Vitamin E Oxidation in Rat Liver Mitochondria†

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ABSTRACT: Antioxidant reactions of α-tocopherol (vitamin E, α-TH) were studied by examining the fate of α-TH during oxidative challenge to mitochondrial membranes. Rat liver mitochondria were exposed to increasing concentrations of the water-soluble radical initiator 2,2′-azobis(2-amidinopropane) dihydrochoride (ABAP), and damage was assessed by monitoring mitochondrial respiration, α-TH oxidation, and lipid peroxidation. Significant lipid peroxidation was observed after 50% of the initial α-TH was depleted. Oxidative damage produced by ABAP-generated peroxyl radicals inhibited mitochondrial use of O_2, as indicated by decreases in the respiratory control ratio and in state 3 and state 4 respiration. Rat liver mitochondria were supplemented with [^{14}C]-α-TH by incubation of liver homogenate with [^{14}C]-α-TH for 30 min at room temperature, followed by isolation of mitochondria by differential centrifugation. This supplementation resulted in a distribution of 83.7% and 14.3% of the added α-TH to the inner and outer mitochondrial membranes, respectively, which is similar to the distribution of endogenous α-TH. [^{14}C]-α-TH-supplemented mitochondria then were treated with ABAP, and α-TH oxidation products were identified by radiochromatographic analysis of mitochondrial extracts. Products observed included α-tocopherolquinone, α-tocopherolquinone-2,3-oxide, and α-tocopherolquinone-5,6-oxide, which were identified by comparing HPLC retention and UV spectra to those of authentic standards. Product identities were verified by GC−MS of product *O*-trimethylsilyl derivatives. Another product, which was identified by HPLC, UV, and mass spectral analysis as 8a-(ethyldioxy)tocopherone, was found to be an artifact of sample workup and was shown to be derived from 8a-hydroperoxytocopherone, which was formed by α-TH oxidation in the mitochondria. These results indicate that α-TH antioxidant reactions in mitochondria are similar to those identified in homogeneous solutions and model liposomal systems.

Vitamin E (α-TH;[1] Figure 1) is the primary lipid-soluble antioxidant in biological membranes. Lipid peroxyl radicals react with α-TH via hydrogen abstraction to produce lipid hydroperoxides and the α-tocopheroxyl radical **2** (eq 1).

$$\text{LOO}^{\bullet} + \alpha\text{-TH} \rightarrow \text{LOOH} + \mathbf{2} \qquad (1)$$

Radical **2** is a resonance-stabilized phenoxyl radical that does not readily propagate radical chain reactions. The effectiveness of α-TH as an antioxidant depends in large part on the fate of the α-tocopheroxyl radical. α-TH may be regenerated by cellular reductants, such as ascorbic acid (Tappel et al., 1961; Packer et al., 1979; Niki et al., 1985). This reaction may account for the synergism between α-TH and other antioxidants *in vitro* and may also extend the effectiveness of α-TH *in vivo*. Tocopheroxyl radicals that are not reduced to α-TH can further react with other peroxyl radicals to form nonradical products (eq 2). These latter reactions then account for oxidative α-TH turnover in biological membranes.

$$\mathbf{2} + \text{LOO}^{\bullet} \rightarrow \text{nonradical products} \qquad (2)$$

Reactions of peroxyl radicals with the α-tocopheroxyl radical follow two main pathways. The first forms 8a-substituted tocopherones and often involves the coupling of peroxyl radicals with the α-tocopheroxyl radical to form 8a-(alkyldioxy)tocopherones **3**, such as those formed when α-TH is oxidized by peroxyl radicals derived from the azo initiator 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) (Liebler et al., 1989; Yamauchi et al., 1989a), by methyl linoleate peroxyl radicals (Yamauchi et al., 1990), or by phospholipid-derived peroxyl radicals (Yamauchi et al., 1994). These products then can hydrolyze to form 8a-hydroxytocopherone (**6**) via the tocopherone cation **5**. 8a-Hydroxytocopherone then rearranges to α-tocopherol quinone (**4**) (α-TQ). 8a-Hydroxytocopherone also may be formed by electron transfer from the tocopheroxyl radical via the tocopherone cation **5** (Liebler & Burr, 1992). The second pathway involves the formation of isomeric epoxy-8a-hydroperoxytocopherones **7/8** and their hydrolysis products, epoxytocopherol quinones **9/10** (Liebler et al., 1990, 1991; Liebler & Burr, 1992). Although the epoxides are not decomposition products of 8a-substituted tocopherones, the mechanism by which they are formed is not known (Liebler et al., 1990). Oxidation of α-TH by the pathways described above results in a net consumption of two peroxyl radicals per molecule of α-TH oxidized.

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[1] Abbreviations: α-TH, α-tocopherol-*d*; α-TQ, α-tocopherol quinone; ADP, adenosine diphosphate; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride; BHT, 2,6-di-*tert*-butyl-4-methylphenol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GC−MS, gas chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; HPLC-ECD, high-performance liquid chromatography with electrochemical detection; MS, mass spectrometry; EI-MS, electron ionization mass spectrometry; NICI-MS, negative-ion chemical ionization mass spectrometry; MS−MS, tandem mass spectrometry; TMS, trimethylsilyl.

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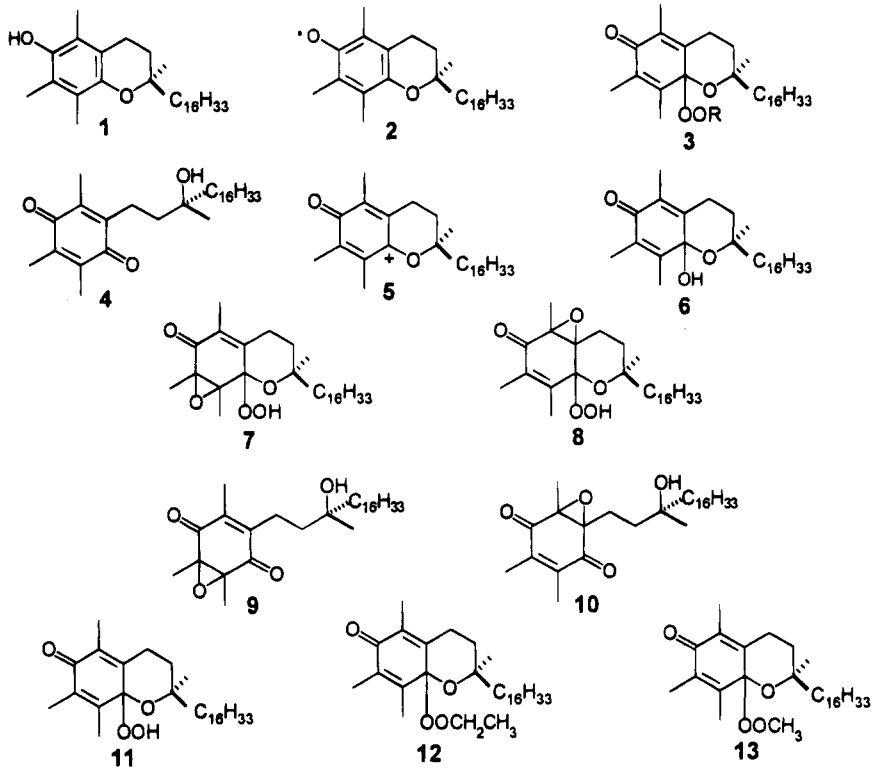

FIGURE 1: Structures of compounds referred to in the text.

In the present studies, our primary objective was to account for the fate of α-TH in a biological membrane system exposed to peroxyl radicals. Mitochondria provide a readily accessible biological membrane model whose functional integrity can be assessed by simple procedures for each experimental preparation. Mitochondria also are the principal site of cellular oxygen metabolism and are targets for oxidative damage (Richter & Frei, 1985; Turrens & Boveris, 1980; Turrens et al., 1985; Richter & Kass, 1991; Jones & Lash, 1993). The antioxidant status of mitochondria has been shown to be critical for cell survival (Meredith & Reed, 1982, 1983; Olafsdottir et al., 1988).

Here we have used a chemically defined oxidative challenge to reproducibly deplete mitochondrial α-TH and produce lipid peroxidation. We have identified oxidation products of α-TH consistent with peroxyl radical scavenging chemistry previously characterized in synthetic model systems. We also have studied the relationship between mitochondrial functional parameters, lipid peroxidation, and α-TH status in this experimental system.

EXPERIMENTAL PROCEDURES

Chemicals and Instrumentation. Sodium succinate, ADP, and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO). ABAP was purchased from Polysciences (Warrington, PA). [5-$^{14}CH_3$]-α-Tocopherol was synthesized as previously described (Urano et al., 1980; Liebler et al., 1990). [5-C^2H_3]-α-Tocopherol (α-TH-d_3) and [5,7-$(C^2H_3)_2$]-α-tocopherol (α-TH-d_6) were synthesized by the reduction of [5-C^2H_3]-α-tocopherol acetate and [5,7-$(C^2H_3)_2$]-α-tocopherol acetate with lithium aluminum hydride in tetrahydrofuran. The deuterated α-tocopherol acetates were generously provided by the Natural Source Vitamin E Association (Kingsport, TN). 8a-Hydroperoxytocopherone (**11**) was synthesized by photochemical oxidation of α-TH as previously described (Clough et al., 1979). HPLC analyses were done with a Spectra Physics 8800 solvent delivery system (Spectra Physics, San Jose, CA) equipped with an ESA Coulochem electrochemical detector with standard ESA guard and analytical cells (ESA Inc., Bedford, MA). Some HPLC analyses were done with a Hewlett-Packard 1050 HPLC system equipped with a 1040A diode-array detector. GC−MS analyses were done either with a Finnegan MAT-90 instrument (Finnegan MAT, Palo Alto, CA) equipped with a Varian 3400 gas chromatograph (Sunnyvale, CA) or with a Fisons MD800 instrument (Beverly, MA) equipped with a Carlo Erba 8035 gas chromatograph and an on-column injector.

Mitochondrial Preparation and Oxidant Challenge. Rat liver mitochondria were isolated from male Sprague-Dawley rats by an adaptation of the procedures of Yohana and Tampo (1987) with additional modifications described by Schnellmann et al. (1989). Livers were perfused *in situ* with ice-cold 0.9% saline, removed, and homogenized in 9 volumes of homogenization buffer (0.25 M sucrose, 1 mM EGTA, 5 mM Tris-HCl buffer, pH 7.4). The homogenate then was centrifuged at 600*g* for 10 min, and the resulting supernatant was centrifuged at 9000*g* for 15 min. The resulting pellet was washed three times with 0.25 M sucrose, and the final pellet was resuspended in homogenization buffer. Mitochondrial incubations contained 2 mg of protein mL^{-1} in an incubation medium containing 130 mM KCl, 9 mM Tris−phosphate buffer (pH 7.4), 4 mM Tris-HCl buffer (pH 7.4), and 1 mM EGTA and were done at 37 °C. Oxidations were initiated with the water-soluble azo initiator ABAP at concentrations varying from 2.5 to 50 mM. Mitochondrial respiration was measured with a Clark-type oxygen electrode (YSI, Inc., Yellow Springs, OH) in 3-mL suspensions containing 0.667 mg of protein mL^{-1}. State 4 respiration was initiated by the addition of 10 μmol of sodium succinate,

and state 3 respiration was initiated by the subsequent addition of 180 nmol of ADP.

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive substances as described by Beuge and Aust (1978) with the modification that 1 mg of BHT in 50 μL of 2-propanol was added to the samples to prevent adventitious oxidation. α-TH was extracted according to the procedure of Burton et al. (1985), with the modification that mitochondria were sonicated for 5 s with a microtip probe sonicator in the presence of all components of the extraction mixture.

Labeling of Mitochondrial α-TH with [^{14}C]-α-TH, α-TH-d_3, and α-TH-d_6. Mitochondria were labeled with [5-$^{14}CH_3$]-α-tocopherol, α-TH-d_3, or α-TH-d_6 by incubating the homogenate for 30 min in homogenization buffer at room temperature with a 100 μM concentration of the labeled α-TH. Mitochondria then were isolated by differential centrifugation as described above. Depletion of α-TH-d_6 and unlabeled α-TH was monitored by GC–MS analysis of TMS derivatives of the tocopherols extracted as described above with 140 pmol of α-TH-d_3 added as an internal standard. Extracts were evaporated under N_2 and converted to TMS derivatives by treatment with 50 μL of pyridine and 50 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, IL) at 65 °C for 1 h. The samples then were evaporated under N_2 and resuspended in 100 μL of hexane. Tocopherol TMS ethers were introduced by splitless injection at 250 °C and separated isothermally at 270 °C on a J&W DB-5 30 m × 0.25 mm column (Folsom, CA) and were analyzed by electron ionization MS at 70 eV on the Finnigan MAT 90 instrument. Tocopherols were detected by selected ion monitoring of the molecular ions at *m/z* 502 (unlabeled α-TH), 505 (α-TH-d_3), and 508 (α-TH-d_6).

To measure the amount of endogenous and supplemented α-TH in the inner and outer membranes of the mitochondria, α-TH-d_3-supplemented mitochondria were subjected to digitonin membrane fractionation by standard methods (Schnaitman & Greenwalt, 1968; Greenwalt, 1974). TMS ethers of endogenous (unlabeled) α-TH and supplemented α-TH-d_3 were quantified by GC–MS with α-TH-d_6 added as an internal standard.

α-TH Oxidation Product Identification. α-TH was analyzed by HPLC–ECD as described by Liebler et al. (1989). The formation of oxidation products was monitored by reverse-phase HPLC analysis of radiolabel from mitochondria supplemented with [^{14}C]-α-tocopherol. HPLC analyses were done on a Spherisorb ODS-2 5-μm 250 × 4.6 mm column eluted with methanol/1 N sodium acetate, pH 4.25 (93:7 v/v), for 30 min, followed by a 15-min gradient to 100% methanol and then by another 5-min gradient to methanol/ethyl acetate (50:50 v/v). Radiolabeled products were detected by collecting 0.8-min fractions, which were assayed for ^{14}C by liquid scintillation counting.

Tocopherolquinone (**4**) and epoxyquinones **9/10** were analyzed by GC–MS on the Fison MD-800 instrument as their TMS derivatives. 8a-(Alkyldioxy)tocopherone products were characterized by HPLC with diode-array detection (see above), by reduction to α-TH with ascorbate, and by hydrolysis to α-tocopherolquinone (**4**) in HCl (Liebler et al., 1989). NICI-MS and MS–MS of the 8a-(alkyldioxy)-tocopherone products were done on the Finnegan MAT-90 instrument described above by direct probe insertion.

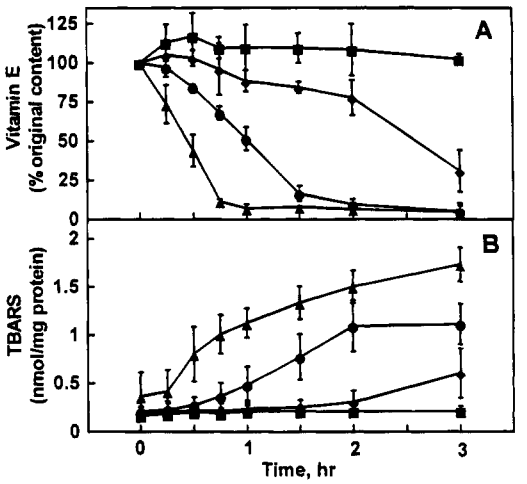

FIGURE 2: (A) Effect of ABAP concentration on α-TH content and lipid peroxidation in rat liver mitochondria incubated at 37 °C. Incubations contained no ABAP (■), 2.5 mM ABAP (◆), 10 mM ABAP (●), or 50 mM ABAP (▲). (B) Lipid peroxidation in rat liver mitochondria measured by the formation of thiobarbituric acid-reactive substances (TBARS). ABAP concentrations used are identical to those used in (A).

RESULTS

ABAP-Induced Vitamin E Depletion and Lipid Peroxidation. This investigation required a reproducible oxidative challenge in which loss of mitochondrial function was accompanied by lipid peroxidation and depletion of α-TH. When mitocondria were incubated under air or O_2, mitochondrial function gradually declined, as assessed by a decline in the respiratory control ratio, without significant α-TH depletion (data not shown). We therefore used the water-soluble azo initiator ABAP to induce oxidative damage through the production of peroxyl radicals. Azo initiators such as ABAP produce peroxyl radicals through thermal decomposition and addition of oxygen (eq 3 and 4). The

$$R{-}N{=}N{-}R \rightarrow 2R^{\bullet} + N_2 \quad (3)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (4)$$

slow decomposition of ABAP at millimolar concentrations produces peroxyl radicals at a nearly constant rate (Niki, 1990) and serves as a kinetically reproducible oxidative insult.

Incubation with increasing concentrations of ABAP decreased mitochondrial α-TH content in a concentration-dependent manner (Figure 2A). In incubations with 2.5 mM ABAP, rapid depletion of α-TH was preceded by a lag phase, during which α-TH was only slightly decreased. Incubations with 10 or 50 mM ABAP caused rapid depletion of α-TH without a lag phase. At all ABAP concentrations, lipid peroxidation, as measured by the formation of thiobarbituric acid-reactive substances, became significant only after approximately 50% of the α-TH had been depleted (Figure 2B).

Labeling and Depletion of the Mitochondrial α-TH Pool. To facilitate detection of the α-TH oxidation products, mitochondria were supplemented with [5-$^{14}CH_3$]-α-TH. The advantage of using a radiolabel was the ability to detect all α-TH products, regardless of their structures and properties,

Table 1: UV and MS Data for α-TH Oxidation Products

compound	UV λ_{max} (nm)	MS fragmentation (*m/z*)
α-TQ (**4**)	262, 268	503 (1.3%), 430 (27.6%), 341 (50.3%), 293 (100%), 165 (57.6%)[a]
α-TQE 1 (**9**)	272	341 (42.2%), 309 (100%), 267 (59.6%)[a]
α-TQE 2 (**10**)	272	341 (46%), 309 (100%), 211 (68.4%)[a]
8a-(ethyldioxy)tocopherone (**12**)	240, 290 sh[b]	490, 476, 461, 446, 429, 165[c] 475, 461, 446, 429, 165[d]
8a-(methyldioxy)tocopherone (**13**)	240, 290 sh[b]	476, 461, 446, 429, 165[c] 461, 446, 429, 165[e]
product from 8a-hydroperoxytocopherone reaction with ethanol	240, 290 sh[b]	490, 476, 446, 429, 165[c] 475, 459, 447, 430, 166[d]

[a] EI-MS of TMS derivative, relative intensities in parentheses. [b] sh represents shoulder peak. [c] NICI-MS of HPLC-purified product. [d] NICI-MS−MS (B/E-linked scan) analysis of *m/z* 490. [e] NICI-MS−MS (B/E-linked scan) analysis of *m/z* 476.

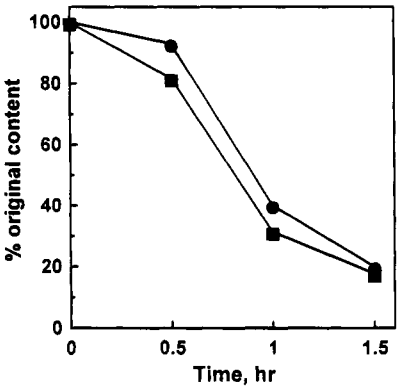

FIGURE 3: Depletion of endogenous α-TH (■) and supplemented α-TH-d_6 (●) in mitochondria treated with 50 mM ABAP. Mitochondria were supplemented with α-TH-d_6 as described under Experimental Procedures.

and to estimate product distribution by radiochromatographic analysis on reverse-phase HPLC. Supplementation was achieved by incubating labeled α-TH with liver homogenate, which contains cytosolic α-TH transport proteins (Sato et al., 1991, 1993) that have been previously shown to facilitate α-TH incorporation into subcellular membrane fractions *in vitro* (Murphy & Mavis, 1981). A potential problem with this approach is that, following supplementation, the added α-TH may be associated with mitochondrial membranes in a different way than the endogenous α-TH. To assess this possibility, we supplemented mitochondria with α-TH-d_6 and monitored the depletion kinetics of both the supplemented α-TH-d_6 and the endogenous α-TH. The assumption was that differences in depletion kinetics between the supplemented α-TH-d_6 and the endogenous α-TH would indicate possible differences in rates and pathways of reactions with peroxyl radicals. Depletion kinetics of both the supplemented α-TH-d_6 and the endogenous α-TH in mitochondria incubated with 50 mM ABAP over 90 min were virtually identical (Figure 3). This suggests that the fate of labeled α-TH in mitochondria supplemented by this method approximates that of endogenous mitochondrial α-TH.

To examine the distribution of the supplemented α-TH relative to the endogenous α-TH, mitochondria supplemented in the same manner with α-TH-d_3 were subjected to membrane fractionation by digitonin solubilization of the outer membrane. The outer membrane contained 91.5% of the endogenous and 83.7% of the supplemented α-TH, respectively, whereas the inner membrane contained 8.5% of the endogenous and 14.3% supplemented α-TH, respectively (Figure 4). The ratio of supplemented α-TH to endogenous α-TH in the inner membrane was slightly greater

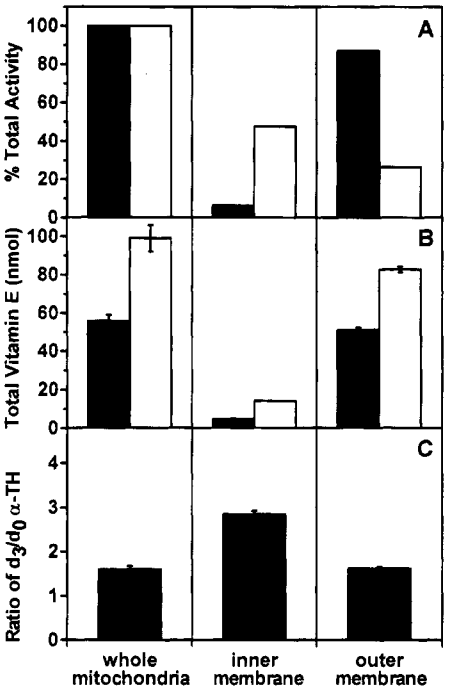

FIGURE 4: Separation of inner and outer membrane fractions of α-TH-d_3-supplemented mitochondria. Mitochondria were fractionated by digitonin solubilization as described under Experimental Procedures. Panel A depicts the distribution of the outer membrane marker monoamine oxidase (solid bar) and the inner membrane marker succinate dehydrogenase (open bar). Panel B depicts distribution of unlabeled, endogenous α-TH (solid bar) and added α-TH-d_3 (open bar). Panel C depicts ratios of α-TH-d_3/unlabeled in mitochondrial membrane fractions.

than in the outer membrane or in whole mitochondria. The distribution of the labeled α-TH indicates that the supplementation procedure labels the α-TH pools of both the inner and outer mitochondrial membranes and that the percentages of total mitochondrial α-TH in the two membrane fractions was similar to that of endogenous α-TH.

Oxidation Products of α-TH Formed during ABAP-Induced Oxidation. Figure 5 shows a representative radiochromatogram of products formed during incubation of [^{14}C]-α-TH-supplemented mitochondria with 10 mM ABAP. Peaks labeled **9** (2,3-epoxy-α-tocopherolquinone), **10** (5,6-epoxy-α-tocopherolquinone), and **4** (α-TQ) eluted at 11.5, 12.5, and 17.5 min, respectively. Identification of these oxidation products was based on chromatographic coelution with authentic standards, on UV spectra, and on GC−MS analysis of TMS derivatives of each product (Table 1). Peaks **9**, **10**, and **4** accounted for approximately 80% of the total products formed.

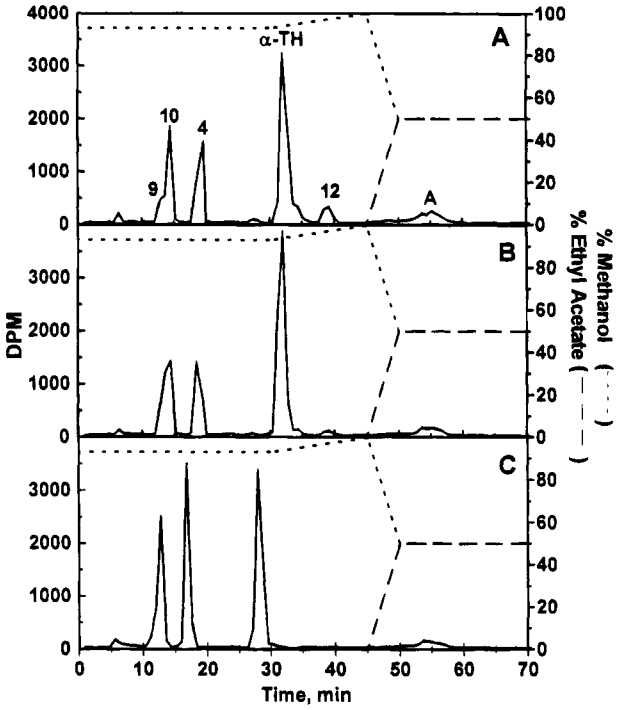

FIGURE 5: HPLC analysis of products formed during incubation of [^{14}C]-α-TH-supplemented mitochondria with 10 mM ABAP for 3 h. Product extracts either were analyzed directly without further treatment (A) or were treated with ascorbic acid (B) or HCl (C) prior to HPLC analysis as described under Experimental Procedures. Dashed lines depict the mobile phase composition. The initial mobile phase was 93:7 methanol:1 M sodium acetate, pH 4.25 (v/v).

Two other product groups eluting after α-TH (peaks **12** and **A**, Figure 5) displayed HPLC retention characteristics similar to that of 8a-substituted tocopherone products (i.e., elution shortly after α-TH) (Liebler et al., 1990). 8a-Substituted tocopherones **3** undergo a characteristic acid-catalyzed hydrolysis to α-TQ and are also readily reduced to α-TH by ascorbic acid at pH 3. When a hexane extract from the incubation mixture was evaporated, redissolved in ethanol, and treated with HCl, peak **12** was converted to α-TQ (Figure 5). On the other hand, treatment of the extract with ascrobic acid in sodium formate/ethanol, pH 3, converted peak **12** to α-TH. Peak **12** thus behaved as an 8a-substituted tocopherone. The UV spectrum of peak **12** exhibited an absorbance maximum at 240 nm with a shoulder at 290 nm, consistent with an 8a-substituted tocopherone chromophore (Table 1).

Peak **12** was collected, extracted into hexane, and analyzed by direct probe NICI-MS (Table 1). Although NICI typically yields only molecular ions, an apparent molecular ion at *m/z* 490 and several fragment ions were observed. Other studies in this laboratory indicate that, in contrast to other α-TH oxidation products, 8a-substituted tocopherones undergo fragmentation in NICI.[2] B/E-linked-scan MS−MS analysis of *m/z* 490 yielded a product ion spectrum similar to that observed in normal NICI-MS analysis. The product thus was assigned as 8a-(ethyldioxy)tocopherone (**12**). By analogy to other 8a-(alkyldioxy)tocopherones, **12** could be formed by addition of an ethylperoxyl radical to a tocopheroxyl radical. However, we felt that the formation of ethylperoxyl radicals was unlikely in this mitochondrial system. Therefore, we hypothesized that **12** instead was formed during sample workup by reaction with ethanol used in the extraction procedure. When sample workup was done with methanol, instead of ethanol, a product with an approximately 1 min shorter HPLC retention time was formed. NICI-MS and MS−MS analysis of this product were consistent with assignment as 8a-(methyldioxy)tocopherone (**13**) (Table 1). This suggested that products **12** and **13** were formed by reaction of some other α-TH oxidation product with the alcohols during sample preparation.

We postulated that 8a-(ethyldioxy)tocopherone was formed by the reaction of 8a-hydroperoxytocopherone (**11**) with ethanol (Figure 6). We synthesized **11** as described by Clough et al. (1979) and then dissolved it in ethanol and analyzed the product by HPLC using the gradient system described above. A product with a retention time and UV spectrum identical to 8a-(ethyldioxy)tocopherone (**12**) was identified and found to have an NICI mass spectrum identical with that of 8a-(ethyldioxy)tocopherone (**12**). B/E-linked-scan MS−MS analysis of *m/z* 490 confirmed this structure (Table 1). 8a-(Ethyldioxy)tocopherone (**12**) apparently results from solvolysis of 8a-hydroperoxytocopherone (**11**) during workup of mitochondrial extracts and isolation of **12** and provides evidence that **11** is a product of ABAP-initiated α-TH oxidation in mitochondria. Since 8a-hydroperoxytocopherone (**11**) elutes just prior to α-TQ (**4**) (~16.8 min), it is possible that this product was not detected because the collection intervals for fractions for radiochemical detection did not allow resolution of these two peaks. Peak **12** accounted for 10−15% of the total products formed during α-TH oxidation (data not shown).

Peak **A** was not affected by treatments with either ascorbic acid or HCl. This fraction, which apparently contained multiple products, displayed elution similar to that of a spirodimer oxidation product of α-TH (Yamauchi et al., 1989b). Peak **A** thus may be a group of α-TH-derived dimers. This group of products, which was not analyzed further, generally accounted for approximately 5% of the total oxidation products formed (data not shown).

Effects of ABAP Treatment on Mitochondrial Respiration. Incubation with ABAP decreased state 3 respiration, state 4 respiration, and the respiratory control ratio (Figure 7). The decrease in state 3 respiration was particularly apparent at 1 h with 50 mM ABAP, in which state 3 respiration was approximately one-tenth that in incubations without ABAP (Figure 7A). At 50 mM ABAP, state 4 respiration increased before it decreased (Figure 7B). A similar result was seen with 10 mM ABAP, except that the increase in state 4 respiration was delayed until 30 min. The ABAP-induced increase in the state 4 respiration rate eventually equaled that for state 3 respiration, so that the respiratory control ratio approached unity (Figure 7C). The increase in state 4 respiration was followed by a decrease in both state 3 and state 4 respiration to levels below those in incubations without ABAP. Incubation with 2.5 mM ABAP depressed respiratory control slightly relative to control incubations. The loss of respiratory control with 2.5 mM ABAP appeared to be due primarily to a decrease in state 3 respiration, which also occurred in control incubations. This differed from the higher ABAP concentrations in which the increase in state 4 respiration appeared to be primarily responsible for the

[2] D. C. Liebler and T. D. McClure, unpublished results.

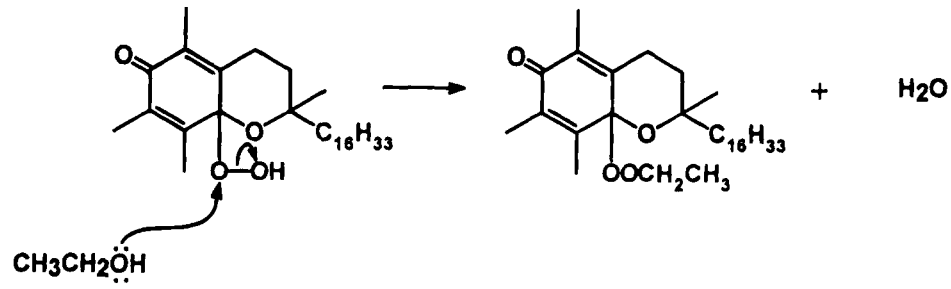

FIGURE 6: Proposed mechanism for solvolysis of 8a-hydroperoxytocopherone (**11**) to 8a-(ethyldioxy)tocophrone (**12**) in ethanol. See text for discussion.

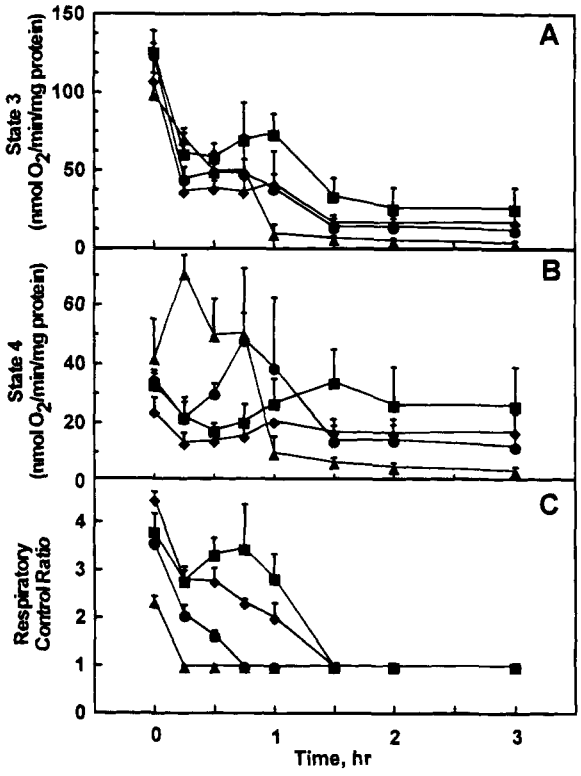

FIGURE 7: Effect of ABAP concentration on state 3 respiration (A), state 4 respiration (B), and the respiratory control ratio (C) in rat liver mitochondria incubated at 37 °C. Incubations contained no ABAP (■), 2.5 mM ABAP (◆), 10 mM ABAP (●), or 50 mM ABAP (▲).

decrease in the respiratory control ratio. Supplementation with unlabeled α-TH, which increased mitochondrial α-TH approximately 3-fold (see above), did not afford any additional protection against oxidative damage by ABAP. Changes in state 3 and state 4 respiration and the respiratory control were similar to those observed in unsupplemented mitochondria (data not shown).

DISCUSSION

The effectiveness of α-TH as an antioxidant depends not only upon its ability to trap peroxyl radicals via reaction 1 but also upon the ability of the resulting tocopheroxyl radical to trap additional peroxyl radicals via reaction 2. Products of this latter reaction may be useful markers for α-TH antioxidant chemistry in tissues. Here we provide the first description of the oxidative fate of α-TH in mitochondria during peroxyl radical oxidation. The distribution of α-TH oxidation products in mitochondria is similar to that previously found in liposome model systems. Thus, peroxyl radical trapping by α-TH in mitochondria apparently proceeds by the same mechanisms previously identified in model systems (Winterle et al., 1984; Yamauchi et al., 1989b; Liebler et al., 1990, 1991; Liebler & Burr, 1992).

Our first goal was to identify the oxidation products of α-TH produced in the mitochondria during ABAP-initiated oxidations. This was achieved by supplementing mitochondria *in vitro* with [^{14}C]-α-TH and monitoring the production of oxidation products by reverse-phase HPLC. The principal products formed were the epoxyquinones **9/10** and α-TQ (**4**), which accounted for approximately 80% of the α-TH oxidized. These products were formed in proportions similar to those in homogeneous solutions (Liebler et al., 1990) and in liposomal systems (Liebler et al., 1991; Liebler & Burr, 1992). The α-TH product distribution seen in our present experiments indicates that two competing peroxyl radical oxidation pathways, which were previously identified in chemical models, account for most α-TH oxidation in mitochondria exposed to peroxyl radicals. The first pathway yields epoxytocopherones **7/8**, which hydrolyze to epoxyquinones **9/10**. The second pathway yields 8a-(alkyldioxy)tocopherones **3**, which then hydrolyze to α-TQ. 8a-(Alkyldioxy)tocopherones **3** or 8a-hydroxytocopherone (**6**) is formed in homogeneous solutions and liposomal systems in which the lipid-soluble azo compound azobis(2,4-dimethylvaleronitrile) was the peroxyl radical initiator (Liebler et al., 1990, 1991). However, no evidence for the formation of analogous ABAP-derived tocopherones was found. These products would incorporate a highly polar 8a-alkyldioxy substituent and would be expected to exhibit different chromatographic and chemical properties from peak **12** or peak **A**. We recently have found that oxidation of α-TH by ABAP in acetonitrile/buffer mixtures yielded epoxyquinones **9/10** and α-TQ as the only products.[3] In addition, we found no evidence for the formation of a phospholipid-derived 8a-(alkyldioxy)tocopherone product analogous to that reported by Yamauchi et al. (1994). This was not unexpected, given that a peroxyl radical chain is not established in mitochondrial phospholipid until α-TH is largely depleted (see above). These considerations suggest that α-TQ and its precursor, 8a-hydroxytocopherone (**6**), are formed via the tocopherone cation **5**. This occurs either by dispropotionation of **2** or by electron transfer from **2** to a peroxyl radical (Liebler & Burr, 1992).

The isolation of 8a-(ethyldioxy)tocopherone (**12**) from ABAP-treated mitochondria provided evidence for the formation of 8a-hydroperoxytocopherone (**11**). This product is formed in low yield during peroxyl radical oxidations of α-TH (Matsuo et al., 1989; Liebler et al., 1990) and can

[3] D. C. Liebler and J. A. Burr, unpublished observations.

also form α-TQ by hydrolysis and rearrangement. Incomplete solvolysis with ethanol during product workup may have led us to underestimate the yield of tocopherone **11** at 10−15%. However, this yield is consistent with that estimated previously in solution oxidations (Liebler et al., 1990). In addition to the products described above, another group of products was formed which displayed retention characteristics in our HPLC system similar to those of dimers of α-TH. This second group of products was not previously observed in liposomal systems (Liebler et al., 1991; Liebler & Burr, 1992) and was only minor products of α-TH oxidation in mitochondria.

The next goal of this study was to investigate the relationship between functional parameters, lipid peroxidation, and α-TH status in this experimental system. ABAP produced concentration-dependent changes in mitochondrial respiration, in α-TH content, and in lipid peroxidation. It is important to distinguish differences in the deterioration of mitochondrial respiratory control with and without ABAP exposure. In incubations under an air atmosphere without ABAP, there was no time-dependent depletion of vitamin E and no lipid peroxidation, but the respiratory control ratio did decrease, due primarily to a decrease in state 3 respiration. This indicates that the decrease in the respiratory control ratio resulted from loss of the linkage of respiration to phosphorylation of ADP. These results are consistent with those observed by Khazanov et al. (1992) in which they demonstrated that mitochondria incubated in a medium saturated with air showed a decline in state 3 respiration, relative to mitochondria incubated in a medium maintained at physiological pO_2. The inhibition of respiratory function caused by ABAP was characterized by concentration-dependent changes in both state 3 and state 4 respiration and accompanied by depletion of α-TH and an increase in lipid peroxidation. At high concentrations of ABAP, the rapid decrease in respiratory control was the result of an initial increase in state 4 respiration, followed by decreases in state 3 and state 4 respiration. In mitochondria incubated without ABAP, loss of respiratory control instead reflected a decline in state 3 respiration. The pattern of damage produced by ABAP suggests an uncoupling of the mitochondria in which the protonmotive force that exists between the matrix and the intermembrane space may be altered before other components of the respiratory chain. This can result from the disturbance of the proton gradient or of the membrane potential across the inner membrane. Since ABAP is not a direct uncoupler of mitochondrial respiration (Kanno et al., 1994), this most likely occurs as a result of an alteration in the membrane fluidity or permeability due to lipid peroxidation. This is consistent with our data, since the lowest concentration of ABAP did not elicit any increase in state 4 respiration, and lipid peroxidation was not observed for 90 min after the loss of respiratory control (Figures 2 and 7). Lipid peroxidation in the 10 and 50 mM ABAP incubations became significant within 15 min after state 4 respiration increased and may not have been detected earlier due to sensitivity limits of the lipid peroxidation assay used.

Supplementation of the mitochondria with α-TH *in vitro* appeared to have no measurable protective effect against damage induced by ABAP. This could be due to several reasons. First, endogenous α-TH may be above a threshold-protective level such that more α-TH would not afford any additional protection. This seems unlikely, as α-TH is readily depleted by ABAP. Second, since the oxidant used in these studies was water-soluble, increased α-TH content in lipid membranes may afford minimal additional protection to critical proteins easily oxidized by a water-soluble oxidant such as ABAP. Water-soluble antioxidants (e.g., glutathione, ascorbate) may be more efficacious in the defense against ABAP-induced damage. Finally, although isolated mitochondria provide a useful model in which to study the peroxyl radical trapping reactions of α-TH, this system may not be appropriate for investigating protective effects of α-TH supplementation. The respiratory function of isolated mitochondria rapidly declines, as demonstrated by the rapid decline of state 3 respiration during the first 15 min of incubation, even in the absence of ABAP (Figure 7A). Further investigation of any protective role of increased α-TH on mitochondrial function will require the use of more physiologically intact systems.

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