Small Molecule Oxidation Products Trigger Disease-Associated Protein Misfolding

JAN BIESCHKE, QINGHAI ZHANG, DARYL A. BOSCO, RICHARD A. LERNER, EVAN T. POWERS, PAUL WENTWORTH, JR., AND JEFFERY W. KELLY*

The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received March 6, 2006

ABSTRACT

Oxidative stress and inflammation are risk factors for both the development of α-synucleinopathies, such as Parkinson's disease and dementia with Lewy bodies, and Alzheimer's disease, the two most common neurodegenerative disorders. These diseases are associated with the neurotoxic deposition of misassembled α-synuclein and amyloid-β (Aβ) peptides, respectively. Both occur sporadically, that is, without detectable disease-related mutations, in the vast majority of cases. Small molecule oxidation products, especially secosterols derived from cholesterol and 4-hydroxynonenal derived from lipid peroxidation, found in afflicted brains, accelerate the misassembly of both Aβ and α-synuclein. This Account explores the mechanism of small molecule oxidation product-mediated protein misassembly and possible intervention strategies.

Introduction

The risk of developing a protein misfolding disease increases with age.[1] The amyloidoses are a major category of misfolding diseases involving the misassembly and deposition of toxic protein aggregates in various morphologies, including amyloid fibrils.[2,3] Protein misfolding diseases are subcategorized as intracellular or extracellular (although this distinction may not be useful if proteotoxicity arises from membrane structure perturbation). The most prominent "extracellular" amyloid disease is Alzheimer's disease (AD), whereas the most common intracellular misfolding diseases are the α-synucleinopathies, diseases including Parkinson's disease (PD) and dementia with Lewy bodies (DLB).[4] The amyloidoses typically exhibit tissue selective deposition and pathology; for example, the transthyretin amyloidoses either affect the peripheral nerves (neuropathies), the heart (cardiomyopathies), or the brain, depending on the mutant undergoing amyloidogenesis.[5−9]

Amyloid is an intermolecular fold available to most peptides and proteins.[10,11] X-ray crystallography, solid-state NMR, and hydrogen−deuterium exchange have recently been used to characterize the cross-β sheet structure of various amyloid fibrils at atomic resolution.[12−14] However, smaller aggregation intermediates are also implicated in the toxicity of amyloidogenic proteins.[1,15,16] Different aggregation conditions favor different aggregate morphologies[17] from small spherical assemblies[18] to protofibrils.[19]

Nucleated Polymerization Mechanism

Our understanding of amyloid fibril formation, while incomplete, suggests that aggregation can either be thermodynamically favored from the outset[7] or be characterized by a pronounced lag phase.[20−22] The lag phase of a nucleation-dependent polymerization[19,23] (Figure 1A) typically corresponds to slow formation of an oligomeric nucleus (Figure 1B). The steps preceding nucleation are energetically unfavorable because entropy loss outweighs the enthalpic gains from burial of hydrophobic surface area and H-bonding. Multivalent interactions occurring postnucleation are thought to render further monomer addition energetically favorable. Aggregates will not form

* Corresponding author. E-mail: jkelly@scripps.edu.

Jan Bieschke received his Ph.D. from the Max-Planck-Institute for Biophysical Chemistry in Göttingen, Germany (2000). He joined the laboratories of Hans Kretzschmar in Munich, Germany (2000−2003), and then Jeffery Kelly (2003−2006) for postdoctoral research focusing on single molecule fluorescence and the mechanisms of protein folding and misfolding. He is now a Research Group Leader at the Max Delbrück Center for Molecular Medicine in Berlin.

Qinghai Zhang received his Ph.D. from the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences (2001). His postdoctoral research in the Kelly laboratory focused on the role of oxidative metabolites in triggering protein misfolding diseases (2001−2004). He is now an Assistant Professor in the Department of Molecular Biology at The Scripps Research Institute (TSRI) investigating membrane chemistry and biophysics.

Daryl Bosco received her Ph.D. in bio-organic chemistry from Brandeis University (2003). Her postdoctoral research (2003−2005) in the Kelly laboratory focused on oxidative metabolite initiated α-synuclein misfolding. Daryl now works at Serono.

Richard A. Lerner, President of The Scripps Research Institute, is the Lita Annenberg Hazen Professor of Immunochemistry, holds the Cecil H. and Ida M. Green Chair in Chemistry, and is a member of The Skaggs Institute for Chemical Biology. His research investigates the conversion of antibodies into enzymes and combinatorial methods for the synthesis of antibodies for therapeutic and catalytic purposes. Furthermore, he is interested in antibody facilitated ozone and reactive oxygen synthesis that can react with molecules in the body, such as cholesterol, to produce toxic compounds that induce protein misfolding.

Evan Powers received his Ph.D. in Organic Chemistry from the Massachusetts Institute of Technology (1999). He joined the Kelly lab in 1999 and became an Assistant Professor at TSRI in 2000. His main interests are the energetics and mechanisms of protein folding and aggregation.

Paul Wentworth, Jr. received his Ph.D. from the University of Sheffield, U.K. (1994). His postdoctoral experience was obtained at The Scripps Research Institute (TSRI) (1994−1997). He is now a Professor of Chemistry at TSRI and a Professor of Medicinal Chemistry at the University of Oxford, U.K. His interests include chemistry and biology of reactive oxygen and nitrogen species, inflammatory diseases, and mechanisms of protein misfolding and disease.

Jeffery W. Kelly received his Ph.D. from the University of North Carolina at Chapel Hill (1986) and performed postdoctoral research at The Rockefeller University. In 1989, he became Assistant Professor at Texas A&M University where he was subsequently promoted to Professor of Chemistry. He joined the Department of Chemistry at TSRI in 1997 as the Lita Annenberg Hazen Professor of Chemistry. He became Dean of Graduate Studies and Vice President of Academic Affairs in 2000. His research focuses on understanding protein folding and misfolding in vitro and in vivo and the influence of aging and small molecules on these processes.

10.1021/ar0500766 CCC: $33.50
Published on Web 08/29/2006

below a critical concentration, K_c.[20,21] At peptide concentrations above K_c, aggregates grow until the monomer concentration decreases to K_c (see Figure 5C). The lag phase is eliminated when preformed fibrils or seeds are introduced into the reaction, obviating nucleation (Figure 1C). Energetically costly unimolecular conformational changes can also be required for monomeric nucleus formation.[22]

Inflammatory Processes Are Implicated in Protein Misfolding Diseases

Familial protein misfolding diseases have provided important insights into pathogenesis. These diseases are typically caused by destabilizing mutations in amyloid prone proteins or in enzymes that process or degrade amyloidogenic peptides, such as γ-secretase or ubiquitin ligases.[24] While familial forms of AD and PD have been informative, the vast majority of AD and PD cases are sporadic, wherein the sequences of the misfolded proteins and interacting proteins are identical to those in persons without disease. Thus, epigenetic, environmental, or aging-associated factors[1] have to be considered. Extending Anfinsen's teachings suggests that either the environment immersing the chain could be different or an unusual post-translational modification could occur in patients with sporadic misfolding diseases.

Amide side chain deamidation is known to enhance amyloidogenicity;[25] however, little is known about the role of established bimolecular protein modifications mediated by aldehydic oxidative metabolites and similar molecules.[26] We hypothesize that aberrant post-translational protein modifications may trigger the onset of sporadic misfolding diseases by making amyloidogenesis more efficient, perhaps by changing the mechanism. Determining the factors exacerbating sporadic amyloidogenesis is key to slowing or preventing these diseases.

Epidemiological evidence suggests that inflammatory processes,[27] for example, those caused by traumatic brain injury[28] or mutations in pro-inflammatory cytokines,[29,30] promote AD. Levels of inflammation-related lipid peroxidation products are higher in AD.[31,32] The clinical use of nonsteroidal anti-inflammatory drugs (NSAIDs) appears to protect against the onset of AD.[33] Inflammation is also linked to PD[34,35] and possibly to DLB.[36] Anti-inflammatory drugs appear to exert a protective effect in mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a drug that typically induces Parkinson's-like symptoms.[34] There is clear evidence for oxidative stress and lipid peroxidation in the blood of PD patients.[37]

Lipid Peroxidation and the Generation of Reactive Aldehydes *in Vivo*

Lipid peroxidation in a biological setting results in the formation of aldehydic products.[38,39] Peroxidation proceeds via lipid alkoxyl radicals and is accelerated by transition metal ions such as Fe^{2+}, Cu^{+}, and Co^{2+} (Figure 1D).[38,40,41] Some of these aldehydes produce pathologic effects *in vivo*. For example, 4-hydroxynonenal (4-HNE),

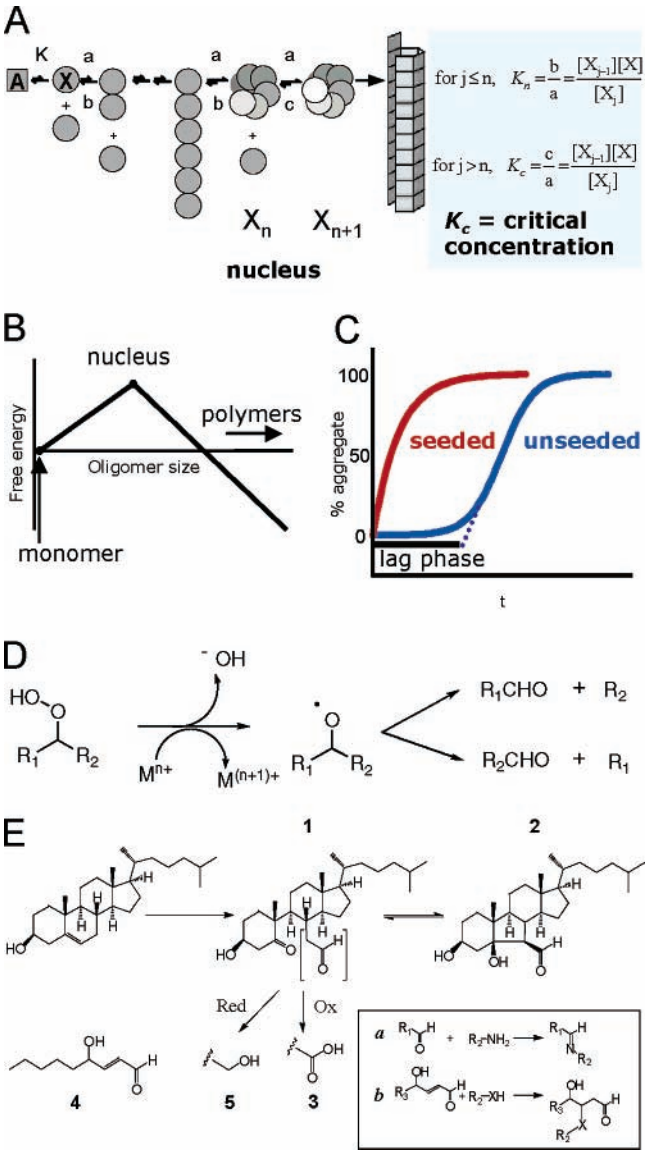

FIGURE 1. Nucleation dependent polymerization. (A) Elementary monomer addition reactions in fibrillogenesis. (B) Free energy diagram for oligomer formation, when the peptide concentration is higher than the critical concentration K_c. The free energy is a maximum for the nucleus. (C) Aggregation kinetics observed in nucleated polymerization. (D) β-Cleavage route of lipid hydroperoxides to aldehyde products. (E) Cholesterol is oxidized to form aldehyde (**1**), which rapidly equilibrates to the aldol (**2**) via Schiff base formation. Further oxidation yields **3**, whereas reduction yields **5**. Reaction a shows that reactive aldehydes can conjugate proteins by Schiff base formation. Reaction b shows that $\alpha-\beta$ unsaturated aldehydes, such as **4**, can additionally link proteins by conjugate addition.

an aldehyde generated from the peroxidation of ω-6 polyunsaturated fatty acids (PUFAs), is cytotoxic, genotoxic, mutagenic, and hepatotoxic.

These aldehydes are significantly elevated in cells, plasma, and organ tissue when an organism is under oxidative stress.[42] Oxidative stress, while complex, can be viewed as an imbalance in the generation and destruction of reactive oxygen species generated by normal or pathological metabolic processes, including infections. The relationship between AD, PD, and 4-hydroxynonenal

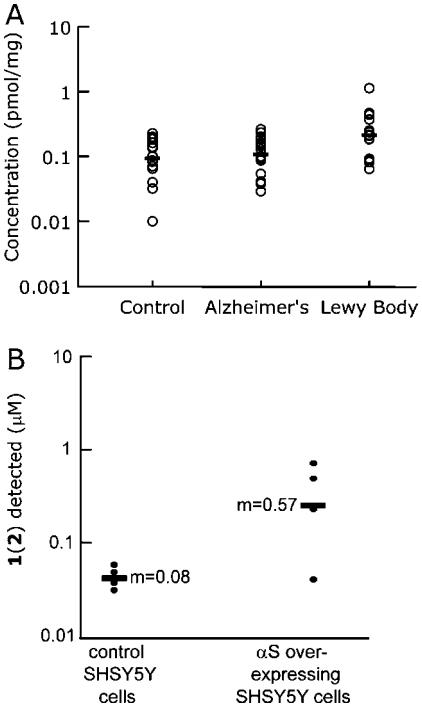

FIGURE 2. Quantification of cholesterol oxidation products in vivo. (A) Extraction of **1(2)** from 19 AD, 15 LBD, and 18 age matched control cortex tissue samples reveals elevated **1(2)** concentration in LBD brains (0.21 vs 0.09 pmol/mg). Concentrations of **1(2)** in AD brains (mean 0.12 pmol/mg) were not significantly elevated. (B) Concentration of **1(2)** in α-synuclein overexpressing SHSY5Y cell culture was elevated (0.57 μM) relative to control cells (0.075 μM).

(Figure 1E, **4**) illustrates this point.[43] The physiological concentration of **4** ranges from 0.1 to 1.0 μM.[44] Its levels are elevated significantly in the brains of individuals with AD relative to age-matched controls.[45] Moreover, it has been detected immunohistochemically in AD amyloid plaques[46] and Parkinsonian Lewy bodies.[47]

Inflammation-Linked Aldehydes Associated with Atherosclerosis Covalently Modify Proteins

We have provided evidence that the aldehyde-containing cholesterol 5,6-secosterols **1** and **2** (Figure 1E), which were recently discovered in the plaque material and plasma of patients with atherosclerosis, also may be important in AD and PD.[48] Because **1** and **2** interconvert by reversible Schiff base-mediated aldolization, the equilibrium mixture will be referred to as **1(2)**. *Ex vivo* activation of residual macrophages within human atheroma leads to a significant increase in secosterol levels. Their formation has been linked to the antibody-catalyzed water oxidation pathway that is itself activated by singlet dioxygen ($^1O_2$), which is generated by activated leukocytes.[49−52]

Secosterols **1(2)** and 4-hydroxynonenal (**4**) contain a large hydrophobic structure attached to an aldehyde that can covalently modify polypeptides and substantially alter their physical properties (**1(2)** are especially hydrophobic). Cholesterol, the precursor of **1(2)**, forms micelles at nanomolar concentrations.[53] Similarly, **1(2)** linked to a peptide could enhance peptide aggregation by mediating micelle-like self-assembly. The aldehydes of **1**, **2**, and **4** can react with the ϵ-amine of lysine, the N-terminal amine, or both to reversibly form Schiff bases (Figure 1E, reaction a). In contrast to **1(2)**, **4** has two reactive sites and can therefore cross-link proteins intra- or intermolecularly.[54] This results when a histidine or cysteine nucleophilic side chain from a protein adds to **4** by a 1,4 (Michael) addition[54] (Figure 1E, reaction b), followed by Schiff base formation utilizing the resulting aldehyde. Whereas the polypeptide−**1(2)** metabolite conjugates are envisioned to lower the critical concentration, **4** could initiate aggregation by intermolecular cross-linking.

Cholesterol Aldehyde-Mediated Misfolding Can Be Traceless in Alzheimer's Disease

We have shown that **1(2)** is present in postmortem human brain samples (Figure 2A);[55] however the levels of **1(2)** in Alzheimer's brains and age-matched controls is not significantly different. Nonetheless, a transient increase in the levels of **1(2)** resulting from inflammation (trauma, infection) could initiate Aβ amyloidogenesis, having effects on pathology long after the levels of **1(2)** have dropped to normal, because the fibrils so formed could trigger sporadic AD by acting as seeds for subsequent fibril formation. Such a scenario would explain the cholesterol- and inflammation-related risk factors associated with AD.

Cholesterol Oxidation Products Are Elevated in Lewy Body Dementia Brains

In contrast to AD patients, Figure 2A demonstrates that DLB patients have significantly higher levels of **1(2)** (0.21 pmol/mg) than aged-matched controls (0.09 pmol/mg).[56] Thus, **1**(**2**) are significantly elevated in α-synucleinopathies, suggesting that these metabolites influence aggregation.

Synucleinopathies have been linked to elevated α-synuclein expression,[57] which in turn has been linked to the formation of reactive oxygen species (ROS),[58] which could lead to a vicious cycle in which oxidative metabolites accelerate α-synuclein aggregation, inducing the generation of more reactive oxygen species. α-Synuclein overexpression in SHSY5Y human dopaminergic neuroblastoma cells leads to an order of magnitude increase in **1(2)** concentration relative to control cells (Figure 2B, 0.57 vs 0.08 μM).[56] Although high cholesterol is not an established risk factor for synucleinopathies, α-synuclein is associated with cholesterol-rich regions of cell membranes.[59] This association, along with the elevated levels of **1(2)**, suggests that these metabolites could play an active role in the Lewy body pathogenesis.

Oxidized Cholesterol Accelerates Aβ Aggregation by Covalent Modification

If oxidized small molecules play a mechanistic role in promoting protein aggregation in humans, triggering misfolding diseases, they should accelerate Aβ and α-synuclein aggregation *in vitro*. We have found this to be the

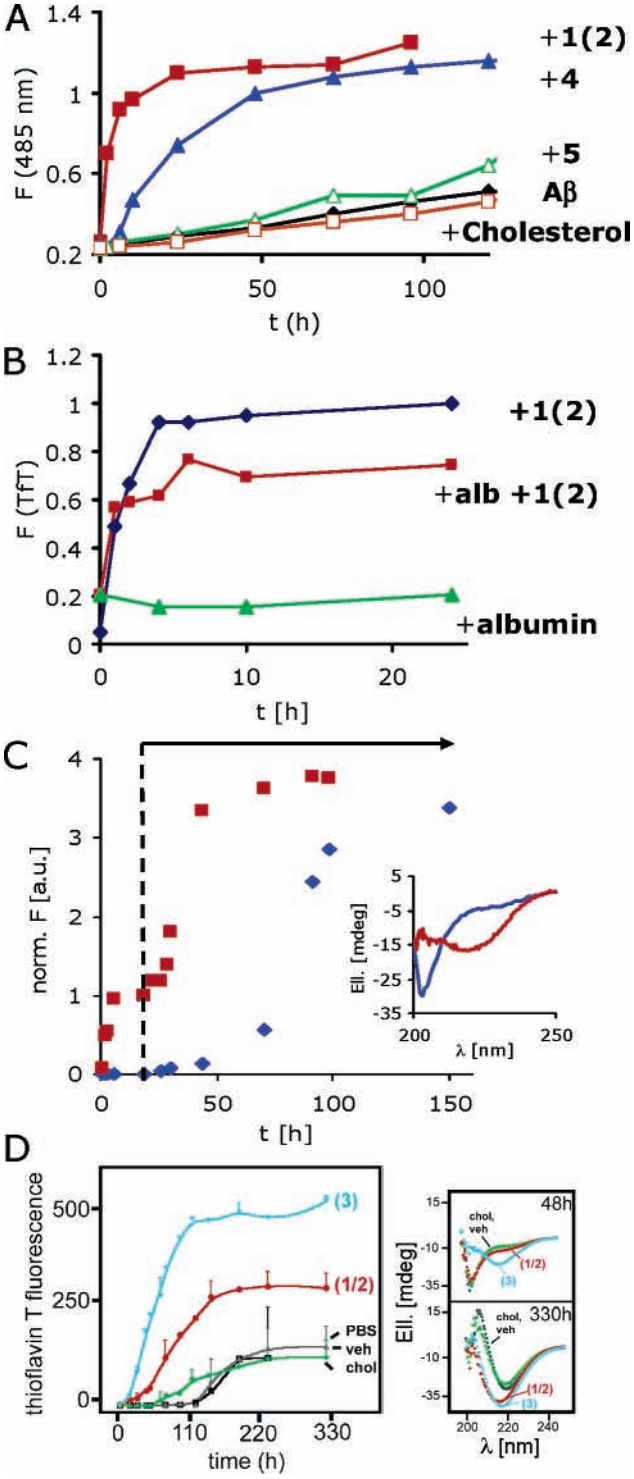

FIGURE 3. Lipid aldehydes accelerate aggregation. (A) Aβ 1−40 (100 μM) aggregation in the absence and presence of cholesterol, **1(2)**, **4**, and **5** (50 μM), monitored by TfT fluorescence at 37 °C (300 mM NaCl, 50 mM sodium phosphate, pH 7.4) in a stagnant assay. (B) Albumin (15 μM, 885 μM in lysine residues) does not significantly inhibit the effect of **1(2)** (50 μM) under the conditions described in panel A. (C) Aβ 1−40 aggregation as described in panel A except that agitation (5 s every 30 min) was initiated after 19 h. The inset shows the CD spectra of Aβ 1−40 after 1 (blue) and 150 h (red). (D) Increase in TfT fluorescence detecting α-synuclein (25 μM) aggregation is accelerated in the presence of **1(2)** (25 μM, red) and **3** (blue) relative to cholesterol (green) and buffer (gray) controls. The inset shows the CD spectra of α-synuclein after 48 and 330 h.

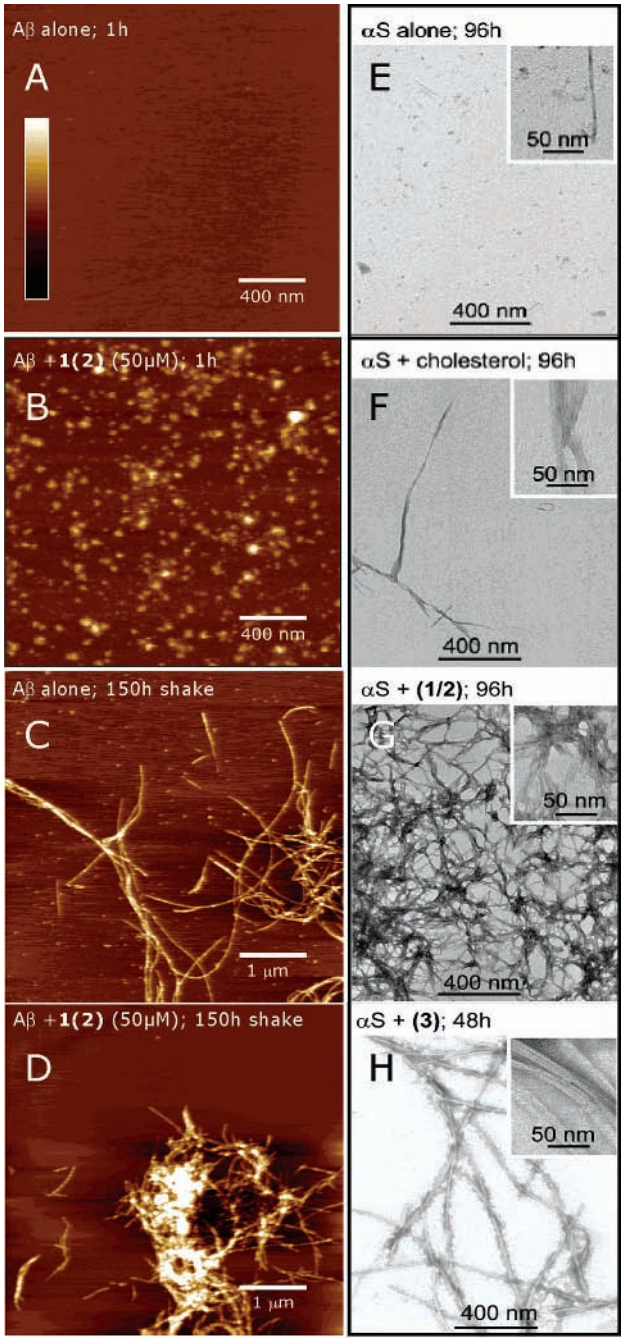

FIGURE 4. Aggregates formed by Aβ 1−40 with agitation as described in Figure 3C. Spherical aggregates form rapidly in the presence (B) but not in the absence of **1(2)** (A). Final aggregates show fibrillar morphology (C, D). (E−H) α-Synuclein forms mature fibrils under conditions described in Figure 3D within 48 h in the presence of **3** (H) and within 96 h in the presence of **1(2)** (G), whereas few, short fibrils are detected in cholesterol (F) and buffer controls (E) after 96 h. Height scale bars were (A, B) 0−20 nm and (C, D) 0−10 nm.

case.[55,56,60] Incubation of seed-free Aβ 1−40 at neutral pH (pH 7.4, 300 mM NaCl, 37 °C) with **1(2)** leads to formation within less than 10 h of Aβ aggregates that bind the amyloidophilic dye thioflavin T (TfT). Under the same conditions, no TfT signal was observed in the absence of **1(2)** (Figure 3A). Likewise, 4-hydroxynonenal (**4**) induced aggregate formation, whereas cholesterol and ketoalcohol

(**5**) did not (Figure 3A). The presence of other prominent cerebrospinal fluid proteins, such as albumin, did not substantially inhibit the hastening of Aβ aggregation, demonstrating that, although **1(2)** likely forms Schiff bases with these competitor proteins, the process is reversible so the metabolites are still available to modify Aβ and accelerate its aggregation (Figure 3B). The physical properties of natively unfolded proteins are dramatically changed by covalent modification, unlike the situation for large globular proteins.

Atomic force microscopy demonstrates that aggregates of Aβ 1−40 (100 μM) were not detected in the absence of **1(2)** (Figure 4A); however spherical Aβ 1−40 aggregates with a diameter of 5−8 nm were formed within 1 h (Figure 4B) in the presence of **1(2)**, which are kinetically stable under quiescent conditions. To observe fibrillization (Figure 4C,D) of the spherical aggregates on a reasonable time scale, the reaction had to be agitated (Figure 3C). Fibrillar aggregates showed a predominantly β-sheet structure based on far-UV circular dichroism spectra (Figure 3C, inset). The spherical Aβ aggregates formed in the presence of **1(2)** under quiescent conditions also converted to fibrils upon addition of fibrillar seeds, indicating that the spherical aggregates are kinetically competent to form fibrils.[55]

Oxidized Cholesterol Accelerates α-Synuclein Amyloidogenesis Apparently Noncovalently

Secosterols **1**(**2**) accelerate α-synuclein aggregation in both stagnant and agitated assays.[56] The TfT signal increases over 72 h in samples of α-synuclein (25 μM) in the presence of **1**(**2**) (25 μM), Figure 3D. In the presence of cholesterol or the vehicle buffer, no increase in TfT fluorescence was observed over 120 h, and the final TfT amplitude was much reduced, consistent with the EM data (Figures 4F,G). Our inability to detect α-synuclein−**1**(**2**) adducts forced us to consider a noncovalent mechanism. To test such a mechanism, compound **3** was prepared by oxidizing the aldehyde functional group in **1**(**2**) to an acid that is incapable of Schiff base formation. Acid **3** hastened α-synuclein aggregation even more than **1(2)** (t_{50} = 48 h, Figure 3D), affording fibrils (cf. Figure 4E,H) with a predominantly β-sheet structure. Although we know that α-synuclein forms a Schiff base with **1**, because α-synuclein catalyzes the conversion of **1** into **2**,[56] this conjugate was not observed by mass spectrometry and was not essential for acceleration of α-synuclein aggregation. The micellar properties of **1**, **2**, and **3** may be responsible for hastening α-synuclein fibrillization. We are currently investigating this possibility.

Hydrophobic Aldehyde Modification of the Aβ Peptide Lowers Its Critical Concentration (K_c)

Increasing the hydrophobicity of a fibrillogenic peptide by metabolite modification could facilitate fibril formation by burial of large hydrophobic groups in the assemblies.[61,62] This would translate into a decrease in K_c, which would allow metabolite-modified peptides or a specific

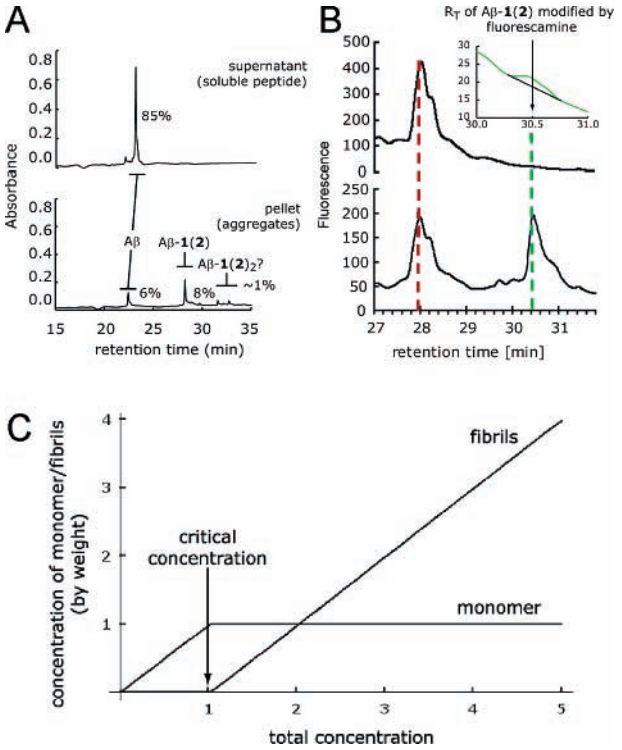

FIGURE 5. Oxidation products lower Aβ critical concentration (K_c). (A) HPLC traces from the $NaBH_4$-reduced supernatant and pellet of Aβ (100 μM) incubated with **1(2)** (100 μM) for 3 h as in Figure 3A. (B) Same incubation (16 h) after which Aβ is derivatized with fluorescamine. Concentration of Aβ−**1(2)** in the supernatant was at most 70 ± 20 nM. (C) Schematic representation of the concentrations of monomers and aggregates as a function of total peptide concentration. The maximal concentration of soluble peptide is equal to K_c.

stoichiometry of modified and unmodified peptides to form fibrils at lower concentrations than their unmodified counterparts. This could explain how Aβ forms fibrils at physiological concentrations (∼1−10 nM),[63] which are much lower than K_c's reported for Aβ aggregation *in vitro* (∼1−10 μM).[64,65]

Since K_c is equal to the concentration of soluble peptide after aggregation is complete, separating aggregated Aβ from soluble Aβ by ultracentrifugation is a convenient way of assessing the upper limit of K_c. Figure 5A shows HPLC chromatograms of supernatant and pellet fractions of an Aβ 1−40 aggregation reaction in the presence of **1(2)** (50 μM).[55] The pellet has equal amounts of modified and unmodified Aβ after $NaBH_4$ reduction of the Schiff base (Figure 5A,B, lower panels). No Aβ−**1(2)** conjugate was detected in the soluble fraction suggesting that the conjugate's K_c is very low. Both fractions also were derivatized with fluorescamine, which allowed detection of Aβ by HPLC down to a concentration of ∼50 nM. The small fraction of soluble Aβ−**1(2)** detected put an upper limit on K_c of 70 ± 20 nM (Figure 5B).[55] Thus, modification by **1(2)** lowered the K_c of Aβ 1−40 by approximately 100-fold. Modified Aβ 1−40 also recruits unmodified Aβ 1−40 to aggregate with it, since the pellet has equal amounts modified and unmodified Aβ peptide after $NaBH_4$ reduction of the Schiff base (Figure 5A,B, lower panels). This

could occur either if aggregates consisting only of modified Aβ acted as a seed on which unmodified Aβ later adds or if modified and unmodified Aβ formed a "solid-solution"-type mixed oligomer in which modified and unmodified Aβ were both included within the fibril structure. The latter explanation would suggest that stabilizing interactions occur between the metabolite substructure on modified Aβ 1−40 and unmodified Aβ 1−40 in the aggregates preventing the dissociation of unmodified Aβ. In either case, the aggregates induced by Aβ modification can serve as templates for further fibril growth by addition of unmodified Aβ, as mentioned above.

Lipid Oxidation Products Obviate Aβ Nucleation by Forming Spherical Aggregates

Increases in amyloidogenic peptide hydrophobicity mediated by Schiff base formation with **1(2)** should cause the equilibrium constant K_n (Figure 1A) to decrease. Since K_n dictates the stability of the nucleus relative to monomers, this could profoundly change the mechanism of fibril formation. If K_n became lower than the peptide concentration, the nucleus would no longer be the highest energy species on the fibril formation pathway; the monomer would be. This would lead to an aggregation mechanism referred to as a downhill polymerization (Figure 6E, dotted line).

To test this hypothesis, we plotted the point of half-maximal TfT fluorescence (t_{50}) on a logarithmic scale versus the starting peptide concentration. Since aggregation time scales with $(n + 1)/2$ for a nucleated polymerization, the resulting slope would be $-(n + 1)/2$,[21] whereas downhill polymerizations would yield a slope of -1.[66] We and others have used this approach to test the mechanisms of aggregation of several proteins.[7,22] Figure 6A shows the TfT fluorescence from a shaken Aβ 1−40 aggregation reaction (5−100 μM) in the presence of **1(2)** (50 μM).[60] A plot of log t_{50} vs log [Aβ] for the first stage of aggregation (Figure 6B) exhibits a slope of -0.96 ($R^2 >$ 0.99) indicating that the first stage of Aβ 1−40 aggregation reaction, in which spherical aggregates are formed and which is the only stage observed in stagnant assays in the presence of metabolite,[55] is a downhill polymerization.[60]

The influence of metabolites **1(2)** on fibril elongation was also probed (Figure 6E, dashed line) by measuring fibril elongation rates, calculated from the initial slopes of aggregate growth in seeded aggregation assays.[67] Figure 6C shows the increase in TfT fluorescence of Aβ 1−40 (20 μM) in the presence of 5%, 10%, and 20% (w/w) aggregation seeds, prepared by sonication of preformed Aβ 1−40 fibrils. The initial slopes (Figure 6C,D) showed no dependence of the fibril growth rate on **1(2)**.[60]

Aldehyde Scavengers Inhibit Schiff Base Hastened Aβ but Not α-Synuclein Amyloidogenesis

Aldehyde-enabled Schiff base formation is critical for **1(2)** and **4** to accelerate Aβ aggregation.[55] Hence, it should be

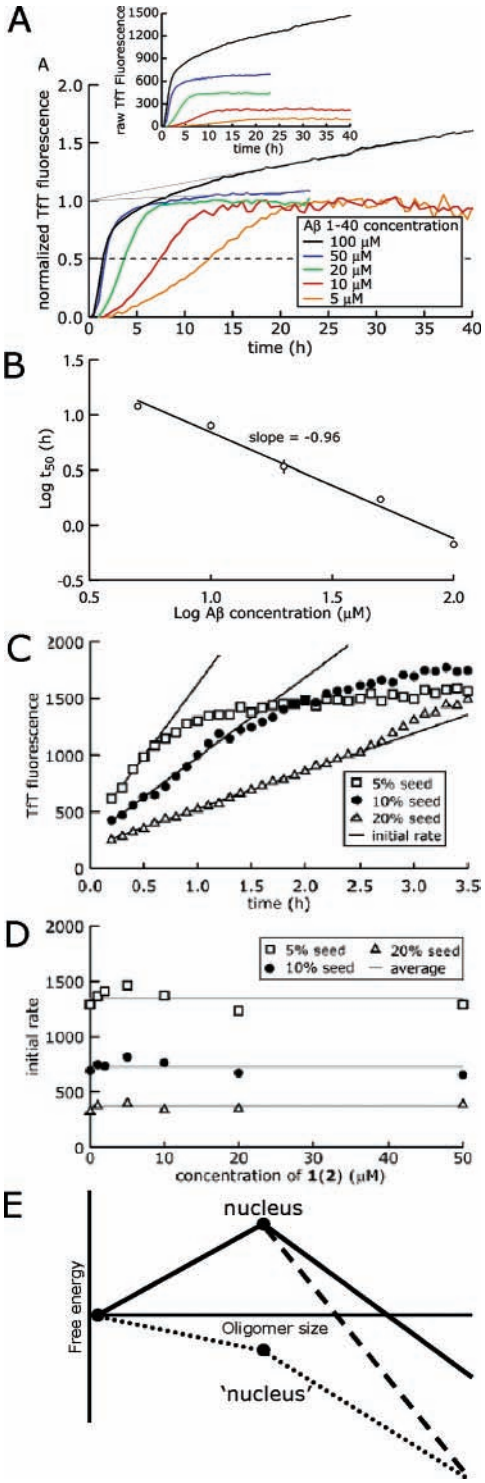

FIGURE 6. Lipid oxidation products induce aggregation by downhill polymerization. (A) Aβ 1−40 concentration (5−100 μM) dependence of the first stage of its aggregation in the presence of **1(2)** (50 μM) under the conditions described in Figure 3A, except that it is agitated by shaking for 5 s every 10 min. (B) Time to 50% completion (t_{50}) is plotted against Aβ concentration on a double logarithmic scale (slope $= -0.96$, $R^2 = 0.99$, $n = 1$). (C) Aggregation of Aβ 1−40 (50μM) as described in Figure 5 seeded by the addition of 5%, 10%, and 20% sonicated fibrillar Aβ 1−40. (D) Initial slopes for different concentrations of **1(2)** show no concentration dependence. (E) Free energy diagram for aggregation by a downhill polymerization mechanism (dotted line), compared to nucleated polymerization (solid line) and facilitated fibril growth (dashed line).

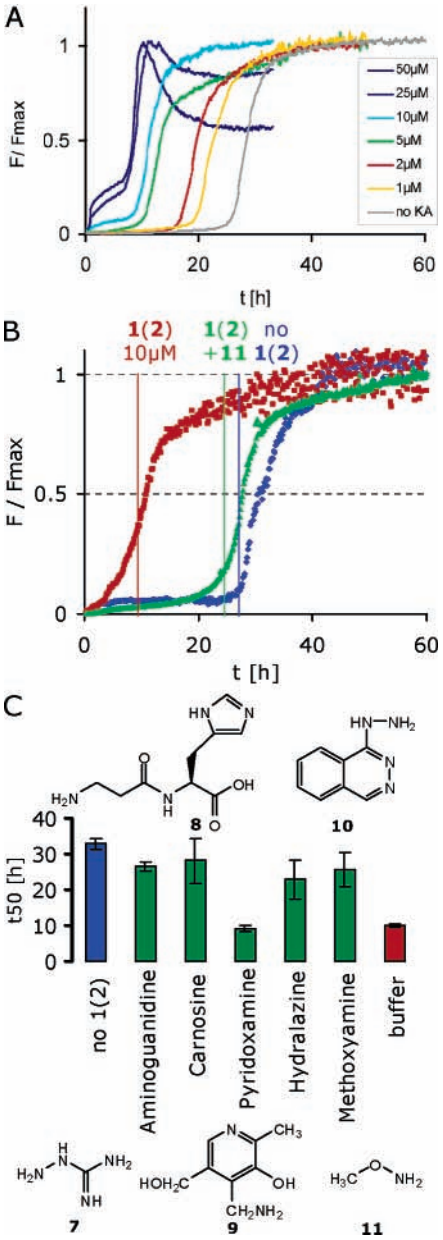

FIGURE 7. Acceleration of fibril formation by oxidative metabolites can be inhibited by aldehyde scavenging compounds. (A) The second stage of Aβ 1−40 (50 μM) aggregation, that is, fibril formation, is accelerated depending on concentration of **1(2)** (assay as in Figure 6A). (B) t_{50} in the presence of **1(2)** (10 μM, red) can be prolonged by methoxyamine (100 μM, green) back to the value observed in the absence of **1(2)** (blue). (C) t_{50} values for different compounds.

possible to eliminate their influence by using aldehyde-sequestering small molecules.[68] Several such molecules were added to an Aβ aggregation reaction at a 10-fold molar excess over **1(2)** (Figure 7B) to test the hypothesis that aldehyde-sequestering compounds can reverse metabolite-accelerated misfolding.[60] Aminoguanidine, carnosine, hydralazine, and methoxyamine (but not pyridoxamine) at 100 μM were able to eliminate metabolite **1(2)** (10 μM) acceleration of Aβ 1−40 (50 μM) aggregation, restoring the aggregation rate to that observed in the absence of **1(2)** (Figure 7A−C). Therefore, aldehyde carbonyl-trapping therapeutic strategies, which are already under examination for other diseases,[68,69] could be effective in combating sporadic Alzheimer's disease. Analogous studies with α-synuclein revealed that the aldehyde sequestering compounds did not eliminate oxidative metabolite accelerated misfolding,[56] supporting the hypothesis that **1(2)**-mediated acceleration of α-synuclein aggregation occurs predominantly by a noncovalent mechanism.

Conclusion

Oxidized hydrophobic membrane components hasten Aβ and α-synuclein aggregation, associated with AD and synucleinopathies, by covalent and noncovalent mechanisms, respectively. Thus, these may be a new class of risk factors and potential prevention and intervention targets in neurodegenerative diseases. The aldehydes discussed herein probably only represent a small fraction of the oxidized small molecule pool that can trigger protein misfolding in sporadic amyloid diseases, which are the clinically most important diseases of the brain.

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AR0500766