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MITOCHONDRIA AND MICROSOMAL MEMBRANES HAVE A FREE RADICAL REDUCTASE ACTIVITY THAT PREVENTS CHROMANOXYL RADICAL ACCUMULATION

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Summary Enzyme-dependent mechanisms which prevent accumulation of chromanoxyl radicals derived from the vitamin E analogue, 2,2,5,7,8-pentamethyl-6-hydroxycromane (PMC), were characterized in rat liver microsomal and mitochondrial membranes. The free radical oxidation product of PMC (chromanoxyl radical) was generated in membranes using either photochemical (uv light) or enzymatic (lipoxygenase and arachidonic acid) methods and detected by ESR. Substrates (NADH or NADPH) prevented accumulation of chromanoxyl radicals until the substrate was fully consumed. In microsomes, reduced glutathione increased the efficacy of NADPH in preventing the accumulation of the chromanoxyl radical, but was without effect in the absence of NADPH. Ascorbate also prevented accumulation of the chromanoxyl radical. It is concluded that rat liver microsomes and mitochondria have both enzymatic and non-enzymatic mechanisms for reducing chromanoxyl radicals.

Vitamin E (tocopherol) protects membranes from oxidative damage by acting as a lipid-soluble free radical chain breaking antioxidant (1). In this process, vitamin E reduces lipid radicals, undergoing one-electron oxidation to form the relatively stable tocopheroxyl radical. Whereas most free radicals react rapidly with each other, dimerization of chromanoxyl radicals has been shown to occur relatively sluggishly (2). The high persistence of the tocopheroxyl radical has made it possible to detect it by ESR in a variety of chemical solvent and liposomal model systems.

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Abbreviations: tocopherol, d-α-tocopherol; PMC, 2,2,5,7,8,-pentamethyl-6-hydroxy-chromane; SMP, sub mitochondrial particles.

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Oxidation of vitamin E to the tocopheroxyl radical in non-biological systems results in a destruction of the vitamin and leads to a variety of oxidation products including dimers, trimers and the tocopherol quinone (3-5). Vitamin C, in the absence of enzymes has been shown to protect vitamin E from oxidative destruction (1) by regenerating tocopherol. Vitamin E has also been shown to act synergistically with glutathione (6-9).

Tocopherol is present physiologically in relatively small concentrations and cannot by synthesized by mammals. The maintenance of useful amounts of vitamin E may involve enzymatic as well as chemical reduction of tocopheroxyl radicals to native forms of vitamin E, which can continue to serve as antioxidants (1,8).

Investigation of an enzyme role in the regeneration of vitamin E was undertaken with both tocopherol and the vitamin E analogue, 2,2,5,7,8,-pentamethyl-6-hydroxy-chromane (PMC) which can be incorporated into membranes *in vitro* to achieve much higher concentrations than of naturally occurring tocopherols. This made it possible to detect and study the generation, stability and destruction of chromanoxyl radicals in liposomes, mitochondrial and microsomal membranes.

Methods & Materials: Mitochondria and submitochondrial particle (SMP) membranes were prepared as in (10,11). Microsomes were prepared by perfusing the liver with ice cold 1.15% KCl. The liver was then removed and homogenized followed by a 10 minute 10,000 x g centrifugation. The supernatant from this fraction was centrifuged at 105,000 x g for 60 minutes. This pellet was resuspended as the microsomal preparation. Protein was measured by the method of Lowry (12). Unilamellar asolectin vesicles containing defined admixtures of tocopherol were prepared by combining asolectin with tocopherol in chloroform and removing the solvent under nitrogen. The lipid was then hydrated with 10mM NaPi, pH 7.0 and sonicated (Branson sonicator) until the liposome suspension became opalescent.

ESR measurements were made on a Varian E-109E spectrometer at room temperature, in either glass capillaries or gas-permeable Teflon tubing (.8mm internal diameter, .013mm thickness, from Zeus Industrial Products, Raritan N.J. USA.). The permeable tube (approximately 8cm in length) was filled with 50 μl of a mixed sample, folded into quarters and placed in an open 3.0mm internal diameter EPR quartz tube such that all of the sample was within the effective microwave irradiation area. The sample was flushed with oxygen. Spectra were recorded at 10mW power and 2.5 gauss modulation, and 50 gauss/minute scan time. Buffer was KPi pH7.5, 50mM. The light source was an Oriel Xenon Arc Lamp, Model 6140, producing both uv and visible

light (>220 nm), which was placed at a distance of 25cm from the cavity. The light intensity was controlled qualitatively by adjusting the focus. Attenuation of short wavelength uv (<310 nm) diminished ESR-detectable radical signals, but otherwise did not alter results. Material sources: Asolectin, (Associated Concentrates, Woodside, New York) tocopherol, or d-α-tocopherol was a gift from the Henkel Co, La Grange, Il, PMC was a gift from the Esai Chemical Company, Tokyo, Japan. NADH, NADPH, arachidonic acid and soybean lipoxygenase were from Sigma Chemical Company, St. Louis Mo.

Results: Initial studies confirmed that the radical formed from tocopherol and PMC had indistinguishable ESR spectra in liposomes, microsomes or SMP. Figure 1 shows the ESR signal from tocopherol formed by uv irradiation. All of the spectra presented in this study were modulated at 2.5 gauss to enhance detection of the signal. Overmodulation caused some distortion of line shapes but this did not affect the quantitation of the signal. Radicals derived from tocopherol and PMC were detected only in aerobic samples and decayed within a few seconds in the dark. Under continuous uv irradiation, the signal decayed after several minutes.

Since tocopherol derived radicals could not be observed in membranes isolated from normal rats, *in vitro* enrichment was attempted by adding an

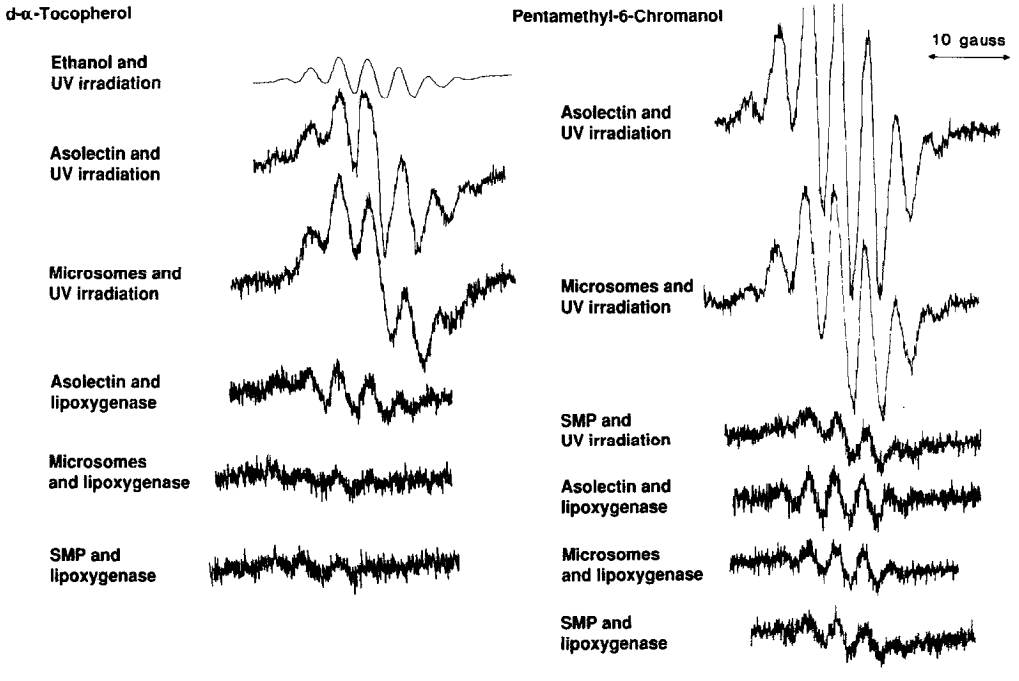

Figure 1 ESR spectra of radicals derived from tocopherol or PMC. Protein concentrations were 0.34 mg/ml for SMP and microsomal protein was 0.42 mg/ml. Arachidonic acid was 0.6mM and lipoxygenase was 20ug/ml, tocopherol and PMC were at 1mM.

ethanolic solution of tocopherol or PMC (0.3M stock solution) directly to subcellular membranes. This procedure (figure 1) shows that tocopherol or PMC added to liposomes, microsomes or SMP and oxidized either by uv irradiation or arachidonic acid and lipoxygenase, results in a measurable EPR signals of these radicals.

Previous studies have shown that ascorbate prevents vitamin E consumption under defined conditions (3). Ascorbate addition to samples of PMC-enriched membranes yielded the characteristic ascorbyl ESR signal. Uv irradiation of the samples resulted in a sharp transient increase in the intensity of the ascorbyl radical signal which decayed rapidly (Figure 2). Ascorbate completely quenches photochemical-induced chromanoxyl ESR signals in liposomes and microsomes and SMP, i.e until the ascorbyl radical disappears. At that time the chromanoxyl radical accumulates to levels detectable by ESR. Figure 2 shows a time course of the formation of chromanoxyl radical in microsomes in the presence of ascorbate. As the ascorbate is consumed by oxidation the ascorbyl radical decreases and the chromanoxyl radicals are detected. At about 13 minutes the chromanoxyl radical begins to decrease in intensity and a "broad radical" centered around g=2.0 is detected. This broad radical is formed within a minute if PMC or ascorbate are not added to the microsomes or SMP.

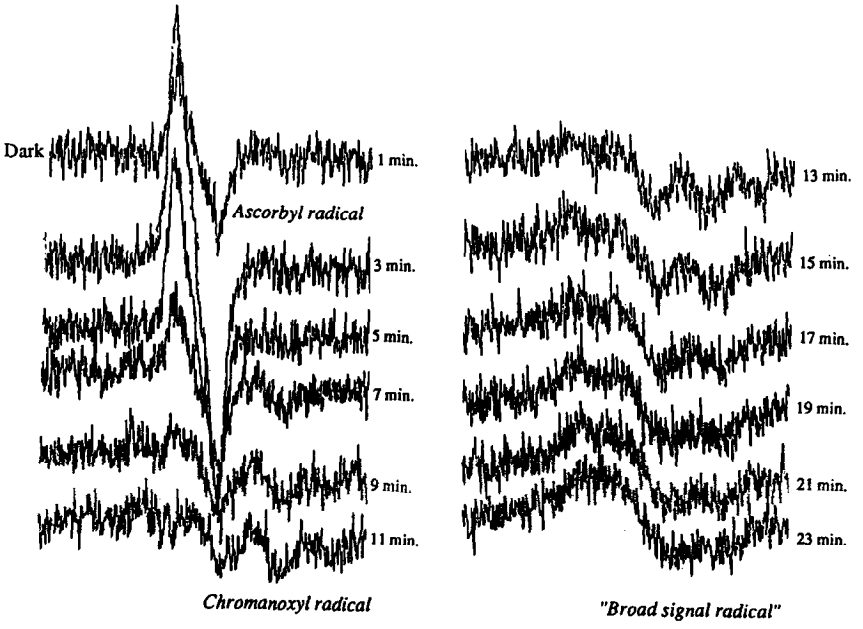

Figure 2 Effect of ascorbate on chromanoxyl radicals in microsomes. Microsomes (0.42 mg/ml) and ascorbate (1mM) were measured initially in the dark. Subsequent spectra were in uv light. ESR spectra were recorded sequentially.

Similar experiments with added tocopherol showed that the tocopheroxyl radical began to form well before the ascorbate was consumed, (data not shown). This observation, taken together with the poor water solubility of tocopherol, suggested that *in vitro* addition of tocopherol to membranes resulted primarily in the formation of an emulsion of tocopherol droplets, with perhaps little incorporation into the membranes. To further analyze the interaction of chromanoxyl radicals with membrane enzymes, we pursued experiments with the vitamin E analogue PMC, whose water solubility is sufficient to allow for *in vitro* membrane incorporation.

Prevention of chromanoxyl accumulation in the presence of NAD(P)H: The addition of substrates to microsomes (NADPH > NADH) or SMP (NADH > NADPH) membranes prevented the formation of the PMC radical signal for a time period that depended on the amount of reductant added and the rate of NAD(P)H consumption (Fig. 3). The NAD(P)H effects, which delayed the accumulation of the chromanoxyl radical were not observed in liposomes or heat-treated (90^0, 30 secs.) subcellular membranes. Succinate addition to SMP also prevents chromanoxyl formation. In microsomes reduced glutathione in a concentration dependent manner, also retarded the accumulation of chromanoxyl radical, but this was strictly dependent on NADPH or NADH being present. Glutathione potentiated the NADPH/NADH delay in chromanoxyl radical accumulation. A direct inhibitory effect of reducing agents or reducing enzymes upon the lipoxygenase/arachidonate radical generating systems could be ruled out by showing that reducing agents did not prevent the formation of conjugated diene products of arachidonic acid oxidation. Depletion of oxygen in the reaction mixture was not significant. This was assessed by observation of added trace quantities of methylene blue in

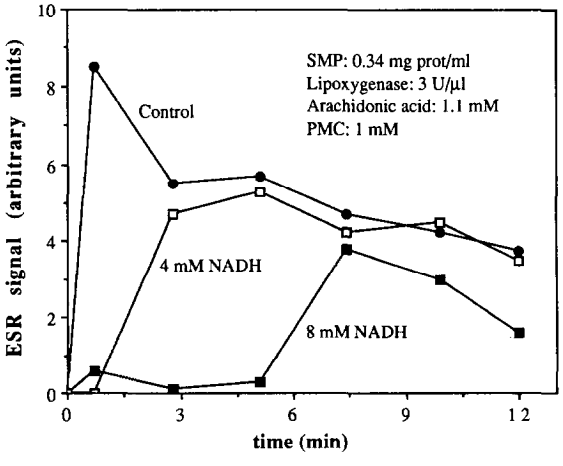

Figure 3 Time dependence of the effect of NADH on the ESR signal height of the chromanoxyl radical in SMP.

parallel experiments. If SMP were placed in a glass capillary in the presence of NADH and methylene blue, the system became colorless and, therefore, anaerobic within seconds. In the Teflon tubing under oxygen, and with substrate, methylene blue remained oxidized.

In microsomes, treatment with the P450 inhibitor metyrapone had virtually no effect on the NADPH-dependent delay in the formation of the chromanoxyl radical. Tocopherol added to the membranes in an ethanol solution at concentrations comparable to those used in the PMC studies, yielded very small or no radical signals with the lipoxygenase system. When added in large excess, (30mM) a small radical signal was observed, but it was insensitive to treatment with NAD(P)H.

Discussion: Chromanoxyl radical accumulation can be prevented by pyridine nucleotides in both microsomal and mitochondrial membranes, suggesting that the radicals are being reduced by NAD(P)H-dependent enzymes. Previous attempts to establish enzymatic sparing of vitamin radical sought to show a glutathione-dependent pathway (6,7) but recent work has shown that glutathione can act indirectly to spare vitamin E consumption by inhibiting processes that initiate lipid peroxidation in the aqueous phase (13). A further complication of studies with glutathione is the established role of glutathione in reducing lipid hydroperoxides (14), which would be expected to affect vitamin E consumption by decreasing the hydroperoxide-mediated oxidation of tocopherol (3). We designed our experiments to distinguish between processes that affect chromanoxyl radical generation and those that quench the radicals, once they are formed. Because the natural abundance of vitamin E in liver membranes is insufficient for ESR studies, we used the analogue PMC, whose solubility characteristics allow for *in vitro* loading to membranes to study enzymatic processes that suppress accumulation of the chromanoxyl radical. Identification of chromanoxyl radicals relied upon the characteristic hyperfine ESR pattern of these radicals. Except for ascorbyl radical, the intensity of the chromanoxyl radical signal was larger than that of any other radical species present in the membranes, thus allowing for measurement of its intensity. The amount of radical detected was in all cases a very small portion of the tocopherol or PMC present.

Formation of chromanoxyl radicals with lipoxygenase and arachidonic acid system provides an experimental system for investigating the metabolism of the radical by ESR. Previous studies had shown that peroxidized lipid can be used to generate the tocopheroxyl radical (4).

Although the quantity of PMC added to the membranes exceeds *in vivo* tocopherol concentrations, enzymatic prevention of the accumulation of the chromanoxyl radical was clearly demonstrated. The chromanoxyl radical is very similar to the tocopheroxyl radical and the reactions of PMC may model the behavior of tocopherol. PMC has the chromane structure of tocopherol, and lacks only the "inert" hydrophobic tail. The important difference between tocopherol and PMC may be that PMC has less restriction of mobility and greater accessibility to enzyme site(s) in the aqueous phase and, perhaps, in the membrane that may be inaccessible to tocopherols. The prevention of chromanoxyl accumulation in the presence of the substrates NAD(P)H and succinate could be due to several enzyme sites, or perhaps by an indirect mechanism e.g. via a hydro- or semiquinone intermediate. In microsomes cytochrome P-450 does not appear to be involved. There is, as yet, no evidence that an enzyme "vitamin E free radical reductase" (8) exists which directly reduces the radical.

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