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Influence of advanced glycation end-products and AGE-inhibitors on nucleation-dependent polymerization of β-amyloid peptide

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Abstract

Nucleation-dependent polymerization of β-amyloid peptide, the major component of plaques in patients with Alzheimer's disease, is significantly accelerated by crosslinking through Advanced Glycation End-products (AGEs) in vitro. During the polymerization process, both nucleus formation and aggregate growth are accelerated by AGE-mediated crosslinking. Formation of the AGE-crosslinked amyloid peptide aggregates could be attenuated by the AGE-inhibitors Tenilsetam, aminoguanidine and carnosine. These experimental data, and clinical studies, reporting a marked improvement in cognition and memory in Alzheimer's disease patients after Tenilsetam treatment, suggest that AGEs might play an important role in the etiology or progression of the disease. Thus AGE-inhibitors may generally become a promising drug class for the treatment of Alzheimer's disease.

Keywords: Advanced glycation end-product; β-Amyloid peptide; AGE-inhibitor; Alzheimer's disease

1. Introduction

Alzheimer's disease is a progressive dementia affecting a large proportion of the aging population. Histological hallmarks of the disease are widespread neuronal cell death and the formation of amyloid plaques and neurofibrillary tangles. The major proteinaceous component of the amyloid deposits which accumulate extracellularly in the Alzheimer's disease brain, is the β/A4-amyloid peptide. This 39–42 amino acid peptide consists of 28 extramembranal amino acids plus 11 to 14 residues of the hydrophobic transmembrane domain of its precursor, the amyloid β precursor protein, APP (for reviews, see [1–3]).

Genetic and biochemical evidence suggest that the β-amyloid peptide is important in the etiology or progression of the disease. Genetic studies have linked familial cases of early-onset Alzheimer's disease to

Abbreviations: AGEs, advanced glycation end-products; APP, amyloid precursor protein; CSF, cerebrospinal fluid; NPY, neuropeptide Y; AD, Alzheimer's disease

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the APP-locus on chromosome 21, where mutations in the APP-gene may influence APP-processing, β-amyloid peptide concentration or its aggregation parameters [4,5]. Further, two major biochemical mechanisms by which β-amyloid peptide or its aggregates may be involved in neuronal cell death have been proposed: direct neurotoxicity and indirect, immune system mediated neurotoxicity of the peptide aggregates. Firstly, several studies have demonstrated that β-amyloid peptide is both toxic to neurons in primary culture and to clonal cell lines [6–8]. Interestingly, the neurotoxicity of the peptide seems to increase with its aggregation state, since preaggregation of the synthetic peptide at concentrations above 50 μg/ml produces a maximal toxic effect [9]. The induction of oxidative stress [10], apoptosis [11] and ion channel formation [12] are the proposed interrelated mechanisms mediating this direct toxicity.

Secondly, the β-amyloid peptide is suggested to stimulate an indirect, immune mediated neurotoxicity by activation of resting microglia to brain macrophages by proteinous plaque deposits. This results in the concomitant release of oxygen free radicals, NO, cytokines and complement proteins, which may damage surrounding neurons through 'bystander lysis' [13–15].

The aggregation of β-amyloid peptide, therefore, may be critical for neuronal cell death in Alzheimer's disease via the stimulation of either direct and indirect neurotoxicity. This suggests that, changes in aggregation rate and plaque formation are likely to be important for the onset and progression of the disease. Aggregation of the β-amyloid peptide has been proposed to follow a nucleation-dependent polymerization process, consisting of two distinctive steps, an initial slow nucleus formation, followed by a rapid growth phase [16]. The formation of a nucleus is a reversible process dependent on the concentration of the peptide monomer, thus it is likely that the initiation of this process at a sub-threshold peptide concentration may only be started by irreversible covalent crosslinking of the monomers. A universal extracellular crosslinking of long-lived proteins by AGEs occurs following the initial reaction of a protein amino group with a monosaccharide, such as glucose or fructose [33,38]. Recently, immunohistochemical studies on post mortem tissue have identified AGEs as major components of amyloid plaques [17,18]. In vitro studies with synthetic β-amyloid peptide suggest that AGE-amyloid seeds can trap soluble peptides and accelerate plaque formation [18]. In addition, AGE-crosslinking inhibitors, such as Tenilsetam, have been shown in clinical trials to reverse impairments of cognitive function in patients with Alzheimer's disease [19].

Our aim in this study was to investigate the extent, to which different monosaccharides accelerate both steps of amyloid plaque formation, nucleation and growth. Secondly, we investigated the ability of AGE-inhibitors to attenuate these reactions. A positive result would posit a biochemical mechanism for their beneficial effect and promise a new approach to rationale drug design for the treatment of Alzheimer's disease.

2. Methods

2.1. Peptide preparation

β-amyloid peptide (1–40) was purchased from Auspep (Melbourne, Australia) or synthesized on a Zinsser simultaneous multiple peptide synthesizer using Fmoc-protection and DIC/HOBT activating chemistry with routine double coupling steps. Deprotection was achieved by TFA/ anisol/ ethandithiol/H_2O (93/5/2.5/2.5). After precipitation in ether, peptide was redissolved in 10% formic acid and purified on a preparative HPLC RP-C18 column. Purity and molecular weight of the peptide was confirmed by comparison with commercial (1–40) β-amyloid peptide standards by analytical HPLC and mass spectrometry.

2.2. Incubation of peptides with sugars

Stock solutions of peptides were dissolved in H_2O at a concentration of 1 mg/ml. For AGE-crosslinking experiments, they were incubated in 0.5 ml microtubes or 1 ml cuvettes at a concentration of 250 μg/ml (60 μM) in 50 mM sodium phosphate buffer pH 7.9 at 40°C or 50°C in the dark for the times indicated. NaN_3 (0.01% w/v) was added to prevent microbiological growth. In long term experiments, water was added every 12 h to compensate for solvent evaporation.

2.3. Quantification of peptide by polyacrylamide gel electrophoresis and image analysis

Prior to electrophoresis, samples were incubated in 4% SDS, 0.01% Bromophenol Blue in 0.1 M Tris, pH 8.9, for 30 min at 42°C. Samples were separated in a 16.5% polyacrylamide gel, topped with a 6% spacer and a 1% stacking gel using a tricine buffer system (pH 8.3) which allows separation of peptides as small as 2 kDa [37]. Gels were fixed in 50% methanol/10% acetic acid for 30 min, stained with 0.025% Coomassie Brilliant Blue G-250 in 10% acetic acid for 30 min and destained with 10% acetic acid overnight. Coomassie Brilliant Blue has been shown to be the most accurate protein determination assay for glycated and AGE-modified proteins [45]. Gels were photographed and protein bands quantified using an Apple Colorone scanner and the NIH Image 1.57 software. The lag time (the end of nucleus formation) was defined as the time, after which more than 10% of the monomer had disappeared.

2.4. Quantification of peptide oligomers > 30 kDa by ultrafiltration

β-Amyloid peptide oligomers > 30 kDa were separated by ultrafiltration through a 30 kDa membrane. Briefly, 30 μl of 60 μM peptide solution containing 6.25 μg of peptide was transferred into a Schleicher and Schuell Centrix UF cartridge and centrifuged at $13\,000 \times g$ for 2 min. After addition of 20 μl of PBS, the centrifugation step was repeated. The retained supernatant was resuspended in 2×20 μl PBS and transferred into a second tube. Protein content was determined in both supernatant and filtrate by the Bradford assay (see Section 2.7).

2.5. Counting of peptide aggregates with a cell counter

β-Amyloid peptide aggregates were counted in a Scherf Casy cell counter equipped with a 150 μm capillary. 10 μl of the samples were diluted in 10 ml 0.9% NaCl and 200 μl of the solution counted in triplicate. The cursor were set at 6 and 24 μm and the number of peptide aggregates and their total volume within this interval was determined.

2.6. Determination of β-sheet content of β-amyloid peptide aggregates by Thioflavin T

β-sheet content of peptide aggregates after ultrafiltration (see Section 2.4) was measured with Thioflavin T as described in [18]. Briefly, 30 μl of sample containing about 7.5 μg of peptide was ultrafiltrated, the supernatant resuspended and added to 1 ml 3 μM Thioflavin T solution in 50 mM sodium phosphate pH 6.0. After gentle vortexing, emission at 480 nm (excitation at 450 nm) was measured in a Spex Fluoromax fluorescence spectrometer with slits set at 1 nm. Values are expressed as arbitrary units (AU).

2.7. Bradford protein assay

Total peptide or protein content in solution was measured by the Bradford assay. The sample (containing up to 7.5 μg of peptide) was added to 400 μl Bradford reagent (0.1% Coomassie Blue in 10% H_3PO_4, 5% ethanol). After 5 min, optical density at 595 nm was measured in a LKB UV-Vis spectrophotometer.

2.8. Western blot

1 μg of β-amyloid peptide was removed from the incubation mixture and incubated in sample buffer (4% SDS, 0.01% Bromophenol Blue in 0.1 M Tris, pH 8.9) for 30 min at 42°C prior to electrophoresis. Samples were separated in a 16.5% polyacrylamide gel, topped with a 6% spacer and a 1% stacking gel using a tricine buffer system (pH 8.3) which allows separation of peptides as small as 2 kDa [37]. After electrophoresis, proteins were transferred onto a nitrocellulose sheet. The nitrocellulose sheet was blocked for 1 h with PBS containing 6% BSA, 1% FCS and 0.05% Tween 20. After incubation for 2 h with an β-amyloid peptide (1–40) rabbit antibody (a gift from Dr. Stauffenbiel, Sandoz AG) in a dilution of 1:2000, alkaline phosphate linked anti rabbit IgG (Boehringer, diluted 1:2000 in PBS) was added and incubated for 1 h. The Western blot was developed with DTNB in glycine buffer (pH 10.4) and photographed.

3. Results

3.1. Assays for monitoring crosslinking and aggregation of β-amyloid peptide

Nucleation-dependent polymerization of amyloid peptide in solution leads to an equilibrium with two components: insoluble amyloid peptide aggregates and a low concentration of soluble monomer in the supernatant [16]. Among different APP derived β-amyloid peptides and fragments, β-amyloid peptide (1–40) has sufficient kinetic solubility at micromolar concentrations, and was therefore used in all experiments shown below. Fructose was used in addition to glucose in AGE-crosslinking experiments, because it has been shown to be abundant in brain and to crosslink proteins up to ten times faster than glucose [21]. The experiments were performed with concentrations of reactants and temperatures well above the physiological levels. However, this enabled us to accelerate the process of plaque formation, which takes decades in vivo [28], to be monitored in vitro in a reasonable time period.

Formation of β-amyloid aggregates from soluble monomer was measured by four different assays. The loss of soluble β-amyloid peptide monomer was monitored by SDS gel electrophoresis, Coomassie Blue staining and image analysis of the 4.2 kDa β-amyloid peptide band in the gel. Protein content of peptide oligomers > 30 kDa were quantified in filtrate and supernatant after ultrafiltration by the Bradford (Coomassie Blue) protein assay. Coomassie Blue binding has been shown to be the less influenced assay for protein determination by glycation and AGE-modification [45]. In agreement with this study, total protein loss measured by this assay in our experiments was less than 25% in 240 h, indicating that loss of monomer is not simply caused by adsorption of peptide to the test tubes or other surfaces during sample handling. In a second assay for protein aggregates with a molecular weight > 30 kDa, β-sheets content was measured by the increase in Thioflavin T fluorescence in the supernatant after ultrafiltration through a 30kDa membrane. The increase in protein concentration measured both by Thioflavin T and Bradford (Coomassie Blue) of the supernatant after ultracentrifugation (aggregates > 30 kDa) solely in the samples containing fructose show that the disappearance of monomer (by AGE crosslinking) is directly coupled to the gain of aggregates. Formation of β-amyloid peptide aggregates was also visualized by Western blot using a specific polyclonal antibody raised against the 1–40 peptide. Finally, number and size of macroscopic peptide aggregates (> 6 μm in diameter) were measured with a cell counter.

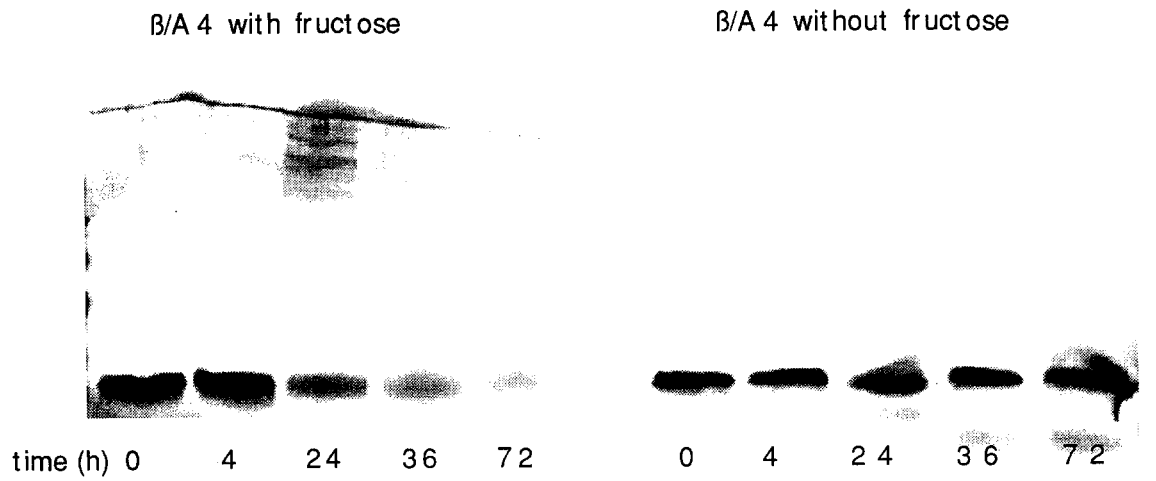

Fig. 1. Loss of soluble β–amyloid peptide (1–40) by glycation/AGE-crosslinking. 60 μM β-amyloid peptide in 50 mM sodium phosphate buffer, pH 7.9, was incubated at 50°C without sugars (right gel) and with 50 mM fructose (left gel). After the times indicated, 20 μl of the sample (5 μg of peptide) was added to 10 μl SDS electrophoresis sample buffer and incubated at 42°C for 30 min. The peptides were separated in a Tris/Tricine polyacrylamide gel and stained with Coomassie Blue G-250.

3.2. Acceleration of β-amyloid peptide aggregation by fructose mediated AGE-crosslinking

β-amyloid peptide (1–40) was incubated at a concentration of 60 μM with and without 50 mM fructose at 50°C. In contrast to other peptides, i.e. neuropeptide Y (see Fig. 9) or model proteins like lysozyme, which show the characteristic oligomer 'ladder' after AGE-crosslinking, crosslinking of β-amyloid peptide (1–40) by fructose derived AGEs leads to a constant reduction of soluble monomer over time, similar to that described for glucose induced crosslinking [18], but with a much faster reaction rate (lag time: 18 h at 50 °C). Faint bands of peptide oligomers are visible at approximately 24 h (Fig. 1, lane 3), but cannot be detected at a later stage. It is possible, that the AGE-crosslinked β-amyloid peptide oligomers are spread over a range of different molecular weights; thus the amount of peptide in one oligomer band is below the sensitivity limit of the Coomassie stain assay. Immunoblotting was used as a more sensitive method to improve the detection of high molecular weight aggregates after 36 h, where no oligomers can be detected in the gel by Coomassie staining. Immunoreactive β-amyloid peptide aggregates could be detected below the border of the spacer and the separating gel; their amount decreases slowly during the timecourse of the reaction. In addition, a significant portion of the aggregates are compressed directly at the gel border, leading to an apparent higher local peptide concentration (Fig. 2). However, one has to be careful with a quantitative interpretation, since adsorption of large aggregates and peptide monomer may be quite different. β-amyloid peptide incubated without sugars seems to form aggregates at a much slower rate; thus the amount of soluble monomer is nearly constant for the first 72 h and no aggregates are formed during this time (Fig. 1). The loss of peptide monomer in the

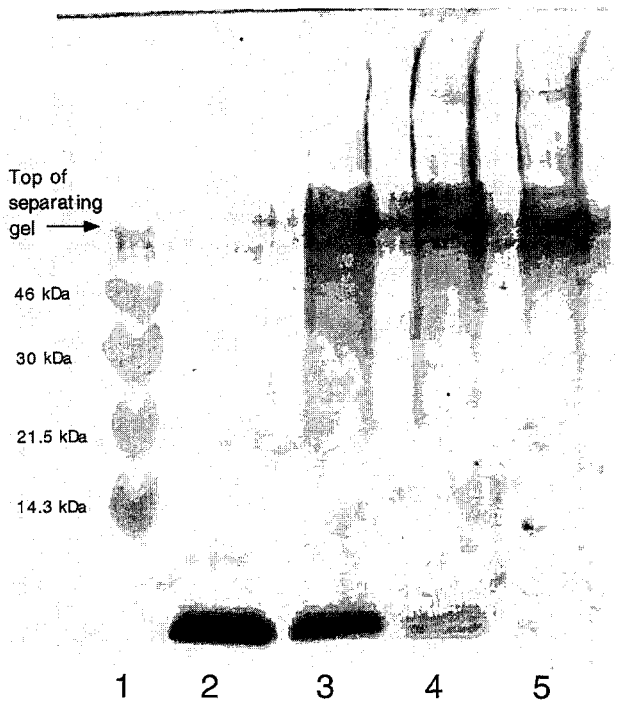

Fig. 2. Immunoblot of β-amyloid aggregates. Western blot was performed using 1 μg of β-amyloid peptide incubated with 50 mM fructose in 50 mM sodium phosphate buffer, pH 7.9. Whereas aggregates are not visible in freshly dissoved peptide (lane 2), a heterogeneous collection of aggregates can be detected after 36 h (lane 3), 72 h (lane 4) and 96 h (lane 5). In lane 1, molecular weight markers are shown.

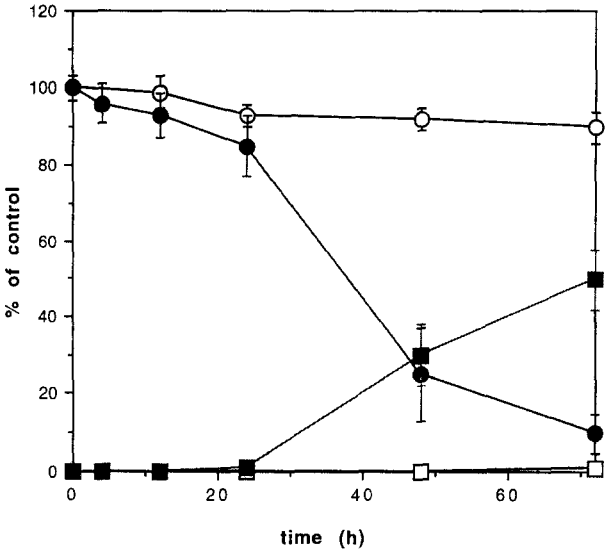

Fig. 3. Timecourse of the disappearance of β-amyloid monomer and formation of peptide aggregates > 30 kDa by AGE-mediated crosslinking. 60 μM β-amyloid peptide in 50 mM sodium phosphate buffer, pH 7.9, was incubated without sugars (open symbols) and with 50 mM fructose (filled symbols). β-amyloid peptide incubated without sugar at 50°C shows no loss of monomer (open circles) and no formation of aggregates (open square), whereas disappearance of monomer (filled circles) and formation of aggregates (filled squares) was enhanced by fructose. Experiments were performed in duplicate; data are shown as mean ± S.E.M.

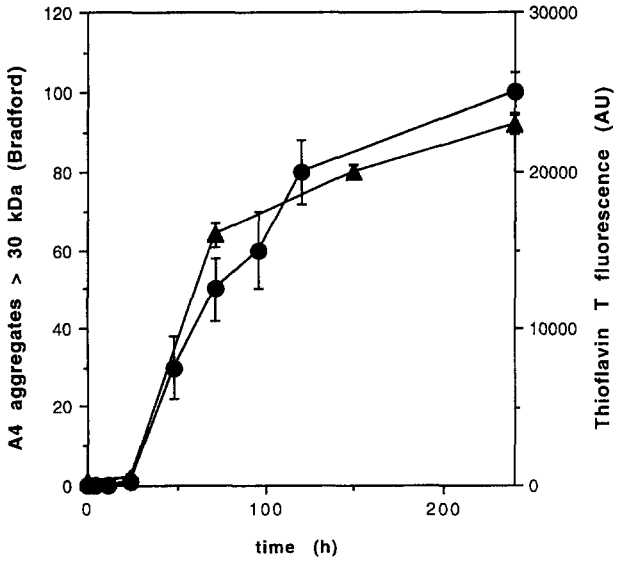

Fig. 4. Formation of thioflavin binding β-amyloid aggregates by AGE-crosslinking. β-amyloid aggregates with a molecular weight above 30 kDa by crosslinking with 50 mM fructose was measured with thioflavin T (as an indicator of β-sheet content, filled triangles) and Coomassie Blue (Bradford assay; as an indicator of total protein content, filled circles) between 0 and 240 h. Experiments were performed in duplicate; data are shown as mean ± S.E.M.

samples containing fructose was accompanied by a parallel increase in peptide aggregates above 30 kDa. When the β-amyloid peptide was incubated without fructose, both the disappearance of β-amyloid monomer and the formation of aggregates > 30 kDa was marginal compared to the samples with fructose (Fig. 3). This is in agreement with the hypothesis of a cooperative aggregation model with an association constant of 1.05×10^4 mol^{-1} [51], which is twice the peptide concentration used in our experiments.

The β-amyloid aggregates over 30 kDa were also measured by Thioflavin T binding, a method assumed to correlate with the β-sheet content of the respective protein [18]. Interestingly, β-sheet formation correlates well with the amount of peptide oligomers, indicating that the change of conformation, from random-coil of the monomer to β-sheet of the oligomer, occurs parallel to crosslinking and oligomerisation (Fig. 4). In addition, Vasan et al have shown by electron microscopy in a very recent study that AGE-mediated crosslinking of β-amyloid leads to formation of β-sheeted fibrils [52].

Large peptide aggregates (in μm size) were counted and analyzed in a cell counter (Fig. 5). The number of β-amyloid aggregates and their total volume is constant for the first 100 h, then increases

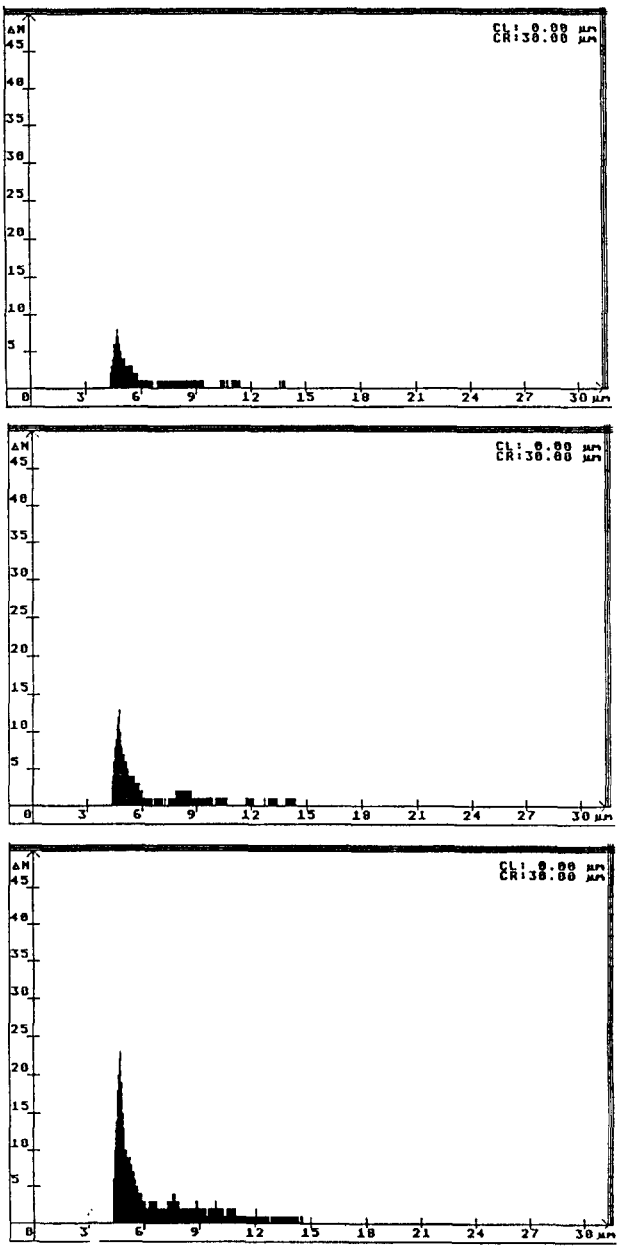

Fig. 5. Analysis of large β-amyloid aggregates with a cell counter. AGE-β-amyloid peptide aggregates with a diameter between 6 and 24 μm were counted in a cell counter. 10 μl of 60 μM peptide solution were diluted in 10 ml 0.9% NaCl; the number of aggregates and their size distribution were analyzed in triplicate. The left chromatogram shows the typical background level of the counter, the middle picture the aggregate size distribution of an β-amyloid/fructose sample at 0 h, the right chromatogram after 180 h.

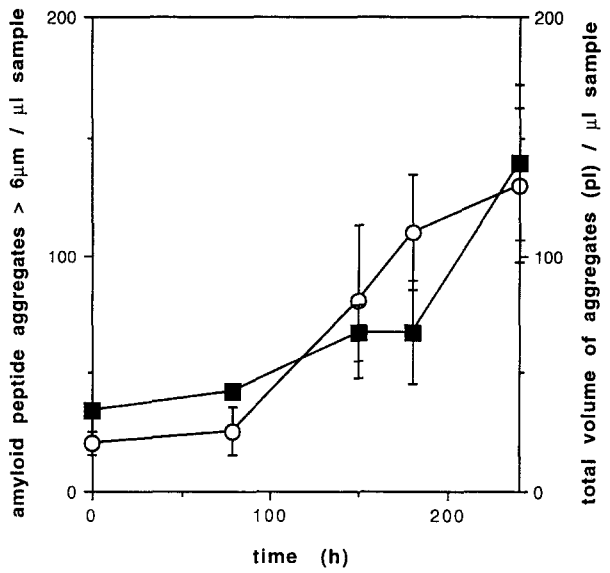

Fig. 6. Timecourse of the formation of large AGE-β-amyloid aggregates. After subtraction of the background level of the counter, the number of AGE-β-amyloid peptide aggregates (open circles) and their total volume (filled squares) are shown per µl of 60 µM peptide solution. Experiments were done in duplicate; data are shown as mean ± S.E.M.

between 100 and 240 h (Fig. 6). Interestingly, a significant number of seeds is already present in a freshly dissolved β-amyloid peptide solution. However, this assay has to interpreted very cautiously, since light microscopy shows also fibrillary assemblies, which may not be counted accurately.

In summary, among the different assays to monitor covalent crosslinking of the β-amyloid peptide by AGEs, the disappearance of the 4.2 kDa band of the monomer was the earliest indication for occurrence of crosslinking; hence this assay was used for the following experiments.

3.3. Influence of temperature and different fructose concentrations on aggregation parameters

Having established the accelerating effect of fructose on β-amyloid nucleation and aggregate growth, this reaction was investigated further. For this purpose, different fructose concentrations (50 mM vs. 200 mM) and different temperatures (40°C vs. 50°C) were used (Fig. 7). At 40°C, the crosslinking reaction, measured by the disappearance of soluble monomer, was significantly slower (52 h at 40°C, 18 h at 50°C). The lag time decreased from 52 h to 32 h, when the concentration of fructose was raised from 50 mM to 200 mM, indicating a faster glycation and subsequent nucleus formation of the peptide. The similar slope of the growth curve indicates that, after the glycation steps were completed, the growth of the aggregate depends mainly on the inherent crosslinking reactivity of the bound AGE. This indicates, that the protein bound Amadori products are the predominant precursors for the crosslinking reaction, as recently reported for collagen in phosphate containing buffers [50].

3.4. Attenuation of seed formation and plaque growth by AGE-inhibitors

Attenuation of β-amyloid peptide aggregation and plaque formation is considered one of the prime targets of drug development for Alzheimer's disease.

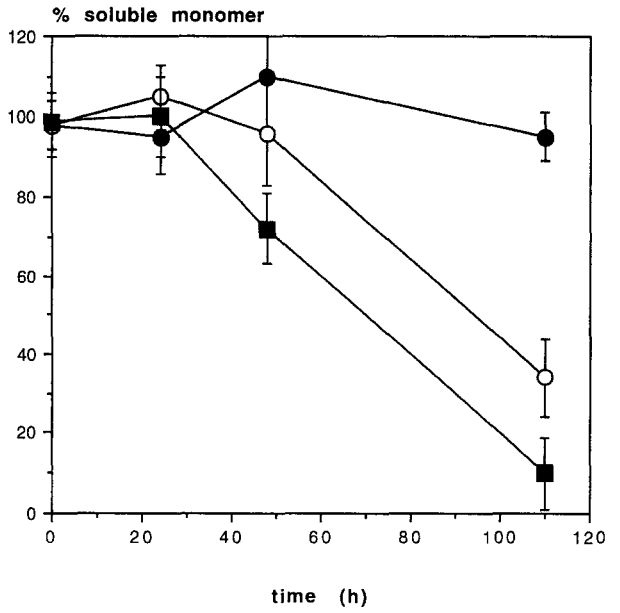

Fig. 7. Acceleration of AGE-mediated β-amyloid monomer crosslinking by increased fructose concentrations. 20 µg of β-amyloid peptide was incubated in 80 µl 50 mM sodium phosphate buffer, pH 7.9, at 40°C without sugars (filled circles), 50 mM fructose (open circles) and 200 mM fructose (hatched squares). After 22, 48 and 110 h, samples containing 5 µg of β-amyloid peptide were taken and the percentage of soluble monomer was quantified by densitometric scanning of the Coomassie Blue G-250 stained band after gel electrophoresis. Experiments were performed in duplicate; data are shown as mean ± S.E.M.

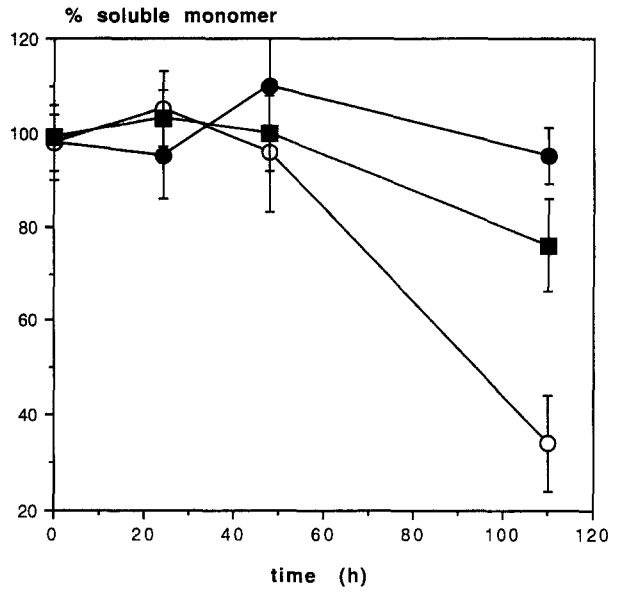

Fig. 8. Inhibition of AGE-mediated β-amyloid peptide crosslinking by the AGE-inhibitor Tenilsetam. 20 μg of β-amyloid peptide was incubated in 80 μl 50 mM sodium phosphate buffer, pH 7.9, at 40°C without sugars (filled circles), 50 mM fructose (open circles) and 50 mM fructose + 50 mM Tenilsetam (hatched squares). After 22, 48 and 110 h, samples were taken and the percentage of soluble monomer left in the supernatant quantified by scanning of the Coomassie Blue G-250 stained band after gel electrophoresis. Experiments were performed in duplicate; data are shown as mean ± S.E.M.

Inhibition of AGE-mediated β-amyloid crosslinking is a promising therapeutic strategy with the potential to slow down β-amyloid plaque formation and the progression of the disease [44]. One AGE-inhibitor of demonstrated clinical benefit in Alzheimer's disease, Tenilsetam, was shown to inhibit glycation and AGE-mediated crosslinking of model proteins [20]. Incorporation of Tenilsetam into glycated, but only marginally into unmodified proteins suggests that it exerts its anti-crosslinking effect by capping reactive aldehyde groups on free and bound sugars [20]. To measure the effect of Tenilsetam on the timecourse of fructose induced crosslinking of β-amyloid peptide, it was added in a 1:1 ratio (50 mM) to the reaction mixture and the time-course was followed for 110 h at 40°C. Seed formation (increased lag time) seems to be only minimally attenuated, whereas the growth of aggregate (loss of monomer/time) was slowed by more than 50% (Fig. 8).

The experiments were extended to other known AGE-inhibitors. Aminoguanidine is a more reactive amine than the lysine ε-amino group and blocks the carbonyl group of the fructosyl moiety by covalent attachment [22]. Carnosine is a naturally occurring dipeptide with strong antioxidant, metal chelating and anti-glycation properties, most likely by mimicking the preferred glycation site on proteins [23]. It was also shown to rejuvenate and prolong the life span of diploid fibroblasts [43]. All three AGE crosslinking inhibitors (aminoguanidine, carnosine and Tenilsetam, Fig. 9) were compared in a system containing 60 μM β-amyloid peptide and 50 mM fructose for 24 h at 50°C. To demonstrate that the inhibitory effect on crosslinking is a general effect, a control peptide with a similar molecular weight, neuropeptide Y, was included. Whereas NPY showed the typical oligomer ladder, β-amyloid peptide incubated without AGE-

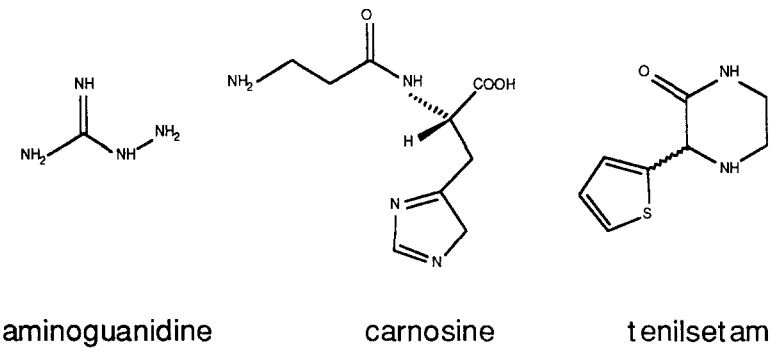

Fig. 9. Chemical structures of the AGE-inhibitors aminoguanidine, carnosine and Tenilsetam.

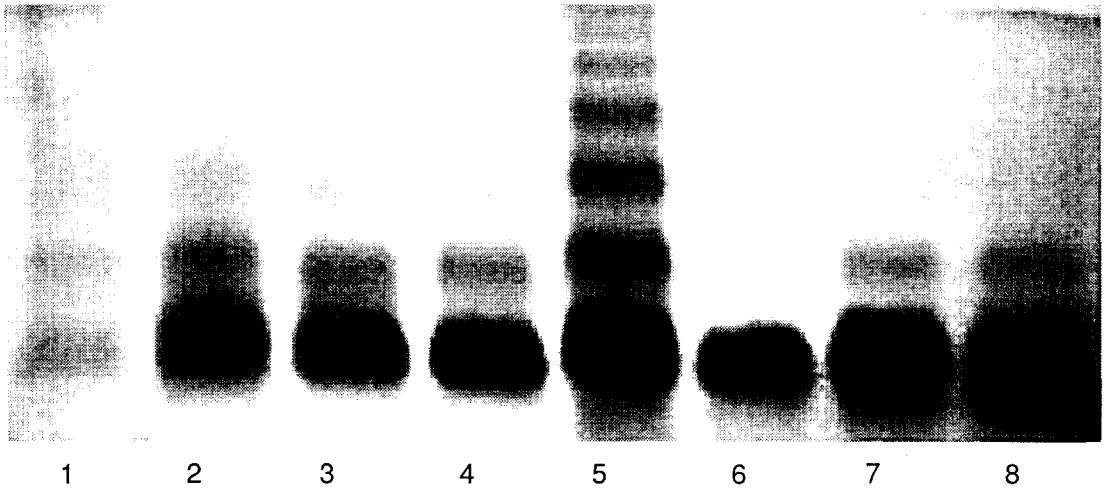

Fig. 10. Comparison of inhibition of AGE-mediated β-amyloid peptide and neuropeptide Y crosslinking by AGE-inhibitors. β-amyloid peptide and NPY (5 μg of peptide in 20 μl 50 mM sodium phosphate buffer, pH 7.9) were incubated with 50 mM fructose (lanes 1, 5), 50 mM fructose + 50 mM aminoguanidine (lane 2, 6), 50 mM fructose + 50 mM carnosine (lane 3, 7) and 50 mM fructose + 50 mM Tenilsetam (lane 4, 8) for 24 h at 50°C. Whereas β-amyloid peptide oligomers (lanes 1–4) precipitate, distinct soluble NPY oligomers (lanes 5–8) are visible under the same conditions.

inhibitors showed the characteristic disappearance of soluble monomer. All three AGE-inhibitors prevented crosslinking of both peptides significantly, and nearly 100% of the β-amyloid peptide was kept in solution (Fig. 10). However, it has to be noted that this experiment utilized a peptide batch from a commercial source (Auspep), which already contains a small amount of crosslinked β-amyloid peptide dimer.

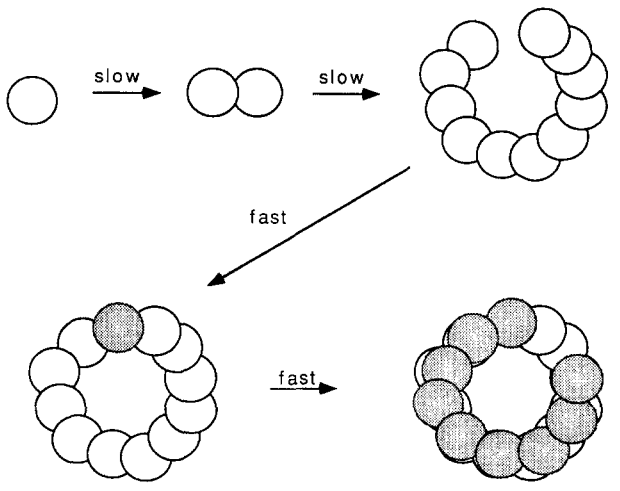

Fig. 11. Mechanism of nucleation dependent polymerization. Aggregation of the β-amyloid peptide monomer follows the mechanism of nucleation dependent polymerization, in which the slow formation of a nucleus from peptide monomers was subsequently followed by a rapid growth phase as soon as the seed is formed.

4. Discussion

4.1. β-amyloid peptide in plaque formation and in Alzheimer's disease

An unequivocal causal relationship between β-amyloid formation and Alzheimer's disease has not been proven so far. However, significant genetic and biochemical evidence supports the hypothesis that β-amyloid peptide is involved in the etiology or progression of the disease [6,9,24,25]. Since age is the major risk factor in Alzheimer's disease [42], the time-course of these aggregation processes is the key to understanding the progress of the disease as well as the therapeutic opportunities for its treatment.

Lansbury and coworkers proposed that aggregation of the β-amyloid peptide occurs by nucleation-dependent polymerization, a process similar to that seen in such as protein crystallization and microtubule formation [16]. The process consists of an initial slow nucleus formation followed by a subsequent rapid growth phase (Fig. 11). Formation of the nucleus requires a series of association steps that are thermo-

dynamically unfavorable and only occur in supersaturated solutions, because the energy gain of the association has to be greater than the entropic costs. Since these association steps are reversible, crosslinking of the monomers, which prevents the dissociation of the formed oligomers, could facilitate the formation of a nucleus at low physiological peptide concentrations. Once the nucleus is formed, further attachment of monomers to the preformed seed at multiple binding sites leads to rapid growth of the aggregate [16].

Both steps of this polymerization process are accelerated by crosslinking of monomers through AGEs. The β-amyloid peptide contains four major crosslinking sites, for AGEs in its sequence (two lysines, one arginine and the N-terminal amino group), allowing the formation of a three-dimensional network. The first step, the formation of the nucleus, is significantly shortened by crosslinking of the monomers by AGEs, with fructose being much more reactive than glucose. Increased concentration of sugar accelerates the formation of a seed, supporting the hypothesis that crosslinking of the β-amyloid peptide by AGEs is the crucial step for formation of a stable nucleus.

For the second step, the growth of the plaque from a preformed seed, AGE-modified seeds are faster reacting nuclei for β-amyloid peptide aggregation than non-modified AGE seeds [18]. We have shown that fructose significantly accelerates the growth of the plaque from a preformed seed in comparison with glucose. However, under these experimental conditions the reaction rate for the aggregate growth is independent of fructose concentration. This suggests that the growing aggregate is saturated with crosslinking-reactive AGEs, which are able to covalently bind additional peptide monomers. Therefore, after the amino groups of the peptide are completely glycated, the conversion of the Amadori products to reactive AGEs might be the rate determining steps for the growth of the aggregate.

In most studies, aggregation experiments with synthetic amyloid peptides utilized supersaturated solutions. In order to obtain a reasonable amount of AGE-crosslinked protein in a reasonable time period, we have worked with high concentrations of both peptide and sugars. However, under physiological conditions, AGE-modification of proteins yields a substantial amount of crosslinked proteins only after a long time span, in the range of months or even years [38], which is similar to the late onset and slowly progressive nature of Alzheimer's disease. Nevertheless, these experiments provide a basis for the planning of long-term studies under physiological conditions. Since the first step, the glycation of the primary amine, is a simple bimolecular chemical reaction, the reaction rate is proportional to concentrations of both partners ($v = k \times [\text{sugar}] \times [\text{protein}]$) [39]. Therefore, glucose concentrations up to 1M and protein concentrations up to 50 mg/ml are generally used in AGE studies to accelerate the reaction [26,27]. In Alzheimer's disease, the average of the plaque proteins, measured by amino acid racemization, was estimated to be at least 20 years [28]. In this time frame, covalent crosslinking of peptide monomers is necessary to allow nucleation and growth in physiological, non-supersaturated peptide solutions, when self aggregation is thermodynamically unfavorable [51].

4.2. Contribution of fructose to AGE-mediated crosslinking in the brain

Previous crosslinking experiments with β-amyloid peptide have used glucose, the major energy source in the brain [18]. However, crosslinking by glucose under non-oxidative conditions is extremely slow. Glycoxidation products, which are produced by transition metal-mediated oxidation of soluble and protein bound sugars have therefore been proposed to be the reactive species [29]. Since our experiments were also performed under oxidative conditions, we suggest a significant crosslinking of β-amyloid peptide by fructose in vivo via a mechanism similar to that proposed for glucose [30].

In human plasma which has a glucose concentration of approximately 4–5 mM and fructose concentration of approximately 0.2 mM (ratio $> 20{:}1$), about 15% of the plasma proteins are glycated by fructose [21], indicative of the higher glycation reactivity of this sugar. However, in human brain, the glucose/fructose ratio is reduced from 20 to about 4 (glucose 2 mM, fructose 0.5 mM), consistent with the proposed higher contribution of fructose to AGE-crosslinking in the brain than in peripheral systems [21]. One might speculate that changes in the concentration of fructose or other AGE reactive sugars should have a significant effect on peptide glycation

and subsequently on the time-course of plaque formation in vivo. In ongoing studies, we are preparing fructose-AGE antibodies to identify these compounds in post mortem tissue. The involvement of sugars other than glucose in AGE crosslinking might be a interesting link to another characteristic feature of Alzheimer's disease, the disturbance of the cerebral glucose metabolism [32].

4.3. AGEs, diabetes and Alzheimer's disease

AGEs have been shown to play an important role in the evolution of vascular complications in normal aging, more so in diabetes and renal failure. In diabetes, accelerated AGE accumulation is caused by a higher level of plasma glucose and, intracellularly, by the activation of the polyol pathway and in hemodialysis by the inability of the dialysis cartridges to remove AGE-modified peptides [46,47]. In AD, other factors are required to explain the elevated level of AGEs and AGE-crosslinked proteins.

Interestingly, there is no increased risk of AD in diabetic patients. This might be due to several reasons: Dementia in patients with diabetes is likely to be a vascular type dementia, which statistically increases the number of AD patients in the non-diabetic group. Further, diabetic patients do not have a greater risk of accumulating AGEs in the brain, since the brain glucose level of diabetic patients is not increased. In contrary, the transport capacity for glucose across the blood brain barrier is reduced, most likely resulting from downregulation of the glucose transporters [48]. On the other hand, Alzheimer's disease patients do not show an increased AGE level in plasma [49], again suggesting that cerebral AGE accumulation in Alzheimer's disease is a very selective, brain specific event.

4.4. AGE-inhibitors as neuroprotective drugs

AGE mediated protein crosslinking can be inhibited by aminoguanidine, the prototype of an AGE-inhibitor not only in vitro, but also in vivo as seen in the prevention of vascular complications of diabetes [33,34]. Tenilsetam, a cognition-enhancing and antidementia drug was also shown to be an effective AGE-inhibitor without any radical scavenging properties [19,20]. Tenilsetam reacts with sugars and glycated proteins and acts as an inhibitor of AGE-derived protein crosslinking in vitro. Carnosine, the third AGE-inhibitor tested, is classified as an antioxidant, a metal-chelator as well as an anti-glycation agent [23,31]. In general, AGE-inhibitors can exert their effects at different levels of the glycation cascade (Maillard reaction). Continuous capping of reactive AGEs on growing amyloid plaques is one of the explanations of the clinical effects of the AGE-inhibitor Tenilsetam [20]. This can feasibly be extended to the inhibition of AGE-mediated microtubule associated protein tau crosslinking in neurofibrillary tangle formation [35].

In this study, Tenilsetam could significantly inhibit the growth of the β-amyloid peptide aggregate by nucleation-dependent polymerization. The other AGE-inhibitors were similarly effective in keeping the β-amyloid peptide in its soluble monomeric form. For both proposed mechanisms of neuronal cell death, direct or indirect toxicity of the β-amyloid peptide, a decreased formation of toxic peptide aggregates may be therapeutic. It is also be possible that AGE-inhibitors modify the structure of AGEs in a way that causes a loss of their binding to cell surface AGE-receptors. This could lead to a diminished inflammatory response and decreased oxidative stress [40,41]. These hypotheses provide a speculative but reasonable explanation for the clinical effects seen with Tenilsetam in patients with Alzheimer's disease, where cognitive abilities and memory improve slowly over a time span of three months [19,20]. However, a direct attenuation of cytotoxic AGE effects by AGE-inhibitors has to be shown at least before proposing this as a valid approach for the treatment of AD patients.

4.5. Physiological role of AGE-inhibitors

One of the obvious disadvantages of AGE-inhibitors in regard to their biological significance and therapeutic efficacy is, that relatively high concentrations (in a equimolar ratio to sugars) seem to be required for an effective inhibition of AGE-formation. This is the result of the underlying chemical stochiometry of the Maillard reaction. However, when sugars more reactive than glucose with a lower physiological concentration are taken into consideration, a 1:1 ratio of AGE-inhibitor and sugar might be

achieved. In tissues such as muscle and olfactory tissue the physiological AGE-inhibitor carnosine and related substances reach a concentration of up to 50 mM [36]. It is likely that mammals have developed strategies to minimize glycation and AGE-formation as a defense mechanism using physiological AGE-inhibitors [23]. Dysfunction of the physiological AGE-defense and clearance mechanisms may be an explanation for a variety of degenerative diseases. On the other hand, supplementing naturally occurring anti-glycation molecules by synthetic drugs like Tenilsetam, especially in tissues with low concentrations of natural AGE-inhibitors may be a promising therapeutic strategy not only for Alzheimer's disease, but also for various AGE-mediated degenerative and AGE-related diseases.

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