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Phospholipid Hydroperoxides and Lipid Peroxidation in Liver and Plasma of ODS Rats Supplemented with α-Tocopherol and Ascorbic Acid

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Forty-five mutant male ODS rats, unable to synthesize ascorbic acid, were fed nine diets containing 5, 50 or 250 mg of vitamin E/kg diet and 150, 300 or 900 mg of vitamin C/kg diet for 21 days. The concentrations of vitamins C and E increased in liver and plasma in relation to the level of these vitamins in the diet. Vitamin C dietary supplementation increased the plasma vitamin E content at low levels of vitamin E intake, supporting the concept of an *in vivo* synergism between both antioxidant vitamins. Vitamin C, at the dietary levels studied, did not affect the lipid peroxidation. Vitamin E decreased liver and plasma endogenous levels of thiobarbituric acid-reactive substances and liver sensitivity to non-enzymatic lipid peroxidation. This was confirmed by a highly specific assay of lipid hydroperoxides using high performance liquid chromatography with chemiluminescence detection. The hepatic concentration of both phosphatidylcholine and phosphatidylethanolamine hydroperoxides decreased as the vitamin E content of the diet increased. The results show for the first time the capacity of vitamin E to protect against peroxidation of major phospholipids *in vivo* under basal unstressed conditions.

Key words: α-tocopherol, vitamin E, lipid hydroperoxides, peroxidation, free radicals, ODS rats

Abbreviations: BHT, butylated hydroxytoluene; CL, chemiluminescence; HPLC, high performance liquid chromatography; MDA, malondialdehyde; ODS, Osteogenic Disorder Shionogi; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; TBARS, thiobarbituric acid-reactive substances.

INTRODUCTION

Free radical mediated peroxidation of biological molecules and tissues, especially lipids, has recently received much attention in connection with a variety of pathological events, such as cardiovascular diseases, rheumatoid arthritis, inflammatory disorders, cancer and even the aging process.[1-3] Lipid peroxidation proceeds by a free radical chain mechanism by which many lipid molecules may be oxidized to lipid hydroperoxides for every initiation event. Ascorbic acid and α-tocopherol are biological antioxidants which scavenge free radicals preventing chain

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propagation reactions. There is a strong interest in these two vitamins since they are promising for *in vivo* protection against oxidative stress through easy and safe dietary supplementation.

Several *in vitro* studies have provided evidence that ascorbic acid may be involved in the regeneration of vitamin E from tocopheroxyl radical.[4–6] Nevertheless, many contradictory results have been reported concerning the existence of such a relationship in biological systems, since vitamin E resides within the membrane while hydrophilic vitamin C resides in the cellular compartment. Thus, both synergistic[7,8] and antagonistic[9,10] interactions, as well as a lack of any kind of relationship[11,12] have been described *in vivo* for several rat and guinea pig tissues.

Since ascorbic acid is synthesized by most mammals, there is a need for new laboratory animal models to test the antioxidant role of these vitamins. A colony of mutant Wistar rats with a hereditary defect in L-ascorbic acid synthesizing ability has been recently established. This characteristic is controlled by a single autosomal recessive gene in the ODS (Osteogenic Disorder Shionogi) rats.[13–15] This rat mutant, like primates and guinea pigs, lacks L-gulonolactone oxidase enzymatic activity (EC 1.1.3.8) which catalyzes the conversion of L-gulono-τ-lactone into L-ascorbic acid in the last step of L-ascorbic acid biosynthesis, and requires a dietary supply of vitamin C.

Previous work relating lipid peroxidation to vitamins C and E has been performed using peroxidation assays showing poor specificity and measuring final products of lipid peroxidation which can be further metabolized. In this work we have studied the effect of supplementation with different amounts of vitamins C and E in the diet on lipid peroxidation and concentration of these two antioxidants in ODS rat liver and plasma. The study included both the classical measurement of thiobarbituric acid-reactive substances (TBARS) and the assay of phosphatidylcholine and phosphatidylethanolamine hydroperoxides (PCOOH and PEOOH) by a recently developed assay using high performance liquid chromatography (HPLC) with chemiluminescence detection.[16] Dietary levels of low C and E groups were designed to obtain non-deficient animals with low liver and plasma levels of these antioxidant vitamins.

MATERIALS AND METHODS

Animals and Diets

Forty-five male, six-week old ODS rats (100–120 g) obtained from Clea Japan, Inc. (Tokyo, Japan) were housed in wire bottomed stainless-steel cages in a room with a temperature of 23 ± 1°C, relative humidity of 55 ± 5% and a 12-h light:dark cycle. The animals were fed on a vitamin C deficient standard diet (Clea Japan, Inc.) and were supplemented with 1 mg ascorbic acid/mL of drinking water for 1 week. Rats were randomly divided into nine groups of five rats each and were fed on a powdered vitamin E and vitamin C free basal diet (Oriental Yeast Co., Tokyo). Table 1 shows the composition of the

TABLE 1 *Composition of the basal diet*

Component	Amount g/100 g
Casein	25.0
Mineral mixture[1]	3.5
Vitamin mixture[2]	1.0
Choline bitartrate[3]	0.2
Soybean oil[4]	5.0
Cellulose powder	5.0
Cornstarch:sucrose (2:1)	60.0
DL-Methionine[5]	0.3

[1]AIN-76 mineral mixture. The basal diet contains the following (mg/kg diet): $CaHPO_4$ 17500, NaCl 2590, $K_3C_6H_5O_7 \cdot H_2O$ 7700, K_2SO_4 1820, MgO 840, $MnCO_3$ 122.5, Fe-citrate (Fe 17%) 210, $ZnCO_3$ 56, $CuCO_3 \cdot Cu(OH)_2 \cdot H_2O$ 10.5, $Na_2SeO_3 \cdot 5H_2O$ 0.35, KIO_3 0.35, $CrK(SO_4) \cdot 12H_2O$ 19.25.

[2]AIN-76A vitamin mixture. The basal diet contains the following (mg/kg diet): vitamin B-12 0.01, vitamin D-3 0.027, vitamin K-3 0.05, D-biotin 0.2, folic acid 2, vitamin B-1-HCl 6, vitamin B-2 6, vitamin B-6-HCl 7, vitamin A acetate 8, pantothenic acid-Ca 16, nicotinic acid 30; α-tocopherol and ascorbic acid were not included.

[3]Choline bitartrate, Wako Chemical, Co. (Osaka, Japan).

[4]Soybean oil, Ajinomoto, Co. (Tokyo, Japan).

[5]DL-Methionine, Wako Chemical, Co. (Osaka, Japan).

basal diet. The experimental diets were obtained by adding 5, 50 or 250 (groups E LOW, E MEDIUM and E HIGH) mg of DL-α-tocopherol/kg of basal diet and 150, 300 or 900 (groups C LOW, C MEDIUM and C HIGH) mg of ascorbic acid/kg of basal diet. Rats were fed nine diets (E LOW C LOW, E LOW C MEDIUM, E LOW C HIGH, E MEDIUM C LOW, E MEDIUM C MEDIUM, E MEDIUM C HIGH, E HIGH C LOW, E HIGH C MEDIUM and E HIGH C HIGH) for 21 days. L(+) Ascorbic acid was obtained from Wako Pure Chemical Co. (Osaka, Japan) and dl-α-tocopherol was from Eisai Co. (Tokyo, Japan). The animals had free access to food and water. Body weight gain and food intake were measured between 17:00 and 18:00 h once a week. After 3 weeks on the experimental diets, following overnight food-deprivation, heparinized blood was withdrawn by heart puncture under light ether anaesthesia. Then, livers were perfused *in situ* with ice cold 150 mM sodium chloride, and they were removed and weighed. Plasma was obtained by centrifuging blood at 1,500 × g for 15 min at 4°C. Liver and plasma samples were immediately frozen and stored at −84°C until performance of biochemical analyses no later than 4 weeks afterwards.

Lipid Extraction

Total lipid was extracted with a mixture of chloroform-methanol 2:1 (v/v) from liver and plasma according to Folch *et al.*[17] Two mL of NaCl 150 mM containing 0.002% butylated hydroxytoluene (BHT) as antioxidant were added to 500 mg of liver, and the mixture was homogenized in a teflon-glass homogenizer under ice-cold conditions. The homogenate was added to 5 mL of chloroform-methanol 2:1 (v/v) and mixed vigorously for 1 min with a vortex mixer. The mixture was centrifuged at 1,500 × g for 15 min at 4°C. The chloroform-methanol extraction was performed to the upper methanol-water layer for a second time and a chloroform extraction was carried out for a third time. The lower chloroform layer containing total lipid was dehydrated with anhydrous sodium sulphate and filtered through a filter paper No. 5C (Advantec, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The solvent was removed in a rotary evaporator and the residue was dried under a nitrogen stream. The total lipid obtained was immediately dissolved in 200 μL of chloroform-methanol 2:1 (v/v) and a 20 μL portion was subjected to hydroperoxide assay by chemiluminescence(CL)-HPLC. It has been previously confirmed that artificial hydroperoxides are not formed during the extraction procedure as described above.[18] Plasma total lipids were extracted similarly to liver. Five mL of chloroform-methanol 2:1 (v/v) containing 0.002% BHT were added to 1 mL of plasma. The extraction (without BHT) was repeated for the upper layer. The total lipids obtained were redissolved in 100 μL of chloroform-methanol 2:1 (v/v) and a 20 μL portion was subjected to CL-HPLC. The remaining was collected to determine phosphorus concentration.

Hydroperoxide Determination by CL-HPLC

Phospholipid hydroperoxides (PCOOH and PEOOH), in the total lipid fraction of liver and blood plasma were determined by CL-HPLC. The analytical system consisting on HPLC and postcolumn chemiluminescence detection was previously described.[16] Analytical conditions were as follows: the HPLC column was JASCO Finepak SIL NH_2-5 (5 μm, 250 × 4.6 mm, n-propylamine column, Japan Spectroscopic Co., Tokyo, Japan). The mobile phase was hexane-2-propanol-methanol-water (5:7:2:1, v/v) with a flow-rate of 1.0 mL/min (JASCO PU-980 pump). After passing through a JASCO UV-970 detector set at 234 nm to monitor conjugated diene, the column eluent was mixed with a chemiluminescence (CL)-reagent at a post-column mixing joint (Y-type; Kyowa Seimitsu, Tokyo, Japan). The CL-reagent was prepared by dissolving 10 μg/mL of cytochrome c (from horse heart, type VI; Sigma Chemical Co., St. Louis, MO) and 2 μg/mL of luminol (3-aminophthaloyl hydrazine; Wako

Pure Chemical Co., Osaka, Japan) in 50 mM borate buffer (pH 10) pumped at 1.1 mL/min with a JASCO PU-980 pump. The CL generated by reacting the hydroperoxide with the CL-reagent was measured with a JASCO 825-CL detector or a CLD-100 CL detector (Tohoku Electronic Industries Co., Sendai, Japan). Calibrations of PCOOH and PEOOH were done by determining the peroxide value of PC standard (Nippon Oil & Fats Co. Ltd., Tokyo, Japan) by iodometric titration.[19] Phosphorus concentration in the total lipid extracted was determined by the method of Bartlett[20] using 72% perchloric acid instead of H_2SO_4 and H_2O_2 to digest the sample.

Measurement of Thiobarbituric Acid-Reactive Substances

Endogenous liver peroxidation was measured by the thiobarbituric acid (TBA) test specially adapted for tissue extracts.[21] The assay was performed in the presence of 10 μM butylated hydroxy-toluene in order to avoid artifactual peroxidation. Sensitivity to peroxidation *in vitro* was estimated by incubating liver supernatants with 0.4 mM ascorbate and 0.05 mM $FeSO_4$ for 180 min at 37°C before performing the TBA assay. The results were represented as TBARS and expressed in nmoles of malondialdehyde (MDA) per gram tissue. Levels of TBARS in plasma were measured according to the fluorometric method of Yagi.[22] The spectrofluorometer used was JASCO FP-770 (Japan Spectroscopic Co., Ltd.).

Measurement of α-Tocopherol

Liver and plasma α-tocopherol was determined by HPLC with fluorometric detection.[23] Liver samples were homogenized in cold 150 mM NaCl (1 g/mL). α-Tocopherol was extracted from 2 mL of liver homogenates or 0.2 mL of plasma samples with 5 mL of n-hexane after addition of 2 mL of ethanol containing 2 μg of 2,2,5,7,8-pentamethyl-6-hydroxychroman (Wako Pure Chemical Co., Osaka, Japan) as internal standard. n-Hexane extracts were evaporated in a rotary evaporator and dried under a nitrogen stream. The residue was redissolved in 100 μl of n-hexane and a 20 μl portion was injected into the HPLC. α-Tocopherol concentration was determined by using a spectrofluorometric detector (Ex. 298 nm Em. 325 nm) Jasco 821-FP (Japan Spectroscopic Co., Ltd.).

Measurement of Ascorbic Acid

Liver samples were homogenized in 2% metaphosphoric acid (Wako Pure Chemical Co., Osaka, Japan) (250 mg/mL) in ice cold conditions. Four mL of 2% metaphosphoric acid were added to 1 mL of plasma. Samples were mixed for 30 s with a vortex mixer and centrifuged at 1,500 × g for 10 min at 4°C. Ascorbic acid concentration of liver and plasma supernatants were measured at 540 nm using a UV/VIS spectrophotometer (Jasco Ubest-35, Japan Spectroscopic Co. Ltd.) by the 2,4-dinitrophenylhydrazine method.[24]

Statistical Methods

Statistical analysis were performed between vitamin C groups in each vitamin E group and between vitamin E groups in each vitamin C group. Data were analyzed by ANOVA followed by Fisher's least significant difference (LSD) test when necessary to analyze significance between paired groups. Data were log transformed before ANOVA when variances were significantly different by Bartlett test. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

RESULTS

The vitamin E dietary treatment led to very different levels of α-tocopherol in the liver and plasma of ODS rats. The increment of vitamin E dietary content from group E LOW to group E MEDIUM resulted in a 225% increase in liver α-tocopherol and in a 190% increase in plasma α-tocopherol in

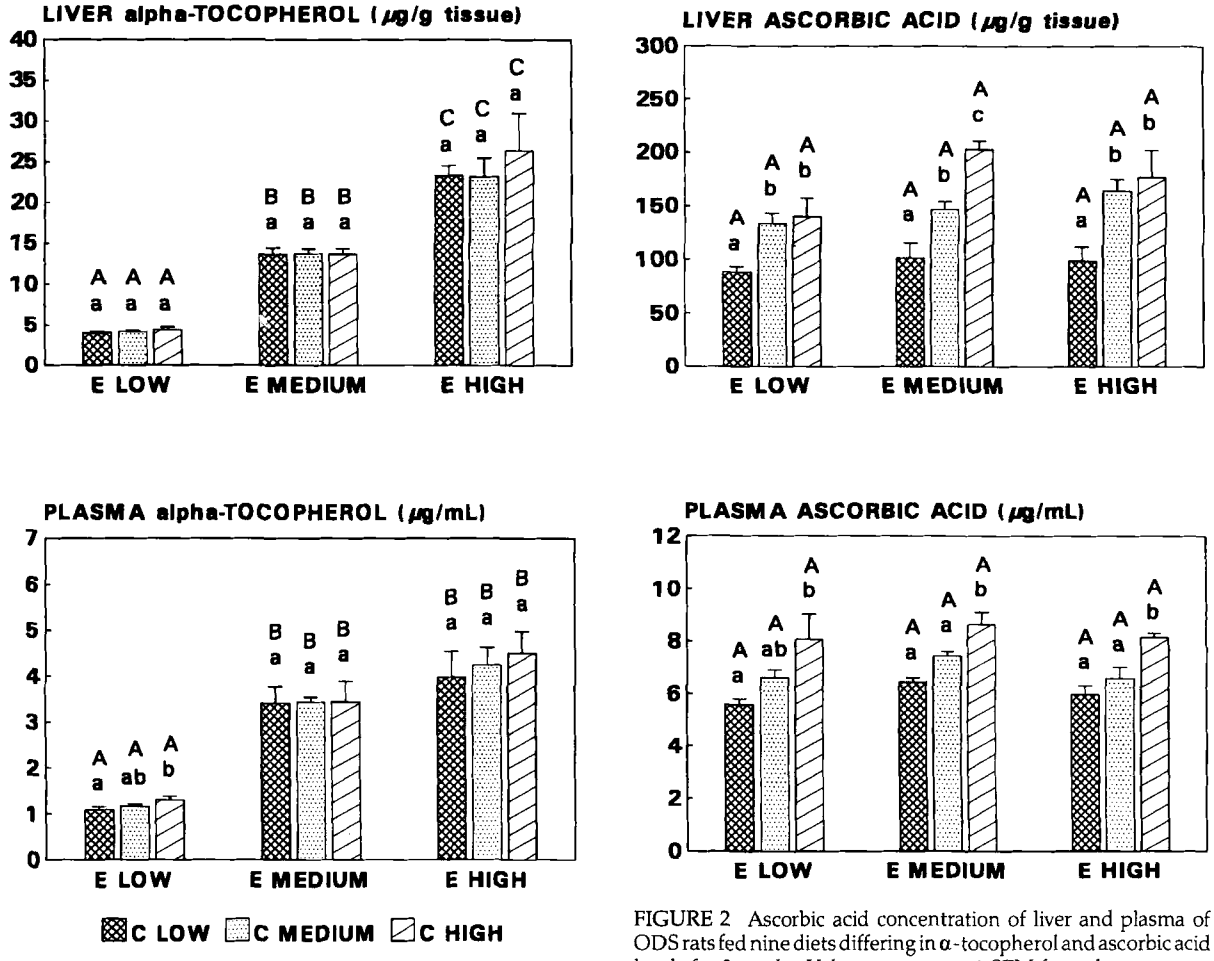

FIGURE 1 α-Tocopherol concentration of liver and plasma of ODS rats fed nine diets differing in α-tocopherol and ascorbic acid levels for 3 weeks. Values are means ± SEM from five rats per group. Means not sharing a common upper-case (α-tocopherol effect) or lower-case (ascorbic acid effect) letter are significantly different at $P<0.001$, except between groups E MEDIUM C HIGH and E HIGH C HIGH in liver and between groups E LOW C LOW and E LOW C HIGH in plasma, which are different at $P<0.05$.

FIGURE 2 Ascorbic acid concentration of liver and plasma of ODS rats fed nine diets differing in α-tocopherol and ascorbic acid levels for 3 weeks. Values are means ± SEM from three rats per group. Means not sharing a common upper-case (α-tocopherol effect) or lower-case (ascorbic acid effect) letter are significantly different at $P<0.05$, except between groups E MEDIUM C LOW and E MEDIUM C HIGH in liver, which are different at $P<0.001$.

all vitamin C dietary groups (Figure 1). When dietary vitamin E was further increased from group E MEDIUM to E HIGH the hepatic α-tocopherol content increased by 75%. These increases showed a high degree of statistical significance. The α-tocopherol concentrations found in group E LOW were low (4-4.4 μg/g in liver and 1.1–1.3 μg/mL in plasma) but substantially higher than those typical of vitamin E-deficient rats. Dietary ascorbic acid supplementation from group C LOW to group C HIGH increased plasma vitamin E levels in group E LOW.

Vitamin C dietary content also changed ascorbic acid concentration in liver and plasma (Figure 2). Liver ascorbic acid significantly increased from group C LOW to group C MEDIUM at all vitamin E levels whereas a significant increase from group C MEDIUM to group C HIGH was detected only in the E MEDIUM group (Figure 2). Significant increases in plasma ascorbic acid were obtained at all vitamin E dietary levels between groups supplemented with the lowest (C

LOW) and the highest (C HIGH) dietary vitamin C (Figure 2). Significant increases in plasma ascorbate were also observed from group C MEDIUM to group C HIGH in intermediate (E MEDIUM) and high (E HIGH) vitamin E groups. Dietary vitamin E did not affect ascorbic acid levels in any case.

The treatment with nine diets differing in vitamins E and C content did not change body weight gain nor the daily food intake of rats at 1, 2 or 3 weeks of supplementation (data not shown). The hepatosomatic index (% liver weight versus body weight) was similar in the nine groups at the end of the experimental period.

Non-stimulated liver TBARS (at 0 min) significantly decreased ($P<0.05$) from group E LOW to group E MEDIUM in the C LOW group, and from E LOW to E HIGH in the C LOW and C MEDIUM groups (Figure 3). *In vitro* incubation of liver supernatants with ascorbate-Fe^{2+} increased lipid peroxidation (TBARS) above basal levels in the nine groups of animals (Figure 3). The increase in lipid peroxidation from 0 to 180 minutes of incubation was specially pronounced in all the E LOW groups. *In vitro* TBA values significantly decreased from group E LOW to group E MEDIUM in all vitamin C groups. Plasma TBARS also decreased from group E LOW to group E MEDIUM at all vitamin C levels (Figure 3). TBA values did not show differences between vitamin C groups either in liver or plasma.

Liver lipid hydroperoxides (PCOOH and PEOOH) did not show significant differences among vitamin C groups in any case (data not shown) whereas a systematic trend to show lower levels as vitamin E dietary content increased was evident. The lack of vitamin C effect allowed pooling of hydroperoxide data from the three vitamin C subgroups in each vitamin E group. When this was done, it was observed that both PCOOH and PEOOH significantly decreased ($P<0.05$) as vitamin E increased (Figure 4). This was found between E LOW and E HIGH for PCOOH and already between E LOW and E MEDIUM for PEOOH.

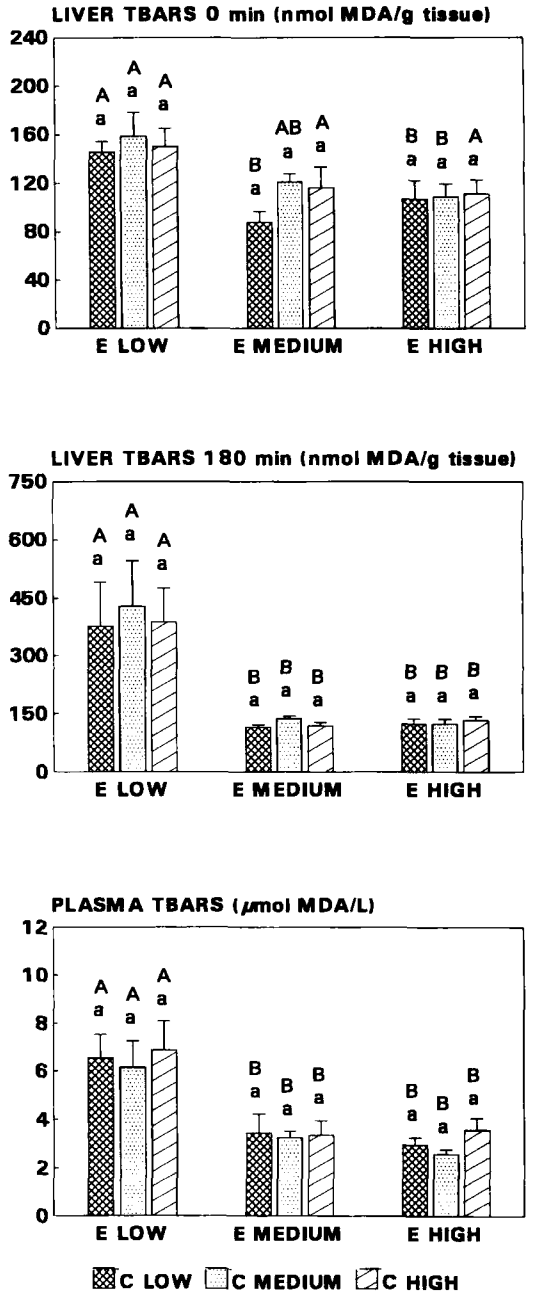

FIGURE 3 Liver (0 and 180 min) and plasma TBARS levels of ODS rats fed nine diets differing in α-tocopherol and ascorbic acid levels for 3 weeks. Values are means ± SEM from four or five rats per group. MDA = malondialdehyde. Means not sharing a common upper-case (α-tocopherol effect) or lower-case (ascorbic acid effect) letter are significantly different at $P<0.05$, except between groups E LOW C MEDIUM and E HIGH C MEDIUM, E LOW C HIGH and E MEDIUM C HIGH, and E LOW C HIGH and E HIGH C HIGH in liver (180 min) which are different at $P<0.001$.

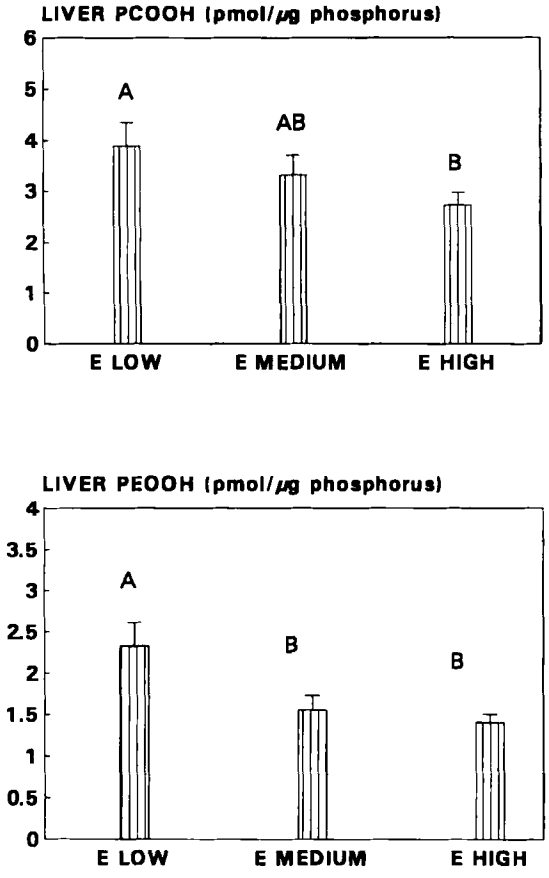

FIGURE 4 Phosphatidylcholine (PCOOH) and phosphatidylethanolamine (PEOOH) hydroperoxides concentration of liver of ODS rats fed three diets differing in α-tocopherol levels for 3 weeks. Values are means ± SEM from 15 rats per group.

DISCUSSION

In this study, liver concentration of vitamin E strongly reflected dietary levels of this vitamin at the three selected doses, whereas plasma vitamin E showed dietary dependence only between low (E LOW) and intermediate (E MEDIUM) vitamin E groups. Vitamin C was diet-dependent at the three supplementation levels studied both in liver and plasma.

Dietary supplementation with vitamin C did not change *in vivo* or *in vitro* TBARS values either in liver or in plasma and this was confirmed by the specific determination of liver PCOOH and PEOOH. A previous study in ODS rats[25] also showed a lack of effect of vitamin C on plasma TBARS levels. Other authors have shown the capacity of dietary vitamin C to decrease liver TBARS[26] or malondialdehyde measured by HPLC[27] in the guinea pig. The difference between these results and those reported here for ODS rats can be due to the use of wider vitamin C dietary ranges or longer times of supplementation in the guinea pig studies, or to intrinsic differences in the response to dietary ascorbate between both animal models.

A synergism between vitamin C and vitamin E in trapping free radicals was first suggested by Tappel.[28] Several *in vitro* studies indicate that ascorbic acid present in the aqueous phase reacts very efficiently with the α-tocopheroxyl radical produced in liposomes undergoing lipid peroxidation, regenerating α-tocopherol and therefore its radical scavenging activity. Scarpa *et al.*[6] demonstrated this synergistic effect in phosphatidylcholine liposomes using an electron paramagnetic resonance method. The reduction of vitamin E radical by vitamin C has also been confirmed by spectroscopy in homogeneous solution.[5] Experiments with lipid-soluble radical initiators using liposomes in aqueous dispersion have also shown this synergistic effect.[4,29] Although this vitamin C-vitamin E radical interaction occurs *in vitro*, it remains to be determined if this type of interaction also takes place *in vivo*. Bendich *et al.*[7] showed that the level of vitamin E found in plasma and lung tissue is higher in guinea pigs supplemented with ascorbic acid than in guinea pigs fed the same diet without ascorbate. An antioxidant synergism of vitamins C and E has been also described in the liver and lung of guinea pigs fed with oxidized oil.[30] The results of our study suggest the existence of some degree of *in vivo* synergism in basal conditions since plasma vitamin E increased as a function of dietary vitamin C in ODS rats subjected to low (E LOW) vitamin E dietary levels. The contrary did not take place: ascorbate levels were independent of vitamin E in the diet. This is consistent with the relative molar ratios of both antioxidants *in vivo* (much

higher for vitamin C) and with the sense of the redox interaction predicted: vitamin C reducing vitamin E radicals.

Various authors have described the capacity of vitamin E to decrease lipid peroxidation. Dietary supplementation with vitamin E decreased *in vivo* TBARS values in rat liver,[31–33] decreased *in vitro* TBARS in rat liver microsomes[34] and in guinea pig liver,[35] and decreased *in vivo* pentane production in rats.[36] Nevertheless, these works were performed using methods poorly specific which are specially problematic for *in vivo* lipid peroxidation values. The results obtained here show that ODS rats also respond to vitamin E dietary treatment with a reduction of *in vivo* TBARS values both in liver and plasma and with a clear decrease in sensitivity to hepatic lipid peroxidation (*in vitro* TBARS), a finding consistent with the antioxidant functioning of vitamin E in the lipophilic region in biological systems. The decrease of *in vitro* TBARS in liver and of *in vivo* TBARS in plasma already occurs from low (E LOW) to intermediate (E MEDIUM) vitamin E levels and further increases of vitamin E levels do not bring additional benefits. Exactly the same has been reported for *in vitro* TBARS in vitamin E supplemented guinea pigs.[35] The capacity of vitamin E to decrease lipid peroxidation *in vivo* was demonstrated in this work using a highly specific HPLC technique. Lipid hydroperoxides are formed in the lipid peroxidation pathway prior to chain fragmentation which leads to highly varied products. They are then ideal lipid peroxidation markers. Both liver phosphatidylcholine and phosphatidylethanolamine hydroperoxides (PCOOH and PEOOH) decrease *in vivo* as the vitamin E dietary content increases. The decrease is already observed at intermediate vitamin E levels (E MEDIUM) for PEOOH but higher vitamin E levels (E HIGH) are needed to observe a significant reduction of PCOOH. Thus, assessment of optimum dietary levels, a main objective of research on antioxidant vitamins, can vary depending on the parameter measured. However, the results obtained here indicate that these optimum levels are much higher than those needed to avoid deficiency syndromes. This is further supported by the concentrations of vitamin E observed in the E LOW group, which were low but substantially higher than those typical of vitamin E deficient rats. The capacity of vitamin E to inhibit phospholipid hydroperoxide accumulation in liver of rats intoxicated with carbon tetrachloride has been reported.[37] The protective effect of vitamin E on lipid hydroperoxide formation *in vivo* in the absence of any experimental manipulation increasing oxidative stress over basal conditions is described here for the first time.

In summary, the ODS rat model was used in order to simultaneously study the capacity of dietary vitamins C and E to protect against liver and plasma peroxidation in unstressed animals. Dietary vitamin C increased plasma vitamin E at low dietary levels of this last vitamin. Dietary vitamin E, in agreement with previous work in other animal models, decreased lipid peroxidation in liver and plasma as measured by the TBA test. This capacity was confirmed by a highly specific determination of PCOOH and PEOOH by HPLC with chemiluminescence detection.

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