubation of Aβ in the presence of Fe(III) or Cu(II). All data points are means ± SD, n = 3 replicate wells, from a single microplate, and are typical of three experiments. (A) Signal from the TBARS assay produced by vitamin C (10 μM + 500 μM H_2O_2 to generate a strong positive control signal); insulin and amylin (10 μM); and variant Aβ (10 μM) species: Aβ1−42, Aβ1−40, rat Aβ1−40, Aβ40−1, Aβ1−28, and Aβ25−35. Incubations were in PBS, pH 7.4, with 1 μM Fe(III) or Cu(II) as indicated, for 1 h, 37 °C, under ambient buffer gas conditions. (B) Effect of OH•-specific scavengers upon TBARS signal generation by vitamin C and Aβ1−42. Mannitol (5 mM) or dimethyl sulfoxide (DMSO, 5 mM) was coincubated with vitamin C (10 μM + 500 μM H_2O_2) or Aβ1−42 (10 μM) (conditions as for Figure 3A).

chelators was assayed (Figure 2D). The presence of BP or DTPA abolished Aβ-mediated H_2O_2 formation in the presence of 1 μM Fe(III), supporting the obligatory presence of redox-active Fe(III) in this reaction. These findings are also indirect evidence that Fe(III) bound to Aβ is reduced to Fe(II) (Figure 1) since Fe(III) will not transfer electrons to O_2.

We then studied the amount of H_2O_2 produced by the various Aβ peptides, and observed that Aβ1−42 produced only slightly more H_2O_2 than Aβ1−40 (Figure 2D) in the presence of 1 μM Fe(III), although it is much more active than Aβ1−40 at reducing Fe(III). Similarly, in the presence of 1 μM Cu(II), Aβ1−42 produced only slightly more H_2O_2 than Aβ1−40 (Figure 2E), and we have previously found that Aβ1−42 is more than twice as active as Aβ1−40 at reducing Cu(II) (Huang et al., submitted). Notably, vitamin C exhibits much stronger Fe(III) (Figure 1A) and Cu(II) (Huang et al., submitted) reducing activity compared to Aβ1−42, but produces little H_2O_2 compared to Aβ1−42 or Aβ1−40 in the presence of 1 μM Fe(III) or Cu(II) (Figure 2E). Therefore, these data further confirm that reduction of metal ions alone is insufficient to generate H_2O_2 and that the peptide plays an essential role in recruiting O_2 into the reaction. The amounts of H_2O_2 produced by Aβ1−42, Aβ1−40, or vitamin C were slightly greater in the presence of Cu(II) than in the presence of Fe(III) (Figure 2E), which may be because these agents produce more Cu(I) than Fe(II) under the same conditions (Figure 1B; Huang et al., submitted). Peptides that did not reduce Fe(III) (rat Aβ1−40, Aβ40−1, Aβ25−35, Aβ1−28, insulin, and amylin;

Figure 1A) did not produce H_2O_2 in the presence of Fe(III) (data not shown).

The Aβ−Metal Complex Generates TBARS Reactivity. Having demonstrated that human Aβ peptides simultaneously produce H_2O_2 and reduced metals, we proceeded to determine whether the hydroxyl radical (OH•) may be consequently formed by Fenton-like or Haber−Weiss reactions. We employed a modified TBARS assay that may detect OH• released from coincubation mixtures of Aβ peptides and 1 μM Fe(III) or Cu(II). Again paralleling the amounts of metal reduction observed for the respective peptides (Figure 1A), the TBARS signals detected were greatest for Aβ1−42 > Aβ1−40 ≫ insulin, amylin, rat Aβ1−40, Aβ40−1, Aβ25−35, and Aβ1−28 ≈ 0 (Figure 3A). The TBARS signal was abolished in the presence of the Cu/Fe chelator DTPA (200 μM) (data not shown). Importantly, the signals generated by Aβ1−42 were as great as those generated by the positive control (vitamin C + 500 μM H_2O_2). In the absence of added H_2O_2, vitamin C alone did not produce a signal in the presence of the metal ions, suggesting that the assay is detecting Fenton-like chemistry. Interestingly, the TBARS signal generated by all reagents was greater in the presence of Fe(III) than in the presence of Cu(II), despite the observation that vitamin C and Aβ were found to produce less H_2O_2 in the presence of Fe(III) than in the presence of Cu(II) (Figure 2E). Increased TBARS reactivity in the presence of Fe(III) may be explained by the Fe(II) that is produced being more chemically stable than Cu(I), and therefore having a greater chance than Cu(I) of causing the Fenton-like generation of OH•.

The effects of the OH• scavengers dimethyl sulfoxide (DMSO) and mannitol upon Aβ1−42-mediated TBARS reactivity were studied. Both of these agents partially inhibited the generation of the TBARS signal by the positive control, vitamin C, in the presence of Fe(III), but only DMSO inhibited the generation of the TBARS signal by vitamin C in the presence of Cu(II), indicating that the radical is difficult to intercept before it reacts with deoxyribose under these conditions. Neither scavenger suppressed the generation of the TBARS signal by Aβ1−42, whether in the presence of Fe(III) or Cu(II) (Figure 3B), which may indicate that the OH• is generated in a structure which preferentially admits deoxyribose but excludes DMSO and mannitol. However, the possibility that the TBARS assay is not selectively detecting OH•, but rather detecting another reactive species (eg $Aβ^{+•}$) in this experiment, cannot yet be excluded.

Generation of H_2O_2 by Aβ Is Not via a Superoxide Intermediate. H_2O_2 can potentially form by a two-electron transfer to O_2 to generate the peroxide anion (O_2^{2-}), or by a one-electron transfer generating the superoxide anion ($O_2^{-•}$) that subsequently undergoes disproportionation to H_2O_2, or by two one-electron transfers from each of two iron atoms to a bridging dioxygen molecule resulting in peroxide. We examined Aβ1−42 (10 μM) incubated with Fe(III) (1 μM in PBS, pH 7.4) for evidence of O_2^- formation over a 1 h incubation. Neither of the O_2^--selective detection reagents hydroethidium (20 μM, Molecular Probes) or Nitro Blue Tetrazolium (NBT, 0.1 mM), detected O_2^- formation from Aβ, using xanthine (1 mM) with xanthine oxidase (0.015 unit/mL) in PBS as a positive control. These findings suggest that the Aβ/Fe-mediated generation of H_2O_2 is not by a superoxide intermediate.

DISCUSSION

Our findings indicate that the Cu and Fe concentrated (≈0.4 mM and ≈1 mM, respectively) in amyloid deposits in AD (*33*) may serve to both assemble Aβ aggregates (*19*, *26*−*29*) and, according to our current findings, engender reactive oxygen species (ROS) generation. These reactions may contribute to the oxidation damage and Aβ oligomerization that is in evidence in AD. Further evidence that the Fe in amyloid is bound to Aβ comes from the observation that treatment of brain sections with diethyl pyrocarbamate (DEPC) releases redox-active Fe from plaque deposits (*34*), a reaction that correlates with the effect of DEPC treatment of Aβ which abolishes Fe(III) interaction by modifying peptide histidines (*29*). Interestingly, transgenic mice that express amyloid neuropathology have been reported to concentrate redox-active Fe in histological amyloid deposits (*20*), suggesting that the recruitment of Fe is a general feature of β-amyloid architecture. The proportion of the total metal content within plaque that is bound to Aβ is not yet known, but the generation of H_2O_2 by Aβ is driven by substoichiometric amounts of Fe(III)/Cu(II) (≤1:10, metal:Aβ; Figure 2), and if less than 0.5% of the total Fe or Cu present in plaque deposits exchanged with Aβ, then the in vitro findings that we currently report would occur in vivo. The concentration of Aβ in AD brain is on the order of 100 μg/g of tissue (*45*), which corresponds to approximately 20 μM, assuming a tissue density of 1 g/ml. Our data indicate that 10 μM Aβ1−40 or Aβ1−42 generates up to 25 μM H_2O_2 in 1 h, depending on the O_2 tension. Therefore, the concentrations of Aβ present in the AD brain have the potential to significantly raise H_2O_2 levels in the brain parenchyma.

Rat Aβ1−40, Aβ1−28, and Aβ40−1 did not reduce Cu(II) (Huang et al., submitted) or Fe(III), which correlates with attenuated Zn(II) binding by the same peptides (*19*, *26*). These data also indicate that the three rat Aβ substitutions, two of which (Y10→F and H13→R) are found in the minimal zinc binding domain (*26*), target one or more residues that mediate the metal-reducing properties of the peptide. However, that Aβ1−28 did not reduce metal ions indicates that the hydrophobic carboxyl-terminal domain is also critical for the reduction properties of Aβ. The mechanism by which the two additional hydrophobic residues (Ile and Ala) on Aβ1−42 so substantially enhance the peptide's redox activity compared to Aβ1−40 is still unclear, but may be related to the observations that the redox-active proteins such as SOD1 bind an active-site metal ion within β-sheet or β-barrel secondary structures (*46*) and that the extra two hydrophobic residues on Aβ1−42 increase the β-sheet content of Aβ (*47*).

Aβ−Fe(II) facilitated the reduction of O_2 to peroxide. Since O_2 is preferentially dissolved in hydrophobic environments (*44*), the hydrophobic carboxyl terminal of Aβ may also act as a reservoir for the O_2 substrate and create a microenvironment that facilitates the electron transfer from the reduced metal ion to the dioxygen molecule. The participation of hydrophobic residues in the O_2-trapping domain may contribute to the enhanced production of H_2O_2 by Aβ1−42 compared to Aβ1−40.

Our findings also indicate that H_2O_2 is produced by the Aβ−Fe complex in great molar excess (≈10-fold) of the amount of metal ion present. This would be possible if the

metal ion cycles between reduced and oxidized states in a mechanism that could involve the repeated transfer of electrons to successive O_2 molecules. Two moles of Fe(II) are required to generate 1 mol of O_2^{2-}, and since 2 mol of Aβ generates 1 mol of Fe(II) (reaction 1), it would require 4 mol of Aβ to generate 1 mol of H_2O_2. As only 10 μM Aβ was present in the experiments in Figure 2, the theoretical maximum amount of H_2O_2 that could be produced would be 2.5 μM if the peptide was irreversibly consumed in the production of H_2O_2. However, up to 25 μM H_2O_2 was observed to be produced by 10 μM Aβ at high O_2 tensions (Figure 2B). Therefore, multiple cycles of Fe(III) reduction must occur. To achieve this, $(A\beta)_2$ must be regenerated from Aβ:A$\beta^{+\bullet}$. However, the source of electrons that regenerates $(A\beta)_2$ in this system is unclear.

Although we found that Aβ generates TBARS reactivity in the presence of metal ions, further analysis is necessary to determine whether OH$\bullet$ is formed by the reaction of the reduced metal ions with the H_2O_2 produced by the peptide. Such a reaction has a precedent in SOD1, which acts as an anomalous peroxidase by reacting Cu(I) with H_2O_2 to generate OH$\bullet$ (*48*). The reactions we have observed for Aβ−metal interactions may explain the elevated TBARS levels directly assayed from AD brain tissue (*49*).

These reactions may have a bearing upon our understanding of Aβ-mediated neurotoxicity. Previously it had been assumed that the elevated H_2O_2 that mediated neurotoxicity in cells exposed to Aβ originated from impaired cellular metabolism (*21*). Our findings suggest that the H_2O_2 produced by extracellular Aβ may contribute to the intracellular H_2O_2 burden, since H_2O_2 can cross lipid boundaries before it reacts. The striking differences observed in the relative redox activities of the various native Aβ peptides to reduce Cu(II)/Fe(III), generate H_2O_2, and produce TBARS reactivity (Aβ1−42 > Aβ1−40 > rat Aβ) also support the possibility that these reactions relate to Aβ-mediated neurotoxicity. The relative redox-competence of Aβ1−42, Aβ1−40, and rat Aβ correlates with the respective participation of the peptides in amyloid neuropathology, and may be expected to correlate with their in vitro neurotoxicity. Aβ1−42 has been reported to be more neurotoxic than Aβ1−40 in cell culture (*15*), while, to our knowledge, the toxicity of rat/mouse Aβ has not been compared in similar experiments. However, rats and mice do not develop Aβ amyloid (*16*) or related neurotoxicity in vivo, even in mice transgenic for familial AD-linked mutant presenilin that overexpress endogenous mouse Aβ1−42 (*50*), which suggests that the rat/mouse Aβ species is not as intrinsically pathogenic as the human peptide. Whether Aβ accumulations cause neuronal demise in AD is still contended, although a recent report describing neurotoxicity associated with human-sequence Aβ deposition in a transgenic mouse model (*51*) supports this possibility. In vitro, we have recently determined that Cu(II) potentiates the neurotoxicity of Aβ1−42 in cell culture (Huang et al., submitted), while the inhibition of Aβ1−40 neurotoxicity in cell culture by Fe(III) chelation has already been shown (*52*).

Our data indicate that metal reduction by Aβ may not be chemically linked to metal-induced Aβ aggregation, and that metal reduction is a property of soluble and aggregated Aβ. However, Aβ accumulations may be sites of intensified redox activity, especially where combined with high concentrations of Fe and Cu, as observed in amyloid deposits (*33*). An intensification of redox activity may possibly contribute to the increased neurotoxicity of Aβ aggregates (*53*).

An inconsistency in the correlation of Aβ-redox competence with reported neurotoxicity is our finding that the non-native Aβ25−35 fragment does not reduce metal ions. However, a recent report has differentiated the cytotoxic mechanisms of the native and non-native peptides in that Aβ25−35 toxicity, unlike Aβ1−42, is not rescued by the antioxidant propyl gallate, indicating that the cytotoxicity of Aβ25−35 is by a mechanism that does not involve oxidative stress (*54*). We found that Aβ25−35 does not generate reduced metals or ROS. However, Aβ25−35 has also been reported to form a radical in a metal-independent manner (*55*). Radicalization may mediate the polymerization or modification of both Aβ25−35 and Aβ1−42, but since Aβ25−35 does not reduce metal ions under these conditions, its radicalization process must be chemically distinct from that described for Aβ1−42 in reaction 1.

Should our findings on the relative redox competence of native Aβ peptides be relevant in vivo, they indicate that interdiction of inappropriate Aβ interaction with biometals could hold promise as a therapeutic intervention in the treatment of Alzheimer's disease.

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