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Advanced glycation end products contribute to amyloidosis in Alzheimer disease

(β-amyloid peptide/aggregation/nucleation-dependent kinetics/seed structure and function)

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ABSTRACT **Alzheimer disease (AD) is characterized by deposits of an aggregated 42-amino-acid β-amyloid peptide (βAP) in the brain and cerebrovasculature. After a concentration-dependent lag period during *in vitro* incubations, soluble preparations of synthetic βAP slowly form fibrillar aggregates that resemble natural amyloid and are measurable by sedimentation and thioflavin T-based fluorescence. Aggregation of soluble βAP in these *in vitro* assays is enhanced by addition of small amounts of pre-aggregated β-amyloid "seed" material. We also have prepared these seeds by using a naturally occurring reaction between glucose and protein amino groups resulting in the formation of advanced "glycosylation" end products (AGEs) which chemically crosslink proteins. AGE-modified βAP-nucleation seeds further accelerated aggregation of soluble βAP compared to non-modified "seed" material. Over time, nonenzymatic advanced glycation also results in the gradual accumulation of a set of posttranslational covalent adducts on long-lived proteins *in vivo*. In a standardized competitive ELISA, plaque fractions of AD brains were found to contain about 3-fold more AGE adducts per mg of protein than preparations from healthy, age-matched controls. These results suggest that the *in vivo* half-life of β-amyloid is prolonged in AD, resulting in greater accumulation of AGE modifications which in turn may act to promote accumulation of additional amyloid.**

Alzheimer disease (AD) is the major cause of dementia and the fourth leading cause of death in the United States, affecting 10% of the population age 65 and older. In combination with gradual cognitive decline, AD is characterized by the progressive accumulation of neurofibrillary tangles, amyloid plaques, and cerebrovascular amyloid deposits in the brain. Typically, 5- to 10-fold greater numbers of amyloid plaques accumulate in the brains of AD patients than in age-matched healthy controls. These amyloid plaques mainly comprise aggregated copies of a 42-amino-acid β-amyloid peptide (βAP; ref. 1), which derives by cleavage from a family of larger amyloid peptide precursor proteins (2, 3). Although the amount of aggregated βAP or β-amyloid may directly relate to the increased release of soluble βAP in some rare genetically linked forms of familial AD (4–6), the levels of soluble βAP in cerebrospinal fluid of sporadic AD cases are similar to those in age-matched healthy counterparts (7, 8). If increased levels of soluble βAP monomers generally fail to account for the increased numbers of amyloid plaques seen in the majority of AD patients, then the increased amyloid in AD must instead reflect an increase in the rate of aggregation and amyloid formation, a decreased rate of amyloid degradation, or some combination of both.

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To better understand the factors which contribute to amyloid formation and stability, we have studied the *in vitro* aggregation of soluble synthetically prepared βAP monomers, a process which displays nucleation-dependent kinetics, especially at physiological (nanomolar) concentrations of monomer (refs. 9 and 10; this report). Although millimolar concentrations of βAP exhibit extensive aggregation within minutes, micromolar and lower concentrations of monomer display a concentration-dependent lag period during which little or no measurable aggregate is formed, followed by a "growth" phase of more rapid aggregation (9–11). This lag period can be eliminated by adding trace amounts of preformed aggregate as "seed" material which serves to immediately induce aggregation (9). Seeding apparently eliminates the need for *de novo* formation of aggregation nuclei, a much slower process than the subsequent growth of nucleated aggregates. Thus, amyloid seeds can be seen to represent critical protein masses with special structural features capable of nucleating the formation of larger insoluble aggregates of amyloid from pools of soluble βAP. The nature of these structural characteristics is unknown, but the net result of seeding is significant acceleration in the rate of aggregation compared with unseeded incubations of soluble βAP. By extrapolation from this simple *in vitro* model to the similar nanomolar concentrations of soluble βAP found in cerebrospinal fluid *in vivo*, it might be expected that the deposition and accumulation of βAP as amyloid plaques would reflect, in part, the amount or availability of seed material able to nucleate aggregation. Some cases of AD might correspondingly arise as a consequence of increased availability of nucleation seeds in the disease-prone brain relative to normal counterparts not destined to suffer AD at a similar chronological age.

In the present communication, we explore the possibility that the formation or stability of amyloid structures which seed βAP aggregation may be enhanced by covalent crosslinks that chemically polymerize components of the aggregate. Under physiological conditions, long-lived proteins become markedly modified and extensively crosslinked by advanced "glycosylation" end products (AGEs). AGEs result from the spontaneous, nonenzymatic glycation reactions between proteins and reducing sugars (12), including for instance the ubiquitous monosaccharide glucose, which is the primary energy source used by the brain. Initially in the process of advanced glycation, condensation reactions between reducing sugars and protein amino groups form a population of readily reversible Schiff bases, some of which rearrange over days into more stable Amadori products. Over

Abbreviations: AD, Alzheimer disease; βAP, β-amyloid peptide; AGE, advanced "glycosylation" end products.

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weeks and months, these early products chemically evolve by additional rearrangement, dehydration, cleavage, and addition reactions into a widely heterogeneous set of covalently bound adducts known collectively as AGEs. The accumulation of such AGEs *in vivo* mainly depends on the bioavailability of susceptible primary amino groups, the ambient concentration of reducing sugars, and the half-life of the modified protein. Accordingly, AGE modifications are strikingly evident on very long-lived proteins such as lens crystallins, nerve myelin, and matrix collagen; are markedly enhanced in diabetic patients (13–15); and might well be expected to accumulate on the aggregated proteins of AD and other amyloidoses.

By accumulating slowly over time, the AGE modifications that appear on proteins *in vivo* not only serve as an index of protein half-life but also can initiate a variety of biological effects mediated both by cellular receptor systems for AGE-modified ligands (16) and by processes which depend on the inherent chemical reactivity of both nascent and late AGE moieties (17). As a first step in understanding the potential consequences of AGE accumulation on β-amyloid in AD, we investigated the effects of advanced glycation on the aggregation of βAP monomers *in vitro*. Our data indicate that advanced glycation of βAP accelerates aggregation of soluble βAP and that small amounts of preformed AGE-modified βAP seeds more efficiently nucleate the further merging of βAP peptides into insoluble aggregates than does nonglycated seed material. We also report that purified plaque fractions from AD brains contain more AGE adducts per mg of protein than do parallel fractions prepared from healthy age-matched controls, further underlining the potential physiological and pathogenic importance of AGE-regulated βAP aggregation and amyloid accumulation.

MATERIALS AND METHODS

Aggregation and Seeding Reactions. Synthetic, HPLC-purified peptides representing the first 28 [βAP-(1–28)] and the first 40 [βAP-(1–40)] amino acids of the 42-amino-acid βAP were obtained from Bachem. Aggregation of soluble βAP-(1–28) or βAP-(1–40) at different concentrations was initiated by addition of 0.1 M sodium acetate and continued for the indicated times. Quantitative aggregate formation at high micromolar βAP concentrations was detected by the procedure of LeVine (18). Briefly, fluorescence of aggregates added to 10 μM thioflavin T (Aldrich) in 50 mM potassium phosphate buffer at pH 6.0 was measured upon excitation at 450 ± 5 nm, with emission detected at 482 ± 10 nm on a Perkin–Elmer LS-50B spectrofluorimeter. Where indicated, small amounts of preformed aggregates ("nucleation seeds") were added to the monomeric βAP and 0.1 M sodium acetate. Lysines at positions 16 and 28 of βAP contain primary amino groups which may react with reducing sugars to generate AGEs. Monomeric βAP-(1–40) (250 μM) and 0.2 M sodium phosphate buffer (pH 7.5) were incubated with or without 1 M glucose at 37°C for 4 months to generate preformed aggregates of glycation-modified βAP, referred to as "AGE-βAP seed" or "βAP seed," respectively. After incubation, protein concentrations of seed preparations were measured and adjusted with buffer to 150 μM final concentration. By competitive ELISA (19), AGE-βAP seed contained 50 AGE units/mg of protein and βAP seed contained <0.5 AGE unit/mg of protein. Indicated amounts of glucose and/or aminoguanidine, a potent inhibitor of advanced glycation and crosslink formation (20), were also added to solutions of βAP before sodium acetate in some experiments.

Aggregation of physiological concentrations of βAP was quantitated by the method of Burdick *et al.* (11). Synthetic preparations of βAP-(1–40) were labeled with ^{125}I (NEN) and chloramine-T (Sigma) for 1 min before the reaction was quenched with 10 mM tyrosine/50 mM sodium metabisulfite. Unincorporated label was removed by filtration through a Sephadex G-10 column equilibrated with 0.5× phosphate buffered saline (PBS) at pH 7.4. The ^{125}I-labeled βAP (specific activity, ≈3 × 10^6 cpm/μg) was immediately diluted to 5 nM final concentration in the presence of various seeds, glucose, and/or aminoguanidine at the indicated concentrations. After various incubation periods at 37°C, aggregation reaction mixtures were underlaid with 20% sucrose/0.1 M sodium acetate at the same pH as the incubation, centrifuged for 30 min at 50,000 × *g*, and frozen in liquid nitrogen. Each microcentrifuge tube was cut and the bottom 5-mm section, representing the aggregated, sedimentable fraction which had pelleted through the sucrose cushion, was analyzed for radioactivity in a γ counter. The remainder of the tube and liquid were also analyzed. The amount of aggregate formed was calculated as a percentage: (pellet cpm/total cpm) × 100.

Measurement of AGEs by Competitive ELISA. Aliquots of frozen samples of prefrontal cortex (Broadman areas 9 and 10) from patients with or without behaviorally and neuropathologically confirmed AD were suspended in 10 volumes (per wet weight) of 2% sodium dodecyl sulfate/0.1 M 2-mercaptoethanol and Dounce homogenized. The homogenates were boiled for 10 min and then centrifuged at 10,000 × *g* for 10 min. Supernatants were aspirated and the resulting pellets were washed and recovered three times with PBS at 10,000 × *g* for 10 min. In some experiments, this crude plaque fraction was further washed twice with 4 M urea and twice more with PBS before protease digestion. PBS-washed pellets were resuspended in 1/10th the original homogenate volume of PBS with 0.1% proteinase K (Boehringer Mannheim) and digested overnight at 37°C. Quadruplicate 5-, 10-, and 20-μl aliquots of the digested plaque-containing pellet fractions were assayed for AGE content by competitive ELISA (19) against standardized preparations of AGE-modified bovine serum albumin (AGE-BSA). Only values in the linear range of the standard curve were included in the analyses. Protein amounts were quantified with a micro-BCA kit (Pierce) and with fluorescamine (21). AGE units per volume of sample were interpolated from a standard dilution curve of AGE-BSA and divided by the sample protein concentration to give AGE units per mg of protein. Statistical analysis using Student's *t* test was performed with the STATWORKS program for MacIntosh.

RESULTS

βAP Aggregation Displays Nucleation-Dependent Kinetics. Aggregation of synthetic βAP was analyzed *in vitro*, where the aggregation rate of soluble βAP was found to depend mainly upon pH and the initial concentration of monomer. We empirically determined kinetics of aggregation and found that at 600 μM, βAP-(1–28) spontaneously and rapidly formed thioflavin T fluorescent aggregates within minutes at pH 7.2 (Fig. 1). At concentrations below 300 μM, however, βAP aggregated very slowly with a considerable lag before fluorescent aggregates were measurable.

At low concentrations of βAP, the lag period preceding measurable aggregation is reminiscent of crystallization reactions where solutions of protein very slowly assume a single conformation and then aggregate in a well-defined, molecular packing arrangement (9, 10, 22). The kinetics of such aggregation in many cases can be significantly accelerated by the addition of a preformed aggregate or "seed" material as nucleation centers for aggregate formation. We prepared stable βAP seed material by incubating 250 μM βAP-(1–40) for 4 months at 37°C. Compared with the small amounts of fluorescent aggregate measured when 300 μM βAP monomer or 75 μM βAP seed was separately incubated in control preparations, incubation of monomer plus βAP

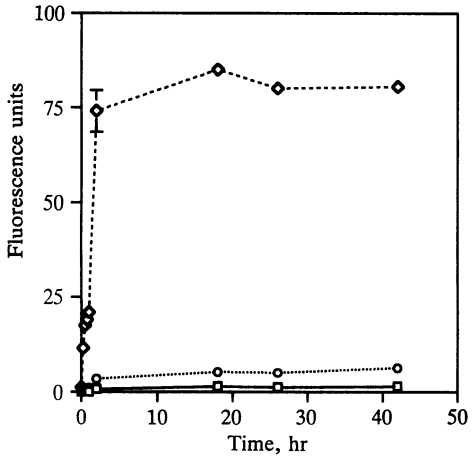

FIG. 1. Fluorescent aggregates formed with synthetic βAP depend on initial concentration of monomer and time. βAP was incubated with 0.1 M sodium acetate at pH 7.2, aliquots were removed at various times, and fluorescence in the presence of thioflavin T was measured. The mean (± SE) of three measurements is plotted for 600 μM (◇), 300 μM (○), and 150 μM (□) βAP.

seed resulted in the accumulation of much larger amounts of fluorescent aggregates (Fig. 2). We also prepared AGE-modified βAP seed by incubating βAP monomer with 1 M glucose in pH 7.5 phosphate buffer at 37°C for 4 months (AGE-βAP seed). AGE formation was confirmed by competitive ELISA, where βAP seed contained <0.5 AGE unit/mg of protein and AGE-βAP seed contained 50 AGE units/mg of protein, which is comparable, within an order of magnitude, to the AGE content of plaques from AD brains. When 75 μM AGE-βAP seed was incubated with 300 μM βAP monomer, much more fluorescent aggregate was detected than in parallel incubations with unmodified βAP seed and monomer (Fig. 2). As with βAP seed, separate incubation of AGE-βAP seed alone did not lead to a change in the small amount of fluorescent aggregate with time.

To test whether this seeding phenomenon occurred at concentrations of βAP typically found *in vivo*, a sedimentation assay was employed to measure aggregation. Aggregation of 10 nM ^{125}I-βAP-(1–40) monomer in 0.1 M sodium acetate at pH 7.0 increased slowly over a 2-day incubation (Fig. 3). When 10 nM labeled monomer was incubated with 200 nM unlabeled βAP seed material, the amount of sedimentable label was similar to the no-seed, monomer-only curve. In contrast, incubation of 10 nM monomer with 200 nM AGE-βAP seed increased the amounts of sedimentable label compared with the βAP-seed and no-seed experimental points. Thus, the amount of labeled βAP associated with sedimentable aggregates observed in incubations of βAP monomer with AGE-βAP seed was greater than that seen in incubations with βAP seed or no seed under conditions in which pH and soluble βAP concentrations were physiological.

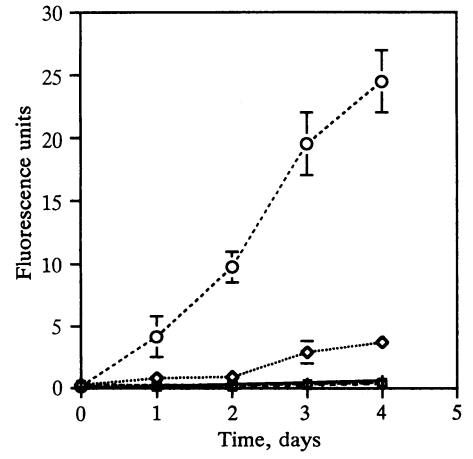

FIG. 2. Fluorescent-aggregate formation displays nucleation-dependent kinetics. Stable aggregates of βAP were formed in the presence or absence of glucose to generate AGE-βAP seed and βAP seed. Seeds were added to 300 μM βAP monomer and incubated for various times. The mean (± SE) of triplicate thioflavin T fluorescence measurements is plotted as a function of time for AGE-βAP seed plus βAP (○) and βAP seed plus βAP (◇). Control incubations included AGE-βAP seed alone (△), βAP seed alone (□), and βAP monomer without seeding (+).

Glucose Modifies Kinetics of Aggregation. In the process of advanced glycation, formation of a Schiff base between glucose and a protein amino group precedes subsequent maturation of Amadori products into AGE-modified βAP. Aminoguanidine is a compound which prevents AGE formation following initial glucose reactions with protein amines (20). Incubation of 400 μM βAP-(1–28) in 0.1 M sodium acetate (pH 7.0) with 100 mM glucose stimulated fluorescent-aggregate formation when compared with parallel incubations of monomer, monomer plus aminoguanidine, or monomer plus aminoguanidine plus glucose (Fig. 4). Separate incubations of glucose or aminoguanidine failed to generate any detectable fluorescent signals.

AGE Content of Brain Fractions. On average, plaque-enriched fractions isolated from samples of frontal cortex of 10 AD brains contained significantly more AGE modifications than did corresponding preparations from 7 healthy controls (8.9 ± 1.4 versus 2.7 ± 0.5 AGE units/mg of protein, $P = 0.002$; see Fig. 5). The average chronological age of both the AD and control groups was 77 years. The presence of AGEs in similar fractions of parietal cortex from AD and normal brains has also been observed (data not shown). Supernatant fractions of sodium dodecyl sulfate-soluble proteins from AD and control brains routinely contained <0.1 AGE unit/mg of protein.

DISCUSSION

Although advanced glycation adducts form spontaneously *in vivo*, their accumulation is slow and becomes most notable with increasing time on long-lived tissue components. Along with time and the availability of susceptible protein amino

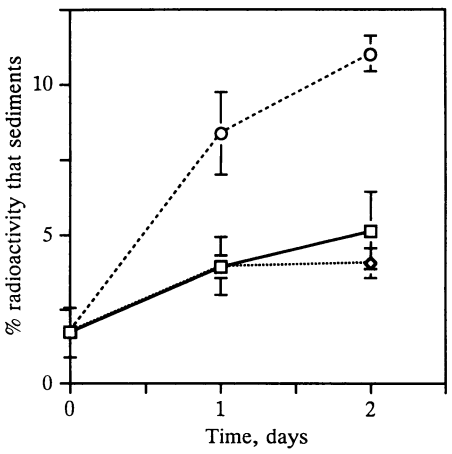

FIG. 3. AGE-βAP seeds nucleate more aggregation at physiological concentrations of βAP than βAP seeds. ^{125}I-βAP-(1–40) (10 nM) was mixed with no seed (□), βAP seed (◇) or AGE-βAP seed (○) and incubated for the indicated times at 37°C. The mean (± SE) of quadruplicate measurements is plotted.

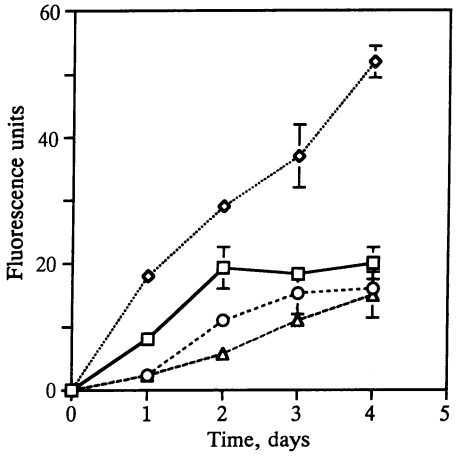

FIG. 4. Glucose modifies the kinetics of fluorescent-aggregate formation. Soluble βAP-(1–28) (400 μM) with 0.1 M sodium acetate in 0.1 M phosphate buffer at pH 7.0 (□) was mixed with 0.1 M glucose (◇), 0.05 M aminoguanidine (△), or glucose and aminoguanidine (○). The mean (± SE) of triplicate thioflavin T fluorescence measurements is plotted.

groups, ambient glucose is the other major determinant of AGE formation. Thus, quantitative analysis of the degree of AGE modification of a single protein species under normoglycemic conditions yields an index of protein half-life *in vivo*. We found that plaque-enriched fractions isolated from AD brain samples contained about 3-fold more AGE modifications than did comparable fractions prepared from age-matched control brains, suggesting that βAP half-life is prolonged in AD. That amyloid components exhibit a prolonged half-life in AD is also supported by studies that demonstrated the time-dependent, nonenzymatic isomerization of aspartic residues occurring at positions 1 and 7 of βAP isolated from AD brain (23); unfortunately, comparable βAP fractions from normal brain were not tested in parallel. To what degree this alteration in βAP turnover accounts for the accumulation of βAP as aggregated amyloid in AD remains to be established. Likewise, it is of interest to determine the normal mechanism of βAP removal during turnover of amyloid components and how this process is slowed in AD.

AD is characterized by progressive dementia and increased numbers of amyloid plaques relative to healthy age-matched

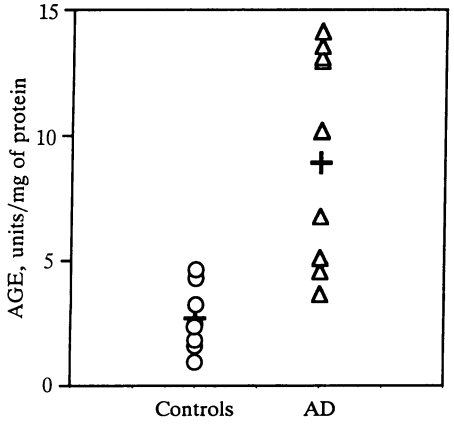

FIG. 5. Amyloid plaque-enriched fractions of AD frontal cortex contain more AGE adducts per mg of protein than equivalently prepared fractions of age-matched, nondemented controls. Each control patient is represented by a circle and AD patients by triangles. Each symbol represents the average of at least four independent measurements of immunoreactive AGE adducts for each sample patient, with the mean of each patient group marked by a cross.

controls. While a causal relationship between increased dementia and plaque numbers has not been proven, the gradual onset of symptoms appears to parallel the progressive deposition of β-amyloid. From *in vitro* studies, it is clear that millimolar concentrations of monomeric βAP will spontaneously aggregate into fibrillar amyloid structures by following a nucleation-dependent mechanism. At lower concentrations, the requirement for nucleus formation introduces a substantial lag period during which a solution of βAP that still requires most of this time to form aggregation nuclei is indistinguishable from one on the verge of rapid aggregation and growth into a "one-dimensional crystal" (10). The effect of this concentration-dependent nucleus formation is extreme as illustrated by published calculations showing that a mutation of amyloid precursor protein in a Swedish form of familial AD which raises soluble βAP concentrations 6-fold (4, 5) should reduce the lag time before aggregate growth occurs from 100 years to about 3 hr (9). Since the cerebrospinal fluid concentration of soluble βAP in AD patients is the same as in age-matched controls (7, 8), the rate of concentration-dependent self-formation of nuclei as reflected by the amounts of amyloid formed and deposited might also be expected to be the same. As this is not the case and AD brains form substantially more aggregated deposits of βAP than their nondiseased counterparts, then one explanation for the increased amount of amyloid present in afflicted brain tissue is that aggregation is more efficiently nucleated than in healthy brain parenchyma.

Although the physical characteristics of such aggregation nuclei are presently unknown, we operationally define nucleation seeds as structures of βAP possessing a specific conformation that promotes the rapid accretion of additional soluble βAP resulting in the growth of insoluble βAP aggregates. Since the spontaneous formation of nuclei is thermodynamically unfavorable (9), we investigated the possibility that processes known to chemically crosslink proteins *in vivo* might stabilize specific conformations of βAP with nucleating characteristics. Advanced glycation is a naturally occurring process of covalent posttranslational modification of proteins that readily occurs extracellularly. AGEs in other contexts are well known as protein–protein crosslinking agents *in vivo*, and AGE accumulation on matrix proteins is associated with increased resistance to proteolysis (24). At physiological concentration and pH *in vitro*, βAP monomers aggregate slowly. Of note, the addition of preformed aggregates of AGE-modified βAP stimulated markedly more rapid aggregation of nanomolar solutions of soluble βAP than did preformed aggregates of unmodified βAP in 2-day incubations. That this acceleration occurred at normal pH, at physiological concentrations of βAP monomer, and with seeds containing amounts of AGE modifications comparable to those found in AD plaque fractions suggests that a similar process could occur *in vivo*. Glycation adducts comprise a structurally heterogeneous family of products that slowly evolve chemically by a variety of rearrangement, condensation, and elimination reactions. The particular AGE species that enhance nucleation remain unknown, but these may be relatively early glycation products, as evidenced by the time course over which glucose accelerated the formation of fluorescent βAP aggregate compared with aggregation in the presence of glucose and aminoguanidine, a specific inhibitor of AGE formation. Given that plaque numbers increase in association with neuronal degeneration and cognitive decline in AD, and that aggregated but not monomeric βAP is actively neurotoxic (25, 26), interference with the processes by which AGE formation enhances βAP aggregation may provide new therapeutic opportunities to reduce the pathophysiological changes associated with AD.

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