Amyloid β Peptides Do Not Form Peptide-derived Free Radicals Spontaneously, but Can Enhance Metal-catalyzed Oxidation of Hydroxylamines to Nitroxides*

(Received for publication, August 7, 1998, and in revised form, December 10, 1998)

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Amyloid β (Aβ) peptides play an important role in the pathogenesis of Alzheimer’s disease. Free radical generation by Aβ peptides was suggested to be a key mechanism of their neurotoxicity. Reports that neurotoxic free radicals derived from Aβ-(1–40) and Aβ-(25–35) peptides react with the spin trap N-tert-butyl-a-phenylnitrone (PBN) to form a PBN/Aβ peptide radical adduct with a specific triplet ESR signal assert that the peptide itself was the source of free radicals. We now report that three Aβ peptides, Aβ-(1–40), Aβ-(25–35), and Aβ-(40–1), do not yield radical adducts with PBN from the Oklahoma Medical Research Foundation (OMRF). In contrast to OMRF PBN, incubation of Sigma PBN in phosphate buffer without Aβ peptides produced a three-line ESR spectrum. It was shown that this nitroxide is di-tert-butyl nitroxide and is formed in the Sigma PBN solution as a result of transition metal-catalyzed auto-oxidation of the respective hydroxylamine present as an impurity in the Sigma PBN. Under some conditions, incubation of PBN from Sigma with Aβ-(1–40) or Aβ-(25–35) can stimulate the formation of di-tert-butyl nitroxide. It was shown that Aβ peptides enhanced oxidation of cyclic hydroxylamine 1-hydroxy-4-oxo-2,2,6,6-tetramethylpiperidine (TEMPONE-H), which was strongly inhibited by the treatment of phosphate buffer with Chelex-100. It was shown that ferric and cupric ions are effective oxidants of TEMPONE-H. The data obtained allow us to conclude that under some conditions toxic Aβ peptides Aβ-(1–40) and Aβ-(25–35) enhance metal-catalyzed oxidation of hydroxylamine derivatives, but do not spontaneously form peptide-derived free radicals.

Plasminogen activator inhibitor (PAI-1) is characterized by a loss of memory and neurons. These characteristics have been associated with brain lesions known as neurofibrillary tangles composed of Tau protein and amyloid plaques, which consist of amyloid β (Aβ) peptide. In a small number of families, mutations in the genes for the amyloid peptide precursor (APP, chromosome 21) to Aβ peptide for presenilin-1 (PSEN-1, chromosome 14), presenilin-2 (PSEN-2, chromosome 1) and for an anonymous gene on chromosome 12 have been associated with AD. In contrast, Saunders and colleagues (1) discovered that a significant percentage of patients inheriting one or more of the epsilon-4 alleles of apolipoprotein-E (APOE4, chromosome 19) were at risk of acquiring AD at an earlier age than their counterparts expressing the more common epsilon-3 allele of APOE. The observation that AD patients with mutant genes for APP, PSEN-1, or PSEN-2 have higher levels of Aβ peptide than non-diseased controls (2, 3) argues that Aβ peptide may cause AD. Further supporting this idea is the observation that APOE4 patients with AD display more amyloid plaques than those with APOE3 (4). Based on neuropathological, genetic, and biochemical associations, the presence of amyloid β peptide is a key component of AD.

A crucial link between Aβ peptide and AD was provided by Yankner and colleagues (5), who showed that fibrillar aggregates of Aβ peptide were toxic to neurons. This seminal observation inspired a global quest to define the mechanism by which fibrillar Aβ peptide mediates neurotoxicity. One of the mechanisms proposed suggests that neurons exposed to Aβ peptide suffer severe oxidative stress that may lead to their death. Clear evidence of oxidative stress in Alzheimer’s disease has been provided by Smith et al. (6), who found that neurons of AD patients contained nitrotyrosine modifications of proteins, which were not detected in age-matched control brains. Increased levels of lipid peroxides (7), reactive aldehydes such as hydroxynonenal (8), and oxidized DNA (9) have also been reported in AD brains, providing additional evidence of an oxidative stress component in the disease.

The exact nature of the radical species generated in Alzheimer’s disease is unknown. In one line of investigation, Bohl et al. (10) demonstrated that cells exposed to fibrillar Aβ peptide responded by releasing hydrogen peroxide and dying, a process that could be inhibited by the application of catalase to degrade the released peroxide. Subsequent reports have demonstrated cellular release of superoxide (11) and nitric oxide (12) in response to Aβ peptide treatment. The effect of these radicals may be potentiated since Aβ peptides also appear to inhibit the cellular redox mechanisms that normally protect cells from oxidative stress (13).

In a provocative hypothesis, Hensley et al. (14) suggest that the Aβ peptide itself spontaneously generates free radicals that can damage cells. By mixing the spin trap N-tert-butyl-a-phenylnitrone (PBN) with neurotoxic forms of the Aβ peptide such as Aβ-(1–40) and Aβ-(25–35), they report the generation of ESR-detectable radical adducts in cell-free solutions (14–17).
They suggest that these radicals are generated by methionine oxidation (14, 15) or by the fragmentation of Aβ peptide into smaller oligopeptide radicals (14, 15) that act as “shrapnel” to damage and eventually kill cells. Recently, these results were described as possibly artifactual due to contaminants in some of the preparations (18).

We have reinvestigated the spin-trapping studies of spontaneous free radical formation by Aβ peptides. In contrast to previous literature (14–17), we now report that neurotoxic Aβ(1–40) and Aβ(25–35) in the presence of the spin trap PBN do not form ESR-detectable radical adducts spontaneously. Amyloid/PBN radical adducts (14–17) reported earlier were found to be di-tert-butyl peroxytrinitroxide and tert-butyl hydroxynitroxide, which are formed by oxidation of the corresponding hydroxylamines. We have investigated the possibility that toxic Aβ may potentiate metal-catalyzed oxidation of hydroxylamine derivatives. Transition metals like iron and copper frequently contribute to reactions where molecules are oxidized, but the transition metal oxidation of hydroxylamine derivatives has not been previously studied. A body of emerging work demonstrates that copper and iron are significantly associated with amyloid plaques in the brains of patients with AD, but not with the neuropil lacking plaques nor with control neuropil from healthy brains (19). Since Aβ is retained on metal chelate columns charged with copper (20), and copper dramatically increases the rate of Aβ aggregation into amyloid fibrils (21), then complexes of Aβ and copper may contribute to the pathological oxidative stress associated with AD. We now report the effects of neurotoxic Aβ(1–40) and Aβ(25–35) and non-toxic Aβ(40–1) on the oxidation of hydroxylamine derivatives in the presence and absence of metals. The data obtained allows us to conclude that the previously reported formation of amyloid/PBN radical adducts (14–17) in the Sigma PBN treated with Aβ peptides can be explained as Aβ peptide-stimulated, metal-catalyzed oxidation of the corresponding hydroxylamine impurities in Sigma PBN.

EXPERIMENTAL PROCEDURES

Reagents—PBN was obtained from the Oklahoma Medical Research Foundation (OMRF) Spin Trap Source (Oklahoma City, OK). Two different lots (101H3696 and 87H36602) of PBN from Sigma were used. Deferoxamine (Desferal) was purchased from Sigma. Di-tert-butyl nitroxide (Aldrich), N-tert-butylhydroxylamine (TCI America, Portland, OR) and analytical grade Chelex-100 (Bio-Rad) were used. Synthetic Aβ(1–40), Aβ(25–35) and Aβ(40–1) peptides were purchased from Bachem (Torrance, CA). These lots of Aβ(1–40) and Aβ(25–35), but not the Aβ(40–1) peptides, have been previously shown to kill neurons in a cell culture system (data not shown). TEMPO-H was obtained from Alexis Corporation (Läufelfingen, Switzerland) (22). All other reagents were analytical grade.

Preparation of PBN Stock Solution—Stock solutions of PBN (150 mM) in HPLC grade water were used to prepare final 50 mM PBN solutions.

Preparation of TEMPO-H Stock Solutions—TEMPO-H was dissolved in oxygen-free HPLC grade water (30-min argon bubbled water) with 1 mM Desferal. Desferal was used to decrease the spontaneous oxidation of hydroxylamines catalyzed by traces of transition metal ions. The concentration of TEMPO-H in the stock solutions was 1 mM. Prior to the experiments, the stock solution was kept under a flow of argon in a cool, air-tight place.

Preparation of tert-Butylhydroxynitroxide Solution—tert-Butylhydroxynitroxide was obtained by auto-oxidation of 1 mM N-tert-butylhydroxylamine in water or by oxidation of 10 µM solution of N-tert-butylhydroxylamine with 10 µM K3[Fe(CN)6] in 0.15 µM phosphate buffer at pH 7.4.

ESR Spin-trapping Experiments—The ESR spectra were recorded using a Bruker E570 spectrometer operating at 9.76 GHz with a modulation frequency of 50 kHz and a TM110 cavity. Spin-trapping experiments were started by solubilizing Aβ peptides into a solution of PBN or TEMPO-H. All ESR samples were placed in the 17-mm flat cell, which contained Aβ peptides (1 mg/ml), PBN (50 mM), or TEMPO-H (0.5 mM) in sodium phosphate buffer (0.15 M) at pH 7.4. In order to perform experiments with a lower concentration of transition metals, phosphate buffer was treated with 5 g of Chelex-100/100 ml of solution for 2 h, followed by filtration using Millex-HA 0.45-µM filters (Millipore Corp., Bedford, MA). The ESR instrumental settings for experiments with PBN were as follows: field sweep, 70 G; microwave frequency, 9.76 GHz; microwave power, 40 milliwatts; modulation amplitude, 0.32 G; conversion time, 1310 ms; time constant, 1310 ms; receiver gain, 1 × 106. ESR signals were monitored for 6 h. The ESR instrumental settings for TEMPO-H experiments were the following: field sweep, 50 G; microwave frequency, 9.76 GHz; microwave power, 20 milliwatts; modulation amplitude, 1 G; conversion time, 656 ms; time constant, 2620 ms; receiver gain, 1 × 107.

Study of Iron-catalyzed Oxidation of Hydroxylamines—The presence of hydroxylamine impurities in Sigma PBN was determined by studying iron’s effect on nitroxide formation in solutions of PBN with K3[Fe(CN)6] and the iron-chelating agent Desferal. Oxidation of hydroxylamine impurities in Sigma PBN was performed using 5 mM K3[Fe(CN)6]. Inhibition of iron-catalyzed oxidation of hydroxylamine impurities in Sigma PBN was carried out in the presence of 2 mM Desferal.

Study of Ferric-catalyzed Oxidation of Hydroxylamines—The oxidation of TEMPO-H by ferric ions was confirmed by the formation of TEMPO nitroxide during the incubation of TEMPO-H with ferric ions. The effect of 1 mM Fe(NH4)2(SO4)2 and chelating agent Desferal on the TEMPO nitroxide formation from 0.1 mM TEMPO-H was studied. Inhibition of iron-catalyzed oxidation of TEMPO-H was carried out in the presence of 1 mM Desferal.

Computer Simulation—Computer simulations and spin-trap data base searches were performed using a computer simulation program, the details of which have been described elsewhere (23).

RESULTS

Reinvestigation of Spontaneous Free Radical Formation by Aβ Peptides—The ability of Aβ peptides to spontaneously form free radicals was studied by ESR using the spin trap PBN from the OMRF Spin Trap Source. Aβ(1–40), Aβ(40–1), and Aβ(25–35) peptides were tested in parallel with a control that did not contain Aβ peptides (Fig. 1). ESR spectra were collected every 40 min over 6 h, and no ESR signals were observed in any mixtures (Fig. 1, A–D). Therefore, Aβ peptides do not form ESR-detectable radical adducts spontaneously.

In contrast to the OMRF PBN results, ESR spectra from mixtures of Sigma PBN alone, plus Aβ(1–40), plus Aβ(40–1), or plus Aβ(25–35) peptides all yielded a triplet ESR spectrum with the Aβ peptide inhibiting radical formation (Fig. 1, E–H). This triplet spectrum has a nitrogen hyperfine-coupling constant of 17.14 G. The formation of this triplet spectrum with Sigma PBN, but not OMRF PBN, is consistent with the spectrum arising from an impurity in Sigma PBN.

Formation of Di-tert-butyl nitroxide and tert-Butyl hydroxynitroxide from Impurities in Sigma PBN—PBN from Sigma was checked for the presence of impurities that might lead to the formation of ESR signals. After a 6-h incubation of 50 mM aqueous Sigma PBN, a strong ESR spectrum was observed (Fig. 2A) that consisted of ESR spectra from two nitroxides (Fig. 2, B–D). One nitroxide has a triplet ESR spectrum (Fig. 2C) with a nitrogen hyperfine coupling constant of 17.16 G, which is very close to the reported nitrogen hyperfine coupling constant for di-tert-butyl nitroxide. The second nitroxide has a four-line ESR spectrum (Fig. 2D) that is consistent with a nitrogen hyperfine coupling constant of 14.61 G and a hydrogen hyperfine coupling constant of 13.93 G, which are very close to...
the reported hyperfine coupling constants for tert-butylhydroxylamine (24, 25).

To confirm the chemical structure of nitroxide radicals observed in the solution of Sigma PBN, we obtained experimental ESR spectra of di-tert-butyl nitroxide (Fig. 2E) and tert-butylhydronitroxide (Fig. 2F). Experimental ESR spectra of di-tert-butyl nitroxide and tert-butylhydronitroxide were identical to the ESR spectrum of the PBN solution shown above (Fig. 2). The ESR spectra from the Sigma PBN solution (Fig. 2A) can be described as a combination of two specific ESR spectra: those of di-tert-butyl nitroxide (Fig. 2E) and tert-butylhydronitroxide (Fig. 2F). Thus, during the incubation of Sigma PBN in water, both di-tert-butyl nitroxide and tert-butylhydronitroxide were formed.

In order to analyze the mechanism of nitroxide formation in the PBN solution, the effects of the chelating agent Desferal and of ferric iron addition were studied. Fresh solutions of Sigma PBN in Chelex-100-treated phosphate buffer contained trace amounts of di-tert-butyl nitroxide as identified by its ESR spectrum (Fig. 3A). After just 2 h of incubation, the ESR signal was increased dramatically (Fig. 3B). A parallel 2-h incubation in the presence of the chelating agent Desferal significantly inhibited formation of di-tert-butyl nitroxide (Fig. 3C). Addition of 5 mM K₃[Fe(CN)₆] to the fresh PBN solution caused greater nitroxide formation (Fig. 3D) than that of the 2-h incubation (Fig. 3B).

**Linewidth Dependence on the ESR Instrumental Settings**—Previously, it was reported that Aβ-(1–40) and Aβ-(25–35) peptides formed radical adducts with identical triplet ESR spectra and nitrogen hyperfine coupling constants of 17.1 G (16), which is actually the same as that reported for di-tert-butyl nitroxide (Fig. 2E). Based on the much greater linewidth of the Aβ peptide/PBN reaction product (1.6 G), these authors concluded that this product was not di-tert-butyl nitroxide (17). However, the linewidth is not a specific parameter of a nitroxide ESR spectrum (26). Linewidth is very dependent on the ESR instrumental settings, mainly modulation amplitude and microwave power. In order to show that authentic di-tert-butyl nitroxide and the nitroxide derived from Sigma PBN can have the same ESR linewidth as has been described for a PBN/Aβ peptide radical adduct, the ESR spectra were obtained using different modulation amplitude settings (Fig. 4). The linewidth of the ESR spectra of di-tert-butyl nitroxide was equal to that of the nitroxide from Sigma PBN (Fig. 4, A and D).
when ESR spectra were collected under identical instrument settings. Moreover, both spectra displayed the same linewidth dependence on the modulation amplitude setting. We observed peak-to-peak linewidths of 0.51, 0.96, and 1.56 G when using modulation amplitudes of 0.32, 1.00, and 1.59 G, respectively (Fig. 4).

Previously, it was also reported that non-toxic A\(\beta\)-(40–1) peptide formed a radical adduct with a quartet ESR spectrum and equivalent nitrogen and hydrogen hyperfine coupling constants of 14.5 G (16, 17), which are very close to those reported for tert-butylhydronitroxide (Fig. 2F). Based on the much greater linewidth of the PBN/A\(\beta\) peptide reaction product, it was concluded that this product was not tert-butylhydronitroxide (17). In order to show that tert-butylhydronitroxide and the nitroxide from Sigma PBN solution could have the same ESR spectrum linewidth as the radical described as the PBN/A\(\beta\) peptide-(1–40) reaction product, the ESR spectra were obtained using different modulation amplitude settings for the ESR spectrometer (Fig. 5). The linewidth of the ESR spectrum of tert-butylhydronitroxide was the same as for the nitroxide species from the Sigma PBN solution (Fig. 5, A and D). Moreover, the dependence of linewidth on the modulation amplitude was the same. Using modulation amplitudes of 0.32, 1.00, and 1.59 G, the widths of the low-field line were observed to be 0.76, 0.98, and 1.53 G, respectively (Fig. 5).

A\(\beta\) Peptide Stimulation of Oxidation of Di-tert-butylhydroxylamine Impurity in Sigma PBN—The ability of A\(\beta\) peptides to stimulate oxidation of di-tert-butylhydroxylamine was studied by ESR using PBN from Sigma. For these experiments, solutions of A\(\beta\)-(1–40), A\(\beta\)-(25–35), and A\(\beta\)-(40–1) with 50 mM PBN and 1 mM Desferal were tested in parallel with a control that did not contain A\(\beta\) peptides (Fig. 6). Incubation of PBN (Sigma) with A\(\beta\) peptides for 2 h in phosphate buffer with Desferal gave rise to the ESR spectra shown in Fig. 1. Neurotoxic A\(\beta\)-(1–40) and A\(\beta\)-(25–35) produced triplet ESR spectra (Fig. 6, A and B).
in agreement with Butterfield and co-workers (14–17). Computer simulation of these ESR spectra (Fig. 6) revealed an ESR spectrum of one nitroxide compound which displays a triplet ESR spectrum with a nitrogen hyperfine coupling constant of 17.16 G, which is identical to the nitrogen hyperfine coupling constant for di-tert-butyl nitroxide. Non-toxic Aβ-(40–1) produced a much weaker nitroxide triplet and the four-line tert-butyl hydroxylamine (Fig. 6C). ESR spectra of a control sample without Aβ peptides did not reveal any nitroxide formation (Fig. 6D). Under these conditions the formation of this triplet spectrum with Sigma PBN was dependent on the presence of toxic Aβ-(1–40) and Aβ-(25–35). It was previously shown that di-tert-butyl nitroxide can be formed in a solution of PBN by the metal-catalyzed auto-oxidation of di-tert-butylhydroxylamine. Therefore, in the presence of 1 mM Desferal in phosphate buffer, Aβ-(1–40) and Aβ-(25–35) stimulate the oxidation of the di-tert-butylhydroxylamine impurity to form di-tert-butyl nitroxide.

**Aβ-stimulated Oxidation of TEMPONE-H**—In order to study the mechanism of Aβ-stimulated hydroxylamine oxidation, we used TEMPONE-H (22). The time course of ESR spectra and the effect of removing transition metals from the phosphate buffer by treatment with chelating resin Chelex-100 were studied.

TEMPONE-H was incubated with Aβ peptides in phosphate buffer with 1 mM Desferal (Fig. 7). It was found that after a 1-h incubation of TEMPONE-H (0.3 mM) with Aβ-(25–35) and Aβ-(1–40), the ESR amplitude of TEMPONE nitroxide increased up to 4 times in comparison with the control (Fig. 7, A, B, and D). Incubation of TEMPONE-H (0.3 mM) with Aβ-(40–1) increased the ESR amplitude in the range of 50–100% compared with the control (Fig. 7 C, D). Therefore, neurotoxic Aβ-(1–40) and Aβ-(25–35) significantly stimulated oxidation of TEMPONE-H to TEMPONE nitroxide (Fig. 7, A and B). Non-toxic Aβ-(40–1) caused relatively minor changes in the formation of TEMPONE nitroxide (Fig. 7C) compared with a control sample without Aβ peptides (Fig. 7D).

In order to clarify the role of transition metals in Aβ-mediated stimulation of hydroxylamine oxidation, phosphate buffer was pretreated for 1 h with chelating resin Chelex-100 to remove trace-metal contaminants. ESR spectra obtained after the incubation of TEMPONE-H (0.3 mM) with Aβ peptides in Chelex-100-treated phosphate buffer are shown in Fig. 7, E–H. In Chelex-100-treated phosphate buffer, Aβ peptides did not stimulate oxidation of TEMPONE-H, implying that Aβ peptides were not the primary source of trace transition metals.

Fig. 8 shows the time dependence of TEMPONE formation during the incubation of TEMPONE-H with Aβ peptides. Formation of TEMPONE nitroxide in the samples with Aβ-(1–40) and Aβ-(25–35) was significantly faster than with Aβ-(40–1) and in the control (Fig. 8A). Removing transition metals from the solution by pretreatment of phosphate buffer with Chelex-100 strongly inhibited Aβ stimulation of TEMPONE formation (Fig. 8B). Therefore, in phosphate buffer with 1 mM Desferal, Aβ-(1–40) and Aβ-(25–35) enhanced TEMPONE-H oxidation, which was inhibited by Chelex-100 treatment.

**Effect of Ferric Ions on TEMPONE-H Oxidation**—The potential role of ferric ion in Aβ-stimulated hydroxylamine oxidation was studied (Fig. 9). ESR spectra of the fresh solution of TEMPONE-H revealed only a relatively small amount of TEMPONE (Fig. 9A). Addition of 1 μM Fe³⁺NH₄(SO₄)₂ to TEMPONE-H led to a 10-fold increase in the ESR amplitude of the TEMPONE spectra (Fig. 9B). The chelating agent Desferal (1
mM) completely inhibited oxidation of TEMPONE-H in solutions containing 1 mM Fe$^{3+}$NH$_4$(SO$_4$)$_2$ (Fig. 9C). Therefore, ferric ion can oxidize hydroxylamine TEMPONE-H to form nitroxide TEMPONE and, presumably, ferrous ion (Reaction 1), which is air-oxidized (Reaction 2) (27). Hydrogen peroxide did not directly oxidize the hydroxylamine TEMPONE-H in solution containing Desferal (data not shown).

\[
\begin{align*}
\text{REACTIONS 1 and 2} \\
\text{Fe}^{3+} + \text{TEMPONE-H} & \rightarrow \text{Fe}^{2+} + \text{TEMPONE} \\
\text{Fe}^{2+} + \text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{O}_2^-
\end{align*}
\]

**Effect of Cupric Ions on TEMPONE-H Oxidation**—The possible role of cupric ions in Aβ-stimulated hydroxylamine oxidation was studied (Fig. 10). Addition of 1 mM Cu$^{2+}$SO$_4$ to TEMPONE-H led to a drastic increase in the ESR amplitude of the TEMPONE spectrum (Fig. 10, A, and D), which was much more pronounced than the effect of 1 mM Fe$^{3+}$NH$_4$(SO$_4$)$_2$ (Fig. 10B). The chelating agent Desferal (1 mM) completely inhibited oxidation of TEMPONE-H by 1 mM Cu$^{2+}$SO$_4$ (Fig. 10C). Therefore, cupric ion can oxidize hydroxylamine TEMPONE-H to form nitroxide TEMPONE and cuprous ion (Reaction 3), which is oxidized by oxygen (Reaction 4) (28).

\[
\begin{align*}
\text{REACTIONS 3 and 4} \\
\text{Cu}^{2+} + \text{TEMPONE-H} & \rightarrow \text{Cu}^{+} + \text{TEMPONE} \\
\text{Cu}^{+} + \text{O}_2 & \rightarrow \text{Cu}^{2+} + \text{O}_2^-
\end{align*}
\]

**Auto-oxidation of Hydroxylamines**—In order to clarify the mechanism of auto-oxidation of hydroxylamine derivatives, formation of TEMPONE was determined in the presence of superoxide dismutase, catalase, or Desferal (Fig. 11). TEMPONE-H was incubated in phosphate buffer for 30 min. ESR spectra of the control sample of TEMPONE-H revealed a small amount of TEMPONE (Fig. 11, A). The addition of superoxide dismutase (100 units/ml) to TEMPONE-H led to a 2-fold decrease in the ESR amplitude of the TEMPONE spectra (Fig. 11, B). The effect of superoxide dismutase supports the role of Reaction 5 in the auto-oxidation of hydroxylamines (22).

\[
\begin{align*}
\text{REACTION 5} \\
\text{O}_2^- + \text{TEMPONE-H} & \rightarrow \text{H}_2\text{O}_2 + \text{TEMPONE}
\end{align*}
\]

Addition of catalase (10 μg) to TEMPONE-H did not significantly change the content of TEMPONE in the sample (Fig. 11C). Addition of the chelating agent Desferal (100 μM) to TEMPONE-H greatly inhibited the TEMPONE formation (Fig. 11D). Therefore, transition metals (M$^{n+}$) can oxidize hydroxylamine TEMPONE-H to form TEMPONE (Reaction 6). Reduced transition metals (M$^{n-}$) are oxidized by oxygen (Reaction 7). Decomposition of hydrogen peroxide by catalase did not affect the oxidation of hydroxylamine TEMPONE-H, excluding a role for a direct oxidation of TEMPONE-H by hydrogen peroxide.
**Fig. 11.** Formation of TEMPO nitroxide during the auto-oxidation of TEMPO-H. ESR spectra obtained after a 30-min incubation of 0.1 mM TEMPO-H in 0.15 M sodium phosphate buffer (pH 7.4). A, control, B, TEMPO-H (0.1 mM) + SOD (100 units/ml). C, TEMPO-H (0.1 mM) + catalase (10 μg/ml). D, TEMPO-H (0.1 mM) + Desferal (0.1 mM). ESR receiver gain was $2 \times 10^7$.

\[
M^{n+} + \text{TEMPO-H} \rightarrow M^{n+} + \text{TEMPONE} \\
M^{n+} + O_2 \rightarrow M^{n+} + O_2^-
\]

**REACTIONS 6 and 7**

**DISCUSSION**

Despite many studies on Aβ peptide-mediated neurotoxicity, its exact mechanism of action is still undefined. One intriguing hypothesis suggests that Aβ peptides in solution spontaneously form free radicals which damage and kill cells (14, 15). We did not observe any radical adducts when the Aβ peptides Aβ(1–40), Aβ(40–1), and Aβ(25–35) were mixed with high quality PBN (OMRF Spin Trap Source).

Previously, it was reported that ESR signals formed during the incubation of Aβ peptides with PBN were due to PBN/Aβ peptide radical adducts (14–17). However, according to the current literature, there are no reported radical adducts of PBN that have similar ESR parameters ($g_N = 17.1$). Using Sigma PBN, both the triplet and quartet spectra of di-tert-butylnitroxide and tert-butyldihydroxynitroxides, respectively, were detected. These nitroxides have been previously misinterpreted as novel radical adducts formed from Aβ radicals (14–17).

Recently, it was suggested that PBN/Aβ peptide radical adducts decomposed to an alkoxy nitroxide (15, 17). This alkoxy nitroxide would have the same structure as the alkoxy adduct of 2-methyl-2-nitrosopropane. This assignment is also an error, because the nitrogen hyperfine coupling constant of the alkoxy adduct of 2-methyl-2-nitrosopropane is about 27 G (29, 30), which is quite different from the 17.1 G for the radical adduct of 2-methyl-2-nitrosopropane. This assignment is also strongly suggested that the ESR spectra reported as PBN/Aβ peptide radical adducts actually result from the formation of di-tert-butylnitroxide and tert-butyldihydroxynitroxide radicals. These nitroxide radicals are formed during metal-catalyzed auto-oxidation of di-tert-butyldihydroxylamine and N-tert-butyldihydroxylamine (Scheme 1), which are impurities in PBN from Sigma.

The spin trap PBN is usually synthesized by condensation of benzaldehyde with N-tert-butyldihydroxylamine (31). Therefore, the trace amount of N-tert-butyldihydroxylamine in preparations of PBN is probably due to incomplete purification.

The appearance of di-tert-butyldihydroxylamine in PBN can be explained by the presence of di-tert-butyldihydroxylamine in the N-tert-butyldihydroxylamine commonly used for the synthesis of PBN. Scheme 2 illustrates the mechanism of di-tert-butyldihydroxylamine formation from N-tert-butyldihydroxylamine. N-tert-butyldihydroxylamine is a good reducing agent, which, after two one-electron oxidations, produces 2-methyl-2-nitrosopropane. 2-Methyl-2-nitrosopropane is an unstable compound, which is readily decomposed by heat or light to the tert-butyl radical and nitric oxide (32, 33). The tert-butyl radical will react with 2-methyl-2-nitrosopropane (which is actually a well known spin trap) to form di-tert-butylnitroxide (32, 33).

Di-tert-butylnitroxide can easily be reduced to di-tert-butyldihydroxylamine even by excess N-tert-butyldihydroxylamine. The presence of di-tert-butyldihydroxylamine in the sample of N-tert-butyldihydroxylamine was supported by the fact that the ESR spectra of N-tert-butyldihydroxylamine oxidized by K$_3$[Fe(CN)$_6$] contained small traces of the triplet signal of di-tert-butylnitroxide (Fig. 3F). Therefore, trace amounts of di-tert-butyldihydroxylamine are present in many preparations of PBN. Due to the intrinsic sensitivity of ESR, oxidation of trace levels of these impurities can lead to readily detected ESR signals. The assignment of the structure of these nitroxides to specific compounds excludes the possibility that these ESR spectra are derived from amyloid β peptides.

In this study we investigated the effect of neurotoxic Aβ-(1–40) and Aβ-(25–35) and non-toxic Aβ-(40–1) on the oxidation of hydroxylamine derivatives. We previously showed that, in the presence of Desferal, incubation of Aβ-(1–40) and Aβ-(25–35) with PBN (Sigma) led to the formation of di-tert-butylnitroxide, which was produced after oxidation of di-tert-butyldihydroxylamine. Stimulation of oxidation of hydroxylamine derivatives by Aβ-(1–40) or Aβ-(25–35) was also demonstrated using TEMPO-H. Oxidation of TEMPO-H to TEMPO nitroxide in the presence of Aβ-(1–40) or Aβ-(25–35) was significantly more intensive than in the control without Aβ. The treatment of phosphate buffer with chelating resin Chelex-100 inhibits the Aβ effect on TEMPO-H oxidation. These data support a catalytic role of phosphate buffer-derived transition metal contaminants in the Aβ-stimulated oxidation of hydroxylamine derivatives.
It is known that ferricyanide is able to oxidize hydroxylamine derivatives (34). Cupric ion is another candidate for the oxidation of hydroxylamines. In this work we checked the possible role of both ferric and cupric ions in oxidation of hydroxylamine derivatives. It was found that both ferric and cupric ions were effective in oxidation of TEMPO-NE. Moreover, oxidation of TEMPO-NE by cupric ions was 40-fold more effective than ferric ions. Therefore, both ferric and cupric ions could be involved in Aβ-stimulated metal-catalyzed oxidation of hydroxylamine derivatives.

The data obtained allow us to conclude that, under some conditions, the toxic Aβ peptides Aβ-(1–40) and Aβ-(25–35) enhance transition metal-catalyzed oxidation of hydroxylamine derivatives. Therefore, the previously reported formation of radical adducts with Aβ peptides can be explained as an Aβ-enhanced, transition metal-catalyzed oxidation of hydroxylamine derivatives found as impurities in commercial PBN. In addition, our data (Fig. 7) demonstrate that Aβ peptides can compete with Desferal for transition metals such as copper, which implies that Aβ peptides may bind redox-active transition metals in vivo where trace metal concentrations are extremely low. Any biological significance of the catalytic role of the toxic Aβ peptides in the transition metal-mediated oxidation of hydroxylamine is yet to be determined. Although these hydroxylamines do not occur naturally, other easily oxidized substances exist in vivo such as ascorbate and GSH.

Moreover, it is known that copper and iron ions catalyze protein damage and may be partly responsible for the alterations of protein damage in vivo (35). Metal-catalyzed protein damage is associated with oxidative modification of amino acids, for example, formation of protein carbonyl groups (35). Therefore, the previously reported inactivation of glutamine synthetase and creatine kinase incubated with Aβ-(25–35) (14) could be explained on the basis of metal-catalyzed protein damage. Previously, it was shown that iron facilitates Aβ toxicity to cultured cells (36). Moreover, it was found that iron metabolism is altered in Alzheimer’s disease (37), which, in combination with accumulation of Aβ peptides, could result in peroxidative stress in the brains of Alzheimer’s disease patients. It was suggested that lowering the level of available iron could provide a therapeutic approach to Alzheimer’s disease (38). Furthermore, it was shown that Aβ-(25–35) enhances iron- and copper-catalyzed generation of reactive oxygen species (39). Therefore, our data concerning toxic Aβ-stimulated metal-catalyzed oxidation of hydroxylamine derivatives could give insight into the synergic toxicity of transition metals and Aβ peptides.

The data obtained lead to the conclusion that Aβ peptides do not form ESR-detectable radical adducts spontaneously. Our spin-trapping results and interpretation differ from the previous investigations (14–17). Our data do not support the conclusion that Aβ peptides spontaneously form free radicals, but demonstrate that hydroxylamine impurities in the spin trap preparations are responsible for the observed ESR spectra. Therefore, the reported spontaneous generation of PBN radical adducts by Aβ peptides has been reinterpreted. Although the spontaneous free radical model of Aβ neurotoxicity has been criticized by Sayre et al. (40), the previous spin-trapping results and interpretations have not been challenged until now.