Site-specific Effects of Peptide Lipidation on β-Amyloid Aggregation and Cytotoxicity

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- Amyloid (Aβ) aggregates at low concentrations in vivo, and this may involve covalently modified forms of these peptides. Modification of Aβ by 4-hydroxynonenal (4-HNE) initially increases the hydrophobicity of these peptides and subsequently leads to additional reactions, such as peptide cross-linking. To model these initial events, without confounding effects of subsequent reactions, we modified Aβ at each of its amino groups using a chemically simpler, close analogue of 4-HNE, the octanoyl group: K16-octanoyl acid (OA)-Aβ, K28-OA-Aβ, and Nα-OA-Aβ. Octanoylation of these sites on Aβ-(1–40) had strikingly different effects on fibril formation. K16-OA-Aβ and K28-OA-Aβ, but not Nα-OA-Aβ, had increased propensity to aggregate. The type of aggregate (electron microscopic appearance) differed with the site of modification. The ability of octanoyl-Aβ peptides to cross-seed solutions of Aβ was the inverse of their ability to form fibrils on their own (i.e. Aβ ≈ Nα-OA-Aβ ≫ K16-OA-Aβ ≫ K28-OA-Aβ). By CD spectroscopy, K16-OA-Aβ and K28-OA-Aβ had increased β-sheet propensity compared with Aβ-(1–40) or Nα-OA-Aβ. K16-OA-Aβ and K28-OA-Aβ were more amphiphilic than Aβ-(1–40) or Nα-OA-Aβ, as shown by lower “critical micelle concentrations” and higher monolayer collapse pressures. Finally, K16-OA-Aβ and K28-OA-Aβ are much more cytotoxic to N2A cells than Aβ-(1–40) or Nα-OA-Aβ. The greater cytotoxicity of K16-OA-Aβ and K28-OA-Aβ may reflect their greater amphiphilicity. We conclude that lipidation can make Aβ more prone to aggregation and more cytotoxic, but these effects are highly site-specific.

Alzheimer disease, the most prevalent neurodegenerative disorder, leads to progressive memory loss, disability and eventually death (1). It is characterized by the accumulation of extracellular plaques of amyloid β (Aβ)² (2). Although aggregation of Aβ appears to be important in the pathogenesis of Alzheimer disease, one of the unanswered questions is how Aβ peptides aggregate at the low concentrations at which they exist in the central nervous system. The concentration of Aβ in the cerebrospinal fluid has been estimated as in the nanomolar range or even lower (3–12). This is below the “critical micelle concentration” for Aβ (13) and at the margin of the critical concentration (Cₘ), at which Aβ solutions can extend fibrillar seeds (14, 15). One recently proposed answer to this question is that an aggregation-prone, covalently modified Aβ could catalyze aggregation of the unmodified peptide. In addition to catalyzing peptide aggregation, aldehyde-modified Aβ could also be cytotoxic to neurons by itself. Oxidative stress, a possible pathogenic factor in Alzheimer disease, can lead to the formation of reactive lipid aldehydes, such as 4-HNE (16–21) and cholesterol oxides (22), among others. Indeed, 4-HNE immunoreactivity is detected in plaques and cerebrospinal fluid of patients with Alzheimer disease (19, 20) and is believed to play a role in many other neurodegenerative diseases, including Parkinson disease (23–26), Lewy body disease (27), amyotrophic lateral sclerosis (28–31), olivopontocerebellar atrophy (32), and progressive supranuclear palsy (33), in all of which protein aggregation is believed to play a pathogenic role.

In this paper, we address two outstanding issues related to the effects of 4-HNE on the aggregation of Aβ. 1) There is little information on site-specific effects of 4-hydroxynonenal. Position-nonspecific addition of this lipid group seems to increase the propensity of Aβ to aggregate. 4-HNE, however, can react with amino, imidazole, guanidino, and hydroxyl groups (and thiols, which are not present in Aβ). These groups comprise nine of the 40 residues in Aβ-(1–40). It is unlikely that the addition of 4-HNE to all of these sites would have identical effects on aggregation. 2) There is little information that distinguishes initial versus subsequent effects of 4-HNE on protein aggregation. The initial addition of 4-HNE to a protein is followed by subsequent reactions of the adduct (21, 34–39). 4-HNE can form Michael adducts and Schiff bases, both of which can lead to protein cross-linking, cyclization, and rearrangements of the adduct. For example, the double bond of Michael adducts or Schiff bases can undergo epoxidation, and the 4-hydroxy group can be further oxidized to an aldehyde, either of which can lead to protein cross-linking by reaction with amino groups on adjacent molecules. Thus, 4-HNE initially increases the hydrophobicity of a protein and subsequently leads to protein cross-linking. Although either of these could increase Aβ aggregation, from the mechanistic point of view, it is important to distinguish between these two effects.

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2 The abbreviations used are: Aβ, β-amyloid; CMC, critical micelle concentration; DPH, diphenylhexatriene; HFIP, hexafluoroisopropl alcohol; 4-HNE, 4-hydroxynonenal; N2A, neuro2A; OA, octanoyc acid; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; HPLC, high pressure liquid chromatography; ThT, thioflavin T.

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To address these questions, we sought a model of 4-HNE that could conveniently be added to specific sites on Aβ, but not undergo subsequent reactions. Such a model would enable us to examine the role of increasing the hydrophobicity of the peptide at specific sites, without confounding effects, such as protein cross-linking or adduct rearrangements. For this reason, we chose to examine Aβ octanoylated at specific sites. The octanoyl group is similar to 4-HNE in chain length and polarity and is thus a good model for the lipidic effects of 4-HNE but, in contrast to the latter group, makes stable adducts. In this paper, we will describe synthetic Aβ with octanoyl adducts at its three amino groups, the side chains of Lys16 and Lys28 and the N-terminal amine, which are prone to modification by aldehydes. We will present evidence that the octanoylated Aβ peptides are more prone to aggregation and are more cytotoxic than unmodified Aβ. The magnitude and type of effect, however, depends strongly on the site of modification. We will also show that for this series of peptides, there is an inverse relationship between the ability of the peptide to form fibrils and its ability to induce mixed aggregates with unmodified Aβ.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis, Lipidation, and Purification**—The human Aβ-(1–40) peptide was synthesized manually by solid phase methods using standard t-butoxycarbonyl chemistry: NH₂-DAAEFRHDSGY10EVHHQKLVFF20AEDVGSNKGA30IIGLMVGGVV40-COOH. Peptides were prepared using preloaded (phenylacetylamido)ethyl resins (AnaSpec). Amino acids were deprotected with trifluoroacetic acid and coupled for 10 min with 0.5 M 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (Peptides International). Peptides were cleaved from the resin with hydrogen fluoride in the presence of p-cresol and purified using a reverse-phase, C18 preparative HPLC column (Rainin Dynamax) with an acetonitrile gradient in 0.1% trifluoroacetic acid at 60 °C. Peptide purity was greater than 95% by analytical HPLC (Rainin C18 column). The molecular masses of the peptides were verified with electrospray ionization and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The addition of octanoyl groups was essentially as described previously (40). For Aβ that was to be octanoylated at Lys16 or Lys28, (α-t-butoxycarbonyl)-L-Lys(ε-9-fluorenylmethoxycarbonyl) (AnaSpec) was incorporated into the peptide chain. The side chain 9-fluorenylmethoxycarbonyl group was then removed with 20% piperidine, and octanoic acid was coupled to the free ε-amino group using 0.2 M O-(7-azabenzotriazole-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (Applied Biosystems). To octanoylate Aβ at its N terminus, the N-terminal t-butoxycarbonyl protecting group was removed, after which octanoic acid was added to the free amino group as described above. Lipidated peptides were purified as described for unmodified Aβ. As predicted, octanoylation of Aβ rendered it more hydrophobic, as shown by later elution from the C18 reverse phase HPLC column (supplemental Fig. 1). After purification, all peptides were lyophilized and then redissolved in hexafluoroisopropylalcohol (HFIP), and the mixture was then sonicated for 15 pulses using a Branson Sonifier 450 probe sonicator (Danbury, CT) at a power setting of 4.5. The HFIP was then evaporated under a
Site-specific Aβ Lipidation, Aggregation, and Cytotoxicity

gentle stream of N₂. Although Me₂SO is preferable for initial solubilization of Aβ, this solvent cannot be used for far UV CD spectroscopy. For measuring spectra, peptides were dissolved in 600 µl of 10 mM sodium phosphate, pH 7.40. The peptide was centrifuged at 13,000 × g for 10 min. The supernatant was withdrawn, and its concentration was measured by absorbance at 274.6 nm, as described above.

CD spectroscopy of the peptides in the presence of phospholipids was performed in two different ways. First, CD spectra were measured of peptides mixed with preformed 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles. Vesicles were formed by dissolving the phospholipids (Avanti Polar Lipids) in CHCl₃/CH₃OH (1:1, v/v) at a concentration of 2.5 mg/ml. Solvent was evaporated by a gentle stream of N₂, and the lipid was suspended in 10 mM sodium phosphate, pH 7.40, by vortexing to yield a final lipid concentration of 1 mM POPC. The suspension was then sonicated as described above. For CD spectroscopy, POPC vesicles were added to peptide to yield a final peptide/POPC ratio of 1:20 (mol/mol).

Second, CD spectra were measured of peptides that were mixed with POPC during the formation of vesicles. Peptide (~0.4 mg) was dissolved in 500 µl of HFIP, and the sample was sonicated as described above. The solvent was then evaporated under a gentle stream of N₂. POPC dissolved to 2.5 mg/ml in CHCl₃/CH₃OH (1:1, v/v) was then added to the peptide film, to give a final peptide/lipid ratio of 1:20 (mol/mol). The solvent was then dried under N₂. Control samples consisted of peptide alone, without the lipid. Samples were then suspended in 600 µl of 10 mM sodium phosphate, pH 7.40, and the mixture was then sonicated for 15 pulses at a sonicator setting of 4.5, after which most of the material appeared to be in solution. The mixture was then filtered using 0.2-µm cellulose acetate filters. Because of the possibility of light scattering from vesicles and/or protein aggregates, protein concentrations were measured by absorbance at 274.6 nm and also confirmed by analytical HPLC. Samples were injected onto a Varian Microbore-MV C18 column (Lake Forest, CA), and the mass of peptide was measured by the area of the peak, compared with Aβ standards. CD spectra of these samples were measured immediately after making them and again at 48, 96, and 168 h.

Surface Isotherms of Peptide Monolayers at the Air-Water Interface—Surface isotherms were measured essentially as described previously (40). More details of these procedures are given in the supplemental materials.

Diphenylhexatriene (DPH) Fluorescence—The critical micelle concentration was estimated using DPH fluorescence (46). Fluorescence measurements were performed using a Hitachi F-2000 fluorescence spectrophotometer. Because Me₂SO interferes with these measurements, peptide samples were dissolved in 1 ml of HFIP to a concentration of ~75 µM and centrifuged at 13,000 × g for 10 min. The supernatant was removed, and the HFIP was evaporated under N₂. Peptides were then redissolved in 10 mM sodium phosphate, pH 7.40, to various concentrations. DPH (Molecular Probes) was dissolved as a stock in tetrahydrofuran to a concentration of 10 mM; for measurements, the stock was diluted with tetrahydrofuran to a concentration of 200 µM. Measurements were made on 300 µl of peptide at various concentrations, to which 7.5 µl of DPH had been added. The samples were kept unsealed to allow evaporation of the tetrahydrofuran but were protected from light by aluminum foil to prevent photobleaching of the DPH. After 5 min of incubation (previous experimentation showed that this time was sufficient to obtain maximum fluorescence), fluorescence of the sample was measured (λₑₓ = 358 nm, λₑₘ = 430 nm) with a bandwidth of 10 nm and the photomultiplier setting at 700 V.

Cross-seeding of Aβ by Seeds of Octanoylated Aβ Peptides—We investigated the extent to which fibrils of octanoylated Aβ were able to cross-seed solutions of Aβ; in these measurements, cross-seeding was always compared with self-seeding of Aβ solutions by Aβ fibrils. Fibrils were made specifically for cross-seeding studies starting with aliquots of HPLC-purified peptides. Aliquots of 0.4 µg of peptide were dissolved directly into 10 mM sodium phosphate, pH 7.40, with no disaggregation procedure, to a final concentration of either 100 or 200 µM; no differences were observed between seeds formed at these two concentrations. For forming seeds, solutions were incubated at 37 °C for 168 h; ThT fluorescence was monitored daily, and by 168 h, this value had plateaued.

For cross-seeding experiments, Aβ was first solubilized in undiluted Me₂SO to a concentration of 5 mM; this was then diluted 50-fold into 10 mM sodium phosphate, pH 7.40, to yield a final concentration of 100 µM. The solution was centrifuged at 13,000 × g for 10 min, and the top ~90% of the supernatant was taken for the experiment. To the soluble Aβ, seeds of the various peptides or no seeds were added. In all cases, seeds consisted of 1.5 nmol of fibrillar peptide, which represented 5% of the mass of peptide. Measurements represent the average of three experiments, each with two replicates. At various times, ThT fluorescence was measured in aliquots of the seeded solutions of Aβ, as described above.

Size Exclusion Chromatography of Aβ and Octanoylated Aβ—Size exclusion chromatography of Aβ and the three octanoylated Aβ peptides was performed using a Superdex 200 (10/300 GL) column on an Agilent 1100 high pressure liquid chromatograph. Column effluent was monitored using a diode array detector, and the absorbances were recorded at 215 and 220 nm; flow rate was 0.5 ml/min. For these experiments, peptides were disaggregated by initially dissolving them in Me₂SO, according to the procedure described previously (41). Size exclusion chromatography was performed on peptide solutions at different concentrations; concentration was varied by serial dilution of the initial solutions, which had peptide concentrations of 200 µM.

Cell Viability Assay—Neuro2A (N2A) cells, generously provided by Drs. Godfrey Getz, Catherine Readon, and Gopal Thianakaran, were maintained at 37 °C, 5% CO₂, in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were seeded into 96-well plates and allowed to reach 70% confluence. Growth medium was replaced with Dulbecco's modified Eagle's medium without serum or phenol red ("treatment medium"), and the cells were allowed to acclimate overnight. On the next day, half of the liquid in each well was replaced with the treatment medium containing one of the four peptides at various concentrations or treatment medium without peptide.
Site-specific Aβ Lipidation, Aggregation, and Cytotoxicity

The cells were then returned to the incubator for 2 days, after which cell viability was assessed by measuring the ability of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt to purple formazen crystals. The crystals were solubilized with 10% Triton X-100, 0.1 N HCl in isopropyl alcohol at 37 °C overnight. The absorbance at 570 nm was measured using a Molecular Devices Versamax tunable microplate reader (Sunnyvale, CA); the background at 690 nm was subtracted from the reading.

RESULTS

Rationale of These Studies—We synthesized three peptides, one octanoylated at each of the three amino groups in Aβ. The rationale was as follows. 1) As discussed above, 4-hydroxynonenal, a major product of unsaturated fatty acid oxidation, reacts with aggregating proteins and peptides in Alzheimer disease and other neurodegenerative diseases. We confirmed this finding immunohistochemically, using an anti-HNE adduct antibody; as shown in supplemental Fig. 2, localization of immunoreactivity against the 4−HNE adduct occurs in a pattern consistent with neuritic plaque deposition. 2) We wanted to examine the effects of adding a hydrophobic group, such as 4-HNE, on the initial stages of aggregation of Aβ. However, 4-HNE both increases the hydrophobicity of proteins or peptides to which it is added and also undergoes numerous subsequent reactions, including rearrangements and protein cross-linking reactions. In order to focus upon the initial stages of protein aggregation and examine the effects of added hydrophobicity, we sought a model of similar chain length and polarity as 4-HNE, but which would not undergo the subsequent rearrangements to which 4-HNE is prone. The octanoyl group fulfills these criteria, because it is of similar chain length and polarity as 4-HNE but has the advantages of chemical stability and synthetic simplicity (40). 3) Although it has been shown that the addition of hydrophobic groups to Aβ increases its aggregation, little information is available on the site-specific effects of modification. There is good reason, however, to expect that modification of different parts of Aβ would have radically different effects. In particular, consider the three amino groups of Aβ, which are readily modified by lipid-derived aldehydes, such as 4-HNE. In the Aβ fibril (47–51), the α-amino group is within an unstructured, hydrophilic N-terminal segment, Lys16 is in the N-terminal β-sheet, and Lys28 is within the “bend region” between the two β-sheets. Accordingly, in this study, we present data showing the effects of octanoylating each of these three sites on aggregation, their ability to associate with and assume structures at lipid surfaces, and their ability to cross-seed unmodified Aβ into fibrils. Finally, since octanoylation significantly altered aggregation and surface properties of Aβ, we assessed the cytotoxicity of these peptides at very low concentrations.

OA-Aβ Peptides Each Have Aggregation Kinetics Distinct from Those of Aβ and from Each Other—The kinetics of aggregation of lipidated peptides were monitored by ThT as described under “Experimental Procedures.” Fig. 1 shows fibrillogenesis of the four peptides as monitored by ThT fluorescence; rate constants and other kinetic parameters for fibrillogenesis are given in Table 1.

In agreement with previous work (e.g. see Ref. 52), unmodified Aβ aggregates into fibrils and shows thioflavin fluorescence following a lag period of approximately 2 days (Fig. 1A). These data could be analyzed using the equation of a stretched exponential (as in Ref. 41),

\[
\text{ThT} = \text{ThT}_0 + (\text{ThT}_\infty - \text{ThT}_0)(1 - \exp(-kt)^n) \tag{Eq. 1}
\]

where ThT, ThT₀, and ThTₐ∞ represent thioflavin fluorescence at various times, t = 0, and at infinite time, respectively, n is a power function of time (54, 55), and k is a rate constant.

Octanoylation of Aβ markedly alters the kinetic pattern of fibrillogenesis, but in a site-specific way. As shown in Fig. 1B, K16-OA-Aβ rapidly reached maximal ThT fluorescence (<5 min), and this maximum was consistently slightly lower than that of Aβ itself. The data shown in Fig. 1B were analyzed using a monoexponential equation, consistent with the lack of a lag period.

\[
\text{ThT} = \text{ThT}_0 + (\text{ThT}_\infty - \text{ThT}_0)(1 - \exp(-kt)) \tag{Eq. 2}
\]

K28-OA-Aβ reached a maximal thioflavin fluorescence rapidly (~15 h), but this maximum was consistently ~5-fold higher than that attained by Aβ, indicating a different mode of
In addition, the maximal value of ThT fluorescence was attained by unmodified \( \text{A} \)-\( \beta \) fibrils, electron microscopy of K16-OA-\( \text{A} \)-\( \beta \) showed that only short, thin protofibrils (9.0 ± 1.0 nm diameter) were present at 48–72 h, and the development of mature fibrils was apparent only after prolonged incubation (~500 h).

In marked contrast to K16-\( \text{OA} \)-\( \text{A} \)-\( \beta \), mature fibrils of K28-\( \text{OA} \)-\( \text{A} \)-\( \beta \) were present by 12 h of incubation. Indeed, some protofibrillar aggregates are apparent immediately after putting this peptide into aqueous buffer, despite attempts to disaggregate the peptide using \( \text{Me}_2\text{SO} \), the same technique as is used for unmodified \( \text{A} \)-\( \beta \). This finding is especially striking in view of the very high levels of ThT fluorescence attained in the same period of time. Fibrils of K28-\( \text{OA} \)-\( \text{A} \)-\( \beta \) are somewhat thinner than those of \( \text{A} \)-\( \beta \) (8.8 ± 0.9 and 11.9 ± 1.2 nm, respectively), and the monoeponential decline of thioflavin fluorescence after the peak at ~15 h appears to coincide with lateral association of the fibrils. In agreement with ThT fluorescence measurements, \( \text{OA} \)-\( \text{OA} \)-\( \text{A} \)-\( \beta \) formed only thin, somewhat irregular fibrils after prolonged incubation periods.

**CD Spectroscopy of \( \text{A} \)-\( \beta \) and the Three Octanoylated \( \text{A} \)-\( \beta \) Peptides in the Presence or Absence of POPC Vesicles**—Fig. 3, A and B, shows CD spectra of \( \text{A} \)-\( \beta \) and the three OA-\( \beta \) peptides in the presence or absence of POPC vesicles, respectively. In these experiments, an aqueous solution of peptide was mixed with preformed POPC vesicles, and the CD spectra was measured immediately after the sample was made. Each peptide was at \( \leq 50 \mu\text{M} \), at which concentration \( \text{A} \)-\( \beta \) is predominantly monomeric. As shown in Fig. 3 and Table 1, the CD spectra of \( \text{A} \)-\( \beta \) were most consistent with a “random coil” (i.e. virtually no \( \alpha \)-helix or \( \beta \)-sheet). In contrast to unmodified \( \text{A} \)-\( \beta \), K16-\( \text{OA} \)-\( \text{A} \)-\( \beta \) showed a pronounced \( \beta \)-sheet signature; as shown below (size exclusion chromatography (Fig. 8B)), of the four peptides, K16-\( \text{OA} \)-\( \text{A} \)-\( \beta \) was most prominently populated with oligomers at this concentration. The spectra of all four peptides remained the same after the addition of POPC vesicles, indicating that under these conditions, the peptides either do not associate appreciably with the vesicles, or if they do, this association induces no conformational change. The preceding experiments differ from those shown in Fig. 4, A–H. In these experiments, POPC vesicles were formed by sonication in the presence of a dried peptide film rather than adding peptide to preformed vesicles as in the previous experiments. CD spectra were recorded over times up to 168 h and compared with peptides treated identically except that no POPC was added. In the absence of POPC, the CD spectra of \( \text{A} \)-\( \beta \) remained

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**TABLE 1**

Properties of \( \text{A} \)-\( \beta \)-(1–40) and three octanoylated peptides

| Property                     | \( \text{A} \)-\( \beta \)-\( \beta \)-(1–40) | \( \text{OA} \)-\( \alpha \)-\( \beta \)-(1–40) | \( \text{OA} \)-\( \text{K} \)-\( \beta \)-(1–40) | \( \text{OA} \)-\( \text{K} \)-\( \beta \)-(1–40) |
|------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Kinetics Rate equation       | Stretched exponential 1.29 × 10⁻⁵ min⁻¹       | Stretched exponential 6.12 × 10⁻⁵ min⁻¹       | Monoeponential 2.36 ± 1.70 min⁻¹              | Biexponential 2.1 × 10⁻⁵ min⁻¹ 4.90 × 10⁻⁵ min⁻¹ |
| Fibril diameters (nm)        | 11.9 ± 1.2                                  | 13.4 ± 1.1                                  | 9.0 ± 1.0                                    | 8.8 ± 0.9                                    |
| Surface properties           |                                             |                                             |                                             |                                             |
| MW (molecules/oligomer)      | 6.03 × 10⁴ (14)                             | 7.42 × 10⁴ (18)                             | 2.47 × 10⁴ (6)                               | 8.18 × 10⁴ (18)                             |
| CD spectra (A²/аминокислота) | 10.5                                       | 11.3                                       | 19.0                                        | 11.3                                        |
| π₀ (мл/м)                    | 9.0                                        | 9.5                                        | 17.7                                        | 9.6                                         |
| CMC (мМ)                     | 50                                         | 50                                         | 10                                          | 8                                           |
| Toxicity (LD₅₀, мМ)          | 42.00 μМ                                   | 25.8 μМ                                   | 0.60 μМ                                     | 1.34 μМ                                     |

**FIGURE 2.** Electron micrographs of negatively stained fibrils formed by \( \text{A} \)-\( \beta \) (first row), K16-\( \text{OA} \)-\( \beta \) (second row), K28-\( \text{OA} \)-\( \beta \) (third row), and \( \text{OA} \)-\( \text{OA} \)-\( \beta \) (fourth row) at various times ranging from 0 to 500 h. Fibrils were grown from 115 μМ peptide in 10 мМ sodium phosphate, pH 7.40, from a peptide stock in \( \text{Me}_2\text{SO} \) (final \( \text{Me}_2\text{SO} \) concentration ~2%). All peptides except \( \text{OA} \)-\( \beta \) eventually form long, unbranched fibrils with the typical amyloid morphology; OOA-\( \beta \) forms somewhat thin, irregular fibrils. There are some differences in diameters, as detailed in Table 1. Magnification of all images is ×137,200. Scale bar, 50 nm.
essentially unchanged throughout this experiment (Fig. 4A). In contrast, the addition of POPC led to a change in the spectrum; there was a minimum at 218 nm and a maximum below 200 nm (Fig. 4B). These changes indicate formation of β-sheet at times and peptide concentrations at which the peptide alone or added to preformed POPC vesicles is unstructured. A similar pattern was observed for Nα-OA-Aβ (Fig. 4, G and H), although this peptide showed a somewhat less prominent β-sheet signature than unmodified Aβ. Light scattering due to vesicles precluded accurate assessment of the maximum.

In contrast to either unmodified Aβ or Nα-OA-Aβ, K16-OA-Aβ and K28-OA-Aβ both showed a minimum at 218 nm, even in the absence of POPC (Fig. 4, C and E, respectively). The ellipticity at 218 increased ~2–3-fold, reaching a maximum after 48 h, remaining constant up to the end of the experiment at 168 h. The magnitude of change was the same in the presence or absence of POPC (Fig. 4, D and F, respectively).

Properties of Aβ and Octanoylated Aβ at the Air-Water Interface—We and others (13, 40, 56, 57) have shown that Aβ peptides are amphiphilic, as would be predicted from the cluster of hydrophobic amino acids near the C terminus. We compared monolayers of OA-Aβ with those of unmodified Aβ. 50 μg of each peptide dissolved in HFIP was spread onto the buffer surface, and the monolayer was compressed. The data represented six replicate monolayers spread onto the surface. As described in the supplemental materials, data for surface pressures (π) ≤ 1 mN m −1 were analyzed using the equation of a two-dimensional gas,

\[ \pi(A - nA_0) = nRT \]  
(Eq. 3)

where π represents surface pressure (mN m −1 ≡ erg cm −2), A is surface area (cm 2), n is the number of moles of peptide spread at the surface, A 0 is the molar exclusion area, R is the gas constant, and T is the temperature (K).

For π > 1, the equation was modified such that the exclusion area, A 0, was a variable dependent on surface pressure,

\[ A_0 = A_{00}(1 - \kappa \pi) \]  
(Eq. 4)

where A 00 represents the molar exclusion area of the peptide extrapolated to π = 0, and κ has the units of a compressibility factor. Thus, the two-dimensional gas equation is altered to the following,

\[ \pi(A - nA_{00}(1 - \kappa \pi)) = kT \]  
(Eq. 5)

where k represents a parameter with the same units as mol × gas constant. The isotherms, with fits to the above equations, are shown in Fig. 5; values of the parameters are shown in Table 1. All of the peptides were amphiphilic, as shown by monolayer collapse pressures (π c). For Aβ, π c was 26 mN m −1; for K16-OA-Aβ, K28-OA-Aβ, and Nα-OA-Aβ, π c was slightly higher: 34, 34, and 32 mN m −1, respectively. Analysis of the isotherms showed that the addition of the octanoyl group to ε-NH 2 of Lys 16 significantly altered the monolayer properties. First, the exclusion areas, A 0 and A 00 (the “footprint” of the peptide at the surface) were 19.0 and 17.7 Å 2/amino acid for K16-OA-Aβ, whereas for the other peptides, these values were much lower, ranging from 10.5 to 11.3 (A 0 ) and from 9.0 to 9.6 (A 00 ). All of these numbers suggest that the peptides assume a secondary or tertiary structure at the surface, since the surface area of a fully extended “random coil” peptide is much larger (~40–50 Å 2/amino acid). K16-OA-Aβ, however, is much less compact at the surface than the other peptides. In addition, the apparent molecular weight of all of the peptides indicated self-association into oligomers at the surface, but again, K16-OA-Aβ differed from the other peptides; the apparent molecular weight suggested approximately hexamers for K16-OA-Aβ, whereas the other peptides had aggregates with ~14–18 peptide molecules.

Octanoylation Decreases the “Critical Micelle Concentrations” of K16- and K28-OA-Aβ but Not Nα-OA Aβ—The oligomerization of these peptides was investigated by two additional techniques: binding of the dye DPH to assess CMC and size exclusion chromatography. Fig. 6 shows fluorescence of DPH as a function of peptide concentration. CMC was estimated as the x intercept of the nearly linear portion of the curve at higher peptide concentrations. The curves for unmodified Aβ and Nα-OA-Aβ are similar and to the right of the curves for K16-OA-Aβ and K28-OA-Aβ. Thus, Aβ and Nα-OA-Aβ have CMCs of ~40–60 μM, whereas K16-OA-Aβ and K28-OA-Aβ have CMCs of ~10 μM. The CMC of K16-OA-Aβ may be slightly lower than that of K28-OA-Aβ.
to cross-seed Aβ solutions. Note that the curve for K28-OA-Aβ is shifted upward on the y axis. This shift can be attributed to the addition of even the small amount (5% by weight) of seed, since K28-OA-Aβ fibrils give very large thioflavin T fluorescence responses (see Fig. 1C). This shift notwithstanding, the addition of K28-OA-Aβ fibrils caused only modest rate enhancement, at most, of Aβ fibril formation.

Size Exclusion Chromatography of Aβ and Octanoylated Aβ Demonstrates the Propensity of Octanoylated Aβ to Form Soluble Oligomers—Results of size exclusion chromatography of Aβ and OA-Aβ are shown in Fig. 8, A–D. Aβ eluted mainly as a low molecular weight species, consistent with monomer. There was also a small peak in the void volume, the relative proportion of which increased with increasing peptide concentration. The elution profile of Nα-OA-Aβ was similar, except that several small, partially included peaks were present, consistent with soluble oligomers. In contrast, K16- and K28-OA-Aβ showed major oligomer peaks even at the lowest concentrations tested (i.e. 0.78 mM). K16-OA-Aβ showed a major oligomer peak that eluted after the void volume, corresponding to an apparent molecular mass of ∼544 kDa; in addition, a smaller peak eluted in the void volume. K28- and K16-OA-Aβ showed a major oligomer peak eluting in the void volume. In addition, both K16- and K28-OA-Aβ showed a loss of ∼20% of the protein on the column, presumably due to precipitation and/or adsorption. These data show the propensity of K16- and K28-OA-Aβ to form soluble oligomers, even after a disaggregation procedure that leaves relatively little oligomer in the solutions of unmodified Aβ. The small amount of residual Aβ oligomer may be present because the solutions were made by serial dilution of a concentrated (200 μM) stock solution.

Effect of Aβ and Octanoylated Aβ Peptides on N2A Cell Viability—Octanoylation of Aβ alters its aggregation, including the concentration at which it forms oligomers, and its surface properties. For these reasons, we assessed the toxicity of Aβ and the three forms of octanoylated Aβ toward N2A cells, a mouse cell line derived from a neuroblastoma. Peptides were incubated with cells for 48 h at 37 °C, after which cell viability

**Na-OA-Aβ Is Extremely Efficient at Cross-seeding Aβ Solutions; Ability to Cross-seed Varies Inversely with Ability to Form Fibrils—**The three octanoylated Aβ peptides were compared for their ability to cross-seed solutions of Aβ; their efficiency of cross-seeding was compared with that of Aβ seeds (self-seeding) and with unseeded fibril formation. Fig. 7 shows that there was an inverse correlation between the efficiency of cross-seeding and the ability of the peptide to form fibrils when present in pure form. Thus, Na-OA-Aβ, although it forms fibrils at a very slow rate, was approximately as efficient at seeding Aβ to form fibrils as seeds of Aβ itself. K16-OA-Aβ was less efficient at cross-seeding. Strikingly, K28-OA-Aβ, which forms fibrils almost instantly and without any lag period, showed little ability...
was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt reduction assay. Fig. 9 shows that K16-OA-Aβ/H9252 and K28-OA-Aβ/H9252 both displayed markedly increased toxicity toward N2A cells, as indicated by the LD_{50} values: 42.00 μM for Aβ, compared with 0.60 and 1.34 μM for K16-OA-Aβ and K28-OA-Aβ, respectively. In fact, the toxicity of these peptides toward these cells was even greater than that of the “positive control” peptide, mellitin, well known as a cytolytic peptide. In addition, the shape of the curve for these two octanoylated peptides was different from that of Aβ. The slope of the nearly linear middle portion of the curve (analyzed as a
rectangular hyperbola) was steeper for the two octanoylated peptides, possibly reflecting differences in concentration dependence of oligomerization by these peptides. Na-OA-Aβ was only slightly more cytotoxic than unmodified Aβ in these assays (LD_{50} = 25.8 μM).

**DISCUSSION**

Oxidative stress, either in the central nervous system during the development of neurodegenerative diseases or in the artery wall in the development of atherosclerosis, can lead to scission of unsaturated fatty acyl chains, and the products of these reactions lead, in turn, to protein modification. In particular, 4-hydroxynonenal, a major product of unsaturated fatty acid oxidations, reacts with amino groups of lysine and N-terminal imidazole groups in a-amino groups of lysine and N-terminal imidazole groups in hydrogenated N-terminal segment, Lys and Lys within the “bend region” between the two β-sheets. Our experiments demonstrate that this modification has strikingly diverse effects on aggregation, surface properties, and cytotoxicity of Aβ.

To recapitulate, the findings in our studies were the following. 1) Octanoylation of the three sites on Aβ had strikingly different effects on fibril formation. K16-OA-Aβ showed maximal thioflavin T fluorescence instantly but formed only irregular “protofibrils” in electron microscopy and formed mature amyloid fibrils only after prolonged (~500-h) incubation. K28-OA-Aβ formed mature amyloid fibrils more rapidly than unmodified Aβ, and these fibrils showed much higher thioflavin T fluorescence levels than unmodified Aβ. Octanoylation of the N terminus did not accelerate development of thioflavin T fluorescence. Furthermore, Na-OA-Aβ formed only thin, irregular fibrils and never formed mature amyloid fibrils in <500-h incubation. 2) The ability of octanoyl-Aβ peptides to cross-seed solutions of Aβ was the inverse of their ability to form fibrils on their own. Thus, although Na-OA-Aβ did not form mature fibrils on its own, it was as efficient as unmodified Aβ at seeding solutions of Aβ. Conversely, K28-OA-Aβ formed fibrils more rapidly than unmodified Aβ but did not seed fibril formation by solutions of unmodified Aβ. K16-OA-Aβ aggregated extremely rapidly, but formation of mature amyloid fibrils was delayed compared with unmodified Aβ. It was able to seed fibril formation by unmodified Aβ much less efficiently than Na-OA-Aβ. 3) K16-OA-Aβ and K28-OA-Aβ carry their own lipid environment that favors β-sheet structure. Modification of either of the two lysine side chains led to rapid development of a β-sheet signature in CD spectra, and this occurred whether or not the peptides were mixed with POPC, either by adding preformed vesicles or co-sonicating the peptide with POPC. In contrast, both unmodified Aβ and Na-OA-Aβ had random coil signatures in CD spectra. The latter two peptides could be induced to form β-sheets by co-sonication with POPC to form mixed vesicles but retained their random coil signatures when they were simply mixed with preformed POPC vesicles. 4) K16-OA-Aβ and K28-OA-Aβ are more amphiphilic than unmodified Aβ or Na-OA-Aβ. This was shown by three sets of findings: the first two peptides had lower CMCs (DPH fluorescence), increased collapse pressure (π_c) of monolayers at the air-water interface, and more oligomers in size exclusion chromatography. 5) K16-OA-Aβ and K28-OA-Aβ were much more cytotoxic to N2A cells than either unmodified Aβ or Na-OA-Aβ. The first two of these peptides were even more cytotoxic than the “positive control” peptide, melittin.

Putting all of these findings together, we conclude the following. 1) The effects of adding an octanoyl group to Aβ depend strongly on the site of modification. 2) Although modification at each led to distinct changes in aggregation and surface properties, to a first approximation, modification of the two Lys side
Site-specific Aβ Lipidation, Aggregation, and Cytotoxicity

chains led to highly aggregative peptides that were structurally distinct from unmodified Aβ. In contrast, Na-OA-Aβ, although delayed in making fibrils (or possibly unable to do so), was structurally closer to unmodified Aβ. It is important, in considering these results, to distinguish between kinetics and structure. Na-OA-Aβ is kinetically trapped in a prefibrillar form, but one upon which unmodified Aβ can grow into fibrils epitaxially. The findings on Na-OA-Aβ are also in agreement with previous observations on a double mutation of Aβ, D1E/A2V, which makes the N-terminal region more hydrophobic and promotes formation of oligomers or protofibrils but does not accelerate fibril formation (58). In contrast, the relatively inefficient cross-seeding by K16- and K28-OA-Aβ suggest more significant structural alterations, as would be expected from the location of these two sites within the sequence.

Finally, our findings on the cytotoxicity raise mechanistic questions about the interactions between these peptides and the cell membrane. The increased cytotoxicity of K16- and K28-OA-Aβ may reflect the increased amphipathicity of these peptides, which could lead to membrane disruption. The interaction of fibril-forming peptides and lipid surfaces is complex, however. Whether lipids promote or inhibit fibril formation depends on peptide/lipid ratio (59), the charge and other electrostatic properties of the lipids, and the structural propensities of the peptides. β-Amyloid interacts with phospholipids, especially but not exclusively anionic ones, through ionic interactions, and this promotes the transition from random coil to β-sheet (60, 61). Initial electrostatic interactions ultimately lead to penetration of the peptide into dipalmitoylphosphatidylcholine bilayers (56). Our observations on POPC indicate minimal interactions of Aβ with a preformed surface composed of this neutral lipid. In contrast, interactions occurred when Aβ was mixed with POPC during formation of vesicles, suggesting that although Aβ is repelled by the electrostatic potential of the POPC vesicle surface, co-sonicating peptide and lipid can bypass these repulsions and allow interactions to occur.

These results may relate to the pathogenesis of Alzheimer disease and are reminiscent of earlier work from the atherosclerosis field, in which 4-HNE, malondialdehyde, and other products derived from oxidation of unsaturated phospholipids were found to form adducts with apolipoprotein B (65–67). Recent studies on Aβ and α-synuclein suggest the possibility of a similar scenario occurring in the brain in neurodegenerative diseases, either through the participation of 4-HNE (26, 68) or oxysterols (22, 53, 69, 70–74). The specifics of these interactions remain to be elucidated. Nevertheless, our data support the idea that modified Aβ could render these peptides both more prone to aggregation and more potent as a cytotoxic and could help explain the deleterious effects of Aβ peptides that occur in patients without gross elevations in the concentrations of these peptides in the brain or cerebrospinal fluid.

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