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Amyloid β-peptide spin trapping I: peptide enzyme toxicity is related to free radical spin trap reactivity

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SYNTHETIC β-amyloid peptides (Aβs) demonstrate lot-to-lot variation in toxicity that has not been adequately explained. Studies from our laboratory have shown that Aβ toxicity may result from the ability of the peptide to promote oxidation reactions. Both Aβ(1–40) and Aβ(25–35) inactivate the oxidation-sensitive enzyme glutamine synthetase (GS) and generate electron paramagnetic resonance (EPR)-detectable products upon reaction with the spin trap phenyl-*tert*-butylnitrone (PBN). We now report that samples of synthetic Aβ(1–40) and Aβ(25–35) with attenuated toxicity with respect to peptide-induced GS inactivation, produce qualitatively different EPR spectra when the peptides are incubated with PBN. The results suggest an interpretation of conflicting observations regarding the toxicity of synthetic Aβs, and that investigators must be careful to assess the reactivity state of Aβ being studied.

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

Introduction

β-Amyloid peptides (Aβs) are neurotoxic peptides postulated to be involved in the etiology of Alzheimer's disease (AD). The mechanism of neurotoxicity of Aβs has remained elusive for some time. We recently reported that synthetic Aβ(1–40) and the fragment Aβ(25–35) both react with the spin trap phenyl-*tert*-butyl nitrone (PBN) to generate a nitroxide product, indicating the presence of a peptidyl radical.[1] In solution, Aβ(1–40) and Aβ(25–35) both react with salicylate to form 2,3-dihydroxybenzoate, implying the presence of a reactive oxygen species (ROS) in the peptide incubate.[1,2] In brain cytosolic extracts, the oxidation-sensitive enzymes glutamine synthetase (GS) and creatine kinase (CK) are rapidly inactivated upon exposure to Aβ, suggesting that Aβ toxicity may be a consequence of its ability to promote oxidation reactions.[1,2] Furthermore, Aβ(1–40) and Aβ(25–35) produce increased levels of oxidized proteins and peroxides in hippocampal neuronal cultures and in cultured PC12 cells and react with peroxidation-sensitive membrane probes in rodent synaptosomal preparations.[1–4]

A troubling aspect of research involving Aβ peptides has been lot-to-lot variability in toxicity of synthetic Aβ(1–40). This variability apparently is not due to peptide impurities.[5,6] We have found that Aβ(25–35), like Aβ(1–40), is variable with respect to its toxic potential. In order to examine peptide variability we performed EPR and enzyme toxicity studies on different aliquots and synthetic lots of Aβ. We observed a striking correlation between toxicity of Aβ toward GS and peptide reactivity towards PBN. Highly toxic samples of both Aβ(1–40) and Aβ(25–35) produced characteristic three-line EPR spectra upon reaction with PBN. Anomalous Aβ samples, which demonstrated reduced toxicity toward GS, reacted with PBN to produce a four-line EPR spectrum. Furthermore, Aβ(25–35) samples which showed essentially no toxicity toward GS, generated no significant EPR signal upon incubation with PBN. These observations may partially explain the observed variability in Aβ toxicity.

Materials and Methods

Aβ peptides: Peptides used in these studies were prepared by t-BOC solid phase synthesis (Bachem Chemical, Torrence, CA) and verified by high performance liquid chromatography (HPLC) and amino acid analysis following synthesis. The primary sequence of Aβ(25–35) is GSNKGAIIGLM. Aβ(25–35) samples used in these experiments originated from Bachem lot ZJ744 or Bachem lot ZL650. Aβ(1–40) was Bachem lot ZL831. Peptides were stored in the dry state below −10°C when not in use, unless otherwise noted. PBN was obtained from Centaur Pharmaceuticals (Sunnyvale, CA).

EPR spin trapping: In each of the spin trapping experiments, 300 μg samples of the peptide were solubilized in 300 μl of 50 mM PBN in phosphate buffered saline (PBS: 150 mM NaCl, 10 mM $NaHPO_4$, 10 mM NaH_2PO_4, pH 7.4). PBS was stirred overnight in the presence of Chelex-100 resin (Sigma) prior to use, in order to minimize trace metal contamination. 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).

Glutamine synthetase assay: GS activity was determined by the method of Rowe *et al*[7] as modified by

Miller *et al*[8] and corrected for non-specific glutaminase activity by comparison of activity in the presence and absence of ADP and arsenate. GS enzyme (sheep brain, Sigma) and Aβ(25–35) were solubilized to a final concentration of 0.014 mg ml^{-1} and 1 mg ml^{-1}, respectively, and coincubated in deionized water for 1 h at 37°C prior to assay. Aβ(1–40) was preincubated in water at 2 mg ml^{-1} and 37°C for 24 h prior to GS addition.

HPLC/amino acid analysis: Amino acid analysis was performed on a Beckman 6300 HPLC amino acid analyzer utilizing orthophthalaldehyde (OPA) post-column fluorescence detection. Peptide samples were prepared for analysis by hydrolyzing in 6 N HCl at 110°C for 24 h. Samples were then vacuum dried and redissolved in citrate analyte buffer (Beckman) before column injection. Norleucine was added to the samples as an internal reference for quantification purposes.

Results

In PBN spin trapping experiments, a nonparamagnetic phenyl nitrone (the trap) reacts with a transient free radical (the spin) to generate a more stable radical species (the spin adduct), which is detectable by EPR spectroscopy. The exact appearance of the PBN adduct spectrum depends upon electronic and steric factors and solvation effects, and often, though not always, allows identification of the trapped radical.[9]

We have found that Aβ(1–40) and its toxic fragment Aβ(25–35) both routinely produce somewhat broadened three-line EPR spectra (relative intensities 1:1:1) upon incubation with PBN at physiologic pH.[1] The Aβ(25–35)/PBN signal appears within minutes of PBN addition to the peptide. In this spectrum, the nitrogen electron-nuclear hyperfine coupling (hfc) a_N = 17.1 G, and the average peak-to-peak line width ΔH = 1.6 G. Without exception, these Aβ peptides rapidly inactivate GS in cell-free incubates (Fig. 1). We designate this reactive, highly toxic class of peptide Aβ(25–35)-A to distinguish it from four-line generating and EPR inactive (and correspondingly non-toxic) peptide variants discussed presently.

In the course of our investigations, we received shipments from Bachem of Aβ(25–35) which reacted with PBN to produce a four-line EPR spectrum (relative intensities 1:2:2:1, Fig. 2B), in contrast to the three-line pattern routinely observed. For this spectrum, a_N = 14.5 G and ΔH = 0.9 G. This variation of Aβ(25–35) was found to be approximately half as toxic as the norm with respect to GS inactivation (Fig. 1). The less active class of peptide was designated Aβ(25–35)-B. Other, nearly coincident shipments of Aβ(25–35), from the same batch, prepared during the same synthesis and lyophilization and differing only in storage and shipment variables, showed essentially no GS toxicity and no EPR signal upon PBN incubation (Figs 1, 2C). In some cases, GS activity actually increased somewhat after coincubation with this EPR-inactive Aβ variant (Fig. 1). This essentially inert Aβ peptide was designated Aβ(25–35)-C.

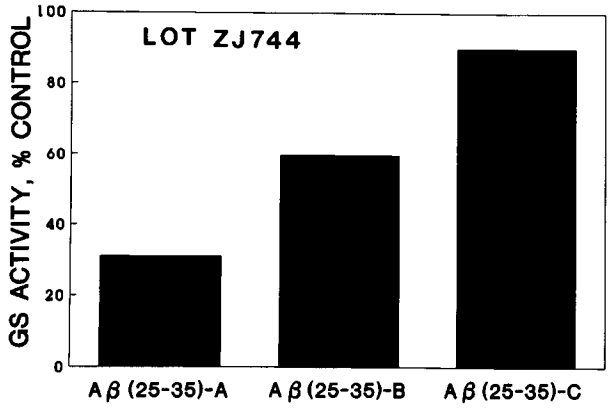

FIG. 1. Inactivation of GS enzyme upon treatment with Aβ(25–35)-A, Aβ(25–35)-B, or Aβ(25–35)-C, as described in the text. Data represent mean of duplicate measurements from each sample. The data in this figure represent different samples of peptide from lot ZJ744.

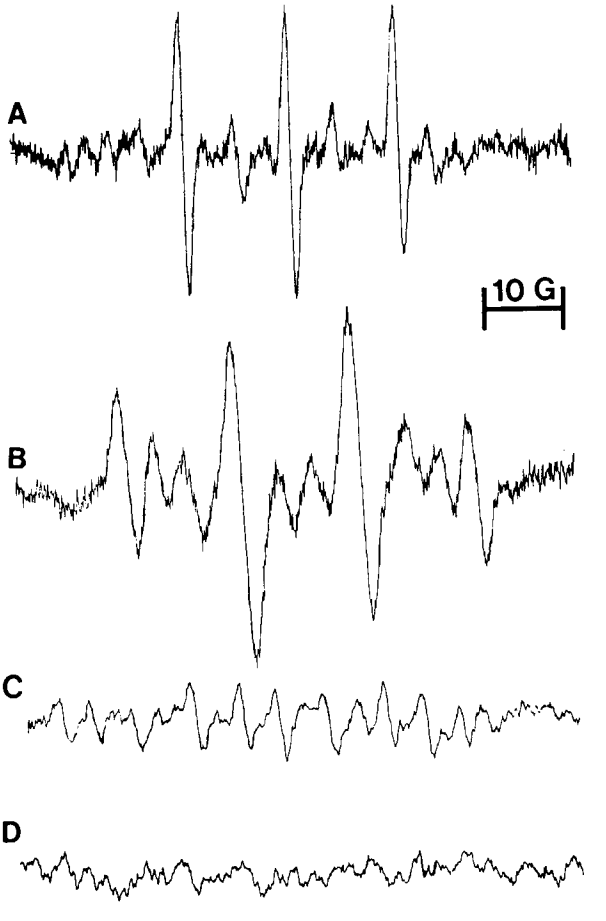

FIG. 2. (**A**) PBN spectrum obtained after 3 h incubation with Aβ(25–35)-A. (**B, C**) PBN spectra obtained after 3 h incubation with Aβ(25–35)-B or Aβ(25–35)-C, respectively. (**D**) PBN/PBS solution incubated in the absence of peptide. The latter spectrum shows that no EPR signal arises from the PBN itself. Spectra represent the same samples of lot ZJ744 used in GS toxicity experiment depicted in Fig. 1.

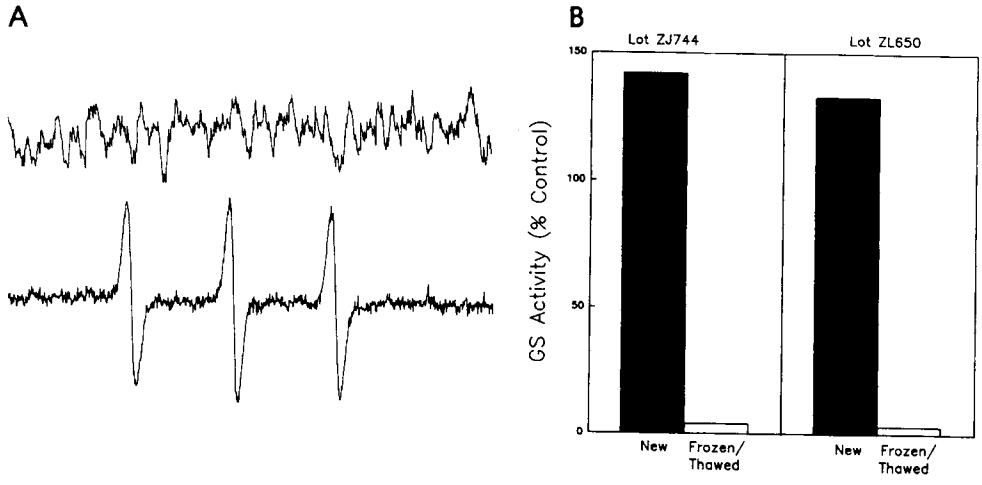

FIG. 3. (**A**) PBN spectrum of Aβ(25–35)-C (a single sample of lot ZJ650) before freeze–thaw procedure (upper spectrum) and following freeze–thaw procedure (lower spectrum). Each spectrum in this figure was acquired within 10 min of solubilization of peptide in PBN/PBS solution. (**B**) GS toxicity of two Aβ(25–35)-C samples before and after freeze–thaw procedure. Note: the sample of lot ZJ744 peptide used in this particular GS toxicity experiment is not the same sample as that depicted in the GS toxicity experiment of Fig. 1.

It was serendipitously discovered that the toxicity of Aβ(25–35)-B and Aβ(25–35)-C toward GS increased upon protracted storage at −10°C. Further investigation of this behavior led to the remarkable observation that ineffectual Aβ(25–35)-B or Aβ(25–35)-C could often be restored to full potency by incubating the lyophilized powder at −80°C overnight, followed by equilibration at room temperature for 5 h prior to use (Fig. 3B). These 'resurrected' peptides, which had produced either no EPR spectra or four-line EPR spectra before the freeze–thaw procedure, then demonstrated three-line spectra upon incubation with PBN (Fig. 3A). We attempted the freeze–thaw reactivation procedure on five samples (obtained from the supplier at different dates over a 5-month period) of Aβ(25–35)-B and two samples of Aβ(25–35)-C. Three of the four-line generating Aβ(25–35)-B samples and both Aβ(25–35)-C samples were converted into the operationally defined class A peptide by the freeze–thaw protocol. Two of the class B peptides remained unchanged despite the procedure.

Although variability in synthetic Aβ(25–35) has not been documented previously, variability in Aβ(1–40) has been well documented and appears to correlate with peptide β-sheet content.[5,6] We have found Aβ(1–40) very reliable with respect to toxicity and PBN reactivity, and have observed only one instance (out of many peptide shipments spin trapped) in which this peptide failed to generate a three-line EPR spectrum upon reaction with the spin trap. A sample from Bachem lot ZL831, which demonstrated attenuated GS toxicity (0% inactivation of pure GS *vs* typical values of 50–70%), was found to generate a four-line PBN reaction product with $a_N = 14.5$ G, similar to the case of Aβ(25–35)-B (Fig. 4). This particular spectrum, however, differed profoundly from the spectrum obtained from the 11-mer peptide in that the peak-to-peak linewidth of the Aβ(1–40)/PBN species was much broader ($\Delta H = 2.4$ G *vs* 0.9 G).

HPLC/amino acid analysis was performed on each of the Aβ(25–35) lyophilates (class A, B, or C) and in all cases the analyses matched those of the supplier: no modified amino acid residues were observed in the HPLC chromatogram, and the residue stoichiometry was maintained (Table 1). Upon incubation of highly toxic Aβ(25–35)-A in 37°C chelexed PBS for 12 h (during which time peptide-derived free radicals were observed in spin trapping experiments), the methionine HPLC peak decreased by approximately 50% with a corresponding increase of a species which co-elutes with methionine sulfoxide (MetOX; Table 1). This methionine oxidation occurred despite the attempts

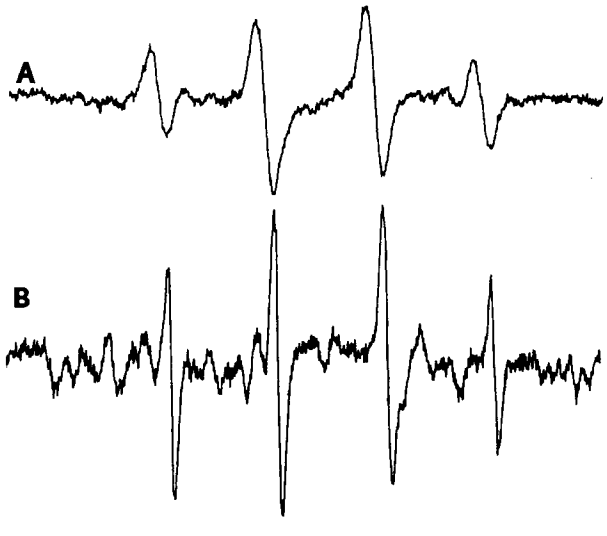

FIG. 4. (**A**) Aβ(1–40) four-line spectrum formed by reaction of non-toxic peptide with PBN. (**B**) Typical Aβ(25–35)-B four-line spectrum for comparison. Note the much greater linewidth of the Aβ(1–40) spectrum.

Table 1. Amino acid analysis

	Theory	Aβ(25–35)-A (dry)	Aβ(25–35)-A (PBS)	Aβ(25–35)-A (PBS/PBN)
GLY	3	2.57	2.81 ± 0.23	3.20 ± 0.21
SER	1	0.76	0.70 ± 0.03	0.58 ± 0.06
ASN	1	1.05	0.98 ± 0.03	0.90 ± 0.08
LYS	1	1.22	1.00 ± 0.13	0.81 ± 0.10
ALA	1	1.28	1.22 ± 0.04	1.27 ± 0.06
ILE	2	1.80	1.64 ± 0.08	1.91 ± 0.10
LEU	1	1.23	1.11 ± 0.06	1.36 ± 0.37
MET	1	0.90	0.62 ± 0.18	1.06 ± 0.08
METOX	—	0.00	0.57 ± 0.24	0.00 ± 0.00

Amino acid content (mol residue per mol peptide) in Aβ(25–35)-A dry lyophilate (before incubation in buffer) or after 12 h incubation in 37°C PBS or PBS/PBN. Numbers in PBS and PBS/PBN columns indicate mean ± s.d. of three independent samples (received on different dates and verified to toxicity and PBN reactivity). As indicated by Bachem and as known from amino acid analysis of peptides, the apparent serine deficit is an artifact of HCl hydrolysis of serine during preparation for analysis. Aβ(25–35)-B and Aβ(25–35)-C dry lyophilates gave similar amino acid compositions as indicated for dry Aβ(25–35)-A.

made to exclude metals from the buffer by chelex-100 treatment. Co-incubation of Aβ(25–35)-A with a 50 mM solution of the free radical scavenger PBN completely prevented the Met-35 conversion (Table 1).

Discussion

The variability in toxicity among different batches of synthetic Aβ peptides has been a matter of contention.[5,6] In this paper we report data obtained from unusual samples of Aβ(25–35) peptide and one sample of Aβ(1–40) peptide which displayed attenuated enzyme toxicity. When incubated with the spin trap PBN, three distinct EPR spectral patterns are observed: three lines in the case of routine, highly toxic peptide; four lines in the samples which display markedly reduced enzyme toxicity; and no significant EPR signal from samples which are essentially non-toxic. HPLC/amino acid analysis showed no detectable chemical alteration in the dry lyophilate, consistent with documentation by the supplier and previous analytical studies regarding purity of the peptide.[5] Oxidation of the Met-35 residue during incubation may indicate the importance of this residue to peptide free radical reactivity.[1,3] As noted, peptide variability observed in our present work occurred within the same synthetic lot of peptide as well as between different synthetic lots, indicating the possibility of post-synthesis modification of the peptide. This is the first documentation of a well-defined difference in chemical reactivity among variably toxic synthetic Aβ peptides. The findings of this study indicate that investigators should perform potency assays to determine the reactivity state of individual Aβ samples prior to further experimentation. These observations also indicate that EPR spectroscopy offers such a means to ascertain the relative potency of Aβ peptides.

It is not yet clear whether the differences in Aβ reactivity stem from differences in the nature of the primary peptidyl radical center or, rather, from variable structural features of the peptide. The observation that less reactive peptide variants can be made more active by freeze–thawing implies a reversible structural component to reactivity and toxicity. 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. One aspect of Aβ reactivity, the mechanism of Aβ(25–35) reaction with the PBN C=N bond, is explored in greater detail in an accompanying paper.[2]

Conclusions

Reactivity of synthetic Aβ peptides toward GS enzyme is related to peptide spin trap reactivity, suggesting that alternative pathways of peptide radicalization may explain previously documented variation in peptide toxicity.

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