Hydrogen Peroxide Is Generated during the Very Early Stages of Aggregation of the Amyloid Peptides Implicated in Alzheimer Disease and Familial British Dementia*

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Alzheimer disease and familial British dementia are neurodegenerative diseases that are characterized by the presence of numerous amyloid plaques in the brain. These lesions contain fibrillar deposits of the β-amyloid peptide (Aβ) and the British dementia peptide (ABri), respectively. Both peptides are toxic to cells in culture, and there is increasing evidence that early “soluble oligomers” are the toxic entity rather than mature amyloid fibrils. The molecular mechanisms responsible for this toxicity are not clear, but in the case of Aβ, one prominent hypothesis is that the peptide can induce oxidative damage via the formation of hydrogen peroxide. We have developed a reliable method, employing electron spin resonance spectroscopy in conjunction with the spin-trapping technique, to detect any hydrogen peroxide generated during the incubation of Aβ and other amyloidogenic peptides. Here, we monitored levels of hydrogen peroxide accumulation during different stages of aggregation of Aβ-(1–40) and ABri and found that in both cases it was generated as a short “burst” early on in the aggregation process. Ultrastructural studies with both peptides revealed that structures resembling “soluble oligomers” or “protofibrils” were present during this early phase of hydrogen peroxide formation. Mature amyloid fibrils derived from Aβ-(1–40) did not generate hydrogen peroxide. We conclude that hydrogen peroxide formation during the early stages of protein aggregation may be a common mechanism of cell death in these (and possibly other) neurodegenerative diseases.

There is mounting evidence for the importance of oxidative damage to the brain in a wide range of neurodegenerative diseases based on detection of markers such as elevated levels of redox-active transition metal ions, lipid peroxidation, DNA and protein oxidation, and the introduction of carbonyl groups into proteins (reviewed, for example, in Refs. 1–6). These are hallmarks of attack by reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and the hydroxyl radical. The β-amyloid peptide (Aβ), which is responsible for senile plaque formation in Alzheimer disease (AD), has been reported to generate hydrogen peroxide from molecular oxygen through electron transfer interactions with the spin-trapping technique, to detect any hydrogen peroxide generated during the incubation of Aβ-(1–40) and ABri and found that in both cases it was generated as a short “burst” early on in the aggregation process. Ultrastructural studies with both peptides revealed that structures resembling “soluble oligomers” or “protofibrils” were present during this early phase of hydrogen peroxide formation. Mature amyloid fibrils derived from Aβ-(1–40) did not generate hydrogen peroxide. We conclude that hydrogen peroxide formation during the early stages of protein aggregation may be a common mechanism of cell death in these (and possibly other) neurodegenerative diseases.

MATERIALS AND METHODS

Peptides and Electron Spin Resonance Spectroscopy—The Aβ-(1–40) peptide was purchased from BIOSOURCE International. The ABri and WT peptides were synthesized using a Milligen 9050 peptide synthesizer (PE Applied Biosystems Ltd., Cheshire, UK) (11). Oxidized and reduced forms of ABri and WT were prepared (11). Solutions (100 μM) of Aβ-(1–40), ABri, and WT in phosphate-buffered saline (PBS), pH 7.4, were prepared as described previously (12, 13) and incubated in Eppendorf tubes at 37 °C in complete darkness. After the required incubation time, a 50-μl aliquot of PBS containing aggregating peptide was removed, and solutions of DMPO (12.5 μM, 50 mM), diethylenetriaminepentaacetic acid (DTPA) (12.5 μL, 125 μM), and then Fe(II) sulfate (12.5 μL, 0.10 mM) were added. Immediately after mixing, the resulting solution was transferred to an ESR sample tube for detection of the DMPO- OH spectrum. All spectrometer settings were as detailed previously (13–15). Addition of catalase or DTPA (as metal ion chelator) to ABri was carried out as described previously for Aβ (13). Because DMPO, DTPA, and Fe(II) concentrations remain the same for the addition to each aliquot of preincubated peptide solution, the intensity of the ESR spectrum reflects the concentration of DMPO-OH formed at each time point. Calibration curves show that the intensity of the ESR spectrum increases with hydrogen peroxide concentration. Mature Aβ-(1–40) fibrils were prepared as described above with incubation for 72 h. At the end of the incubation period, the sample was centrifuged in a MSE HAWK 15/05 refrigerated microcentrifuge at 20,000 × g for 15 min at 5 °C. The resulting supernatant was removed, and the fibril pellet was resus-
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pended in fresh PBS and washed. This procedure was repeated before resuspension in the required final volume of fresh PBS solution.

ELISA for Aβ Oligomers—Non-biotinylated 6E10 antibody (initial concentration, 1 mg/ml; Signet Pathology Systems Inc.) was diluted 1:1000 in 200 mM NaHCO₃ (Sigma), pH 9.6, containing 0.02% (w/v) sodium azide, and 100 μl of this solution was dispensed to each well of a 96-well microplate (Fisher Scientific). It was then covered with a plate sealer and stored at 4 °C overnight. (Antibody 6E10 recognizes amino acid residues 1–17 of human Aβ.) The plate was then washed four times with PBS (Sigma) containing 0.5% Tween 20 (Sigma) (PBST) on a microplate washer. Blocking buffer (PBS, pH 7.4, containing 2.5% gelatin (Sigma) and 0.05% Tween 20) was added (200 μl/well), and the plate was incubated at 37 °C for 2 h. After washing four times with PBST, 100 μl/well aliquots of the samples to be tested (Aβ solutions were diluted to 1 μl/sample) were added to each well, and the plate was again incubated at 37 °C for 2 h. After washing four times with PBST, 100 μl/well biotinylated 6E10 antibody (1 mg/ml), diluted 1:1000 in blocking buffer, was added, and the plate was incubated at 37 °C for a further 2 h. The plate was then washed four times with PBST, incubated with 100 μl/well ExtrAvidin-alkaline phosphatase (Sigma) diluted 3:5000 in blocking buffer, and incubated for 1 h at 37 °C. Finally the plate was washed four times with PBST before adding 100 μl/well p-nitrophenyl phosphate substrate (Sigma). Absorbance values at 405 nm were measured after allowing the color to develop for 30 min at room temperature. Results are the mean of triplicate samples.

Thioflavin T Assay—Triplicate 10-μl samples of Aβ-(1–40) and Aβri (100 μM in PBS) were removed after incubation for various times at 37 °C. These samples were diluted into 190 μl of PBS containing 20 μM thioflavin T. Fluorescence was measured in black 96-well plates using a Victor 1420 multi-label microtiter plate reader with excitation at 440 nm and emission at 490 nm. To allow for background fluorescence, the fluorescence intensity of a blank PBS solution was subtracted from all readings. Results are the mean of triplicate samples.

Atomic Force Microscopy—Representative samples were selected for AFM analysis at various time points as required. These samples were diluted in ultrapure (Milli-Q) water so as to optimize the concentration of each sample prior to imaging. The best results were found with Aβ-(1–40) when the samples were diluted 300–600 times and with Aβri when the samples were diluted 20 times; no dilution was required with the 0-h samples. Each sample was prepared for analysis by pipetting either 5 μl of the Aβ-(1–40) solution or 10 μl of the Aβri solution onto a freshly cleaved mica-covered metal sample stub. This was placed into a Petri dish and allowed to dry in air prior to imaging. All of the AFM imaging was undertaken using a multimode AFM with a nanoscope IIIa controller in tapping mode in air with SiN₄ high spring constant cantilevers (TESP, Veeco, Cambridge, UK). A high resolution scanner (E-type scanner, Veeco) was used to allow the best possible resolution of deposits at early experiment times. Five randomly chosen areas were imaged on each sample, and the subsequent images then were carefully analyzed for ultrastructural differences.

Curve-fitting Procedure—The best-fit curves to the sigmoidal experimental aggregation data were obtained from the kinetic equation for a seeded growth process. Integration of this rate equation yields the standard solution,

\[
\left[ N \right] = \frac{\left[ M \right]_0 + \left[ N \right]_0}{1 + \left( \frac{\left[ M \right]_0}{\left[ N \right]_0} \right) e^{-k_1 t (\left[ M \right]_0 + \left[ N \right]_0)}} \tag{Eq. 1}
\]

where \([M]\) and \([N]\) represent the instantaneous concentrations of monomeric peptide and nucleated seeds, respectively, and \([M]_0\) and \([N]_0\) represent their initial concentrations at the commencement of incubation. The best-fit curves were obtained by optimizing these concentrations and the rate constant \(k_1\) for the reaction process at different times \(t\). (For a more detailed description of the seeded process see, for example, Ref. 20.)

The same kinetic equation was used to obtain the best-fit curves for the relative hydrogen peroxide concentration. Hydrogen peroxide decay was modeled assuming catalytic decomposition induced by redox-active transition metal ions in the solutions. The curves showing the “burst” of hydrogen peroxide production were obtained by differentiation of the above relative concentration curves (assuming no decay).

RESULTS

Correlation of Aggregation and Hydrogen Peroxide Formation for Aβ-(1–40)—Aβ-(1–40) was incubated (at 100 μM) in PBS for periods of up to 80 h. We focused most of our ESR measurements on the first few hours of incubation because preliminary experiments, together with our own previously published results (13, 17, 18), indicated that the DMPD-OH spectrum was usually observable after ~1 h. Aggregation was monitored by immunoassay (Fig. 1, A and B; curve a) and thioflavin T methods (Fig. 1D) and showed the typical sigmoidal profile observed previously with Aβ and other amyloids (20, 21), reaching a plateau after ~48–72 h. The early time lag in these profiles reflects the kinetic nature of the nucleation process and the sensitivity of the assay techniques. Later the curve rises rapidly with the formation of elongating fibrils before eventually reaching a plateau as more mature fibrils are formed and as the supply of monomeric species becomes exhausted. The time course for hydrogen peroxide formation was monitored by ESR spectroscopy (Fig. 1, A and B, curve b) and, again, showed three characteristic phases in DMPD-OH spectral intensity, i.e. a brief time lag before a spectrum was observed, followed by a rapid growth in spectral intensity (which for our samples reached a maximum after 2–3 h) and then a subsequent decay. A comparison of the two profiles (compare curves a and b in Fig. 1, A and B) shows them to be in complete contrast and that the generation of hydrogen peroxide (Fig. 1, A and B, curve c) occurs very early during the aggregation process and precedes the formation of mature amyloid fibrils.

Alongside our ESR measurements, samples of the incubated peptide were also examined at selected time points by AFM to obtain more detailed information on the nature of the fibrils present. Initially, Aβ-(1–40) (100 μM) was completely soluble in PBS, and no distinct AFM images were observed. However, further analysis at periods of significant hydrogen peroxide production (see Fig. 2B for appearance after 90 min) revealed the presence of small “protofibrils,” which were only the right size and at the right positions for amyloid fibrils to form. By 50 h, some clear fibrils were formed. By 70 h, most of the fibrils had extended, and only a few protofibrils were left. By 110 h, fibrils were not seen, and the remaining signal was the result of a molecular interaction between fibrils that we have not yet been able to correlate with fibril formation.

To examine the ability of fully formed amyloid fibrils to generate hydrogen peroxide, the Aβ-(1–40) peptide was incubated under the same conditions as before for a period of 72 h. The characteristic DMPD-OH spectrum was again detected in samples removed after 1 and 2 h, and mature amyloid fibrils were observed by electron microscopy (EM) and AFM at the end of this incubation period (data not shown). The fibrils were washed by repeated centrifugation followed by re-suspension of the pellet, and a sample of fibrillar peptide was incubated for up to 72 h at 37 °C in PBS at 100 μM. No DMPD-OH spectrum was obtained from samples removed during this incubation period and tested using our standard ESR protocol.

Correlation of Aggregation and Hydrogen Peroxide Formation for Aβri—Both the oxidized and reduced forms of Aβri and of the shorter WT peptide were incubated (at 100 μM) in PBS as described above. ESR spectra were only obtained from the oxidized form of Aβri with an intact intramolecular disulphide bond (for typical spectra see Fig. 3). No spectra were observed with the other three related peptide treatments even after prolonged incubation over a 48-h period. As for Aβ (13), the formation of the DMPD-OH spectrum from the oxidized form of Aβri was blocked by catalase or DETAPAC, showing that hydrogen peroxide is formed by a metal-dependent mechanism. With this peptide, detailed ESR measurements were undertaken over a 2-h period because preliminary experiments employing this peptide indicated that this was the period during which the DMPD-OH ESR spectrum was observable. The time course for hydrogen peroxide formation and decay again followed the characteristic three phases noted above. In this case, however, the production and decay of the DMPD-OH spectrum was far more rapid than that observed during the aggregation of Aβ-(1–40) (see Fig. 1C) with the ESR spectrum no longer observable after 2 h of incubation.

AFM images were again obtained at selected time points to gain more detailed information on the nature of the aggregates present after various incubation periods (see Fig. 2, D–F). After 30 min, correspondingly to relatively high hydrogen peroxide levels, only small, roughly spherical forms (some of which were aggregated into short chains (120–170 nm in length) were present (Fig. 2E). After longer incubation periods (90 min, see Fig. 2F), when hydrogen peroxide levels declined, large amorphous “clumps” of aggregates were seen with no evidence of protein fibrils. The thioflavin T binding assay was used to investigate if these aggregates contain the characteristic β-sheet pleated sheet structure of amyloid. As shown in Fig. 1D, the aggregates formed by Aβri in PBS (pH 7.4) did not bind to thioflavin T. This was found to be the case even after a prolonged incubation period of up to 72 h (see Fig. 1D).

DISCUSSION

The major objective of these investigations was to obtain information on how the self-generation of hydrogen peroxide during incubation of both Aβ-(1–40) and Aβri relates to the extent of peptide aggregation.
Fig. 1, A and B (curve c), clearly shows that for Aβ-(1–40) the time period over which the generation of hydrogen peroxide occurs corresponds to a period when the AFM images (Fig. 2 B) indicate the presence of structures closely resembling previously described protofibrils (22). The failure to detect a DMPO-OH spectrum during the incubation of mature Aβ/H9252-(1–40) amyloid fibrils and the reduced levels of hydrogen peroxide observed during the later stages of amyloid fibril growth both strongly support the concept that hydrogen peroxide generation is an early event. Our results, therefore, show that hydrogen peroxide is not generated continuously throughout the aggregation of Aβ-(1–40) but as a short “burst” comparatively early on during the peptide incubation period when mature amyloid fibrils are not present.

Numerous previous studies have shown that Aβ that has been allowed to aggregate at concentrations similar to those employed here followed by addition to cultured cells at low micromolar concentrations is toxic. The molecular mechanisms responsible for this effect are unclear, but the addition of Aβ to cells causes calcium ion influx, oxidative free radical damage, and apoptosis, and it has been reported that toxicity can be inhibited by catalase, free radical scavengers, or antioxidants (see Ref. 23 for review). This is consistent with exposure of cells to hydro-
gen peroxide or other ROS. There is still no clear consensus on the precise nature of the toxic form of Aβ, but attention has focused recently on early protein assemblies (protophilobol, soluble oligomers, Aβ-derived diffusible ligands (ADDLs), or globular neurotoxins) as the potential culprit (22, 24–28). In accord with this, our data suggest that Aβ toxicity could be because of the generation of hydrogen peroxide by an early form of protein aggregate. However, it is also possible that hydrogen peroxide is generated as a “by-product” of the early stages of the aggregation process itself. The latter would be compatible with a recent report closely relating Aβ toxicity to protein aggregation (29). The formation of hydrogen peroxide during early aggregation could be due, at least in part, to the formation of ditryosine cross-links (30).

The relevance of the data on Aβ toxicity to neurodegeneration in vivo in AD is uncertain. However, there is now substantial evidence indicating that oxidative damage to the brain is one of the earliest pathological events in AD (23). Our results raise the intriguing possibility that this could be because of the generation of hydrogen peroxide during the early stages of Aβ oligomerization before the formation of substantial numbers of “mature” senile plaques. In the presence of redox-active metal ions, hydrogen peroxide is readily converted via the Fenton reaction into the highly reactive hydroxyl radical, which could be responsible for much of this early oxidative damage. Because oxidative stress can increase the neuronal production of Aβ (23), the presence of hydrogen peroxide (and the hydroxyl radical) could set up a potentially catastrophic positive feedback mechanism whereby Aβ oligomers stimulate their own production. The amount of hydrogen peroxide generated by Aβ can be considerably enhanced by co-incubation with a reducing substrate (31–33). If this substrate is cholesterol, the resulting oxidation product is 7β-hydroxycholesterol, which is neurotoxic at nanomolar concentrations and so could also contribute to oxidative brain damage (33). The average concentrations of “soluble” and “immobile” Aβ in the brain in AD have been reported to be around 75 μM and 5 μM, respectively (34). The focal concentrations of Aβ in the vicinity of the “primitive” senile plaque would be expected to be considerably greater than this. Aβ is capable of generating equimolar concentrations of hydrogen peroxide (7). Because submicromolar concentrations of hydrogen peroxide can kill cells over long exposure times (35), the quantities of ROS formed during Aβ aggregation could be sufficient to induce neurodegenerative changes in the brain over the extended time periods involved in the development and progression of AD.

Recently, it has been reported that very low concentrations (1–2 nM) of cell-derived soluble oligomers of Aβ can inhibit long term potentiation and disrupted learning behavior in rats (36, 37). However, these conditions have not been shown to lead, ultimately, to neuronal cell death, and so the relevance of our observations to these reports is not clear. However, we note that hydrogen peroxide can affect synaptic plasticity by acting as a diffusible signaling molecule (38).

We have also discovered that hydrogen peroxide is generated by the oxidized form of ABri but not by the reduced form of this peptide or by the corresponding WT peptide. This finding correlates well with the toxicity of these peptides to neuronal cells. Recent studies with the same batches of ABri and WT peptides as those used here have shown that only the oxidized form of ABri is toxic to the neuronal cells and the redox properties of ABri being non-toxic (11, 12). Our experiments with other amyloidogenic proteins and peptides, including Aβ, α-synuclein, and various forms of the prion protein (PrP), have also demonstrated a good correlation between the generation of hydrogen peroxide and cytotoxicity (13–18). Previous studies on the oxidized form of Aβ have indicated that it is more toxic during the early stages of aggregation when soluble oligomers are present than after prolonged incubation periods (11, 12). Our results showing that only small oligomers of Aβ are present during the active phase of hydrogen peroxide formation are in accord with this. The ultrastructural appearance of the ABri oligomers detected by AFM (Fig. 2F) is very similar to that observed previously by EM (12). After prolonged incubation in PBS, we found that ABri formed large, irregularly shaped clumps (Fig. 2F) that did not bind to thioflavin T, indicating a lack of β-sheet fibrillar structure. This agrees with previous reports that, at neutral pH, Aβi does not aggregate into typical amyloid fibrils but instead forms amorphous aggregates with reduced thioflavin T binding properties (9) and a lack of Congo red birefringence (12). Again, the AFM appearance of the aggregates seen here is very similar to that reported previously using EM (compare Fig. 2F with Fig. 8c in the paper by El-Agnaf et al. (12)). The fact that hydrogen peroxide was still formed from ABri oligomers under these conditions emphasizes the fact that hydrogen peroxide formation does not appear to be associated with the presence of mature amyloid fibrils. Finally, we stress that many of the features discussed above may not be unique to AD and FBD but could well have relevance to other neurodegenerative diseases associated with protein aggregation.

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