Effect of vitamin E- and selenium-deficiency on rat liver chemiluminescence

Cesar G. FRAGA,*† Rosa F. ARIAS,† Susana F. LLESUY,* Osvaldo R. KOCH† and Alberto BOVERIS*

*Instituto de Química y Fisicoquímica Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, and †Catedra de Patología I, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

The role of vitamin E and selenium as protective agents against oxidative stress was evaluated by measuring liver chemiluminescence *in situ*. Weanling rats fed a vitamin E- and selenium-deficient diet showed liver chemiluminescence that was increased 60 and 100% over control values at 16 and 18 days respectively after weaning. At day 21, the double deficiency led to hepatic necrosis, as observed by optical and electron microscopy, and increased serum levels of lactate dehydrogenase and alanine aminotransferase. Single deficiencies, in either vitamin E or selenium, did not produce liver necrosis but increased liver chemiluminescence. Vitamin E deficiency led to a 23 and 50% increase in liver emission at days 18 and 20 respectively; selenium deficiency produced a 64% increase at day 16. The activity of liver selenium-glutathione peroxidase diminished to 13% of the control value in the rats fed doubly deficient and selenium-deficient diets. Activities of superoxide dismutase, catalase and non-selenium-glutathione peroxidase were not modified by the different diets. These results suggest that oxy-radical generation may play a major role in hepatic necrosis in vitamin E- and selenium-deficiency.

INTRODUCTION

The radical chain reaction of lipid peroxidation appears to be a continuous physiological process that, if out of control, can alter essential cell functions and lead to cell death (Tappel, 1973; Chance *et al.*, 1979). Vitamin E, as a lipid soluble antioxidant, and selenium, as part of the active site of glutathione peroxidase, play major protective roles against oxidative stress (Tappel, 1980; Witting, 1981; Burton *et al.*, 1983; Diplock, 1983; Wilson, 1983). Vitamin E, in membrane hydrophobic domains, scavenges free radicals (Witting, 1981) and/or quenches excited species (Forman & Fisher, 1981). Selenium-containing glutathione peroxidase catalyses the reduction of hydrogen peroxide, and of lipid and organic hydroperoxides (Mills, 1960; Rotruck *et al.*, 1973), mainly in hydrophilic cell domains (Chance *et al.*, 1979; Tappel, 1980) and at membrane interfaces (Mills, 1960). A double deficiency of vitamin E and selenium in rats leads to liver necrosis (Schwarz, 1958; Porta *et al.*, 1968). In pre-necrotic stages, changes in activities of membrane-bound enzymes and pro-oxidant balance is related to increased rates of lipid peroxidation (Machado *et al.*, 1971; Hafeman & Hoekstra, 1977). Since liver chemiluminescence *in situ* affords an organ-specific and organ-non-invasive method to evaluate oxy-radical and organic-peroxy-radical generation (Boveris *et al.*, 1980, 1981, 1983; Cadenas & Sies, 1984; Jamieson *et al.*, 1986; Fraga *et al.*, 1987), measurements of liver chemiluminescence were performed in parallel with morphological and biochemical studies in vitamin E- and selenium-deficient rats to establish a possible cause-effect relationship between lipid peroxidation and cell injury and death.

MATERIALS AND METHODS

Animals

Weanling male Wistar rats weighing 42 ± 3 g were placed into four groups of 40 animals each. One group was fed the control diet (Table 1); a second group was fed a vitamin E- and selenium-deficient diet; a third group was fed a vitamin E-deficient diet; and a fourth group was fed a selenium-deficient diet. All animals were provided water *ad libitum*. The experiments were carried out for 21 days after weaning. Growth rates were 1.53 ± 0.08 ($P < 0.05$ as compared with the other three groups), 1.23 ± 0.10 , 1.28 ± 0.08 and 1.29 ± 0.11 g/day for control, vitamin E- and selenium-deficient, vitamin E-deficient, and selenium-deficient groups respectively. Before development of necrosis, liver weights (3.3 ± 0.2 g) in the four groups were not significantly different.

Table 1. Composition of the control diet

Component	Composition (g/100 g of diet)
Torula yeast	30
Cod liver oil	5
Sucrose	59
Salt mixture	5
Vitamin mixture (without vitamins D, A and E)	1
Vitamin E	0.003
Sodium selenite	0.00036

† Present address and address for correspondence and reprint requests: Department of Food Science and Technology, 1480 Chemistry Annex, University of California, Davis, CA 95616, U.S.A.

Liver chemiluminescence

Rats fasted 20 h were anaesthetized with Nembutal (5 mg/100 g, intraperitoneally). The liver surface was exposed by laparotomy, and chemiluminescence was measured as previously described (Boveris *et al.*, 1980, 1981, 1983). The emission was expressed as c.p.s./cm² of liver surface.

Marker enzymes of liver necrosis

Immediately after chemiluminescence determinations, blood samples were obtained by cardiac puncture. The activities of alanine aminotransferase and lactate dehydrogenase were measured in serum samples.

Optical and electron microscopy

After chemiluminescence determinations, the liver was removed from the animal and a section of a median lobe was fixed in 10% (v/v) formaldehyde and stained for optical-microscopy studies (Lillie, 1965). For electron microscopy, small blocks of tissue from the same lobe were fixed for 2 h at 4 °C in 3% glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, and postfixed in Caulfield's osmic mixture for 2 h at 4 °C (Sabatini *et al.*, 1963). The sections were embedded in Epon 812 (Luft, 1961).

Antioxidative enzyme activities

The activities of selenium-glutathione peroxidase, non-selenium-glutathione peroxidase, catalase and superoxide dismutase were measured. The livers were homogenized 1:9 (w/v) in 140 mM-KCl/10 mM-phosphate, pH 7.4, and centrifuged at 600 g for 10 min at 4 °C to remove nuclei and cell debris (Boveris *et al.*, 1983). Glutathione peroxidase was determined after NADPH oxidation at 340 nm (Paglia & Valentine, 1967; Flohe & Gunzler, 1984) in a reaction medium consisting of the isolation buffer, 0.17 mM-GSH, yeast glutathione reductase (0.2 unit/ml), 0.3 μ M-NADPH, and 0.5 mM-hydrogen peroxide for determination of selenium-glutathione peroxidase activity, or 0.5 mM-t-butyl hydroperoxide for the determination of selenium-glutathione peroxidase plus non-selenium-glutathione peroxidase. Catalase was determined by measuring the decrease in A_{240} in a reaction medium consisting of 50 mM-phosphate buffer, pH 7.3, 2 mM-hydrogen peroxide, 1% Triton X-100 and 0.1–0.3 mg of protein/ml. Catalase content was expressed as nmol/g of tissue by dividing the obtained k value by the second-order reaction constant for pure catalase, $k = 4 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Sies *et al.*, 1973). Superoxide dismutase was determined by measuring the inhibition of the adrenochrome formation rate (Misra & Fridovich, 1972) in a reaction medium containing 1 mM-adrenaline (epinephrine) and 50 mM-glycine/NaOH, pH 9.6 (Boveris *et al.*, 1983).

Chemicals

Adrenaline bitartrate, NADPH, GSSG, yeast glutathione reductase, Triton X-100, and alanine aminotransferase and lactate dehydrogenase kits were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Hydrogen peroxide was obtained from Baker Chemical Co., Phillipsburg, NJ, U.S.A.; *t*-butyl hydroperoxide from Aldrich Chemical Company, Milwaukee, WI, U.S.A.; Nembutal from Abbott Laboratories, North

Fig. 1. Spontaneous chemiluminescence *in situ* of liver of rats fed vitamin E- and selenium-deficient diets

The dietary groups were: ○, control; ●, vitamin E- and selenium-deficient; ▲, selenium-deficient; and △, vitamin E-deficient. The abscissa indicates time after weaning. Symbols and bars indicate mean values $\pm$ S.E.M.

Chicago, IL, U.S.A.; vitamin E, vitamin mixture and salt mixture from ICN Life Science Group, Cleveland, OH, U.S.A.; sodium selenite from BDH, Poole, Dorset, U.K.; formaldehyde from Merck, Darmstadt, Germany; and glutaraldehyde, cacodylate, and Epon 812 from Polysciences, Warrington, PA, U.S.A.

Statistics

Values given in the text and in the Figures represent mean values $\pm$ S.E.M. The significance of differences were analysed by one-way analysis of variance.

RESULTS

Liver chemiluminescence

Spontaneous liver chemiluminescence of control rats fed the vitamin E and selenium-supplemented diet was 43 ± 3 c.p.s./cm²; this value was not modified during the 21-day period during which the experiments were performed (Fig. 1). The non-necrotic livers of rats fed the vitamin E- and selenium-deficient diet showed an increase in emission of about 60% at day 16 and an increase of 100% at day 18 (Fig. 1). The emission of livers from rats fed the vitamin E-deficient diet was increased 23 and 50% at days 18 and 20 respectively (Fig. 1). There was no difference between the emission from livers of selenium-deficient and doubly deficient rats until day 16, when the chemiluminescence from selenium-deficient rat liver was increased 64% over control values and then remained the same until day 20 (Fig. 1).

To control changes in the transmittance properties of the outer surface of the liver that could modify the emission, the absorbance of liver slices was measured. Determinations at 500, 600, and 650 nm showed that the different diets did not significantly change the transmittance of the liver surface.

Fig. 2. Spontaneous chemiluminescence and necrosis in the liver of vitamin E- and selenium-deficient rats

The closed circles (●) indicate the percentage increase in liver chemiluminescence, and the hatched area (▨) represents the cumulative percentage of the rats that developed necrosis. The relationship between chemiluminescence and necrosis, up to day 18, fits a straight line with a correlation coefficient (r) of 0.98 ($P < 0.05$). The abscissa indicates time after weaning. Symbols and bars indicate mean values $\pm$ S.E.M.

Morphological studies

Optical and electron microscopy did not show alterations in tissue organization in non-necrotic livers. In the rats fed the doubly deficient diet, either submassive or massive hepatic necrosis was observed in 90% of the animals 20 days after weaning (Fig. 2). At day 18, only 34% of the rats had developed hepatic necrosis. Neither rats fed the control diets nor rats fed vitamin E-deficient or selenium-deficient diets showed signs of hepatic necrosis during the 21-day course of the experiments.

Necrosis was diagnosed by evidence of two of the following three criteria: (a) macroscopic haemorrhagic foci; (b) microscopic replacement of liver cells by erythrocytes and leucocytes; and (c) 6-fold increased serum levels of both alanine aminotransferase and lactate dehydrogenase.

Enzyme activities

Liver selenium-glutathione peroxidase was greatly diminished as a function of time in rats fed the selenium-deficient diets. Activity in control rats was 7.6 ± 0.7 units/g of liver, and was diminished to 39, 21, and 13% of this value at days 16, 18 and 20 respectively in selenium-deficient and vitamin-E-and-selenium-deficient rats. A deficiency only of vitamin E did not modify the enzyme activity. An inverse correlation between spontaneous liver chemiluminescence and selenium-glutathione peroxidase activity was observed in the livers of the vitamin-E-and-selenium-deficient and the selenium-deficient rats (Fig. 3). The activities of non-selenium-glutathione peroxidase (6.9 ± 1.5 units/g) and superoxide dismutase (900 ± 35 units/g) and the content of catalase (6.8 ± 0.1 nmol/g) were not modified in the livers of rats fed the different diets.

Fig. 3. Correlation between spontaneous liver chemiluminescence and hepatic selenium-glutathione peroxidase activity

Dietary groups were: ○, vitamin E- and selenium-deficiency; ●, selenium-deficiency. The correlation coefficient (r) was 0.92 ($P < 0.05$).

DISCUSSION

A deficiency in vitamin E and selenium in rats leads to hepatic necrosis (Schwarz, 1958; Porta *et al.*, 1968) and increased peroxidation, as measured by ethane and pentane exhalation (Hafeman & Hoekstra, 1977; Dillard *et al.*, 1978). Lipid peroxidation may lead to membrane disruption and cell death, which initiates the necrotic process.

The determination of liver chemiluminescence *in situ* in vitamin E- and selenium-deficient rats allowed assessment of the liver as a target organ for increased lipid peroxidation. At day 18 after weaning, the liver emission of doubly deficient rats was twice that of control values in animals without necrosis (65% of the rats assayed). At this time, the observed decrease in selenium-glutathione peroxidase activity and the diminished membrane content of vitamin E may have contributed to a higher production of oxy radicals and excited species (singlet oxygen and carbonyl groups), as measured by liver chemiluminescence. At 18 days, it was possible to define a pre-necrotic stage with a high rate of excited-species generation, without observable morphological changes, with unmodified catalase and superoxide dismutase activity, and with a further decrease in selenium-dependent glutathione peroxidase activity. Necrosis appeared 24–48 h after this pre-necrotic stage, and the close relationship observed between chemiluminescence and necrosis (Fig. 2) suggested lipid peroxidation as a promoter of cell death. Similar observations were made by Fee *et al.* (1975), who used red cells and measured malonaldehyde production preceding haemolysis. The decreased activity of glutathione peroxidase would probably lead to an increased rate of free-radical generation in the cytosol, which would deplete the membrane of vitamin E, thus promoting membrane disruption.

Either dietary vitamin E or selenium prevented the necrotic process, probably by controlling excessive free-radical generation, since the liver emission in each single deficiency was lower than that produced by the

double deficiency. In single deficiencies, the kinetics of emission as a function of dietary feeding time were different, depending on which nutrient was supplemented. In vitamin E deficiency, the enhancement in free-radical production should occur in the hydrophobic membrane domains. Vitamin E would be slowly oxidized to a critical level to allow an increase in emission by 16 days after weaning. Normal levels of glutathione peroxidase and other antioxidant enzymes seem to maintain the low level of emission. In selenium deficiency, the increase in liver emission was observed earlier and was higher than in vitamin E-deficiency. Chemiluminescence in livers of selenium-deficient rats indicates that there was production of a high level of free radicals. The presence of vitamin E, however, maintained free radicals at a level that did not allow the development of necrosis.

Rats fed selenium-deficient diets had decreased selenium-glutathione peroxidase activity and concurrent increased liver emission (Fig. 3). In rats fed the selenium-deficient diet, chemiluminescence reached a maximum by 16 days, but glutathione peroxidase had not decreased to its lowest level at this time. Other investigators (Lawrence *et al.*, 1978) have shown that, as selenium-glutathione peroxidase activity decreases, the activity of non-selenium-glutathione peroxidase increases. The non-selenium enzyme measured by difference between activities with hydrogen peroxide and t-butyl hydroperoxide substrates did not show increased activity of this enzyme. Because the method used may not have accurately detected non-selenium-glutathione peroxidase, it is possible that an undetected increase in glutathione S-transferase was responsible for stopping the rise in chemiluminescence by 16 days. If increased activity did occur, it had no influence on the development of necrosis in livers of the doubly (vitamin E- and selenium-) deficient rats.

Our results on spontaneous liver chemiluminescence strongly suggest that free-radical damage to vital cell membranes plays a major role in hepatic necrosis in rats deficient in vitamin E and selenium.

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