Prolonged α-Tocopherol Deficiency Decreases Oxidative Stress and Unmasks α-Tocopherol-dependent Regulation of Mitochondrial Function in the Brain*

Received for publication, March 26, 2007, and in revised form, December 6, 2007 Published, JBC Papers in Press, January 7, 2008, DOI 10.1074/jbc.M702572200

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Vitamin E is the major lipid-soluble chain-breaking antioxidant in mammals and plays an important role in normal development and physiology. Deficiency (whether dietary or genetic) results in primarily nervous system pathology, including cerebellar neurodegeneration and progressive ataxia (abnormal gait). However, despite the widely acknowledged antioxidant properties of vitamin E, only a few studies have directly correlated levels of reactive oxygen species with vitamin E availability in animal models. We explored the relationship between vitamin E and reactive oxygen species in two mouse models of vitamin E deficiency: dietary deficiency and a genetic model (tocopherol transfer protein, Ttp+/− mice). Both groups of mice developed nearly complete depletion of α-tocopherol (the major tocopherol in vitamin E) in most organs, but not in the brain, which was relatively resistant to loss of α-tocopherol. F4-neuroprostanes, an index of lipid peroxidation, were unexpectedly lower in brains of deficient mice compared with controls. In vivo oxidation of dihydroethidium by superoxide radical was also significantly lower in brains of deficient animals. Superoxide production by brain mitochondria isolated from vitamin E-deficient and Ttp+/− mice, measured by electron paramagnetic resonance spectroscopy, demonstrated a biphasic dependence on exogenously added α-tocopherol. At low concentrations, α-tocopherol enhanced superoxide flux from mitochondria, a response that was reversed at higher concentrations. Here we propose a mechanism, supported by molecular modeling, to explain decreased superoxide production during α-tocopherol deficiency and speculate that this could be a beneficial response under conditions of α-tocopherol deficiency.

α-Tocopherol was first discovered as a micronutrient indispensable for reproduction in female rats (1). The view that α-tocopherol functions solely as a chain-breaking antioxidant has prevailed, reflecting a large body of accumulated experimental evidence showing that α-tocopherol can reduce lipid peroxidation in many models and the observation that α-tocopherol inhibits lipid peroxidation by scavenging peroxyl radicals faster (106 M−1 s−1) than they can react with other lipids (102 M−1 s−1) or proteins (2). Recently, however, a broad array of “nonantioxidant” cellular functions for α-tocopherol have emerged (reviewed in Refs. 3–8, but see Ref. 9 for the opposing view that supports a pure antioxidative function for α-tocopherol). The mechanism by which these “nonantioxidant” actions factor into the observed biological effects of α-tocopherol is not yet known. A systematic study of the relationship between levels of α-tocopherol and ROS production is one approach to begin to differentiate the documented ability of α-tocopherol to act as a lipid peroxidation inhibitor and antioxidant versus its other potential activities in vivo.

In the current study, we used two mouse models of vitamin E deficiency: 1) dietary deficiency (VED) in C57BL6 mice and 2) a genetic model, α-tocopherol transfer protein (TTP) knock-out mice. Although a number of studies have previously investigated the effect of dietary vitamin E deficiency, they have generally concentrated on liver, skeletal muscle, or heart (10–18). Most have not maintained deficiency for an extended period. In our study, we maintained mice on deficient diets for >9 months to obtain a high degree of α-tocopherol depletion, allowing us to carry out “add-back” experiments on depleted tissues. We focused primarily on the brain rather than peripheral tissues, because α-tocopherol deficiency has such profound effects on nervous system integrity and function. The two human congenital syndromes that result in severe vitamin E deficiency, abetalipoproteinemia, caused by deletion mutations in the apolipoprotein B gene, and AVED (ataxia of vitamin E deficiency), resulting from deletion mutations in the α-tocopherol transfer protein (TTP) gene, result in severe progressive cerebellar degeneration, ataxia, loss of deep tendon reflexes, and a number

* This work was supported by National Institutes of Health (NIH) Grant N541796 and a gift from the family of Selma I. Hartke (to L. L. D.); NIH Grants GM15431, DK48831, and CA77839 and a Burroughs Wellcome Award (to J. D. M.); and a Canby Robinson Society Award (to E. S. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 The abbreviations used are: ROS, reactive oxygen species; DEPMPO, 5-diethoxypyloseryl-5-methyl-1-pyrroline N-oxide; DHE, dihydroethidium; ETC, electron transport chain; HPLC, high performance liquid chromatography; RCR, respiratory control ratio; TTP, tocopherol transfer protein; VED, vitamin E-deficient; F2-Isop, F2-isoprostane.
of other neurological manifestations, with minimal pathology outside the nervous system (19). This indicates that the brain may be uniquely sensitive to α-tocopherol deficiency. Both of these deficiency syndromes can be improved by high dose vitamin E therapy, confirming that α-tocopherol deficiency is key to the nervous system pathology.

Here we examined the effects of α-tocopherol deficiency on levels of brain neuroprostane and isoprostanes, reported to be sensitive and specific indices of in vivo lipid peroxidation (20), superoxide radical production in vivo using confocal fluorescence imaging, mitochondrial respiratory function, and superoxide generation from isolated mitochondria using electron paramagnetic resonance spectroscopy and then correlated these findings with tissue levels of α-tocopherol.

MATERIALS AND METHODS

Chemicals and Reagents—(R,R,R)-α-Tocopherol (≥99.0% purity) was obtained from Sigma. Complete™ protease inhibitor mixture tablets were obtained from Roche Applied Science. L-α-Phosphatidylcholine type III/S, lipoxidas type IV, sodium deoxycholate, GSH, glutathione reductase, NADPH, Triton X-100 (peroxide-free), sodium azide, and other chemicals were obtained from Sigma. Dihydroethidium (DHE) was obtained from Molecular Probes, Inc. (Eugene, OR). 5-Diethoxysulfonyl-5-methyl-1-pyrroline N-oxide (DEPMPO) was purchased from Alexis Biochemicals (AXXORA, LLC, San Diego, CA). The primary antibody against mouse glutathione peroxidase 4 was a generous gift of Dr. Qiato Ran (University of Texas Health Science Center, San Antonio, TX).

Mouse Vitamin E Deficiency Models: Dietary Deficiency and TTP Knock-out Mice—Male C57B6J mice at 6–8 weeks of age were purchased from Jackson Laboratories and housed under standard conditions. Upon arrival, mice were randomly assigned to either control or vitamin E-deficient diets ad libitum. Diets were obtained from Bio-Serv (Frenchtown, NJ). Both diets were based on their AIN-93G formulation, with the vitamin E-deficient chow prepared from vitamin E-stripped corn oil. Ttp−/− (tocopherol transfer protein) breeders were obtained from Jackson Laboratories, and a colony was established to generate Ttp−/− mice. Ttp−/− mice were genotyped as described (21). Male knock-out mice were maintained on standard rodent chow starting at 3 weeks of age until sacrifice. Care and use of animals was approved by the Washington University School of Medicine Animal Studies Committee.

HPLC Determination of α-Tocopherol Tissue Concentrations—α-Tocopherols were quantified using a hexane extraction method and HPLC. Briefly, at sacrifice, animals were perfused with phosphate-buffered saline, and tissue samples were stored at −80 °C until analysis. Tissue samples (~75 mg) were homogenized in 1 ml of Tris-radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.4, 1% Igepal CA-630, 1% deoxycholate, 0.5% SDS, 150 mM NaCl, 1 mM EGTA, 1 mM molybdc acid, Complete™ protease inhibitor, and 1 μg/ml peptatin). For tocopherol quantification, 25 μl of 10 mg/ml butylated hydroxyl toluene in ethanol, 2 ml of ethanol, and 2 ml of hexane were added to the homogenate. The mixture was vortexed for 10 min at 4 °C and then spun at 3000 rpm for 2 min to separate the phases. 1 ml of the top hexane layer was removed to a new tube and dried under N2, and the lipid film was resuspended with a 500-μl mobile phase (88% methanol, 12% water, 5 mM sodium dihydrogen phosphate). The sample was then filtered through 3 μm Empore C8-high density 1-ml cartridges, with one 500-μl mobile phase wash, and injected onto an Agilent 1100 high performance liquid chromatograph. Samples were maintained at 4 °C. Separations were performed on a Zorbax SB-C8 4.6 × 150-mm column maintained at 38 °C, using the above mobile phase at 1 ml/min. Tocopherols were detected by an electrochemical detector (ESA Coulomb II model 5010 analytical cell with a model 5020 guard cell, ESA Inc., Bedford, MA). The electrochemical detector settings were as follows: guard cell, −750 mV; detector 1, −250 mV; detector 2, +550 mV. Quantification was done using peak areas and commercial standards.

Quantification of Neuroprostanes—F4-neuroprostanes and F2-isoprostanes were hydrolyzed and purified from the neocortex and quantified by gas chromatography/mass spectrometry as previously described (22, 23).

Confocal Imaging of Cell-specific in Vivo DHE Oxidation—To identify cell-specific superoxide formation, we employed systemic administration of the superoxide-sensitive fluorescent dye DHE (Molecular Probes) as described (24, 25). Control and vitamin E-deficient mice were injected intraperitoneally with DHE, and mice were then anesthetized and sacrificed 16 h after DHE injection. Mice were perfused with 4% paraformaldehyde in phosphate-buffered saline (50 ml/animal). Brains were then removed and postfixed in 2% paraformaldehyde for ~2 weeks. Cerebellar sections or coronal sections through the cortex at the level of the substantia nigra pars compacta (100 μm) were cut, washed with phosphate-buffered saline, and mounted. Fluorescence images were obtained using excitation λ of 488 nm and emission λ of >560 nm (590-nm long-pass filter) on a Zeiss LSM510 META confocal laser-scanning microscope equipped with 488- and 543-nm laser lines using a ×63 C-Apochromat (numerical aperture 1.2) water immersion objective. At least 15 cells/slice (3 slices/animal) were analyzed by an individual blind to the experimental group, and the treatment group “code” was only revealed after all analyses were complete.

Analysis of Mitochondrial Respiratory Function—Nonsynapsosomal brain mitochondria were isolated from phosphate-buffered saline-perfused whole brain using a Percoll gradient (26). Oxygen consumption was measured using a Clark-type oxygen electrode (Oxygraph™, Hansatech, Norfolk, UK). Purified mitochondria (~200 μg of protein) were added to the oxygraph chamber in a 1-ml solution containing 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM H3PO4, 0.05 mM EDTA, and 10 mM Tris-HCl, pH 7.4. After 2 min of equilibration, 5 mM pyruvate and 2.5 mM malate were added, and oxygen consumption followed for 2 min. ADP (250 μM) was added to measure state 3 (phosphorylating) respiration. Oligomycin (2.5 μg/ml) was added 2 min later to inhibit the F0F1-ATPase and determine state 4 (resting) respiration. The maximal uncoupled respiratory rate was obtained by adding 0.2 μM carbonylcyanide p-trifluoromethoxyphenyl-hydrazone to the chamber. Oxygen utilization traces and rate determinations were obtained using Oxygraph™ software. The respiratory control ratio (state 3
respiration/state 4 respiration) was calculated. Protein concentrations were quantified using the Pierce BCA microassay.

EPR Spectrometry on Isolated Brain Mitochondria—A Bruker e-scan benchtop EPR spectrometer was used to detect superoxide and hydroxyl radical generation by mitochondria, as described (25, 27). EPR spectra were acquired after the incubation of 1–2 mg of protein/ml of brain mitochondria isolated from different groups for 1 h at 37 °C. In each incubation, 10 mM pyruvate, 5 mM malate, and 1 mM ADP were included along with the desired concentration of (R,R,R)-α-tocopherol dissolved in absolute ethanol. In order to exclude the possibility that ethanol changed the free radical production from the isolated mitochondria, we included compensatory amounts of ethanol so that the final volumes of ethanol were always the same. In some experiments, 50 or 100 units/ml superoxide dismutase was included to confirm that the observed signal derived from superoxide. Each mixture was injected into the EPR cavity of the Bruker e-scan benchtop spectrometer via a Teflon tube with an inner diameter of ~0.4 mm. The EPR settings were as follows: receiver gain, 1 × 10³; scan width, 200 G centered at 3484.9 G; modulation amplitude, 2 or 4 G; time constant, 5.16 ms; modulation frequency, 86 kHz; microwave power, 5.04 milliwatts; sweep time, 5.24 s; spectrometer operating frequency, 9.784 GHz. Each spectrum was the average of 100 scans. To quantify free radicals, EPR intensities were normalized for protein contents.

Calculations and Modeling—Molecular model constructions, geometry optimization, and energy minimization for all molecules were carried out using the MM2 force field provided by CS Chem3D Ultra® version 7.0 (CambridgeSoft, Cambridge, MA) and then verified and refined using the BioMedCACHI WorkSystem Pro version 6.02 (Fujitsu America Inc.). Energy minimization of α-tocopherol and CoQ derivatives were performed under the Project Manager environment using the following procedures. First, a thorough conformational search was carried out within Mechanics using CAChe Augmented MM2 or MM3 parameters through the conjugate gradient method until the gradient was below 0.001 kcal/mol within 3000 steps. Then the unique minimum was fully optimized with MOPAC (PM3 or occasionally PM5 force fields). The PRECISE keyword was also used in order to increase the geometric and electronic convergence criteria. In some cases, the GEO-OK keyword was used to prevent calculation routines from unexpected termination.

Molecular docking studies of α-tocopherol and α-tocopheryl quinone into the yeast cytochrome cb (QCR) subunit using the BioMedCACHI suite of algorithms were done using the binding pocket of CoQ as a model for the binding mode. The crystal structure of the yeast QCR was selected as a model because of the availability of the x-ray structure of QCR binding with CoQH₂, which enabled the definition of the binding pocket hence used to adopt α-tocopherol. The x-ray structure of the monomeric unit of QCR at 2.3 Å resolution was downloaded from the Protein Data Bank site on the World Wide Web (code 1EZV) (28). After correcting the crystal structure of QCR for vanety and hydrogen bonding, CoQH₂ was redocked into its identified pocket in the cytochrome b (COB) subunit by holding the residues of the protein fixed and allowing the ligand to be flexible. The orientation and hydrogen bonding of the redocked complex were close to that in the crystal structure, and the flexibility of the protein residue of the active site was consequently allowed. The docking score was then calculated using a genetic algorithm (1000 generations) with a fast, simplified potential of mean force (grid spacing, 0.3 Å) (29).

The optimized structure of α-tocopherol (or α-tocopheryl quinone) was then superimposed on CoQH₂ in the binding pocket, the model of CoQH₂ was removed, and the model of α-tocopherol was docked into the pocket by holding the protein rigid and the ligand flexible before allowing a flexible active site and recalculation. In the final optimized complex, α-tocopherol and α-tocopheryl quinone were solely stabilized by hydrophobic interactions in the transmembrane domain of COB while retaining close positions around the heme b₅ group.

RESULTS

Brain α-Tocopherol Levels in VED and Ttp⁻/⁻ Mice—We observed an age-dependent increase in α-tocopherol levels in control brain, increasing from 0.01 nmol/mg protein in 1-week-old mice to 0.170 nmol/mg in 46- and 54-week-old mice (Fig. 1a). Accumulation of α-tocopherol in brain during development has been previously reported (11, 30); taken together, these results suggest that there is an active and age-dependent process for accumulating α-tocopherol in the brain.

While our study was under way, two separate groups published studies on Ttp⁻/⁻ mice. Both groups reported that liver α-tocopherol levels in Ttp⁻/⁻ mice were 30% of Ttp⁺/⁺ controls but that Ttp⁻/⁻ mice never accumulated α-tocopherol in brain, with levels <5% of controls (21, 31, 32). This suggests that tocopherol transfer protein may be critical for brain uptake of α-tocopherol, an observation consistent with data from humans with the AVED syndrome, who also show greater depletion of α-tocopherol in the brain compared with other tissues. In our study, we confirmed that brain α-tocopherol was nearly absent in Ttp⁻/⁻ mice (Ttp⁻/⁻: 0.094 ± 0.004 nmol/mg versus Ttp⁺/⁺: 0.005 ± 0.002 nmol/mg, p < 0.00001) despite moderate levels remaining in liver and other tissues (data not shown).

Neuroprostanes, a Specific Marker of Lipid Oxidation, Are Reduced in Vitamin E-deficient Mice—If vitamin E is acting solely as a chain-breaking antioxidant in vivo, we would predict higher levels of lipid peroxidation products during deficiency. We therefore analyzed the effect of vitamin E deficiency on F4-neuroprostane concentration in our two models of vitamin E deficiency. F4-neuroprostanes are derived from the radical-induced oxidation of docosahexanoic acid (22:6), which is
abundant in the brain. Surprisingly, F4-neuroprostanes in two brain regions, cortex and cerebellum, were significantly positively correlated with α-tocopherol levels (i.e., lower α-tocopherol levels were associated with lower F4-neuroprostanes) (Fig. 2a, r = 0.83, p = 0.04). Interestingly, the cerebellum, which is the brain region most affected in human α-tocopherol deficiency syndromes, had significantly higher levels of neuroprostanes in both deficient and control mice compared with other brain regions (boxed area, p = 0.001, t test), suggesting that the cerebellum may be uniquely vulnerable to ongoing lipid peroxidation, even under α-tocopherol-replete conditions.

Figure 1. α-Tocopherol content in brain (cortex) in control mice of various ages and in genetic (Ttp+/−) and dietary (VED) mouse models of deficiency. The concentration of α-tocopherol (nmol/mg protein) in control C57BL/6J mice maintained on normal chow at various ages is shown in a. b, mice maintained on VED for 38 or 54 weeks had significant depletion compared with age-matched controls. Values are mean ± S.E., *, p < 0.01. c, levels in Ttp+/− and Ttp−/− mice, also maintained on standard chow, were compared. By 10 weeks of age, less than 5% of α-tocopherol remained in Ttp−/− brain, as previously reported (21, 31, 32).

Figure 2. F4-Neuroprostanes in cortex and cerebellum are significantly correlated with α-tocopherol concentration. a, a positive correlation between F4-neuroprostanes and α-tocopherol concentration was observed in cortex (■, r = 0.83, S.D. = 2.86, p = 0.04) and cerebellum (box, △). b, F4-neuroprostanes increase with age (p = 0.02, analysis of variance). Data are mean ± S.E.

Isoprostanes Are Not Elevated in Vitamin E-deficient Mice—As an additional measure of lipid peroxidation, we analyzed F2-isoprostanes (F2-IsoPs), prostaglandin-like compounds formed from peroxidation of arachidonic acid independent of cyclooxygenase. Brain levels of F2-IsoPs in control and VED at 40 weeks of vitamin E deficiency were as follows (in pg/mg; mean ± S.D., n = 3): 1.3 ± 0.1 (control) and 1.2 ± 0.1 (VED) for cortex, 1.5 ± 0.3 (control) and 1.2 ± 0.1 (VED) for cerebellum, and 1.4 ± 0.2 (control) and 1.4 ± 0.1 (VED) for basal ganglia. There was a nonsignificant trend toward lower F2-IsoP levels in cortex and cerebellum from VED mice, but in reviewing the literature reporting F2-IsoPs in brain, we found that our values were at the lowest limits of sensitivity for this assay, thereby limiting our ability to detect a further decrease in F2-IsoP levels.

Effects of α-Tocopherol on Mitochondrial ROS
proteins between control and VED mice (data not shown). Unexpectedly, we also failed to observe a difference in these antioxidant expression for Sod1, Sod2, catalase, or GPx1 (glutathione peroxidation. Either mechanism would result in less lipid peroxidation. To antioxidant defenses or decreased levels of cellular ROS production, we carried out Western immunoblot analysis on brain extracts but found no differences in protein assessment antioxidant systems, we carried out studies to expression, we determined the maximal carbonylcyanide oxygen consumption was observed (Table 1), indicating that α-tocopherol deficiency does not alter mitochondrial yield or viability during the isolation procedure, we compared the wet weight of brain, total brain protein, and mitochondrial protein between control and VED groups. Brain weight (mean ± S.D.) was 460 ± 22 mg (control) and 455 ± 61 mg (VED). Total protein (mean ± S.D.) was 11.0 ± 1.3 mg/ml (control cortex) and 12.7 ± 1.2 mg/ml (VED cortex), 3.52 ± 0.53 mg/ml (control cerebellum), and 3.64 ± 0.53 mg/ml (VED cerebellum). Mitochondria protein was 1.3 ± 0.44 mg/ml (control) and 1.24 ± 0.22 mg/ml (VED). It is clear from these data that there was no significant difference between control and VED mitochondria yield. We then compared the activity of individual ETC complexes of cortical mitochondria from VED versus control mice. No significant change was observed upon VE deficiency for any of the complexes. The data, as a percentage of control (mean ± S.E.) at 10 weeks of age. Mitochondrial respiration was measured in an oxymetry chamber equipped with a Clark-type O2 electrode and standard respiratory substrates and electron transport chain inhibitors.

In Vivo Superoxide Production Is Lower in VED Mice—Our observation that neuroprostane levels are lower with vitamin E deficiency could reflect either a compensatory enhancement of antioxidant defenses or decreased levels of cellular ROS production. Either mechanism would result in less lipid peroxidation. To assess antioxidant systems, we carried out Western immunoblot analysis on brain extracts but found no differences in protein expression for Sod1, Sod2, catalase, or GPx1 (glutathione peroxidase). Between control and VED mice (data not shown). Unexpectedly, we also failed to observe a difference in these antioxidant proteins between Ttp<sup>+/+</sup> and Ttp<sup>−/−</sup> mice. Because not only GPx1 but GPx4 is important for the repair of oxidized lipids, we also measured the activity of GPx4 but again failed to observe any difference in activity between groups (data not shown).

Since antioxidant defenses were not overtly different between control and deficient mice, we carried out studies to look at ROS generation. Superoxide radical is a major product of many ROS-producing biological systems (e.g. mitochondria, various oxidases, P450 enzymes) (33) and can cause lipid peroxidation through a number of established reaction pathways. Cellular production of superoxide in vivo, evaluated by confocal fluorescence imaging of DHE oxidation in control and VED mice after 38 weeks of deficiency or in Ttp<sup>−/−</sup> mice versus wild-type controls indicated that both VED and Ttp<sup>−/−</sup> mice had significantly less superoxide production in hippocampus than their respective controls (Fig. 3) (38 weeks of VED versus control, p = 0.0004; Ttp<sup>−/−</sup> versus Ttp<sup>+/+</sup>, p = 0.004). Superoxide production in Ttp<sup>−/−</sup> mice was also significantly reduced in cortex (p = 0.046).

Mitochondrial Respiratory Response to Vitamin E Deficiency—Mitochondria are generally assumed to be the major source of ROS production in cells. Because changes in mitochondrial respiratory function and coupling may modify ROS production, we evaluated respiration in isolated brain mitochondria from all groups of mice. Uncoupling results in O₂ consumption that is not associated with ATP production and is often expressed as a lower respiratory control ratio (RCR) (state 3/state 4 respiration). Mitochondria were isolated by Percoll gradient from the brains of control and vitamin E-deficient mice after 38 weeks of deficiency and from Ttp<sup>+/+</sup> control and Ttp<sup>−/−</sup> mice at 10 weeks of age. Mitochondrial respiration was measured in an oxygraph chamber equipped with a Clark-type O₂ electrode and standard respiratory substrates and electron transport chain inhibitors.

To verify that vitamin E deficiency did not alter mitochondrial yield or viability during the isolation procedure, we compared the wet weight of brain, total brain protein, and mitochondrial protein between control and VED groups. Brain weight (mean ± S.D.) was 460 ± 22 mg (control) and 455 ± 61 mg (VED). Total protein (mean ± S.D.) was 11.0 ± 1.3 mg/ml (control cortex) and 12.7 ± 1.2 mg/ml (VED cortex), 3.52 ± 0.53 mg/ml (control cerebellum), and 3.64 ± 0.53 mg/ml (VED cerebellum). Mitochondria protein was 1.3 ± 0.44 mg/ml (control) and 1.24 ± 0.22 mg/ml (VED). It is clear from these data that there was no significant difference between control and VED mitochondria yield. We then compared the activity of individual ETC complexes of cortical mitochondria from VED versus control mice. No significant change was observed upon VE deficiency for any of the complexes. The data, as a percentage of control (mean ± S.E.) were as follows: complex I, 139 ± 36%; complex II/III, 110 ± 3%; complex IV, 95 ± 8%). Finally, we determined the maximal carbonylcyanide p-trifluoromethoxyphenyl-hydrazone-induced mitochondrial respiration, which provides a specific measure of the activity, number, and viability of the ETC components (complexes I, II, and III) by driving the ETC to maximal activity due to dissipation of the proton gradient by carbonylcyanide p-trifluoromethoxyphenyl-hydrazone. Again, no significant differences between control and VED mice in carbonylcyanide p-trifluoromethoxyphenyl-hydrazone-induced oxygen consumption was observed (Table 1), indicating that α-tocopherol deficiency does not alter yield, active content, or viability of mitochondria isolated from brain.

**TABLE 1**

| Respiratory parameters of mitochondria isolated from cortex of C57BL/6J mice with α-tocopherol deficiencies |
|-------------------------------------------------|
| State 3 | State 4 | RCR | FCCP |
|---------|--------|-----|------|
| Control | 539 ± 99 | 98.0 ± 17 | 6.1 ± 0.5 | 403 ± 94 |
| VED (46 weeks) | 503 ± 157 | 116 ± 32 | 4.4 ± 0.1 | 397 ± 63 |
| Ttp<sup>−/−</sup> | 341 ± 15 | 82 ± 8 | 4.5 ± 0.5 | 153 ± 67 |
| Ttp<sup>−/−</sup> | 278 ± 13 | 119 ± 14 | 2.7 ± 0.2 | 117 ± 81 |

* Statistically significant differences in comparison with control groups (p < 0.05, analysis of variance).
The Readdition of α-Tocopherol to Deficient Mitochondria Causes a Biphasic Increase in Superoxide Production—As discussed above, vitamin E deficiency resulted in altered mitochondrial respiratory function. Changes in respiratory coupling can, in turn, modulate mitochondrial ROS production. In order to explore the relationship between mitochondrial ROS production and vitamin E deficiency, we employed spin-trapping EPR spectroscopy to measure mitochondrial ROS directly. We used a relatively new nitroxide spin trap, DEPMPO, currently the most efficient spin trap reported for superoxide due to the lifetime of its superoxide adduct, which is sufficiently long to allow detection and quantification of superoxide radical production by mitochondria (24, 25, 34). In previous EPR studies on mitochondria, electron transport chain (ETC) inhibitors, such as rotenone (of complex I) or antimycin A (complex III), were required to produce an observable EPR signal. However, in our studies, with the improved sensitivity provided by our system, we were able to avoid the use of these inhibitors, allowing basal production of ROS from the mitochondrial respiratory chain to be assessed under conditions that may better match the physiology of in situ mitochondria. In Fig. 5, we show the effect of exogenously added (R,R,R)-α-tocopherol on mitochondrial superoxide generation from the brains of all studied groups. Fig. 5a shows a representative EPR response to α-tocopherol. For these experiments, to eliminate any effect of the vehicle (ethanol), α-tocopherol was always dissolved in the same final volume of ethanol. The ethanol-alone condition was
also included. We also confirmed that there was no effect of the order in which samples were analyzed by randomizing the order of EPR measurement after a fixed incubation time. Finally, from spectral simulations, we concluded that the observed signal is attributable to the DEPMPO-OH adduct, which in turn originated from superoxide, since the signal was completely abolished upon the inclusion of 100 units/ml superoxide dismutase in the incubation (data not shown).

As shown in Fig. 5b, exogenously added α-tocopherol affected mitochondrial superoxide production in a biphasic manner. In other words, superoxide signals increased upon the addition of up to 1.8 ± 0.2 (control), 2.2 ± 0.3 (VED), or 3.8 ± 0.8 (Ttp−/−) nmol of α-tocopherol/mg of mitochondrial protein but decreased with higher exogenous α-tocopherol concentrations (Fig. 5c). Although a relationship between initial α-tocopherol concentration and the amount of exogenous α-tocopherol required to reach maximal superoxide production was noted (Fig. 5b, Ttp−/− > VED > control), this did not quite reach significance. However, the concentration of α-tocopherol producing the maximum EPR signal (Cαmax, Fig. 5, b and c) from mitochondria isolated from Ttp−/− mice was significantly higher than that isolated from either control or VED mice. The value of Cαmax for Ttp−/− mice normalized for mitochondrial protein content (3.8 ± 0.8 nmol of α-tocopherol/mg of mitochondrial protein) represents the threshold concentration below which α-tocopherol appears to promote superoxide production. The further addition of α-tocopherol above that value reversed the increase in superoxide.

Finally, to determine whether α-tocopherol might be functioning as a pro-oxidant in these studies (e.g. by direct redox cycling) instead of by altering ROS produced by the mitochondrial electron transport chain, a series of experiments were performed. Using increasing concentrations of α-tocopherol in a superoxide-generating system (hypoxanthine/xanthine oxidase), we failed to observe enhanced superoxide production by EPR, and in fact, α-tocopherol decreased the superoxide signal in a dose-dependent manner, as expected and as previously reported (35). Thus, our data support an effect of α-tocopherol on mitochondrial superoxide production when α-tocopherol is extremely depleted, but as levels increase, this response is reversed.

**DISCUSSION**

Although it has been previously noted that brain is more resistant to vitamin E depletion than most other tissues (36), those studies suggested a lag in depletion rather than a complete resistance to α-tocopherol loss. In contrast, we found that even in a prolonged dietary model of vitamin E deficiency, the nervous system avidly retained α-tocopherol. In our study, even after 54 weeks of deficiency, ~55% of brain α-tocopherol remained (Fig. 1), whereas other tissues retained only 5–20% of control levels. Our findings support the idea that the brain is especially efficient at conserving α-tocopherol and may partially explain why neurological dysfunction is rarely observed in patients who have impaired uptake of vitamin E (due to intestinal bypass or other surgeries that limit transport) even after many years of chronic deficiency (37). It is also consistent with the known importance of α-tocopherol for normal neurological development and function (i.e. nervous system α-tocopherol levels appear to be preserved at the expense of other tissues in the body), and when brain levels are ultimately depleted, neurological impairment and degeneration ensue. We also observed an age-dependent increase in brain α-tocopherol in mice on a normal diet (Fig. 1), a phenomenon that has been previously reported (30). Interestingly, in humans, brain α-tocopherol also increases with age, with relatively high levels in the elderly (30).

We compared tissue concentrations of α-tocopherol and F4-neuroprostanes in two brain regions (Fig. 2) and found a significant correlation between lower α-tocopherol and decreased F4-neuroprostanes. Our results are supported by a study by Reich et al. (38), who investigated the interaction of apolipoprotein E and dietary vitamin E and included data indicating that dietary vitamin E deficiency for 39 weeks did not lead to an increase in isoprostanes or neuroprostanes in control (wild-type) mice. In that study, as expected, vitamin E supplementation caused a decrease in isoprostanes and neuroprostanes. Our results only indicate that lack of vitamin E reduces neuroprostanes below control levels, not that α-tocopherol elevates lipid peroxidation. In this view, levels of lipid peroxidation in control animals should be viewed as the “normal” or baseline levels to which levels in the deficient animals are compared. Taken together, these observations are not consistent with α-tocopherol acting solely as a chain-breaking inhibitor of lipid peroxidation in vivo and suggest that α-tocopherol depletion unmasks other, non-chain-breaking actions that affect ROS homeostasis in brain.

We found that brain superoxide production, measured by DHE oxidation in vivo, was also lower in deficient mice, suggesting that lower lipid peroxidation was the result of less ROS production “upstream.” Interestingly, of the three brain regions examined, the cerebellum had the highest levels of F4-neuroprostanes while simultaneously having the highest superoxide production. This is despite the fact that the concentration of α-tocopherol in cerebellum is similar to that in the hippocampus. The presence of higher levels of lipid peroxidation in cerebellum correlates well with previous studies showing that cerebellum has the highest rate of α-tocopherol turnover (39) and is the primary brain region functionally affected in patients with the AVED syndrome (40, 41). Based on these observations, we speculate that metabolism of α-tocopherol may differ between cerebellum and forebrain and that this difference underlies the unique vulnerability of cerebellum to α-tocopherol deficiency.

EPR results on the effect of α-tocopherol on superoxide release from isolated mitochondria, complemented by the RCR measurements, suggested a possible mechanism to explain the observed decrease in levels of oxidative stress in vitamin E-deficient mice. The fact that readdition of vitamin E to mitochondria increased levels of superoxide in all groups (control, VED, and TTP mice) strongly suggested that α-tocopherol can modify mitochondrial ROS production. The concentration of α-tocopherol added to mitochondria to enhance ROS production was higher for Ttp−/− versus VED mice (Fig. 5b), probably reflecting the much lower basal concentrations of α-tocopherol in Ttp−/− mice versus VED mice versus controls. We also
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observed a similar systematic and significant increase in the α-tocopherol concentration required to reach maximum mitochondrial superoxide flux and found that this was also predicted by the base-line level of α-tocopherol (compare the values of $C_{\text{max}}$ in Fig. 5c).

Our EPR results directly confirm that exogenously added α-tocopherol modulates the superoxide production from mitochondria, albeit in an unexpected biphasic manner (Fig. 5b). In other words, the inclusion of roughly 0–5 nmol of α-tocopherol/mg of mitochondria protein gradually increased the superoxide yield from mitochondria (Fig. 5). This increase was then reversed upon the inclusion of additional amounts of α-tocopherol. It should be stressed here that the exact amount of the α-tocopherol added back that actually intercalates into the mitochondrial membrane cannot be inferred from these experiments, making quantitative correlations to physiological concentrations difficult. However, these observations led us to propose a specific mechanism to account for the observed enhancement of superoxide production by brain mitochondria upon the back-addition of α-tocopherol.

The generation of superoxide radical (O$_2^-$) as a byproduct of oxidative phosphorylation is known to involve ubisemiquinone (CoQ$^-$) due to the highly reducing midpoint potential (−160 mV) of the redox couple (CoQ/CoQ$^-$). CoQ$^-$ occurs at three different sites along the ETC: one site at complex I in addition to two sites at complex III. As a result, complexes I and III are proposed to be the main sources of superoxide leakage in mitochondria (42–45). Intuitively, any factor that increases the lifetime of CoQ$^-$ (i.e. populating the reduced state) is expected to increase the chance of electron transfer from CoQ$^-$ to oxygen. Complex I is known to release superoxide toward the matrix, whereas complex III releases superoxide radical both to the matrix and into the intermembrane space (46). Since DEPMPO/superoxide signals were very sensitive to exogenously added superoxide dismutase, an enzyme not capable of penetrating mitochondria inner membrane, we take this as an evidence that complex III is the more likely source of superoxide leakage upon the back-addition of α-tocopherol. We were inspired by the structural similarity between ubiquinone and α-tocopherol and tested the hypothesis that these two molecules may be competing for binding into the active site close to the heme $b_L$.

Based on the above arguments, we assumed that the effect of vitamin E on mitochondrial produced superoxide is likely to involve the cytochrome $bc_1$ (QCR, EC 1.10.2.2) components of the mitochondrial complex III. QCR is a multisubunit membrane protein complex that catalyzes electron transfer from ubiquinol to cytochrome $c$ and couples this process with electrogenic translocation of protons across the membrane (47, 48). As shown in Fig. 6, each monomer of the homodimeric complex contains three essential catalytic subunits: cytochrome $c_1$ (CYT1), cytochrome $b$ (COB) spanning the inner membrane and contains two $b$-type heme groups that interact with the CoQ and CoQH$_2$ (blue in b) and mobile Rieske protein (RIP1, purple), containing a [2Fe-2S] cluster, which delivers electrons to the $c$-type heme on the third component, cytochrome $c$. To illustrate their structural similarity, α-tocopherol was superimposed on CoQH$_2$ (UQ6) in the binding pocket identified from the x-ray structure containing CoQH$_2$ (28). Blue marks a portion of the surface where the ligand needs hydrogen bonds, red marks a portion of the surface where the ligand needs hydrogen bond donors, and cream marks places where it should have a hydrophobic surface to achieve good binding to the protein.

FIGURE 6. α-Tocopherol can partially block binding between CoQH$_2$ and the COB subunit of the QCR component of the mitochondria complex III. a, binding of α-tocopherol (magenta; enlarged window) close to the heme $b_L$ group (green). The complex contains three essential catalytic subunits: cytochrome $b$ (COB; green) spans the inner membrane and contains two $b$-type heme groups that interact with the CoQ and CoQH$_2$ (blue in b) and mobile Rieske protein (RIP1, purple), containing a [2Fe-2S] cluster, which delivers electrons to the $c$-type heme on the third component, cytochrome $c_1$ (CYT1, blue). b, to illustrate their structural similarity, α-tocopherol was superimposed on CoQH$_2$ (UQ6) in the binding pocket identified from the x-ray structure containing CoQH$_2$ (28). Blue marks a portion of the surface where the ligand needs hydrogen bonds, red marks a portion of the surface where the ligand needs hydrogen bond donors, and cream marks places where it should have a hydrophobic surface to achieve good binding to the protein.
structure was corrected and refined before calculating the docking score of CoQH$_2$, which was found to be $-122.6$ kcal/mol. An $\alpha$-tocopherol model was then created and structurally optimized using semiempirical quantum mechanical calculations and superimposed on CoQH$_2$ molecule (see the two ligands superimposed at the binding pocket close to the heme $b_L$ site in the COB subunit) (Fig. 6b). After the deletion of CoQH$_2$, the docking score for $\alpha$-tocopherol was calculated to be $-29.4$ kcal/mol. In the proton motive Q cycle suggested by Mitchell (50), electrons on heme $b_L$ are donated to CoQ$^-$ with a concomitant translocation of two protons from the matrix side to generate CoQH$_2$. Our modeling results indicate that $\alpha$-tocopherol is stabilized in the binding pocket for both CoQH$_2$ and CoQ$^-$ and thus may limit electron transfer from the heme $b_L$ to CoQ$^-$ to form CoQH$_2$. Moreover, the localization of electron on the heme $b_L$ is expected to dissipate the redox potential difference between the two heme groups and retard the electron transfer from CoQ$^-$ and heme $b_L$. Since the redox potential difference between the two heme groups is opposed by the membrane potential (51), the dissipation of the redox potential difference between the two heme groups is predictable in the light of our model. Sohal et al. (58) concluded that concurrent supplementation of $\alpha$-tocopherol with CoQ is more likely to be effective as a potential treatment for age-related learning deficits than supplementation with CoQ or $\alpha$-tocopherol alone, which suggests that coenzyme Q and $\alpha$-tocopherol act in concert. Along the same line, Sumien et al. (59) reported recently that short term vitamin E intake fails to improve cognitive or psychomotor performance of aged mice and may even cause detrimental effects on brain functions. Interestingly, Lass and Sohal (57, 60) showed that in submitochondria particles from bovine heart, $\alpha$-tocopherol retards the succinate-induced oxidation of CoQH$_2$ back to CoQ in a dose-dependent manner. These authors explained their results in terms of redox-cycling mechanism, although the CoQH$_2$/CoQ $\alpha$-tocopherol stoichiometry is 1:1 (Fig. 8 in Ref. 54), and that in some cases retardation of this oxidation was seen although no parallel decline in $\alpha$-tocopherol was observed (Fig. 7 in Ref. 54; $b$ versus $c$ at 1 and 2 nmol of $\alpha$-tocopherol/mg of SMP protein). We believe that binding competition between CoQ and $\alpha$-tocopherol would provide more plausible accounts of all of these observations.

Our study investigated only the situation of $\alpha$-tocopherol deficiency and cannot be extrapolated to $\alpha$-tocopherol-replete conditions. In fact, our data confirmed that at higher levels of $\alpha$-tocopherol, mitochondrial ROS production is decreased. However, although global $\alpha$-tocopherol deficiency per se is rare, there are a number of situations in which our findings might be relevant, including prior oxidative stress in specific tissues, which could lead to localized depletion of $\alpha$-tocopherol or inadequate dietary availability of $\alpha$-tocopherol in vulnerable populations (e.g., surgical patients and smokers). Interestingly, a recent report indicated that treatment of a human cell line (HL-60) with $\alpha$-tocopherol enhanced mitochondrially derived ROS production and cytotoxicity in cells under chronic oxidative stress, but not in unstressed cells (61). In another study, treatment of splenocytes with low versus high concentrations of $\alpha$-tocopherol produced a biphasic change in interleukin-2 expression, a response that was mediated through NFkB (62), a transcription factor known to be regulated by ROS. These studies suggest that $\alpha$-tocopherol may have effects on cellular redox status at low concentrations that are not predicted by its conventional anti-peroxidation actions. Further studies will clarify how $\alpha$-tocopherol effects on mitochondria contribute to its observed biological effects in vitro and in vivo.

**Acknowledgment—**We thank Dr. Margarita M. Behrens for valuable discussions.

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