Plaque-Derived Oxidative Stress Mediates Distorted Neurite Trajectories in the Alzheimer Mouse Model

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Abstract
Alzheimer disease (AD) is characterized both by senile plaques and neurodegeneration, although the details of the relationship between the 2 are not well understood. We postulated that oxidative stress resulting from senile plaques may mediate plaques’ effects on local neuronal processes. Using multiphoton microscopy, we directly demonstrate the generation of reactive oxygen species by senile plaques. After screening of several natural antioxidants ex vivo, we assessed in vivo the effect of 2 orally administered antioxidants in APPswc/PS1d9 transgenic mice. Both Ginkgo biloba extract and vitamin E reduced the oxidative stress resulting from senile plaques in vivo as monitored with intracranial imaging. Both treatments also lead to a progressive reversal of the structural changes in dystrophic neurites associated with senile plaques. These results suggest a causal relationship between plaque-associated oxidative stress and neuritic alterations and demonstrate for the first time that the focal neurotoxicity associated with the senile plaques of AD is partially reversible with antioxidant therapies. The quantitative ex vivo screen combined with in vivo monitoring of efficacy should lead to more effective clinical therapies for the prevention of oxidative stress and neurotoxicity in AD.

Key Words: Alzheimer disease, Ginkgo biloba extract (Egb 761), Multiphoton microscopy neurite, Oxidative stress, Senile plaque, Vitamin E (trolox)

INTRODUCTION
Alzheimer disease (AD) is the most common cause of dementia characterized by progressive memory loss, cognitive impairment, and behavioral deterioration. AD is diagnosed in postmortem analysis by the presence of neurofibrillary tangles, senile plaques, and neuronal loss. Strong evidence from genetic, histologic, and biochemical studies support the idea that the accumulation of amyloid-β (Aβ) aggregates in the brain plays a seminal role in the pathogenesis of AD (1). A specific morphologic subset of senile plaques, the compact or dense core plaque, is a source of focal neurotoxicity both in AD and transgenic mice (2). Compact senile plaques have also been associated with synaptic loss and neuritic dystrophy (3–5). Dense core plaques are associated with curvature of nearby neurites (6–8) and are capable of disrupting cortical synaptic integration (9). Recent studies in cultured neurons have also shown that soluble Aβ leads to a progressive degeneration that begins in neurites and axons and ends with cell death (10). It is thus well-established that the altered microenvironment around plaques is responsible to some extent for the pathologic neurites present in AD brains (11).

Although the toxic factors in the microenvironment of the plaque are not known, indirect evidence is consistent with a role for oxidative stress (12). It has been reported that Aβ peptides can produce H2O2 (13) and provoke protein and lipid peroxidation (14). Aβ also seems to have a significant effect on neuronal DNA fragmentation, loss of neuritic networks, and cell viability (15). Following this idea, it has been suggested that free radicals associated with plaques may mediate or contribute to plaque-induced toxicity (2, 16, 17). Given these considerations, new efforts are being directed toward combating oxidative stress damage. In this sense, natural antioxidants have a special relevance and several studies have shown positive effect of vitamins, proanthocyanidins, and bioflavonoids in cell culture experiments (18, 19) in animal models of AD (20, 21) or in clinical trials with patients with AD (22, 23).

In the present work, we used multiphoton microscopy and Amplex Red (AR), a well-established reporter of free radicals and reactive oxygen species, to evaluate therapeutic approaches based on antioxidant treatment that could diminish or alleviate both the levels of oxidative stress and the focal pathologic neuronal alterations in APP mice. We first developed a screen to determine if a series of natural antioxidants could diminish plaque-associated reactive oxygen species formation ex vivo. Not all antioxidants were effective at reducing this plaque-associated oxidative stress. We then determined in vivo if effective antioxidants cross the blood–brain barrier sufficiently well to diminish reactive oxygen species generation in living animals. Finally, we imaged neurites and plaques in living APP mice to test whether antioxidant treatment affects plaque size and number as well as...
neuritic dystrophy. Remarkably, the degree of morphologic alterations surrounding plaques resolved after treatment with orally active antioxidants despite no effect on plaque size or number, supporting a role for oxidative stress in the local neuritic changes that accompany plaques in AD.

MATERIALS AND METHODS

Animals

APPswe/PS1d9 mice aged 6 to 8 months, YFP or APPswe/PS1d9xYFP aged 8 to 10 months (24) obtained from Jackson Lab (Bar Harbor, Maine), and Tg-2576 (25) aged 22 to 24 months were used in the experiments. All studies were conducted with approved protocol from the Massachusetts General Hospital Animal Care and Use Committee and in compliance with National Institutes of Health guidelines for the use of experimental animals.

Reagents

Amplex Red (10-actyl-3,7-dihydroxyphenoxazine) and Texas Red dextran 70,000 D and fluorescein dextran 3,000 D molecular weight (Molecular Probes, Eugene, OR), AK-Fluor (Akorn, Inc., Decatur, IL), methoxy-XO4 (gift from Dr. Klunk, University of Pittsburgh), Ginkgo biloba extract (EGb 761) (Beaufour Ipsen, Paris, France), grape seed extract (Interhealth Nutraceuticals, Benicia, CA), peroxidase, β-carotene, vitamin E, vitamin C, quercetin, chrysins, hesperitin, flavone, rutin, resveratrol, rosmarinic acid, N-tert-butyl-α-phenylnitrone (PBN), thioflavin S, and common chemical reagents were obtained from Sigma (St. Louis, MO).

Ex Vivo Screening of Natural Antioxidants

Paraformaldehyde-fixed brain sections of Tg2576 or APPswe/PS1d9 mice were used for the ex vivo assays (17). Mounted tissue was dehydrated and treated for 45 minutes with the antioxidants: 10 nM to 1 mM for quercetin, chrysin, hesperitin, flavone, rutin, rosmarinic acid, resveratrol, β-carotene, vitamin C, and trolox and 0.01 to 1 mg/mL in the case of EGb 761 and grape seed extract. All compounds were prepared weekly and stored at −20°C. No differences were observed in the activity of the compounds between batches. Quercetin, chrysin, hesperitin, flavone, and rutin were diluted in DMSO/phosphate-buffered saline (PBS) (1/10) and trolox, rosmarinic acid, and resveratrol were diluted in DMSO/PBS (1/100). The rest of the compounds used in the ex vivo studies were diluted in PBS. Control tissue was incubated in PBS without drug treatment, and PBN (100 μM) was introduced in each experiment as a positive control. Sections were carefully washed and incubated for 45 minutes with 200 μM AR (in the presence of 0.5 mg/mL peroxidase) with the previously used antioxidants at the same concentrations, whereas control tissue was incubated in AR and peroxidase only. The tissue was covered to minimize light and air exposure. Sections were washed in PBS to rinse excess reagent, aqueously coverslipped, and imaged. Afterward, brain tissue was washed in PBS and incubated for 20 minutes in thioflavin S (0.01%). After washing, the sections were covered and imaged again.

In Vivo Treatment and Surgical Preparation

Acute treatments were as follows: APPswe/PS1d9 mice received gavage administrations of EGb 761 (100 mg/kg in water) or trolox (210 mg/kg 25% Cremophor in distilled water) 24 hours and 30 minutes before the surgical procedure. During the surgical procedure, 200 μL of trolox (100 μM) or EGb 761 (1 mg/mL) were locally applied on the surface of the cortex of the animals for 20 minutes. AR (10 mM) in the presence of peroxidase (0.5 mg/mL) and the antioxidants was applied afterward on the surface of the brain for 20 more minutes.

Chronic treatment included APPswe/PS1d9 and APPswe/PS1d9-YFP mice. Animals received gavage administrations once a day for 15 days of EGb 761 (100 mg/kg in water) or trolox (210 mg/kg 25% Cremophor in distilled water). No topical drug treatment was applied. Control animals followed similar procedures: 2 of them received distilled water and 2 received 25% Cremophor in distilled water. Because no differences were observed between them they were considered as a single control group.

Surgery was performed as previously described (17) with minor modifications. APPswe/PS1d9 mice were treated as follows: mice were anesthetized with isoflurane. After removing the dura, the antioxidants were topically applied for 20 minutes in the case of acute treatments. AR in the presence of the antioxidants, or alone in the case of the control animals, was applied afterward for 20 more minutes, covering the head to protect from light and air exposure. The site was then washed, a coverslip was attached over the site, and the animal was imaged. Before the second imaging session, the coverslip was removed, animals were treated with thioflavin S for 20 minutes, and the coverslip was replaced and fixed with dental cement. Angiograms were performed with 10% intravenous injection of fluorescein dextran 3,000 D. Chronically treated animals followed similar procedures avoiding the topical application of the antioxidants.

Multiphoton Imaging and Processing

As previously described (17), 2-photon fluorescence was generated with 800 nm excitation from a mode-locked Ti:sapphire laser (MaiTai, Spectra-Physics, Mountain View, CA) mounted on a multiphoton imaging system (Bio-Rad 1024ES; Bio-Rad, Hercules, CA). A custom-built external detector containing 3 photomultiplier tubes (Hamamatsu Photonics, Bridgewater, NJ) collected emitted light in the range 380 to 480, 500 to 540, and 560 to 650 nm. Ex vivo imaging was performed using the normal scan speed and multiple z-series were collected after adding AR and again after adding thioflavin S using a 20× water immersion objective (615 × 615 μm, z-step, 2 μm, depth 50 μm approximately). In vivo imaging was conducted under the same conditions (615 × 615 μm; z-step, 2 μm, depth 200 μm approximately). Images were analyzed with Image-J software (National Institutes of Health freeware) and the intensity of the immediate surroundings of the dense core plaques was subtracted from the dense core fluorescence for AR and thioflavin S to correct for background levels. AR is a very sensitive probe for H2O2 (26), although H2O2 can lead to the production of many other reactive oxygen species. A ratio between AR intensity and thioflavin S intensity was
calculated for each plaque to normalize across images and mice. This ratio provides a quantitative, dimensionless index of plaque-associated oxidative stress. Results are expressed as a percentage of control values.

In the case of YFP or APPswe/PS1d9xYFP mice used to study neuritic abnormalities, low-resolution images (615 × 615 μm; z-step, 5 μm, depth, 200 μm approximately) were acquired to provide a map of the area. Higher-resolution images were captured to identify single neurites (125 × 125 μm; z-step, 0.8 μm, depth, 20 μm approximately). Images for neurite analysis were further processed using AutoDeblur (AutoQuant, Watervliet, NY) as previously described (7). Neurite curvature ratio was calculated by dividing the end-to-end distance of a neurite segment by the total length between the 2 segment ends (5, 6, 8). To monitor amyloid deposits, mice received an intraperitoneal injection of methoxyX-O4 (5 mg/kg) (27) 24 hours before the surgical procedure to label plaques. Angiograms were performed with 12.5 mg/mL intravenous injection of Texas Red dextran (70 kD).

To determine the effect of proximity to plaques, the average distance between the nearest methoxy-XO4 stained amyloid plaque and each dendritic segment was calculated using the average of the distance from the plaque edge to each end and the midpoint of the dendritic segment. The mean areas of dystrophic neurites within a plaque were also determined using Image-J software.

RESULTS

Ex Vivo Activity of Natural Antioxidants

Tissue sections from Tg2576 or APPswe/PS1d9 mice brain were used for ex vivo screening by measuring the fluorescence of AR resulting from oxidative activity from individual senile plaques. AR is nonfluorescent until oxidized. The fluorescent product can be measured quantitatively, and imaging allows spatial discrimination of the oxidative signal. Our previous results demonstrated that dense core plaques lead to significant local accumulation of fluorescent signal. Multiphoton microscopy was necessary for these experiments because visible light excitation leads to direct generation of oxidative stress and therefore fluorescence of the probe (17). A ratio of AR fluorescence to subsequent thioflavin S fluorescence normalized for plaque-to-plaque variations in intensity.

We first used the compound PBN to evaluate the plaque-associated production of oxygen radicals. PBN is a well-characterized spin trap compound that was able to neutralize oxidative stress both ex vivo and in vivo in the Tg2576 mouse model (17). PBN showed a statistically significant reduction (approximately 45%) in AR fluorescence intensity associated with dense core plaques when compared with control values in all performed experiments. No substantial differences were observed between Tg2576 and APPswe/PS1d9 mice. PBN was then used as a positive control for subsequent evaluation of other putative antioxidants.

We next evaluated the plaque-associated antioxidative ability of naturally occurring compounds in APPswe/PS1d9 brain tissue. We selected a variety of agents that are present in fruits, vegetables, juices, and dietary supplements. Each compound was obtained from a commercial source and compared with vehicle alone (Table). The first set was naturally occurring compounds. Quercetin (F5,209 = 22.574; *, p < 0.001) and chrysin (F5,142 = 13.248; *, p < 0.001) showed a statistically significant dose-dependent decrease in AR intensity compared with controls at all concentrations used (10 nM–1 mM). Hesperitin (F5,153 = 10.159; *, p < 0.001; †, p < 0.001 vs 1 mM) and flavone (F5,146 = 7.990; *, p < 0.001) showed significant reductions of AR intensity but with a slightly erratic profile. Surprisingly, neither rutin (after post hoc Tukey B test [F5,125 = 2.454; p > 0.5]) nor rosmarinic acid (F5,144 = 6.677; p > 0.05) showed an effect in reducing AR

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxidative Stress Index (% control)</th>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>N-tert-butyl-α-phenylnitrone (100 μM)</td>
<td>54.68 ± 1.863*</td>
</tr>
<tr>
<td>Natural compounds</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>56.83 ± 3.64*</td>
</tr>
<tr>
<td>Chrysin</td>
<td>65.03 ± 2.56*</td>
</tr>
<tr>
<td>Hesperitin</td>
<td>54.55 ± 1.73†</td>
</tr>
<tr>
<td>Flavone</td>
<td>55.97 ± 2.81*</td>
</tr>
<tr>
<td>Rutin</td>
<td>78.23 ± 20.28</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>96.20 ± 14.20†</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>130.97 ± 16.32</td>
</tr>
<tr>
<td>Complex extracts</td>
<td>0.01 mg/mL</td>
</tr>
<tr>
<td>Ginkgo biloba (EGb 761)</td>
<td>84.08 ± 4.66†</td>
</tr>
<tr>
<td>Grape seed extract</td>
<td>84.05 ± 15.34</td>
</tr>
<tr>
<td>Vitamins</td>
<td>10 nM</td>
</tr>
<tr>
<td>Vitamin E (trolox)</td>
<td>78.24 ± 5.59†</td>
</tr>
<tr>
<td>Beta carotene</td>
<td>100.33 ± 10.23</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>98.51 ± 13.91</td>
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</table>

Fluorescence intensity of Amplex Red, resulting from plaque-mediated oxidation is expressed as percentage of control values. Data are representative of 10 to 96 plaques from 3 animals. Significant differences were determined by one-way analysis of variance followed by Tamhane or Tukey B test. (†, p < 0.001 vs 1 mM or 1 mg/mL).
not at lower concentrations \(F_{6,109} = 4.581; \ast, p < 0.001; \dagger, p < 0.001 \text{ vs } 1 \text{ mM})

We also evaluated 2 standardized plant extracts that contain complex mixtures of some of these polyphenols as well as other compounds. We observed a statistically significant reduction in AR intensity for grape seed extract at 1 mg/mL but as other compounds. We observed a statistically significant reduction in oxidative stress with trolox \(F_{5,149} = 19.779; \ast, p < 0.001\) vs control; \(\dagger, p < 0.001 \text{ vs } 1 \text{ mM})

Lastly, a few well-characterized vitamins were analyzed and we measured statistically significant dose-dependent reductions in oxidative stress with trolox \(F_{5,149} = 19.779; \ast, p < 0.001 \text{ vs control group; } \dagger, p < 0.001 \text{ vs } 1 \text{ mM} \text{ trolox and } 100 \mu\text{M trolox}) \text{ and vitamin C } \left(F_{5,155} = 10.799; \ast, p < 0.001\right). \text{ Surprisingly, } \beta\text{-carotene } \left(F_{5,134} = 4.512; \ast, p > 0.05\right) \text{ did not reduce plaque-derived oxidative stress. All ex vivo antioxidant experiments are summarized in the Table. These results demonstrate that known antioxidants have unpredictable protective abilities for the oxidative stress resulting from the senile plaques of AD and that direct, quantitative screening may thus be the most relevant assay for evaluating therapeutic potential.}

In Vivo Effect of Natural Antioxidants on Oxidative Stress

The ex vivo assay permits quantitative characterization of the antioxidative effects of known compounds with direct relevance to AD. The next step was characterizing the protective ability in vivo in the mouse models. Two compounds from the ex vivo screen were chosen based on the potency of protection in the ex vivo assay. The first in vivo experiments evaluated the acute effects (combined oral and topical administration, 24 hours) of EGb 761 and trolox. Acute experiments required in vivo imaging of the fluorescence of AR generated by plaque-associated reactive oxygen species followed by thioflavin S staining. To allow reliable registration of plaques within each imaging session, a fluorescent angiogram was used to provide 3-dimensional fiduciary markers. Representative images of in vivo angiograms using fluorescein and AR or thioflavin S staining of dense core plaques are shown in Figures 1A and B, respectively. Acute treatments with both EGb 761 and trolox in vivo showed statistically significant reductions in AR intensity when compared with animals receiving saline only (Fig. 2). These results demonstrate that the ex vivo assay translates to in vivo efficacy with a combination of oral and direct application of the antioxidant. These experiments were proof in principle that in vivo modulation of oxidative stress was possible with these reagents.

We next evaluated chronic treatment (15 days) of these compounds using only oral administration. Ginkgo biloba extract EGb 761 (100 mg/kg) and trolox (210 mg/kg) were administered daily by gavage. After the treatment period, AR fluorescence followed by thioflavin S fluorescence was measured on a plaque-by-plaque basis using a fluorescence angiogram for image registration. A ratio of AR fluorescence to subsequent thioflavin S fluorescence provided an index of oxidative stress that normalized for plaque-to-plaque variations in intensity. This normalization is supported by significant statistical correlations between AR and thioflavin S plaque intensity measurements (Pearson’s correlation >0.298; \(p < 0.05\) within all analyzed groups: control, Ginkgo biloba, and Trolox acute and chronic treated). EGb 761 led to a reduction in plaque-associated oxidative stress during this time. Trolox showed a trend toward a reduction in oxidative stress that did not achieve statistical significance (Fig. 2). These results demonstrate quantitatively that orally administered natural antioxidants can reduce the oxidative stress resulting from parenchymal plaques in living animal models of AD.

FIGURE 1. Illustrative example of Aβ plaque oxidation of free radical indicators in vivo. Dense-core Aβ plaques activate the fluorogenic free radical indicator Amplex Red (A), histochemically confirmed by thioflavin S (B). Fluorescein containing blood vessels (green) were used to register sites shown in both images. Scale bar = 20 μm.

FIGURE 2. Quantitative inhibition of oxidative stress from senile plaques in living mice. Fluorescence intensity of Amplex Red, resulting from plaque-mediated oxidation, was reduced by Ginkgo biloba extract (EGb 761) acutely and chronically. Acutely administered trolox reduced Amplex Red intensity, although no statistical differences were detected when trolox was chronically administered. Data are representative of 41 to 63 plaques from 4 to 5 animals. Significant differences were determined by one-way analysis of variance followed by Tukey B test \(F_{4,233} = 4.559; \ast, p < 0.001 \text{ vs control group}\).
In Vivo Effect of Natural Antioxidants on Neuronal Abnormalities

We used YFP to allow multiphoton imaging of neuronal processes and APPswe/PS1d9xYFP mice injected with MeO-XO4 to allow simultaneous multiphoton imaging of neuronal processes and Aβ deposits. Fluorescent neurites were imaged in vivo and the pathologic “curvy” neurites and dystrophies were observed near dense core plaques. When we compared YFP and untreated APPswe/PS1d9xYFP mice, we observed a very significant increase in neurite curvature in the APPswe/PS1d9xYFP mice (Student t test F = 26.915, p < 0.001), demonstrating the deleterious effect of senile plaques on neuronal processes. Mean curvature ratios were as follows: YFP = 0.9934 ± 0.0008, APPswe/PS1d9xYFP = 0.9433 ± 0.035 (n = 30–114).

After having established the toxic effect of senile plaques on neurites in the APPswe/PS1d9 model and the efficacy of orally administered antioxidants at reducing the plaque-derived oxidative stress, we next examined the effect of the selected antioxidants on local neurotoxicity in vivo. With multiphoton imaging, we measured the size of individual senile plaques as well as the curvature of neurites and size and number of dystrophies quantitatively in vivo. We assessed the effect of the chronic treatment of EGb 761 extract and trolox on neurite curvature when neurites up to 50 μm in the trolox-treated group were compared YFP and untreated APPswe/PS1d9xYFP mice, we observed a very significant increase in neurite curvature in the APPswe/PS1d9xYFP mice (Student t test F = 26.915, p < 0.001), demonstrating the deleterious effect of senile plaques on neuronal processes. Mean curvature ratios were as follows: control = 372.52 ± 466.61, EGb 761 = 385.28 ± 29.11, and trolox = 358.63 ± 37.57 μm², respectively (n = 33–49). When neurite morphology was examined, a restorative effect of both compounds on curvature ratio was found when all neurites traced, up to 50 μm from the senile plaque borders, were included in the analysis (Fig. 3). We also compared the curvature ratios of neurites in even closer proximity to the plaques (<20 μm) (Fig. 4). The curvature ratio showed a statistically significant straightening effect in the case of EGb 761, and although trolox showed a tendency toward reducing neurite curvature, it did not reach statistical significance (Fig. 3). We also analyzed dystrophic neurites associated with plaques in APPswe/PS1d9xYFP mice by measuring the areas of swelling (3) and we did not detect any treatment effect (F2,11445 = 2.943; p = 0.053). Mean area of swellings were as follows: control = 7.30 ± 0.34, EGb 761 = 7.87 ± 0.31, and trolox = 6.66 ± 0.38 μm², respectively (n = 299–488).

**DISCUSSION**

Although the neurotoxic mechanisms in AD have not been fully elucidated, there is substantial evidence that the microenvironment surrounding plaques is a local source of oxidative stress (28). Moreover, plaques contain a myriad of molecules apart from Aβ, including apolipoprotein E, phosphorylated tau, and many others (29). The role of these various bioactive components is difficult to dissect. Although the mouse models of plaque deposition show very limited neuronal death (2, 30), there is substantial neurotoxicity exhibited.
by focal dystrophies and distorted neurites (5, 7, 9). To test whether this toxicity is mediated at least in part by oxidative stress, we treated animals with antioxidant compounds that were shown to reduce the oxidative stress resulting from mature plaques.

Our results demonstrate that orally administered antioxidants can reduce the oxidative stress resulting from senile plaques but do not reduce the size of the deposits. However, the treatment partially restores the pathologic neuritic abnormalities. Together, these results suggest that the abnormal neuritic morphology results from focal oxidative stress and that effective antioxidant therapy can restore neuron structure.

This study used multiphoton microscopy together with the fluorogenic oxidation reporter agent Amplex Red (17) to allow direct, quantitative examination of oxidative stress resulting from senile plaques both ex vivo and in vivo. Using this approach, it is possible to systematically screen potential antioxidants for the ability to protect against free radical mediated toxicity in AD and select those with the best profile for in vivo testing. Because the oxidation occurs in fixed, dead tissue, the plaques themselves were the source of oxidation, not surrounding cellular activity (17). This does not, however, preclude a cellular or diffuse contribution to oxidative stress in the AD models.

Among the available compounds, vitamins and polyphenols have been of special relevance because of their wide range of biologic, pharmacologic, and therapeutic activities against free radicals and oxidative stress (31, 32). Although many of these compounds have shown antioxidant and/or protective effects in other assays (18), no study has systematically assessed all of the antioxidants used here in the same paradigm. Many of the selected compounds showed some capacity to reduce oxidative stress associated with senile plaques ex vivo such as quercetin, chrysin, or resveratrol. Surprisingly, some well-known antioxidants such as β-carotene had no effect. Among the selected compounds, Ginkgo EGb 761 and the water-soluble version of vitamin E (trolox) showed very promising profiles in the ex vivo assay and abundant literature supports the neuroprotective effect of both compounds. EGb 761 is a standardized extract from Ginkgo biloba leaves that contains considerable amounts of natural bioflavonoids with antioxidant activity (33, 34). EGb 761 has been shown to protect hippocampal cells against reactive oxygen species and Aβ-induced toxicity (33, 35). EGb 761 also seems to modestly improve cognitive function in patients with dementia (22), regardless of the stage of dementia (36).

Our results with trolox confirm the protective actions attributed to vitamin E by indirect in vitro paradigms (37–39). More importantly, in one clinical trial, vitamin E appeared to slow the progression of the disease (40), and it is commonly prescribed for patients with AD. Recent controversy regarding the safety of vitamin E at high doses (41) and the lack of efficacy (42), however, has led to a reevaluation of the use of vitamin E in AD.

Taking the current ex vivo results into account as well as the preponderance of evidence for Ginkgo biloba and vitamin E in animal models and clinical trials, we tested both compounds in vivo to assess the ability to reduce oxidative stress associated with senile plaques as well as their possible neuroprotective activity on neurites surrounding plaques. Although PBN showed a good ex vivo profile, previous studies have already assessed the effect of the spin trap in vivo (17). Acute treatments followed previous studies (17) using oral doses of the antioxidants in the range of concentrations previously used for EGb 761 (43) and trolox (44). The acute treatment showed a significant reduction in oxidative stress both for EGb 761 and trolox when compared with control values, providing an initial promising approach to reduce oxidative stress related to senile plaques. Although the main goal of the acute experiments was to ensure a maximal amount of antioxidants in the brain of the treated mice to correlate with our ex vivo findings, the topical administration of the antioxidants to the brain surface limits the use of this approach. Therefore, we also performed a chronic study of 15 days of oral administration. EGb 761 produced similar results to those provided by the acute treatment, reducing oxidative stress related to senile plaques by approximately 30%, suggesting that EGb 761 effectively crosses the blood–brain barrier and retracts its antioxidant capacity. When we administered trolox chronically, we observed a tendency to reduce reactive oxygen species associated with thioflavin S-positive amyloid plaques. The reduction observed after chronic treatment was not statistically significant, perhaps reflecting bioavailability issues; the ex vivo data and the statistical trend seen in vivo suggest that longer treatments or higher doses could prove effective in plaque-mediated oxidative stress.

To our knowledge, this is the first time that the effect of senile plaques in surrounding neurites is assessed in this animal model. As previously described in other APP mice (7), we have demonstrated that senile plaques in APPswe/PS1d9 mice also induce abnormal neuritic curvature. When we assessed the effect of chronic treatments with Ginkgo biloba and trolox on neurite curvature in APPswe/PS1d9xYFP, we observed a neuroprotective effect of both antioxidant treatments that lead to a significant straightening of the distorted neurites when we included all the neurites imaged (up to 50 μm from the plaque border). Neurites in very close proximity to the plaques showed similar profiles, although trolox treatment did not reach statistical significance. These data are consistent with the apparent in vivo potency of these agents as antioxidants in the doses used. Alternatively, these slight differences may be attributed to different mechanisms of action involved for EGb 761 and vitamin E, as has been suggested (45). The fact that vitamin E when administered is preferentially taken up in the cerebellum rather than the cerebral cortex (46) may also account for the differences observed between both compounds.

Previous studies have shown alterations in the characteristic straight shape of neurites when they pass near Aβ deposits (6–8). It seems likely that Aβ itself contributes because the use of anti-Aβ antibodies not only removed the existing plaques, but also led to restoration of dendritic architectures (5), prevented synaptic degeneration (47), and reduced neuritic dystrophies associated with senile plaques (3). However, whether the Aβ effect is direct or indirect is
unknown and our current study tested the idea that plaque-associated oxygen radicals contribute to local alterations in neurite trajectories. Interestingly, no effect on plaque size or on neuritic dystrophies was observed, suggesting that the underlying mechanisms of (primarily dendritic) alterations in trajectories and (primarily axonal) dystrophies may differ.

In our study, the straightening effects were observed in the areas immediately surrounding the plaques in the absence of an effect on plaque size. These data support the possibility of a diffusible agent that mediates the pathologic changes. However, because free radicals themselves would be expected to have a diffusion range considerably less than 50 μm, we postulate that the effect is the result of prevention of secondary changes resulting from local oxidative damage. After this, our data implicate oxidative stress in the neuronal damage associated with AD, although other mechanisms, including toxic oligomeric species of Aβ or secondary inflammatory responses that may lead to injury of neurons, cannot be excluded. Moreover, this experiment supports, as previously shown (5), that a remarkable degree of plasticity occurs in the adult mouse brain within 15 days of treatment. All together, these results show for the first time the contribution of antioxidant therapy to straighten distorted neurites, suggesting that antioxidants can partially restore the damage experienced by patients with AD, providing support for the treatment of patients with AD with this class of agents.

REFERENCES