NeuroReport 6, 493–496 (1995)

Amyloid β-peptide spin trapping II: evidence for decomposition of the PBN spin adduct

Kenneth Hensley,[1] Marina Aksenova,[1] John M. Carney,[1] Marni Harris[1] and D. Allan Butterfield[CA]

Department of Chemistry and Center of Membrane Sciences and [1]Department of Pharmacology, University of Kentucky, Lexington, KY 40506, USA

[CA] Corresponding Author

SYNTHETIC β-amyloid peptides (Aβs) react with the spin trap phenyl-*tert*-butyl nitrone (PBN) to form products detectable by electron paramagnetic resonance (EPR) spectroscopy. At least two EPR-detectable products can be distinguished from the Aβ/PBN reaction, and peptide toxicity towards glutamine synthetase enzyme correlates with the type of PBN reaction product observed. We have reacted synthetic Aβ(25–35) peptide with [^{12}C]- or [^{13}C]PBN to demonstrate that the two products represent alternate pathways of spin adduct decomposition. Results indicate that the C=N bond of PBN is cleaved by Aβ in what we hypothesize is a radical addition–fragmentation reaction.

Key words: Amyloid; Spin trapping; Phenyl-*tert*-butyl nitrone; Oxidation

Introduction

Synthetic amyloid beta peptides (Aβs) generate reactive oxygen species (ROS), produce free radical spin adducts and promote oxidation of enzymes and cell membrane components.[1–4] No mechanism has been proposed to explain this unique reactivity inherent in toxic Aβ peptides. We report in the accompanying paper that samples of Aβ(25–35) and Aβ(1–40) which generate three-line EPR spectra upon reaction with PBN are much more toxic to pure glutamine synthetase (GS) than peptide samples which generate four-line spectra, and that rare peptide samples which do not react with PBN to generate EPR spectra have no GS toxicity. This is the first well-defined chemical difference in reactivity among variably toxic synthetic Aβs, and may provide valuable insight into the radicalization of synthetic and possibly natural Aβ.

We now explain the three- and four-line PBN reaction products as nitroxide decomposition products of a peptidyl radical spin adduct. Reaction of Aβ(25–35) with [^{13}C]PBN indicated cleavage of the ^{13}C=^{14}N bond upon reaction with the peptide. Reaction of the four-line generating Aβ(25–35) variant with PBN in D_2O medium resulted in spectroscopic ^{2}H–^{14}N coupling indicative of a hydronitroxide. Another common nitrone spin trap, dimethyl-pyrroline-N-oxide (DMPO), was also found to yield a three-line EPR spectrum upon treatment with Aβ(25–35). These results suggest that Aβ reaction with C=N bonds may be significant steps in Aβ peptide-mediated free radical toxicity.

Materials and Methods

Aβ peptides: Peptides were prepared by t-BOC solid phase synthesis (Bachem Chemical, Torrence, CA) and verified by HPLC and amino acid analysis following synthesis. The sequence of Aβ(25–35) is GSNKGAIIGLM. Peptides were stored in the dry state below – 10°C when not in use.

EPR spin trapping: PBN spin trapping experiments were conducted as previously described (accompanying paper). [^{12}C]- and [^{13}C]PGN were provided by Centaur Pharmaceuticals (Sunnyvale, CA). In DMPO spin trapping experiments, DMPO (Sigma) was repurified by filtration through charcoal and 50 mM DMPO solution was then made by addition of DMPO to chelexed PBS, and added to peptide to give 1 mg ml^{-1} Aβ. EPR spectra were obtained with a Bruker 300 EPR spectrometer (gain = 5 × 10^5 modulation amplitude = 0.3 G, time constant = 1.28 ms, conversion time = 10.28 ms).

Results

A spin trap is a non-paramagnetic species which reacts with a transient free radical to form a more stable, EPR-detectable species termed a spin adduct. PBN is a phenyl nitrone spin trap (Fig. 1A). Most PBN spin adducts give six-line EPR spectra due to magnetic coupling of the unpaired electron with the ^{14}N nucleus (giving three lines) and further electron-nuclear coupling to the adjacent β-H nucleus (splitting each ^{14}N line into a doublet).[5] The EPR linewidth of a paramagnetic species is exquisitely sensitive to the rate of molecular tumbling and the size of the paramagnetic molecule. Slow isotropic tumbling of very large radicals (e.g. protein-bound nitroxides) engenders EPR line broadening. If the size of the PBN spin adduct is extremely large and β-H coupling is very small, motional line

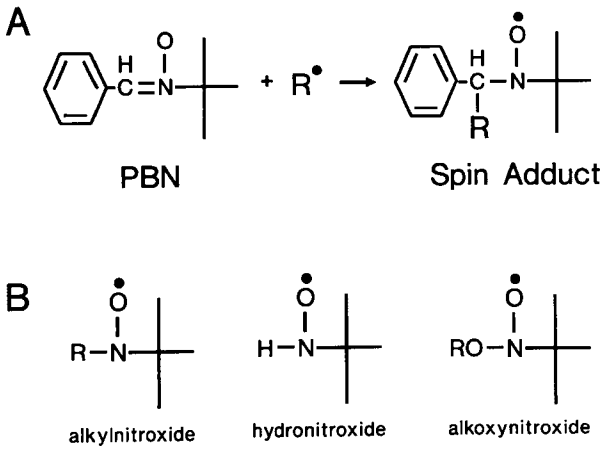

FIG. 1. (A) Reaction of PBN with a transient free radical R* to form a spin adduct (typically characterized by a six-line EPR spectrum). (B) Possible decomposition products of the spin adduct include alkyl- or arylnitroxide (three or more lines depending on the nature of R), alkoxynitroxide (three lines), or hydronitroxide (four lines).

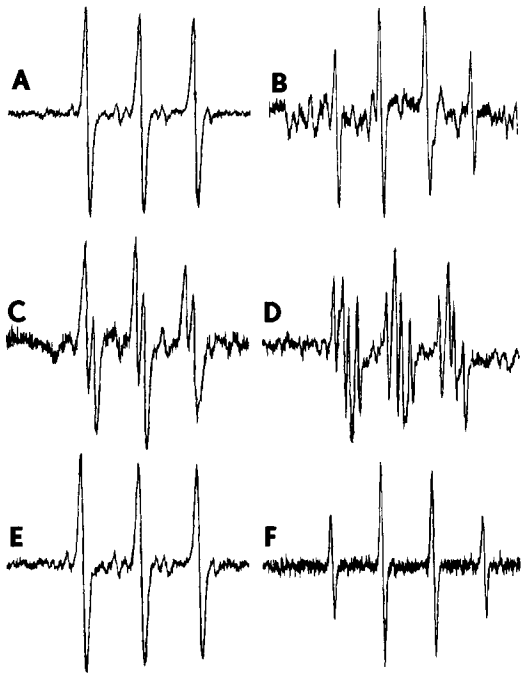

FIG. 2. (A) Aβ(25–35)-A/[^{12}C]PBN reaction product. (B) Aβ(25–35)-B/[^{12}C]PBN reaction product. (C) [^{12}C]PBN *OH adduct generated in 1 mM H_2O_2/100 μM Fe^{2+}/50 mM PBN incubate. (D) [^{13}C]PBN *OH adduct. (E) Aβ(25–35)-A/[^{13}C]PBN reaction product. (F) Aβ(25–35)-B/[^{13}C]PBN reaction product. Note that spectra A and E are indistinguishable, as are spectra B and F.

broadening may produce an apparent three-line spectrum. Alternatively, deviation from the expected six-line PBN spectrum may indicate decomposition of the spin adduct to yield secondary reaction products such as alkyl-, aryl-, alkoxy-, or hydronitroxides (Fig. 1B). In each case, the number of spectral lines and the splitting constants depend on the identity of the nuclei in the immediate vicinity of the ^{14}N center. EPR linewidth, as noted, depends primarily on the size of the nitroxide substituents.

Reaction of PBN with EPR-active Aβ(25–35) yields either a three-line EPR spectrum within 10 min (Fig. 2A, highly GS-toxic peptide) or a four-line spectrum after several hours (Fig. 2B, moderately GS-toxic peptide). The yield of EPR-detectable product is approximately 1 mol product per 25 mol peptide based on comparison of spectral intensities with stable nitroxide standards. We have operationally defined the three-line generating and highly toxic peptide variant Aβ(25–35)-A, and the four-line generating less toxic variant Aβ(25–35)-B. EPR inactive and non-toxic peptides were termed Aβ(25–35)-C. The ^{14}N electron-nuclear hyperfine coupling (hfc) in the three-line spectrum is a_n = 17.1G, the linewidth is broad (ΔH = 1.6 G), and there is no resolvable β-H coupling. Loss of β-H coupling could result from fragmentation of the spin adduct and substitution of the β-proton with a magnetically inactive nucleus.

In order to determine that the three-line nitroxide product results from PBN decomposition rather than pure motional effects, we reacted Aβ(25–35)-A and Aβ(25–35)-B with [^{13}C]PBN in which the carbon of the C=N nitrone bond was isotopically substituted. ^{13}C, unlike ^{12}C, has a non-zero nuclear spin (I = 1/2) and, therefore, can magnetically couple to the nitroxide center increasing the number of spectral lines by a factor of two (a_{13C} = 4 G). This fact was verified by comparison of [^{12}C]PBN and [^{13}C]PBN *OH adducts generated by the Fenton reaction in an Fe^{2+}/H_2O_2 incubate (Fig. 2C, D). Cleavage of the ^{13}C=^{14}N bond is the only means of uncoupling the two nuclei and reducing spectral multiplicity. The class A and class B Aβ(25–35) [^{13}C]EPR spectra were identical to corresponding [^{12}C]PBN products (Fig. 2), demonstrating that in both cases the C=N nitrone bond in PBN is broken during reaction with Aβ.

We considered that the three-line Aβ(25–35)-A/PBN product may simply be di-*tert*-butylnitroxide (di-tBN). This possibility was ruled out by comparison with authentic di-tBN formed by incubating nitroso-*tert*-butane dimer in H_2O at 37°C overnight until the solution turned light blue, indicating di-tBN presence.[5–7] The authentic di-tBN EPR spectrum consisted of three lines with nitrogen hfc a_N = 17.1 G and extremely narrow linewidth (ΔH = 0.5 G), exactly as reported in the literature (Fig. 3B).[5–7] Based on the much greater linewidth of the Aβ(25–35)-A/PBN reaction product, we conclude that this product is not di-tBN.

We have found that three-line generating Aβ(25–35) reacts with nitrone bonds in molecules other than PBN. Incubation of 1 mg ml^{-1} Aβ(25–35)-A with 50 mM DMPO for 1 h produced a weak three-line EPR spectrum with a_N = 17.1 G, similar to the PBN/Aβ

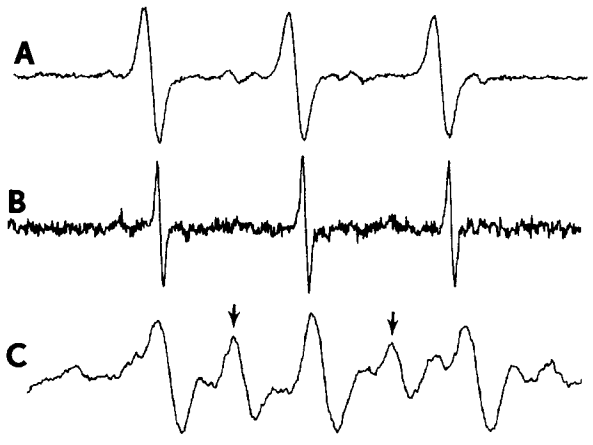

FIG. 3. (**A**) EPR spectrum of Aβ(25–35)-A/PBN reaction product. (**B**) EPR spectrum of authentic di-*tert*-butylnitroxide. (**C**) EPR spectrum of Aβ(25–35)-A/DMPO reaction product. Arrows indicate unremovable DMPO impurity.

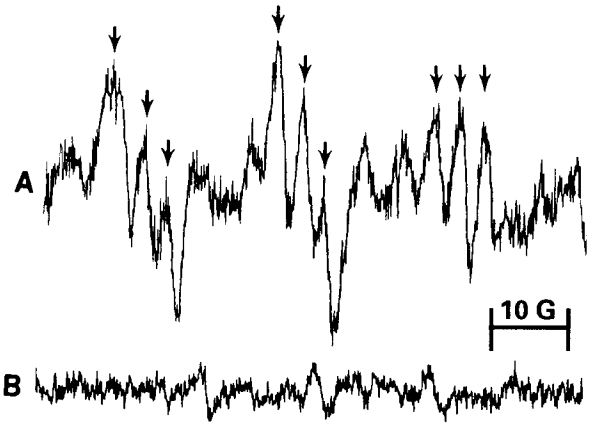

FIG. 4. (**A**) EPR spectrum of PBN incubated for 3 h at 37°C with 1 mg ml^{-1} Aβ(25–35)-B in phosphate buffered D_2O. Arrows indicate resonance peaks. (**B**) EPR spectrum of PBN/D_2O background.

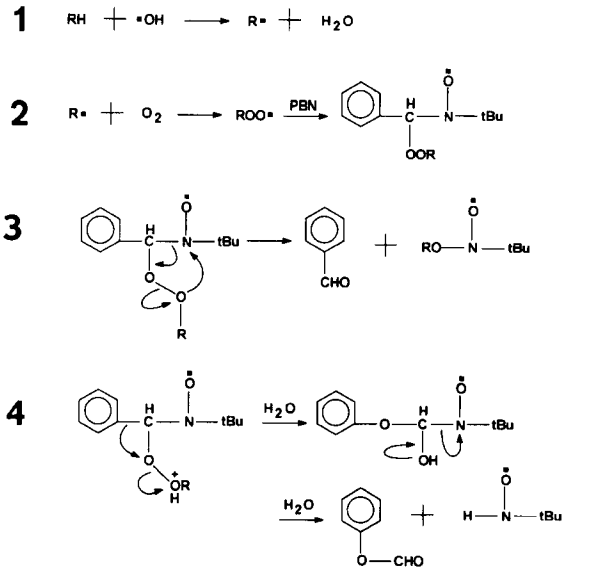

FIG. 5. Possible mechanisms for decomposition of a putative peptidyl peroxy-PBN adduct. **1**. Metal-catalyzed formation of peptidyl radical center. **2**: Addition of O_2 to form peroxyl radical and subsequent reaction with PBN. **3**: Rearrangement/decomposition to yield an alkoxynitroxide (three-line EPR spectrum). **4**: Decomposition pathway yielding *tert*-butyl-hydronitroxide (four-line EPR spectrum).

reaction product (Fig. 3C). In our experience, Aβ is the only reagent which can produce identical PBN and DMPO EPR spectra.

The hpc and linewidth of the 4-line Aβ(25–35)-B/PBN reaction product were consistent with those reported for *tert*-butyl-hydronitroxide ($a_N = a_H = 14.5$ G, $\Delta H = 0.9$ G).[5,6] In order to validate further the hypothesis of a hydronitroxide presence, Aβ(25–35)-B was reacted with PBN in D_2O. Under these conditions deuterium exchange with the putative hydronitroxide proton would result in a spectrum consisting of a triplet of triplets. The nine-line EPR spectrum was observed as predicted (Fig. 4). This spectrum is expected to be very weak due to perturbation by the electric quadrupole of the ^2H nucleus.

We have found Aβ(1–40) to be more reliable than the Aβ(25–35) fragment with respect to PBN reactivity. We have identified only one shipment of Aβ(1–40) (out of many peptide shipments spin trapped) which failed to generate a three-line spectrum. This particular Aβ(1–40) peptide (Bachem lot ZL831) was not toxic to GS, and generated a four-line EPR spectrum when reacted with PBN (accompanying paper). Although the four-line Aβ(25–35)-B/PBN reaction product resembles *tert*-butyl-hydronitroxide, this is not the case for the four-line spectrum observed when inactive Aβ(1–40) is reacted with PBN. The latter spectrum has the same hfc as the Aβ(25–35)-B spectrum; however the linewidth is almost three times greater ($\Delta H = 2.4$ G *vs* 0.9 G). Hence, the alkyl group in the Aβ(1–40)/PBN reaction product is probably much larger than a *tert*-butyl moiety.

Discussion

We present evidence in this work that EPR-detectable Aβ/PBN reaction products are stable nitroxides or hydronitroxides formed from peptide-mediated cleavage of the PBN nitrone bond. This novel pattern of reactivity is consistent with the hypothesis of an Aβ-peptidyl peroxy radical species.

An explanation for the unusual three- and four-line Aβ/PBN signals is decomposition of the Aβ/PBN spin adduct to form an aryl- or alkoxynitroxide (three lines, 1:1:1 intensity) or a hydronitroxide (four lines, 1:2:2:1 intensity). Consideration of the [^{2}H]-exchangeability of the four-line generating Aβ/PBN product and the uncoupling of the [^{13}C]PBN $^{13}C{=}^{14}N$ bond upon Aβ reaction supports the decomposition hypothesis. The existence of two decomposition pathways implies that either two different peptidyl radicals

can exist (one which reacts with PBN to give a three-line species and one which reacts to give a four-line product), or that peptide structural considerations (i.e. the stereoelectronic environment about the PBN reaction site) determine the decomposition pathway. The structural features which influence reactivity towards PBN could also effect peptide toxicity (accompanying paper).

It is not possible at this point to identify the peptide residue on which the primary peptidyl radical exists. One likely position for this radical center is a branched-chain leucine (Aβ residue 34) or isoleucine residue (Aβ residues 31 and 32), wherein a tertiary carbon-centered radical could be stabilized by hyperconjugation and steric protection from adjacent methyl or ethyl groups. Formation of a putative quasi-stable leucyl radical would require H abstraction, perhaps by *OH radical inadvertently generated during peptide synthesis and lyophilization procedures (i.e. before the peptide is shipped to the researcher). Generation of *OH in the peptide/PBN incubate is unlikely give the inability of metal chelators to inhibit the Aβ/PBN reaction.[1] We have also considered the possibility of methionine sulfoperoxy radical presence stemming from reaction of the Aβ Met-35 residue.[1,3] A radical center on Leu-34 of the Aβ sequence could catalyze oxidation of Met-35 to the sulfoxide which forms during incubation of Aβ(25–35).[2] There would be no way to directly detect small amounts of a peptidyl radical in the solid lyophilate.

In Figure 5 we present plausible schemes that are consistent with EPR data regarding the Aβ/PBN reaction. As previously reported,[1] the Aβ/PBN reaction depends on the presence in buffer of dissolved O_2. Addition of O_2 to a quasi-stable peptidyl radical would form a reactive peroxyl radical capable of adding to the PBN C=N bond. The nucleophilic oxygen on the resulting peroxide moiety is primed to attach the nitroxide center, causing adduct fragmentation and yielding a *tert*-butyl-alkoxynitroxide (three lines) and benzaldehyde (reaction 3). The rearrangement would be driven by carbonyl formation and an entropic factor; the estimated reaction enthalpy for this process is – 22 kcal mol^{-1} based on summation of bond dissociation enthalpies. Alternative decomposition pathways (e.g. reaction 4) could yield *tert*-butyl-hydronitroxide (four lines). Rearrangement of phenyl-peroxides similar to reactions 3–4 have been previously documented.[8] Other rearrangements not presented in Figure 5, including aryl-shift to the nitroxide center, may be possible. In particular, Janzen *et al* have proposed a variety of mechanisms by which PBN oxy-radical adducts may decompose to hydronitroxide species.[7,9]

Further study of Aβ solution chemistry, including investigations of inactive variant forms of the peptide, may yield important insights into Aβ peptide reactivity and toxicity. Such studies are currently under way in our laboratory.

Conclusions

PBN fragments upon reaction with synthetic Aβ peptides. The three-line EPR spectrum generated from PBN reaction with Aβ(25–35) or Aβ(1–40) probably represents a *tert*-butyl-alkoxynitroxide species, but is not di-*tert*-butylnitroxide. The four-line species formed from Aβ(25–35)/PBN reaction is EPR-spectroscopically identical to *tert*-butyl-hydronitroxide. The four-line Aβ(1–40)/PBN reaction product represents a different hydronitroxide species. C=N bonds in other molecules, such as DMPO, are also susceptible to Aβ attack.

References

1. Hensley K, Carney JM, Mattson MP *et al. Proc Natl Acad Sci USA* **91**, 3270–3274 (1994).
2. Harris ME, Hensley K, Butterfield DA *et al. NeuroReport* **6**, 489–492 (1995).
3. Butterfield DA, Hensley K, Carney JM *et al. Biochem Biophys Res Commun* **200**, 710–715 (1994).
4. Behl C, Davis JB, Lesley R *et al. Cell* **77**, 817–827 (1994).
5. Buettner GR. *Free Rad Biol Med* **3**, 259–303 (1987).
6. Chamulitrat W, Jordan SJ, Mason RP *et al. J Biol Chem* **268**, 11520–11527 (1993).
7. Kotake Y and Janzen EG. *J Am Chem Soc* **113**, 948–951 (1991).
8. Yablokov VA. *Russ Chem Rev* **49**, 833–842 (1980).
9. Janzen EG, Lin CR and Hinton RD. *J Org Chem* **57**, 161–163 (1992).

ACKNOWLEDGEMENTS: We thank Dr Kirk Maples of Centaur Pharmaceuticals for synthesis and provision of [^{13}C]PBN. This work was supported in part by grants from NSF (EHR-9108764; CTS-9307518) and NIG (AG-10836).

Received 31 October 1994; accepted 9 December 1994