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ORIGINAL CONTRIBUTION

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Chronic vitamin E inadequacy and thermally treated oils affect the synthesis of hepatic metallothionein isoforms

Summary *Background:* Metallothionein (MT)[#] synthesis can be stimulated in many organs not only by various metals such as cadmium, zinc, and copper, but also by many nonmetallic compounds or experimental conditions such as oxidative stress. The latter lead to the hypothesis that MT is induced in response to free radicals formed in tissues and lipid peroxidation.

Aims of the study: Whether the relationship between lipid peroxidation and MT synthesis is a common phenomenon also valid for lipid peroxidation induced by dietary factors such as chronic vitamin E inadequacy and autoxidation products of polyenoic fatty acids derived from thermally oxidized oil was investigated in the present study.

Methods: The relationship between the induction of metallothionein isoforms I and II (MT-I and MT-II) in response to diet-induced lipid peroxidation using a rat model system in which lipid peroxidation was examined *in vivo* by chronic vitamin E inadequacy or by administration of lipid peroxidation products from a thermally treated polyenoic-rich oil with either basal (dietary zinc concentration: 48 mg/kg; experiment 1) or Zn-stimulated MT levels (dietary zinc concentration: 305 mg/kg; experiment 2) was studied. In both experiments, growing male rats were fed diets containing either a fresh or a thermally treated soybean oil with deficient or sufficient amounts of vitamin E (14 and 11 vs. 648 and 560 mg α-tocopherol equivalents per kg diet) over 40 days according to a bifactorial experimental design. Plasma and liver concentrations of tocopherols and hepatic levels of thiobarbituric acid-reactive substances (TBARS) were measured by high performance liquid chromatography. MT isoform concentrations in rat liver were isolated and quantified by ion-exchange high performance liquid chromatography and atomic absorption spectrometry.

Results: Irrespective of the zinc supply, rats receiving inadequate amounts of vitamin E with the diet had markedly lower plasma and liver concentrations of α-tocopherol and total tocopherols than vitamin E-sufficient rats. ANOVA also revealed an interaction between the diet factors vitamin E and oil on tocopherols in plasma and liver of rats from both experiments. In experiment 1, where rats received normal amounts of dietary zinc, ingestion of the thermally treated oil impaired the tocopherol status compared to the treatment with the fresh oil, although this effect was only obvious in the vitamin E-deficient groups. In experiment 2, where rats received excessive amounts of zinc, the thermally treated oil did not contribute to a reduction of the tocopherol status in plasma and liver. In both experiments a significant increase in TBARS level, indicative of lipid peroxidation, was observed in the liver at chronic vitamin E inadequacy, but no effect of the oil was observed. Here, we show that the dietary treatments had some effects on the synthesis of liver metallothionein isoforms. In groups, receiving normal amounts of zinc, there was a significant interaction between the dietary treatments on the levels of MT-I and MT-II in liver. Chronic vitamin E inadequacy which was accompanied by diminished tocopherol levels in liver induced the synthesis of MT-I and MT-II. When vitamin E inadequacy was combined with the ingestion of a thermally treated polyenoic acid-rich oil hepatic levels of MT-I and MT-II remained low. In experiment 2, where rats were fed the high zinc diet, vitamin E inadequacy caused an increase of hepatic MT-I level just as in experiment 1, although this MT stimulating effect was irrespective of the oil. For MT-II there was a 43 % increase in the vitamin E-deficient group fed the fresh oil compared to all the other groups, although this ef-

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fect was not statistically significant. The liver MT isoform response to stress was similar in rats with basal MT levels and Zn-induced liver MT levels. The failing effect of the thermally treated oil on MT levels which were stimulated by vitamin E deficiency in experiment 2 was possibly due to the low oxidation grade of the thermally treated oil.

Conclusion: The present results are strongly indicative of an apparent induction of MT isoform synthesis in response to an impaired antioxidant defence system in the lipid regions of liver cells induced by vitamin E inadequacy. In contrast, thermally treated polyenoic-rich oils with a certain oxidation grade seem to restrain the induction of MT isoform synthesis under the present experimental conditions.

Key words Vitamin E inadequacy – thermally treated soybean oil – metallothionein isoforms – liver – rat

Abbreviations
GSH-Px = glutathione peroxidase; MT = metallothionein; PUFA = polyenoic fatty acids; TBARS= thiobarbituric acid-reactive substances; Teq = tocopherol equivalents.

Introduction

Metallothioneins (MT) are a class of low-molecular-weight, cysteine-rich metal-binding proteins that function in detoxification of heavy metals, zinc and copper homeostasis, and in the acute phase response. Recently, studies have suggested an additional role for MT as a free radical scavenger, because lipid peroxidation of erythrocyte ghosts induced by xanthine-xanthine oxidase in the presence of iron is inhibited by MT (50), and preinduction of MT by injection of bismuth, zinc or cadmium prevented the lethal toxicity and lipid peroxidation caused by the anti-cancer drug, adriamycin (33, 43). MT synthesis can be stimulated in many organs not only by various metals such as cadmium, zinc, and copper, but also by many nonmetallic compounds such as ethanol, indomethacin, alkylating agents, chelators, and hormones including glucagon, glucocorticoid hormones, and catecholamines (5, 25) or by experimental conditions such as food restriction (6). MT is also induced in cells and tissues of mammals exposed to radical generating agents or conditions such as producers of reactive oxygen species like tert-butyl hydroperoxide (3), the redox cycling agents, paraquat or adriamycin (3, 14, 34, 41), the lipid peroxidation enhancer, cisplatin (3), glutathione consumers such as diethyl maleate (3), high oxygen tension (19), X-irradiation (44) and UV-irradiation (16). In these studies, the liver has been found to be very responsive to these agents.

This evidence leads to the hypothesis that MT is induced in response to free radicals formed in tissues and lipid peroxidation. However, the mechanism of increased MT accummulation in certain tissues induced by physiological factors and experimental conditions is unclear. Whether the relationship between lipid peroxidation and MT synthesis is a common phenomenon also valid for lipid peroxidation induced by dietary factors such as chronic vitamin E inadequacy and autoxidation products of polyenoic fatty acids (PUFA) derived from thermally oxidized oil was investigated in the present study. Thermally oxidized soybean oil was used as dietary fat, because typical Western diets contain large quantities of oxidized fat derived from fried foods, and recent studies could demonstrate that the primary and secondary autoxidation products of PUFA as a component of fried foods affect the vitamin E status (12, 13, 27, 28) and promote lipid peroxidation in tissues (15, 18, 20). In order to avoid differences in food intake between groups fed the fresh oil and the thermoxidized oil, because restriction of food intake led to an increase in the concentration of Zn-metallothionein in rat liver (6), the thermally treated oil used in this study was only moderately oxidized to preserve its palatability. Additionally, oils with equalized levels of tocopherols were used in this study to limit the observed differences to the action of autoxidation products alone. The antioxidative status was determined by monitoring the individual tocopherols in plasma and liver. The formation of thiobarbituric acid-reactive substances (TBARS) in liver was measured to demonstrate the sensitivity of biological lipid structures to autoxidation under the conditions of the thiorbarbituric acid assay.

We also considered the possibility that the liver MT response to stress may be different in rats with zinc-induced liver MT because if MT induction by one or both dietary stress factors is due to lipid peroxidation in liver membranes, animals with much preexisting MT would not be expected to synthesize more MT in the liver when exposed to further oxidative stress because MT may scavenge free radicals prior to the production of hydrogen peroxide in polyenoic-rich membranes of liver cells. Therefore, we carried out a second experiment in which liver MT was simultaneously induced by a high dietary zinc administration. Two major isoforms of MT are found in most vertebrate tissues and are designated as MT-I and MT-II (25). It is known that the relative proportions of the isoproteins depend on the particular stimulus, although the physiological significance of this variation is still unknown. To further elucidate the relationship between tissue levels of MT isoforms and dietary-induced oxidative stress, we used a sensitive method based on ion-exchange high performance liquid chromatography and atomic absorption spectrometry for determination of non-stimulated and stimulated metallothionein isoform (MT) levels in rat liver (36), which was

used as the target organ, because the liver is one of the most important tissues with regard to some of the putative MT functions.

Materials and methods

Animals and diets

In experiment 1 forty male SPF Sprague-Dawley rats (WIGA GmbH, Sulzfeld, Germany) with an average body weight of 51.1 (±0.8 g, SEM) were divided into four groups of ten each and were fed semisynthetic diets with 48 mg zinc/kg (analyzed value) based on the AIN–93 formulation (40) with low (E–, 13.8 mg/kg DM) or high levels (E+, 648 mg/kg DM) of α-tocopherol equivalents containing either fresh soybean oil (FO) or thermally oxidized soybean oil (OO) in amounts of 100 g/kg diet for 40 days. In experiment 2, liver MT was simultaneously induced by zinc administration during the last 8 days of the experiment. Forty male SPF Sprague-Dawley rats (WIGA GmbH, Sulzfeld, Germany) with an average body weight of 53.4 (±0.8 g, SEM) received 36 mg zinc/kg diet (analyzed value) for the first 31 days of the experiment and 305 mg zinc/kg (analyzed value) from day 32 to day 40 of the experiment. As in experiment 1, the rats were divided into four groups of ten each and were fed similar diets as in experiment 1 with either deficient or adequate amounts of dietary vitamin E (E–, 11.2 mg/kg DM; E+, 560 mg/kg DM) and with fresh or thermally oxidized soybean oil for 40 days. The semisynthetic diets used in both experiments consisted of (in g/kg) casein (200), corn starch (320), sucrose (287), fresh/thermoxidized soybean oil (100), minerals (40), fiber (30), vitamins (20), coconut oil (16.7), DL-methionine (2), and choline chloride (1). The diets were stored at +4 °C during the experimental period. The thermoxidized oil used in both experiments was prepared by heating fresh soybean oil for 22 h at a constant temperature of 130 °C. Throughout the heating process, air was bubbled through the oil for 8 h. The extent of peroxidation was determined by assaying the peroxide value (1), the fatty acid composition of the diet lipids (47), and the concentrations of individual tocopherols (2). The heat treatment caused a considerable loss of individual tocopherols and a moderate but concomitant increase of the peroxide value (Table 1). Prior to inclusion in the diet, the oils were standardized to an identical vitamin E activity with all-rac-α-tocopheryl acetate (considering that the biopotency of all-rac-α-tocopheryl acetate is 67 % of that of α-tocopherol). The oils were included in the diet either in this form or after supplementation with all-rac-α-tocopheryl acetate. For further induction of peroxidation, and for preparation of diets that could be offered in defined portions, dietary components were mixed with water, homogenized, and dried at a temperature of 40 °C for 4 days. During the drying procedure, the peroxide values of the dietary oils moderately increased (Table 2). The tocopherol concentrations of the diets are presented in Table 2. For assessing the peroxide values, aliquots of the diets were analysed at different times within the experimental period. Average peroxide values of the diet lipids are also shown in Table 2. The fatty acid composition of the diets with the thermoxidized soybean oil in experiment 1 and 2 was not clearly different from those with fresh soybean oil. The diet lipids used for both experiments consisted of about 60 mol % of polyenoic acids, linoleate (18:2 n–6) and linolenate (18:3 n–3) and 24 mol % of monoenoic fatty acids such as 16:1 n–9 and 18:1 n–9. The fatty acid composition of the diets with the thermally treated soybean oil was characterized by a 1.7 % lower proportion of polyenoic fatty acids than the fatty acid composition of the diets with the fresh soybean oil. The fatty acid composition and tocopherol concentrations of the diets were not altered during storage at a temperature of 4 °C.

Table 1 Characteristics of the dietary oils used in experiments 1 and 2

	experiment 1		experiment 2	
	fresh oil	oxidized oil	fresh oil	oxidized oil
Tocopherols (mg/kg)				
α-tocopherol	46.8	8.0	106	75.7
γ-tocopherol	585	40	622	386
δ-tocopherol	266	164	231	187
D-α-Teq[1] before alignment	113	17	175	120
All-rac-α-tocopherol acetate	–	143	–	82
D-α-Teq after alignment	113	113	175	175
Peroxide value (meq O_2/kg)	4.0	23.0	4.1	26.1

[1] Teq = Tocopherol equivalents

Table 2 Analyzed concentrations of tocopherols and peroxide values of the diets used in experiments 1 and 2

	fresh oil		thermoxidized oil	
	Vit E–	Vit E+	Vit E–	Vit E+
Experiment 1				
α-Tocopherol (mg/kg)	7.1	623	13.2	665
γ-Tocopherol (mg/kg)	58.0	58.0	3.8	3.8
δ-Tocopherol (mg/kg)	27.0	27.0	13.6	13.6
Peroxides (meq O_2/kg lipid)	9.4	9.6	74	75
Experiment 2				
α-Tocopherol (mg/kg)	5.9	571	5.8	539
γ-Tocopherol (mg/kg)	60.4	59.2	37.1	33.0
δ-Tocopherol (mg/kg)	23.0	33.0	10.0	12.3
Peroxides (meq O_2/kg lipid)	15.6	14.4	76	80

The rats in experiments 1 and 2 were housed individually in a controlled environment, in Macrolon cages (Becker GmbH, Castrop-Ruxel, Germany), in a room maintained at 24 °C with a humidity of 60 %. All rats were kept under conditions of controlled lighting with a daily 12-h light:dark cycle. Food and water were provided ad libitum. Care and treatment of the rats followed recommended guidelines (35). At the end of the experimental period of 40 days, 12 h after the last feeding, rats from both experiments were killed by decapitation after light anesthesia with diethyl ether.

Analytical determinations

Blood for determination of plasma concentrations of zinc, individual tocopherols, glutathione peroxidase, and thiols was collected into heparinized tubes (Sarstedt, Nümbrecht, Germany). The livers were promptly excised for determination of MT, tocopherols and TBARS. Liver samples used for MT analysis were immediately processed. Plasma and liver samples used for other measurements were stored at –80 °C until analyzed.

The plasma concentration of zinc was measured directly by its absorbance at 213.9 nm by aspirating a dilute solution (1:5) into the flame of the atomic absorption spectrophotometer (model 5100, Perkin-Elmer, Überlingen, Germany). The zinc concentration of each sample was calculated from standard curves using various dilutions of zinc standard solutions (1000 ± 2 mg/l, Merck, Darmstadt, Germany). The specimens were analyzed in duplicate, and the coefficient of variation for duplicate analysis was typically below 2 %.

The concentrations of individual tocopherols in the diets, plasma, and liver were determined by high performance liquid chromatography (2). Liver homogenate and plasma samples were saponified with sodium hydroxide in the presence of pyrogallol as an antioxidant. Individual tocopherols extracted with n-hexane were separated on a LiChrosorb Si 60 column (5 µm particle size, 250 mm length, 4 mm internal diameter, Merck) with a n-hexane and 1,4 dioxane-mixture (94:6, v/v) as an eluent (isocratically) and detected by fluorescence (excitation wavelength: 295 nm, emission wavelength: 320 nm).

The formation of TBARS in the liver was measured by high-performance liquid chromatography (17). Therefore, liver homogenates were mixed with 1 mL thiobarbituric acid reagent (0.4 % solution of TBA in 0.1 M HCl, pH 1) and 50 µL of a 0.5 % solution of butylated hydroxytoluene (in ethanol, absolute) (49). This mixture was heated for 45 min at 100 °C. TBARS were extracted with n-butanol and injected into the HPLC system. TBARS were separated using a LiChrosorb RP–18 Cartridge (5 µm particle size, 250 mm length, 4 mm internal diameter, Merck). A water/acetonitrol mixture (80:20, v/v) was used as mobile phase (isocratically); the fluorescence was measured at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. 1,1,3,3 tetramethoxypropane was used as internal standard.

The concentration of extractable liver lipids needed for the expression of liver tocopherols and liver TBARS as nmol/g lipids, were measured gravimetrically by extraction of the liver lipids with hexane/isopropanol (3:2, v/v) overnight. The weight of residue obtained after exhaustive lipid extraction of a 1 g aliquot of the liver, followed by evaporation of the solvent, provided the measure of lipid content. Fat analyses were run in duplicate.

The determination of total thiol groups in plasma (from protein and glutathione) was done by a spectrophotometric method (21). The normalization of total thiol groups for total protein was done to even out possible differences in plasma protein content. The concentration of plasma protein was determined by a standard procedure using an autoanalyzer (model 704, Hitachi, Tokyo, Japan) and a kit reagent (Boehringer, Mannheim, Germany, ref. 1553836). The activity of glutathione peroxidase (GSH-Px) in plasma was determined by a spectrophotometric method (38). Protein in plasma aliquot used for the GSH-Px determination was measured by a method of Smith et al. (46) using bicinchoninic acid, and bovine serum albumin as a standard.

Metallothionein isoform levels in rat liver were isolated and quantified by ion-exchange high performance liquid chromatography and atomic absorption spectrometry (AAS) using a method of Klaassen and Lehman-McKeeman (26) which was modified in several steps (36). Therefore, fresh rat liver was homogenized with three volumes 10 mM Tris-HCl, pH 7.4 (sparkled with N_2), followed by two centrifugation steps at 10 000 × g (20 min) and 114 000 × g (1h). The resulting supernatant was portioned in 1 mL quantities and mixed with 50 µL of 1000 mg Cd/L $CdCl_2$ solution to achieve complete cadmium saturation of metallothionein. The mixture was then heat-treated for 2 min in a 80°C water bath and chilled immediately in ice water. Following a further centrifugation at 13 000 × g (5 min) and membrane filtration (0.22 µm), a 500 µL aliquot was applied to an ion-exchange column and MT-I and MT-II were separated by a HPLC elution procedure. A Merck L–6200 high-performance liquid chromatograph consisting of one single L–6200 intelligent pump and a Rheodyne 7125 injector equipped with a 1000 µL sample loop was used for MT analysis. MT separations were achieved on a tentacle anion-exchange column (Fractogel EMD DEAE- 650 (S), 20–40 µm; 70 mm × 10 mm I. D., Merck). Gradient elution was performed using 20 mM Tris-HCl, pH 7.4 (buffer A) and 200 mM Tris-HCl, pH 7.4 (buffer B). MT-I and MT-II were eluted with a linear gradient from 0 to 60 % B in 18 min at a flow rate of 1 mL/min. For regeneration the column was rinsed for 7 min with 1 M NaCl and then for 25 min with buffer A. All solutions were saturated with nitrogen. According to this elution procedure MT-I and MT-II were eluted at concentrations of 55 and 90 mM Tris-HCl buffer, respectively. The column efflux was collected with

Table 3 Effect of vitamin E and thermoxidized oil on oxidative/antioxidative balance and concentrations of hepatic metallothionein isoforms of rats fed adequate amounts of dietary zinc (experiment 1)[1]

	fresh oil		thermoxidized oil		ANOVA		
	Vit E–	Vit E+	Vit E–	Vit E+	oil	Vit E	oil × vit E
Plasma tocopherols (μmol/L)							
α-tocopherol	2.75 ± 0.21[a]	38.1 ± 2.4[c]	5.24 ± 0.31[b]	42.8 ± 1.1[c]	0.00	0.00	0.00
γ-tocopherol	5.99 ± 0.52	n. d.[2]	0.06 ± 0.01	n. d.	-	-	-
δ-tocopherol	0.37 ± 0.04	n. d.	0.04 ± 0.01	n. d.	-	-	-
Total tocopherols	9.11 ± 0.69[b]	38.1 ± 2.4[c]	5.30 ± 0.32[a]	42.8 ± 1.1[c]	0.00	0.00	0.00
Plasma zinc (μmol/L)	20.6 ± 0.7	20.6 ± 0.5	19.0 ± 0.4	19.8 ± 0.8	0.07	0.92	0.83
Liver tocopherols (nmol/g lipids)							
α-tocopherol	99.1 ± 11.4[a]	2997 ± 193[c]	251 ± 11[b]	2597 ± 159[c]	0.00	0.00	0.00
γ-tocopherol	221 ± 18	n. d.	n. d.	n. d.	-	-	-
δ-tocopherol	14.8 ± 1.6	n. d.	n. d.	n. d.	-	-	-
Total tocopherols	335 ± 27[b]	2997 ± 193[c]	251 ± 11[a]	2597 ± 159[c]	0.06	0.00	0.00
Liver TBARS (nmol/g lipids)	2012 ± 300[b]	759 ± 44[a]	1760 ± 286[b]	716 ± 86[a]	0.49	0.00	0.63
Liver metallothionein (μg/g)							
Metallothionein-I	6.6 ± 0.9[b]	4.1 ± 0.5[a]	3.2 ± 0.3[a]	4.0 ± 0.4[a]	0.01	0.16	0.01
Metallothionein-II	4.0 ± 0.6[b]	2.3 ± 0.4[a]	2.3 ± 0.2[a]	2.5 ± 0.2[a]	0.06	0.09	0.03
Total metallothionein	10.7 ± 0.5[b]	6.4 ± 0.8[a]	5.5 ± 0.5[a]	6.5 ± 0.5[a]	0.01	0.10	0.01

[1]Data are represented as means ± SEM, n = 10; [2]n. d., not detectable; [a,b,c]Values without a common superscript letter are significantly different at $P < 0.05$ (Fisher's multiple range test)

a Gilson M 201 fraction collector (Abimed, Langenfeld, Germany) in 1 mL fractions.

The quantitation of the MT isoforms was based on the peak height of cadmium detection by AAS at a wavelength of 228.8 nm (model 5100, Perkin-Elmer). To generate calibration curves cadmium saturated MT-I and MT-II standards were applied to anion-exchange HPLC, eluted, separated, and fractionated as described above until each fraction was measured for cadmium content by AAS. The total cadmium content of the fractions corresponding to MT-I and MT-II was plotted against the different concentrations of MT injected on the column and linear regression analysis performed to determine the type of relationship. For sample analysis, a 500 μL aliquot of the filtered sample was injected into the anion-exchange column, the MT isoforms were eluted in the manner described and collected for cadmium analysis by AAS. The MT concentration of the liver sample results from the final cadmium content of the MT-I or MT-II fractions, the linear regression of the standard curves, sample weight, dilution, and recovery of the method. The concentrations of MT-I and MT-II were calculated by the following regression equations:
MT-I (μg/0.5 mL) = 0.0413 × (ng Cd/0.5 mL) + 0.210
MT-II (μg/0.5 mL) = 0.0403 × (ng Cd/0.5 mL) + 0.067
The validation of this method, which was carried out by the standard addition method, revealed a good linear relationship of MT-I and MT-II with regression coefficients of R^2 = 0.986 and 1.00, respectively. The recovery obtained was 96 % (MT-I) and 110 % (MT-II). The reproducibility of five preparations of fresh liver provided relative standard deviations of 9.6 % for MT-I and 11.6 % for MT-II. The detection limit was 2.0 μg/g liver for MT-I and 1.3 μg/g liver for MT-II.

Statistics

Treatment effects were evaluated by ANOVA with the factors diet fat, vitamin E supply and their interaction. For statistical significant F-values ($P < 0.05$), means were compared by the Fisher's multiple range test. Data in the present text are expressed as means ± SEM. Data were subjected to logarithmic transformation where necessary to achieve homogeneity of variances.

Results

Experiment 1: without Zn stimulation

The daily feed intake and weight gain did not differ between the groups in experiment 1 where rats received normal amounts of dietary zinc (feed intake: FO,E–: 17.7 ± 0.6 g/d; FO,E+: 18.1 ± 0.3 g/d; OO,E–: 18.1 ± 0.6 g/d; OO,E+: 18.2 ± 0.4 g/d; weight gain: FO,E–: 7.97 ± 0.26 g/d; FO,E+: 7.98 ± 0.17 g/d; OO,E–: 8.23 ± 0.23 g/d; OO,E+: 8.33 ± 0.20 g/d). There were also no differences in the plasma concentration of zinc between the groups (Table 3). Rats receiving inadequate amounts of vitamin E with the diet had markedly lower plasma and liver levels of α-

Table 4 Effect of vitamin E supply and thermoxidized oil on oxidative/antioxidative balance and concentrations of hepatic metallothionein isoforms of rats fed excessive amounts of zinc (experiment 2)[1]

	fresh oil		thermoxidized oil		ANOVA		
	Vit E–	Vit E+	Vit E–	Vit E+	oil	Vit E	oil × vit E
Plasma tocopherols (μmol/L)							
α-tocopherol	2.70 ± 0.28[a]	42.8 ± 1.9[c]	4.13 ± 0.22[b]	43.6 ± 3.3[c]	0.00	0.00	0.00
γ-tocopherol	2.21 ± 0.25[d]	0.16 ± 0.01[b]	0.30 ± 0.03[c]	0.03 ± 0.01[a]	0.00	0.00	0.73
Total tocopherols	4.91 ± 0.48[a]	42.9 ± 1.9[b]	4.43 ± 0.23[a]	43.6 ± 3.3[b]	0.67	0.00	0.68
Plasma zinc (μmol/L)	20.9 ± 1.0	23.4 ± 0.8	23.1 ± 0.8	21.1 ± 0.6	0.87	0.34	0.28
Plasma GSH-Px[2]	92.7 ± 3.0	96.8 ± 3.0	91.5 ± 2.9	90.8 ± 2.1	0.20	0.55	0.39
Plasma thiols (μmol/ g protein)	2.55 ± 0.21	2.93 ± 0.27	2.66 ± 0.07	2.74 ± 0.05	0.81	0.20	0.40
Liver tocopherols (nmol/g lipids)							
α-tocopherol	53.8 ± 5.3[a]	4943 ±408[c]	118 ± 8[b]	4792 ±417[c]	0.00	0.00	0.00
γ-tocopherol	35.8 ± 3.5[d]	16.8 ± 1.7[c]	5.8 ± 0.6[b]	2.8 ± 1.0[a]	0.00	0.00	0.20
Total tocopherols	89.6 ± 7.9[a]	4959 ±409[c]	124 ± 8[b]	4795 ±417[c]	0.18	0.00	0.00
Liver TBARS (nmol/g lipids)	1167 ±134[b]	259 ± 11a	1023 ±184[b]	296 ± 15[a]	0.71	0.00	0.14
Liver metallothionein (μg/g)							
Metallothionein-I	9.5 ± 1.6[b]	6.2 ± 0.6[a]	8.2 ± 1.6[b]	4.5 ± 0.7[a]	0.25	0.01	0.88
Metallothionein-II	7.6 ± 1.5	5.3 ± 0.7	5.4 ± 1.1	5.2 ± 1.1	0.34	0.28	0.38
Total metallothionein	17.1 ± 3.1[b]	11.4 ± 1.1[a]	12.7 ± 2.4[b]	9.1 ± 1.7[a]	0.15	0.04	0.66

[1]Data are represented as means ± SEM, n = 10; [2]One unit of GSH-Px (glutathione peroxidase) is defined as one μmol NADPH oxidized per min per g protein; [a,b,c,d]Values without a common superscript letter are significantly different at $P < 0.05$ (Fisher's multiple range test)

tocopherol and total tocopherols than rats fed excessive amounts of vitamin E (Table 3). ANOVA revealed that the oil also effected levels of tocopherols in plasma and liver, although significant differences were only obvious in groups fed inadequate amounts of vitamin E. Vitamin E-inadequate rats receiving the thermoxidized oil had lower concentrations of total tocopherols in plasma and liver than vitamin E-inadequate rats fed the fresh oil. Liver concentrations of γ and δ-tocopherol were detectable only in the group fed the vitamin E-deficient diet with fresh oil. Liver levels of TBARS were raised significantly by vitamin E inadequacy, but no effect of the oil was observed (Table 3). MT isoforms were detectable in all rat livers. ANOVA revealed a significant interaction between the diet factors fat and vitamin E. Individual comparisons between means indicated that chronic vitamin E inadequacy significantly increased the hepatic MT-I and MT-II levels, but only in rats fed the fresh oil, not in the rats fed the thermally treated oil. The increase of MT-I levels in the vitamin E-deficient group with fresh oil was about 75 % and that of MT-II levels 68 % compared to all the other treatment groups. In the vitamin E-deficient group fed the thermoxidized oil, the MT levels were reduced by 52 % compared to the vitamin E-deficient group fed the fresh oil.

Experiment 2: Zn stimulation

The daily feed intake did not differ between the groups in experiment 2 where rats received excessive amounts of dietary zinc during the last 8 days of the experiment (FO,E-: 19.2 ± 0.6 g/d; FO,E+: 18.4 ± 0.4 g/d; OO,E–: 18.8 ± 0.6 g/d; OO,E+: 18.7 ± 0.6 g/d). Daily weight gain also was similar in these groups (FO,E–: 8.73 ± 0.34 g/d; FO,E+: 8.13 ± 0.19 g/d; OO,E–: 8.47 ± 0.35 g/d; OO,E+: 8.38 ± 0.30 g/d). There were no differences in the plasma concentration of zinc between the groups (Table 4). Vitamin E inadequacy significantly diminished the levels of α-tocopherol and total tocopherols in plasma and liver relative to a high vitamin E supply (Table 4). Under conditions of chronic vitamin E inadequacy, rats fed the thermoxidized oil had similar concentrations of total tocopherols in plasma and even higher concentrations of total tocopherols in liver than rats fed the fresh oil. The plasma activity of glutathione peroxidase and the thiol density of plasma proteins were not influenced by the dietary treatments. When vitamin E, a natural antioxidant, was given to the rats excessively, the TBARS value was decreased significantly compared with the vitamin E deficient group, but no effect of the oil was observed (Table 4). Animals fed the high zinc diet in the last 8 days of the experiment had higher liver levels of MT-I and MT-II than rats from experiment 1 fed normal amounts of zinc. Individual comparisons between means indicated that vitamin E-deficient rats had significantly higher hepatic MT-I levels by 53 and 82 % than rats fed excessive amounts of vitamin E with either fresh or thermoxidized oil. Level of MT-II was increased by 43 % in the vitamin E-deficient group fed the fresh oil compared to all the other groups, although this effect was not statistically significant.

Discussion

We studied the induction of MT-I and MT-II in response to dietary-induced lipid peroxidation using a rat model system in which lipid peroxidation was induced *in vivo* by chronic vitamin E inadequacy or by administration of lipid peroxidation products from a thermally treated polyenoic-rich oil with either basal or Zn-stimulated MT levels. In order to diminish vitamin E storage in liver, because the biological half-life of hepatic vitamin E has been shown to be 10 days (23), both experiments were extended to 40 days.

The increase of TBARS value in the liver as an expression of an enhanced sensitivity of hepatic lipid structures to autoxidation under the conditions of the thiobarbituric acid assay indicates an acceleration of lipid peroxidation in this tissue after chronic vitamin E inadequacy which confirms previous reports on the effect of chronic vitamin E depletion on lipid peroxidation (15, 37, 52). From the present data it is obvious that the administration of a moderately oxidized soybean oil over an experimental period of 40 days did not contribute to an enhanced lipid peroxidation in liver. If lipid peroxidation is a factor involved in MT synthesis only rats depleted of vitamin E are expected to synthesize more MT, but not those fed the thermoxidized oil. In order to comply this theory, it was necessary to detect small levels of MT isoforms as they occur in untreated animals. The high sensitivity of the HPLC/AAS assay used in this study to measure MT isoform levels permits the detection of subtle changes that otherwise would be ignored.

Here, we show that MT-I and MT-II levels were elevated as much as 50–80 % in rats fed the vitamin E-deficient diet. It was remarkable that this effect only occurred in the vitamin E-deficient group fed the fresh oil but not in the group fed the thermally treated oil. The reason for MT suppression in vitamin E-deficient rats fed the thermally treated oil is completely unknown. We suppose that special compounds in the thermally oxidized oil such as cyclic monomeric acids might well have a crucial impact on the magnitude of the stress-induced MT synthesis and on the type of response. The failing effect of the thermally treated oil on MT synthesis in experiment 2, therefore, was possibly due to the low oxidation grade of polyenoic-rich oil, accompanied by the relatively high γ-tocopherol concentration of the soybean oil, which failed to diminish the vitamin E status of the rats. Since induction of metallothionein synthesis is mediated by transcription factors which require heavy metals such as zinc for binding, it is also possible that thermally oxidized oils make zinc less available for the thionein molecule. Consequently, further studies will be needed to better understand the mechanism by which thermally treated oils inhibit MT synthesis.

However, the present results strongly suggest an interrelationship between dietary vitamin E supply, lipid peroxidation, and MT. Our work confirms previous data from Hidalgo et al. (19) who reported that pretreatment with vitamin E reduced both lipid peroxidation and the induction of liver metallothionein synthesis in stressed rats. It is an interesting aspect that the vitamin E-related effect in the study of Hidalgo et al. (19) was even higher in rats treated acutely with this vitamin, suggesting that a partial metabolic adaptation to the excess of vitamin E was present in the chronically treated animals. Thus, it is possible that MT induction by vitamin E depletion is only a temporary process which is mainly present at the beginning of the vitamin E storage emptying. In contrast, data from another study indicated that enhanced lipid peroxidation in liver is not necessary for induction of metallothionein synthesis by carbon tetrachloride or paraquat, because an increase in hepatic concentration of MT was observed in the carbon tetrachloride-treated and paraquat-treated rats, but this was not affected by pretreatment with vitamin E (42). It was, on the contrary, suggested that superoxide or other radicals are essential for metallothionein synthesis and the development of toxicity, but accelerated lipid peroxidation is not directly linked with the induction. Thus, the effect of lipid peroxidation on MT synthesis remains a subject of some controversy.

However, this is one of the first studies showing that dietary factors alone, without adding any other stress factor such as an prooxidans, are sufficient stimuli for changes in MT isoform synthesis. Vitamin E is a powerful antioxidant in the lipid matrix where it is located that protects tissue lipids from free radical attack because active oxygen species are continually produced in tissues by the action of the mitochondrial electron transport system (9), and of NADPH oxidase (51) in leukocytes and macrophages under normal physiologic conditions. Recent results show that vitamin E can directly regulate hydrogen peroxide production in mitochondria and suggest that the overproduction of mitochondrial reactive oxygen species is the first event leading to the tissue damage observed in vitamin E deficiency syndromes (11). Disruption of mitochondrial ultrastructure is one of the earliest pathologic events during vitamin E depletion. It is also evident that lipid peroxidation reactions destroy the biological membranes in which they occur. Plasma membranes of cells as well as intracellular membranes lose their ultrastructural architecture, leading to changes in fluidity and permeability (7, 8, 45). Lipid peroxidation also inactivates a number of membranal enzymes and protein receptors (22, 32). From this study it cannot be consequently concluded that MT synthesis was induced directly by lipid peroxidation or by secondary processes such as those mentioned above.

Another aspect of this study was to test the MT response to vitamin E depletion and lipid peroxidation in rats treated with high amounts of dietary zinc. As expected, the liver MT levels were greatly increased by Zn administration. The short time of zinc administration was chosen because it is known that chronic administration of high zinc diets may reduce both the body zinc levels as well as the MT levels as a consequence of homeostatic control (24,39,53). Zn stimulation increased the MT-I level by 66 % and the MT-

II levels by 115 % compared to the rats fed normal amounts of zinc. The stronger reaction of MT-II compared to MT-I observed in this study is a typical phenomenon observed with zinc stimulation (10). It was an interesting result of this study that the liver MT response to oxidative stress was nearly the same for animals fed excessive and normal amounts of dietary zinc. Here, we show that induction of liver MT by vitamin E inadequacy is not affected by pre-existing zinc induced MT levels.

A possible explanation for the increased MT in liver of vitamin E-depleted rats fed the fresh soybean oil could be that zinc derived from these tissues that were damaged by free radicals and peroxides could accumulate in the liver, thus stimulating the induction of MT synthesis. Thomas et al. (50) have demonstrated that Zn-metallothionein, the physiologically prevalent form of metallothionein, is an efficient antagonist of cell-damaging lipid peroxidation. Mobilization of zinc from MT by an oxidative reaction may either constitute a general pathway by which zinc is distributed in the cell or tissues may be restricted to conditions of stress where zinc is needed in antioxidant defence systems (4, 29, 30). Although TBARS levels are known to be a relative unspecific measure for lipid peroxidation, it is possible that the lower hepatic TBARS levels observed in experiment 2 may be due to the high dietary zinc, although this would be in contrast to results from Markant and Pallauf (31) and Hidalgo et al. (19), who found no significant reduction in liver lipid peroxidation levels of Zn-treated rats compared to non-treated animals.

It can be concluded from this study that a dietary-induced shift in the prooxidant/antioxidant balance in favor of the former may induce the production of MT isoforms in liver. The liver MT response to stress was similar in rats with basal MT levels and Zn-induced liver MT levels. Although it is known that the iso-MTs differ in their amino acid composition and isoelectric point there is little information regarding physiological significance of MT-I or MT-II. Further investigations are required to establish the exact stimuli by which MT synthesis is increased or decreased. Although an extrapolation of these findings from rats to humans might be complex, it cannot be excluded that diet-induced changes of the radical defence mechanism may have some influence on MT regulation in the liver.

References

1. Association of Official Analytical Chemists (1980) Official methods of analysis. Horwith W (ed) 13th ed. AOAC, Airlington, VA, pp 440–441
2. Balz MK, Schulte E, Thier HP (1993) Simultaneous determination of α-tocopheryl acetate, tocopherols and tocotrienols by HPLC with fluorescence detection in foods. Fat Sci Technol 95:215–220
3. Bauman, JW, Liu J, Liu YO, Klaassen CD (1991) Increase in metallothionein production by chemicals that induce oxidative stress. Toxicol Appl Pharmacol 110:347–354
4. Bray TM, Bettger WJ (1990) The physiological role of zinc as an antioxidant. Free Radical Biol Med 8:281–291
5. Bremner I (1987) Interactions between metallothionein and trace metals. Prog Food Nutr Sci:1–37
6. Bremner I, Davies NT (1975) The induction of metallothionein in rat liver by zinc injection and restriction of food intake. Biochem J 149:733–738
7. Bridges JW, Benford DJ, Hubbard SA (1983) Mechanisms of toxic injury. Ann NY Acad Sci 407:42–63
8. Bruch RC, Thayer WS (1983) Differential effect of lipid peroxidation on membrane fluidity as determined by electron spin resonance probes. Biochim Biophys Acta 733:216–222
9. Cadenas E, Boveris A, Ragan CI, Stoppani AOM (1977) Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome C reductase from beef-heart mitochondria. Arch Biochem Biophys 180:248–257
10. Chan HM, Cherian MG (1993) Metallothionein isoforms in different mammalian species. Toxicol 13:165
11. Chow CK, Ibrahim W, Wei ZH, Chan AC (1999) Vitamin E regulates mitochondrial hydrogen peroxide generation. Free Rad Biol Med 27:580–587
12. Corcos Benedetti P, D'Aquino M, Di Felice M, Gentili V, Tagliamonte B, Tomassi G (1987) Effects of a fraction of thermally oxidized soy bean oil on growing rats. Nutr Rep Int 36:387–401
13. D'Aquino M, Di Felice M, Tomassi G (1985) Vitamin E status and effects of thermoxidized fats on structural alpha-tocopherol and fatty acid of different rat tissues. Nutr Rep Int 32:1179–1186
14. Eck P, Pallauf J (1999) Induction of metallothionein by paraquat injection in zinc-deficient rats. J Anim Physiol Anim Nutr 81:203–211
15. Eder K, Kirchgessner M (1998) The effect of dietary vitamin E supply and a moderately oxidized oil on activities of hepatic lipogenic enzymes in rats. Lipids 33:277–283
16. Fornace AJ Jr, Schalch H, Alamo I Jr (1988) Coordinate induction of metallothionein I and II rodent cells by UV irradiation. Mol Cell Biol 8:4716–4720
17. Fukunaga K, Suzuki T, Takama K (1993) Highly sensitive high-performance liquid chromatography for the measurement of malondialdehyde in biological samples. J Chromatogr 621:77–81
18. Hayam I, Cogan U, Mokady S (1995) Dietary oxidized oil and the activity of antioxidant enzymes and lipoprotein peroxidation in rats. Nutr Res 15:1037–1044
19. Hidalgo J, Campamany L, Borras M, Garvey J, Armario A (1988) metallothionein response to stress in rats: role in free radical scavenging. Am J Physiol 255:E518–E524
20. Hochgraf E, Modady S, Cogan U (1997) Dietary oxidized linoleic acid modifies lipid composition of rat liver microsomes and increases their fluidity. J Nutr 127:681–688
21. Hu ML (1994) Measurement of protein thiol groups and glutathione in plasma. Methods Enzymol 233:380–385
22. Imagawa T, Kasai S, Matsui K, Nakamura T (1982) Methyl hydroperoxy-epoxy-octadecenoate as an autoxidation product of methyl linoleate: a new inhibitor-uncoupler of mitochondrial respiration. J Biochem (Tokyo) 92:1109–1121
23. Ingold KU, Burton GW, Foster DO, Hughes L, Lindsay DA, Webb A (1987) Biokinetics of and discrimination between dietary RRR- and SRR-α-tocopherol in male rat. Lipids 22:163–172
24. Irato P, Sturniolo GC, Giacon G, Magro A, D'Inca R, Mestriner C, Albergoni V (1996) Effect of zinc supplementation on metallothionein, copper, and zinc con-

centration in various tissues of copper-loaded rats. Biol Trace Elem Res 51:87–96
25. Kägi JHR, Kojima Y (1987) Chemistry and biochemistry of metallothionein. Experientia Suppl 52:25–61
26. Klaassen CD, Lehman-McKeeman LD (1991) Separation and quantification of isometallothioneins by high-performance liquid chromatography – atomic absorption spectrometry. Methods Enzymol 205:190–198
27. Liu J-F, Huang C-J (1995) Tissue α-tocopherol retention in male rats in compromised by feeding diets containing oxidized frying oil. J Nutr 125:3071–3080
28. Liu J-F, Huang C-J (1996) Dietary oxidized frying oil enhances tissue α-tocopherol depletion and radioisotope tracer excretion in vitamin E-deficient rats. J Nutr 126:2227–2235
29. Maret W (1994) Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange. Proc Natl Acad Sci USA 91:237–241
30. Maret W, Vallee BL (1998) Thiolate ligands in metallothionein confer redox activity on zinc clusters. Proc Natl Acad USA 95:3478–3482
31. Markant A, Pallauf J (1997) metallothionein and zinc as potential antioxidants in radical-induced peroxidation in cultured hepatocytes. J Trace Elem Med Biol 10:88–95
32. Marshansky VN, Novgorodov SA, Yaguzhinsky LS (1983) The role of lipid peroxidation in the induction of cation transport in rat liver mitochondria. The antioxidant effect of oligomycin and dicyclohexylcarbodiimide. FEBS Lett 158:27–30
33. Naganuma A, Satoh M, Imura N (1988) Specific reduction of toxic side effects of adriamycin by induction of metallothionein in mice. Jpn J Cancer Res 79:406–411
34. Nakagawa I, Suzuki M, Imura N, Naganuma A (1998) Involvement of oxidative stress in paraquat-induced metallothionein synthesis under glutathione depletion. Free Radical Biol Med 24:1390–1395
35. National Research Council (1985) Guide for the care and use of laboratory animals. Publication no. 85–23 (rev.), National Institute of Health, Bethesda, MD
36. Nöstelbacher K, Kirchgessner M, Stangl GI (2000) Separation and quantitation of metallothionein-isoforms from liver of untreated rats by ion-exchange high performance liquid chromatography and atomic absorption spectrometry. J Chromatogr (in press)
37. Nolan MR, Kennedy GG, Blanchflower WJ, Kennedy S (1995) Feeding corn oil to vitamin E- deficient pigs increases lipid peroxidation and decreases tissue glutathione concentrations. Internat J Vit Nutr Res 65:181–186
38. Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 40:158–169
39. Reeves PG (1995) Adaption responses in rats to long-term feeding of high-zinc diets: emphasis on intestinal metallothionein. J Nutr Biochem 6:48–54
40. Reeves PG, Nielsen FH, Fahey G C Jr (1993) AIN–93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN–76A rodent diet. J Nutr 123:1939–1951
41. Sato M, Apostolova MD, Hamaya M, Yamaki J, Choo KHA, Michalska AE, Kodama N, Tohyama C (1996) Susceptibility of metallothionein-null mice to paraquat. Environmental Toxicol Pharmacol 1:221–225
42. Sato M, Sasaki M (1991) Enhanced lipid peroxidation is not necessary for induction of metallothionein-I by oxidative stress. Chem Biol Interact 78:143–154
43. Satoh M, Naganuma A, Imura N (1988) Involvement of cardiac metallothionein in prevention of adriamycin induced lipid peroxidation in the heart. Toxicol 53:231–237
44. Shiraishi N, Aono K, Utsumie K (1983) Increased metallothionein content in rat liver induced by X-irradiation and exposure to high oxygen tension. Radiat Res 95:298–302
45. Slater TF (1982) Lipid peroxidation. Biochem Soc Trans 10:70–71
46. Smith K, Krohn RJ, Hermanson GT, Mallia AK, Garnter FH, Provenzano MD, Fujimoto EK, Goeke M, Olson BJ, Klenk DC (1975). Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85
47. Stangl GI, Müller H, Kirchgessner M (1999) Conjugated linoleic acid effects on circulating hormones, metabolites and lipoproteins, and its proportion in fasting serum and erythrocyte membranes of swine. Eur J Nutr 38:271–277
48. Suzuki KT, Yamamura M (1980) Induction of zinc-thionein in rat liver and kidneys by zinc loading as studied at isometallothionein levels. Toxicol Lett 6:59–65
49. Tatum VL, Changchit C, Chow CK (1990) Measurement of malondialdehyde by high performance liquid chromatography with fluorescence detection. Lipids 25:226–229
50. Thomas JP, Bachowski GJ, Girotti AW (1986) Inhibition of cell membrane lipid peroxidation by cadmium- and zinc-metallothionein. Biochim Biophys Acta 884:448–461
51. Wakeyama H, Takeshige K, Takayanagi R, Minakami S (1982) Superoxide-forming NADPH oxidase preparation of pig polymorphonuclear leukocytes. Biochem J 205:593–601
52. Wang S-T, Kuo JH, Chou R-G, Lii C-K (1996) Vitamin E protection of cell morphology and protein thiols in rat hepatocytes treated with tert-butyl hydroperoxide. Toxicol Lett 89:91–98
53. Windisch W, Kirchgessner M (1994) Zinkexkretion und Kinetik des Zinkaustauschs im Ganzkörper bei defizitärer und hoher Zinkversorgung: Zum Effekt einer unterschiedlichen Zinkversorgung auf den quantitativen Zinkumsatz im Stoffwechsel adulter Ratten. J Anim Physiol Anim Nutr 71:123- 130