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Metalloenzyme-like Activity of Alzheimer's Disease β-Amyloid

Cu-DEPENDENT CATALYTIC CONVERSION OF DOPAMINE, CHOLESTEROL, AND BIOLOGICAL REDUCING AGENTS TO NEUROTOXIC H_2O_2*

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β-Amyloid (Aβ) 1–42, implicated in the pathogenesis of Alzheimer's disease, forms an oligomeric complex that binds copper at a CuZn superoxide dismutase-like binding site. Aβ·Cu complexes generate neurotoxic H_2O_2 from O_2 through Cu^{2+} reduction, but the reaction mechanism has been unclear. We now report that Aβ1–42, when binding up to 2 eq of Cu^{2+}, generates the H_2O_2 catalytically by recruiting biological reducing agents as substrates under conditions where the Cu^{2+} or reducing agents will not form H_2O_2 themselves. Cholesterol is an important substrate for this activity, as are vitamin C, L-DOPA, and dopamine (V_{max} for dopamine = 34.5 nM/min, K_m = 8.9 μM). The activity was inhibited by anti-Aβ antibodies, Cu^{2+} chelators, and Zn^{2+}. Toxicity of Aβ in neuronal culture was consistent with catalytic H_2O_2 production. Aβ was not toxic in cell cultures in the absence of Cu^{2+}, and dopamine (5 μM) markedly exaggerated the neurotoxicity of 200 nM Aβ1–42·Cu. Therefore, microregional catalytic H_2O_2 production, combined with the exhaustion of reducing agents, may mediate the neurotoxicity of Aβ in Alzheimer's disease, and inhibitors of this novel activity may be of therapeutic value.

Aβ[1] characteristically collects in the neocortex in AD. Aβ1–40 is the major soluble species (1), and Aβ1–42 is a minor species but is enriched in plaque amyloid (2). Familial AD-linked mutations of amyloid protein precursor, presenilin-1 and presenilin-2, increase the concentration of Aβ1–42 (3). Aβ1–42 is toxic in primary neuronal culture at μM concentrations (4), by an unclear mechanism likely to be mediated by reactive oxygen species generation (5, 6). We recently reported that Aβ binds Cu^{2+} with very high affinity ($K_{Aβ1-42}$ = 8 attomolar) (7, 8), forming an allosterically cooperative Cu^{2+} coordination site that resembles superoxide dismutase 1 (9). The Aβ·Cu^{2+} complex is redox-active and produces H_2O_2 from O_2 through the reduction of Cu^{2+} (10, 11). The H_2O_2 that Aβ directly generates contributes to the neurotoxicity of the peptide in primary neuronal cultures (11). This is important because of the marked H_2O_2-mediated damage to neocortical tissue in AD (12, 13) and in amyloid-bearing transgenic mice (Tg2576) (14).

The metal dependence of the generation of H_2O_2 by Aβ may be a target for AD therapeutics. Cu and Zn are markedly elevated in amyloid plaques (15, 16). Therefore it is significant that H_2O_2 generation by Aβ *in vitro* is abolished by chelators (10, 11), such as clioquinol, which we have reported recently has *in vivo* efficacy in blocking brain Aβ accumulation in Tg2576 mice (17).

The chemical origin of the electrons in the Aβ-mediated reduction of O_2 to generate H_2O_2 (10, 11, 18) remained to be clarified. Here we elaborate this reaction mechanism, demonstrating the donation of electrons from biological reducing agents (*e.g.* cholesterol, catecholamines, vitamin C). We further demonstrate that this reaction is catalytic and that the Aβ·Cu complex is a cuproenzyme that can be purified from the human brain. The neurotoxicity of Aβ in cell culture is shown to be completely dependent upon both the binding of Cu^{2+} and the simultaneous presence of a reducing agent. Taken together, these data argue that Aβ toxicity is because of abnormal enzymatic activity, which represents an important target for medical chemistry approaches for the treatment of AD.

EXPERIMENTAL PROCEDURES

Reagents—Aβ peptides were synthesized by the W. Keck Laboratory, Yale University, New Haven, CT. Confirmatory data were obtained by

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[1] The abbreviations used are: Aβ, β-amyloid; AD, Alzheimer's disease; DA, dopamine; DCF, 2′,7′-dichlorofluorescin diacetate; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; L-DOPA, levo-dihydroxyphenylalanine; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; DTPA, diethylene triamine pentaacetic acid; DETC, diethyl dithiocarbamate.

employing Aβ peptides synthesized in the laboratory of Dr. C. Glabe, University of California, Irvine, CA. Aβ peptide stock solutions were prepared in water treated with Chelex (Bio-Rad) and quantified according to established procedures (19). Cu^{2+}-Gly (1:6) was prepared as described previously (8). Monoclonal anti-Aβ antibodies 4G8 (which detects Aβ residues 17–21) and 6E10 (which detects residues 9–16) were obtained from Senetek, and 10H3 (immunogen is residues 1–28 of Aβ) were obtained from Pierce Endogen. The other reagents were obtained from Sigma unless otherwise noted.

Hydrogen Peroxide Assay—Dichlorofluorescein diacetate (DCF; Molecular Probes, Eugene, OR) was dissolved (5 mM) in 100% dimethyl sulfoxide (argon purged for 2 h at 20 °C), deacetylated with 0.25 M NaOH for 30 min, and then neutralized, pH 7.4, to a final concentration of 1 mM. Horseradish peroxidase (HRP) stock solution was prepared to 1 μM in PBS, pH 7.4. The reactions were carried out in PBS, pH 7.4, under ambient conditions in a 96-well plate (250 μl/well) containing freshly prepared synthetic peptide (up to 1 μM), Cu-Gly (up to 2 μM), reducing agents (up to 10 μM), deacetylated DCF (100 μM), and HRP (0.1 μM), incubated at 37 °C. 10 μM EDTA was included to prevent reactions with contaminating concentrations (<0.2 μM) of free Cu^{2+}. The concentrations of Aβ used varied to bring readout values into a convenient target range. For example, in studies to determine which reducing agents would promote H_2O_2 production (see Fig. 1) relatively higher peptide concentrations (1 μM) were studied so that low production of H_2O_2 would not be overlooked. In most experiments, though, Aβ was used at 200 nM to reflect the soluble concentration in the AD brain (20). Studies were completed on the day of reagent preparation. Reactions were conducted in the dark to avoid photodynamic effects. The signal specific for H_2O_2 was the decrease in fluorescence of parallel samples coincubated with catalase (4000 units/ml; 10 μM). Fluorescent readings were recorded by a Packard Fluorocount plate reader (485 nm excitation, 530 nm emission), against a standard curve of reagent grade H_2O_2 in PBS, pH 7.4.

Primary Neuronal Cultures—Cortical neuronal cultures were prepared as described previously (18) with some modifications. Briefly, embryonic day 18 BL6Jx129sv mouse cortices were removed, dissected free of meninges, and dissociated in 0.025% (w/v) trypsin. Dissociated cells were plated in poly-L-lysine-coated 48-well culture plates (Nunc) at a density of 1×10^5 cells/cm^2 in plating medium (minimum Eagle's medium with 10% fetal calf serum and 5% horse serum). Cultures were maintained at 37 °C in 5% CO_2 for 2 h before the plating medium was replaced with Neurobasal growth medium containing B27 supplements. This method resulted in cultures highly enriched for neurons (≈5% glia). After 6 days in culture, the medium was replaced with fresh Neurobasal medium supplemented with B27 lacking antioxidants (to minimize scavenging of H_2O_2 generated in the medium) or Locke's buffer, which contained the following (in mM): NaCl, 154; KCl, 5.6; $CaCl_2$, 2.3; $MgCl_2$, 1; $NaHCO_3$, 3.6; glucose, 5; Hepes, pH 7.4, 5, into which experimental compounds were added.

For treatment of neuronal cultures fresh Aβ stock solution was diluted (final concentration 0.2–2.5 μM) in Neurobasal medium or Locke's buffer and coincubated (15 min, 20 °C) ± Cu-Gly (0.4–5 μM), DA (5–20 μM), catalase (1000 unit/ml), or Me_2SO (98 μM). The mixtures were then added to neuronal cultures for up to 24 h, and cell viability was then assayed.

Cell Viability Assays—Cell survival was monitored by phase contrast microscopy, and cell viability was quantitated using the MTS assay as described previously (21). Briefly, cells were washed two times with 250 μl of Locke's buffer, then placed in Neurobasal medium (250 μl), and 25 μl of MTS (Promega) was added to each well and incubated for 4 h in a 48-well plate. Absorbance (490 nm) was determined using a Wallac Victor Multireader and compared with MTS incubated in cell-free medium. The data were calculated as a percentage of values from untreated cells.

RESULTS

TCEP, used in our previous studies to detect H_2O_2, may have served as the source of electrons to form H_2O_2 from O_2, because it is also a reducing agent (10, 11). To evaluate this possibility we coincubated Aβ complexed with Cu^{2+} (Aβ·Cu) ± TCEP using the DCF assay to measure H_2O_2. A ratio of 2Cu:Aβ was chosen for initial studies, because Aβ1–42 can bind up to two Cu atoms at pH 7.4 (8). TCEP was indeed found to be a significant substrate for H_2O_2 formation by Aβ·Cu (Fig. 1*A*); however H_2O_2 formation by Aβ·Cu alone was not observed. HRP is used in the DCF-based H_2O_2 assay to catalyze the reaction of H_2O_2 with the DCF detection reagent. As negative controls, we confirmed that Aβ·Cu did not increase DCF fluorescence in the absence of HRP and that catalase abolished DCF fluorescence in these and subsequent assays (not shown). Therefore, DCF is a suitable reagent to detect H_2O_2 production by Aβ. Furthermore, in the absence of Aβ, the Cu^{2+} (≤2 μM) alone did not generate H_2O_2 in these and subsequent experiments, indicating that EDTA at this concentration (10 μM, present in all subsequent experiments) blocks H_2O_2 generation by any possible free Cu^{+}.

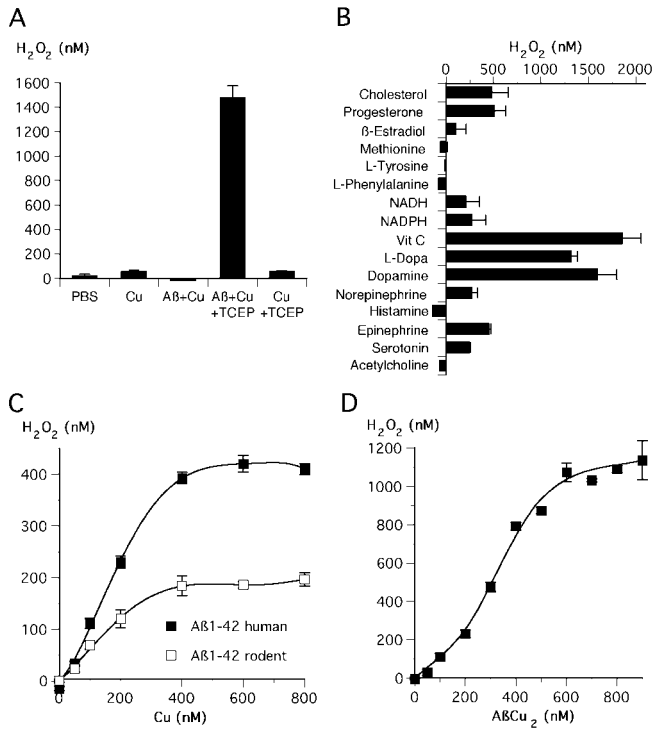

FIG. 1. **Characterization of H_2O_2 production by Aβ·Cu.** *A*, TCEP is a substrate for H_2O_2 production by the Aβ·Cu complex. Aβ1–42 (1 μM) and Cu^{2+}-Gly (2 μM) (AβCu_2) were incubated ± TCEP (10 μM), and H_2O_2 levels were measured by DCF assay. Control incubations were performed in the absence of Aβ. *B*, differential effect of reducing agents. AβCu_2 was incubated with different reducing agents (10 μM), and H_2O_2 levels were measured. *C*, optimal stoichiometry of Aβ1–42·Cu complex. Synthetic human or rat Aβ1–42 (200 nM) was incubated with increasing concentrations of Cu^{2+}-Gly (0–800 nM) in the presence of DA (5 μM), and H_2O_2 levels were measured. *D*, dependence of H_2O_2 production upon AβCu_2 concentration. Increasing concentrations of AβCu_2 were incubated with DA (5 μM), and H_2O_2 produced was measured as a product of Aβ concentration. All incubations were in PBS, pH 7.4, 10 μM EDTA for 60 min at 37 °C. Data are means ± S.E.

Having established that a non-biological reducing agent (TCEP) will serve as a reservoir substrate for the generation of H_2O_2 by Aβ·Cu, we next surveyed a variety of neurochemicals to identify candidate biological substrates for the same reaction (Fig. 1*B*). H_2O_2 production was greatest for the stronger biochemical reducing agents, vitamin C (1.9 μM) > DA (1.7 μM) > L-DOPA (1.3 μM). Other agents that promoted significant H_2O_2 production included cholesterol, progesterone (0.5 μM), epinephrine (0.4 μM), norepinephrine, serotonin, and NADPH (0.3 μM). Equivocal H_2O_2 levels were generated by NADH and β-estradiol. Acetylcholine, histamine, L-phenylalanine, L-tyrosine, and methionine were all unable to generate H_2O_2 in the presence of Aβ·Cu.

To characterize the enzymology of catalytic H_2O_2 production by Aβ·Cu *in vitro*, we elected DA as the test substrate, because its redox chemistry is well understood. We first optimized H_2O_2 production as a product of the stoichiometry of Cu bound to Aβ1–42. Under the same conditions as in Fig. 1*B*, we incubated

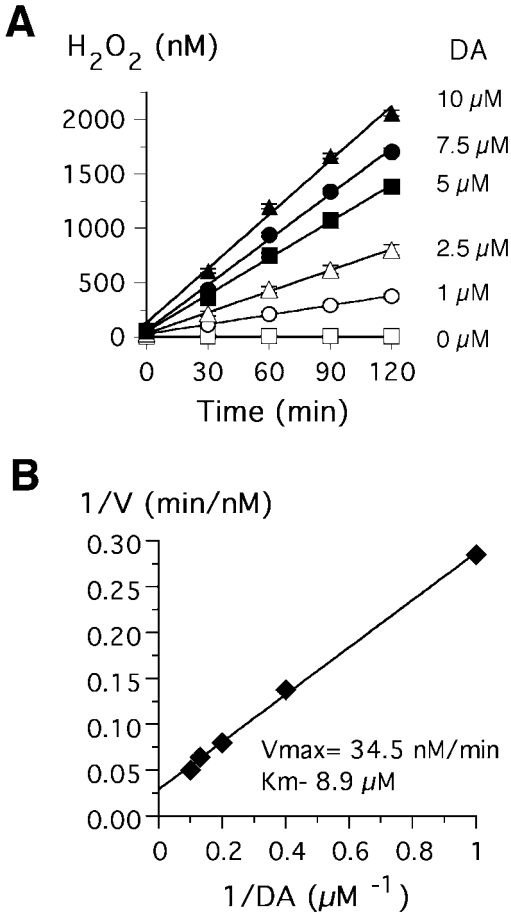

FIG. 2. **Catalytic H_2O_2 production by AβCu_2 using dopamine as substrate.** *A*, AβCu_2 (200 nM) was incubated with increasing DA concentrations for 120 min at 37 °C in PBS, pH 7.4, 10 μM EDTA, and H_2O_2 levels were measured at 30-min intervals. Data are means ± S.E. *B*, Lineweaver-Burk transformation of the data obtained at 60 min from the kinetic experiment described in *A*.

Aβ1–42 (200 nM) with Cu^{2+} (0 to 1000 nM) and assayed resultant H_2O_2 levels generated in the presence of DA (5 μM) and EDTA (Fig. 1*C*). H_2O_2 production by Aβ·Cu saturated at a molar ratio of 2Cu:1Aβ (AβCu_2) suggesting that there the two Cu binding sites coordinated by each Aβ monomer at pH 7.4 (8) both support redox activity. The saturation of H_2O_2 production in this experiment confirms that only the Cu^{2+} bound to Aβ, and not free Cu^{2+}, contributed to the production of H_2O_2.

We studied the dynamic range of H_2O_2 production from AβCu_2 by incubating the complex (0–900 nM Aβ) with DA (5 μM). H_2O_2 production was linearly dependent on AβCu_2 concentrations between 100 and 600 nM (Fig. 1*D*). To determine the Michaelis-Menten relationships between AβCu_2 and DA, we demonstrated that the rate of H_2O_2 production by AβCu_2 (200 nM Aβ) was dependent upon DA concentration (0 to 10 μM) (Fig. 2*A*). The data obtained at 60 min of incubation were plotted using a Lineweaver-Burk equation (Fig. 2*B*). This revealed a linear relationship ($R^2 = 0.998$) between 1/v and 1/DA and a V_{max} of 34.5 nM/min and K_m of 8.9 μM. 60 min was chosen as the interval for the Lineweaver-Burk transformation, because it was midpoint of the 120-min study, and because H_2O_2 production was occurring at a constant rate for all DA concentrations during the incubation period. The transformation yielded similar values for data from the 30-, 90-, and 120-min incubation values (not shown).

Because reaction with DA may alter the redox activity of Aβ·Cu, we repeated some previous observations of Aβ-mediated H_2O_2 production, this time in the presence of DA. AβCu_2 (0.5 μM) + DA (5 μM) generated no detectable superoxide ($O_2^{\cdot-}$) using dihydroethidium (20 μM; Molecular Probes) as an indicator following 1 h of incubation. Catalytic H_2O_2 was readily detected from AβCu_2 at 200 nM (Fig. 1, *B* and *C*), so lack of detectable $O_2^{\cdot-}$ from a 2.5-fold greater concentration of AβCu_2 suggests that H_2O_2 is not generated by disproportionation of $O_2^{\cdot-}$, in agreement with our previous report (10). The H_2O_2 catalytic activity of variant Aβ peptides (prepared as AβCu_2), using DA as substrate, was again found to exhibit the same relative redox activities (Aβ1–42 > Aβ1–40 > rat Aβ1–42 > rat Aβ1–40) (see Figs. 1*C* and 3*A*) as reported previously, correlating with the respective abilities of the peptides to reduce Cu^{2+} and their toxicity in neuronal culture (10, 11). The lower H_2O_2 catalytic activity of rat Aβ1–42 compared with human Aβ1–42 did not appear to be because of decreased Cu^{2+} affinity or stoichiometry, because excess Cu^{2+} added to rat Aβ1–42 did not enhance its activity to the same level as human Aβ1–42, saturating at a binding ratio of 2Cu:1Aβ (Fig. 1*C*). Importantly, cerebral amyloid deposits are scarce in aged rats and mice (22), which possess a homologue with three amino acid substitutions (23). Aβ1–28 and Aβ25–35, as well as control peptides insulin and apotransferrin, were again found to be redox inert in the presence of DA (Fig. 3*A*) (10, 11).

To appraise the affinity of the active Cu^{2+} in catalytic H_2O_2 production, we studied the effects of chelators of higher Cu^{2+} affinity than EDTA. DTPA and CDTA have both been observed previously to deplete Cu^{2+} from Aβ1–42 (8) and were found to abolish H_2O_2 production in the presence of DA (Fig. 3*B*). Similarly, DETC, which inhibits Cu-mediated superoxide dismutase activity, inhibited H_2O_2 production. In contrast, melatonin, which has little affinity for Cu^{2+}, was unable to inhibit H_2O_2 production (data not shown).

The effects of stereochemical occlusion of the AβCu_2 active site were studied using anti-Aβ monoclonal antibodies. AβCu_2 was preincubated (30 min) with various antibodies, and H_2O_2 generation upon incubation with DA (5 μM) was determined. All three anti-Aβ antibodies significantly inhibited H_2O_2 production (10H3 > 4G8 > 6E10), whereas a nonspecific anti-IgG antibody present at 5-fold greater excess than the anti-Aβ antibodies had no inhibitory effect (Fig. 3*C*).

Cytotoxic H_2O_2 production by Aβ, which is augmented by Cu in cultures (11, 18), could potentially be driven by reducing agents in the culture medium. To test this, we studied the effects of Aβ1–42, Cu^{2+}, and DA together and individually, upon the survival of E18 primary neurons studied in media that lacked H_2O_2 scavengers. To achieve this, the cells were first grown in Neurobasal medium plus B-27 supplement with antioxidants, and after 6–8 days the medium was changed to Locke's buffer without antioxidants for the experiments. We treated the cultures with Aβ1–42 (2.5 μM) ± Cu^{2+}-Gly (5 μM), in the presence or absence of DA (20 μM), and assayed for survival at 14 and 16 h (Fig. 4*A*). Because DA rapidly oxidizes in culture medium, it was necessary to abbreviate the onset of toxicity, which was achieved by using higher concentrations of reagents than were employed in the H_2O_2 assay studies. Aβ1–42 by itself was not toxic (Fig. 4*A*), but when bound to Cu Aβ1–42 became rapidly toxic (85% survival at 14 h, 60% survival at 16 h; intervals chosen after pilot studies showed that cell death commenced after 12 h and was near total by 24 h). The time dependence of toxicity in the absence of DA was compatible with the catalytic H_2O_2 production by AβCu_2 utilizing reducing agents released by the cells and accumulating in the culture medium. Compatible with H_2O_2 production mediating cell death, AβCu_2 toxicity was markedly exaggerated by the addition of exogenous DA. Neither Cu alone nor DA

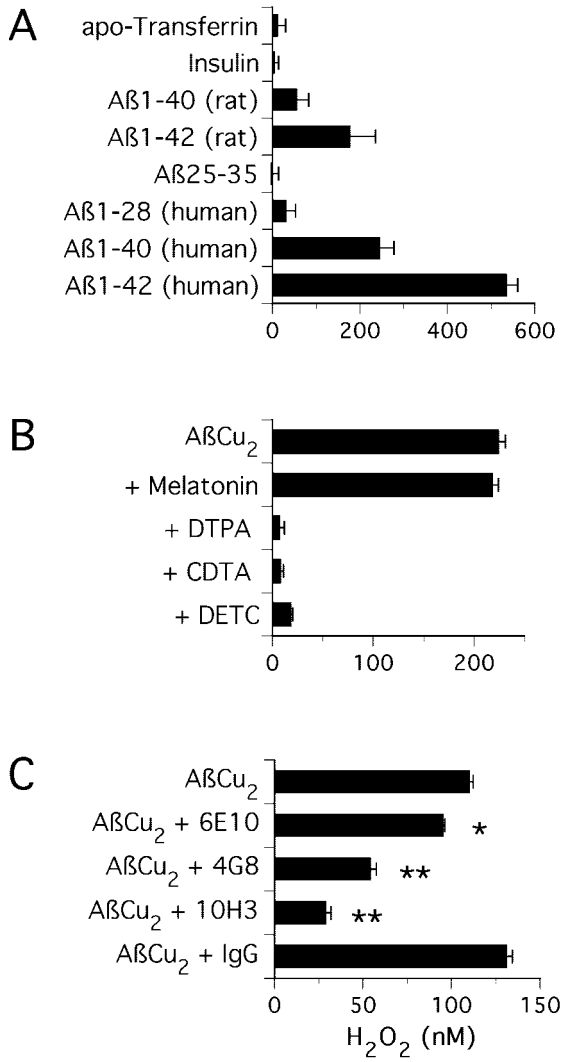

FIG. 3. **Characterization of AβCu$_2$-mediated catalytic H$_2$O$_2$ generation by activity mapping and inhibition.** *A*, synthetic Aβ fragments and variants or control peptides (apotransferrin and insulin) were incubated (200 nM) with Cu^{2+}-Gly (400 nM) in the presence of DA (5 μM) for 60 min at 37 °C in PBS, pH 7.4, and H_2O_2 levels were measured. *B*, inhibition by copper chelators. AβCu$_2$ (200 nM) was incubated in PBS, pH 7.4, containing DA (5 μM), EDTA (10 μM) ± melatonin or chelators DETC, CDTA, or DTPA (10 μM) for 60 min at 37 °C, and H_2O_2 levels were measured. *C*, inhibition by anti-Aβ antibodies. AβCu$_2$ (100 nM) was preincubated for 30 min in PBS ± anti-Aβ antibodies (100 nM) or excess purified mouse IgG (500 nM). The mixtures were then incubated with DA (5 μM) for 60 min at 37 °C in PBS, pH 7.4, 10 μM EDTA, and H_2O_2 levels were measured. Data are means ± S.E. *t* test results of effects of antibody treatment are indicated by * ($p < 0.05$), ** ($p < 0.01$).

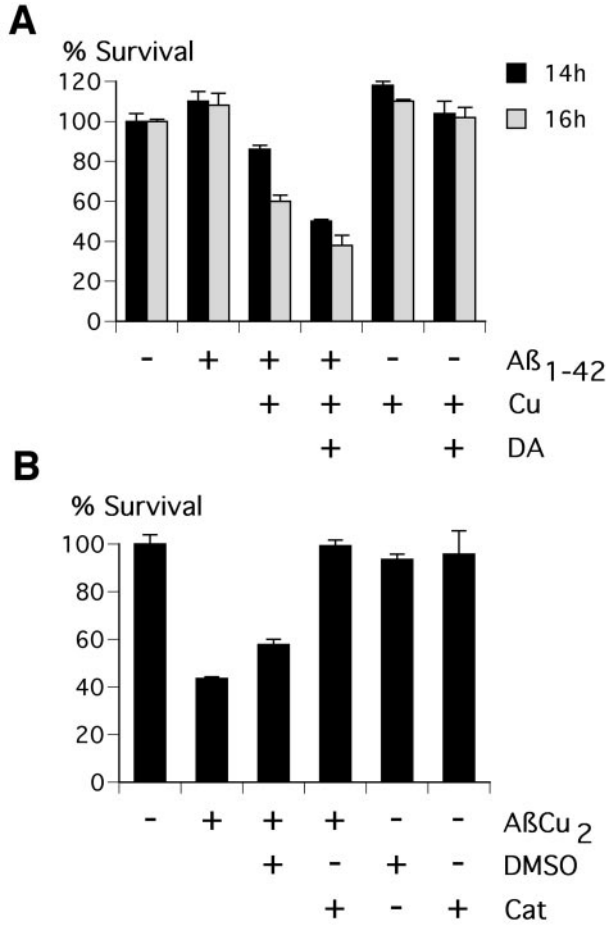

FIG. 4. **Catalytic H$_2$O$_2$ production by AβCu$_2$ mediates toxicity in neuronal culture.** *A*, dopamine potentiation of AβCu$_2$ neurotoxicity. Primary cortical neurons were incubated in Locke's buffer + EDTA (10 μM) for 14 or 16 h. The effects of Aβ1–42 (2.5 μM), Cu^{2+}-Gly (5 μM), and DA (20 μM), alone and in combination, upon cell viability compared with untreated cells were determined. *B*, catalase rescue of AβCu$_2$-dopamine neurotoxicity. Primary cortical neurons in Neurobasal medium + EDTA (10 μM) were incubated with AβCu$_2$ (200 nM) + DA (5 μM) ± catalase (*Cat*; 1000 units/ml) or Me$_2$SO (98 μM) for 24 h, and cell viability was then determined. Data are means ± S.E.

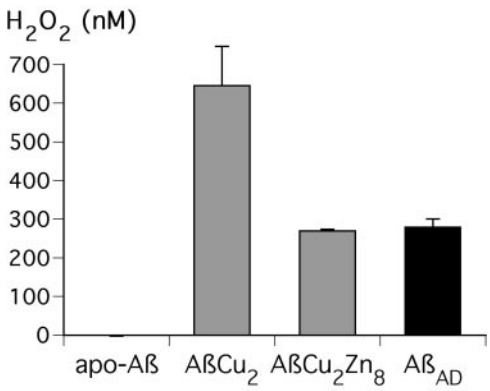

FIG. 5. **Catalytic H$_2$O$_2$ generation by Aβ metalloprotein purified from Alzheimer's disease amyloid.** Synthetic Aβ (200 nM) with no added metals (*apo-Aβ*), AβCu$_2$ (200 nM), 200 nM AβCu$_2$ + 1600 nM Zn^{2+} ($A\beta Cu_2Zn_8$), and Aβ purified from AD-affected brain tissue ($A\beta_{AD}$; 200 nM, which is metallated with 1.7 eq Zn, 0.4 eq Cu, 0.2 eq Al, 0.1 eq Fe) were incubated with DA (5 μM) + EDTA (10 μM) in PBS, pH 7.4, and H_2O_2 levels were assayed after 1 h.

alone was toxic (Fig. 4*A*). To ensure that the neurotoxicity was not because of unbound Cu^{2+}, EDTA (10 μM) was present in the incubation medium.

We investigated the H_2O_2-related toxicity of Aβ1–42 at lower peptide concentration (200 nM) to test whether toxicity will occur at physiological peptide concentrations (20) and when the cells are under less acute stress. Because toxicity evolved more slowly at this lower concentration, a longer incubation was needed. Therefore, to avoid toxicity due to serum deprivation, Neurobasal medium (lacking B27) was employed instead of Locke's buffer. Under these conditions, we observed that 200 nM AβCu$_2$ was markedly toxic in the presence of 5 μM DA (<45% survival after 24 h; see Fig. 4*B*). The toxicity of AβCu$_2$ was completely rescued (100% viability) by the selective H_2O_2 scavenging enzyme, catalase, but only partially rescued by the radical scavenger Me$_2$SO (Fig. 4*B*). Hence, the toxicity of Aβ could be attributed entirely to catalytic H_2O_2 production.

To ascertain whether these *in vitro* reactions could occur in

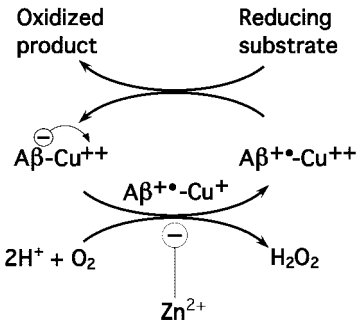

FIG. 6. **Model for catalytic H_2O_2 production by AβCu_2.** Aβ reduces Cu^{2+} to Cu^{+} by transferring an electron from the peptide backbone, generating a peptide radical. The Cu^{+} donates two electrons to O_2, generating O_2^{2-}, which becomes protonated to form H_2O_2. The Aβ radical is reconstituted by reaction with a reducing agent. Zn^{2+} inhibits the generation of H_2O_2 possibly by inhibiting Cu reduction.

AD, we measured catalytic H_2O_2 production from Aβ purified from AD plaque (24). This Aβ preparation is neurotoxic in neuronal primary cell culture (24). We found that the purified Aβ generated a significant amount of H_2O_2 (≈270 nM; see Fig. 5). Inductively coupled plasma mass spectrometry found that the only metals it contained were Zn (1.7 eq), Cu (0.4 eq), Al (0.2 eq), and Fe (0.1 eq). By comparison, synthetic AβCu_2 generated ≈630 nM H_2O_2, whereas AβCu_2 incubated with a 4-fold excess of Zn^{2+} generated a similar amount of H_2O_2 as the human-derived Aβ (which also bound an ≈4-fold excess of Zn^{2+} to Cu^{2+}). Zn^{2+} binding inhibits Aβ-mediated H_2O_2 production (18). However, the Aβ within amyloid is not bound to sufficient Zn^{2+} to prevent Cu^{2+}-mediated H_2O_2 production.

DISCUSSION

The major finding in this report is that Aβ forms an H_2O_2-producing cuproenzyme-like activity utilizing O_2 and biological reducing agents as substrates. The K_m for DA as the substrate (8.9 μM) indicates that this enzyme-like reaction is likely to occur under physiological conditions. Because H_2O_2 could not be generated by free Cu in this assay system (which was complexed by EDTA), Aβ must therefore generate a specific structure that presents redox-active Cu to O_2 and certain reducing substrates. This structure could be similar to the Cu binding site on CuZn-superoxide dismutase (9), which also catalyzes the reduction of O_2 (25).

A proposed reaction pathway for the sequence of electron transfers that converts O_2 into H_2O_2 is described in Fig. 6. In this model, an electron is initially transferred from Aβ to reduce Cu^{2+} to Cu^{+}. It is also possible that two electrons reduce two Cu^{2+} atoms bound to a single molecule of Aβ (11). After transferring the electron(s) to Cu^{2+}, the Aβ peptide forms a positively charged radical ($A\beta^{+\bullet}$). The oxidized site of Aβ that supports this electron transfer is uncertain, but the sole Met residue at position 35 is a candidate for this reaction. This residue is essential for Cu^{2+} reduction, which is abolished when the peptide buries this residue by membrane insertion (9), and is also abolished in Aβ1–28, which lacks Met (11). Met-35 is also essential for the toxicity of Aβ (26). The strong reducing potential of the A$\beta \cdot Cu^{2+}$ complex (+550 mV) (11) appears to impel the reduction of O_2 to O_2^{2-}. This step occurs as a double electron transfer from $2A\beta^{+\bullet}Cu^{+}$ or from $A\beta^{+\bullet}Cu^{+}{}_2$, although the formation of Cu^{3+} has also been considered possible (11). The dependence of this reaction step upon the concentration of O_2 has been demonstrated previously (11). The formation of a O_2^{-} intermediate, and subsequent disproportionation to H_2O_2, was not observed currently or previously (10, 11). After electron donation to O_2, the radicalized $A\beta^{+\bullet} \cdot Cu^{2+}$ complex might be restored to A$\beta \cdot Cu^{2+}$ by electron transfer from biological reducing agents (*e.g.* vitamin C and DA; see Fig. 1*B*). It is possible that the oxidized products of this reaction step might themselves be neurotoxic (*e.g.* dopaminochrome).

Our findings indicate that DCF is more suitable than TCEP as a detection reagent for H_2O_2 production by Aβ·Cu, because, unlike TCEP, DCF does not act as an electron donor in this system (Fig. 1*A*). Our current findings therefore provide a more complete explanation for the reaction sequence in our original report (10); H_2O_2 was generated by Aβ·Cu using TCEP as a substrate for O_2 reduction (the formation reaction), whereas a proportion of the TCEP (which was abundant in the incubation) reacted simultaneously with H_2O_2 (in the detection reaction).

Our data also indicate that several biological reducing agents could potentially drive the generation of H_2O_2 by Aβ in the neocortex in extracellular and intracellular compartments. Cholesterol was found to be a significant substrate for H_2O_2 production (Fig. 1*B*), suggesting a mechanism for how statin use might reduce the risk for AD (27). Aβ penetrates lipid membranes when bound to Cu^{2+} or Zn^{2+} (9) where it could potentially convert membrane cholesterol into H_2O_2. Scavenging of cholesterol by Aβ·Cu activity may also contribute to the membrane thinning and pathology seen in AD cortical tissue (28). Similarly, the availability of progesterone as a substrate for H_2O_2 generation by Aβ (Fig. 1*B*) is intriguing because of the increased incidence of AD in women. Vitamin C, L-DOPA, and DA were excellent substrates for Aβ·Cu-mediated production of H_2O_2 (Fig. 1*B*). Aβ could readily encounter vitamin C in intracellular and extracellular compartments *in vivo*. Although Aβ accumulation is principally an extracellular feature of AD neuropathology, intraneuronal Aβ accumulation has been proposed as an early pathophysiological event in AD (29) where it may contribute to Aβ accumulation in neurofibrillary tangle-bearing neurons (2). Intracellular Aβ could potentially recruit catecholamine precursors, L-DOPA and DA, to produce cytotoxic H_2O_2 within cortical noradrenergic neurons. This may contribute to the loss of noradrenergic input to the hippocampus in AD, where noradrenergic axonal abnormalities are associated with β-amyloid deposits (30). Several other untested biological reducing agents could also be substrates for Aβ-mediated H_2O_2 production, and they await further investigation. A systematic survey of concentrations of the reducing agents in vulnerable brain regions in AD and control tissue also awaits investigation. Cholesterol, however, has already been found in Aβ plaques (31).

Aβ1–42 has such high affinity for Cu^{2+} (attomolar) (8) that it is likely to be metallated *in vivo*, and indeed we found that Aβ purified from AD plaque is mainly bound to Zn (1.7 eq per mol of Aβ) and Cu (0.4 eq). The excess of Zn binding to the human Aβ probably reflects the plaque environment where Zn levels are markedly elevated (15, 16, 32). The binding of Zn and Cu to human Aβ is in agreement with our previous finding that there are selective sites on Aβ (8) that favor binding Zn and Cu over other metals (7).

Our findings are the first to link the toxicity of Aβ solely to the generation of a neurotoxin (H_2O_2) catalytically generated by the peptide itself, by a mechanism that is driven by the consumption of normal metabolites (*e.g.* dopamine and vitamin C). This would appear to be a simple explanation for the toxicity of Aβ and provides a novel alternative target for a drug that would neutralize only toxic (rogue enzyme) forms of what is otherwise a normal protein. We found that Aβ can be neurotoxic at much lower concentrations (*e.g.* 200 nM; see Fig. 4*B*) than those usually reported (usually micromolar). Our data would be germane to the neurochemical environment of the brain in AD, where the toxicity of Aβ has been linked to soluble

forms of the protein that are present at concentrations of about 200 nM (20, 33). Aβ toxicity was completely rescued by catalase (Fig. 4*B*), which does not penetrate the cells. Therefore, the origin of the neurotoxic H_2O_2 is probably extracellular $A\beta Cu_2$ rather than from a downstream intracellular event such as mitochondrial dysfunction. H_2O_2 is freely permeable across tissue boundaries, so the H_2O_2 formed by Aβ could be the primary source of the abundant intracellular and lipid peroxidative damage generated in AD-affected brain (12, 13, 34). A secondary neurochemical stress that could be caused by Aβ·Cu enzyme activity could be the consumption and depletion of normal metabolites such as vitamin C, catecholamines, cholesterol, and progesterone.

A caveat with our experimental paradigm, as with most reports of Aβ toxicity in cell culture, is that toxicity is acute (<24 h), whereas neuronal demise in actual AD is a far slower process. In actual AD brain tissue, up-regulated H_2O_2 scavenging enzymes (35) may help protect the brain from acute damage. The induction of these defenses may be gradual, because the buildup of Aβ takes many years. However, in our experiments, the increase of H_2O_2 caused by Aβ may be too rapid for cells in culture to induce a sufficient up-regulation of their antioxidant systems. The cell death that we observed was particularly abrupt because of the removal of reactive oxygen species scavengers and glutathione precursors from the culture medium. Some level of H_2O_2 can be cleared by neuronal catalase, but beyond the threshold of what can be cleared by catalase, glutathione will be consumed to clear H_2O_2. Energy is expended to reconstitute reduced glutathione. To some extent the cell can also provide cysteine, required for glutathione synthesis, by breakdown of polypeptides like metallothionein. However, in the presence of continuous catalytic H_2O_2 production by Aβ, the nutriture of the cell may be insufficient to maintain glutathione levels and keep up with clearing the H_2O_2. H_2O_2 then permeates into all tissue compartments causing oxidative toxicity and inducing apoptosis. This model would explain why the threshold for cellular resistance to Aβ toxicity in cell culture has been reported to be proportional to the activities of catalase and glutathione peroxidase (36). A further caveat with our cell toxicity data is that we performed studies using embryonal neuronal cultures, whereas the AD process affects adult neurons. Adult neurons might be even more vulnerable to H_2O_2-mediated toxicity, because there is evidence that as neurons differentiate they lose glutathione peroxidase and catalase activity and become markedly more vulnerable to oxidative stress in cell culture (37). Finally, intact cortical tissue may have a qualitatively different resistance compared with cells in culture to Aβ-mediated H_2O_2 production. Nevertheless, injection of synthetic Aβ into brain parenchyma in mutant tau transgenic mice induces AD-like tau cytopathology (38). It is possible that catalytic H_2O_2 production may mediate that result following recruitment of parenchymal Cu and reducing agents by the peptide. The role of H_2O_2 in the toxicity of Aβ might be further tested in transgenic models of AD, where genetic or pharmacological ablation of peroxide scavengers (*e.g.* catalase and glutathione peroxidase) would be expected to exaggerate the neuropathology associated with Aβ accumulation.

Critical neurochemical factors in AD that could contribute to the abnormal generation of H_2O_2 by Aβ would include the increases in cortical Cu (15) and reducing agents (39). Elevated brain-reducing equivalents in AD are thought to represent a compensation for increased oxidative stress, which leads to up-regulation of glucose-6-phosphate dehydrogenase (39, 40). Our current data suggest that this compensatory increase in reducing equivalents could paradoxically promote H_2O_2 generation by Aβ·Cu, leading to a vicious biochemical cycle.

The inhibition of H_2O_2 production from $A\beta Cu_2$ by anti-Aβ antibodies (Fig. 3*C*) is also of interest, because vaccination with Aβ1–42, or passive vaccination with anti-Aβ antibodies, is being studied currently with a view to clinical utility, following the success of the approach in preventing amyloid accumulation in transgenic mice (41). The mechanisms by which these Aβ-centered immune reactions achieve this effect are still unclear. Our data indicate that antibody binding to Aβ could inhibit abnormal H_2O_2 production by the peptide. This would make the antibody act, in a similar manner to a Cu^{2+} chelator (Fig. 3*B*), by blocking the active site on Aβ from transferring electrons to O_2.

The role of Zn^{2+} in the pathophysiology of Aβ is pleiotropic (42). Cobinding of Zn^{2+} inhibited catalytic H_2O_2 production by AβCu, but not completely, despite the presence of Zn^{2+} in 4-fold excess to Cu^{2+} (Fig. 5). 10-Fold excess of Zn^{2+} was observed previously to abolish H_2O_2 formation completely by Aβ (18). Hence the ability of Zn^{2+} to quench the cuproenzyme activity of Aβ appears to be relatively inefficient and may explain why coincubation of synthetic Aβ with Zn^{2+} rescues the neurotoxicity of Aβ·Cu incompletely (18). Similarly, the Aβ purified from AD brain was found to bind Zn in molar excess to Cu but in insufficient excess to abolish the catalytic generation of H_2O_2 (Fig. 5). Previous studies have shown that similar preparations of purified AD Aβ are markedly neurotoxic in cell culture (43). Zn^{2+}, released during neurotransmission from cortical glutamatergic synapses, precipitates Aβ to form amyloid plaques (16, 44, 45). Because Zn^{2+} suppresses H_2O_2 production by Aβ (18) (Fig. 5), we proposed that plaque might be less damaging than soluble Aβ or diffuse deposits of Aβ. However, our current data indicate that the Aβ from plaque is insufficiently loaded with Zn^{2+} to completely abolish catalytic activity (Fig. 5), and therefore, Zn^{2+}-induced plaque formation might not be a wholly effective means of preventing H_2O_2 production by Aβ. Indeed, despite an inverse correlation between oxidative damage and plaque burden in AD, oxidative adducts are still abnormally elevated even in the cases where plaque burden is heaviest (18). Precipitation of Aβ by Zn^{2+} may also inhibit clearance and catabolism of Aβ (46). Therefore, interdiction of both the Zn^{2+} and the Cu^{2+} interactions with Aβ may be beneficial in a therapeutic compound, such as clioquinol, which is markedly effective at inhibiting Aβ accumulation *in vivo* (17) and has shown efficacy in a recent phase 2 clinical trial of AD (47). Future studies will determine whether inhibition of catalytic H_2O_2 production by Aβ *in vitro* fulfills its potential to become a readout system that predicts efficacy of small molecules like clioquinol in clinical trials.

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Metalloenzyme-like Activity of Alzheimer's Disease β-Amyloid: Cu-DEPENDENT CATALYTIC CONVERSION OF DOPAMINE, CHOLESTEROL, AND BIOLOGICAL REDUCING AGENTS TO NEUROTOXIC H2O2

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