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Mitochondrial Respiratory Chain Dysfunction in Ageing; Influence of Vitamin E Deficiency

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The causes and consequences of ageing are likely to be complex and involve the interaction of many processes. It has been proposed that the decline in mitochondrial function caused by the accumulation of oxidatively damaged molecules plays a significant role in the ageing process. In agreement with previous reports we have shown that the activities of NADH CoQ_1 reductase and cytochrome oxidase declined with increasing age in both rat liver and gastrocnemius muscle mitochondria. However, only in the liver were the changes in lipid peroxidation and membrane fluidity suggestive of an age-related increase in oxidative stress.

After 12 weeks on a vitamin E deficient diet, vitamin E levels were undetectable in both gastrocnemius muscle and liver. In skeletal muscle, this was associated with a statistically significant increase in lipid peroxidation, a decrease in cytochrome oxidase activity after 48 weeks, and an exacerbation in the age-related rate of decline of NADH CoQ_1 reductase activity. This was consistent with the suggestion that an imbalance between free radical generation and antioxidant defence may contribute to the mitochondrial dysfunction with age. In contrast to this, vitamin E deficiency in the liver caused a significant increase in mitochondrial respiratory chain activities with increasing age despite evidence of increased lipid peroxidation. Comparison of other features in these samples suggested vitamin E deficiency; did not have a significant impact upon mtDNA translation; induced a compensatory increase in glutathione levels in muscle, which was less marked in the liver, but probably most interestingly caused a significant decrease in the mitochondrial membrane fluidity in muscle but not in liver mitochondria.

These data suggest that while increased lipid peroxidation exacerbated the age-related decline in muscle respiratory chain function this relationship was not observed in liver. Consequently other factors are likely to be contributing to the age-related decline in mitochondrial function and specific stimuli may influence or even reverse these age-related effects as observed with vitamin E deficiency in the liver.

Keywords: Vitamin E deficiency; Mitochondrial function; Ageing; Lipid peroxidation

INTRODUCTION

Continuous exposure of aerobic organisms to reactive oxygen species is an inevitable consequence of their greatly enhanced energy production. While enzymatic (glutathione peroxidase, glutathione reductase, Mn and Cu/Zn superoxide dismutase, catalase) and non-enzymatic (glutathione, ascorbate, vitamin E) antioxidant defences have evolved, cellular defences are not perfect. The accumulation of damage to DNA, protein, lipid and other biomolecules can be observed under normal physiological conditions and forms the basis of the free radical theory of ageing.[1]

Mitochondria play a central role in cellular metabolism and have been proposed to play an important role in the ageing process.[2] In addition there is evidence that mitochondrial respiratory chain function, particularly of complexes I and IV, declines with age.[3] However, this phenomenon has not been reported by all groups.[4] The respiratory chain is an important source of cellular free radicals as a by-product of its normal function[5] and free radical

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damage has been proposed to underlie this age-related decline in function. In addition an age-related increase in reactive oxygen species (ROS) production by mitochondria has been reported in insects and mammals.[6] These observations have led to the modification of the free radical theory of ageing to incorporate the mitochondrial contribution.[2] The proximity of this free radical production to mitochondrial membranes and mitochondrial DNA (mtDNA) makes respiratory chain components particularly vulnerable to damage. Increased lipid peroxidation, Mn-SOD activity and mtDNA deletions in mitochondria from aged human tissues[7] are consistent with the proposal that mitochondrial ROS generation and lipid peroxidation occur simultaneously with mtDNA mutations, and represent early causative molecular events in the ageing process.[8]

We have determined mitochondrial respiratory chain function in skeletal muscle and liver mitochondria from normal rats up to 12 months of age to investigate the effect of ageing upon mitochondrial respiratory chain function. To evaluate whether a chronic model of antioxidant deficiency could accelerate any age-related changes in respiratory chain function we have studied rats fed a vitamin E deficient diet for 12 months.

METHODS

Materials

All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK), Merck (Dagenham, Essex, UK) or Boehringer Mannheim Ltd (Lewes, Sussex, UK). Weanling male Wistar rats (21 days old) were placed on a vitamin E deficient diet (Hoffmann-La Roche 814 Tocopherol Deficient Diet; Dyets Inc., Pennsylvania, USA). A second control group received the same diet to which 40 mg/kg of *all-rac*-α-tocopheryl acetate 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.

Mitochondrial Preparation and Analyses

After 12, 24, 36 and 48 weeks rats were killed by cervical dislocation and the gastrocnemius muscle and liver removed immediately and mitochondria prepared as described previously.[9] All mitochondrial enzyme assays were performed at 27°C on Hitachi U-3210 (Hitachi Scientific Instruments, Wokingham, Berks, UK) or Kontron Uvikon 940 (Kontron Instruments Ltd, Watford, Herts., UK) recording spectrophotometers. Mitochondrial samples were subjected to 3 successive freeze-thaw cycles prior to assaying; rotenone-sensitive NADH CoQ_1 reductase (complex I), succinate cytochrome c reductase (complexes II/III), and cytochrome oxidase (complex IV) using methods previously described.[10]

Mitochondrial Protein Synthesis

Freshly isolated mitochondria, prepared under aseptic conditions, were incubated at a final concentration of 1.5 mg/ml in a medium containing 50 mM bicine/KOH buffer pH 7.6, 90 mM KCl, 5 mM $MgCl_2$, 10 mM potassium phosphate, pH 7.6, 1 mM K^+EDTA, 100 μM complete amino acid mixture minus L-methionine, 100 μg/ml cycloheximide, 1 mM ATP, 25 mM glutamate and 50 μM atractyloside.[11] The reaction mixture containing the mitochondria was initiated by the addition of 50 μCi of L-[^{35}S]methionine (1000 Ci/mmol specific activity) and shaken at 30 °C. L-[^{35}S]methionine incorporation was determined by spotting 5 μl aliquots onto Whatman 2.1 cm GF/C glass fibre discs at 0, 10, 20, 30 and 60 min. The discs were placed in ice-cold 10% trichloroacetic acid containing methionine (0.5%) for 1 h. The discs were boiled for; 15 min in 10% trichloroacetic acid/0.5% methionine followed by a further 15 min in 5% trichloroacetic acid/0.5% methionine. The discs were washed in ethanol, air dried, placed in 7 ml of Ultima Gold scintillant and counted on a Beckman LS5801 liquid scintillation counter. The remaining translation mixture was centrifuged at 13,000*g* for 10 min, the supernatant removed and the pellets retained for electrophoretic separation.

Analysis of Mitochondrial Translation Products

Mitochondrial translation products were analysed by urea-SDS-PAGE using the Tricine gel system,[12] except that a linear gradient of 12–16.5% was employed to improve the overall separation. Samples were corrected for half-life decay and equal radioactivity loaded, (10,000–30,000 dpm). Rainbow [^{14}C] methylated molecular weight protein markers (14.3–220 kDa, Amersham International plc, Bucks, UK) were routinely loaded on each gel. The gels were processed for fluorography and dried under vacuum before visualisation of the translation products by autoradiography.

Mitochondrial Membrane Fluidity

Mitochondrial membrane fluidity was determined using the lipophilic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) as described previously.[13]

Vitamin E Levels

Vitamin E (α-tocopherol) concentrations were determined by normal phase, high performance liquid chromatography (HPLC) as described previously.[13]

Lipid Peroxidation

Lipid peroxidation was determined by measuring the tissue malondialdehdye (MDA) content using the HPLC-based thiobarbituric acid (TBA) assay.[14] Tissues were homogenised, extracted and reacted with TBA in the presence of the chain-breaking antioxidant, butylated hydroxytoluene (BHT). Fifty μl aliquots of the TBA-reacted samples were injected onto a 5-μm, 25 cm Techsphere ODS-2 column (HPLC Technology Ltd, Cheshire, UK) and eluted at a flow rate of 1 ml/min with 65% (v/v) 50 mM potassium phosphate buffer, pH 7.0/35% (v/v) HPLC grade methanol. The absorbance of the pink-coloured $(TBA)_2$-MDA adduct was read at 532 nm and the concentrations of MDA estimated by direct comparison against a standard solution of hydrolysed 1,1,3,3-tetraethoxypropane (TEP).

Glutathione Levels

Reduced glutathione (GSH) and oxidised glutathione (GSSG) were assayed by the enzymatic recycling procedure, using 2-vinylpyridine as the GSH trapping reagent.[15]

Protein

Protein concentrations of homogenates and of mitochondrial suspensions were determined by the method of Lowry *et al.*,[16] with bovine serum albumin as a standard.

Statistics

Statistical analysis was performed using the non-parametric Mann-Whitney *U* Test (Instat™, Graph Pad Software).

RESULTS

The effects of ageing on various biochemical parameters of oxidative stress and on mitochondrial function were determined in both gastrocnemius muscle and liver of rats maintained on a vitamin E replete or deficient diet for up to 51 weeks of age.

At 51 weeks, age-related morphological changes were observed in the muscle ultrastructure, characterized by the accumulation of myelin figures, the presence of abnormal degenerating mitochondria and the proliferation of components of the transverse (T) tubular system, whilst the liver remained unaltered (data not shown). Vitamin E levels increased with increasing age in both gastrocnemius muscle and liver (Fig. 1A). While there was a tendency for malondialdehyde (MDA) levels to increase with age in both control skeletal muscle and liver (Fig. 1B), the values were not significantly different to the 15 week values at any time point. In addition there was a fall in MDA levels in muscle at 51 weeks of age. In both tissues the glutathione levels were fairly stable between 15 and 51 weeks of age (Fig. 1C). While the GSH/GSSG ratios increased in both muscle and liver between 15 and 51 weeks of age the values were only statistically significant for muscle (Fig. 1C). Membrane fluidity tended to increase with increasing age in the muscle mitochondria (decreased anisotropy), but decrease in liver mitochondria (increased anisotropy) (Fig. 1D). However, the values were not significantly different to the 15 week values at any time point.

In vitro mtDNA translation rates and the profile of synthesised polypeptides were analysed to obtain information regarding the functional integrity of the mtDNA. The rate of [^{35}S]-methionine incorporation (15 weeks of age; $3.71 \pm 0.47 \times 10^4$ DPM/min/mg protein: 51 weeks of age; $3.39 \pm 0.65 \times 10^4$ DPM/min/mg protein), and the profile of the subunits incorporating radioactivity (data not shown) were not influenced by the age of the rats.

Spectrophotometric measurements of mitochondrial respiratory chain function were assessed at 15, 27, 39 and 51 weeks of age. In both the liver and gastrocnemius muscle mitochondria the activities of NADH CoQ_1 reductase (muscle: $r = -0.52$, $p = 0.01$; liver: $r = -0.84$, $p = 0.0001$) and cytochrome oxidase (muscle: $r = -0.36$, $p = 0.09$; liver: $r = -0.60$, $p = 0.003$) declined with increasing age (Figs. 2 and 3). However, succinate cytochrome c reductase activities were essentially unchanged over time in both tissues.

Vitamin E levels were undetectable in both the gastrocnemius muscle and liver from animals on a vitamin E deficient diet for 12, 24, 36 and 48 weeks. Morphological analysis revealed ultrastructural abnormalities in the muscle of rats from as early as 12 weeks on the vitamin E deficient diet and included the presence of dense residual bodies, myelin figures, degenerating mitochondria and honeycomb structures derived from proliferated T tubules. After 48 weeks of vitamin E depletion, atrophic, degenerating and necrotic (macrophage-infiltrated) fibres were prevalent in the muscle. In contrast, no morphological abnormalities were observed in the vitamin E deficient liver (data not shown). At all time points on the deficient diet MDA levels were increased in both the liver and gastrocnemius muscle when compared to the age-matched controls, indicating increased lipid peroxidation (Fig. 1B). Glutathione levels showed a compensatory increase of 177% ($p = 0.0025$) in muscle and 46% ($p = 0.005$) in liver after 48 weeks on the vitamin E deficient diet (51 weeks of age, Fig. 1C). This was associated with a decrease in the GSH/GSSG ratio in muscle indicative of oxidative stress, but this was not apparent in liver (Fig. 1C).

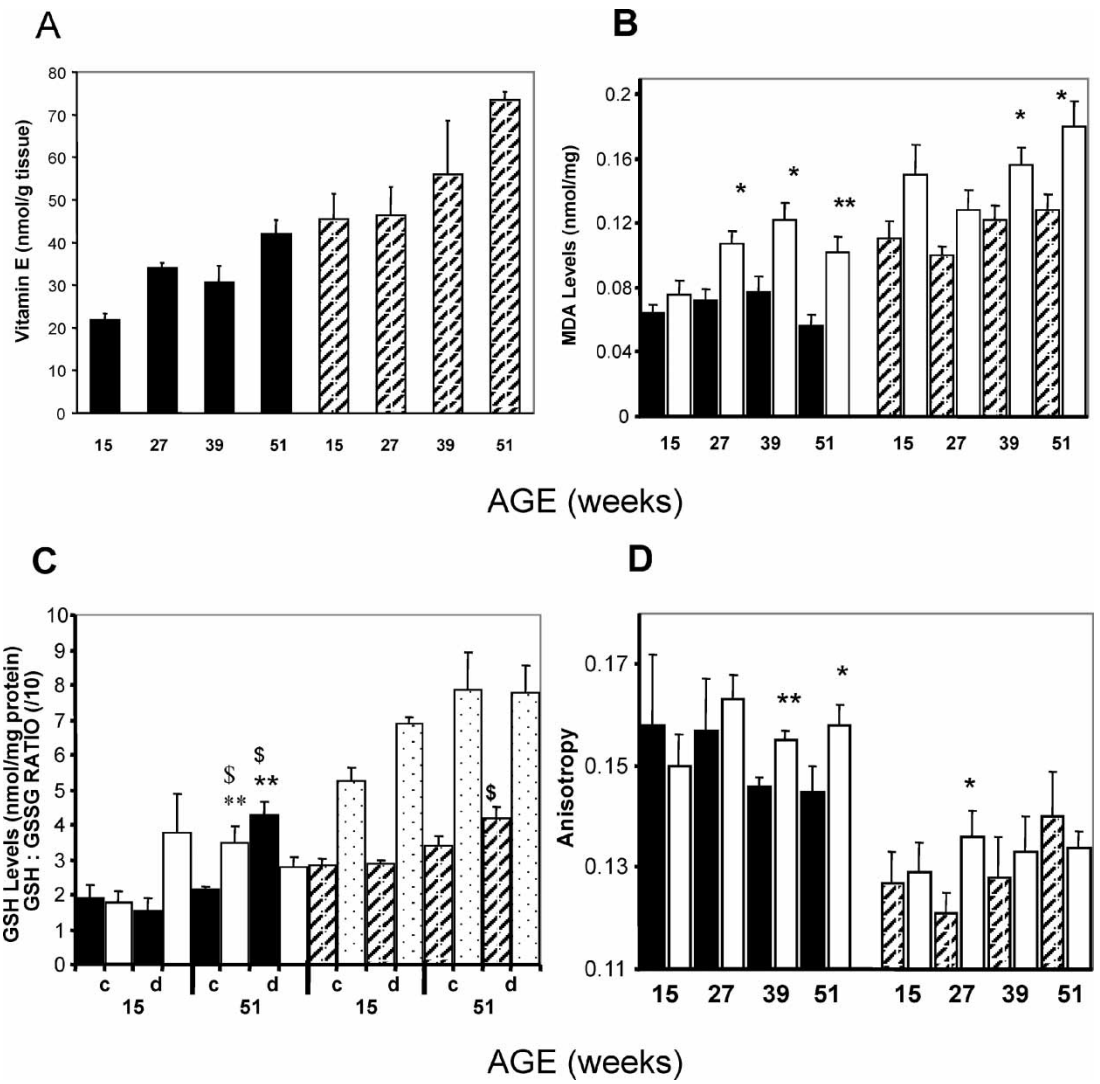

FIGURE 1 Analysis of gastrocnemius muscle (solid bar) and liver (hatched bar) mitochondria from rats fed a normal or vitamin E deficient (open bar) diet. Effect of increasing age on (A) vitamin E levels ($n = 3$). Comparison of increasing age in normal and vitamin E deficient rats upon; (B) malondialdehyde levels ($n = 6$); (C) glutathione levels (solid and hatched bars) and GSH/GSSG ratios (open and stippled bars) for control (c) or vitamin E deficient (d) animals respectively (the data for GSH/GSSG ratios and liver GSH have been divided by 10), and (D) mitochondrial membrane anisotropy ($n = 6$). Values are expressed as mean ± SEM. Statistical difference Mann-Whitney U test; compared with controls $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; compared with 15 week data $^{\$}$ $p < 0.01$.

Muscle mitochondrial membrane fluidity in the animals on the deficient diet decreased slightly over time in contrast to the age-related increase in fluidity in normal animals. Consequently there was a significant decrease in membrane fluidity after 36 and 48 weeks on the diet, (39 and 51 weeks of age) in comparison to the control mitochondria (Fig. 1D). However, in the liver mitochondria, although there was a significant decrease in fluidity after 24 weeks on the deficient diet, overall there were no consistent changes from the normal values (Fig. 1D).

Long-term vitamin E deficiency did not significantly affect either the rate of mtDNA *in vitro* translation ($3.62 \pm 0.94 \times 10^4$ DPM/min/mg protein) or the profile of proteins synthesised by the muscle mitochondria after 48 weeks on the deficient diet (data not shown) indicating the mtDNA was not functionally impaired.

To determine whether exposure to increased lipid peroxidation exacerbated the age-related decline in mitochondrial function observed in both muscle and liver, the respiratory chain activities were correlated with age (Figs. 2 and 3). In vitamin E deficient muscle the age-related rate of decline in NADH CoQ_1 reductase activity (gradient $= -0.82$, $p = 0.0001$) was greater than that seen with normal ageing (Fig. 2A). However, the age-related decline of cytochrome oxidase (gradient $= -0.47$, $p = 0.04$) activity paralleled that seen with increasing age, although the activities were decreased at all time points (Fig. 2C). This was consistent with the suggestion that increased free radical damage

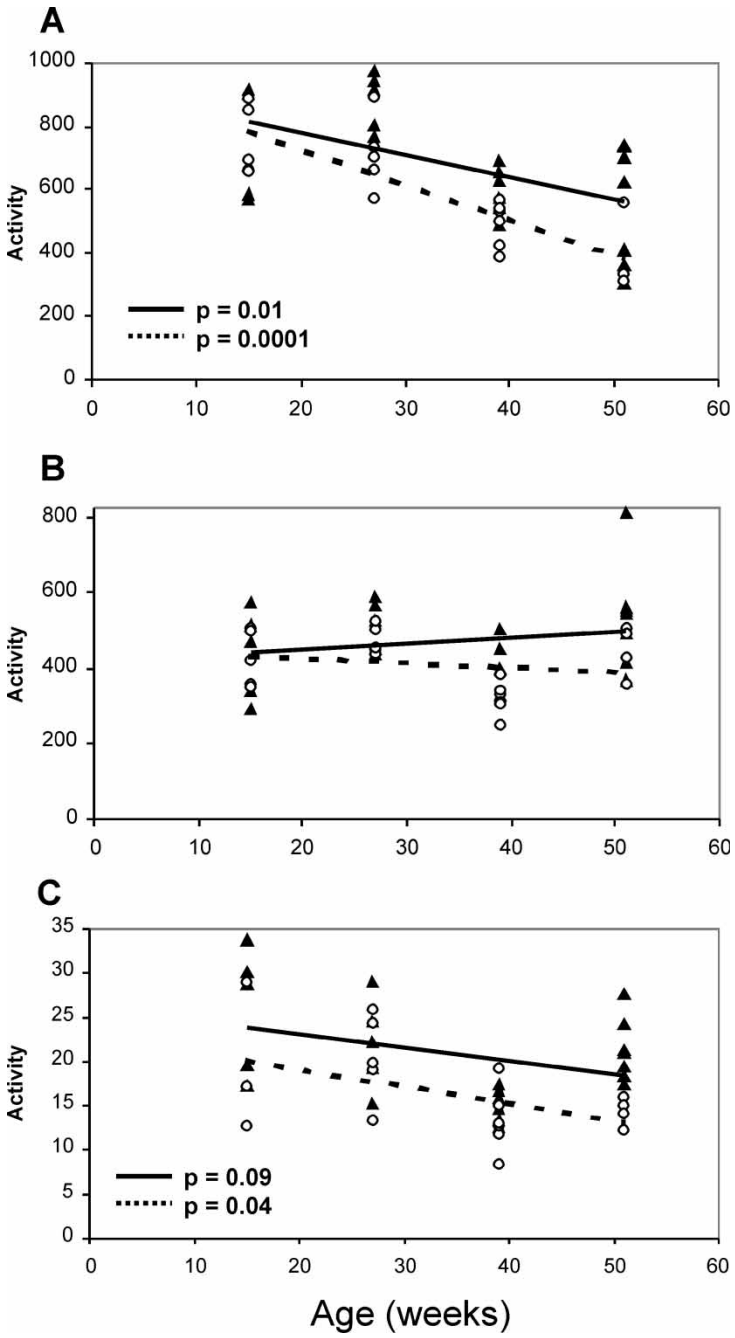

FIGURE 2 Mitochondrial respiratory chain activities in isolated mitochondria from the gastrocnemius muscle of rats fed a vitamin E supplemented (closed triangle) and vitamin E deficient (open circle) diet from 3 weeks of age. Spectrophotometric analysis of: (A) NADH coenzyme Q_1 reductase; (B) succinate cytochrome c reductase and (C) cytochrome oxidase. Values are nmol/min/mg protein except cytochrome oxidase which is k/min/mg protein. The linear regression lines are shown for the control rats (solid line) and vitamin E deficient rats (dotted line), only the p values <0.1 are stated.

exacerbated the age-dependent mitochondrial dysfunction. The opposite was observed in the vitamin E deficient liver, where the age-related decline in NADH CoQ_1 reductase activity was diminished (gradient $= -0.43$, Fig. 3A) and the decline in cytochrome oxidase activity completely reversed (Fig. 3C).

The age-independent effect of vitamin E deficiency on mitochondrial respiratory chain function was determined by comparing the activities of the vitamin E deficient mitochondria with the age-matched controls. After 48 weeks on the vitamin E deficient diet, gastrocnemius muscle mitochondria NADH CoQ_1 reductase, succinate cytochrome c reductase and cytochrome oxidase activities were decreased by 32%, 16% and 33% respectively, but only the latter was statistically significant ($p < 0.01$). However, this contrasted with an increase in NADH

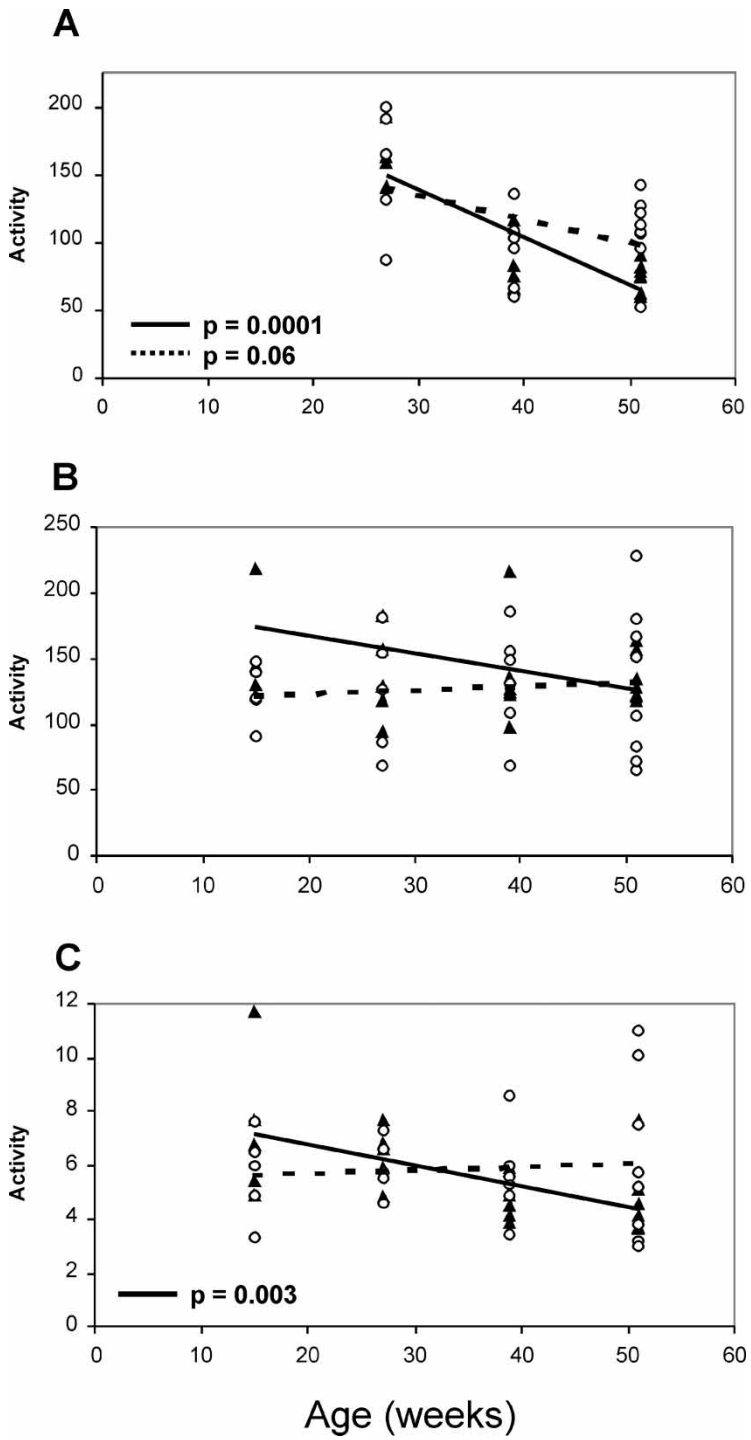

FIGURE 3 Mitochondrial respiratory chain activities in isolated mitochondria from the liver of rats fed a vitamin E supplemented (closed triangle) and vitamin E deficient (open circle) diet from 3 weeks of age. Spectrophotometric analysis of: (A) NADH coenzyme Q_1 reductase; (B) succinate cytochrome c reductase and (C) cytochrome oxidase. Values are nmol/min/mg protein except cytochrome oxidase which is k/min/mg protein. The linear regression lines are shown for the control rats (solid line) and vitamin E deficient rats (dotted line) and the p values < 0.1 are stated.

CoQ_1 reductase (31%) and cytochrome oxidase (25%) activities in the liver mitochondria from these rats, although it was only statistically significant for NADH CoQ_1 reductase activity ($p = 0.014$). This indicated that the long-term effects of vitamin E deficiency on mitochondrial function were markedly different between muscle and liver. In comparison with the liver the decreased respiratory chain activities in the muscle mitochondria were associated with a greater percentage increase in lipid peroxidation and GSH levels and a decrease in membrane fluidity (Fig. 4).

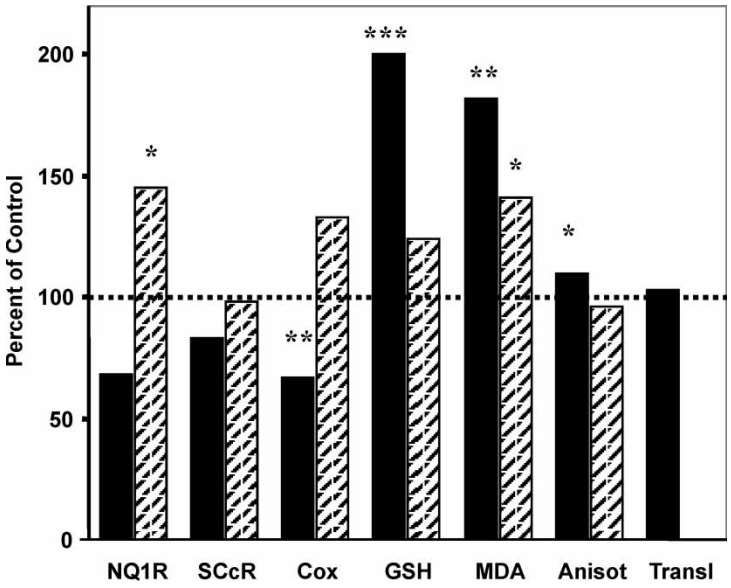

FIGURE 4 Comparison of the effects of 48 weeks on a vitamin E deficient diet upon markers of mitochondrial function in gastrocnemius muscle (filled bars) and liver (hatched bars). The mean values for the vitamin E deficient rats are expressed as a percentage of the mean value for the control rats for; the spectrophotometric assessment of NADH coenzyme Q_1 reductase (NQ1R); succinate cytochrome c reductase (SCcR) and cytochrome oxidase (Cox) activity; glutathione (GSH), malondialdehyde (MDA), mitochondrial anisotropy (anisot) and mtDNA *in vitro* translation rates (Transl). Mean values were obtained from Figs. 1–3. Mann-Whitney *U* test $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

DISCUSSION

While old age in Wistar rats is generally accepted as 24 months of age, limitations imposed by the health of the vitamin E deficient animals restricted this study to 12 months. However, ultrastructural changes observed in control muscle at 51 weeks, were indicative of the ageing process.[17] Liver did not reveal any morphological changes suggesting it may require a more prolonged period of time to manifest age-related changes.

Over the time period of this study the decrease in the activities of complexes I and IV, and normal complex II/III activity in both rat gastrocnemius muscle and liver is consistent with previous reports of age-associated reductions in mitochondrial respiratory chain function.[3,18] The cause of this decline and what role it may play in the ageing process remains the focus of much debate.

The accumulation of oxidatively damaged molecules with age has been suggested to play not only an important role in the decline in respiratory chain activities with age but also in the ageing process itself. However, we were unable to identify a clear relationship between the age related decline in respiratory chain activities and increase in oxidative damage. At 12 months of age there was no evidence of oxidative damage in either rat muscle or liver samples. However, vitamin E levels increased with age in both the muscle and liver samples, suggesting an enhanced protection against age-dependant increases in lipid peroxidation, and may contribute to the relatively stable MDA levels. Antioxidants were not generally increased as the levels of glutathione remained relatively stable in both skeletal muscle and liver. The age related increase in vitamin E levels has been noted before,[19] while glutathione levels have been reported to decrease,[20] increase,[21] or remain unchanged.[22] While increased oxidative damage with increasing age is a common observation,[22] it is not universal[23] and may require the study to be extended to 24 months for more definitive data.

The function of integral membrane proteins can be influenced by the physiochemical state of the lipid environment of membranes.[24] In particular the respiratory chain complexes are embedded in the membrane and their enzymatic activity has an absolute catalytic requirement for cardiolipin, which is particularly sensitive to ROS.[25] The fluidity of the mitochondrial membranes showed a tendency to increase in muscle but decrease in liver with increasing age and was therefore not a common age-related phenomenon. A decrease in mitochondrial membrane fluidity with increasing age has been reported previously,[26] and correlated with increased mitochondrial ROS generation, lipid peroxides and impaired respiratory chain function.[24] While the increase in vitamin E in both tissues would result in a decrease in fluidity, the tissue specific effects on fluidity may be influenced by the fall in lipid peroxidation (MDA) at 51 weeks in muscle, contributing to an increase in mitochondrial membrane fluidity, while in liver the mild increase in lipid peroxidation with age will contribute to a decline in mitochondrial membrane fluidity.

The *in vitro* rate of mtDNA translation in skeletal muscle mitochondria was not influenced by age suggesting the functional integrity of mtDNA was unchanged. While these data do not rule out more subtle oxidative damage to random mtDNA bases, we can exclude a significant decline in mtDNA translation or the down regulation of specific mtDNA subunits due to deletions.

To study if the age associated decline in mitochondrial respiratory chain function was related to increased free radical damage, we turned to vitamin E deficient rats, as a chronic model of decreased antioxidant defence, to determine if the rate of decline was enhanced.

Vitamin E depletion gave rise to the predicted increase in lipid peroxidation in both tissues. Morphologically, vitamin E deficiency accelerated the age related changes in skeletal muscle, suggesting the loss of vitamin E may accelerate the natural process of ageing in skeletal muscle. In contrast, the liver maintained normal morphology in the absence of vitamin E suggesting it was more resistant to the effects caused by the loss of vitamin E. Changes to mitochondrial function mimicked the morphological findings. Muscle showed an increase in the age related decline in complex I activity, suggesting vitamin E deficiency accelerated a process in common with normal ageing. Although, the activity of cytochrome oxidase decreased with vitamin E deficiency, the age-related rate of decline paralleled that observed in the control rats. This suggests that loss of vitamin E resulted in decreased complex IV activity, but the increased lipid peroxidation did not enhance its age-related rate of decline. These changes possibly reflect changes to membrane fluidity and the fact that complex IV is less sensitive to lipid peroxidation than complex I.[13] In contrast, vitamin E deficiency in liver attenuated the age-related decline in complex I and IV activities, despite the liver showing an increase in lipid peroxidation. This suggests that lipid peroxidation per se was not sufficient to cause the decline in respiratory chain activities and additional changes are required. It is not clear whether muscle is more prone to this damage or if liver has induced additional mechanisms to counteract this damage.

In liver it is possible the changes in cytochrome oxidase may be in response to elevated oxidative stress as cytochrome oxidase activities have been shown to increase in rat liver after exposure to increased lipid peroxidation induced by adriamycin,[27] or vitamin E deficiency.[28] It is not known why muscle and liver respond differently, however, cytochrome oxidase activities have shown tissue-specific responses with increases in liver in response to exercise which were not observed in heart.[29]

In both the muscle and liver from the vitamin E deficient rats glutathione levels increased with age, presumably acting as a compensatory protective mechanism. In the muscle there was a decrease in the GSH/GSSG ratio suggesting this was associated with increased oxidative stress, but this was not apparent in the liver. It is unlikely that GSH alone was sufficient to protect the liver mitochondrial function as muscle showed a greater increase in GSH but failed to prevent a decline in respiratory chain function.

The most important difference between muscle and liver was in the mitochondrial membrane fluidity. Muscle mitochondria showed a significant decrease in fluidity after depletion of vitamin E which was not apparent in the liver mitochondria. This is not likely merely to reflect differences in lipid peroxidation because levels were increased in both tissues. Vitamin E has a membrane stabilising effect, consequently, the vitamin E deficient rats would be expected to have more fluid membranes. However, the increased lipid peroxidation in the vitamin E deficient rats would have an opposite influence causing a decrease in fluidity. The combination of these influences could be responsible for the overall decrease in fluidity in muscle, while in the liver the overall increase in fluidity may reflect changes in membrane composition as previously reported.[28]

We have previously shown liver mitochondria depleted of vitamin E did not exhibit an increased sensitivity to lipid peroxidation,[13] suggesting other protective mechanisms are present in liver mitochondria. These could be responsible for protecting the respiratory chain activities in the absence of vitamin E, and if induced following vitamin E deficiency, may protect the liver mitochondria from oxidative damage induced by normal ageing. Similar data is not available for muscle mitochondria, but it is possible these mechanisms are less efficient or absent in muscle.

In conclusion respiratory chain function declined with increasing age in both muscle and liver mitochondria, however, increased lipid peroxidation only caused an accelerated decline in complex I activity in muscle. From the comparison of the liver and muscle data it is clear that increased lipid peroxidation *per se* does not necessarily affect mitochondrial respiratory chain function *in vivo* and that other factors are possibly contributing to the age-related decline in mitochondrial function. We clearly demonstrate that tissue-specific responses to specific stimuli like vitamin E depletion may influence or even reverse these age-related deficits.

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