Sensitivity of respiratory chain activities to lipid peroxidation: effect of vitamin E deficiency

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Rats fed a vitamin E-depleted diet for 48 weeks had undetectable levels of vitamin E in the gastrocnemius muscle and liver, leading to elevated malondialdehyde levels in both tissues and an elevated GSH level in muscle. Skeletal-muscle mitochondria showed decreased mitochondrial respiratory chain (MRC) activities, whereas liver MRC activities were increased. Exposure of normal rat liver submitochondrial particles (SMPs) to an *in vitro* NADPH-dependent lipid peroxidation system resulted in a dose-dependent increase in lipid peroxidation and inhibition of complex I and complex IV activities. Complex I exhibited greater sensitivity to lipid peroxidation than complex IV. At low and high NADPH concentrations, the rate of lipid peroxidation and the level of enzyme inhibition were essentially the same in

liver SMPs from both vitamin E-deficient and control rats, suggesting that under these conditions, the loss of vitamin E did not exacerbate the effects of either lipid peroxidation or enzyme inhibition. These results indicate that normal vitamin E levels in liver mitochondria are not required for protection against lipid peroxidation and are consistent with the normal liver mitochondrial function in vitamin E-deficient animals. This suggests other antioxidants, such as ubiquinol and GSH, may be more important in protecting liver mitochondria and MRC from lipid peroxidation.

Key words: liver mitochondria, mitochondrial function, mitochondrial respiratory chain, muscle mitochondria.

INTRODUCTION

Oxidative stress and damage have been implicated in an increasing number of disease states, including Parkinson's disease [1], motor neurone disease [2] and arthritis [3], although the role they play in disease mechanisms is not known. Cells have a variety of antioxidant defences, including GSH, glutathione peroxidase and glutathione reductase, catalase, ascorbate, superoxide dismutase (SOD; Mn and Cu/Zn) and vitamin E (also known as tocopherol), which, when in balance with the generation of free radicals, protect the cell from superoxide, hydrogen peroxide and lipid peroxidation reactions in particular. A variety of *in vivo* models of oxidative stress have revealed that mitochondria, and in particular the components of the mitochondrial respiratory chain (MRC), are especially sensitive to inhibition by free radicals. These *in vivo* models have tended to investigate the effect of decreased antioxidant defences on MRC function. The mitochondrial activities that are most affected depend on the model. Whereas long-term vitamin E deficiency caused defects of complexes I and IV in rat skeletal muscle [4], MnSOD knockout mice had a severe defect of complex I and II activities in muscle [5], and L-buthionine sulfoximine ('BSO') depletion of GSH caused defects of complexes I, IV and II in neuronal cells in culture [6]. Increasing oxidative stress by loading PC12 cells with iron also caused inhibition of complex I and IV activities [7]. In contrast *in vitro* experiments have shown complex I to be the most vulnerable site when exposed to various free radical-generating systems. Treatment of bovine heart submitochondrial particles (SMPs) with lipoxygenase resulted in marked inhibition of complexes I and II [8], whereas exposure of rat brain mitochondria to hypoxanthine and xanthine in the presence of an iron chelate caused severe inhibition of complex I, but only mild reductions in the activities of complexes II and III [9]. Similarly, Narabayashi et al. [10] found that complex I was more

sensitive to inhibition by NADPH-dependent lipid peroxidation than complex IV in bovine heart SMPs. In a separate study, whereas exposure of beef heart SMPs to hydroxyl radicals caused inhibition of complexes I and II, the superoxide anion radical effectively inhibited complex I, but only mildly inhibited complex II activities, and complex IV activity was highly resistant to both radicals [11].

The vulnerability of the MRC to oxidative stress/damage may reflect certain features specific to the MRC. As a significant source of cellular superoxide generation, under conditions of decreased cellular or mitochondrial antioxidant capacity, the MRC is vulnerable to damage by free radicals generated by the MRC itself. The MRC is located within the mitochondrial inner membrane and is sensitive to the lipid environment, especially the cardiolipin content of the membrane [12], and is therefore sensitive to lipid peroxidation, and its products [13]. In addition mitochondrial DNA (mtDNA) is located in the vicinity of the MRC and is therefore vulnerable to free radical damage. As mtDNA codes for only MRC components this damage may contribute to MRC dysfunction.

Vitamin E is a fat-soluble vitamin that is sequestered in the hydrophobic interior of membranes, where it acts as an important antioxidant, quenching lipid peroxidation [14]. It has been suggested that the distribution of vitamin E reflects the degree of saturation of the membrane lipids [15]. However, there is dispute as to the exact subcellular distribution of vitamin E. Mitochondria are generally found to be relatively rich in vitamin E, but its distribution between the inner and outer mitochondrial membranes is unclear [15–17].

Vitamin E deficiency in humans gives rise to a specific neurological syndrome characterized by ataxia, areflexia, large fibre sensory neuropathy and muscle weakness clinically similar to Friedreich's ataxia [18]. The genetic mutation causing Friedreich's ataxia has now been identified and it affects a

Abbreviations used: BHT, butylated hydroxytoluene; DPH, 1,6-diphenyl-1,3,5-hexatriene; MDA, malondialdehyde; MRC, mitochondrial respiratory chain; mtDNA, mitochondrial DNA; SMPs, submitochondrial particles; SOD, superoxide dismutase.

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mitochondrial protein thought to be involved with mitochondrial iron handling [19,20], and results in decreased MRC function [21]. This gives increased support to the idea that vitamin E deficiency may have a major effect on mitochondrial function. This has been further supported in rats where 12 months of vitamin E deficiency resulted in a necrotizing myopathy, a central-peripheral distal axonopathy and inhibition of complexes I and IV of the MRC in skeletal muscle [4].

To investigate the selective pathological effects of vitamin E deficiency in rats we have compared the sensitivity of gastrocnemius muscle and liver mitochondria with long-term vitamin E deficiency *in vivo*. Using an *in vitro* system to induce lipid peroxidation, we investigated the rates of lipid peroxidation and the sensitivity of the MRC complexes to lipid peroxidation in normal and vitamin E-depleted rat liver SMPs.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), Merck (Dagenham, Essex, U.K.) or Boehringer Mannheim Ltd (Lewes, East Sussex, U.K.).

Animals

Weanling, specific pathogen-free, male Wistar rats (21-days-old) were placed on a vitamin E-deficient diet (Hoffmann-La Roche 814 Tocopherol Deficient Diet; Dyets Inc., Bethlehem, PA, U.S.A.). A second control group received the same diet to which 40 mg of *all-rac- α* -tocopheryl acetate/kg had been added. The rats were maintained on these diets for a period of 48 weeks, and fed *ad libitum* with unrestricted access to drinking water.

Isolation of rat muscle and liver mitochondria

After 48 weeks, rats were killed by cervical dislocation and the gastrocnemius muscle and liver were removed immediately. Muscle and liver mitochondria were freshly prepared as described by Cooper et al. [22].

Preparation of liver SMPs

Liver mitochondria were used to prepare SMPs according to the method of Ragan et al. [23]. Mitochondria were resuspended at a concentration of 5–10 mg/ml in ice-cold isolation medium (225 mM mannitol, 75 mM sucrose, 10 mM Tris and 100 μ M K⁺ EDTA, pH 7.2) and sonicated at full power with a probe sonicator for six 5 s bursts, interspersed with 30 s cooling periods on ice. The sonicated samples were spun at 15000 *g* for 10 min at 5 °C to pellet the intact mitochondria. The SMPs were recovered by centrifugation of the translucent supernatant at 100000 *g* for 45 min at 5 °C, and the pellet was resuspended in 100–200 μ l of isolation medium at a final protein concentration of 5–10 mg/ml and stored at –70 °C until required. The activity of the matrix enzyme citrate synthase was measured [24] in the SMPs, and was consistently found to be < 10 % of the activity in the mitochondria indicating that the mitochondria were effectively inverted.

Vitamin E (α -tocopherol) analysis

Vitamin E levels were measured by normal phase HPLC with fluorescence detection. Isolated liver mitochondria (50–100 mg) were extracted into 4 ml of hexane. The hexane phase was evaporated under nitrogen, and the residue was dissolved in 150–200 μ l of HPLC-grade hexane. Aliquots (50 μ l) of the hexane

extract were injected on to a 3 μ m $\times$ 25 cm Apex I silica column (Jones Chromatography Ltd, Mid Glamorgan, U.K.) and were eluted at a flow rate of 1 ml/min with 12 % (v/v) HPLC-grade methyl *t*-butyl ether in HPLC-grade hexane. Fluorescence was monitored with a PerkinElmer LS50B luminescence spectrometer (PerkinElmer, Beaconsfield, Bucks., U.K.) using an excitation wavelength of 290 nm and an emission wavelength of 325 nm. Concentrations of α -tocopherol were estimated by direct comparison against standard solutions of *all-rac- α* -tocopherol.

GSH and GSSG levels

GSH and GSSG were assayed by the enzymic recycling procedure described by Griffith [25], using 2-vinylpyridine as the GSH blocking reagent.

Membrane fluidity

Steady-state fluorescence polarization was used to evaluate the fluidity of the mitochondrial membranes using the lipophilic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Mitochondrial samples were resuspended at a final protein concentration of 0.1 mg/ml in Hepes buffer (10 mM Hepes, 120 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 0.1 mM MgCl₂, 5 mM NaHCO₃, 6 mM glucose and 1 mM CaCl₂, pH 7.4) and labelled with a 1 μ M solution of DPH (dissolved in tetrahydrofuran) to give a final molar ratio of probe/mitochondrial phospholipid of approximately 1:150–200. Samples were incubated at 37 °C for 1 h to allow full incorporation of the probe into the membrane. Fluorescence intensities were measured at 37 °C in a PerkinElmer LS50B luminescence spectrometer, equipped with a thermostatic cell-holder and a polarization accessory. Fluorescence excitation was performed at 360 nm (2.5 nm slit width) and emission was detected at 430 nm (10 nm slit width). Fluorescence polarization (*P*) was obtained by consecutive measurements of *I_{VV}* and *I_{VH}*, where *I_{VV}* and *I_{VH}* are the fluorescence intensities detected through a polarizer orientated parallel and perpendicular respectively, to the direction of polarization of the excitation beam. The degree of fluorescence polarization was expressed as the fluorescence anisotropy (*r*) and was calculated from the following equation:

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$$

A high degree of fluorescence polarization or anisotropy indicates a high degree of structural order, and both are inversely proportional to membrane fluidity [26].

Lipid peroxidation

NADPH was used to support enzymically induced lipid peroxidation in SMPs in the presence of an iron chelate, according to the method of Narabayashi et al. [10]. Unless otherwise specified in the Figure legends, SMPs were incubated at a final concentration of 0.25 mg/ml in a reaction mixture containing 25 mM Hepes (pH 7.4), 1 mM ADP and 0.1 mM FeCl₃·6H₂O in a final volume of 1 ml, allowing for the addition of initiator. The reaction mixture was incubated in a shaking water bath for 3 min to equilibrate the temperature to 25 °C. Lipid peroxidation was initiated by the addition of NADPH (0.1 or 0.5 mM) and aliquots were removed from the reaction mixture at various time intervals (0–30 min) and added to 20 μ M butylated hydroxytoluene (BHT) to terminate the reaction. Controls included omitting NADPH or adding 20 μ M BHT prior to the addition of NADPH. To determine the rate of lipid peroxidation the malondialdehyde (MDA) content was assayed by the HPLC-

based thiobarbituric acid ('TBA') assay described by Halliwell and Chirico [27].

MRC function

The activities of rotenone-sensitive NADH CoQ₁ reductase (complex I), succinate cytochrome *c* reductase (complexes II/III) and cytochrome *c* oxidase (complex IV) were determined as previously described by Krige et al. [28]. In the peroxidized SMP samples, the complex IV assay was modified by the addition of 1.2 mM dodecyl β -D-maltoside to fully activate the enzyme [29].

Protein concentrations were determined by the method of Lowry et al. [30], with BSA as a standard.

RESULTS

MRC enzyme activities were analysed in the gastrocnemius muscle and liver mitochondria from rats fed either a diet deficient in or supplemented with vitamin E for 48 weeks. Whereas the activities of complexes I, II/III and IV were decreased (only

Figure 1 Effect of vitamin E deficiency on liver and gastrocnemius muscle mitochondrial activities and markers of oxidative stress and damage

MRC enzyme activities and anisotropy (Anisot) values of isolated mitochondria, and MDA and GSH levels in whole tissue from gastrocnemius muscle (A) and liver (B) from rats fed a diet supplemented with (black bars) or deficient in (hatched bars) vitamin E. Values are means $\pm$ S.D. Complex (Cx) activities are given in terms of nmol/min per mg of protein, except complex IV, which is the first order rate constant expressed as k/min per mg of protein (left-hand axis). MDA and GSH are expressed as nmol/mg of protein, and anisotropy is expressed as *r* (right-hand axis). Using the Mann-Whitney unpaired U test **P* < 0.05 and ***P* = 0.01.

Figure 2 Effect of [NADPH] on lipid peroxidation, and [BHT] on complex I and IV activities

(A) MDA formation as a function of time in control rat liver SMPs incubated with the lipid peroxidation system and various concentrations of NADPH (○, 0 mM; ●, 0.1 mM; ▽, 0.2 mM; ▼, 0.4 mM; □, 0.5 mM). (B) Effect of BHT concentrations on complex I (○) and complex IV (●) activities in control rat liver SMPs. Activities were determined after a 5 min incubation of SMPs at 25 °C in the presence of BHT. Mean activities in the absence of BHT were: complex I, 198 nmol/min per mg of protein; complex IV, 10.8 k/min per mg of protein (first order rate constant). Values represent means for at least two experiments.

significantly in the latter) in the gastrocnemius muscle from the rats fed the vitamin E-deficient diet (Figure 1A), these activities were increased or unaffected in the liver mitochondria from the same rats (Figure 1B). Vitamin E was undetectable in both the gastrocnemius muscle and liver from the rats fed a vitamin E-deficient diet (results not shown). In the vitamin E-depleted rats the MDA levels were increased by 82 % in the gastrocnemius muscle compared with a 41 % increase in the liver (Figure 1), indicating that lipid peroxidation was increased in both of these tissues. There was an apparent compensatory rise in GSH levels in both the gastrocnemius muscle (100 % increase, *P* < 0.001) and liver (24 % increase, not significant) from the vitamin E-depleted rats (Figure 1). Mitochondrial membrane anisotropy values were mildly, but significantly, increased in the muscle mitochondria, indicating a decrease in mitochondrial membrane fluidity. This change was not observed in the liver mitochondria (Figure 1).

An *in vitro* lipid peroxidation system was used to investigate the effect of lipid peroxidation on mitochondrial function in liver mitochondria. Using control SMPs the rate of lipid peroxidation increased with increasing concentrations of NADPH, reaching a maximum at 0.4 mM NADPH. The rate of MDA formation increased linearly at all NADPH concentrations over the first

Figure 3 Relative sensitivities of complexes I and IV to lipid peroxidation

(A) Effect of the lipid peroxidation system on complex I ($\square$, $\blacksquare$) and complex IV ($\triangle$, $\blacktriangle$) activities and MDA levels ($\circ$, $\bullet$) in control rat liver SMPs. SMPs were incubated in the presence (open symbols) or absence (solid symbols) of 0.5 mM NADPH. (B) Correlation of MDA formation with inhibition of complex I ($\circ$; slope of -1.626), or complex IV ($\bullet$; slope of -0.847), activities following incubation of control rat liver SMPs with increasing concentrations of NADPH (0–0.5 mM). The values represent the means of at least two separate experiments.

30 min (Figure 2A). At 0.5 mM NADPH, no further MDA was formed after 30 min. In the control system, without NADPH, basal levels of MDA were detectable, but these did not exceed more than 13% of the values obtained in the test system.

SMPs were incubated with increasing concentrations of BHT in order to determine its effect on the activity of complexes I and IV. Complex IV activity was essentially unaffected by BHT, whereas complex I activity was progressively inhibited giving approximately 50% inhibition at a concentration of 1 mM. At the lowest BHT concentration used (20 μ M), the inhibitory effects on the respiratory enzymes were minimal (approximately 7%) (Figure 2B), whereas the formation of MDA in the presence of 0.5 mM NADPH was inhibited by 95% (results not shown), indicating that lipid peroxidation was effectively suppressed at this concentration of BHT. Based on these findings, a final concentration of 20 μ M BHT was used to terminate lipid peroxidation in subsequent experiments.

At a standard rate of NADPH-induced lipid peroxidation (0.5 mM NADPH) MDA formation increased to a maximum at 30 min, and complex I and IV activities were inhibited progressively over a 30 min incubation period, with the inhibition of complex IV being less severe than that observed with complex I (Figure 3A). The activities in the absence of NADPH were only partially affected, consistent with the basal rate of lipid per-

Figure 4 Sensitivity of complex I and IV activities in normal and vitamin E-depleted SMPs to lipid peroxidation

Effect of (a) 0.1 mM NADPH or (b) 0.5 mM NADPH on the rate of MDA formation (nmol of MDA formed/min per mg of protein) and the activity of complexes I and IV in SMPs from control (solid bars) or vitamin E-deficient (hatched bars) rat livers. Incubations were 10 min for complex I and 30 min for complex IV. Control (100%) activities were determined in the absence of NADPH and are as follows. Control SMPs: complex I, 256 ± 57.9 nmol/min per mg of protein; complex IV, 38.6 ± 4.7 k/min per mg of protein (first order rate constant). Vitamin E-deficient SMPs: complex I, 256 ± 46.7 nmol/min per mg of protein; complex IV, 41.3 ± 4.7 k/min per mg of protein. Values are means $\pm$ S.D. ($n = 5$).

oxidation. In agreement with this, when exposed to increasing concentrations of NADPH the relationship between lipid peroxidation (MDA levels) and the decrease in complex I activity (slope of -1.626) showed a greater level of inhibition compared with complex IV activity (slope of -0.847 , Figure 3B). This suggests that complex I is more sensitive to NADPH-induced lipid peroxidation and that it is independent of the rate of lipid peroxidation.

SMPs were generated from liver mitochondria prepared from control rats or rats fed a vitamin E-deficient diet for 48 weeks. Vitamin E levels in the control liver mitochondria were 0.093 ± 0.015 nmol of vitamin E/mg of protein ($n = 5$), but were below the limits of detection in the mitochondria from rats fed a vitamin E-deficient diet, indicating that the vitamin E levels were $< 5\%$ of control values. Control and vitamin E-deficient rat liver SMPs were subjected to two different rates of lipid peroxidation using the lipid peroxidation system in the presence of either 0.1 or 0.5 mM NADPH. Incubation times of 10 min for complex I and 30 min for complex IV were used to compensate for the differential susceptibility of complexes I and IV to different levels of lipid peroxidation.

At low NADPH concentration (0.1 mM) the rate of lipid peroxidation was approximately 19% higher in the vitamin E-deficient SMPs compared with controls (Figure 4). However, this difference was not statistically significant and was not accompanied by increased inhibition of either complex I or complex IV activity. At 0.5 mM NADPH, the rate of lipid peroxidation increased approximately 3-fold, but was essentially the same in the control and vitamin E-deficient SMPs (Figure 4). No significant difference was observed in the inhibition of complex I between the control and vitamin E-deficient SMPs at this rate of lipid peroxidation. Complex IV inhibition was 10% greater in the deficient SMPs, however, this was not statistically significant (Figure 4). Hence, the vitamin E-deficient SMPs were not more susceptible to peroxidation or to MRC inhibition at either low or

high rates of NADPH-dependent lipid peroxidation, compared with control SMPs containing normal amounts of vitamin E.

DISCUSSION

Prolonged vitamin E deficiency appears to disproportionately affect neuronal and skeletal muscle function, giving rise to a neurological syndrome in humans [18], and a necrotizing myopathy and a central-peripheral distal axonopathy in rats [4]. The underlying reason for the susceptibility of neuronal and skeletal muscle tissues is not clear. The fact that these tissues along with testes generally have lower levels of vitamin E than spleen, liver, lung and heart [31], may make them more vulnerable to loss of vitamin E in the diet. However, this contrasts with the higher rate of vitamin E loss from the liver and spleen in comparison with the heart, skeletal muscle and brain in the absence of dietary vitamin E [32,33]. The decreased activity of complex IV and complex I in the vitamin E-deficient rat muscle mitochondria is in agreement with that published previously [4]. The selective clinical involvement of these tissues is consistent with MRC dysfunction playing a role in the pathology of vitamin E deficiency [34]. The underlying cause of the mitochondrial abnormalities is not known, but could relate to an altered membrane environment, caused by loss of vitamin E, increased lipid peroxidation, oxidatively damaged components of the complexes or damage to mtDNA. The increase in anisotropy (decrease in fluidity) of the muscle mitochondrial membranes may contribute to the decrease in mitochondrial activities. Whereas cross-linkage of the lipids following lipid peroxidation will result in a decrease in membrane fluidity the real decrease due to lipid damage is likely to be more severe than indicated from the present data, as loss of vitamin E itself will cause an increase in membrane fluidity [35]. Modification of components of the complexes by products of lipid peroxidation has been previously reported [13], and is, therefore, also likely to be playing a role. However, neither qualitative nor quantitative changes were detected in the *in vitro* translation of the mtDNA (results not shown), suggesting that gross changes in mtDNA were not responsible for the changes in mitochondrial function.

In contrast with the muscle there was an increase in the MRC activities in the vitamin E-depleted liver mitochondria. This was despite the fact that vitamin E levels had been undetectable since 3 months of age (results not shown) and lipid peroxidation was increased, although this was not as marked as in the muscle. Mitochondrial membrane fluidity was not changed in the liver mitochondria, but, as mentioned for the muscle mitochondria, this may not reflect the overall effect on membrane structure. The liver is morphologically normal in these animals and is associated with normal MRC function. This clearly demonstrates that long-term loss of vitamin E neither affects MRC function nor causes cell damage. The lower levels of MDA in the liver may reflect the much higher levels of antioxidant enzymes in the liver than in the muscle [36], which may mean that liver is less dependent on vitamin E to prevent lipid peroxidation. In order to induce liver damage, depletion of both vitamin E and selenium is required [37], presumably reflecting the higher levels of antioxidant protection in the liver, although it is not known what effect this has on MRC function. The increase in GSH levels in the vitamin E-depleted muscle appears to represent a protective mechanism to prevent the higher level of lipid peroxidation, a feature less marked in the liver.

In agreement with a previous report, liver SMPs from normal rats were susceptible to lipid peroxidation generated using an *in vitro* lipid peroxidation system [10], and complex I, and to a

lesser degree complex IV, were vulnerable to this lipid peroxidation. This clearly indicated that lipid peroxidation of liver SMPs can cause inhibition of MRC function, although this was not seen in the *in vivo* vitamin E-depleted model. At both a low and high rate of lipid peroxidation there were no differences between the rates of MDA formation in normal and vitamin E-deficient liver SMPs, suggesting that vitamin E was not playing a major role in preventing lipid peroxidation in liver SMPs. In addition, the levels of inhibition of MRC enzyme activities were essentially the same in liver SMPs from both vitamin E-deficient and control rats. These results suggest that loss of vitamin E does not make the liver mitochondria more vulnerable to lipid peroxidation and could explain the normal mitochondrial activities in liver mitochondria in rats fed a vitamin E-deficient diet for 48 weeks.

Whereas mitochondria are generally found to be relatively rich in vitamin E it may not be the only defence against lipid peroxidation damage as ubiquinol has also been implicated as an antioxidant not only in the mitochondria but also the cytosol [38]. Although its role may be to recycle the antioxidant-derived reaction product of vitamin E [39,40], there is evidence that ubiquinol protection against lipid peroxidation is not dependent on the presence of vitamin E [41]. Within the MRC, reduced ubiquinone can act as an antioxidant under normal physiological conditions [42,43], where it may protect against lipid peroxidation and free radical-mediated MRC inactivation [44]. The fact that the level of the ubiquinone pool has been shown to increase in liver cells in response to vitamin E depletion [45], together with the observation that ubiquinone can protect hepatocytes from free radical damage in the absence of vitamin E [46], may explain why both liver mitochondrial function and liver morphology were not affected in the long-term vitamin E-deficient rat. However, this does not explain why muscle and muscle mitochondria were more vulnerable to vitamin E deficiency *in vivo*. This needs to be addressed by analysing the susceptibility of normal and vitamin E-depleted muscle mitochondria to lipid peroxidation.

This work was supported by the Muscular Dystrophy Group of Great Britain (to R.R.), grant number RA2/307. Ubiquinone-1 was a gift from Eisai Co., Ltd (Tokyo, Japan).

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Received 1 December 2000/30 April 2001; accepted 30 May 2001