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Neurobiology of Aging 22 (2001) 993–1005

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NEUROBIOLOGY
OF
AGING

Phenolic anti-inflammatory antioxidant reversal of A β -induced cognitive deficits and neuropathology

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Received 1 May 2001; received in revised form 17 July 2001; accepted 23 July 2001

Abstract

Both oxidative damage and inflammation have been implicated in age-related neurodegenerative diseases including Alzheimer's Disease (AD). The yellow curry spice, curcumin, has both antioxidant and anti-inflammatory activities which confer significant protection against neurotoxic and genotoxic agents. We used 22 month Sprague-Dawley (SD) rats to compare the effects of the conventional NSAID, ibuprofen, and curcumin for their ability to protect against amyloid β -protein (A β)-induced damage. Lipoprotein carrier-mediated, intracerebroventricular infusion of A β peptides induced oxidative damage, synaptophysin loss, a microglial response and widespread A β deposits. Dietary curcumin (2000 ppm), but not ibuprofen, suppressed oxidative damage (isoprostane levels) and synaptophysin loss. Both ibuprofen and curcumin reduced microgliosis in cortical layers, but curcumin increased microglial labeling within and adjacent to A β -ir deposits. In a second group of middle-aged female SD rats, 500 ppm dietary curcumin prevented A β -infusion induced spatial memory deficits in the Morris Water Maze and post-synaptic density (PSD)-95 loss and reduced A β deposits. Because of its low side-effect profile and long history of safe use, curcumin may find clinical application for AD prevention. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: NSAIDs; Alzheimer; Neuroinflammation; Morris water maze; Acquisition; Isoprostane; Oxidative damage; Antioxidant; PSD-95; Synaptophysin; Synapse

1. Introduction

Alzheimer's Disease (AD) involves the pathological buildup of extracellular vascular and parenchymal deposits of a 40–42 amino acid peptide called amyloid β -protein (A β). Genetic studies of familial AD causally implicate A β because multiple mutations in genes on 3 different chromosomes that cause early onset autosomal dominant AD have one common effect in increasing the production of A β , notably A β (1–42) [65]. Other studies of the major identified genetic risk factor for AD, apolipoprotein E4 allele (ApoE4), have shown that ApoE and its alleles regulate A β deposition [33]. How A β drives the pathogenesis of AD remains an area of active investigation. Proteins, lipids and nucleic acids that have been damaged by free radicals accumulate in the brain and other organs with age, and are implicated in many age-related diseases, including neurodegenerative diseases such as Parkinson's and Alzheimer's

disease [6]. Oxidative damage has been hypothesized to play a central role in AD pathogenesis [6], may involve two known pathways. In vitro, direct A β application to neuronal cells increases hydrogen peroxide production [8], leading to A β neurotoxicity that can be prevented by vitamin E and other antioxidants [7,24]. Indirect A β neurotoxicity from microglia stimulates NADPH (nicotinamide adenine dinucleotide phosphate) oxidase and superoxide [14,45] and iNOS (inducible nitric oxide synthase) and NO (nitric oxide) [48]. Oxidative damage is increased in the brains of AD patients [21,51,66,68] and in β -amyloid plaque-forming transgenic mouse models for AD which lack tangles [56, 57,67] suggesting that elevated A β is sufficient to stimulate oxidative damage.

Because of strong evidence for oxidative damage in AD brain and because anti-oxidants can protect from in vitro β -amyloid toxicity [7,8,46], a clinical trial testing the ability of high dose α -tocopherol to slow AD progression was carried out [63]. The modest success of this trial in slowing the decline in activities of daily living has stimulated interest in antioxidant approaches. However, vitamin E supplements did not slow cognitive decline, and it remains unclear

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to what extent it affected the oxidative damage, neuronal dysfunction, synapse loss and other pathogenic events. The form of vitamin E used in these trials and found in most supplements, α -tocopherol, is a potent inhibitor of lipid peroxidation, but a poor scavenger of the nitric oxide based free radicals produced during inflammation [11]. Although there are reports that iNOS is not upregulated in activated human microglia, NO from A β -treated human glia may be largely from astrocytes [1,15] or even NOS in neurons. Biochemical data show 5–7 fold elevated nitrotyrosine in AD brain compared to age-matched controls [31]. Whether the source of this damage is nNOS (neuronal nitric oxide synthase) or iNOS, more effective scavengers of NO/peroxynitrite need to be evaluated in inflammatory neurodegenerative conditions.

One natural alternative antioxidant to vitamin E is the polyphenolic antioxidant, curcumin, found in turmeric, the yellow curry spice with a long history of use in the traditional Indian diet and herbal medicine [39]. Age-adjusted Alzheimer's prevalence in India is roughly one quarter the rates in the United States, for example 4% vs. 15.7% in those aged 80 years or older [22]. Curcumin was reported several times more potent than vitamin E as a free radical scavenger [82] and effective against nitric oxide based radicals [69]. Oral administration of curcumin has been shown to be centrally neuroprotective [38,61].

AD pathogenesis involves a CNS inflammatory response, and AD risk is reduced in those consuming non-steroidal anti-inflammatory drugs (NSAIDs) [2,9,70]. Although CNS inflammation may contribute to oxidation, aspects of inflammation unrelated to oxidation, such as complement activation, are likely to contribute to AD pathogenesis. Therefore intervention strategies for AD may require targeting both oxidation and inflammation. Curcumin is both a potent antioxidant and an effective anti-inflammatory agent capable of inhibition of nuclear factor kappa B (NF κ B)-mediated transcription of inflammatory cytokines [79], inducible nitric oxide synthase (iNOS) [10], and cyclooxygenase 2 (Cox-2) [58]. Because of anti-tumor activity, relative safety, and its long history of use, curcumin is currently being developed for clinical use as a cancer chemopreventive agent [39]. We hypothesized that acting as a combined antioxidant and NSAID, dietary curcumin might also have significant preventive activity against A β -induced neurotoxicity and cognitive deficits.

Because current transgenic model alternatives have limited neurodegeneration, we have employed a rat A β infusion paradigm using aging retired breeder female rats and both A β (1–40) and A β (1–42) to induce neurodegeneration and A β deposits [39]. While A β (1–42) has frequently been considered the more pathogenic species because of its rapid aggregation and genetic data, other studies suggest that both A β peptides and lipoprotein chaperones may play a critical pathogenic role in AD [35,54,77]. Therefore, we infused both the more soluble A β (1–40) and the more rapidly

aggregating A β (1–42) solubilized with a lipoprotein chaperone into the brains of aging female rats.

2. Materials and methods

2.1. Study design and diets

Study A: The goals of Study A were to: 1) obtain more effective penetration of A β and a more global neurotoxic response than in previous injection studies where vehicle and/or surgery alone appeared toxic [76,78] and 2) determine whether curcumin or ibuprofen could block in vivo toxicity. Aged female animals have been used as recipients in our previous studies in order to optimize chances for success because aged females have 2 known risk factors for AD. Based on the premise that greater toxicity might be obtained, we used the oldest animals we could access: 19 month old female rats were placed on diets, infused at 21 months and sacrificed at 22 months. *Study B:* In the follow up behavioral study, we wanted to use a different paradigm that could be better replicated, since aged animals are not readily available. For this purpose we first performed a dose-response and time course to determine sensitivity of the more readily available 9 month old female retired breeders to A β -induced memory deficits and neurodegeneration. We determined that higher doses of A β (1–42) and A β /HDL ratios were required to detect synaptic and behavior defects (Frautschy et al., in preparation). The time course study showed that A β -dependent behavioral impairments were not detectable until 7 weeks post-infusion. (Frautschy et al., in preparation), so we also extended the time period between infusion and sacrifice. In Study A 19 month female Sprague-Dawley rats were fed one of the following diets (Purina test Diets, Richmond, IN): control chow (cat #5753C-L), curcumin chow (2000 ppm, cat #5753C-G) and ibuprofen chow (375 ppm, cat #5753C). Relatively high initial doses for both drugs were chosen based on published reports of in vivo efficacy without overt toxicity [76]. The curcumin (#C1386) and ibuprofen (#I4883) were both purchased from Sigma Chemicals (St. Louis, MO). In Study B of behavior, groups of ten, 9 month old female Sprague-Dawley rats were placed on diets (500 ppm curcumin, TD99373) or control chow (TD99370-NIH 31 open formula purchased from Harlan Teklad, Madison, WI) for 2 months prior to infusion of A β . The lower curcumin dose was chosen for the second study based on evidence of efficacy at lower doses on amyloid, oxidative and synaptic parameters in a parallel study using APP^{sw} transgenics [43b].

2.2. Pump contents—study A—aged rats

In pilot studies without lipoprotein carrier, A β 42 tended to aggregate within the pump and to penetrate poorly. Therefore, human plasma high density lipoprotein, HDL (Calbiochem, La Jolla, CA), which normally carries A β in

plasma, was used in the pump to reduce aggregation of A β 42 and act as an A β chaperone, insuring better tissue delivery. The method for use of HDL to deliver A β 42 in vivo has been published previously [18,29]. Vehicle contained the HDL carrier and 4 mM HEPES, pH 8.0 buffer. Immediately after pump implantation, rats were injected with buprenorphine (Buprenex, 0.03 mg/kg, sc) to minimize post-surgical pain and ampicillin (100 mg/kg, sc) to reduce chances of infection; each drug was administered twice daily for the first two days postsurgically twice daily. In the aged rats, per day delivery was 5 μ g HDL (150 μ g total), 0.7 μ g of A β 40 (20 μ g total), and 0.2 μ g A β 42 (5 μ g total) in 4 mM HEPES, pH 8.0.

2.3. Pump contents—study B—middle aged rats

Based on encouraging results with the pathology in the aged rats, a pilot study was then set up to determine the doses of A β and duration of study to detect A β -induced pathology and possible behavioral deficits using younger, more readily available 9 month old retired breeders. Higher levels of A β 42 were needed to reliably detect persistent behavioral deficits than the doses used to produce pathology in aged rats. Therefore, in this second study on behavior, per day delivery was 2.7 μ g HDL (80 μ g total), 0.7 μ g of A β 40 (20 μ g total), and 1 μ g A β 42 (30 μ g total) in 4 mM HEPES, pH 8.0. The HDL was reduced and the ratio of A β to HDL was increased to diminish the possible protective effects of high doses of HDL observed in pilot studies (not shown). Protective effects were attributed to solubilization and prevention of aggregation-dependent toxicity with high HDL/A β ratios. Due to the delayed effects of A β on behavioral deficits, infusion of A β during the first, but not during the second or third month, impacted observed deficits (not shown). Rats were sacrificed 3 months post pump implantation (14 months of age), after completion of behavioral studies.

2.4. Surgery

All studies employed groups of 10 female Sprague-Dawley retired breeders placed on diets for 2 months prior to infusions and maintained on diets until sacrifice. Aged female animals have been used as recipients in our previous studies in order to optimize chances for success because aged females may be more at risk for AD. Prior to pump implantation, rats were anesthetized with a cocktail of 1.14 mg/kg acepromazine, 3 mg/kg xylazine and 54 mg/kg ketamine, ip. After placing rats on a Deltaphase isothermal pad (Braintree, MA), and wrapping body in a Saran Wrap™ thermoprotector, the surgical site was scrubbed with 4% chlorohexidine and rinsed with 0.5% chlorohexidine tincture in 70% ethanol. Eyes were protected from drying with Duratears (Alcon Upsonic, Fort Worth, TX) and from light with a gauze drape. To maintain aseptic conditions, the surgeon used a sterile gown, mask and gloves. Once anes-

thesia was established, the rats were placed in a David Kopf stereotaxic instrument (Tujunga, CA), and holes drilled for initial hippocampal injection of 10 ng TGF β 1 (R&D Systems, Minneapolis, MN, coordinates -1.3 posterior to Bregma, ± 1.9 mm mediolateral and -4.0 ventral Dura). TGF β 1 was used to reduce clearance of injected A β , and has been shown to enhance A β deposition in vivo [19] and in organotypic slice cultures [27]. The same holes used for TGF β injection were then used for implantation of a custom made stainless steel double connector cannulas (Plastics One, Roanoke, VA, Cat 3280PD/SPC separated by 3 mm center to center) at a depth 4.0 mm from skull that terminated dorsal to hippocampal injection in the ventricles. After securing cannula with acrylic dental cement attached to a screw that did not completely penetrate the skull, the cannula was connected to a single Alzet pump (#5004, Durect Corporation, Cupertino, CA) via polyethylene tubing with a cannula bifurcation connector (#21/22Y, Plastics One, Roanoke, VA). The pump was placed in a subcutaneous pocket under the dorsal neck.

2.5. Morris water maze

Middle aged rats in the second group (study B) were subjected to the Morris water maze using a 2 meter diameter tank as described [78]. Water temperature was maintained at 21°C. Distal stationary cues were placed around the walls of room, and no proximal or mobile cues were present as experimenter was not visible to rats during trials. Prior to first trial of each day, rats were placed on platform for 30 seconds for spatial orientation. Then rats were removed from platform, heads covered with a drape until they were placed in a random start site, facing tank wall. Initially rats were trained for three days in a visible platform test (3 trials per day), followed by 7 days of acquisition in a hidden platform position (2 trials per day, 90 seconds maximum). Path lengths and acquisition were determined by HVS image software and video tracking system HVS Image Ltd. (Buckingham, UK, www.hvsimage.com).

2.6. Sacrifice and tissue collection

Prior to sacrifice, rats were anesthetized and perfused with a non-fixative protease inhibitor buffer as previously described for transgenic mice [43]. Half the brain was immersion-fixed in 4% paraformaldehyde and paraffin-embedded, and the remaining half of the brain was dissected and snap frozen for biochemical analysis as described [43].

2.7. Histology/immunohistochemistry

A β deposits were labeled with 10G4 anti-A β antibody recognizing residues 5–13 [43,81]. This was followed by ABC ELITE-peroxidase kit (Vector) and metal diaminobenzidine (DAB, Pierce, Rockford, IL) for single labeling. Microglia were labeled with anti-phosphotyrosine (PT) an-

tibody (1:2000, Sigma, St. Louis, MO) [40] which specifically labels microglia in fixed brain sections [37] (see Fig. 3C for example of microglial-specific PT staining). For double labeling, after microglia were labeled using DAB, sections were formic acid treated and washed to remove PT antibody and enhance subsequent A β staining using ABC-alkaline phosphatase and Vector Blue chromagen (Vector, Burlingame, CA).

2.8. Microscopic image analysis

Analysis of histochemical sections was performed on three consecutive 8 μ m thick sections at a constant distance 2 mm posterior to the A β -infusion cannula (A β -ir, PT). All histological and immunohistochemical images were acquired from an Olympus Vannox-T (AHBT) microscope with an Optronix Engineering LX-450A CCD video camera system onto a Macintosh computer via an averaging frame grabber (AG-5, Scion, MD) and then analyzed with NIH-Image public domain software (developed at the NIH and available at <http://rsb.info.nih.gov/nih-image/>). Custom Pascal macro sub-routines were written for A β and PT labeled slides to calculate average plaque size for A β -ir (μ m²). PT-ir and ring analysis of PT-ir was calculated as percent area stained and described in more detail elsewhere [18,20]. In 3 rats, A β -ir was detected every 5 sections (40 μ m) to determine the number of deposits as a function of the distance posterior to the cannula. Deposit number was relatively constant out to 2 mm posterior to the cannula and then declined precipitously by \sim 25% in sections by 3 mm from the cannula. All image analysis was performed at 2 mm posterior to cannula within the area of maximum A β deposition. A distal region to cannula within area of maximum deposition was chosen to analyze immunohistochemistry to minimize effects of cannula induced glial, neuronal, and synaptic alterations. Although the gradient of soluble A β in relation to distance from cannula could not be measured, all analysis was conducted at equidistance from cannula so one could control for gradient effects in soluble A β concentrations and A β deposits in relation to cannula proximity. Presumably, the lipoproteins enabled deeper penetration of infused A β .

2.9. Biochemistry

Buffer perfusion allows us to remove most serum contaminants (ApoE, complement, IgG etc.). Snap frozen cerebral cortex was powderized and split in half. Half of the powder was extracted for isoprostane analysis and half was homogenized with complete protease inhibitor cocktail [43]. Aliquots were assayed for protein content and used for biochemical analysis. Western analysis with 4% stack and 6–20% Tris Tricine gradient gels blotted to Immobilon PSQ was used to detect PSD-95 (Upstate, Lake Placid, NY) and to validate the synaptophysin ELISA. Internal standards, β -actin and glyceraldehyde 3 phosphate dehydrogenase

were used to assess loading and processing losses on blots. ECL detection was on X ray film without saturation followed by scanning on a Biorad scanning densitometer [43].

2.10. ELISAs for synaptophysin and isoprostanes

Synaptophysin was assayed as previously described [28]. Briefly, we coat plates with 2 μ g of homogenate in triplicate and detect with SY38 (Boehringer) and anti-mouse alkaline phosphatase and Attophos detection.

Isoprostanes are prostaglandin-like lipid peroxidation products; their concentrations can be used to assess oxidative stress. We utilized the 8EPI-F2 Immunoassay Kit (EA84; Oxford Biomedical Research, Oxford, MI) to measure an arachidonate product, 8-epi-prostaglandin F₂ α (8-EPI) also known as 8-iso-PGF₂ α . According to the manufacturer's data, values with gas chromatography combined with mass spectrometry (GS/MS) correlate closely with values obtained by this ELISA ($r^2 = 0.976$). Instructions were provided with the kit. Briefly, the kit is a competitive enzyme-linked immunoassay (ELISA) for determining 8-EPI levels. Frozen brain samples were ground on dry ice using a mortar and pestle. Total lipids were extracted using a modification of the method of Hara and Radi [25].

2.11. Statistical analysis

Percentage microglial area stained in cortical layers 2 versus layer 3–4 were assessed using a region x treatment ANOVA. Plaque parameters included multifactorial ANOVA with repeated measure, using day values of behavioral parameters as repeated measure (average of two trials for swim path and swim latency from start to platform (90 seconds maximum) and the Fishers LSD post-hoc test for determining differences between pre-planned comparisons (StatView 4.5, Abacus, Berkeley). The repeated measure analysis is useful because it controls for similarities within animal between different days. ANOVA analysis of PSD-95 values in curcumin-fed rats versus control rats and analysis of isoprostane values between A β infused and vehicle infused rats, required logarithmic transformation for establishing homogeneity of variance as required by ANOVA.

3. Results

3.1. Oxidative damage

In Study A with aged rats, we tested the impact of A β -infusion on global cortical oxidative damage evaluated by immunoassay for a stable lipid peroxidation product, 8-EPI-F2 isoprostane. Intraventricular A β -infusion into 22 month old rats resulted in a 2.3 fold increase in cortical F2 isoprostane levels (Fig. 1A). We compared A β -infused animals on control chow with A β -infused animals fed diets

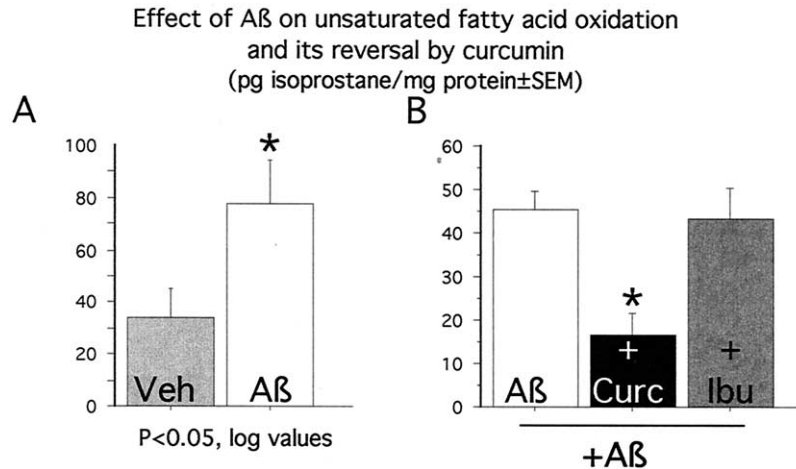


Fig. 1. Dietary curcumin decreases elevated isoprostanes induced by CNS A β infusion. Panel A. Aged rats (21 months) were infused with A β and HDL or vehicle (HDL) alone for one month and extracts of cortex analyzed for F2-isoprostanes at 22 months of age. Isoprostanes \pm SEM in the A β + HDL (vehicle) group on control diet were significantly increased relative to the group receiving vehicle alone on control diet ($p < 0.05$). ANOVA analysis required logarithmic transformation to establish homogeneity of variance, but actual means and SEM and shown. Panel B. Groups of rats were placed on control or experimental diets 2 months prior to one month A β + HDL infusions and sacrificed at 22 months. Isoprostanes in A β -infused animals on 2,000 ppm curcumin diet but not ibuprofen (375 ppm) were significantly reduced ($p < 0.05$) relative to A β -infused rats on control diet.

containing 375 ppm ibuprofen or 2,000 ppm curcumin. Unlike ibuprofen, dietary curcumin completely suppressed the elevated levels of isoprostanes in A β -treated controls ($p < 0.05$, Fig. 1B).

3.2. Synaptophysin loss

Small, but statistically significant overall cortical synaptophysin loss was measured by ELISA in triplicate samples comparing A β -infused animals with HDL alone (vehicle, $p < 0.05$). This loss was significantly reduced in A β -infused rats fed curcumin ($p < 0.05$), but not in rats fed ibuprofen (Fig. 2). Synaptophysin loss at one month post infusion with the same dose of A β does not always occur in younger animals, and may even increase, although by 3 months and 6 months a delayed reduction of synaptophysin occurs even if A β is infused for only one month (not shown).

3.3. Inflammatory cells

Microgliosis was evaluated using morphometric NIH image analysis of phosphotyrosine (PT) staining in regions and in rings around anti-A β labeled plaques [20]. An example of PT staining is shown in Fig. 3B. In the regional analysis, we focussed on cortical layer 2, an AD vulnerable region, but also evaluated cortical layer 3/4 for comparison. We have previously observed that cortical layer 2 microglial staining is heavily affected by our infusion paradigm [29]. Similar to the previous study, we observed that A β infusion induced microgliosis occurred in layer 2 of the cortex ($p < 0.05$), but did not reach significance in layer 3/4. Compared to A β -infused rats fed control diet, A β -infused rats fed ibuprofen or curcumin showed >60% reductions in PT in

layer 2 and slightly smaller but significant reductions in layers 3/4 (Fig. 3A).

Because of the robust curcumin effects on both oxidative damage and microgliosis in layers, additional studies of PT staining in the curcumin group were carried out using ring analysis. A schematic diagram of analysis and results are presented in Fig. 3C, indicating %PT area DAB labeling of

**Effects of ibuprofen and curcumin on synaptophysin
in cerebral cortex of 22 month old rats**
Percent Synaptophysin loss \pm SEM

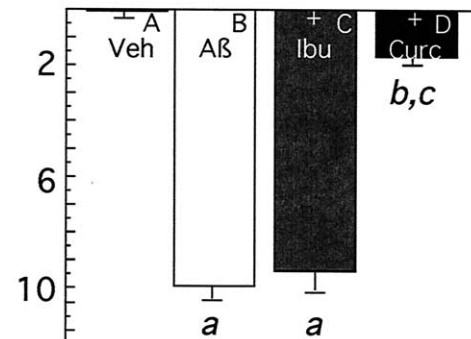


Fig. 2. Effects of ibuprofen and curcumin on synaptophysin in cerebral cortex of 22 month old rats. Treatment groups and diets are those described in Fig. 1. Panel A shows the percent cortical synaptophysin loss in rats treated with: A β /HDL + control diet (Bar B), A β /HDL + ibuprofen diet (Bar C) and A β /HDL + curcumin diet (Bar D) relative to vehicle infused rats on control diet (Bar A). Small case letters associated with the bar indicate which treatment is significantly different ($p < 0.05$). Specifically, the planned treatment comparisons (Fisher's least squares difference) demonstrated that synaptophysin in the A β /HDL-infused groups on control or ibuprofen diets was slightly but significantly ($p < 0.05$) reduced relative to the vehicle-infused group, while synaptophysin in A β /HDL-infused animals on the curcumin diet did not differ from that of vehicle-infused rats.

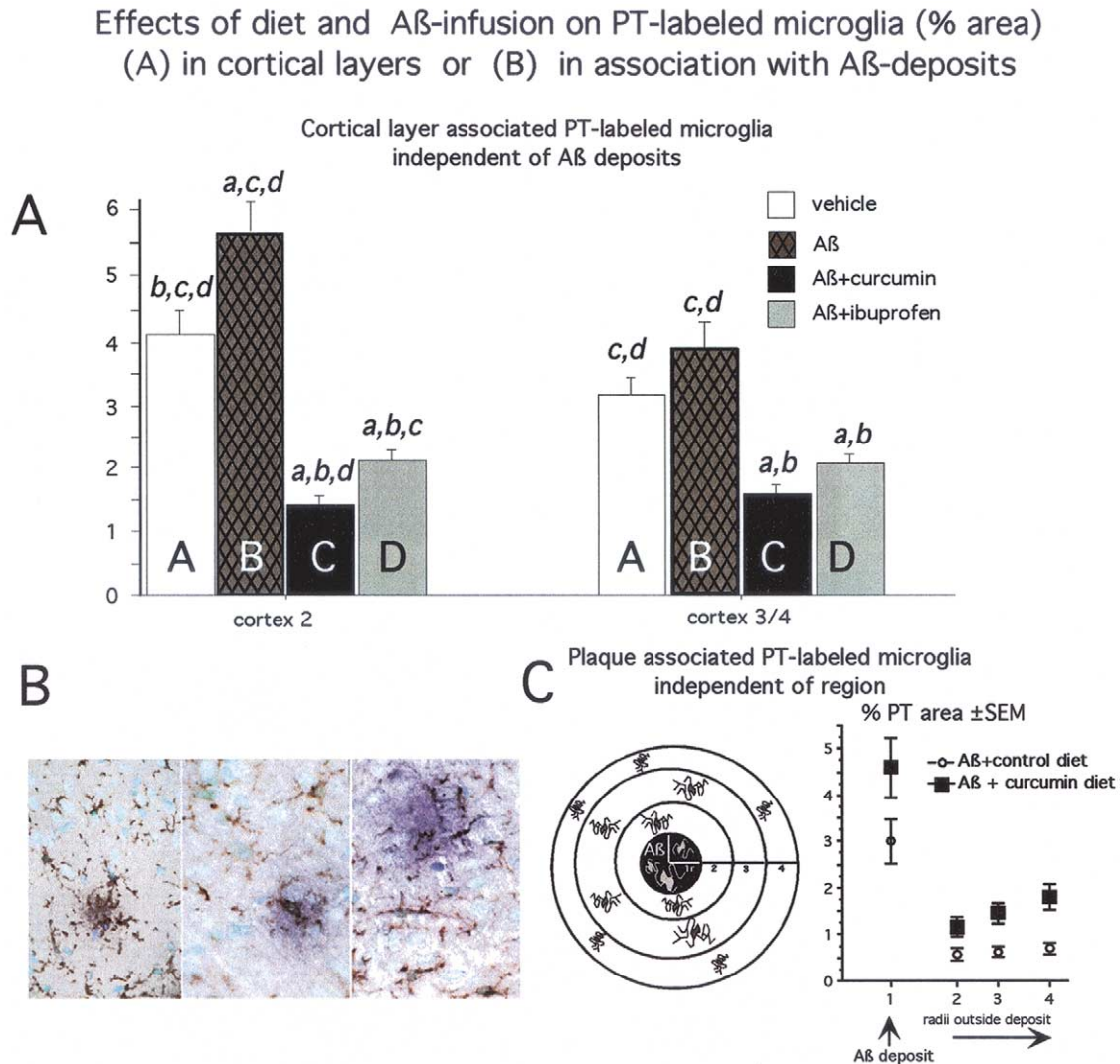


Fig. 3. Image analysis of effects of NSAIDs on phosphotyrosine (PT) labeled microglial immunoreactivity (ir) % area \pm SEM in cortical layers 2 and 3/4 of 22 month old A β infused rats (combined parietal, occipital, temporal, entorhinal). Analysis of these regions was performed at 200 \times optical magnification, to ensure appropriate resolution of cells. Treatment \times region ANOVA was performed on % microglial-ir area stained per region. The treatment effect and region effects were significant ($p < 0.01$), but there was no treatment \times region interaction. Fisher's least square difference analysis between planned comparisons was used to evaluate detailed statistical comparisons. Treatments are labeled with bold capital letters, and small case letters associated with the bar indicate which treatment is significantly different ($p < 0.05$). These data showed that A β significantly increased PT-ir area compared to vehicle in layer 2, but not layer 3–4. Curcumin and ibuprofen reduced microglial area in both cortical layers ($p < 0.05$). Panel B shows type of images that were analyzed to determine microglial reaction to deposits (blue is A β and brown is PT). Panel C. Whether curcumin had an effect on focal microglial reactions to A β -ir deposits independent of region was determined by ring analysis. Ring \times treatment ANOVA analysis of % phosphotyrosine labeled, plaque-associated microglia in rings of 1–4 plaque diameters show a significant treatment and ring effect ($p < 0.001$) without a treatment \times ring interaction. This analysis suggested that there was an effect of A β deposits on increasing microglial-ir within deposit relative to outside deposit. These data also demonstrate that there was a statistically significant more robust effect of curcumin on enhancing microglial-ir associated with A β deposits. This ring analysis demonstrates that despite the net reduction in microglia in curcumin fed rats indicated in Panel A, A β -ir deposits in A β -infused curcumin-fed rats showed more microglial-immunoreactivity within and around deposits than A β -infused control-fed rats.

microglia within 4 plaque radii of lightly vector blue labeled A β deposits. Microglia were concentrated in the deposits similar to microglia in deposits in AD and APP transgenics. Surprisingly, the total plaque-associated microglial area labeled by PT within and surrounding A β deposits was increased in the curcumin treated animals.

3.4. Cognitive deficits

Having found that 2,000 ppm curcumin had significant antioxidant and neuroprotective effects in rats with CNS A β infusion, we sought to test the impact of a lower, more clinically feasible dose of 500 ppm on a clinically

Effects of dietary curcumin (500 ppm) on reversing
A β induced behavioral deficits in Morris Water Maze and PSD-95 loss

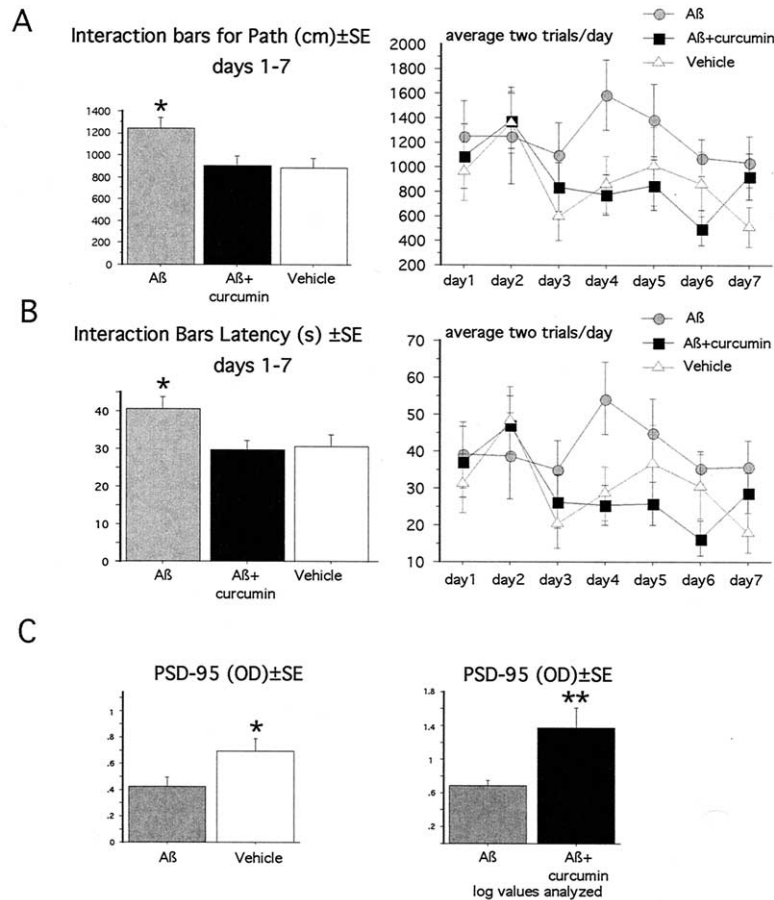


Fig. 4. Acquisition in Morris water maze (A and B) and (C) curcumin restoration of A β induced loss of post-synaptic density protein (PSD-95). Left side of panels A and B indicates repeated ANOVA analysis interaction bars for swim path (cm) and latency (seconds), respectively. On right side of Panels A and B, treatment means of the two trials per day are indicated for each day \pm SEM for swim path (cm) and latency (seconds), respectively. Rats infused with A β traveled longer paths (A) and had longer latencies (B) than rats infused with vehicle ($p < 0.05$). Dietary curcumin (500 ppm) suppresses these A β -induced spatial memory deficits in the Morris water maze (A, B). The cortex from these rat brains were analyzed for synaptic proteins. The two graphs depicted in Panel C are results of the two Western blots (20 lanes), each blot containing tissue from A β -infused group, ran against vehicle (left) or A β + curcumin (right). Despite memory impairment, and the fact that high doses of A β were used, these middle-aged rats showed no synaptophysin loss (not shown). Log values were analyzed in right graph to establish homogeneity of variance required for ANOVA. Regression analysis for path and latency day means, showed significant learning for vehicle-infused rats and curcumin-fed A β -infused rats ($p < 0.001$) but not for control diet-fed A β -infused rats (not shown). Panel C. In contrast to lack of effect on synaptophysin, data showed that the post-synaptic protein PSD-95 was reduced more than 50% in A β infused rats ($p < 0.05$), unless they were fed curcumin ($p < 0.001$).

relevant endpoint, spacial memory. Twenty-two month animals were not available and pilot studies suggested higher doses of A β 42 were required to produce water maze deficits in available 9 month retired breeders. This would be consistent with other pilot observations showing that the same doses of A β 40 + 42/HDL resulted in significant neuron loss in the older animals, but not in the younger group (Frautschy et al., in preparation). Explanations for increased sensitivity in aging animals remain hypothetical but might include reduced clearance, degradation, and antioxidant factors and alterations in neurotrophic factors. Therefore the middle-aged rats used in study B ($n = 10$) were evaluated for spatial memory

deficits in a standard Morris water maze using a A β infusion paradigm that employed higher levels of A β 42. Fig. 4AB shows the result of repeated measures (day means of average of two trials) ANOVA analysis with left graph reflecting interaction bars and right graph showing learning curves. A β -infusion induced a longer path length (4A, $p < 0.05$) and greater latency (4B, $p < 0.05$) to find the platform relative to vehicle infused animals. The effect of A β was evident by day 3 and persisted throughout the remaining 4 days of hidden platform testing consistent with spatial memory deficits. Compared to A β -infused rats fed control diet, A β infused rats fed curcumin (500 ppm) show reduced path length

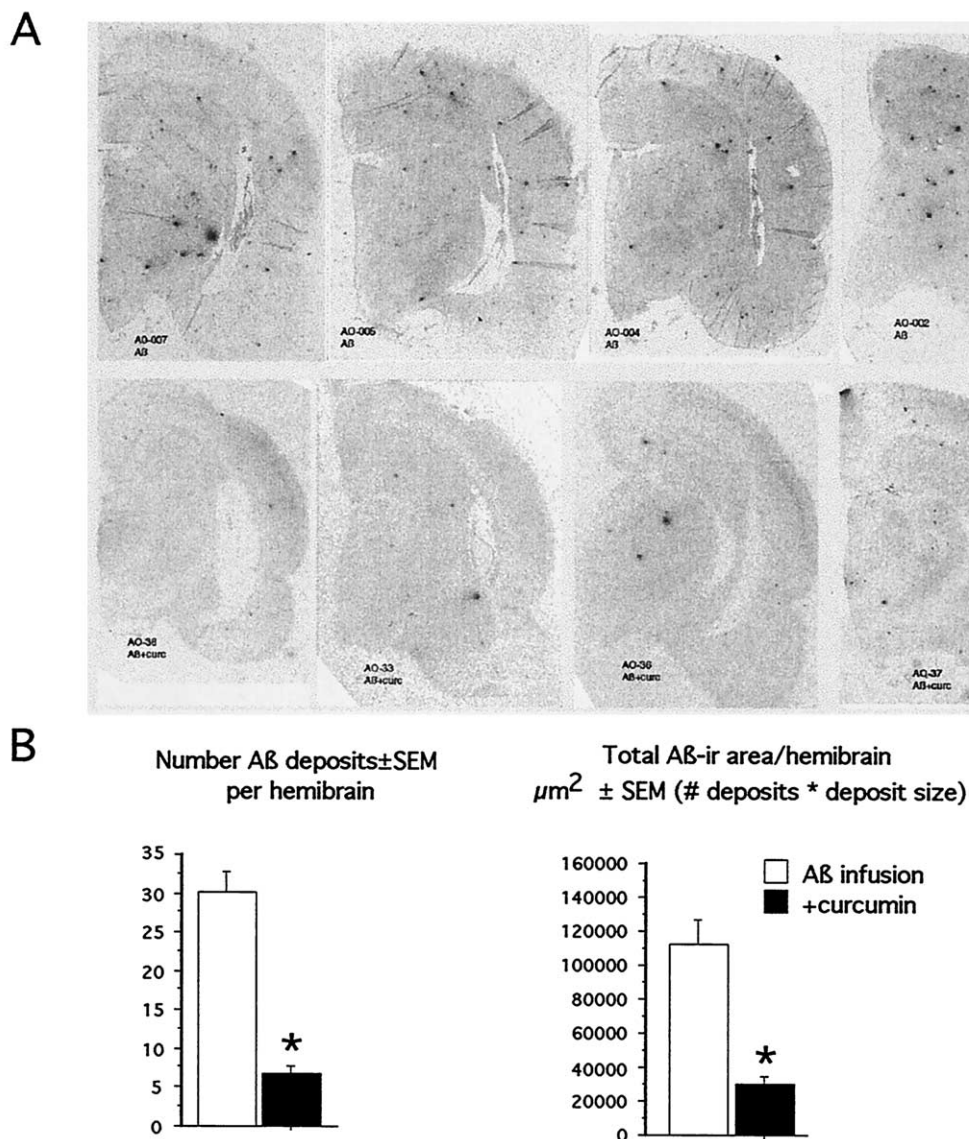


Fig. 5. Dietary curcumin suppresses Aβ-ir deposition. Panel A shows low magnification of immunohistochemical staining for Aβ in four representative rats infused with Aβ (A, top four panels) and in 4 representative rats infused with Aβ and fed a curcumin diet (500 ppm, A, lower four panels). Rat identification number and treatment are shown at the bottom of each subpanel. Although the effect was obvious by eye, image analysis of these deposits revealed that curcumin markedly reduced deposit numbers (left graph, B, $p < 0.01$) and total Aβ-ir area (right graph, B). There were no small deposits in curcumin fed rats (deposits $< 2000 \mu\text{m}^2$). The average size of deposits in curcumin-fed rats showed a trend for being larger than control fed rats infused with Aβ.

and latency in finding the hidden platform, restoring performance to the levels found in the vehicle-infused controls. Regression analysis for path and latency day means, showed significant learning for vehicle-infused rats and curcumin-fed Aβ-infused rats ($p < 0.001$) but not for control diet-fed Aβ-infused rats.

3.5. PSD-95

Because spatial memory in rodents involves postsynaptic factors associated with NMDA receptor function, PSD-95 levels (relative O.D.) were assessed on Western blots comparing Aβ-infused rats on control and curcumin

diets versus vehicle-infused rats on control diets. Because all three groups ($n = 10$) could not be contained on a single 20 lane gel, separate blots were used to compare Aβ-infused versus vehicle on control diets and Aβ infused on control and 500 ppm curcumin diets. As shown in Fig. 4C, Aβ treatment significantly reduced PSD-95 protein levels; this effect was suppressed in animals on the curcumin diet.

3.6. Aβ deposits

Our lipoprotein carrier assisted Aβ infusion protocol resulted in numerous widespread diffuse Aβ deposits (Fig. 5A). Low magnification of representative hemi-

brains stained for A β from A β -infused rats fed control diet (top 4 panels) or fed curcumin diet (bottom 4 panels) are shown. Analysis of number and size of these deposits was performed double-blind. Using NIH image, total A β -ir area and number of A β -ir deposits per hemibrain section (A β burden) were calculated. Curcumin treatment (500 ppm) markedly reduced total A β plaque numbers 80% compared to control diet fed animals (Fig. 5). The higher dose of curcumin reduced plaques 45% (not shown). Curcumin seemed to impact smaller plaques more than larger plaques giving rise to a trend ($p = 0.13$) for a modest increase in plaque size in the few remaining deposits in the curcumin treated group.

4. Discussion

We have previously used an A β infusion approach to induce A β deposits associated with a glial response [19]. Because of the propensity of A β 1–42 to aggregate in the infusion pump and resulting in poor penetration, A β 40 was the primary A β species infused in earlier experiments. Consistent with results from other investigators [32], neurotoxicity was limited in our earlier experiments. However, neurotoxicity could be enhanced by co-infusing protease inhibitors that appeared to elevate neuronal A β [17]. In order to achieve greater toxicity and more effective penetration of the rapidly aggregating and putatively more toxic A β 42 peptide, we have employed a lipoprotein carrier, HDL, as a vehicle for A β 1–42 [18,29]. Prevention of rapid A β aggregation in the pump with low dose lipoprotein carrier may allow the dose-dependent formation of soluble, but toxic A β oligomers [42,44]. Although not identical to CSF HDL, plasma HDL was chosen as a carrier for these experiments because it is similar to CSF HDL in many respects, readily available and has been identified as a major A β transporter in plasma [13,41]. Widespread diffuse A β -ir deposits with associated glia have been consistently produced with this protocol [18,29].

Infusion of A β with the lower 5 μ g dose of A β 42/HDL was sufficient to significantly increase F2-isoprostane levels compared with HDL vehicle. F2-isoprostanes represent stable lipid peroxidation products of arachidonate that are elevated in AD CSF and brain [50,60]. The levels of individual F2-isoprostanes are roughly 2-fold elevated in AD patients with total levels of the 3 major F2 isoprostanes reaching approximately 2–3 ng per gram tissue or 20–30 pg/mg protein, assuming 10% protein. The AD measurements used gas chromatography combined with mass spectrometry (GC/MS). In our study, levels of 8-epi-F2 isoprostane in the A β treated control diet group averaged 74 pg/mg protein giving an approximately 2-fold increase over vehicle treated controls (average 33 pg/mg). Levels in the A β -infused curcumin-fed group were reduced to below the level of the vehicle-infused control diet-fed group, while ibuprofen failed to control the oxidative damage indexed by iso-

prostanes of A β -infused rats. Our data suggests that traditional NSAIDs will not be sufficient to control this class of oxidative damage in AD patients. However, oxidative damage indexed by isoprostanes appears clinically relevant. F2-isoprostane elevations in AD patients were reported to correlate with neurodegeneration (Braak stage, reduction in brain weight and degree of cortical atrophy) better than with numbers of neuritic plaques or tangles [51]. In another study, F2 isoprostane levels correlated with cognitive and functional impairment in AD as well as CSF correlates (tau, A β 42) of disease severity [59].

In AD brain, synaptophysin loss varies greatly by region and layer, but is a close correlate of clinical decline [72,73]. Used as a measure of pathological severity in our animals, global cortical synaptophysin loss with the low dose of A β 42 was only 10% relative to untreated controls or vehicle. This global loss was alternated by curcumin, but not ibuprofen treatment. Synaptophysin levels may be inversely related to F2 isoprostane levels, but this could not be convincingly demonstrated with the number of animals used in the present study. Thus, our results with small numbers of animals suggest that reducing oxidative damage may prevent A β -induced synaptophysin loss. Because elevated CSF and plasma F2 isoprostanes levels can be monitored in AD patients, our results support the idea that isoprostanes may be a useful surrogate endpoint in an AD prevention trial.

Using different paradigms, several groups have reported A β infusion or injection can induce memory deficits [26, 53,80]. In the current study, A β infusion with higher doses of A β 42 than those found to induce the small, but significant, synaptophysin loss were shown to result in spatial memory deficits in the Morris water maze. These memory deficits could be prevented by curcumin treatment. Unlike A β -infused aged rats, A β -infused middle-aged rats showed no loss of the pre-synaptic protein synaptophysin (not shown). In fact, except in aged rats, synaptophysin loss is never observed at one month, but small reductions are observed over 3 and 6 months, even if A β is only infused for the first month (unpublished observations). Even with a 10% loss synaptophysin, this would be unlikely to account for the observed behavioral deficits. Therefore we examined other molecules involved in cognitive function.

PSD-95 is a postsynaptic marker playing a basic role in synaptic transmission by anchoring NMDA receptors, notably NR2B, and interacting with nNOS [12,74]. PSD-95 loss could be relevant to spatial memory deficits because mice lacking PSD-95 have severe spatial memory deficits [49]. In contrast, mice exposed to enriched environments with improved learning and memory have elevated PSD-95 and related postsynaptic proteins [62]. In our study, the levels of the postsynaptic marker, PSD-95 declined by 40% in the A β -infused, memory-impaired group. PSD-95 loss and cognitive deficits were prevented in A β -infused rats on the curcumin diet. Reductions in hippocampal NR2B (~40%) correlate with cognitive deficits in AD [71]. Thus, curcumin's protection against A β -induced reductions in

PSD-95 and possibly related proteins involved in excitatory neurotransmission may underlie cognitive effects.

Compared to A β -infused rats fed control diet, A β -infused rats fed curcumin showed overall reductions in microglial stained area (microglia not associated with plaques) in cortex layer 2, consistent with curcumin's known anti-inflammatory activity. However, a very different microglial reaction was observed in relation to the A β deposits. Ring analysis showed that microglial staining within and around plaques was in fact increased by curcumin diet. Typically, hypertrophied microglia with larger cell bodies or thicker processes are more activated, but not necessarily phagocytic [23]. In fact, ultrastructured analysis suggests that the majority of "activated" microglia in AD brain are not actively phagocytosing amyloid [16]. In contrast, treatments with antibodies to A β or an A β vaccine have been reported to increase microglial activation and phagocytosis and clearance of amyloid [5,64]. Preliminary data for similar results with reductions in plaque numbers and increased plaque-associated microglia activation have been presented for a different NSAID, nitroflurbiprofen [36]. This suggests that an increase in microglial activity in plaques in NSAID treated animals may, while reducing chronic inflammation, allow increased phagocytic degradation of plaque amyloid.

Image analysis of plaque counts showed an approximate 70–80% reduction in A β plaque numbers and burden in the curcumin-treated group. In the rat a 4-fold higher dose of curcumin reduced A β burden 45%, similar to ibuprofen. Preliminary data suggest that curcumin, ibuprofen and the A β vaccine reduce A β deposits by different mechanisms, raising the possibility that combining these agents may optimize efficacy. We have also obtained large reductions in plaque numbers and A β burden in curcumin treated APP^{sw} transgenic mice [43b]. Similar results in the rat A β infusion and APP transgenics suggest that the reduced A β burden observed is not unique to our rat A β infusion model.

Several factors might explain the reduction in plaques with curcumin treatment. Because in the rat model, A β deposition is induced by infusion of exogenous human A β , it is unlikely that a reduction in plaques can be caused by an effect on the expression level of the rodent amyloid precursor protein (APP) or on its processing to A β . Therefore curcumin-mediated reductions in amyloid most likely involve factors affecting amyloid formation or clearance, including apolipoprotein E [3] and alpha 1 antichymotrypsin [52,55]. Possibilities include known pro-amyloidogenic factors that might be reduced by an antioxidant/NSAID like curcumin include apolipoprotein E [3] and α 1 antichymotrypsin [52,55]. There is also evidence that oxidative damage may have direct effects on A β accumulation [21,30,34]. Finally there may be an effect on microglial function. While we observed a reduction in microgliosis away from plaques, microglia in the plaques were not reduced and when individual cell bodies could be identified, they frequently appeared significantly larger in the curcumin group. Whether

this reflects increased amyloid phagocytosis and clearance requires further study.

The question of whether the doses used in the present study might be practically effective in humans remains unresolved and requires clinical trials. Ibuprofen shows similar plasma levels and half lives (1.7–2.5 hrs) in rodents and humans after acute oral ibuprofen dosing. Ibuprofen is lipophilic and appears to enter the human CSF in relatively large amounts. CSF concentrations are higher than their concurrent free plasma concentrations from 1.5 to 8 hrs reflecting a longer half-life in CSF [4]. This is consistent with previously discussed data showing similar CNS activity in humans and rodents [43]. Although the ibuprofen dose used in the models is high and could cause unacceptable side-effects with chronic use in humans, lower doses may also be effective.

Curcumin is also highly lipophilic and should have no trouble entering the CNS. Acute oral or other dosing in rodents or humans result in poor bioavailability, due to rapid conversion to glucuronides (reviewed in [39]). However, this alleged poor bioavailability is at odds with the CNS effects reported here, in other cited results and in an extensive literature on cancer chemoprevention and treatment in many tissues in rodents [39]. For example, systemic curcumin inhibits NF κ B and promotes skeletal muscle regeneration in vivo with an optimum dose of 20 μ g/kg body weight in rats [75]. This would correspond to a dose of 1.5 mg/75 kg body weight in humans and suggests that despite concerns raised in pharmacokinetic studies, curcumin or a derivative may be effective in humans at practical doses. Efficacy and safety at relevant doses need to be established in controlled clinical trials.

NSAIDs appear to be able to reduce risk for AD, but most available NSAIDs have unpleasant gastrointestinal side-effect issues associated with cyclooxygenase-1 (Cox-1) inhibition that limit their use. Specific Cox-2 inhibitors have been proposed as a safer alternative to mixed function inhibitors, but experimental and clinical results have raised questions about their efficacy [47]. Most Cox-1 and Cox-2 inhibitors also lack significant direct antioxidant activity. Curcumin has a long history of safe use and is well-tolerated in humans with limited or no side-effects reported at effective anti-inflammatory and antioxidant doses [39]. The data reported here argue that curcumin or another combined antioxidant/NSAID approach may prove useful for AD prevention or treatment.

Acknowledgments

Supported by grants from K.K. Siegel (UCLA Center on Aging), VA Merit (SAF), AG10685 (SAF), AG16570 (SAF), Bruce and Katherine Kagan Alzheimer Treatment Program, Elizabeth and Thomas Plott. We thank Dr. Fusheng Yang and Dr. Giselle Lim for help with this project, and Dr. Glabe (UC Irvine) for providing A β peptides. We thank

Mychica Simmons, Darrell Nash, Zerlinde Balverde, An-keen Thomasian, Michael Oh and David Horn, Ping Ping Chen for technical support. This paper is dedicated to the memory of Tura Bear.

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