The Nitration Product 5-Nitro-γ-tocopherol Is Increased in the Alzheimer Brain

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Oxidative stress and quasi-inflammatory processes recently have been recognized as contributing factors in the pathogenesis of Alzheimer’s disease (AD). Reactive nitrating species have specifically been implicated in AD based on immunochemical and instrumental detection of nitrotyrosine in AD brain protein. The significance of lipid-phase nitration has not been investigated in AD. This study documents a significant two- to threefold increase in the lipid nitration product 5-nitro-γ-tocopherol in affected regions of the AD brain as determined by high-performance liquid chromatography with electrochemical detection. In a bioassay to compare the relative potency of α-tocopherol and γ-tocopherol against nitrosative stress, rat brain mitochondria were exposed to the peroxynitrite-generating compound SIN-1. The oxidation-sensitive Kreb’s cycle enzyme α-ketoglutarate dehydrogenase was inactivated by SIN-1, in a manner that could be significantly attenuated by γ-tocopherol (at < 10 μM) but not by α-tocopherol. These data indicate that nitric oxide-derived species are significant contributors to lipid oxidation in the AD brain. The findings are discussed in reference to the neuroinflammatory hypothesis of AD and the possible role of γ-tocopherol as a major lipid-phase scavenger of reactive nitrogen species. © 2001 Elsevier Science (USA)

Key Words: Alzheimer; nitric oxide; γ-tocopherol; vitamin E; HPLC; free radical.

Abundant data suggest that oxidative stress is an important factor in normal age-related organ deterioration, as well as a contributing variable in age-related neurodegenerative conditions such as Alzheimer’s disease (AD).2 Biomarkers of protein, lipid, and DNA damage repeatedly have been found elevated in AD brain relative to age-matched brains obtained from neurologically normal individuals (1–5). Unfortunately, most indications of oxidative stress in the AD brain are relatively nonspecific and do not illuminate the biochemistry underlying the enhanced brain oxidation. For instance, protein carbonyl load is reliably increased in the AD brain (1) but such an increase could reflect diverse biochemistry ranging from metal-catalyzed free radical generation to nonenzymatic glycation. In an attempt to circumvent these ambiguities, we have sought to develop analytical methods for measuring oxidative damage that might be indicative of specific alterations in brain redox chemistry.

Toward this goal, we have used high-performance liquid chromatography with electrochemical array detection (HPLC-ECD) to demonstrate significantly increased quantities of 3-nitrotyrosine and 3,3'-dityrosine in the AD brain (5). Both analytes are significantly increased in affected regions of the AD brain (5). These findings suggest that nitric oxide (NO) and its redox congeners may contribute substantially to oxidative stress in AD. In an effort to corroborate and extend earlier findings that NO-dependent oxidative stress is increased in the AD brain, we have developed HPLC-ECD techniques for determining nitration products in the lipid phase. We now report

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2 Abbreviations used: AD, Alzheimer’s disease; HPLC-ECD, high-performance liquid chromatography with electrochemical array detection; 5-NO2-γ-tocopherol, 5-nitro-γ-tocopherol; α-KGDH, α-ketoglutarate dehydrogenase; RNS, reactive nitrogen species; IPL, inferior parietal lobule; SMTG, superior and middle temporal gyri; CBL, cerebellum; SIN-1, 5-amin-2-(4-morpholinyl)-1,2,3-oxadiazolium.
that 5-nitro-γ-tocopherol (5-NO₂-γ-tocopherol; Fig. 1) is significantly elevated in cortical regions of the AD brain in which we have previously found elevated levels of protein-bound nitrotyrosine (5). The cerebellum, which is relatively spared from the histopathological correlates of AD, does not display an increased 5-NO₂-γ-tocopherol content. Complementary investigations were undertaken to compare α-tocopherol and γ-tocopherol protection against nitrative stress, using the enzyme α-ketoglutarate dehydrogenase (α-KGDH) as a biomarker of nitrative damage after in vitro exposure to peroxynitrite (ONOO⁻). In this system, γ-tocopherol significantly protected α-KGDH from peroxynitrite-mediated inactivation, whereas α-tocopherol did not protect. These findings lend further credence to the concept that •NO contributes to oxidative stress in the AD brain and suggest that γ-tocopherol may perform a unique function as a protectant against reactive nitrogen species (RNS).

MATERIALS AND METHODS

Brain tissue. Brain specimens were obtained at rapid postmortem from a total of 10 neuropathologically normal subjects and 15 subjects who demonstrated clinically defined AD according to NINCDS-ADRDA Work Group Criteria and who met standard criteria for the histopathological diagnosis of AD (6–9). Normal individuals had no history of dementia, other neurologic diseases, or systemic diseases likely to affect the brain. Normal subjects were members of a volunteer group who had undergone mental status testing; all subjects used in this study had tested within the normal range. Mean age ± standard deviation of normal subjects was 80 ± 6 years (5 male, 5 female) and of AD subjects was 78 ± 4 years (10 male, 5 female). Postmortem intervals were 2.8 ± 0.8 h (normal subjects) and 2.7 ± 1.2 h (AD subjects). Tissue was collected from three brain regions: inferior parietal lobule (IPL), superior and middle temporal gyri (SMTG), and cerebellum (CBL). All brain regions were not available from every patient. Tissue was frozen at −80°C pending analysis. Brain specimens (approximately 200–500 mg) were briefly sonicated in 1 mL of 10 mM sodium acetate (NaOAc) buffer, pH 6.5, containing 0.1% Triton X-100. Samples were centrifuged at 14,000g for 10 min at 4°C. The supernatant was discarded. Ethanol (0.500 mL of 200 proof) and 13 µL of ethanolic butylated hydroxytoluene (10 mg/mL) were transferred as an antioxidant to each sample. The pellets were vortex mixed, then extracted with 2 × 5 mL of HPLC-grade hexane followed by 1 × 3 mL of HPLC-grade hexane, vortex mixed again for approximately 1 min, and sonicated briefly after each hexane addition. Samples were then centrifuged for 10 min at 10,000g. Pooled hexane fractions were evaporated to dryness under high-purity nitrogen gas and reconstituted in 0.500 mL of HPLC-grade methanol. Samples were filtered through a 0.2 µm × 13 mm polyvinylidene difluoride syringe filter and analyzed by HPLC-ECD.

Chromatography. Unless otherwise stated, all reagents and HPLC-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Company (St. Louis, MO). HPLC methods were similar to those described previously (5, 10, 28). Chromatography was conducted using a Tosohaas (Montgomeryville, PA) ODS-80, reverse-phase C₁₈ column (5-µm particle size, 4.6 × 250 mm). The isocratic mobile phase contained 83% acetonitrile, 12% methanol, 0.2% acetic acid, and 30 mM lithium acetate. Normally, 60 µL of each sample was injected from a Waters Associates (Milford, MA) Model 717 refrigerated autosampler (maintained at 4°C), using a Waters Associates Model 625 pump with a flow rate of 2 mL/min. A photodiode array detector (Waters Associates Model 996) was connected in line with the HPLC column but preceding a 12-channel coulometric electrode detector (ESA, Chelmsford, MA). The optimal wavelength employed for α/γ/5-NO₂-γ-tocopherol detection was 302 nm. The 12 consecutive electrochemical cells were operated in the oxidizing mode and were assigned the following potentials: 200, 300, 400, 525, 600, 625, 650, 675, 700, 750, 825, and 900 mV. Analyte concentrations were determined using a 6-point external calibration curve. During automated analyses, standards were interspersed regularly among samples. Spike recovery and stability studies were performed to ensure analyte stability and accuracy of the analysis.

Standards. 5-NO₂-γ-Tocopherol was synthesized from γ-tocopherol by exposing a hexane solution of γ-tocopherol to a stream of NO₂ gas (11). The nitration product was purified by preparative thin-layer chromatography over silica gel using 99% dichloromethane/1% methanol as the eluent. Identity was confirmed by mass spectrometry and UV-visible spectroscopy. All other tocopherol standards were purchased commercially from Matreya Lipids (Pleasant Gap, PA), Re-
search Organics (Cleveland, OH), or Sigma Chemical Company.

Mitochondrial preparations, ONOO\(^{-}\) generation, and \(\alpha\)-KGDH assays. Rat brain mitochondria were prepared as previously described (22) and suspended in KHP buffer (20 mM HEPES, 80 mM KCl, 5 mM sodium phosphate, 5 mM MgCl\(_2\), pH 7.3) at a protein concentration of 10 mg/mL. \(\alpha/\gamma\)-Tocopherols were diluted into mitochondrial preparations from an ethanolic stock solution and the samples were briefly sonicated; samples not receiving tocopherol received ethanol only (total ethanol consumption, 1%). Peroxynitrite was generated in situ by use of the compound SIN-1 [5-amino-2-(4-morpholinyl)-1,2,3-oxadiazolium], which spontaneously decomposes to liberate the ONOO\(^{-}\) precursors \(\cdot\)NO and \(\cdot\)O\(_2\) (15). SIN-1 (Alexis Neurochemicals, San Diego, CA, or Oxis International, Portland, OR) was dissolved in KHP to a 10 \(\mu\)M final concentration and immediately diluted into the mitochondrial samples. The reaction volume was typically 0.300 mL. Samples were incubated at 37°C for 1 h on a slow rotary mixer (approximately 30 rpm with a 20-cm diameter). At the end of this incubation period, samples were diluted with 1 volume of 2% Triton X-100 solution in KHP and sonicated briefly, and 50-\(\mu\)L aliquots were withdrawn for \(\alpha\)-KGDH assays. The \(\alpha\)-KGDH activity was assayed as previously detailed (23, 24) by following \(\alpha\)-ketoglutarate-dependent NAD reduction at 340 nm and 37°C using a ThermoMax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). For each independent experiment, the assay was performed in quadruplicate at each tested tocopherol concentration. Each experiment was internally controlled with a positive control (mitochondria treated with 0.5 mM SIN-1 without \(\alpha\)-tocopherol or \(\gamma\)-tocopherol) and a negative control (mitochondria incubated without SIN-1 or tocopherol added). Results were tabulated and the percentage of protection afforded by tocopherol against the SIN-1 challenge was calculated as

\[
\text{% Protection} = 100\% - 100 \times \frac{(A_0 - A_{\text{SIN-1, tocopherol}})}{(A_0 - A_{\text{SIN-1}})}
\]

where \(A_0\) is the activity in the negative control.

Statistics. Differences in normalized 5-NO\(_2\)-\(\gamma\)-tocopherol content between AD and normal brain regions were assessed by Student’s \(t\) tests. Differences between \(\alpha\)-tocopherol and \(\gamma\)-tocopherol with respect to \(\alpha\)-KGDH protection were assessed by ANOVA using a homolog \(\times\) concentration model for tocopherol concentrations \(<100 \mu\)M. Subsequent Student’s \(t\) tests were performed to assess significance of tocopherol protection for each congener at each tested concentration.

RESULTS

Figure 1 illustrates the chromatographic resolution of tocopherols extracted from human brain tissue. 5-NO\(_2\)-\(\gamma\)-Tocopherol was identified based on coelution with an authentic standard in both the spectral and the electrochemical dimensions. The proportion of

![FIG. 1. Expanded HPLC-ECD chromatogram of tocopherol variants extracted from the brain of an individual with Alzheimer’s disease. Only the 600-mV channel is illustrated. \(\alpha\)T, \(\alpha\)-tocopherol; \(\gamma\)T, \(\gamma\)-tocopherol; and 5-NO\(_2\)-\(\gamma\)T, 5-NO\(_2\)-\(\gamma\)-tocopherol.](https://example.com/figure1)
γ-tocopherol to α-tocopherol ranged from 2 to 10% in both normal and AD brain samples. This ratio is similar to the γ-tocopherol/α-tocopherol ratio in human plasma (20 and unpublished data). In bulk tissue extracts, the proportion of 5-NO$_2$-γ-tocopherol to γ-tocopherol was between 10 and 60% (Fig. 2). In contrast, the previously reported ratio of nitrotyrosine to tyrosine was 10–100 times lower in AD and normal cortical tissue (Table I and Ref. 5). The concentrations of nitrotyrosine and nitrotocopherol in AD brain are approximately equivalent when considered in terms of moles analyte/mass of tissue. 5-NO$_2$-γ-Tocopherol was increased significantly in cortical regions of the AD brain relative to normal brain (Fig. 2). This was true whether expressed as a ratio to γ-tocopherol (Fig. 2) or as a ratio to protein concentration in the original homogenate (data not shown).

Bulk concentrations of γ-tocopherol were highly variable but generally diminished in the AD brain (mean values ± SE were as follows in normal vs AD tissue: 3.98 ± 0.42 vs 3.24 ± 0.28 pmol/mg protein in the IPL, P < 0.10; 1.85 ± 0.82 vs 1.05 ± 0.21 pmol/mg in the SMTG, not significant; and 0.52 ± 0.09 vs 0.25 ± 0.04 pmol/mg in the CBL, P < 0.05). In the IPL, the loss of γ-tocopherol in the AD brain was almost numerically equal to the increase in 5-NO$_2$-γ-tocopherol (net increase in mean 5-NO$_2$-γ-tocopherol 0.77 pmol/mg protein; net loss of γ-tocopherol 0.74 pmol/mg). The greatest difference in 5-NO$_2$-γ-tocopherol content between AD and normal brain was seen in the IPL, followed by the SMTG, with no significant differences being noted in the CBL. The ratio of 5-NO$_2$-γ-tocopherol/γ-tocopherol in the cerebellum was relatively high, due to the relatively low concentration of the precursor γ-tocopherol in this brain region (Fig. 2). This brain regional pattern of tocopherol nitration is similar to the pattern observed in previous measurements of nitrotyrosine distribution (5).

### TABLE I

Nitrotyrosine/Tyrosine Ratios in Three Regions of the Alzheimer's Diseased Brain

<table>
<thead>
<tr>
<th>Region</th>
<th>3-NO$_2$-Tyr/Tyr ($\times$1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPL</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>SMTG</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>CBL</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>16 ± 5</td>
</tr>
<tr>
<td></td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Note. Data represent means ± SEM (condensed from Ref. 5).
50–70% in affected regions of the Alzheimer’s brain (25).

This enzyme may therefore provide a relevant tool for the in vitro comparison of putative RNS scavenging compounds. As illustrated in Fig. 3A, rat brain α-KGDH was inactivated by the peroxynitrite generator SIN-1 in a dose-dependent fashion, with approximate linear inactivation in the range of 0–0.5 mM SIN-1. A dose of 0.5 mM SIN-1 was therefore chosen in subsequent experiments to compare the potency of α-tocopherol and γ-tocopherol. Partial protection of enzyme activity was obtained with 0.1–10 μM γ-tocopherol (P < 0.05; Fig. 3B). Statistical assessment by ANOVA indicated a significant difference in efficacy between the two tocopherols (P < 0.05). Statistical assessment of individual tocopherol concentrations by Student’s t test indicated that α-tocopherol was significantly protective only at the 10 μM level (Fig. 3B). At higher tocopherol concentrations (above 10 μM α-tocopherol or 100 μM γ-tocopherol) assay solutions became turbid and accurate enzyme analysis was not possible.

DISCUSSION

The neuroinflammatory hypothesis of AD originated from seminal observations by McGeer et al. (13) that subjects taking nonsteroidal anti-inflammatory drugs had a lower incidence or slower progression of the disease than might be anticipated in the general population. The current version of the neuroinflammatory hypothesis states that a type of quasi-inflammation occurs in AD, perhaps originating as a result of amyloid accumulation in senile plaques. According to this model, the AD brain then undergoes an autocrine cycle of cytokine expression, glial activation, and oxidant production that facilitates progressive neurodegeneration. Unlike classical inflammation, diapedesis, edema, and humoral events are not considered part of neuroinflammation. One of the major predicted correlates of neuroinflammation is the expression of inducible nitric oxide synthase, which generates profligate quantities of NO and derived species such as peroxynitrite.

These reactive intermediates then attack proteins and lipids, causing cell damage. As expected, both protein and lipid nitration can be induced by exposure of cell cultures to inflammatory cytokines such as interleukin-1β (11, 14). Increased protein (5) and lipid nitration (this work) in the AD brain is therefore consistent with the neuroinflammatory concept. The findings in this and previous studies raise the issue of whether tocopherol content, particularly the distribution of tocopherol variants, might influence peroxynitrite chemistry in such a way as to affect cell viability during an inflammatory challenge. While α-tocopherol possesses a phenolic structure that accounts for its free radical scavenging capacity, there is no obvious space on the chromane ring system where an electrophilic nitration reaction might occur (Fig. 1). In contrast, the lack of a methyl group in the 5 position of γ-tocopherol might facilitate its ability to scavenge nitrating species. It has been suggested that peroxynitrite will preferentially localize to, and react with, lipid-phase components rather than aqueous tyrosine residues when both targets are available (15). One study reports that γ-tocopherol is preferentially consumed over α-tocopherol when both compounds are exposed to a peroxynitrite generator (16). A contradicting report indicates no sparing of α-tocopherol under similar conditions of peroxynitrite treatment (15). Few studies have

FIG. 3. (A) Inactivation of rat brain α-KGDH activity by the peroxynitrite generator SIN-1. Data represent the means ± standard error for a typical dose–response experiment (N = 6 reactions per dose). (B) Comparison of α-tocopherol (solid line) and γ-tocopherol (dashed line) as inhibitors of SIN-1 inactivation of α-KGDH. Data points with error bars represent means ± standard error for five or six experiments; *P < 0.05 relative to the positive control (SIN-1 only) by Student’s t test.
been published which address the relative efficacies of γ-tocopherol and α-tocopherol as inhibitors of peroxynitrite-induced cell or tissue damage. The experimental results presented in this study suggest that γ-tocopherol may protect some nitration-sensitive enzymes, particularly α-KGDH, against peroxynitrite-mediated inactivation in a manner quantitatively (if not qualitatively) different from the protection afforded by α-tocopherol.

These data add to a growing body of evidence that γ-tocopherol serves biochemical functions different from those of α-tocopherol. For instance, Jiang et al. recently reported that low micromolar concentrations of γ-tocopherol inhibit cyclooxygenase-2 activity in macrophages and epithelial cells, whereas α-tocopherol has little to no effect (26). In a very different work, Saldeen et al. reported that oral γ-tocopherol supplementation may be significantly more effective than α-tocopherol supplementation as a means of inhibiting thrombogenesis in a rat model of arterial thrombosis (27). Taken together, this small but nascent body of data argues for a closer examination of the relative biological effects of naturally occurring tocopherol homologs, particularly in systems in which nitrative stress is thought to be a relevant factor. The importance of γ-tocopherol may be grossly underestimated when considering nutritional influence on chronic diseases wherein lipid nitration may occur. Although γ-tocopherol is the principal tocopherol variant in the U.S. diet (17), α-tocopherol is preferentially packaged into lipoproteins in the liver (18). Human plasma concentrations of γ-tocopherol are typically 10% of the corresponding α-tocopherol concentration (19). Dietary supplementation with α-tocopherol will decrease plasma levels of γ-tocopherol (20), which may have significant importance for AD because AD patients are routinely given α-tocopherol in an attempt to slow disease progression. One clinical trial has shown a subtle but significant delay in AD progression with α-tocopherol supplementation (21), but no studies have specifically addressed the influence of γ-tocopherol supplementation in any human disease. It is conceivable that the beneficial effects of α-tocopherol supplementation are confounded by a diminution of γ-tocopherol pools in AD and other diseases in which lipid-phase nitration chemistry is a salient phenomenon. A better clinical paradigm might entail cosupplementation with γ-tocopherol. These issues will need to be addressed carefully in future work.

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REFERENCES


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