J Bioenerg Biomembr (2006) 38:309–317
DOI 10.1007/s10863-006-9050-1

ORIGINAL PAPER

Adaptations to oxidative stress induced by vitamin E deficiency in rat liver

Rafael de Cabo · John R. Burgess · Placido Navas

Received: 31 May 2006/ Accepted: 20 June 2006 / Published online: 11 October 2006

Abstract Vitamin E deficiency in rats led to a sequence of antioxidant defense adaptations in the liver. After three weeks, α-tocopherol concentration was 5% of control, but ascorbate and ubiquinol concentrations were 2- to 3-fold greater than control. During the early phase of adaptation no differences in markers of lipid peroxidation were observed, but the activities of both cytochrome *b5* reductase and glucose-6-phosphate dehydrogenase were significantly greater in deficient livers. By nine weeks, accumulation of lipid peroxidation end products began to occur along with declining concentrations of ascorbate, and higher NQO1 activities. At twelve weeks, rat growth ceased, and both lipid peroxidation products and cytosolic calcium-independent phospholipase A2 reached maximum concentrations. Thus, in growing rats the changes progressed from increases in both ubiquinol and quinone reductases through accumulation of lipid peroxidation products and loss of endogenous antioxidants to finally induction of lipid metabolizing enzymes and cessation of rat growth.

Keywords Antioxidants · Adaptations · Coenzyme Q · Oxidative stresss · Rat livers · Vitamin E

R. de Cabo· J. R. Burgess
Department of Foods and Nutrition, Purdue University, West Lafayette, IN 47907, USA

R. de Cabo
Present address: National Institute of Aging, NIH, Baltimore, MD 21224, USA

P. Navas (✉)
Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide-CSIC, 41013 Sevilla, Spain
e-mail: pnavas@upo.es

Introduction

All aerobic organisms are exposed to reactive oxygen species (ROS), which have a great ability to damage cellular structures, by acting as initiators of the lipid peroxidation chain reaction (Kehrer, 1993). Intracellular defense against ROS-induced damage includes molecules and enzymes with antioxidant properties. Primary antioxidants are ascorbate, coenzyme Q (CoQ) and α-tocopherol, which function to inactivate ROS and to stop the propagating reactions that are characteristic of lipid peroxidation (Turunen et al., 2004) (Machlin & Bendich, 1987). Antioxidant enzymes such as superoxide dismutase, catalase, selenium-dependent and selenium-independent glutathione peroxidases, and glutathione transferases, involve radical scavenging which detoxify ROS. Another level of defense involves metabolizing enzymes such as endonucleases, lipases, and proteases that remove the ROS-induced damage in cellular macromolecules (Crawford et al., 1994). A fourth level of defense involves adaptive responses that boost antioxidant defense systems to allow cells to withstand chronic assaults of oxidative stress. The role of this fourth level of adaptation in protecting cells from oxidative stress is not clearly understood.

We have previously characterized a series of adaptive responses to oxidative stress that occur when rats are severely deficient in both vitamin E and selenium (Navarro et al., 1998). Vitamin E has been proposed to be the major antioxidant in plasma membrane of all cells functioning as a chain-breaking antioxidant (Sokol, 1988; Kohlschutter et al., 1988; Ingold et al., 1987). In the absence of vitamin E another lipid-soluble antioxidant, CoQ, is implicated in protecting membrane fatty acids from oxidative stress (Turunen et al., 2004; Schultz et al., 1996). We have shown previously that plasma membranes from rat livers fed with a deficient diet

in both vitamin E and selenium had a higher concentration of CoQ_9 and CoQ_{10} than those membranes from rat liver fed with the two nutrients (Navarro et al., 1998). Additionally, both plasma membranes showed similar levels of resistance to induced lipid peroxidation in the presence of NAD(P)H. This result suggests that membrane protection may be mediated by CoQ via its enrichment in plasma membrane and an enhanced recycling mechanism facilitated by electrons supplied by pyridine nucleotides (Beutler et al., 1996; Beyer, 1994; Pandolfi et al., 1995). Vitamin E deficiency mimics aging process because shows an increase of oxidative stress and accumulates membrane damage (Harman, 2003). In fact, NQO1 activity is increased and this increase is partially prevented by the supplementation and membrane incorporation of CoQ_{10} (Bello et al., 2005). Calorie restriction also contributes to extend longevity in mammals by coordinating ubiquinone reductases such as NQO1 and the increase of CoQ_{10}/CoQ_9 ratio (De Cabo et al., 2004).

Although these data are quite intriguing and suggest an important role for CoQ and its recycling machinery in protecting cells from ROS, the results were obtained from one time point in severely deficient rats (Navarro et al., 1998). Thus, it is not clear what contribution adaptive changes make to the preservation of liver membrane integrity in vivo. These adaptive changes may result from severe cellular damage and occur at a late stage in the progression of the vitamin E and selenium deficient state. Rats suffering this severe deficiency experience lost weight and enhanced mortality compared to control rats. Adaptive responses that occur late in the development of oxidative stress may make little contribution to the protection of cells from damage as the stress continues to intensify. On the other hand, adaptive responses that occur early in the development of the deficiency may make a significant contribution in protecting cells. Thus, characterizing the sequence of adaptive changes that occur in cells as oxidative stress develops is important to understand the contribution that the different defense systems make to overall cellular defense. Additionally, defining specific adaptive changes that are characteristic of different stages of oxidative stress may be helpful in establishing markers of progression for diseases in which ROS are implicated as playing a major role.

The purpose of the study reported here was to characterize the sequence of adaptive changes to oxidative stress induced in rats by a deficiency in vitamin E with a constant level of selenium. This model will result in a slower development in oxidative stress severity, and control for the confounding variable of the effects of selenium deficiency independent of its role as a component of the antioxidant enzyme glutathione peroxidase. Our results show that adaptations in CoQ and its recycling machinery occur early in the development of the vitamin E deficiency and make a contribution to the protection of liver cells from oxidative stress.

Material and methods

Animals

Long-Evans hooded male weaning (40–50 g) rats (Harlan, Indianapolis, IN) were used in this study. They were housed individually in stainless steel cages on a 12 h light-dark cycle. The rats had access to deionized water from a sterilized bottle *ad libitum* during the study period. Control animals were fed a normal torula yeast-based diet containing all-rac-alpha-tocopherol acetate (150 mg/kg) and sodium selenite (0.05 mg/kg). This is a level of dietary selenium that is marginal in terms of nutritional status. In order to induce a vitamin E deficiency, rats were then fed a torula yeast-based diet without vitamin E as previously described (<0.1 mg/kg for α-tocopherol). The composition of both diets was as described elsewhere (Kuo et al., 1995). The animals were housed individually and maintained at 22 °C with 12 h light–dark cycles. Rats had free access to food and water at all times and were fed with treatment diets for 12 wk. The protocol for these studies was approved by the Purdue University Animal Care and Use Committee.

Chemicals and reagents

CoQ_{10}, CoQ_9, CoQ_7, α-tocopherol, δ-tocopherol, cytochrome *c*,ascorbate oxidase, and anti-rabbit antibody conjugated to alkaline phosphatase were obtained from Sigma (St. Louis, MO). Dextran T 500 was from Pharmacia (Sweden) and PEG from Fisher Scientific (Pittsburgh, PA).

Preparation of plasma membrane fractions

Liver was homogenized in ice cold homogenization buffer (Golgi grind) containing 38 mM Tris-HCl pH 6.4, 35 mM maleic acid, 10 mg/ml dextran T-500, 50 mM magnesium chloride, 0.5 M sucrose, 28 mM sodium hydroxide, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM PMSF (Phenylmethyl sulfonyl flouride) and 5 mM DTT (Dithiothreitol). This homogenate was then centrifuged at 5000 g for 15 min. After the first centrifugation at 5000 g the upper half of the pellet produced was resuspended in 5 ml of 1 mM sodium bicarbonate, homogenized in a conical Teflon-glass tissue homogenizer, and centrifuged at 5000 g for 15 min. The supernatant was then discarded and the light brown, top portion of the pellet was considered as the microsomal fraction and used to isolate plasma membranes in the two-phase partition as previously described (Navas et al., 1989). Membranes were resuspended in 50 mM Tris-HCl, pH 7.6, containing 10% glycerol, 1 mM PMSF, 1 mM EDTA, and 0.1 mM DTT, and stored at −70°C until needed. The purity of plasma membrane fractions was analyzed by measuring the activity of marker enzymes using ouabain-sensitive

ATPase for PM, glucose-6-phosphatase for endoplasmic reticulum, and cytochrome c oxidase for mitochondria (Navas et al., 1989).

CoQ and α-tocopherol determinations

Lipids were extracted with hexane from purified plasma membrane and then a high-performance liquid chromatography separation was performed at 0.8 ml/min using a reverse phase LC-18-DB column (25 cm $\times$ 5 mm, 5 μm particle size) Supelco (Bellefonte, Pa.). The column was equilibrated in mobile phase (buffer A) composed of 50 mM sodium perchlorate in a mixture of ethanol:methanol:water(9.1:0.4:0.5). α-Tocopherol, CoQ_9, and CoQ_{10} were gradient-eluted with 100% ethanol containing 50 mM sodium perchlorate (buffer B) as follows: buffer A 100%, 5 min; gradient to 100% buffer B, 2 min; buffer B 100%, 10 min; gradient to 100% buffer A, 2 min; buffer A 100%, 10 min. Monitoring was carried out simultaneously with an electrochemical detector (amphoteric 0.7 V, BioAnalytical Systems, West Lafayette, IN) for α-tocopherol (Pascoe et al., 1987), and a UV monitor (Waters 484 variable wavelength detector) set at 275 nm for CoQ_9 and CoQ_{10}. Eluted compounds were quantified by integration of peak areas and comparison with internal standards (δ-tocopherol and CoQ_7, respectively).

Assays for lipid peroxidation

Malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) were measured as end products derived from the breakdown of polyunsaturated fatty acids (Esterbauer et al., 1991). The kit supplied by OxisResearch (Portland, OR) utilizing the chromogenic reagent, N-methyl-2-phenylindole was used for quantifying MDA and HAE, as described in the accompanying literature.

Enzyme activities

All assays were carried at 37°C with constant gentle stirring in 50 mM Tris-HCl, pH 7.6, containing 30–100 μg of plasma membrane in a final volume of 1 ml. NADH-ascorbate free radical (AFR) reductase was assayed as describe previously (Navarro et al., 1998). NAD(P)H: (quinone acceptor) oxidoreductase (NQO1, DT-diaphorase) activity was assayed measuring the dicumarol-sensitive reduction of cytochrome *c* as described elsewhere (Lind et al., 1990). Glucose-6-phosphate dehydrogenase assays were performed measuring the conversion of $NADP^+$ to NADPH by either G6PD or by the second enzyme 6-phosphogluconate dehydrogenase (PGD) as described previously (Beutler et al., 1996; Pandolfi et al., 1995). Phospholipase A2 (PLA2) activity was measured in liver cytosol using L-γ-dipalmitoyl{2-palmitoyl-9,10–3H}phosphatidylcholine (specific activity 1850 GBq/mmol; New England Nuclear, Boston, Mass.) as substrate by the method previously described (Kuo et al., 1995). The glutathione S-transferase (GST) activity was assayed spectrophotometrically as previously described using 1-Cl-2, 4-dinitrobenzene as substrate (Habig et al., 1974). Specific Activity for all enzyme measurements was expressed as nmoles product formed or substrate used per min per mg of protein. Protein concentrations in liver samples were determined as described elsewhere (Bradford, 1976).

Ascorbate determination

We measured ascorbate concentrations in the liver by two methods. For the enzymatic assay, one gram of frozen tissue was homogenized in 5% ice-cold perchloric acid and centrifuged at 10000 $\times$ g. For each assay, 200 μL of the supernatant were added to 1.8 ml of ice-cold 0.2 M phosphate/0.1 M citrate buffer (pH 6.5) and monitored at 265 nm. After recording the readings, 1 unit of ascorbate oxidase (From *Cucurbita*, EC 1.10.3.3) was added and incubated for 30 min at 25°C. After the incubation period the samples were measured again at 265 nm. The amount of ascorbate present in the sample was calculated extrapolating from a standard curve (Nakagawa et al., 1975). Concentrations were verified by an HPLC procedure with equipment as described for tocopherol and ubiquinone concentrations. A sample (20 μl) of the acidified, clarified, and neutralized liver homogenate was injected onto an HPLC, and the separation was performed at a flow rate of 1.2 ml/min using a Supelco LC-18-T column (15 cm $\times$ 4.6 mm, 3 μm particle size, Supelco, Bellefonte, PA). The column was equilibrated in mobile phase (buffer A) composed of 10 mM tetrabutylammonium hydroxide, 10 mM KH2PO4, 0.25% methanol, pH 7.0, and eluted with buffer B which was composed of 2.8 mM tetrabutylammonium hydroxide, 100 mM KH2PO4, 30% methanol, pH 5.5. Pyridine nucleotides and ascorbate were gradient-eluted as follows: buffer A 100%, 5 min; gradient up to 50% buffer B, 5 min; buffer B 50% for 5 min; 5 min up to 100% buffer B; 5 min gradient up to 100% buffer A and 5 min buffer A. Eluted compounds were detected by uv and electrochemical detection and quantified by integration of peak areas by comparison to a standard curve generated by injected standards containing 1–50 μM of ascorbate.

Glutathione determination

One gram of frozen tissue was homogenized in 5% ice-cold perchloric acid and centrifugated at 10000 $\times$ g. For each assay, 25 μL of the supernatant were added to 96-well microtiter plate wells containing 140 mM sodium phosphate buffer (pH 7.5), 5 mM EDTA, 25 μl of 6 mM 5,5′-dithio-bis-(2-nitrobenzoic acid), and 10 μl of 100 units/ml glutathione reductase type III (Sigma, St. Louis, MO). The reaction was

started with the addition of 50 μl of 1.1 mM NADPH, and the change in absorbance at 405 nm was monitored for 3 min. A standard curve for glutathione was run with each of the assays. This methodology allows for the determination of total glutathione including both oxidized and reduced.

Statistical analysis

All results are expressed as mean ± SEM. Serial measurements were analyzed by using two-way ANOVA with Tukey's post hoc test using SigmaStat software from SPSS Science (Chicago, IL). The level of significance was set at $p < 0.05$.

Results

Effect of diet on rat growth

A significant difference in body weight between rats consuming the control and vitamin E deficient diet was not observed until rats were consuming the vitamin E deficient diet for nine weeks. Body weight gain was significantly less after this week compared to that of animals fed the control diet (Fig. 1A). Growth failed by the eleventh week, and by twelve weeks vitamin E deficient animals had an average body weight 25.6% lower than the control group.

The liver weight to body weight ratio was also measured as an indicator of the effects of vitamin E deficiency on liver function. Rats consuming the deficient diet showed a significantly higher liver/body weight ratio from week nine on indicating a clear hepatomegalia (Fig. 1B).

Antioxidants and lipid peroxidation in plasma membrane

Concentrations of α-tocopherol in the plasma membrane of liver were significantly lower in the vitamin E deficient versus the control throughout the study (Fig. 2A). Feeding a vitamin E deficient diet induced a significant depletion of α-tocopherol in the plasma membranes, reaching a minimum level of 5.4% of the α-tocopherol concentration of the control group. Thus, these animals were appropriate to study vitamin E deficiency-induced changes.

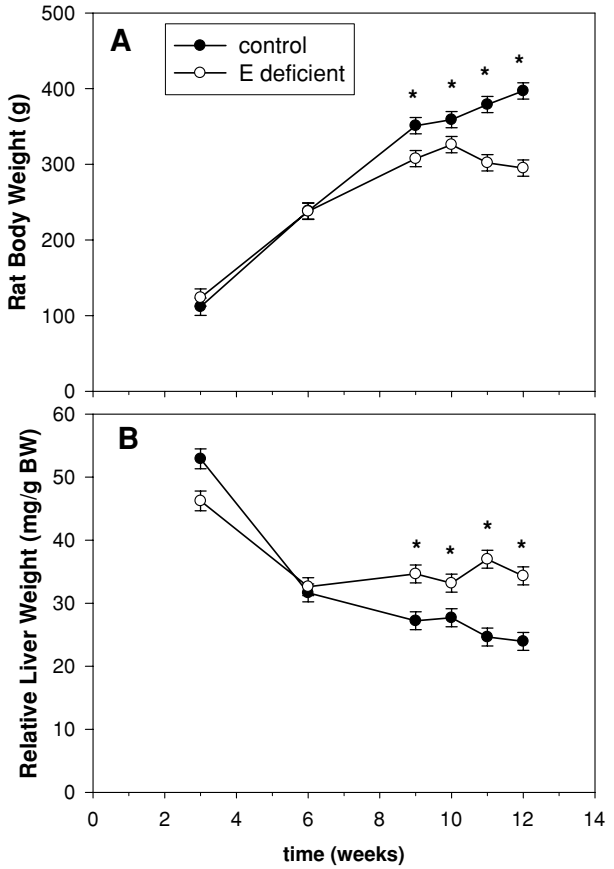

Fig. 1 Effect of vitamin E deficiency on average weight gain (A) and liver to body weight ratio (B) in Long-Evans hooded rats. Values plotted are means ± SEM ($n = 5$). $^*p < 0.05$ as compared to −E at each time point

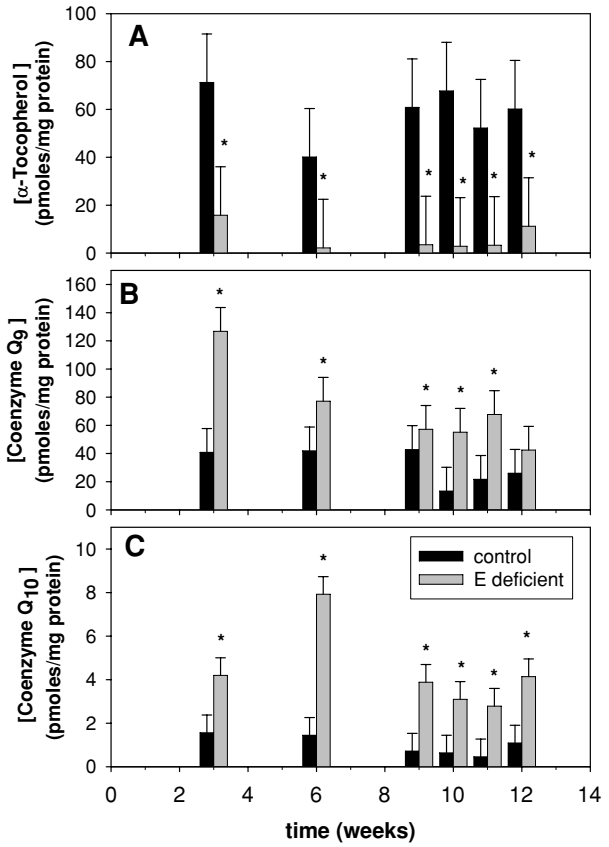

Fig. 2 The effect of consuming a vitamin E-deficient diet on α-Tocopherol (A), CoQ_9 (B) and CoQ_{10} (C) concentrations in the liver plasma membrane of Long-Evans hooded rats. Lipid-soluble antioxidant concentrations were determined on extracted samples using HPLC with electrochemical detection. Values plotted are means ± SEM ($n = 4$). $^*p < 0.05$ as compared to +E at each time point

We measured the plasma membrane concentrations of both CoQ_9 and CoQ_{10}, and found that CoQ_9, the major form of CoQ synthesized in rat tissues, was significantly higher in plasma membrane of the vitamin E deficient animals from week three through week 11 (Fig. 2B). The maximum difference was reached at week three, in which tissue concentrations of CoQ_9 were four times higher than those from the control group. At weeks 10 and 11 plasma membrane concentrations were about three times those of the control group. We observed a general trend toward a significant decrease in the CoQ_9 concentration as the vitamin E deficiency progressed. There was no statistical difference between the two groups at 12 weeks.

CoQ_{10} concentrations in the plasma membrane of animal livers fed the vitamin E deficient diet was also significantly higher throughout the study. Animals fed the vitamin E deficient diet, reached a maximum difference at six weeks in which CoQ_{10} plasma membrane concentrations were about six times those of the control group (Fig. 2C).

In order to determine the effects of vitamin E deficiency on the magnitude of oxidative stress experienced by living tissue two common markers of lipid peroxidation, MDA and HAE, were analyzed (Figs. 3A and B). Both lipid peroxidation markers showed a significantly greater concentration in the vitamin E deficient liver compared to the control but not until the rats had been consuming diets for at least nine weeks. These results indicate that after week nine, rats fed the vitamin E deficient diet were suffering a greater degree of oxidative stress than the control group. Thereafter, both aldehyde compounds that are metabolic products of lipid peroxidation increased reaching a maximum at 12 weeks.

Water-soluble antioxidants in vitamin E deficiency

Animals fed the vitamin E deficient diet showed a significantly greater concentration of ascorbate in liver as early as three weeks of diet consumption (Fig. 4A), but no significant

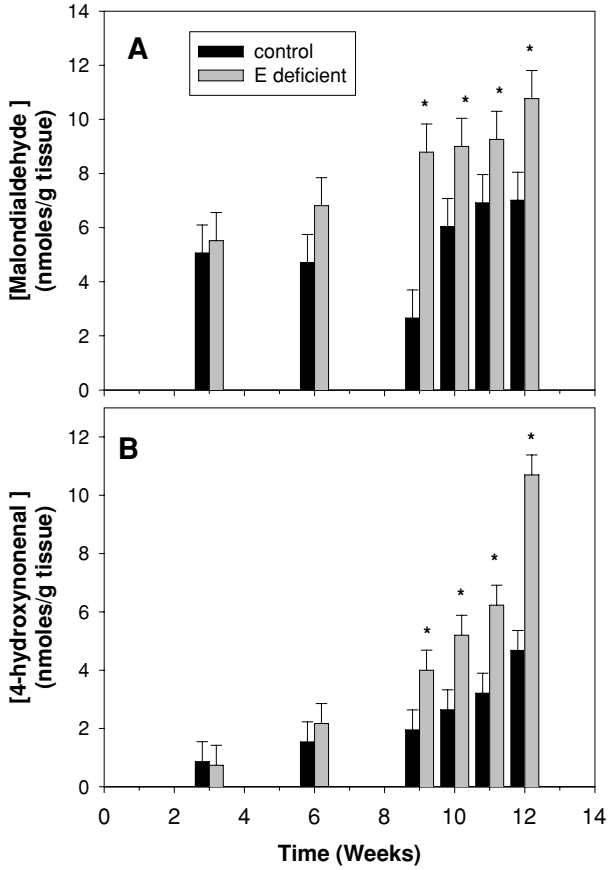

Fig. 3 The effect of consuming a vitamin E-deficient diet on the liver microsomal concentrations of MDA (A) and 4-hydroxynonenal (B) in Long-Evans hooded rats. These products of lipid peroxidation were measured colorimetrically using N-methyl-2-phenylindole as the active reagent. Values plotted are means ± SEM ($n = 4$). $^*p < 0.05$ as compared to +E at each time point

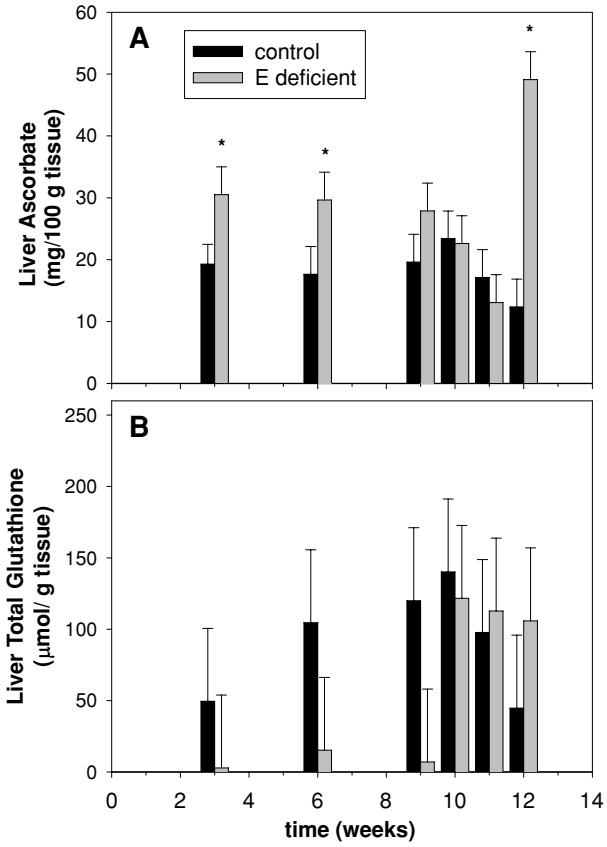

Fig. 4 The effect of consuming a vitamin E-deficient diet on the liver cytosolic concentrations of ascorbate (A) and glutathione (B) in Long-Evans hooded rats. Ascorbate concentrations were determined by 265 nm absorbance of extracted samples using ascorbate oxidase to confirm specificity. Concentrations were verified by HPLC of extracted samples using electrochemical detection. Glutathione concentrations were quantified using 5,5′-dithio-bis-(2-nitrobenzoic acid) as the active reagent. Values plotted are means ± SEM ($n = 4$–5). $^*p < 0.05$ as compared to +E at each time point

differences were observed after week six. However, at week 12 there was a dramatic 3-fold increase in the ascorbate concentration of the liver from vitamin E deficient rats that was not observed in the control group. The ascorbate liver concentrations among the control group animals did not exhibit significant changes throughout the study.

The data for glutathione concentrations of liver was quite variable, and although the mean GSH concentrations appeared to be lower in the rats consuming the vitamin deficient diet until the ninth week, no significant differences were found (Fig. 4B).

Enzyme activities in liver of vitamin E-deficient rats

We have shown previously that during severe oxidative stress induced by deficiency of both vitamin E and selenium, the amounts of cytochrome *b5* reductase were greater in the deficient animals (Navarro et al., 1998). In this study, we have also observed that the activity appeared to increase step-wise, from week 3 through week 11 in samples from rats consuming the deficient diet. The control rats showed a much smaller gradual increase throughout the rest of the study (Fig. 5A).

NAD(P)H: (quinone acceptor) oxidoreductase (NQO1, DT-diaphorase) is a cytosolic enzyme that participates in a 2-electron reduction of CoQ. It has been shown to increase in activity during the treatment of rats with xenobiotics (Lind et al., 1990). In the cytosol of vitamin E deficient animals, NQO1 was significantly greater than in the control group starting at week nine and continuing through week twelve (Fig. 5B). We previously showed that a portion of this NQO1 activity was membrane-bound in rats deficient in vitamin E and selenium (Navarro et al., 1998; Navarro et al., 1999). A smaller amount of NQO1 activity was detected in the membranes of the control group, but membrane-bound NQO1 activities were significantly increased from week nine through week twelve in vitamin E deficient rats (Fig. 5C).

Glucose-6-phosphate dehydrogenase is the first rate-limiting enzyme of the pentose phosphate pathway. The activities of this enzyme were significantly higher—as much as 6-fold—throughout the study in animals fed the vitamin E deficient diet (Fig. 6A).

We measured the activities of two enzyme systems that could contribute to the detoxification of lipid peroxidation metabolites. Glutathione-S-transferase, an abundant enzyme in rat liver, was significantly greater than control at 10 weeks in vitamin E deficient animals, but it was then significantly lower than the control at 11 and 12 weeks (Fig. 6B).

The calcium independent PLA2 has been shown in previous studies to participate in the metabolism of lipid hydroperoxides induced by the combined deficiency of both vitamin E and selenium (Kuo et al., 1995). Animals fed the control or the vitamin E deficient diet exhibited similar activities from week three through week 10. However, at week 11 through week 12, the animals consuming the vitamin E deficient diet showed markedly greater activity of this PLA2 with a maximum at 12 weeks (Fig. 6C).

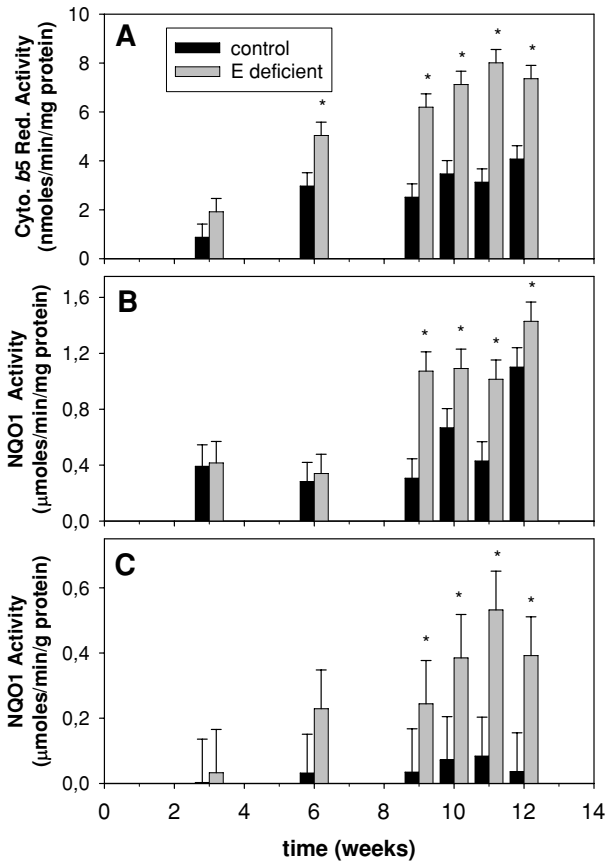

Fig. 5 The effects of consuming a vitamin E-deficient diet on the cytochrome *b5* reductase activity in plasma membranes (A), and cytosolic (B) and plasma membrane bound (C) NQO1 activities in livers of Long-Evans hooded rats. Enzyme activities were determined spectrophotometrically by following NAD(P)H oxidation with appropriate electron acceptors. Values plotted are means ± SEM ($n = 4$–5). $^{*}p < 0.05$ as compared to + E at each time point

Discussion

A deficiency in both vitamin E and selenium leads to a very quick response in which the animals progress to a pathological state within six weeks, and this makes it difficult to separate different events involved in providing cellular resistance to stress (Crawford et al., 1994). The dietary regimen used in this study, a vitamin E deficiency with marginal levels of selenium, resulted in a more gradual development of oxidative stress. This statement is based on the growth rate of rats in comparison to previous studies (Kuo et al., 1995), and the time frame of the increase in concentration

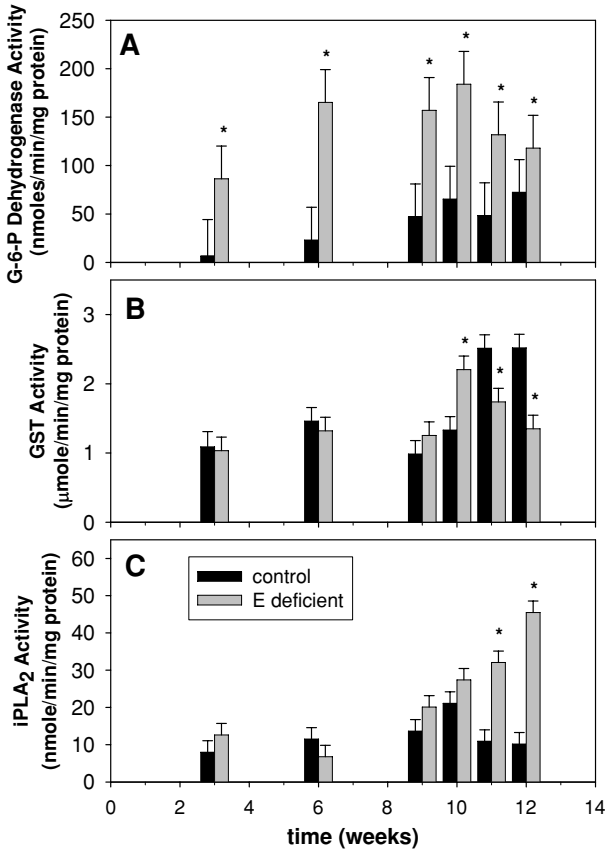

Fig. 6 The effects of consuming a vitamin E-deficient diet on the liver cytosolic activities of glucose-6-phosphate dehydrogenase (A), glutathione-S-transferase (B), and calcium-independent phospholipase A2 (C) in Long-Evans hooded rats. Glucose-6-phosphate dehydrogenase and glutathione-S-transferase activities were measured spectrophotmetrically at 340 nm by following NADP reduction or 1-Cl-2,4-dintrobenzene conjugation respectively. Phospholipase A2 activity was measure radiometrically using 1–^{14}C dipalmitoyl phosphatidylcholine as substrate. Values plotted are means ± SEM ($n = 3$–5). $^{*}p < 0.05$ as compared to +E at each time point

of lipid peroxidation products in liver. Plasma membranes from vitamin E deficient rat livers contained concentrations of α-tocopherol that were only 5% of that of control animals, which is similar to results reported elsewhere (Habig et al., 1974). The time frame difference between the loss of liver membrane α-tocopherol (three weeks), the appearance of lipid peroxidation products above the control values (nine weeks), and the loss of the ability of the rats to maintain growth (11–12 weeks), allowed us to separate events in the adaptation process to oxidative stress.

A first stage of the adaptive process began to occur by week three as α-tocopherol concentrations reached a minimal level in the liver. At this time point, concentrations of both CoQ_9 and CoQ_{10} were 2- to 3-fold greater in the plasma membrane of deficient rats compared to control rats. This large difference suggested that liver cells concentrated CoQ_9 and CoQ_{10} in plasma membrane as an adaptive response to the lack of vitamin E. An accumulation of CoQ in membranes has been described previously for rats treated with chemical agents (Aberg et al., 1994), and cells stimulated to undergo apoptosis (Barroso et al., 1997; Slater et al., 1995). This accumulation could be explained by either an increase in the rate of CoQ biosynthesis or a greater delivery of newly synthesized CoQ to the plasma membrane. Alternatively, a redistribution of ubiquinol among subcellular membanes may occur. A previous study reported a significant decrease in mitochondrial CoQ_9 and CoQ_{10} in rats fed a vitamin E and selenium deficient diet for nine weeks (Scholz et al., 1997). This evidence along with the data reported here may support the hypothesis that ubiquinones move from the intracellular compartments to the plasma membrane in response to oxidative stress. Furthermore, the same group reported no difference in CoQ_9 in total liver microsomes from rats fed a diet deficient in vitamin E versus a control diet (Scholz et al., 1994). These two results are supportive of the relocation of CoQ from one compartment to another. However, it is also known that ubiquinones present in the diet become reduced after their uptake (Stocker and Suarna, 1993) The *de novo* synthesis of the hydroquinone has been proposed to contribute, at least partially, to the high levels of $CoQH_2$ observed in vivo (Stocker and Suarna, 1993; Schultz et al., 1996). Moreover, recent evidence suggests that oxidative stress can change the intracellular distribution of CoQ in mammalian and yeast cells (Barroso et al., 1997). The distribution of CoQ among cellular membranes is an active process that involves the endomembrane pathway and it suggests that the membrane content of ubiquinone may be influenced by the physiological status of the cell (Fernandez-Ayala et al., 2005). The relocation of CoQ to the plasma membrane during induced oxidative stress supports the idea of its involvement in the cellular antioxidant defense system (Navas and Villalba, 2004).

As CoQ plays a central role in the plasma membrane electron transfer system, we measured the activity of two enzymes responsible for catalyzing the reduction of this quinone, cytochrome *b5* reductase and NQO1. We had previously demonstrated that both of these enzyme activities were increased in the liver plasma membrane during the later stages of the development of a combined vitamin E and selenium deficiency (Navarro et al., 1998), and that cytochrome *b5* reductase protein levels were elevated soon after depletion of both nutrients from the liver (Navarro et al., 1999). However, the timeframe for changes in both quinone reductase systems was not clear. In this study we show that only cytochrome *b5* reductase appeared to respond during the early phase of adaptation to oxidative stress. The activity of this enzyme was almost 2-fold greater than the control in plasma membrane from deficient rat livers at six weeks. In contrast, NQO1 activity in the liver plasma membrane was not significantly greater until nine weeks of rats consum-

ing the deficient diet. This result suggests that the earliest stages of adaptation to withstand oxidative stress involve enhancements in one electron transfer reactions (Villalba et al., 1996; Villalba et al., 1995), rather than two electron-dependent reductions(Beyer et al., 1996). This is apparently a general aspect to response to endogenous oxidative stress (Bello et al., 2005).

The pattern of changes in the concentrations of ubiquinone differed between CoQ_9 and CoQ_{10}. Although both CoQ forms were higher in concentration in liver plasma membrane of deficient rats at three weeks compared to controls, at six weeks CoQ_9 concentrations appeared to be declining whereas CoQ_{10} concentrations were increased. Also, at this point in time, cytochrome *b5* reductase activity became significantly greater than the control group. Since NADH is the specific electron donor for this enzyme (Villalba et al., 1995), the observed changes in CoQ and its enzymatic recycling mechanism suggest that one-electron transfer system utilizing NADH as the electron donor may help prevent lipid peroxidation as indicated by no accumulation of MDA or HAE above control levels at these two time points. At nine weeks a marked increase in the activity of NQO1, a two-electron quinone reductase, was observed. This enzyme can utilize both NADH and NADPH. Studies have shown that NADH-dependent one-electron reduction of ubiquinone courses with the production of superoxide, while NQO1 avoids this oxygen radical (Arroyo et al., 2004; Arroyo et al., 2000). Thus, the differential reduction of the one and two-electron systems may be programmed to compensate for the supply of electron sources or the degree of oxidative stress. In agreement with these results, it has been recently shown that the expression of the NQO1 gene was induced after seven weeks of consuming a vitamin E and selenium deficient diets in rat liver (Fischer et al., 2001).

We report here significant changes in the concentrations of the soluble redox molecule, ascorbate. Liver ascorbate concentrations were greater in vitamin E deficient rats at early and later stages of the deficiency. Intracellular ascorbate is extensively oxidized under conditions of oxidative stress (Loke at al., 2006). It is then reasonable that a higher biosynthesis of ascorbate would occur in liver cells after a long-term of vitamin E deficiency-induced oxidative stress. Low concentrations of intracellular glutathione have been associated with increased activity and expression of G6PDH although the majority of these studies were carried out in vitro (Pandolfi et al., 1995; Salvemini et al., 1999; Ursini et al., 1997). We have observed that G6PDH activities were several-fold higher than controls at three, six and nine weeks. These results suggest that the requirement for reducing equivalents during the early stages of oxidative stress were being met by up-regulation of reduced pyridine nucleotide generating systems. It has been suggested that G6PDH is part of an inducible mechanism of cellular response to oxidative stress, and it plays a central role in the control of the intracellular reductive potential (Pandolfi et al., 1995). Evidence for a role of G6PDH in the oxidative stress response mechanism comes from experiments conducted in eukaryotic cells that possess alternative pathways for the production of NADPH. Yeast with a null mutation in the G6PDH gene were able to live in normal media conditions, but underwent programmed cell death upon exposure to trace levels of hydrogen peroxide (Pandolfi et al., 1995). Also, it has been demonstrated that elevated CoQ concentrations in plasma membrane were associated with increased oxidative metabolism in response to peroxisome proliferators and oxidative stress-induced in vitro (Turunen et al., 2004).

After nine weeks of consuming the deficient diet, the rats began to show the symptoms of vitamin E deficiency. These include a statistically significant lower rate of body weight gain in the deficient rats compared to the control group, as well as, an increased liver to body weight ratio. At this point we also observed for the first time significantly higher amounts of lipid peroxidation markers in the livers of vitamin E deficient animals. Thus, the animals were progressing into the second phase of oxidative stress, characterized by increased lipid peroxidation and a decreased ability to sustain growth. There was also a change in the activity of NQO1. We observed that the cytosolic NQO1 activity was significantly higher at nine weeks and the proportion of this enzyme associated with the plasma membrane was greater. This result is consistent with our expectation, because NQO1 has been associated with detoxification activities in rat liver (Lind et al., 1990), and suggests that this phase of the adaptation to oxidative stress might involve radical scavenging enzyme systems.

HAE has been implicated in the regulation of activity and gene expression of a large array of enzymes (Esterbauer et al., 1991). One such enzyme is G6PDH, and we have found that after nine weeks this enzyme declined as the levels of HAE increased. Thus, most of antioxidant components appeared to be decreasing at the same time that the lipid peroxidation products such as MDA and HAE began to accumulate.

A third stage of the adaptive response to oxidative stress in this model was characterized by the increased activity of calcium-independent PLA2, an enzyme involved in metabolism of damaged lipids. Also, there was a re-establishment of higher than control concentrations of ascorbate. Despite these changes, this third stage was associated with more pathological complications such as failure to grow and a greater frequency of the occurrence of vitamin E deficiency symptoms. A calcium-independent form of PLA2 with peroxidase activity has been implicated in the metabolism of oxidized phospholipids (Chen et al., 2000), and enhanced activity of this enzyme may allow for repair and replacement of membrane phospholipids. Thus, the third stage in adaptation to oxidative stress involves many

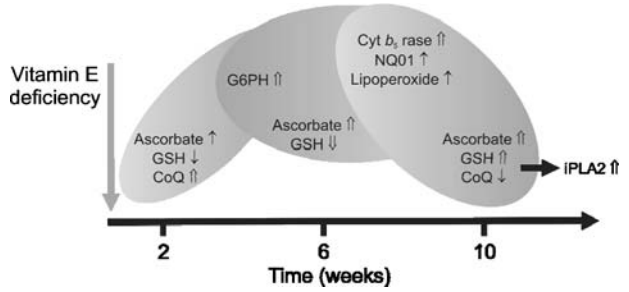

Fig. 7 Scheme of time course of events happening as an adaptation process to vitamin E deficiency

components of the antioxidant defense system, and is the point in which initiation of damage removal becomes most evident.

Our results indicate that the antioxidant defense mechanism invoked in response to oxidative stress induced by vitamin E is an organized hierarchy of events that has three clear but overlapping stages as it is summarized in Fig. 7. The first phase involves the induction of the primary and radical scavenging antioxidants such as ascorbate, CoQ_9 and CoQ_{10} and their recycling machinery.

The second phase is characterized by the onset of accumulating lipid peroxidation products and by an increase of the activity of the two-electron ubiquinone reductases in the plasma membrane. The third phase involves components of all levels of defense and is highlighted by the initiation of enhanced metabolizing enzyme systems such as PLA2.

Acknowledgements Grant support: U.S.D.A. CSREES project no. IND080849 to JRB.

References

Aberg F, Zhang Y, Appelkvist EL, Dallner G (1994) Chem Biol Interact 91:1–14
Arroyo A, Navarro F, Gomez-Diaz C, Crane FL, Alcain FJ, Navas P, Villalba JM (2000) J Bioenerg Biomembr 32:199–210
Arroyo A, Rodriguez-Aguilera JC, Santos-Ocana C, Villalba JM, Navas P (2004) Methods Enzymol 378:207–217
Barroso MP, Gomez-Diaz C, Villalba JM, Buron MI, Lopez-Lluch G, Navas P (1997) J Bioenerg Biomembr 29:259–267
Bello RI, Gomez-Diaz C, Buron MI, Alcain FJ, Navas P, Villalba JM (2005) Exp Gerontol 40:694–706
Beutler E, Vulliamy T, Luzzatto L (1996) Blood Cells Mol Dis 22:49–56
Beyer RE (1994) J Bioenerg Biomembr 26:349–358
Beyer RE, Seguraaguilar J, Dibernardo S, Cavazzoni M, Fato R, Fiorentini D, Galli MC, Setti M, Landi L, Lenaz G (1996) Proc Natl Acad Sci USA 93:2528–2532
Bradford MM (1976) Anal Biochem 72:248–254
Chen JW, Dodia C, Feinstein SI, Jain MK, Fisher AB (2000) J Biol Chem 275:28421–28427
Crawford DR, Edbauer-Nechamen CA, Lowry CV, Salmon SL, Kim YK, Davies JM, Davies KJ (1994) Methods Enzymol 234:175–217
De Cabo R, Cabello R, Rios M, Lopez-Lluch G, Ingram DK, Lane MA, Navas P (2004) Exp Gerontol 39:297–304
Esterbauer H, Schaur RJ, Zollner H (1991) Free Rad Biol Med 11:81–128
Fernandez-Ayala DJ, Brea-Calvo G, Lopez-Lluch G, Navas P (2005) Biochim Biophys Acta 1713:129–137
Fischer A, Pallauf J, Gohil K, Weber SU, Packer L, Rimbach G (2001) Biochem Biophys Res Commun 285:470–475
Habig WH, Pabst MJ, Jakoby WB (1974) J Biol Chem 249:7130–7139
Harman D (2003) Antiox Redox Signal 5:557–561
Ingold KU, Webb AC, Witter D, Burton GW, Metcalfe TA, Muller DP (1987) Arch Biochem Biophys 259:224–225
Kehrer JP (1993) Crit Rev Toxicol 23:21–48
Kohlschutter A, Hubner C, Jansen W, Lindner SG (1988) J Inherit Metab Dis 11 Suppl 2:149–152
Kuo CF, Cheng S, Burgess JR (1995) J Nutr 125:1419–1429
Lind C, Cadenas E, Hochstein P, Ernster L (1990) Methods Enzymol 186:287–301
Loke WM, Proudfoot JM, McKinley AJ, Croft KD (2006) Biochem Biophys Res Commun 345:1039–1043
Machlin LJ, Bendich A (1987) Faseb J 1:441–445
Nakagawa H, Asano A, Sato R (1975) J Biochem (Tokyo) 77:221–232
Navarro F, Arroyo A, Martin SF, Bello RI, de Cabo R, Burgess JR, Navas P, Villalba JM (1999) Biofactors 9:163–170
Navarro F, Navas P, Burgess JR, Bello RI, De Cabo R, Arroyo A, Villalba JM (1998) Faseb J 12:1665–1673
Navas P, Manuel Villalba J (2004) Methods Enzymol 378:200–206
Navas P, Nowack DD, Morre DJ (1989) Cancer Res 49:2147–2156
Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, Luzzatto L (1995) Embo J 14:5209–5215
Pascoe GA, Duda CT, Reed DJ (1987) J Chromatogr 414:440–448
Salvemini F, Franzé A, Iervolino A, Filosa S, Salzano S, Ursini MV (1999) J Biol Chem 274:2750–2757
Scholz RW, Minicucci LA, Reddy CC (1997) Biochem Mol Biol Int 42:997–1006
Scholz RW, Saini AK, Reddy PC, Reddy CC (1994) Biochem Mol Biol Int 34:1215–1225
Schultz R, Ellerby LM, Gralla EB, Valentine JS, Clarke CF (1996) Biochemistry 35:6595–6603
Slater AF, Nobel CS, Orrenius S (1995) Biochim Biophys Acta 1271:59–62
Sokol RJ (1988) Annu Rev Nutr 8:351–373
Stocker R, Suarna C (1993) Biochim Biophys Acta 1158:15–22
Turunen M, Olsson J, Dallner G (2004) Biochim Biophys Acta 1660:171–199
Ursini MV, Parrella A, Rosa G, Salzano S, Martini G (1997) Biochem J 323:801–806
Villalba JM, Gómez-Díaz C, Navarro F, Navas P (1996) Trends Comp Biochem Physiol 2:65–72
Villalba JM, Navarro F, Córdoba F, Serrano A, Arroyo A, Crane FL, Navas P (1995) Proc Natl Acad Sci USA 92:4887–4891