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Antioxidants significantly affect the formation of different classes of isoprostanes and neuroprostanes in rat cerebral synaptosomes

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Abstract

Lipid peroxidation has been implicated in the pathogenesis of a number of diseases, including neurodegenerative disorders. Evidence that antioxidants can affect the clinical course of neurodegenerative diseases is limited. In the present study, we examined the ability of five common antioxidants or antioxidant combinations, α-tocopherol, γ-tocopherol, ascorbic acid, GSH ethyl ester, and a combination of ascorbate and α-tocopherol, to modulate lipid peroxidation in peroxidizing rat cerebral synaptosomes, a well-characterized model of oxidant injury. In these studies, we quantified isoprostanes (IsoPs) derived from arachidonic acid as an index of whole tissue oxidation and neuroprostanes (NeuroPs) formed from docosahexaenoic acid as a marker of selective neuronal peroxidation. We report that these various antioxidants displayed markedly different capacities to inhibit IsoP and NeuroP formation with the most potent effects on IsoPs observed for ascorbate, GSH ethyl ester, and the α-tocopherol–ascorbate combination. α-Tocopherol was slightly less potent and γ-tocopherol significantly less effective. The concentration–response relationships were significantly different for NeuroP formation with the antioxidants being significantly less potent than for IsoP generation. In particular, α-tocopherol did not inhibit NeuroP formation at concentrations up to 100 μM. We also determined that tocopherols, in particular α-tocopherol, act *in vitro* as reducing agents to convert IsoP and NeuroP endoperoxides to reduced F-ring compounds, a finding we have observed previously *in vivo* in brain. These studies are of importance because they have further defined the role of antioxidants to modulate the formation of lipid peroxidation products in peroxidizing brain tissue. In addition, they suggest that α-tocopherol may not be a particularly effective agent to inhibit oxidant stress in the terminal compartment of neurons in the central nervous system.

Keywords: Oxidation; Brain; Isoprostane; Neuroprostane; Eicosanoid; Lipid

1. Introduction

Lipid peroxidation is a major consequence of oxidative damage to a variety of tissues *in vivo*, including the central nervous system [1,2]. A number of research groups have associated increased levels of biomarkers of lipid peroxidation in diseased regions of brain from patients with late-stage neurodegenerative diseases, including AD, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease, implicating oxidative injury in these disorders [3]. Various biomarkers of lipid peroxidation were employed in these studies, among them the measurement of IsoPs and NeuroPs, which are highly accurate quantitative markers of oxidant stress *in vivo* [4,5]. Indeed, in addition to their utility as tools to quantify oxidant stress in autopsy studies, we and others [6–9] have shown that members of one class of IsoPs, the F_2-IsoPs, are increased significantly in cerebrospinal fluid obtained from the lumbar cistern of humans with probable AD early in the course of their illness and in humans with mild-to-moderate Huntington's disease.

IsoPs and NeuroPs are formed from the free radical-initiated peroxidation of AA and DHA, respectively, with subsequent insertion of molecular oxygen followed by cyclization to form endoperoxide intermediates (Fig. 1) [4,5]. These intermediates can be reduced to form com-

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Abbreviations: AA, arachidonic acid; AAPH, 2,2′-azobis(2-amidinopropane)hydrochloride; AD, Alzheimer's disease; DHA, docosahexaenoic acid; IsoP, isoprostane; NeuroP, neuroprostane; and PG, prostaglandin.

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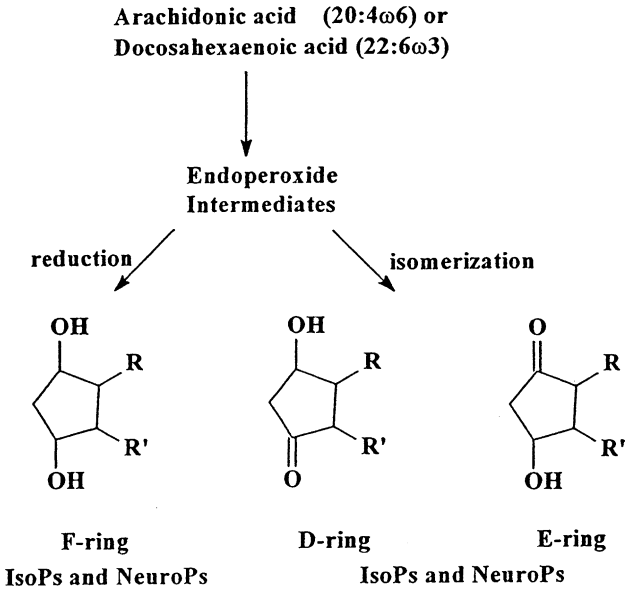

Fig. 1. Formation of different classes of IsoPs and NeuroPs generated from the peroxidation of arachidonic acid or docosahexaenoic acid, respectively. Reduction of endoperoxide intermediates yields F-ring compounds, while isomerization generates D/E-ring compounds.

pounds with an F-ring cyclopentane diol analogous to cyclooxygenase-generated $PGF_{2\alpha}$, or can isomerize to cyclopentane ketoalcohols with structures similar to PGD_2 and PGE_2 [5,10]. AA is distributed evenly among different cell types and regions of the brain, and we have shown that quantification of F_2-IsoPs plus D_2/E_2-IsoPs reflects oxidative damage to brain tissue as a whole. In contrast, DHA is highly concentrated in neuronal membranes, and the quantification of F_4- plus D_4/E_4-NeuroPs uniquely provides a selective tool to examine neuronal membrane oxidative damage [11]. While quantifying F- plus D/E-IsoPs and NeuroPs assesses the magnitude of oxidative damage to membranes, calculation of the F-ring to D- and E-ring (F/D+E) ratio of IsoPs and NeuroPs provides information regarding the reducing microenvironment in which oxidation occurs. In this regard, we have shown previously that the ratio of F-ring to D/E-IsoPs in peroxidizing rat liver microsomes is approximately 0.2 and that addition of GSH increases this ratio in a concentration-dependent manner [12]. In addition to providing biochemical information on lipid microenvironments, the F/D+E-IsoP ratio also may have physiologic significance since different classes of IsoPs have been shown to possess markedly different biological activities [13]. Thus, it is possible that modulation of the relative amounts of the different IsoP classes *in vivo* could alter biological responses to oxidative injury significantly.

The association between oxidative damage and neurodegenerative diseases has served as the basis for clinical trials of antioxidants, primarily α-tocopherol. Overall, the studies have shown a modest therapeutic effect of 2000 IU α-tocopherol/day in slowing the progression of AD, but no therapeutic effect from 2000 IU α-tocopherol/day in humans with Parkinson's disease [14,15]. The lack of a dramatic therapeutic response has dampened enthusiasm for the use of these antioxidants despite the correlation of enhanced lipid peroxidation in the central nervous system with progression of AD. A potential limitation of the studies noted above is that the dose of α-tocopherol required to maximally suppress lipid peroxidation in neurons is unknown. Further, the dose–effect relationship is not known for other commonly used antioxidants or combination of antioxidants.

We have studied the effects of α-tocopherol supplementation in a mouse model of age-related increases in oxidative damage to the cerebrum [16]. Interestingly, we observed that dietary supplementation with α-tocopherol significantly suppressed cerebral F_2-IsoPs plus D_2/E_2-IsoPs but not cerebral F_4-NeuroPs plus D_4/E_4-NeuroPs, suggesting that α-tocopherol may not be an effective antioxidant for neuronal membranes and potentially explaining, at least in part, the minimal effectiveness of α-tocopherol supplementation in patients with neurodegenerative diseases. Herein, we have investigated further the potency of α-tocopherol and other antioxidant supplements to limit oxidative damage to neuronal membranes *in vitro* by exploring the effects of these agents to modulate the formation of IsoPs and NeuroPs in peroxidizing rat cerebral synaptosomes.

2. Materials and methods

2.1. Materials

Dimethylformamide and undecane were purchased from the Aldrich Chemical Co. AAPH was from Kodak. [$^2H_4$]-8-*iso*-$PGF_{2\alpha}$ and [$^2H_4$]-PGE_2 were from Cayman Biochemicals. All other chemicals were obtained from the Sigma Chemical Co. *RRR*-α-Tocopherol (natural) was used in all studies. C-18 and Silica Sep-Pak cartridges were from Waters Associates. 60ALK6D TLC plates were from Whatman Ltd.

2.2. Preparation and incubation of synaptosomes

Synaptosomes were prepared according to previously published methods [10]. The final synaptosomal pellet was resuspended in PBS. For time–course experiments, synaptosomes were incubated at 37° with 1 mM AAPH for up to 3 hr. Without AAPH, lipid peroxidation products were less than 10% of those generated in the presence of AAPH. For experiments with antioxidants, synaptosomes were incubated with vehicle or vehicle plus antioxidant for 15 min at 37°, followed by the addition of AAPH to a final concentration of 1 mM, and subsequent incubation for an additional 2 hr. At the indicated time points, aliquots were removed, and the reaction was stopped by freezing at −80° until being analyzed for lipid peroxidation products. Synaptosomal protein was quantified as described. All studies

were performed at least in quadruplicate using different synaptosomal preparations.

2.3. Quantification of IsoPs and NeuroPs

Following extraction and addition of an internal standard, F-ring and D/E-ring IsoPs and NeuroPs were quantified using previously published stable isotope dilution methods employing gas chromatography/negative ion chemical ionization mass spectrometry with selective ion monitoring [5,10].

2.4. Statistics

Statistical analyses were performed using GraphPad Prism software.

3. Results

3.1. Formation of IsoPs and NeuroPs in peroxidizing rat synaptosomes

We first determined the time course for the generation of esterified (bound) and free IsoPs and NeuroPs in rat cerebral synaptosomal preparations (Fig. 2). Data represent the sum of F-ring and D/E-ring IsoPs and NeuroPs. Incubation of syntapsomes at 37° in buffer saturated with air in the presence of AAPH (1 mM) maximally increased esterified IsoPs 18.5-fold and esterified NeuroPs 14.6-fold in a time-dependent manner. ANOVA for bound NeuroPs in the presence of AAPH had a $P < 0.0001$, and Bonferroni's corrected repeated comparison tests showed that bound NeuroPs at 1, 2, and 3 hr were significantly greater than at time 0 ($P < 0.01$ at all time points); however, there was no significant difference between values at 2 and 3 hr, suggesting that the formation of NeuroPs had reached a maximum at about 2 hr, perhaps due to substrate depletion.

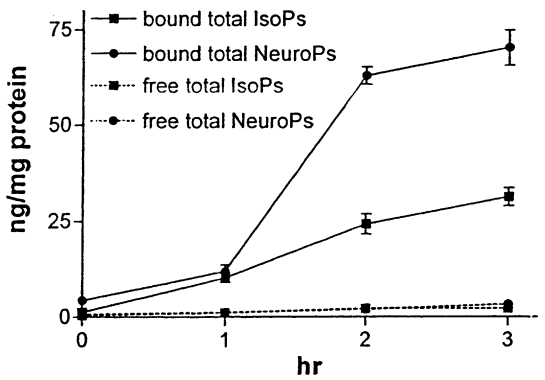

Fig. 2. Formation of bound (esterified) and free IsoPs and NeuroPs in peroxidizing rat cerebral synaptosomes over time. Total IsoPs and NeuroPs represent the sum of F-ring and D/E-ring compounds. Data are expressed per milligram of synaptosomal protein. Values are means ± SEM, N = 12 experiments.

ANOVA for bound IsoPs in the presence of AAPH was similar with $P < 0.0001$, and Bonferroni's repeated comparisons showing significant differences between all subsequent time points and time 0 ($P < 0.01$ for all tests), but not for 2 hr versus 3 hr.

Free IsoPs and NeuroPs were less than 5% of bound IsoPs and NeuroPs, respectively, at all time points. Although the concentration of free IsoPs and NeuroPs was small, levels of free compounds were correlated significantly with their bound counterparts: for IsoPs, $r^2 = 0.93$ and for NeuroPs, $r^2 = 0.90$.

In addition to comparing the absolute amount of IsoPs and NeuroPs, which provides information on the degree of lipid peroxidation in a particular biological fluid or tissue exposed to oxidative stress, calculating the relative amounts of F-ring to D/E-ring compounds, the F/D+E ratio, provides information on the reducing environment in which fatty acid oxidation occurs [12]. Thus, we examined the formation of various classes of IsoPs and NeuroPs in the above experiments. For both IsoPs and NeuroPs, at all times examined, generation of D/E-ring compounds predominated over F-ring compounds. Using the F/D+E ratio data for bound IsoPs and NeuroPs from the time–course experiments described in Fig. 2, two-way ANOVA for the F-ring to D/E-ring ratio at times 0, 1, 2, and 3 hr after initiating AAPH oxidation did not vary over time. The average ratio of F- to D/E-ring IsoP for all time points was 0.30 ± 0.03 (N = 12). The average ratio of F- to D/E-ring NeuroP for all time points was 0.27 ± 0.03 (N = 12). These results show that while large increases in IsoP and NeuroP formation occurred over time, there was no significant change in the relative amounts of different IsoPs and NeuroPs formed.

3.2. Effect of antioxidants on IsoP and NeuroP formation in rat cerebral synaptosomes

We next investigated how supplementation with antioxidants altered the amount and ratio of IsoPs and NeuroPs following incubation with AAPH (Figs. 3 and 4). Incubations were performed for 2 hr since the time–course study noted maximum IsoP and NeuroP generation at that time. Antioxidants studied included α-tocopherol, γ-tocopherol, ascorbic acid, the ethyl ester of GSH, and the combination of α-tocopherol and 100 μM ascorbate. Concentrations of each agent employed varied over the range of 1–100 μM and, thus, included physiologically relevant concentrations [12,16]. The concentration–response relationships for five antioxidants or combination of antioxidants on total IsoPs (Fig. 3A) and total NeuroPs (Fig. 3B) are expressed as the percent of IsoPs or NeuroPs formed from AAPH alone versus the log of antioxidant concentration ranging from 1 to 100 μM.

Ascorbate, GSH ester, and α-tocopherol plus ascorbate each had EC_{50} concentrations for suppression of AAPH-induced IsoPs that were <1 μM; the rank order for potency

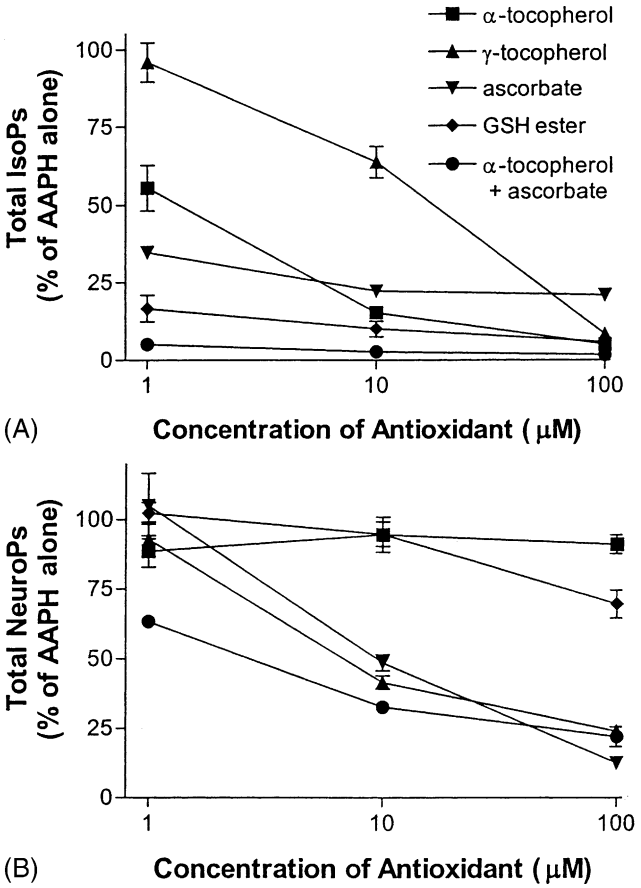

Fig. 3. Effect of different concentrations of antioxidants on the formation of total IsoPs (A) and NeuroPs (B) in peroxidizing rat cerebral synaptosomes. Data are expressed as a percent of oxidation without added antioxidants. Values are the mean percent ± SEM, N = 4 experiments. For isoprostanes, 100% = 24 ± 7 ng/mg protein; for neuroprostanes, 100% = 91 ± 29 ng/mg protein.

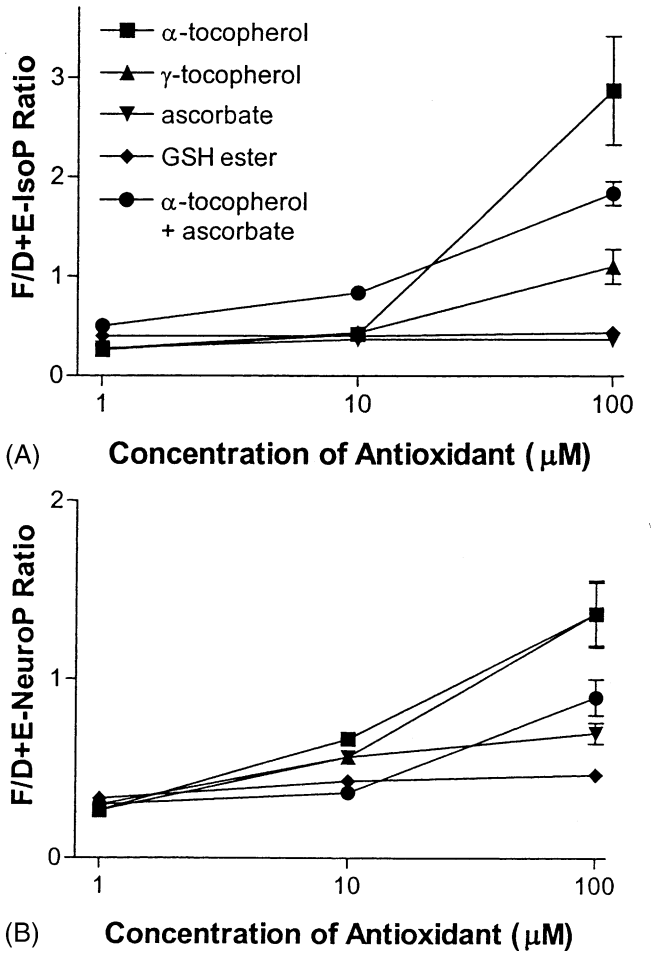

Fig. 4. (A) Effect of different concentrations of antioxidants on the formation of F-ring IsoPs to D/E-ring IsoPs in peroxidizing rat cerebral synaptosomes. Data are expressed as the ratio of F-ring to D/E-ring IsoPs. (B) Effect of different concentrations of antioxidants on the formation of F-ring NeuroPs to D/E-ring NeuroPs in peroxidizing rat cerebral synaptosomes. Data are expressed as the ratio of F-ring to D/E-ring NeuroPs. Values represent the mean percent ± SEM, N = 4 experiments.

at 1 μM was α-tocopherol plus ascorbate > GSH ester > ascorbate. Increasing concentrations of any of these antioxidants did not significantly suppress AAPH-induced IsoP formation further ($P > 0.05$). In contrast, α-tocopherol alone had an EC_{50} concentration for suppression of IsoPs that was 2 ± 1 μM, with a suppression of approximately 80% achieved at 10 μM ($P < 0.001$, compared to untreated controls). Interestingly, γ-tocopherol was the least potent antioxidant with respect to AAPH-induced IsoP formation; indeed, of all the antioxidants investigated, only γ-tocopherol did not suppress IsoP formation significantly at 1 μM. Increasing concentrations of γ-tocopherol displayed significant further suppression of IsoPs with an EC_{50} of 43 ± 6 μM and total suppression of IsoP formation at 100 μM ($P < 0.001$, compared to untreated controls). The concentration–response relationships for antioxidant suppression of AAPH-induced NeuroP formation (Fig. 3B) were quantitatively and qualitatively different from IsoPs. First, only the combination of α-tocopherol plus 100 μM ascorbate achieved significant NeuroP suppression at 1 μM ($P < 0.05$). Ascorbate, γ-tocopherol, and the combination of α-tocopherol plus ascorbate all resulted in the suppression of NeuroP formation with increasing concentrations; the EC_{50} value for each was approximately 10 μM. However, maximal suppression of NeuroP formation varied from 76 to 82%, less than that observed for IsoPs. Finally, both α-tocopherol and GSH ethyl ester were ineffective or much less effective at suppressing AAPH-induced NeuroP than IsoP formation. Indeed, α-tocopherol at concentrations up to 100 μM did not diminish NeuroP formation compared to untreated controls ($P > 0.05$). GSH ethyl ester also was ineffective at limiting NeuroP formation at 1 and 10 μM, but did suppress NeuroP formation at 100 μM by 30% compared to untreated controls ($P < 0.05$).

3.3. *Effects of antioxidants on the ratio of F-ring and D/E-ring IsoPs and NeuroPs*

The ratio of F/D+E-ring IsoPs was not significantly different when synaptosomes were incubated with any of the antioxidants at a concentration of 1 μM (Fig. 4A). The

average F/D+E ratio for IsoPs at a concentration of 1 μM for each antioxidant was 0.36 ± 0.05 ($N = 15$). Of the five antioxidants, only α-tocopherol plus ascorbate in combination increased the F/D+E-IsoP ratio at 10 μM compared to 1 μM ($P < 0.05$). At 100 μM, the rank order for increasing the F/D+E-IsoP ratio was α-tocopherol > α-tocopherol plus ascorbate > γ-tocopherol; all three of these antioxidants shifted the relative amounts of F-ring to D/E-ring IsoPs significantly when compared to AAPH alone (Bonferroni's corrected repeated pair comparisons all had $P < 0.01$). GSH ethyl ester and ascorbate alone only slightly altered the IsoP ratio at any concentration examined.

The effects of the antioxidants on the F/D+E-NeuroP ratio were qualitatively similar to the effects on the IsoP ratio (Fig. 4B); however, the shift toward F-ring NeuroPs was somewhat smaller than that observed for IsoPs. No antioxidant or combination of antioxidants at 1 μM significantly altered the F/D+E-NeuroP ratio from that observed with AAPH alone ($P > 0.05$). Effects, albeit fairly small, were observed at a concentration of 10 μM for all agents except GSH ethyl ester, with α-tocopherol exhibiting the largest effect. At concentrations of 100 μM, however, α-tocopherol, γ-tocopherol, ascorbate, and the combination of α-tocopherol and ascorbate significantly increased the F/D+E-NeuroP ratio (Bonferroni's corrected repeated pair comparisons all had $P < 0.01$). This finding is particularly interesting regarding α-tocopherol since this compound also markedly altered the F/D+E-NeuroP ratio *in vivo* in mouse brain tissue but did not affect the total NeuroPs formed in oxidizing rat cerebral synaptosomes or *in vivo* in mice [16]. The ability of α-tocopherol to alter the F/D+E-NeuroP and IsoP ratio appears to be a direct effect of the agent itself rather than by it acting as a co-factor for an enzymatic mechanism since the ratio was not altered when synaptosomes were boiled prior to incubation with AAPH and α-tocopherol. Similar to that observed for the IsoPs, the GSH ethyl ester only minimally altered the F/D+E-NeuroP ratio at concentrations up to 100 μM, and this effect was not statistically significant ($P > 0.05$). The rank order of antioxidants at 100 μM for increasing the F/D+E-NeuroP ratio was α-tocopherol ≥ γ-tocopherol > α-tocopherol plus ascorbate > ascorbate > GSH ethyl ester.

4. Discussion

The goal of the experiments reported herein was to determine the concentrations of commonly used antioxidant supplements required to alter lipid oxidation in cerebral tissue. In addition, we sought to further explore, *in vitro*, observations that we had made regarding the ability of α-tocopherol to reduce IsoP and NeuroP endoperoxides to F-ring compounds *in vivo* in brain tissue [16]. Therefore, we utilized a model of oxidation of rat brain synaptosomes to quantify IsoPs derived from AA that is evenly distributed in all regions of the brain and NeuroPs derived from DHA that is highly concentrated in neuronal membranes [17].

For these studies, we measured F-ring and D/E-ring IsoPs and NeuroPs that are formed from common endoperoxide intermediates of AA and DHA, respectively. In this way, quantification of the sum of F-ring plus D/E-ring compounds reflects the magnitude of oxidative damage while the F/D+E ratio reflects factors influencing the endoperoxide-reducing capability of the environment in which lipid peroxidation occurs [12]. The studies reported herein have determined the effective concentrations of commonly used antioxidants to suppress lipid peroxidation in the brain. Results from our experiments demonstrate that the antioxidants examined had significantly different potencies for suppressing the oxidation of AA and DHA. Only γ-tocopherol displayed a similar concentration–response relationship for the suppression of formation of IsoPs and NeuroPs. The other antioxidants and the α-tocopherol/ascorbate combination all were better at suppressing IsoP than NeuroP formation. Some of these differences spanned orders of magnitude; GSH ethyl ester and ascorbate had EC_{50} values <1 μM to limit IsoPs but approximately >100 and 10 μM, respectively, to limit NeuroP formation. Particularly striking was the difference in α-tocopherol activity for limiting IsoP and NeuroP formation. Alone, α-tocopherol potently suppressed IsoP generation with an EC_{50} approximating 2 μM and totally suppressed IsoP formation at 100 μM, concentrations ranging from physiologic to supplemented human plasma levels [16]. However, the same concentrations of α-tocopherol were completely ineffective at suppressing NeuroP formation. The same relative difference was observed when α-tocopherol was combined with 100 μM ascorbate; this combination completely suppressed IsoP formation to basal levels at 1 μM but achieved only approximately 80% suppression of NeuroPs at 100 μM. The reason for this marked difference between the ability of α-tocopherol to limit IsoP formation but not NeuroP generation is not clear, but these results confirm our observation made *in vivo* that while α-tocopherol does suppress oxidative damage to AA, even high concentrations of the agent do not limit oxidative damage to neuronal membranes as quantified by measuring NeuroPs [16]. One potential reason for the difference observed between the ability of α-tocopherol to inhibit IsoP but not NeuroP formation may be related to the primary location in the nervous system of each fatty acid. As noted, arachidonate is distributed evenly in all cell types of the nervous system, whereas DHA is predominantly found in neurons. In the present studies, an observed lack of an effect of α-tocopherol to inhibit DHA oxidation may be the result of its inability to enter and prevent oxidation selectively in the neuronal component of synaptosomes. Such a precedence exists for other tissue types, such as atherosclerotic vascular tissue, in which lipoprotein oxidation can occur despite the presence of large amounts of tocopherol [18]. It has been proposed that the Vitamin E

may be sequestered in a location in the vessel wall separate from lipoproteins. Of note from the data presented in Fig. 3B was the observation that lower concentrations of α-tocopherol (1 and 10 μM) combined with ascorbate (100 μM) resulted in the suppression of total NeuroPs to a lesser extent than found using ascorbate alone (100 μM). Such an effect did not occur when α-tocopherol was present at a concentration of 100 μM. One explanation for this observation may be related to the ability of α-tocopherol to act as a pro-oxidant at certain concentrations by generating the tocopheroxyl radical which is capable of promoting lipid peroxidation [19].

Our results examining the effects of antioxidants to modulate the ratio of F/D+E-IsoPs and NeuroPs also showed that higher concentrations of α-tocopherol, and to a lesser extent γ-tocopherol, can enhance the reduction of IsoP and NeuroP endoperoxides. In peroxidizing rat brain synaptosomes, as we reported in mouse brain *in vivo*, α-tocopherol markedly enhances the relative formation of F-ring IsoPs and NeuroPs in relation to D/E-ring IsoPs and NeuroPs [16]. Also of note, we found that the endoperoxide-reducing effect of α-tocopherol was blunted, to some extent, in the presence of ascorbate, suggesting that ascorbate may interfere with the endoperoxide-reducing capability of α-tocopherol.

The observation that tocopherols, and in particular α-tocopherol, appear to act to reduce IsoP and NeuroP endoperoxides is novel. It has been shown previously that the reduction of the cyclooxygenase-derived endoperoxide intermediate PGH_2 is catalyzed by reducing agents, including GSH and the NADPH-requiring enzyme PGF synthase [20,21]. A proposed mechanism to explain the reduction of endoperoxides by α-tocopherol is shown in Fig. 5. Electron transfer from tocopherol to the PGH-like IsoP and NeuroP endoperoxide intermediates gives a tocopheryl radical, a proton, and the radical-anion formed by O–O bond rupture. The intermediate radical-anion is subsequently converted to the F-series compounds by a second electron transfer or an H atom abstraction from α-tocopherol or lipid. We note that the tocopheryl radical itself may be an electron transfer donor capable of initiating endoperoxide reduction, a process that would occur with ring-opening of the tocopherol chroman ring to give a quinone product. In addition to α-tocopherol, γ-tocopherol is capable of reducing IsoP and NeuroP endoperoxides to F-ring compounds, albeit with less efficiency. It is hypothesized that γ-tocopherol is less active than α-tocopherol in this respect since α-tocopherol is better able to donate electrons owing to the increased electron density contributed by the methyl substitutions of the phenolic ring.

The data we report herein are to be contrasted somewhat with those we have published previously regarding compounds that modulate the oxidation of rat liver microsomes with Fe/ADP/ascorbate [12]. In those studies, we did find an effect of GSH on F-ring plus D/E-ring IsoP formation and also on the ratio of F/D+E-IsoPs, with GSH increasing the ratio of F-ring IsoPs to D/E-ring compounds. In the present studies, GSH ethyl ester significantly reduced total IsoP and NeuroP formation in oxidizing rat cerebral synaptosomes but increased the ratio of F/D+E-IsoPs and NeuroPs to a minimal extent. It is of interest to note that the effective concentration of GSH ethyl ester to inhibit oxidation in synaptosomes was in the micromolar range. This concentration of GSH is significantly lower that that present in tissues (millimolar range). It is possible that the potent antioxidant effect of GSH ethyl ester observed was related, in part, to its enhanced lipophilicity compared to free GSH, making it more likely to penetrate and act in lipid bilayers. In our previously reported studies using microsomes, only non-esterified GSH was employed. Another reason for the difference in the ability of GSH to alter the ratio of F/D+E-IsoPs in synaptosomes versus microsomes may

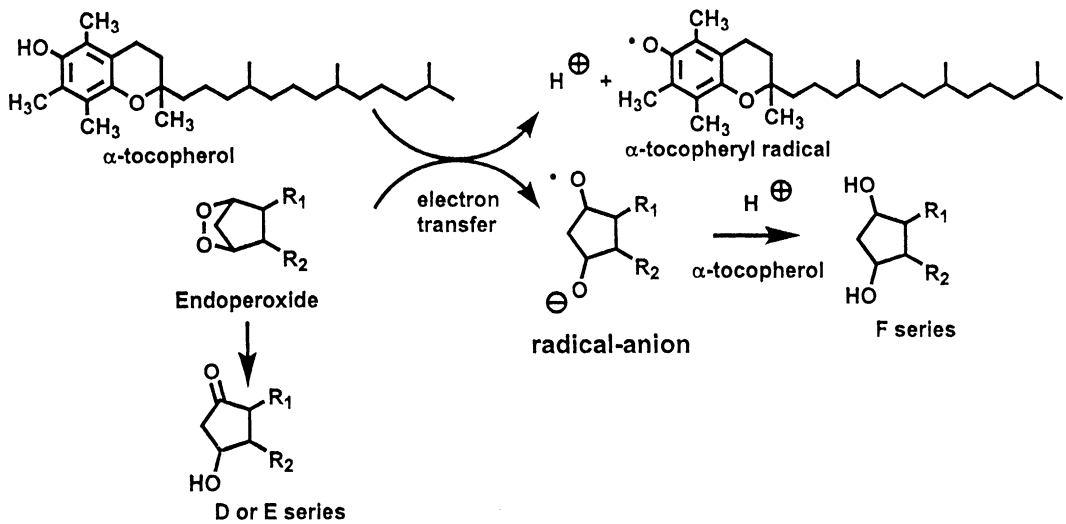

Fig. 5. Mechanism for reduction of IsoP and NeuroP endoperoxides by α-tocopherol. An electron transfer from tocopherol to the endoperoxide gives the tocopheryl radical and the reduced endoperoxide radical-anion. A second reduction of the radical-anion by electron transfer or hydrogen atom abstraction gives the F-series IsoPs or NeuroPs.

relate to the presence of different endogenous antioxidants present in the two membrane preparations that may have affected basal oxidant tone. In addition, our previous studies examining IsoP formation in microsomes utilized iron as the oxidizing agent, while the present studies employ AAPH.

The initial experiments reported herein investigated the effect of AAPH on oxidative damage to AA and DHA in rat cerebral synaptosomes. Using a concentration of AAPH that generates a constant, low level radical flux at 37°, we observed time-dependent increases in esterified IsoPs and NeuroPs. Previously, we have shown that AAPH-induced lipid peroxidation in rat cerebral synaptosomes is also concentration-dependent over the range of 1 to 10 mM AAPH [10]. Importantly, we have demonstrated here for the first time in tissue derived from the brain that free levels are only a small fraction (<5%) of esterified IsoP and NeuroP levels, establishing that bound levels are an accurate reflection of IsoP and NeuroP formation in this system. Moreover, we showed that concentrations of free IsoPs and NeuroPs are highly correlated with the levels of their bound counterparts, providing further support for the measurement of free IsoPs and NeuroPs in cerebrospinal fluid as surrogate markers of lipid peroxidation in brain.

In summary, we report that different antioxidants possess significantly different potencies for suppressing the oxidation of AA and DHA and the subsequent formation of IsoPs and NeuroPs. In addition, these compounds display marked variations in their ability to reduce IsoP and NeuroP endoperoxides. Of particular note, we found that α-tocopherol potently suppressed IsoP formation but not NeuroP formation in peroxidizing synaptosomes, as we had reported previously *in vivo* [16], suggesting that this agent may not be a particularly effective antioxidant to limit neuronal injury. In addition, the fact that tocopherols appear to act efficiently to reduce IsoP and NeuroP endoperoxides suggests that their presence in biological tissues may serve to favor the formation of different classes of IsoPs and NeuroPs over others.

Acknowledgments

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References

[1] Gutteridge JMC, Halliwell B. The measurement and mechanism of lipid peroxidation in biological systems. Trends Biochem Sci 1990;15:129–35.
[2] Smith MA, Perry G, Richey PL, Sayre LM, Anderson VE, Beal MF, Kowall N. Oxidative damage in Alzheimer's. Nature 1996;382:120–1.
[3] Montine TJ, Neely MD, Quinn JF, Beal MF, Markesbery WR, Roberts II LJ, Morrow JD. Lipid peroxidation in aging brain and Alzheimer's disease. Free Radic Biol Med 2002;33:620–6.
[4] Morrow JD, Roberts II LJ. Mass spectrometric quantification of F_2-isoprostanes in biological fluids and tissues as measure of oxidant stress. Methods Enzymol 1999;300:3–12.
[5] Roberts II LJ, Montine TJ, Markesbery WR, Tapper AR, Hardy P, Chemtob S, Dettbarn WD, Morrow JD. Formation of isoprostane-like compounds (neuroprostanes) *in vivo* from docosahexaenoic acid. J Biol Chem 1998;273:13605–12.
[6] Montine TJ, Kaye JA, Montine KS, McFarland L, Morrow JD, Quinn JF. Cerebrospinal fluid $A\beta_{42}$, tau, and F_2-isoprostane concentrations in patients with Alzheimer disease, other dementias, and age-matched controls. Arch Pathol Lab Med 2001;125:510–2.
[7] Montine TJ, Beal MF, Cudkowicz ME, O'Donnell H, Margolin RA, McFarland L, Bachrach AF, Zackert WE, Roberts LJ, Morrow JD. Increased CSF F_2-isoprostane concentration in probable AD. Neurology 1999;52:562–5.
[8] Montine TJ, Beal MF, Robertson D, Cudkowicz ME, Biaggioni I, O'Donnell H, Zackert WE, Roberts LJ, Morrow JD. Cerebrospinal fluid F_2-isoprostanes are elevated in Huntington's disease. Neurology 1999;52:1104–5.
[9] Praticò D, Clark CM, Lee VM-Y, Trojanowski JQ, Rokach J, FitzGerald GA. Increased 8,12-*iso*-iP$F_{2\alpha}$-VI in Alzheimer's disease: correlation of a noninvasive index of lipid peroxidation with disease severity. Ann Neurol 2000;48:809–12.
[10] Reich EE, Zackert WE, Brame CJ, Chen Y, Roberts II LJ, Hachey DL, Montine TJ, Morrow JD. Formation of novel D-ring and E-ring isoprostane-like compounds (D_4/E_4-neuroprostanes) *in vivo* from docosahexaenoic acid. Biochemistry 2000;39:2376–83.
[11] Moore SA. Cerebral endothelium and astrocytes cooperate in supplying docosahexaenoic acid to neurons. Adv Exp Med Biol 1993;331:229–33.
[12] Morrow JD, Roberts LJ, Daniel VC, Awad JA, Mirochnitchenko O, Swift LL, Burk RF. Comparison of formation of D_2/E_2-isoprostanes and F_2-isoprostanes *in vitro* and *in vivo*—effects of oxygen tension and glutathione. Arch Biochem Biophys 1998;353:160–71.
[13] Morrow JD, Roberts LJ. The isoprostanes: unique bioactive products of lipid peroxidation. Prog Lipid Res 1997;36:1–21.
[14] The Parkinson Study Group. Effect of deprenyl on the progression of disability in early Parkinson's disease. New Engl J Med 1989;321:1364–71.
[15] Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, Woodbury P, Growdon J, Cotman CW, Pfeiffer E, Schneider LS, Thal LJ for the Members of the Alzheimer's Disease Cooperative Study. A controlled trial of selegiline, alpha-tocopherol, or both as a treatment for Alzheimer's disease. New Engl J Med 1997;336:1216–22.
[16] Reich E, Montine KS, Gross MD, Roberts LJ, Swift LL, Morrow JD, Montine TM. Interactions between apolipoprotein E gene and dietary α-tocopherol influence cerebral oxidative damage in aged mice. J Neurosci 2001;21:5993–9.
[17] Salem N, Kim HY, Yergey JA. Docosahexaenoic acid: membrane function and metabolism. In: Simopoulos AP, Kifer RR, Martin RE, editors. Health effects of polyunsaturated acids in seafoods. New York: Academic Press; 1986. p. 263–317.
[18] Niu X, Zammit V, Upston JM, Dean RT, Stocker R. Coexistence of oxidized lipids and α-tocopherol in all lipoprotein density fractions isolated from advanced human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 1999;19:1708–18.
[19] Garner B, Witting PK, Wakdeck AR, Christison JK, Raftery M, Stocker R. Oxidation of high density lipoproteins. I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can be enhanced by α-tocopherol. J Biol Chem 1998;273:6080–7.
[20] Morrow JD, Harris TM, Roberts LJ. Noncyclooxygenase oxidative formation of a series of novel prostaglandins: analytical ramifications for measurement of eicosanoids. Anal Biochem 1990;184:1–10.
[21] Hayashi H, Fujii Y, Watanabe K, Urade Y, Hayaishi O. Enzymatic conversion of prostaglandin H_2 to prostaglandin $F_{2\alpha}$ by aldehyde reductase from human liver: comparison to the prostaglandin F synthetase from bovine lung. J Biol Chem 1989;264:1036–40.