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Treatment with a γ -ketoaldehyde scavenger prevents working memory deficits in hApoE4 mice

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Abstract

Both inflammation and oxidative injury are features of Alzheimer's disease (AD), but the contribution of these intertwined phenomena to the loss of working memory in this disease is unclear. We tested the hypothesis that highly reactive γ -ketoaldehydes that are formed both by non-enzymatic free radical catalyzed lipid peroxidation and by cyclooxygenases may be causally linked to the development of memory impairment in AD. We found that levels of γ -ketoaldehyde protein adducts were increased in the hippocampus of brains obtained postmortem from patients with AD compared to age-matched controls, but that levels of γ -ketoaldehyde protein adducts in the cerebellum were not different in the two groups. Moreover, immunohistochemistry revealed that adducts localized to hippocampal pyramidal neurons. We tested the effect of an orally available γ -ketoaldehyde scavenger, salicylamine, on the development of spatial working memory deficits in hApoE4 targeted replacement mice, a mouse model of dementia. Long-term salicylamine supplementation did not significantly alter body weight or survival, but protected against the development of age-related deficits in spatial working memory in 12–14 month old ApoE4 mice. These findings suggest that γ -ketoaldehyde adduct formation is associated with damage to hippocampal neurons in patients with AD and can contribute to the pathogenesis of spatial working memory deficits in hApoE4 mice. These data provide a rational basis for future studies exploring whether γ -ketoaldehyde scavengers may mitigate the development of cognitive dysfunction in patients with AD

Keywords

Aldehydes; Alzheimer's disease; inflammation; isolevuglandin; oxidative stress; salicylamine; working memory

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INTRODUCTION

No effective therapies exist for Alzheimer's disease (AD). Biomarkers of oxidative injury and inflammation increase in AD and in mouse models of AD, suggesting their potential involvement as instigators of neuronal damage [1–7]. Therefore, identifying critical mediators of inflammatory and oxidative injury may provide therapeutic targets for the prevention of neuronal damage and memory impairment.

Reactive aldehydes have been suggested as potential mediators of neuronal damage because of their capacity to covalently modify, crosslink, and aggregate proteins and DNA [8–12]. Both oxidative injury and inflammation generate highly reactive lipid γ -ketoaldehydes via oxygenation of polyunsaturated fatty acids (Supplemental Fig. 1A). These γ -ketoaldehydes react exceedingly rapidly with lysyl residues of proteins to form stable lactam adducts, as well as crosslinks (Supplemental Fig. 1B). These γ -ketoaldehydes crosslink proteins and induce cytotoxicity at much lower concentrations than the well-studied lipid peroxidation product 4-hydroxynonenal [13].

Formation of γ -ketoaldehydes in AD is not well characterized. In a small sample set of AD brains, levels of γ -ketoaldehyde adducts correlated with disease severity [14]. While the γ -ketoaldehydes measured in this study were interpreted to primarily derive from cyclooxygenase, the mass spectrometry assay used did not directly distinguish between γ -ketoaldehydes derived from cyclooxygenase (levuglandins) and their regioisomers derived from the isoprostane pathway of lipid peroxidation (isoketals). Docosahexaenoic acid, which is highly enriched in neurons in the brain, is not a substrate for the cyclooxygenase enzyme. Accordingly, γ -ketoaldehydes formed from oxidation of docosahexaenoic acid (neuroketals) are unambiguously generated via non-enzymatic free radical mediated peroxidation. We therefore sought to measure levels of neuroketal adducts in postmortem brains from patients with AD and aged-matched controls, to determine if lipid peroxidation was also a source of γ -ketoaldehyde formation in AD.

Inflammation and oxidative injury generate numerous injurious mediators besides γ -ketoaldehydes. To specifically define the contribution of γ -ketoaldehydes to disease processes, we have previously identified small molecule scavengers, including salicylamine (SA), that rapidly and preferentially react with γ -ketoaldehydes and prevent their adduction to cellular proteins and other amines [15]. Administration of SA in drinking water results in SA concentrations in mouse tissues equivalent to what is required in cultured cells to inhibit γ -ketoaldehyde adduct formation and γ -ketoaldehyde mediated cellular injury [15–17]. Therefore, we sought to assess the contribution of γ -ketoaldehydes to the development of memory impairment in a mouse model of AD. Studies suggest that the ϵ 4 allele of ApoE (ApoE4) significantly increases the vulnerability to oxidative and inflammatory injury compared to ϵ 2 (ApoE2) and ϵ 3 (ApoE3) alleles [18–24] and that ApoE4 target replacement mice have deficits in working memory [25]. We found that administration of 1 g/L SA prevented the development of working memory deficits in ApoE4 targeted replacement mice.

MATERIALS AND METHODS

Mice

All procedures were approved by Vanderbilt University's Institutional Animal Care and Use Committee and the mice were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, U.S. Department of Health and Human Services. Mice with targeted replacement of the coding elements of the mouse apoE gene with those of human ApoE4 gene were originally developed by Dr. Nobuya Maeda at the University of North Carolina

[26–28]. We purchased homozygous ApoE4 mice from Taconic and bred them through a use agreement with Dr. Maeda. Wild-type (C57BL/6) mice were purchased from Jackson Labs (Bar Harbor, ME) and then bred in the same facility and conditions as the ApoE4 mice. Because we did not have the capacity to breed (or test) as a single cohort enough animals to sufficiently power our studies, we performed testing with four separate cohorts. Each cohort consisted of approximately 20 mice with approximately equal number of wild-type and ApoE4 animals. All the animals within a cohort were born within 4 weeks of each other. At 4 months of age, wild-type and ApoE4 were randomly assigned to receive either regular drinking water, or drinking water supplemented with 1 g/L SA. Animals were generally housed with one or two of their litter mates of the same gender, with large litters being divided as evenly as possible between regular drinking water and SA supplementation groups. All animals received a defined L-amino acid diet supplemented with choline and iron (Dyets, Inc. Bethlehem, PA, Diet #518754). After behavioral testing, animals were anesthetized with isoflurane and euthanized. Brains were flash frozen for analysis of SA levels.

Tissue

Regions of human brain were obtained at autopsy from six patients with AD (2 males, 4 females; mean age at death 80.83 ± 1.82 years) and six age-matched controls (4 males, 2 females; mean age at death 87.33 ± 4.5 years). Materials were obtained from the Alzheimer's Disease Research Center at the Sanders-Brown Center on Aging, University of Kentucky. All AD patients were diagnosed as having probable AD [29–31]. Controls died from non-neurological disease, so that their tissue represents brain samples relatively unaffected by the neurological damage associated with AD. Autopsies were performed within 4 h after death and all tissue sections dissected at the autopsy were kept frozen at -80°C until used.

Neuroketal protein adducts purification and analysis

The purification and analysis of neuroketal (NeuroK) protein adducts were performed as previously described [32, 33]. Briefly, tissue samples were ground in cold ethanol solution (containing 5mg of butylated hydroxytoluene and 50 mg of triphenylphosphine per each 100 ml) (Aldrich, Milwaukee, WI). 0.05 ml cold ethanol solution/mg tissue was added and proteins were precipitated by centrifugation at 2000 rpm at 4°C for 10 min. Proteins were then resuspended in 3ml of cold MeOH (containing butylated hydroxytoluene and triphenylphosphine) and 3ml of 0.4N potassium hydroxide (containing Trolox) and hydrolyzed under argon for 2 h at 37°C . Proteins were then reprecipitated in cold ethanol (0.05 ml/mg tissue, containing butylated hydroxytoluene and triphenylphosphine) and subsequently with 0.05 ml cold Folch (2:1 chloroform/methanol) solution/mg tissue, and washed with 0.05 ml cold methanol/mg tissue (containing butylated hydroxytoluene and triphenylphosphine). Proteins were resuspended in 1X phosphate-buffered saline and heated at 95°C for 10 min. After cooling, pronase (Calbiochem, La Jolla, CA) was added (1 g/g of starting tissue weight) and incubated overnight at 37°C . The digest was then heated at 95°C for 10 min to inactivate the pronase and after cooling, aminopeptidase M (Calbiochem, La Jolla, CA) was added (400 $\mu\text{l/g}$ of starting tissue weight) and incubated 18 h at 37°C . The digest was applied to a 1 g Oasis cartridge (Waters Associates, Milford, MA), filtered and purified by HPLC using a gradient consisting of 20 mM ammonium acetate with 0.1% acetic acid to 5mM ammonium acetate/methanol/acetic acid (10:90:0.1, v/v/v) on a 4.6 $\times$ 250mm Macrosphere 300 C18 column (MacMod Analytical, Chadds Ford, PA). HPLC fractions containing radioactivity from NeuroK-lysyl adduct internal standard were combined, re-extracted with an Oasis cartridge and analyzed by liquid chromatography/electrospray ionization/tandem mass spectrometry (LC/ESI/MS/MS). Internal standards were synthesized as follows: 25 mg of docosahexaenoic acid (Nu-Chek-Prep, Inc., Elysian, MN) was oxidized in presence of [$^{13}\text{C}_6$]-lysine (2 mg, Cambridge Isotope Laboratories, Inc., Andover, MA)

and [^3H]-lysine (50×10^6 cpm, NEN, Boston, MA). Adducts were extracted with Oasis cartridge and HPLC as describe above. One min fractions were collected and aliquots containing radioactivity were analyzed and quantified by LC/ESI/MS/MS using selective reaction monitoring of the fragmentation of the $[\text{MH}]^+$ ion to a specific daughter ion for the lactam adduct [33].

Anti- γ -ketoaldehyde immunostaining

Immunostaining was performed with a single-chain antibody, D11 ScFv, that we had originally isolated by a screening strategy utilizing peptides modified with 15- E_2 -isoketal [34]. We considered the possibility that D11 ScFv might also recognize the lysyl lactam adduct of other isoketal regioisomers and neuroketals, because all γ -ketoaldehydes lysyl lactam adducts share a rigid lactam moiety and several of the γ -ketoaldehydes lactam (e.g., 12- E_2 -isoketal lysyl lactam and 17- E_2 -neuroketal) share similar positioning of the hydroxyl group on the lower alkyl chains, even though their alkyl chains differ in length. Therefore, to determine if D11 ScFv could also recognize other γ -ketoaldehyde lysyl lactam adducts in addition to the 15- E_2 -isoketal lysyl lactam adduct, we generated 12- E_2 -isoketal-lysyl-lactam and a neuroketal-lysyl-lactam. Our synthesis of 12- E_2 -isoketal (alternatively designated iso[4]levuglandin) paralleled the synthesis described by Subbanagounder et al. [35], except that we utilized a slightly different strategy to protect the aldehyde (Supplemental Fig. 2), as we had previously successfully used this strategy to generate 15- E_2 -isoketal [36]. In our synthesis, ethyl 5-acetyl-7,7-dimethoxy-5-hetenolate (obtained from ethyl 5-(diethylphosphono)-6-oxoheptanoate and 2,2-dimethoxyacetaldehyde) was treated with the cyano cuprate of 3-(*tert*-butyldimethylsilyloxy)-1(*E*)-5(*Z*)-undecadiene prepared in situ at -78°C to generate the fully protected 12- E_2 -isoketal. Desilylation, ester hydrolysis, and treatment with montmorillonite K-10 yielded the required γ -ketoaldehyde, which was then reacted with [$^{13}\text{C}_6$]lysine containing tracer amounts of [^3H]lysine to form 12- E_2 -isoketal-lysyl-lactam adduct which was then purified by HPLC. Because we did not have synthetic neuroketal readily available to us, we generated neuroketal-lysyl-lactam by oxidizing docosahexaenoic acid with *tert*-butylhydroperoxide in the presence of [$^{13}\text{C}_6$] $^{15}\text{N}_2$]lysine and tracer amounts of [^3H]lysine, and then purified the resulting lysine adducts by HPLC. One HPLC fraction that contained primarily neuroketal-lysyl-lactam adduct by mass spectrometric analysis (Supplemental Fig. 3B) was used for determination of D11 ScFv affinity. The precise regioisomer(s) of this purified neuroketal lysyl-lactam was not determined. To test whether 12- E_2 isoketal-lysyl-lactam adduct or neuroketal-lysyl-lactam could compete with 15- E_2 -isoketal-lysyl-lactam for recognition by D11 ScFv, the ability of the three antigens to compete for D11 ScFv binding to immobilized 15- E_2 -isoketal adducted peptide was tested using the competitive ELISA method we have previously described [34]. Although 15- E_2 -isoketal was slightly more competitive, all three lactams effectively competed for D11 ScFv binding (Supplemental Fig. 4), suggesting that D11 ScFv can recognize the lactam adduct of a variety of γ -ketoaldehydes adducts.

Hippocampal and cerebellum sections from six AD and six subjects who had died from non-neurological disease were then analyzed by D11 ScFv immunostaining. Sections were obtained after rapid autopsy and a postmortem interval less than 5 h. Age of death for all subjects ranged from 70–90 years old. Immunohistochemistry was performed using D11 ScFv anti- γ -ketoaldehyde antibody as previously described [34]. Peroxidase activity of the secondary antibody was detected by applying 3,3-diaminobenzidine tetrahydrochloride resulting in a brown stain. Sections were counterstained with hematoxylin, resulting in a blue stain, and examined under a light microscope. Representative photomicrographs were recorded at 400x magnification.

Motor and coordination skills tests

At age 12–14 months, the motor and coordination skills of mice were tested in a battery of tests. In the rotarod test, mice were placed on a rotating rod that increased in speed from 4–40 rpm over 3 min, and latency to fall measured. Three daily trials were performed for three consecutive training days, and then the average latency to fall in three trials recorded on the fourth day. In the pole climb test, mice were placed on the center of a horizontal rod marked in thirds and supported by platforms on either side. Scoring was indexed by the section of the rod the animal was able to move to without falling and the speed of movement. A score of 0 represents successful escape onto either one of the support platform within 30 s and a score of 4 represents an immediate fall from the pole. Three trials were performed in a single day. In the inverted screen test, the mice were allowed to fully grip a wire screen placed on an invertible frame. When the animal had gripped the screen with all four paws, the screen was inverted and the time to fall recorded. Three trials were performed in a single day. To measure swimming speed, the exact position of the animal in the pool was continuously monitored by video recording for the first 30 s of the Morris water maze probe trial (described below) and the relative change in position per frame used to calculate the swim speed (cm/sec) of each animal.

Morris water maze

Mouse spatial learning and reference memory were assessed using the Morris hidden platform water maze. Mice were trained to use external visual clues to find a hidden platform within the pool that remained in the same position for all nine of the training days. Each training day consisted of 4 trials, with the mice released from each of four different starting positions randomized in order each day and the time to reach the platform (escape latency) measured. All animals of the cohort completed the trial for the same start position before beginning the trials from the next start position. If an animal failed to find the platform in 60 s, latency was scored as 60 s, and the animal was gently guided to the platform. All animals were allowed to remain on the platform for 10 s before being removed to a warming cage. After the 4th trial of the 9th training day, the ability of the mice to remember the platform location was assessed in a probe trial by removing the platform and allowing the mouse to freely swim for 60 s. The percent time spent in the quadrant where the platform previously was positioned was then measured.

Water radial arm maze

Mouse spatial working memory was determined in a water radial arm maze essentially as previously described [37]. In this working memory task, the target platform was moved to a new arm each day, so that memory of the previous day's platform location must be suppressed and the memory of the new platform location acquired in four training trials. The test was run on nine consecutive days. During each of the training trials, the animal was released from a different start (non-platform) arm, with the order of the five potential start arms semi-randomized for each day. If the mouse entered an arm without the platform or failed to enter any arm after 10 s, the animal was gently pulled back to the start arm by the tail and an error recorded. Animals that failed to reach the platform after 60 s were gently guided to platform. 30 min after the fourth training trial, the working memory test trial was performed by placing the mouse in the fifth and final start arm for the day and measuring both the time to reach the platform (latency time) and the number of errors. The individual score for the animal was the average of the test trials for the final three day block of testing (days 7–9). Individual scores were normalized to the average for untreated WT group (control mice) in the cohort.

Statistical analyses

Data are presented as mean $\pm$ SEM. GraphPad Prism 4.0 was used for statistical analysis. Analysis of variance (ANOVA) was performed to evaluate the statistical significance of difference between groups with the Bonferroni correction. Statistical significance was assigned to $\alpha = 0.05$.

RESULTS

Neuroketal protein adducts increase in the hippocampus, but not cerebellum of AD brains

Protein adducted by the γ -ketoaldehydes presumed to be generated by the cyclooxygenase pathway increased with severity of disease in AD [14]. To investigate whether lipid peroxidation pathways also contributed to the formation of γ -ketoaldehyde protein adducts in AD, we analyzed levels of docosahexaenoic acid peroxidation derived γ -ketoaldehyde protein adducts (NeuroK) in regions of the brain that are (hippocampus) or are not (cerebellum) extensively damaged by AD from individuals who died with AD or without AD according to current consensus criteria [31] (Fig. 1). Levels of NeuroK protein adducts were found to be significantly higher in the hippocampus, a disease-affected area of brain in AD (44.04 ± 5.87 ng/g tissue) than in controls (27.06 ± 2.68 ng/g tissue; $p < 0.02$). In the cerebellum by contrast, an area of the brain that is not affected by AD, levels of NeuroK adducts in AD brains (10.87 ± 1.94 ng/g tissue) were no different from controls (14.69 ± 2.74 ng/g tissue). Although isoketal/levuglandin adducts were not simultaneously measured in these brains, subsequent measurement in two additional AD brains showed that NeuroK adducts were about five-fold higher than isoketal/levuglandin adducts (not shown), in keeping with a previous report that oxidized docosahexaenoate products (neuroprostanes) were higher than oxidized arachidonate products (isoprostanes) in various regions of AD brains [2].

γ -Ketoaldehyde protein adducts localize to pyramidal neurons

To examine the localization of γ -ketoaldehyde adducts in AD hippocampus, we performed immunohistochemistry staining using a single chain antibody, D11 ScFv. D11 ScFv was originally isolated by a screening strategy that utilized lysyl lactam adducts formed by the reaction of peptides with 15-E₂-isoketal. As described in the experimental methods, we have subsequently determined that D11 ScFv binds with only slightly lower affinity to the lactam adducts of at least two other γ -ketoaldehyde as well. We therefore utilized D11 immunoreactivity to examine the localization of γ -ketoaldehyde adducts in AD and age-matched control brains. D11 ScFv intensely immunostained the hippocampal pyramidal neurons of AD brains, with staining being primarily focused in neuron soma and neuropil (Fig. 2A). In contrast, there was very little immunostaining in the same regions of age-matched control brains (Fig. 2B) or in the cerebellum of AD brains (Fig. 2C).

Treatment with salicylamine, a γ -ketoaldehyde scavenger, does not alter growth, strength, or survival

To determine the potential contribution of γ -ketoaldehyde protein adducts to the loss of working memory associated with neurodegeneration and dementia, we examined the effect of SA in mice with targeted replacement of their ApoE gene with the human e4 allele. Wild-type and ApoE4 mice were given either normal drinking water or water supplemented with 1 g/L SA beginning at 4 months of age and continued through the life of the animal. Supplementation with SA did not result in significant changes either in body weight of aged animals (14 months) (Fig. 3A) or in survival (Fig. 3B) compared with mice receiving normal drinking water. SA levels in the brain of SA supplemented wild-type and E4 mice were 22 μ M and 33 μ M respectively.

To determine if SA impacted the physical performance of ApoE4 mice, we performed a battery of tests to measure coordination, muscle strength, and speed. ApoE4 mice had decreased ability to maintain balance on an accelerating rotarod and therefore had a diminished latency to fall from the apparatus as compared to C57 control mice (Fig. 4A). SA supplementation did not affect performance in this test regardless of genotype (Fig. 4A). ApoE4 mice also scored significantly more poorly in a horizontal pole escape task, and again, SA supplementation did not affect performance (Fig. 4B). The time to fall in an inverted screen task was not significantly different across either genotype or SA supplementation groups (Fig. 4C). Swim speed was similarly unaltered (Fig. 4D). Thus long-term treatment with salicylamine does not markedly alter the physical performance of ApoE4 mice.

SA protects against changes in spatial working memory

The effect of genotype (WT versus ApoE4) and SA supplementation (water versus SA) on spatial reference memory was assessed using hidden platform training in the Morris water maze. All four groups of mice were able to learn the hidden platform maze as indicated by shorter escape latency time on successive training days (Fig. 5A) and by preference for the target quadrant during the probe trial (Fig. 5B). Although escape latency was slightly longer for untreated ApoE4 mice and both treated and untreated ApoE4 mice spend slightly less time in the target quadrant of the pool, these differences were not statistically significant.

We then examined the effect of genotype and SA supplementation in a water radial arm maze task which stringently tests spatial working memory [38, 39]. Untreated ApoE4 mice made significantly more errors than controls (200% of control, $p < 0.01$) (Fig. 6A) and had more prolonged latency times (147% of control, $p < 0.01$) (Fig. 6B). In contrast, the number of errors and latency time of SA-treated ApoE4 mice did not significantly differ from untreated wild-type controls (errors, 113% of control, $p = \text{n.s.}$; latency, 104% of control, $p = \text{n.s.}$), and were significantly better than the untreated ApoE4 mice ($p < 0.01$ for both errors and latency versus untreated ApoE4). These data suggest that γ -ketoaldehyde scavenging ameliorates the deleterious effects of the ApoE4 mutation on spatial working memory.

DISCUSSION

Our findings that levels of γ -ketoaldehyde adduct increased in the hippocampal neurons of AD brains and that SA protected aged ApoE4 mice from loss of spatial working memory provide evidence of a previously unknown mechanism, formation of γ -ketoaldehyde protein adducts in pyramidal neurons, whereby oxidative/inflammatory stress may result in working memory deficits.

Pyramidal neurons contribute to formation of spatial working memory. ApoE4 mice showed a significant deficit in the water radial arm maze task, but not in the Morris water maze task. The Morris water maze task primarily tests reference memory, while the water radial arm maze task also tests working memory because the animals must remember the new platform location for each day for 30 minutes after the training trials before beginning the test trial. This spatial working memory requires participation of both prefrontal cortex and hippocampal neurons. Accumulation of γ -ketoaldehyde protein adducts in pyramidal neurons, as we found in AD brain, might therefore disrupt the appropriate formation of hippocampal-dependent memory formation. SA protected against spatial working memory deficits in ApoE4 mice but did not increase survival or protect against deficits in coordination, consistent with its beneficial effects being directed against γ -ketoaldehydes formed in the pyramidal neurons. SA did not improve escape latency in control C57 mice, suggesting that SA acted on processes related to the disease. Beneficial effects of SA may

have been detectable only for spatial working memory and not for coordination simply because the impairment of working memory is relatively mild in ApoE4 mice and therefore more amenable to modification.

While our studies do not define any specific mechanisms whereby γ -ketoaldehydes exert deleterious effects on spatial working memory or whereby salicylamine modulates these effects, studies in cultured cells suggest potential mechanisms by which γ -ketoaldehydes might mediate neuronal dysfunction including induction of neuronal death, loss of synaptic connections, or inhibition of ion channels. For instance, adduction of γ -ketoaldehyde to amyloid- β (A β) greatly increases both the ability of A β to inhibit proteasome activity [40], and the neurotoxicity of A β [41], consistent with the finding that inhibition of the proteasome with pharmacological agents is cytotoxic. This mechanism of neurotoxicity may be particularly relevant because proteasome activity is reduced in AD [42]. Interestingly, proteasome complexes isolated from AD brain have increased immunoreactivity with an antibody that putatively recognizes neuroketal adducts [43]. Unfortunately, the specificity of the commercial antibody used in that study was poorly characterized, so that more substantial evidence for γ -ketoaldehyde modification of proteasomes in AD is needed. An alternative mechanism for γ -ketoaldehydes to induce neuronal dysfunction to inhibition of proteasomes might be inhibition of ion channels. Although γ -ketoaldehyde inhibition of ion channels has primarily been demonstrated in myocytes [17, 44, 45], in principle, ion channels in other cell types are also vulnerable. Because SA blocks γ -ketoaldehyde and oxidant induced ion channel dysfunction in cultured myocytes [17], it seems reasonable to speculate that SA might similarly protect ion channels in neurons, which would provide a plausible mechanism for the effect of SA of spatial working memory.

Our findings implicating a role for γ -ketoaldehydes in dementia also suggest a potential mechanism to rationalize the effects of other experimental interventions that have shown partial efficacy in preventing dementia. For instance, the reduced risk of AD associated with long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) [46], might be mediated by a reduction of γ -ketoaldehyde adducts derived from cyclooxygenase. Increased expression of cyclooxygenases early in AD is well documented [47] and transgenic overexpression of cyclooxygenase-2 has been shown to dramatically increase the amount of γ -ketoaldehyde protein adduct in the brains of mice [48]. Thus, long-term NSAID use would be expected to partially reduce γ -ketoaldehyde levels and therefore spare pyramidal neurons. NSAIDs are unlikely to completely block γ -ketoaldehyde formation, however, because the increased NeuroK adducts we found in AD brain demonstrate that lipid peroxidation is also a significant source of γ -ketoaldehydes in AD. Interestingly, overexpression of mitochondrial superoxide dismutase (SOD2) significantly reduces superoxide levels and prevents learning and memory deficits in Tg2576 mice [49]. We would anticipate that reducing superoxide levels would also significantly reduce levels of γ -ketoaldehydes and thereby prevent memory deficits.

In summary, our results suggest that γ -ketoaldehyde scavengers are a novel therapeutic approach for preventing neuronal damage and memory deficits in AD. They also provide the rationale for the many additional studies needed to determine the mechanism(s) whereby γ -ketoaldehydes contribute to changes in working memory and that SA prevents these changes. Use of γ -ketoaldehyde scavengers would be anticipated to have fewer long-term complications than NSAIDs, which increase risk of gastrointestinal bleeds and vascular disease. Whether SA itself would be suitable for human use requires additional studies, but the oral bioavailability and safety of SA clearly make it a powerful tool for investigating the role of γ -ketoaldehydes in mouse models of neurodegenerative disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Levels of neuroketal protein adduct increase in hippocampus of AD brain. Hippocampus and cerebellum obtained by rapid post-mortem autopsy of AD patients and age-matched control were analyzed for neuroketal protein adducts after complete proteolytic digestion by LC/MS/MS.

Fig. 2.

γ -ketoaldehyde adducts localize to pyramidal neurons in hippocampus of AD brain. Rapid postmortem autopsy brain section of aged adults (70–90 years old at death) who died of Alzheimer's disease (AD brain) or non-neurological disease (age-matched control) were immunostained with anti- γ -ketoaldehyde lysyl adduct single chain antibody (D11 ScFv) and visualized with a peroxidase conjugated secondary antibody using DAB as substrate to produce a brown colored stain. Nuclei were counterstained with hematoxylin to produce blue stain. All photomicrographs are at 400x original magnification. A) Hippocampal section from AD brain. (n) designates a typical pyramidal neuron B) Hippocampal section from age-matched control brain. (n) designates a typical pyramidal neuron C) Cerebellum of AD brain. (pc) designates a typical Purkinje cell and (g) designates a typical granular cell.

Fig. 3.

Long-term supplementation with SA does not alter growth or survival. Wild-type C57BL6 mice (C57) or mice with targeted replacement with human ApoE4 (hApoE4) were given untreated drinking water (-SA) or drinking water containing 1 g/L SA (+SA) beginning at 4 months old and continuing for the life of the animal. A) Body weight of animals at 14 months of age. Groups did not significantly differ. B) Percent of mice in each group that survived to 14 months of age. Groups did not significantly differ.

Fig. 4.

Long-term supplementation with SA does not improve motor performance of hApoE4 mice. At 12–14 months of age, C57 and hApoE4 mice treated with or without SA were subjected to a battery of test to measure their motor and coordination skills. A) Rotorod test. Mice were placed on a rod rotating at gradually increasing speed and the length of time to fall recorded. The hApoE4 mice fell off the rod significantly earlier than C57 mice and treatment with SA did not significantly alter the time to fall in either group. B) Pole climb. Mice were placed on a horizontal pole supported by two platforms and the ability of mice to successfully escape from the pole unto either platform was scored from 0 (escape within 30 sec) to 4 (immediate fall from pole.) C57 mice scored significantly better than hApoE4 mice and treatment with SA did not significantly improve the score of either group. C) Inverted screen. Mice were placed on a wire screen which was then inverted and the time to fall recorded. There were no significant differences in the time to fall between the four groups. D) Swim speed. The swim speed of mice was determined in an open pool of water using video recording. Swim speed did not significantly differ between any of the four groups.

Fig. 5. Effects of long-term supplementation with SA in Morris Water Maze performance. At 12–14 months of age, C57 and hApoE4 mice treated with or without SA were tested in the Morris hidden platform water maze. Animals were released into a pool of water from four different start positions during each of the four daily training trials and allowed to find a submerged platform. After the last training trial on the 9th training day, the platform was removed and a 60 sec probe trial conducted. The amount of time each individual animal spent in the pool quadrant where the platform had been located was determined by video recording. A) Average latency to find platform on each training day. All groups of animals showed significantly decreased latency from the initial to final training day. B) %time spent in target quadrant during the probe trial. The four groups did not significantly differ from each other.

Fig. 6.

Effects of long-term supplementation with SA on performance in water radial arm maze. C57 and hApoE4 mice treated with or without SA were tested in the water radial arm maze where a hidden platform was placed in the target arm of a 6-radial arm maze. The target arm was changed each of the nine testing days. The final (5th) daily trial for each of the last three testing days (days 7–9) was used for comparison to the control group (untreated C57 mice). A) The average number of errors (non-target arms entered). Untreated (–SA) hApoE4 mice made significantly more errors than untreated C57 mice. Treated (+SA) hApoE4 mice made significantly fewer errors than untreated hApoE4 mice and did not significantly differ from untreated C57 mice. Untreated and treated C57 mice did not significantly differ from one another. B) The average latency to find the platform. Similar significant differences were seen with latency as were found with errors.