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PERTURBATIONS IN CEREBRAL OXYGEN RADICAL FORMATION AND MEMBRANE ORDER FOLLOWING VITAMIN E DEFICIENCY

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Summary: The effects of dietary vitamin E deficiency on mouse cerebral membrane order and oxygen reactive species were studied. Quantitation of vitamin E levels in several brain regions showed greatest deficiencies in striatum and cerebellum, followed by substantia nigra, and cortex. Vitamin E deficiency increased central-core membrane order in cerebral P_2 fraction, but was without effect in the superficial hydrophilic membrane domain. Oxygen radical formation was studied using the probe 2',7'-dichlorofluorescin diacetate. Basal generation rates of oxygen reactive species were 2.5-fold higher when compared to control animals. While hepatic levels of vitamin E are much more reduced than brain levels, in deficient mice, the rate of oxygen radical formation in the liver was unaltered. This implies an especial susceptibility of the brain to deficiency of this lipophilic antioxidant vitamin. Data demonstrate that endogenous levels of free radical scavengers, such as vitamin E, may play an important role in maintaining basal oxygen radical levels and membrane integrity. The dietary vitamin E depletion paradigm suggests that a relation exists between elevated levels of oxygen radicals and more rigid hydrophobic central-cores in cerebral membranes, effects that may play a role in mechanisms underlying the neuropathologic lesions observed following vitamin E deficiency.

Vitamin E, α-tocopherol (Vit E), is a membrane associated phenolic antioxidant that functions by reacting with oxygen radicals and lipid hydroperoxides in a radical chain breaking fashion [1]. The significance of the protective role that Vit E plays in nerve tissue has been investigated by means of Vit E deficiency studies. Vit E deficiency enhances the changes caused by oxidative challenge [2], as well as the acute toxicities of paraquat [3,4] and ozone [5]. The nervous system appears exquisitely sensitive to Vit E deficiency as evidenced by the occurrence of neuropathologic lesions [6,7] and increased susceptibility to lipid peroxidation [8].

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Abbreviations used: DCFH-DA, 2',7'-dichlorofluorescin diacetate; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1 4-(trimethylamino)-phenyl -6-phenyl-1,3,5-hexatriene; DCF,2',7'-dichlorofluorescein.

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While several investigations have explored either the membrane stabilizing role of Vit E [9,10] or it's antioxidant activity [11-15], few have concentrated on the relation between these two parameters in biological membranes [16]. Furthermore, to our knowledge no studies exist regarding the effects of Vit E deficiency on antioxidant and membrane order parameters in neural tissue, although oxygen radicals are thought to play a major role in events leading to biological membrane damage [for review see 17].

Two recent studies have reported direct measurement of neural oxygen radical formation, using the probe DCFH-DA, in rat synaptosomes [18] and N18-RE-105 cells (neuroblastoma-primary retina hybrid)[19]. The present study investigates the effects of Vit E deficiency on cerebral oxygen radical formation, using DCFH-DA, and membrane order in mice. These studies utilized two fluorescent probes, one of which is lipophilic and primarily reprts from the lipid core of the membrane DPH [20], while the other TMA-DPH possesses a cationic group which confines the dye to the superficial hydrophilic membrane domain [21].

MATERIALS AND METHODS

Male C57 BL/6 mice (Charles River Breeding Labs, Wilmington, MA) were raised from 3 week old weanlings and fed Vit E deficient diets (Teklad Diets, Madison, WI) for 16 weeks. Animals were cervically dislocated and brains and livers were rapidly excised, and stored at -80°C or -20°C (membrane order) until analysis. For oxygen radical and membrane order studies, the cerebellum and pons-medulla were removed, and the remaining tissue was split for radical and membrane order analyses.

Vit E content in hexane extracts of brain regions was measured by HPLC with fluorescence detection [22].

Oxygen radical formation was determined by loading brain or liver homogenates (0.5% in 40mM Tris, pH 7.4) with 5μM DCFH-DA (Molecular Probes, Eugene, OR) in methanol for 15 min at 37°C. Dye-loaded samples were then centrifuged at 12,500xg for 8min (4°C), and P_2 fraction was resuspended in 5ml of Tris. After resuspension in the same buffer, P_2 fractions were incubated in the presence or absence of ascorbate (0.1mM)/$FeSO_4$ (5μM) for 30 minutes at 37°C. These two incubations represented basal and stimulated oxygen radical fromation. The formation of the fluorescent probe DCF (Polysciences Inc., Warrington, PA) was monitored at excitation wavelength 488nm (bandwidth 5nm), and emission wavelength 525nm (bandwidth 20nm). The cuvette holder was thermostatically maintained at 37°C. Autofluorescence was subtracted prior to DCF fluorescence calculations, and DCF formation was quantified from a standard curve in methanol (0.5-1.0μM).

A crude cerebral P_2 fraction consisting of mitochondria, synaptosomes and myelin fragments [23] was employed to determine membrane order (fluorescence polarization) using the probes DPH (Sigma Chemcial Co., St. Louis, MO) and TMA-DPH (Molecular Probes, Eugene, OR). Five ml of HEPES buffer (mM: NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0 and HEPES, 10) was added to 1ml aliquots of P_2 fractions (0.037g equiv/ml) which were centrifuged at 12,500xg for 8min, and resuspended in 4ml HEPES, 2ml of which were then incubated for 15 min at 37°C with either 5μM DPH or TMA-DPH. Fixed excitation and emission polarization filters were used to measure fluorescence intensity both parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarization phase of the exciting light. $I_{\parallel}$ corresponds to both vertically polarized excitation and emission while $I_{\perp}$ corresponds to vertically polarized excitation and

horizontally polarized emission. Excitation and emission wavelengths of 360nm and 430nm, respectively, were used with the band width of both monochromators at 10nm. Cuvette temperature was maintained at 37°C with a circulating water bath. Fluorescence anisotropy (r) was determined by the formula: $r = (I_{\parallel}) - (I_{\perp})/(I_{\parallel}) + 2(I_{\perp})$ [24].
Statistical analysis on membrane order and oxygen radical studies was performed using a Student's t test. The accepted level of significance was $p<0.05$ using a two-tailed distribution.

RESULTS AND DISCUSSION: The concentration of cerebral Vit E levels following Vit E deficient diets showed cerebellum and striatum as the regions with greatest deficiency, followed by substantia nigra and cortex (Table 1). These data confirm the findings of others [8,25], and support the concept that cerebellum, relative to other brain regions, is an area particularly vulnerable to damage following Vit E deficiency. Evidence of neuropathologic lesions [26] and enhanced levels of lipid peroxidation in the cerebellum following Vit E deficiency [8] further substantiate the cerebellar susceptibility hypothesis.

Cerebral membrane order was measured in P_2 fractions following Vit E deficiency. Central-core membrane order, measured via DPH anisotropy, was significantly increased in Vit E deficient brains, while membrane order in the hydrophilic (exofacial, TMA-DPH labeled) domain remained unchanged (Fig. 1). It is known that Vit E distributes preferentially in membranes enriched in polyunsaturated fatty acids [27], components that are highly concentrated in the brain. Furthermore, Vit E is believed to localize in the hydrophobic central-core membrane region [28], the domain that contains phospholipid fatty acyl side chains and is labeled by DPH [20]. The high cerebral content of polyunsaturated lipids leaves the brain highly susceptible to peroxidative attack, particularly in the case of Vit E deficiency [8]. Lipid peroxidation has been shown to increase membrane order using a pyrene probe [16]. A lack of central membrane-core associated Vit E, as a result of dietary deficiency, may thus lead to increased central-core order, either directly or as a result of increased levels of lipid peroxides [8].

The effects of Vit E deficiency on cerebral oxygen radical formation by the P_2 fraction was also studied, using the non-fluorescent probe DCFH-DA, which is

TABLE 1. The effect of dietary vitamin E deficiency on brain regional vitamin E concentrations

Brain Region	Control	Vit E Deficient (μg Vit E/g tissue)
Striatum	6.08 ± 1.70	3.53 ± 0.32
Substantia Nigra	5.19 ± 0.79	3.26 ± 0.58
Cortex	4.32 ± 0.49	2.85 ± 0.82
Cerebellum	4.36 ± 1.93	2.62 ± 0.32

The concentration of Vit E was measured in hexane extracts by an HPLC method with fluorescence detection [22]. Data are expressed as mean Vit E level in μg/g tissue ± SEM from 4-5 mice.

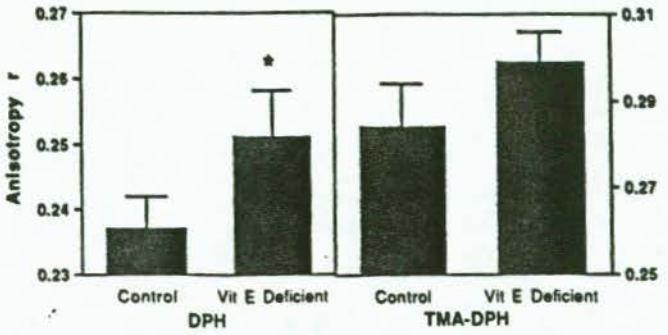

Figure 1
The effects of Vit E deficiency on membrane order of a cerebral P_2 fraction using the fluorescent dyes DPH and TMA-DPH. Data are expressed as the mean anisotropy ± SEM in 6 mice. Asterisk denotes significant difference from controls ($p<0.05$) according to a Student's t test.

oxidized in the presence of oxygen radicals to the highly fluorescent dye DCF [18,19,29]. Basal rates of generation of oxygen reactive species in the Vit E deficient brain were 2.5-fold higher ($p<0.05$) when compared to controls, while the velocity of ascorbate/iron-induced oxygen radical formation was unaffected (Fig. 2). Endogenous Vit E levels may thus be critical in the regulation of intracellular oxygen radical formation in the brain. A recent study concerning this issue reported that in vitro addition of Vit E primarily scavenges lipid-derived oxygen radicals rather than free oxygen radical initiators in liposomal preparations [14]. Differences may then exist between endogenous pools of Vit E, and those added exogenously.

Regulation of oxygen radicals by Vit E after dietary deficiency appears to be organ selective since both basal and ascorbate/iron-stimulated hepatic oxygen radical formation in Vit E deficient mice remained unchanged (Fig. 3). There may be several reasons for especial sensitivity of the central nervous system to oxidative damage following Vit E deficiency. Following Vit E deficient diets, little hepatic Vit

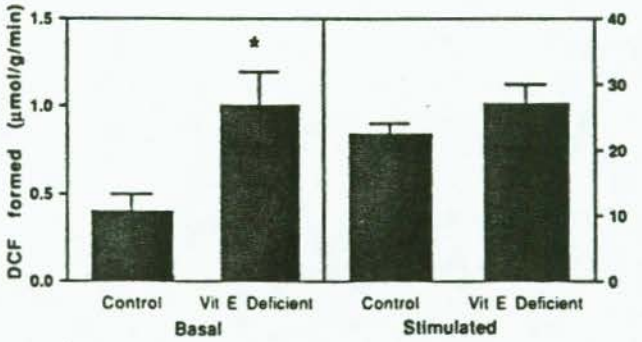

Figure 2
The effects of Vit E deficiency on oxygen radical formation by a cerebral P_2 fraction. Oxygen radical formation was determined in either basal (unchallenged) or stimulated (challenged with ascorbate/iron) samples as described in the materials and methods section. Data are expressed as the mean DCF formed in µmol/g/min ± SEM in 6 mice. Asterisk denotes significant difference from controls ($p<0.05$) according to a Student's t test.

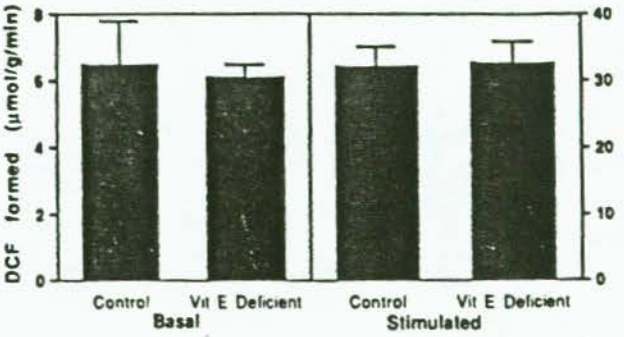

Figure 3
The effects of Vit E deficiency on oxygen radical formation by a hepatic mitochondrial fraction. Experimental details are as in figure 2.

E remains while measurable cerebral amounts are present [25]. A relatively minor reduction of cerebral Vit E levels (34-42%, Table 1) had a much more drastic effect on formation of oxygen radicals than did a larger loss of this vitamin in the liver (99%) [25], despite the fact that basal hepatic oxygen radical generation rates are approximately 6-fold higher than in brain (Figs. 2,3). Although most tissues remove oxygen radicals by superoxide dismutase, glutathione peroxidase, and catalase [30], the brain is deficient in the latter two [31]. This susceptibility of the brain may be confined to lipid soluble oxidative events since Vit E deficiency does not affect cerebral levels of glutathione peroxidase or reductase, enzymes that are much lower in the brain than the liver [32]. Cellular depletion of Vit E has been associated with diminished intracellular levels of glutathione [33], another major antioxidant. While total hepatic glutathione levels are approximately 4-fold higher than brain levels [34], Adams et al [35] demonstrated that t-butylhydroperoxide-induced oxidative stress did not deplete hepatic gluthathione. It is therefore possible that the brain may lack the compensatory properties to adapt to oxidative radical fluxes as effectively as the liver.

These data demonstrate that the generation of oxygen radicals by the brain is regulated by endogenous antioxidants, such as Vit E, which appear to be responsible for maintaining both a homeostatic oxidative status, and physiologically effective membrane order. Decreases in Vit E levels selectively elevate basal cerebral levels of oxygen radicals (Fig. 2) and increase membrane order (Fig. 1). This study suggests a relation between cerebral oxygen reactive species and membrane rigidity, and is the first report to employ a fluorescent dye in cerebral homogenates to directly quantitate oxygen radical generation. This method, provides the groundwork for future studies on brain oxygen radical formation in a direct manner, rather than quantitation of secondary changes resulting from oxidative damage to biological macromolecules.

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