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Cu(II) Potentiation of Alzheimer Aβ Neurotoxicity

CORRELATION WITH CELL-FREE HYDROGEN PEROXIDE PRODUCTION AND METAL REDUCTION*

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Oxidative stress markers as well as high concentrations of copper are found in the vicinity of Aβ amyloid deposits in Alzheimer's disease. The neurotoxicity of Aβ in cell culture has been linked to H_2O_2 generation by an unknown mechanism. We now report that Cu(II) markedly potentiates the neurotoxicity exhibited by Aβ in cell culture. The potentiation of toxicity is greatest for Aβ1–42 > Aβ1–40 ≫ mouse/rat Aβ1–40, corresponding to their relative capacities to reduce Cu(II) to Cu(I), form H_2O_2 in cell-free assays and to exhibit amyloid pathology. The copper complex of Aβ1–42 has a highly positive formal reduction potential (≈+500–550 mV *versus* Ag/AgCl) characteristic of strongly reducing cuproproteins. These findings suggest that certain redox active metal ions may be important in exacerbating and perhaps facilitating Aβ-mediated oxidative damage in Alzheimer's disease.

Oxidative damage in the neocortex coincides with Aβ accumulation both in Alzheimer's disease (AD)[1] (1) and in Aβ amyloid-bearing transgenic mice (2), but the mechanisms of oxidation are unknown. The possibility that Aβ accumulation causes oxidation, perhaps by radical formation (3), has been explored, but the nature of the chemistry involved in generating Aβ-associated oxidation products such as lipid peroxides (4) remains to be elaborated. In culture, Aβ-induced neurotoxicity is characterized by elevated cellular H_2O_2 and is combated by antioxidants such as vitamin E and catalase (5). The origin of the toxic H_2O_2 is unknown.

Recently, we reported that Fe(III) interacts directly with Aβ1–42 and Aβ1–40 to produce H_2O_2 and TBARS formation in a cell-free manner *in vitro*, through reduction of the metal ion (6), suggesting that a source of the H_2O_2 that mediates toxicity in cell cultures exposed to Aβ is extracellular. Cu(II)and Fe(III) have been found in abnormally high concentrations in amyloid plaques (≈0.4 and ≈1 mM, respectively) and AD-affected neuropil (7), and copper-selective chelators have been shown to dissolve Aβ deposits extracted from AD post-mortem brain specimens (8). Therefore, these metal ions may be important cofactors in Aβ-associated oxidative damage. Importantly, we have also reported that the generation of both Cu(II) and Fe(III)-mediated TBARS is greatest for Aβ1–42 > Aβ1–40 ≫ rat Aβ1–40 (6). This rank order is of interest because it mirrors the relative participation of the peptides in amyloid neuropathology, and because the most active one (Aβ1–42) is overproduced in familial AD (9). Rats and mice do not develop amyloid (10), even in mice transgenic for familial-AD linked mutant presenilin that overexpress endogenous mouse Aβ1–42 (11), probably due to the three amino acid substitutions in their homologue of Aβ ($Arg^5 \rightarrow Gly$, $Tyr^{10} \rightarrow Phe$, and $His^{13} \rightarrow Arg$) (12).

Although Fe(III) mediates and potentiates Aβ1–40 toxicity in cell culture (13), it is not clear whether this is due to metal interaction with the peptide or due to a nonspecific increase in reactive oxygen species (ROS) generation within the cell. Redox active metal ions, such as Cu(II) and Fe(III), play an obligatory role in generating ROS, and in mediating ROS-induced damage (*e.g.* the Fenton reaction) (14, 15). Similarly, the Cu(II) and Fe(III) enhancement of dichlorofluorescein (DCF)-reactive oxygen species generated by Aβ25–35 treatment of post-mitochondrial rat cerebrocortex (16) could be due to catalytically enhanced ROS generation within the tissue, rather than due to metal interaction with the peptide.

Cu(II) causes the peptide to aggregate to a greater extent than Fe(III) (Aβ1–42 > Aβ1–40 > rat Aβ1–40) (17), a property that may be related to the relative affinities of the metal ions

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[1] The abbreviations used are: AD, Alzheimer's disease; TBARS, thiobarbituric acid-reactive substance; ROS, reactive oxygen species; DCF, dichlorofluorescein; BC, bathocuproine disulfonic acid; BCA, bicinchoninic acid; PBS, phosphate-buffered saline.

for Aβ. We hypothesize that if such redox active metal ions bind to Aβ peptides with high affinity and become more oxidizing, they may potentiate Aβ-induced cytotoxicity. Furthermore, if the redox competence of Aβ is responsible for its neurotoxicity, then toxicity should be greatest for Aβ1–42 > Aβ1–40 > rat Aβ1–40. Aβ1–42 has been reported to be more neurotoxic than Aβ1–40 (18), but a comparison of the neurotoxicity of these three peptides, or the effects of Cu(II) upon the potentiation of their respective toxicities in culture, has not yet been reported to our knowledge.

Here we report that, in the presence of Cu(II), Aβ is indeed redox-competent (Aβ1–42 > Aβ1–40 ≫ rat Aβ1–40), and that a series of electron transfer reactions occur when Cu(II) binds to Aβ, including reduction to Cu(I) and consequent O_2-dependent, cell-free peroxide formation. These changes correlate with a striking potentiation in the neurotoxicities of the respective Aβ species in cell culture, supporting an extracellular origin for the H_2O_2 that mediates Aβ-induced toxicity. These data suggest that formation of an Aβ-copper complex may be a pathophysiological interaction, and a new target for therapeutic interdiction in AD.

EXPERIMENTAL PROCEDURES

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: Glabe Laboratory, University of California, Irvine, CA; Multhaup Laboratory, University of Heidelberg; U.S. Peptides, Bachem (Torrance, CA); and Sigma. Rat Aβ1–40 was synthesized and purified by the Multhaup Laboratory. For the EPR experiments, Aβ1–40 was synthesized at the University of Queensland. 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 in water treated with Chelex-100 resin (Bio-Rad) and quantified, according to established procedures (17). Cu(II)-Gly stock solutions were used to prevent metal-hydroxy and metal-oxy polymers that form in neutral metal ion solutions and were prepared by mixing National Institute of Standards and Technology (NIST) standard copper with glycine at metal/ligand molar ratio of 1:6. Other reagents are from Sigma, unless otherwise mentioned.

Metal Reduction Assays—Assays were performed using a 96-well microtiter plate (Costar), based upon a modification of established protocols (19). Polypeptides (10 μM) or vitamin C (10 μM), Cu(II)-glycine and Cu(I) indicator (250 μM), either bathocuproine disulfonic acid (BC) or bicinchoninic acid (BCA, 4,4′-dicarboxy-2,2′-biquinoline), were coincubated in Dulbecco's phosphate-buffered saline (PBS: 1.19 mM $CaCl_2$, 0.6 mM $MgCl_2$, 2.7 mM KCl, 1.4 mM KH_2PO_4, 137 mM NaCl, 7.68 mM Na_2HPO_4, pH 7.4), at 37 °C. Absorbances were then measured using a plate reader (SPECTRAmax Plus, Molecular Devices). In control samples, both metal ion and indicator were present to determine the background buffer signal. Absorbance 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 (A) were obtained by deducting the absorbances from these controls from the absorbances generated by the peptide and metal in the presence of the indicator. Cu(I) concentrations (μM) were calculated as $A \times 10^6/M$, where M is the known molar absorption coefficient (M^{-1} cm^{-1}). For Cu(I)-BC, M = 12,250 at 483 nm; and for Cu(I)-BCA, M = 7700 at 562 nm.

Cyclic Voltammetry—Cyclic voltammograms were obtained at room temperature (22 ± 2 °C) on air-equilibrated solutions using an EG&G PARC potentiostat, model 263A. The electrochemical cell incorporated an indium/tin oxide working electrode (Donnelly Corp.) of 0.32-cm^2 area, a platinum auxiliary electrode, and a Ag/AgCl (1 M KCl) reference electrode. Indium/tin oxide electrodes were pretreated by successive 30 min sonications in Alconox solution (8 g/liter), 95% ethanol, Milli-Q water, and PBS, pH 7.3, followed by overnight equilibration in PBS. The peptide solution was prepared by first dissolving Aβ1–42 with sonication in double-distilled water to 300 μM, after which the peptide solution was added to Dulbecco's phosphate-buffered saline without calcium or magnesium (Sigma) to a final concentration of 100 μM. Background voltammograms were first acquired in buffer on each new electrode used, followed by examination of Aβ1–42 (100 μM) in buffer, $CuCl_2$ (17 μM) in buffer, and Aβ1–42 (100 μM) with added $CuCl_2$ (17 μM) in buffer. The possibility that the formal potentials measured were for surface-adsorbed complexes cannot be excluded, and, in fact, the wave shapes (Fig. 3, *lines c* and *d*) are suggestive of involvement by adsorbed species. Further investigation is planned to resolve this issue.

Electron Paramagnetic Resonance—Aβ1–40 (0.42 mg) was dissolved in 300 μl of PBS buffer (20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) to which $CuCl_2$ (as indicated) was later added. Q-, X-, and S- band EPR spectra were recorded on a Bruker ESP300E EPR spectrometer; the magnetic field and microwave frequency were calibrated with a Bruker ER-035M gaussmeter and an EIP 548B microwave frequency counter. Quantitation of the Cu(II)-Aβ1–40 EPR resonance was performed by a comparison method (20) using an X-band dual TE_{104} rectangular cavity with TEMPO as reference sample. The individual cavities have different modulation amplitudes, which can be calibrated, and different microwave magnetic strengths (B1), which was overcome by measuring standard and reference samples in each cavity. The doubly integrated areas are proportional to the spin concentration and were normalized for the anisotropic probability $<g_1^2>$ and instrument settings. The relative areas can then be used to calculate the concentration of Cu(II)-peptide that produces the residual spectrum. The anisotropic spectra of the Cu(II)-AB1–40 complexes were measured at 130 K using a flow-through cryostat. Spin Hamiltonian parameters were extracted from the spectra with the XSophe/Sophe computer simulation software suite (21). Buffers were demetallated with Chelex 100 resin.

Cell Culture—Rat embryonic day 17 forebrain neuronal cultures were grown at 95% O_2, 5% CO_2, 85% humidity for 4 days in serum-free Neurobasal™ medium with B-27 supplement (Life Technologies, Inc.), 20 μM L-glutamate, 100 units/ml penicillin, 0.1 g/ml streptomycin, and 2 mM L-glutamine. On the fifth day (treatment day), the medium was replaced with serum-free Neurobasal™ plus L-glutamine without B-27 supplement (vehicle medium). Stock solutions were mixed in vehicle medium to a final concentration of peptide or copper-glycine. Experimental trials were done in triplicate wells. Viable cells were counted manually using a 1-mm^2 grid (10× objective) stained with either calcein-AM (Live/Dead™ assay, Molecular Probes) or trypan blue. Data were analyzed using one-way analysis of variance followed by *post-hoc* Student-Newman-Keuls method and/or Student's t test. Significance level was set at $p < 0.05$.

Hydrogen Peroxide Assays—The colorimetric H_2O_2 assay was performed in a 96-well microtiter plate (SpectraMax Plus, Molecular Devices), according to a modification of an existing protocol (22). Polypeptides (10 μM) or vitamin C (10 μM), Cu(II) (1 μM), and a H_2O_2 scavenging agent, tris(2-carboxyethyl)phosphine hydrochloride (Pierce, 50 μM), were co-incubated in PBS buffer (300 μl), pH 7.4, for 1 h at 37 °C. Following incubation, the unreacted tris(2-carboxyethyl)phosphine hydrochloride was detected by 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma, 50 μM). The amount of H_2O_2 produced was quantified based on the formula: H_2O_2 (μM) = $A \times 10^6/(2 \times L \times M)$, where A is the absolute absorbance difference between a sample and catalase-only (Sigma, 100 units/ml) control at 412 nm; L = the vertical pathlength, corrected automatically by the plate reader to 1 cm; M is the molecular absorbance for 2-nitro-5-thiobenzoate (14,150 M^{-1} cm^{-1} at 412 nm).

For the DCF assay, 5 mM of 2′,7′-dichlorofluorescin diacetate (Molecular Probes) in 100% ethanol was de-acetylated 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 Cu(II)-glycine were co-incubated 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 were 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 anerobic conditions, prior to the addition of vitamin C or polypeptide.

RESULTS

To establish whether Aβ reduces Cu(II) to Cu(I), we used three independent methods. In the first approach various Aβ peptides, vitamin C (as a positive control), and other control polypeptides were incubated with Cu(II)-glycine chelate. Cu(I) formation was monitored using a chromogenic Cu(I)-trapping agent, BC. Aβ1–42 and Aβ1–40 were the only tested peptides found to reduce significant amounts of Cu(II) to Cu(I) (gener-

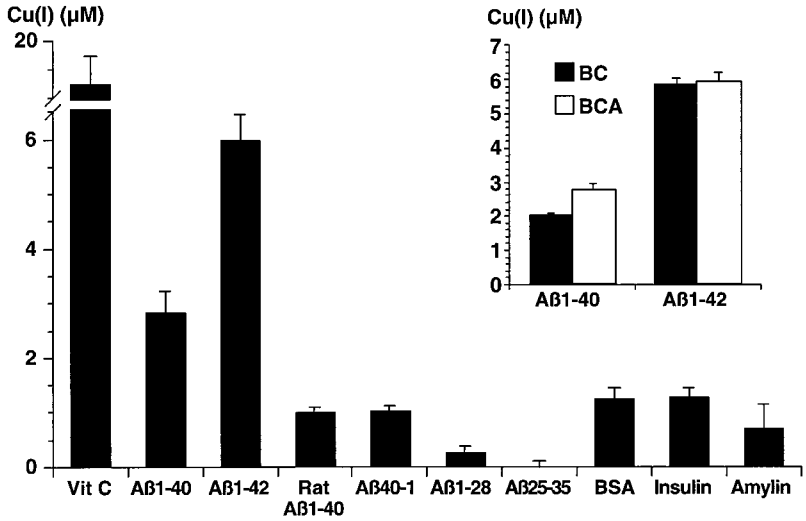

FIG. 1. **Aβ-mediated Cu(II) reduction.** Production of Cu(I) (BC assay) from Cu(II)-glycine (25 μM) by Aβ species, compared with vitamin C, insulin, human amylin (all 10 μM) in PBS, pH 7.4, after 1 h co-incubation, 37 °C. Data indicate means ± S.D., $n = 3$. *Inset*, Cu(I) assay validation. The amounts of Cu(I) generated by Aβ1–42 and Aβ1–40, under the same conditions, were compared by two different assays, BC with BCA.

ating 6 and 3 μM, respectively) during the 1-h incubation period (Fig. 1). Cu(II) was not significantly reduced (<1 μM) by rat/mouse Aβ1–40, reverse sequence human peptide (Aβ40–1), amylin, insulin, Aβ1–28, or Aβ25–35 (Fig. 1). Since it has been pointed out that BC could also bind to Cu(II), potentially altering its reduction potential and cause artifactual estimates of Cu(I) (23), we corroborated the apparent Cu(II)-reducing properties of Aβ1–40/2 by comparing the assay results using BC to those obtained with another Cu(I) detection agent, BCA. The apparent amounts of Cu(I) generated by Aβ1–42 and Aβ1–40 using either BC or BCA were in excellent agreement (Fig. 1, *inset*).

Secondly, EPR spectroscopy was used to measure residual Cu(II) remaining after incubating stoichiometric ratios of $CuCl_2$ with Aβ1–40. This peptide caused a loss of the Cu(II) signal (76%), in relative agreement with the corresponding Cu(I) detected in the bioassays above. The detection of Cu(I) by BC assay, at levels roughly comparable to those estimated from loss of the EPR signal for Cu(II), suggests that the EPR signal was not disappearing due to formation of antiferromagnetically coupled (S = 0) dicopper species. Experimental EPR S-band spectra (Fig. 2) and X- and Q-band spectra (data not shown) establish that copper binds very tightly to these peptides.

Fig. 2*A* shows that an approximately equimolar mixture of Aβ1–40 and $CuCl_2$ produces a single Cu(II)-peptide complex. The multiple resonance signal shown in the EPR spectrum for copper-peptide (Fig. 2*A*) is for a single Aβ-bound Cu(II) species that is paramagnetic. Spin quantitation employing a dual mode TE_{104} X-band rectangular cavity revealed that this EPR signal accounted for 24% of the added Cu(II), consistent with 76% of the Cu(II) being converted to EPR-silent species, very likely Cu(I). There was no evidence of free, uncomplexed Cu(II) remaining after addition of the peptide, since unbound Cu(II) itself gives a different multiple resonance signal. The loss of 76% of the Cu(II) signal upon incubation with Aβ1–40 is compatible with peptide-mediated reduction of Cu(II) to diamagnetic Cu(I), which is undetectable.

Computer simulation of the experimental spectrum of Aβ1–40 with an axially symmetric spin Hamiltonian and the g and A matrices ($g_\parallel$, 2.295; $g_\perp$, 2.073; $A_\parallel$, 163.60, $A_\perp$, 10.0×10^{-4} cm^{-1}) yielded the spectrum shown in Fig. 2*B*. Expansion of the $M_I = -1/2$ resonance revealed nitrogen ligand hyperfine coupling. Computer simulation of these resonances indicated the presence of at least three nitrogen atoms. The magnitude of the $g_\parallel$ and $A_\parallel$ values also suggest a tetragonally distorted geometry, which is commonly found in type 2 copper proteins (24), and together with the Blumberg Peisach plots, are consistent with

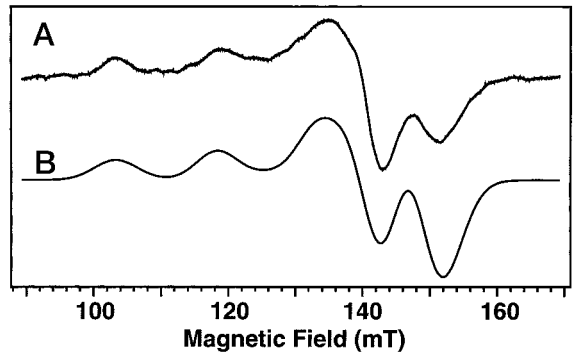

FIG. 2. **Electron paramagnetic resonance spectroscopy of a Cu-Aβ1–40 complex.** *A*, experimental spectrum generated by adding $CuCl_2$ (280 μM) to Aβ1–40 (323 μM) in PBS, pH 7.4. $\nu = 4.0663$ GHz. *B*, computer simulation of A.

a fourth equatorial ligand binding to copper via an oxygen rather than a sulfur donor atom. Thus, the coordination sphere for the copper-peptide complex is CuN_3O_1.

The third line of independent evidence to support Aβ-dependent reduction of Cu(II) was the electrochemical behavior of Cu(II), assessed in the presence and absence of Aβ by cyclic voltammetry (Fig. 3). This revealed that Aβ1–42 (*line c*) gives rise to a voltammetric response with a formal reduction potential of approximately +500–550 mV (*versus* Ag/AgCl) in phosphate-buffered saline alone. This is an extraordinarily high positive potential, which suggests a Cu(I) oxidation state that is highly stabilized by the peptide. Our buffers, even after careful preparation and filtration through Chelex-100 resin, were found routinely to possess ≈0.1 μM copper background contamination, as assessed by inductively coupled plasma analysis (8). To determine whether interaction with this trace quantity of metal ion was responsible for the electrochemical response of Aβ, we added $CuCl_2$ (17 μM) to the solution. This increased the magnitude of the response from the Aβ1–42 solution (*line d*), consistent with this potential being characteristic of a copper-Aβ complex. Background voltammograms of the PBS vehicle did not produce such peaks (*line a*), and Cu(II) in PBS showed only a Cu(II/I) peak in the reduction wave at ≈−80 mV (*line b*) along with the return oxidation wave. This reduction process was not observed when Cu(II) was added to Aβ1–42, consistent with the complete reaction of Cu(II) with Aβ.

Since Cu(I) can in principle reduce O_2, we tested Aβ peptides in the presence of Cu(II) (1 μM) for direct production of H_2O_2. We found that peroxide was indeed formed in these solutions,

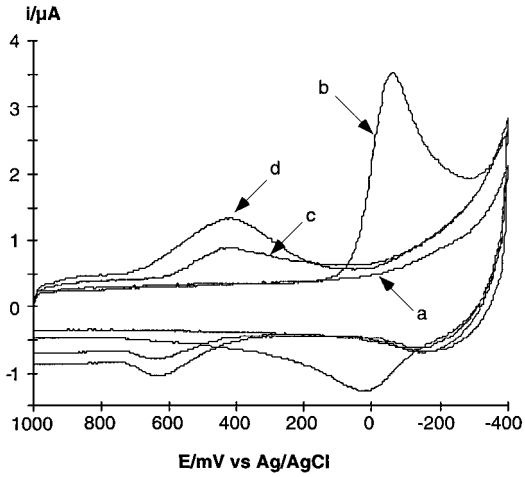

FIG. 3. **Electrochemical characterization of a complex formed between Aβ1–42 and copper.** Electrochemical analysis by cyclic voltametry of Aβ1–42 with and without added Cu(II). Response obtained from: *a*, Dulbecco's PBS, pH 7.3; *b*, Cu(II) (17 μM) in buffer; *c*, Aβ1–42 (100 μM) in buffer; *d*, Aβ1–42 (100 μM) with added Cu(II) (17 μM) in buffer. All cyclic voltammograms were obtained at an indium/tin oxide electrode with a Ag/AgCl (1 M KCl) reference electrode.

the amount of H_2O_2 produced in 1 h by the various Aβ and control peptides was greatest for Aβ1–42 (10 μM) > Aβ1–40 (7.5 μM) ≫ rat Aβ1–40, Aβ40–1, Aβ25–35, Aβ1–28, insulin, and amylin (≈0 μM) (Fig. 4*A*), paralleling the amounts of metal reduction by the same peptides (Fig. 1). Validation of these results was achieved by coincubating Aβ with catalase, which abolished the H_2O_2 signal in a dose-dependent manner, and also by performing a corroborating assay using dichlorofluorescein (data not shown).

To investigate whether the formation of H_2O_2 by Aβ was due to the specific 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 Cu(II) (Fig. 4*B*). 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 non-protein generation of Cu(I). This experiment confirmed that there was a significant dependence of H_2O_2 production upon the O_2 tension. The presence of either Aβ1–42 and Aβ1–40 generated significantly more H_2O_2 (Aβ1–42 > Aβ1–40) than vitamin C under any O_2 tension studied, and generated H_2O_2 under low O_2 tension where vitamin C produced none. Under ambient and argon-purged conditions in this system, the reduction of Cu(II) alone by the positive control, vitamin C, was insufficient to produce detectable H_2O_2. Therefore, Aβ facilitated the reduction of O_2 more than would be expected by the interaction of the Cu(I) 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 form H_2O_2. These data also suggest that copper cycles between the oxidized and reduced forms when bound to Aβ, since the presence of 1 μM Cu(II) was sufficient to produce a catalytic amount (10 μM) of H_2O_2 in 1 h, consistent with redox cycling and multiple electron donation events from Cu(I) to molecular O_2.

We examined Aβ1–42 (10 μM) in Cu(II) (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), nor nitro blue tetrazolium (NBT, 0.1 mM) detected O_2^- formation from Aβ, using xanthine (1 mM) with xanthine oxidase (0.015 units/ml) in PBS as a positive control.

To prove that Aβ-mediated H_2O_2 formation is metal-ion dependent, H_2O_2 production by Aβ1–42 in the presence of copper-selective chelators was assayed (Fig. 4*C*). The presence of 200 μM BC or diethylenetriaminepentaacetic acid abolished Aβ-

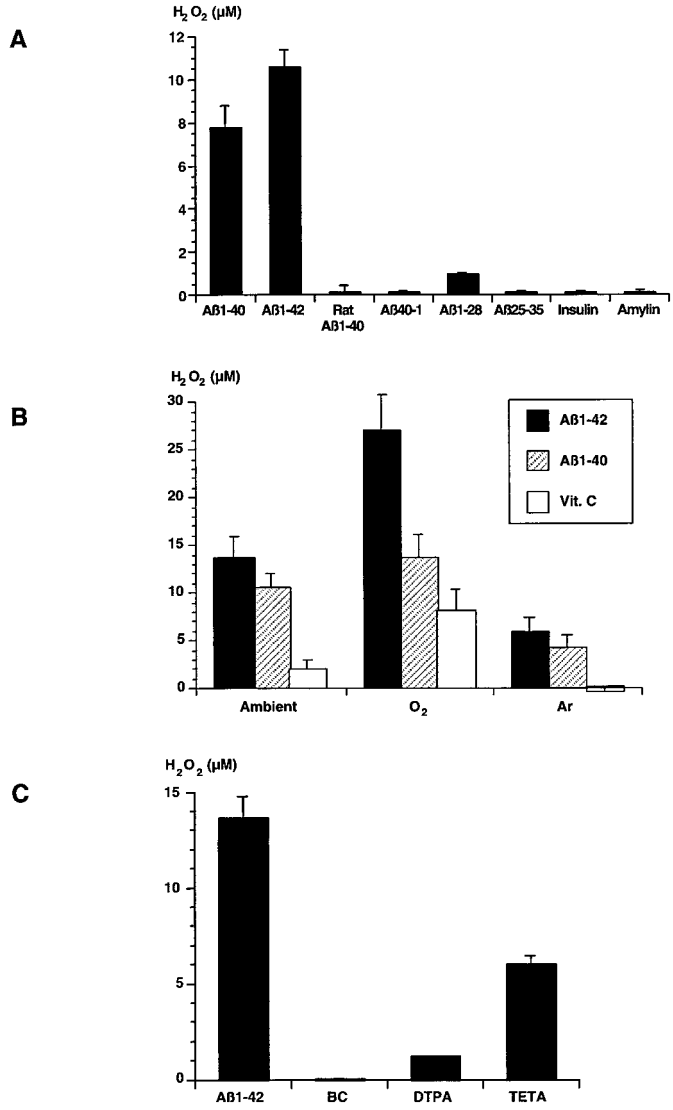

FIG. 4. **Production of H_2O_2 from Aβ in the presence of Cu(II).** *A*, comparison of H_2O_2 generation in 1 h by variant Aβ species: Aβ1–42, Aβ1–40, rat Aβ1–40, Aβ40–1, Aβ1–28, and Aβ25–35, and control peptides insulin and amylin (vehicle conditions as in Fig. 3*a*). *B*, H_2O_2 produced by Aβ1–42, Aβ1–40, and vitamin C in the presence of Cu(II) (1 μM) (in PBS, pH 7.4, 1 h, 37 °C) under various dissolved gas conditions. *Ambient*, no efforts were made to adjust the gas tension in the bench preparations of the buffer vehicle; O_2 and *Ar*, 100% O_2 or Ar were continuously bubbled through the PBS vehicle for 2 h (at 20 °C), before the remainder of the incubation components were added. *C*, metal ion dependence 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 Cu(II)-glycine (1 μM) was measured by the tris(2-carboxyethyl)phosphine hydrochloride assay (vehicle conditions as in *A*). All data points are means ± S.D., $n = 3$.

mediated H_2O_2 formation in the presence of 1 μM Cu(II). Triethylenetetramine dihydrochloride only decreased the formation of H_2O_2 by ≈50%, indicating that high affinity copper chelators may only be able to interrupt these interactions if they have sufficient stereochemical access to the bound copper atom.

We tested the consequences of Cu(II)-Aβ interaction upon the survival of primary neuronal cultures. We found that the combination of Aβ1–42 with Cu(II)-glycine (each at 10 μM) significantly potentiated Aβ neurotoxicity (70% cell death in the presence of copper, 40% in its absence) (Fig. 5*A*). The presence of catalase slightly rescued the toxicity of the peptide alone (30% cell death), but entirely rescued the fraction of Aβ1–42 toxicity that was enhanced by Cu(II), indicating that

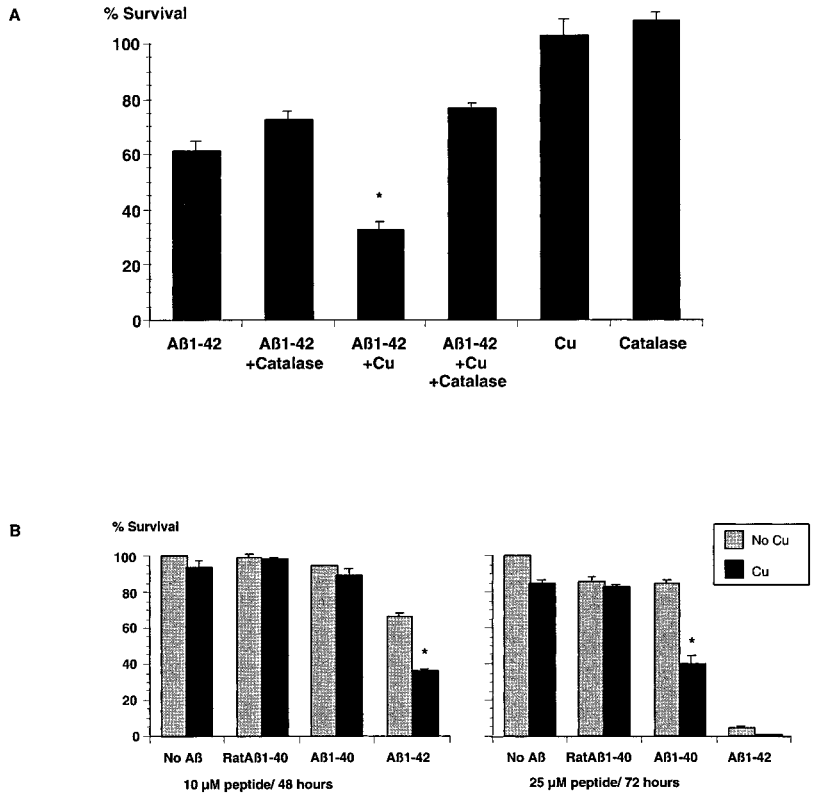

FIG. 5. **Cu(II) potentiates Aβ neurotoxicity through hydrogen peroxide formation.** *A*, Aβ1–42 (10 μM), Cu(II)-glycine (10 μM) and/or catalase (1000 units/ml) were applied to embryonic day 17 rat forebrain primary neuronal cultures, as indicated, and cell viability assayed after 48 h. *B*, Aβ peptide variants (concentrations indicated) were applied to embryonic day 17 rat forebrain primary neuronal cultures and cell viability assayed after 48 (*left panel*) or 72 (*right panel*) h. A longer incubation at a higher peptide concentration was required to exhibit the copper-potentiated neurotoxicity of Aβ1–40. Data indicate mean cell survival (percentage of untreated cultures) ± S.D., $n = 3$. *Asterisk* indicates a significant ($p < 0.01$) increase in Aβ neurotoxicity induced by Cu(II). Results are each typical of four experiments.

the potentiation of toxicity induced by the presence of Cu(II) was mediated by H_2O_2. Catalase could not completely rescue the toxicity of Aβ1–42, even when catalase was used at higher concentrations (2000 and 3000 IU, data not shown). Since Cu(II)-glycine alone was not neurotoxic, these results strongly support the possibility that Cu(II) interaction modifies Aβ leading to enhanced H_2O_2-mediated neurotoxicity. The inability of catalase to completely rescue the neurotoxicity caused by Aβ1–42 suggests that other neurotoxic mechanisms that are not mediated by H_2O_2 may contribute up to 25% of the lethality observed.

To confirm that Cu(II)-enhanced toxicity of Aβ was mediated by extracellular H_2O_2 production, we studied the effects of Cu(II)-glycine supplementation upon the toxicity of the other biologically occurring Aβ species in primary neuronal culture, comparing human Aβ1–42 and Aβ1–40 to rat Aβ1–40 (Fig. 5*B*). In the absence of additional Cu(II), Aβ1–42 was observed to be more neurotoxic than both human and rat Aβ1–40, whose lethal effects were indistinguishable and marginal at the concentrations tested (10–25 μM). However, whereas additional Cu(II) (10 μM) dramatically increased the toxicities of human Aβ1–42 and Aβ1–40, additional Cu(II) did not enhance the toxicity of rat Aβ1–40. The Cu(II)-induced potentiation of Aβ toxicity therefore followed the relationship of Aβ1–42 > Aβ1–40 ≫ rat Aβ1–40, which parallels both Cu(II) reduction, and the Cu(II)-mediated generation of H_2O_2, by the same peptides. Taken together, these data argue that Cu(II) enhances the neurotoxicity of Aβ substantially through the cell-free generation of H_2O_2.

DISCUSSION

The experiments described above establish that Cu(II) is reduced by Aβ peptides, that Cu(I) mediates O_2-dependent cell-free H_2O_2 generation, and that these properties directly correlate with the Cu(II)-mediated potentiation of Aβ neurotoxicity in cell culture. Previously, we showed that concentrations of Cu(II) at = 1 μM induce the aggregation of Aβ (17) and generation of TBARS reactivity (6). The amounts of aggregation (17), TBARS reactivity (6), production of Cu(I) and H_2O_2, as well as Cu(II)-enhanced neurotoxicity, are all greatest when generated by Aβ1–42 > Aβ1–40 ≫ rat/mouse Aβ1–40. This relationship also correlates with the relative participation of these peptides in amyloid pathology (10, 25), and in familial AD-associated Aβ processing (9, 26).

The absence of free Cu(II), determined by our EPR and electrochemical studies following addition of Aβ (Figs. 2 and 3), indicates that both Aβ1–40 and Aβ1–42 have a high affinity for Cu(II). In agreement with these findings, we recently estimated the K_d for Cu(II) binding to Aβ1–42 ($\approx 10^{-15}$ M) and Aβ1–40 ($\approx 10^{-10}$ M).[2] These observations strongly suggest the possibility of a mediating role for even traces of redox active metal ions in the neurotoxic properties of Aβ1–40 and Aβ1–42. Addition of Cu(II) and Aβ to cell cultures (Fig. 5, *A* and *B*) certainly increased the observed neurotoxicity. Since most of the added Cu(II) would be bound by the peptide within the culture medium, we can conclude that neurotoxicity was mediated via peptide-Cu(II) interactions rather than the effects of free Cu(II) upon the cells.

The capacities of biological Aβ peptides (Aβ1–42, Aβ1–40, and rat Aβ1–40) to reduce Cu(II) and to generate cell-free H_2O_2 (Figs. 1 and 4) correlated with Cu(II)-enhancement of the peptides' respective toxicities (Fig. 5*B*). This suggests that the enhanced toxicity caused by Cu(II) supplementation was mediated by cell-free H_2O_2 production, especially since the increase in toxicity caused by the presence of additional Cu(II) was rescued by catalase (Fig. 5*A*). However, we also observed that there was a component of neurotoxicity that could not be rescued by catalase (≈25%) and therefore may not have been mediated by cell-free H_2O_2 production (Fig. 5*A*). Our data indicate, therefore, that Aβ1–40/2 toxicity has a lethal compo-

[2] C. S. Atwood, R. C. Scarpa, R. E. Tanzi, and A. I. Bush, unpublished observations.

nent that is H_2O_2-mediated, and a lethal component that is not H_2O_2-mediated.

Recently, we found that Fe(III) is also reduced by Aβ (1–42 > 1–40 > rat Aβ1–40) (6), but our current data indicate that Cu(I) production under the same conditions, from the same concentrations of metal ions and peptide, occurs to a significantly greater extent than Fe(II) production. The stronger ability of Aβ to reduce Cu(II) to Cu(I) is supported by an unusually positive formal reduction potential for the Cu/Aβ1–42 complex, which is similar to that of small blue copper proteins (*e.g.* laccase type 1-blue, +555 mV, *versus* Ag/AgCl) and oxidases (24), placing it among the extremes of copper-based biological reducing activities.

The observation that Aβ1–28 did not reduce Cu(II) (Fig. 1) indicates that at least part of the hydrophobic carboxyl-terminal domain (residues 29–40, possibly the methionine at residue 35) is critical for the reduction properties of Aβ. Redox active cuproenzymes like superoxide dismutase usually bind the active-site Cu(II) within β-sheet and β-barrel structures (24). The increased β-sheet content caused by the two extra hydrophobic residues (Ile and Ala) on Aβ1–42 (30) may therefore increase the redox activity of Aβ by enhancing Aβ-copper interaction. Aβ-copper reduces O_2 to peroxide under conditions of low O_2 tension (Fig. 4*B*), such as those expected in the brain parenchyma. Since O_2 is preferentially dissolved in hydrophobic environments (14), the hydrophobic carboxyl terminus of Aβ may create a microenvironment that facilitates electron transfer from Cu(I) to O_2, thereby promoting more H_2O_2 production by Aβ1–42 than by Aβ1–40.

There are three ways in which H_2O_2 can form. A two-electron transfer to O_2 to generate the peroxide anion (O_2^{2-}), a one-electron transfer generating the superoxide anion ($O_2^{\cdot-}$) that subsequently undergoes dismutation to H_2O_2, or two one-electron transfers from each of two coppers to a bridging dioxygen molecule resulting in peroxide. The absence of a superoxide intermediate indicates that, as with the case Fe(III)-mediated H_2O_2 production (6), Cu(II)-mediated H_2O_2 production from Aβ must occur by either a double electron transfer from Cu(I) to O_2 (generating Cu(III)), or by two successive one-electron transfers. The simultaneous production of Cu(I) and H_2O_2 by Aβ raises the possibility of the hydroxyl radical ($OH^{\cdot}$) being formed by Fenton chemistry as reported for superoxide dismutase 1 (31), and we have recently determined that Aβ is carbonylated by the decay of $OH^{\cdot}$ in the presence of excess H_2O_2 and Cu(II).[3]

A key novel finding of this work is that the cell-free generation of H_2O_2 by Aβ depends upon only substoichiometric (catalytic) amounts of metal ion (d1:10, copper:Aβ), which lends credence to the possibility of these reactions occurring *in vivo*. The concentration of copper in the neocortex is ≈80 μM, and that released during synaptic transmission is deduced to be ≈15 μM (33). While free Cu(II) does not exist in the cytosol (34), extracellular Cu(II) may be more readily exchangeable (24). Cu(II) interaction with Aβ may be exaggerated in AD where copper levels are abnormally elevated in the neuropil (7, 36), cerebrospinal fluid (37, 38), and especially in amyloid where the concentration of copper has been measured at 30 μg/g (≈0.5 mM, dry weight) (7). Extraction of copper may be the basis of our recent discovery that copper-selective chelators, such as bathocuproine, facilitate the solubilization of Aβ from deposits in post-mortem AD brain specimens (8). Therefore, the abnormally high concentrations of copper in AD neuropil and in amyloid may reflect a pathogenic neurochemical milieu that both aggregates Aβ and induces H_2O_2 production. Since H_2O_2 freely crosses cell boundaries, it is likely that its dissemination from Aβ deposits will contribute to oxygen radical-mediated damage (39, 40), and inappropriate apoptotic signaling in AD brain. Copper-dependent production of H_2O_2 from Aβ accumulations in the brain should now be examined in the context of potential therapeutic intervention in AD.

[3] C. S. Atwood, X. Huang, R. E. Tanzi, and A. I. Bush, unpublished observations.

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Cu(II) Potentiation of Alzheimer Aβ Neurotoxicity: CORRELATION WITH CELL-FREE HYDROGEN PEROXIDE PRODUCTION AND METAL REDUCTION

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