Electron spin resonance spin trapping studies of the effects of dietary zinc deficiency on free radical production in vitro and in vivo under acute oxidative stress

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The objective of this study was to investigate the effect of dietary zinc deficiency on free radical production in the rat liver homogenate and whole liver under acute oxidative stress in vitro and in vivo. Three-week-old male Wistar rats were given free access to either a zinc-adequate diet or a zinc-deficient diet for 3 weeks. A second control group, the pair-fed control, was also included. The oxidative stress was imposed in vitro by incubating whole-liver homogenate prepared from rats with CCl_4, ethanol, or FeNTA. The same oxidants were used to impose oxidative stress in vivo. Incubation of liver homogenate with CCl_4 or ethanol resulted in the detection of their free radical metabolites, a CCl_3· radical (α_N = 13.9 G, α_β^H = 1.9 G) and a 1-hydroxyethyl radical (α_N = 15.1 G, α_β^H = 4.6 G), respectively. A free radical (α_N = 14.9 G, α_β^H = 4.8 G) was detected in the whole-liver homogenate incubated with FeNTA. A CCl_3· radical was detected in the livers of CCl_4-challenged rats in vivo. However, no free radicals were detected in the livers of ethanol- or FeNTA-challenged rats regardless of dietary treatment. The production of free radicals both in vitro and in vivo was not affected by dietary zinc deficiency as indicated by electron spin resonance signals of α-phenyl-N-tert-butyl nitrone-spin adduct. It seems that dietary zinc-deficient rats are capable of coping with an increased free radical production in liver, both in vitro and in vivo, under acute oxidative stresses. (J. Nutr. Biochem. 5:490–494, 1994.)

Keywords: zinc deficiency; free radical production; CCl_4; ethanol; FeNTA;

Introduction

It has been proposed that zinc has a function as an antioxidant. The antioxidant role of zinc is primarily demonstrated in vitro and in animals given pharmacologic doses of zinc. Zinc inhibits hydroxyl radical-induced lipid peroxidation in erythrocyte membranes.[1] The physiological role of zinc as an antioxidant has yet to be elucidated. Carbon tetrachloride (CCl_4) manifests its toxicity in liver through the formation of CCl_3· radical.[2] A pharmacologic dose of zinc protects rats from CCl_4-induced hepatic toxicity.[3] Some evidence indicates that there is an increased oxidative stress in dietary zinc-deficient animals. In rats[4] and mice,[5] dietary zinc deficiency increases the susceptibility of hepatic microsomes to oxidative stress. Dietary zinc deficiency results in a decreased stability of hepatic microsomal P_{450} in rats.[6] This effect of zinc deficiency on microsomal P_{450} appears to be mediated by free radical-mediated damage. Moreover, there is an accumulation of prooxidants, including Fe and H_2O_2 in lung and liver microsomes,[7] and a carbon-centered free radical in lung microsomes,[8] isolated from dietary zinc-deficient rats. However, the physiological consequence of the increase in the concentration of these prooxidants remains unclear.

Free radicals, either generated from cellular metabolism or xenobiotic detoxification,[9] are implicated in the etiology of various diseases.[10] The cellular free radical defense systems have a crucial function in protection against free radical-induced oxidative damage.[11] An altered free radical metabolism, either an increased free radical production or a decreased free radical defense capability, may result in an increased oxidative stress. It is possible that free radical metabolism is altered in dietary zinc-deficient animals, resulting in an increased oxidative stress.

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The objective of this study was to investigate the effect of dietary zinc deficiency on liver free radical production in rats under acute oxidative stress in vitro and in vivo. The electron spin resonance (ESR) signal of α-phenyl-N-tert-butyl nitrone (PBN) spin adduct extracted from whole-liver homogenate and from fresh livers was used as a measure of free radical production in vitro and in vivo, respectively. The oxidative stress in vitro and in vivo was imposed with free radical generating compound, CCl_4 and ethanol, and the general oxidant, ferrous nitrilotriacetate (FeNTA).

Methods and materials

Animals and diets

Three-week-old male Wistar rats with an average initial body weight of 49 g (Charles River, St. Constant, Canada) were given free access to either a zinc-adequate diet (ZnAL, 100 mg Zn/kg diet) or a zinc-deficient diet (ZnDF, <1.0 mg Zn/kg diet) for 3 weeks. A second control group, the pair-fed control (ZnPF), was also included due to the severe depression of feed intake caused by dietary zinc deficiency. The ZnPF rats were individually paired to ZnDF rats. Zinc deficiency status in ZnDF rats was developed by the feeding regimen[7] and the animal care procedures described previously.[6] Plasma zinc concentration was determined using flame atomic absorption spectroscopy.[6]

ESR spin trap studies in vitro

Fresh whole-liver (1 g) from ZnAL, ZnPF, and ZnDF rats was homogenized in 2 mL phosphate buffer (0.2 mol/L, pH 7.4) with a Tekmar homogenizer (Model SDT-1810, Tekmar Company, Cincinnati, OH USA) for five strokes. The whole-liver homogenate was incubated with or without the presence of an oxidant compound. Each reaction mixture contained whole-liver homogenate (400 μL, 250 mg liver), NADPH (0.5 mmol), an NADPH regenerating system (4 mmol glucose-6-phosphate and 5 units of glucose-6-phosphate dehydrogenase), and PBN (200 mmol) in phosphate buffer (80 μmol) with a final volume of 1 mL. One set of reaction mixtures was incubated with PBN alone at 37° C for 30 min as controls. To impose oxidative stress, the reaction mixture prepared from each dietary group was incubated with CCl_4 (20 μL, 30 min), ethanol (25 μL, 15 min), or FeNTA (20 μmol, 15 min) at 37° C. In the FeNTA study, one set of whole-liver homogenates prepared from ZnAL and ZnDF rats was incubated with FeNTA under the same conditions, except in the absence of PBN. At the end of the incubation period, PBN was added, mixed, and left at 37° C for 5 min before terminating the reaction. All incubations were stopped by adding 20 mL of chloroform to the reaction mixture.

ESR spin trap studies in vivo

At the end of the feeding trial, one group of ZnAL, ZnPF, and ZnDF rats was injected i.p. with PBN alone as controls. To impose oxidative stress, one group of ZnAL, ZnPF, and ZnDF rats was given a single dose of CCl_4 (intubation, 1 mL/kg body weight, 40% (vol/vol) corn oil-CCl_4 emulsion), while another group was given ethanol (intubation, 5 mL/kg body weight, 50% [vol/vol] ethanol-distilled water) 1 hr prior to the sampling. A third group of rats was fasted overnight, followed by i.p. injection of freshly prepared FeNTA (18 mg Fe/kg body weight) 24 hr prior to the sampling. In all ESR spin trapping studies in vivo, PBN was i.p. injected at a dose of 75 mg/kg body weight 30 min prior to the sampling. At the end of the challenge period, livers were quickly removed and a sample of liver (5 g) was immediately homogenized in 5 mL phosphate buffer (0.2 mol/L, pH 7.4). Each liver sample from ZnPF and ZnDF rats represents three rat livers pooled.

Sample preparation and ESR spectroscopy

The reaction mixtures in the study in vitro and the liver homogenates in the study in vivo were extracted with chloroform through two consecutive extractions, with a total volume of 40 mL. After centrifugation at 1,000*g* for 10 min, the organic phase was transferred into a new centrifuge tube and dried under N_2. Each extract was redissolved in 0.3 mL toluene, transferred to a round quartz ESR cell, and further gassed with N_2 for 10 min to remove O_2 before ESR reading.

ESR readings were done with a Bruker ESR ER 200D spectrometer (Bruker Analytische Messtechnik GMBH, Karlsruhe, Germany). Data accumulation was done using a Bruker ER-140 (ASPECT) data system. The intensity of the ESR signal of the PBN spin adduct was measured by the total area of the first doublet in arbitrary units (mm on paper). The instrumental settings were as follows: center field 3475 G; microwave frequency, 9.75 GHz; time constant, 100 msec; scan range 100 G; scan time, 50 sec; and microwave source power, 20.5 mW. The modulation frequency was 100 KHz for the untreated and ethanol-treated whole-liver homogenate and 50 KHz for the CCl_4 treated whole-liver homogenate and all in vivo studies. The ESR spectrum obtained in all in vivo studies represents an accumulation of six scans.

Statistical analysis

Data are expressed as means ± SEM. One-way analysis of variance (ANOVA) with 95% confidence intervals was used to test the source of the variation. The least significant difference (LSD) then was used to determine the statistical significance according to the preplanned comparisons. A *P*-value of less than 0.05 was considered significant.[12]

Results

The severe zinc deficiency status of ZnDF rats was confirmed by the development of characteristic zinc deficiency signs: severe growth depression and significantly lower plasma zinc concentration *(Table 1)*. Acute oxidative stress imposed by CCl_4, ethanol, or FeNTA had no effect on body weight gain regardless of dietary treatment. CCl_4 treatment, but not ethanol or FeNTA treatment, elevated plasma zinc concentration in ZnDF and ZnPF rats.

The results of ESR spin trapping studies in vitro and in vivo are summarized in *Table 2*, while the representative ESR signals of PBN spin adducts are shown in *Figure 1*. In the study in vitro, there was no ESR signal of PBN spin adduct in the chloroform extract from the untreated whole-liver homogenate *(Table 2, Figure 1a)*. When whole-liver homogenate was incubated with CCl_4, an ESR signal of PBN spin adduct (α_N = 13.9 G, α_β^H = 1.9 G) was recorded in all dietary treatment groups *(Table 2, Figure 1b)*. The hyperfine splitting constants of the free radical trapped was typical of the PBN spin adduct of CCl_3· radical, a metabolite of CCl_4, in toluene.[13] When whole-liver homogenate was incubated with ethanol, the ESR spectra of PBN spin adduct (α_N = 15.1 G, α_β^H = 4.6 G) was recorded *(Table 2, Figure 1c)*. Based on the hyperfine splitting constants,[14] the free radical trapped appeared to be a 1-hydroxylethyl radical, a metabolite of ethanol. The results of FeNTA study in vitro is shown in *Figure 2*. When NTA alone was added to the

Table 1 Effects of dietary zinc deficiency and oxidative stress on body weight and plasma zinc concentration

Oxidative stress	Diets	Final body weight (g)	Plasma Zn (μg/mL)
Dietary zinc deficiency	ZnAL	188 ± 6[a]	1.36 ± 0.11[a]
	ZnPF	92 ± 6[b]	1.27 ± 0.07[a]
	ZnDF	65 ± 3[c]	0.30 ± 0.04[b]
CCl_4	ZnAL	165 ± 11[a]	1.23 ± 0.12[a]
	ZnPF	73 ± 3[b]	1.55 ± 0.09[a,*]
	ZnDF	59 ± 2[c]	0.79 ± 0.06[b,*]
EtOH	ZnAL	188 ± 5[a]	1.36 ± 0.11[a]
	ZnPF	80 ± 4[b]	1.39 ± 0.16[a]
	ZnDF	66 ± 3[c]	0.31 ± 0.06[b]
FeNTA	ZnAL	190 ± 7[a]	0.93 ± 0.21[a]
	ZnPF	82 ± 5[b]	0.70 ± 0.17[a]
	ZnDF	63 ± 4[c]	0.22 ± 0.05[b]

Values are means ± SEM, n = 3 to 6 rats. Means of different dietary treatments within the same oxidative stress group with different letters are statistically different ($P < 0.05$). Means with * indicates the significant difference ($P < 0.05$) between the oxidative stress control group and the oxidative stress group.
ZnAL: zinc-deficient with free access group; ZnPF: zinc-adequate pair-fed group; ZnDF: zinc-deficient group; EtOH: ethanol group; FeNTA: ferrous nitrilotriacetate group.

Table 2 Summary of the effects of zinc deficiency on free radical production using ESR spin trapping studies

Oxidative stress	Diets	ESR signal in vitro	ESR signal in vivo
Zn deficiency	ZnAL	− (3)	− (6)
	ZnPF	− (3)	− (5)
	ZnDF	− (3)	− (6)
CCl_4	ZnAL	+ (3)	+ (5)
	ZnPF	+ (3)	+ (5)
	ZnDF	+ (3)	+ (5)
EtOH	ZnAL	+ (3)	− (5)
	ZnPF	nd	− (5)
	ZnDF	+ (3)	− (5)
FeNTA	ZnAL	+ (3)	− (5)
	ZnPF	+ (3)	− (5)
	ZnDF	+ (3)	− (5)

Each liver sample contained one liver (ZnAL) or was pooled from three livers (ZnPF and ZnDF).
The number of replications is indicated in parentheses. −: no ESR signal; +: ESR signal recorded; nd: not determined.
ZnAL: zinc-deficient with free access group; ZnPF: zinc-adequate pair-fed group; ZnDF: zinc-deficient group; EtOH: ethanol group; FeNTA: ferrous nitrilotriacetate group.

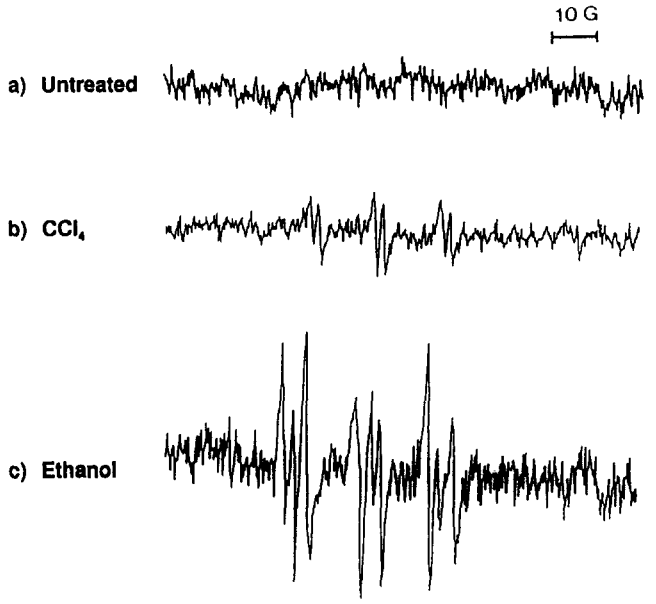

Figure 1 The representative ESR spectrums of PBN spin adducts extracted from the whole-liver homogenate prepared from ZnDF rats. Sample preparation procedures were as described in methods and materials. (a) the untreated whole-liver homogenate incubated at 37° C for 30 min. (b) the whole-liver homogenate was incubated with CCl_4 (20 μl) at 37° C for 30 min. The hyperfine splitting constants of the PBN spin adduct detected were: α_N = 13.9 G, α_β^H = 1.9 G; (c) the whole-liver homogenate was incubated with ethanol (25 μL) at 37° C for 15 min. The hyperfine splitting constants of the PBN spin adduct detected from ZnAl and ZnDF rats were: α_N = 15.1 G, α_β^H = 4.6 G.

reaction mixture, there was no ESR signal recorded *(Figure 2a)*. Incubation of whole-liver homogenate with FeNTA resulted in a detection of a PBN spin adduct (α_N = 14.9 G, α_β^H = 4.8 G, *Figure 2a*). This free radical was detected in whole-liver homogenates prepared from all dietary treatment groups, indicating dietary zinc deficiency had no effect on the production of this free radical. When PBN was initially omitted and then added back to the reaction mixture (which contained FeNTA) at the end of the incubation period, the ESR signal of the PBN adduct was greatly enhanced *(Figure 2b)* in all groups. Thus, the spin trap, PBN, behaved as an antioxidant.

In the ESR spin trapping study in vivo, there was no ESR signal detected in the untreated ZnAL, ZnPF, and ZnDF rats *(Table 2)*. When rats were orally dosed with CCl_4, a similar ESR spectra of PBN spin adduct as obtained in the CCl_4 study in vitro was recorded in the chloroform extract of the livers *(Table 2)*. It also appeared to be a CCl_3· radical. The intensity of the ESR signal was not significantly affected by dietary zinc deficiency *(Table 3)*. There was no ESR signal of PBN spin adduct recorded in the livers of ethanol-

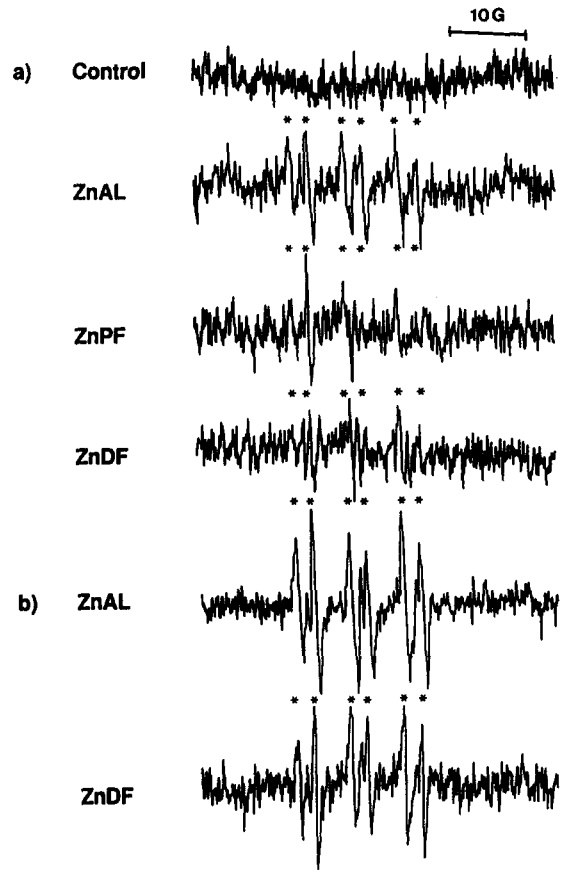

Figure 2 ESR spectrum of PBN spin adduct extracted from whole-liver homogenate incubated with FeNTA. (a) PBN was initially included in the incubation mixture. In the control, only NTA was included in the reaction mixture. The whole-liver homogenate was incubated with FeNTA (20 μmol) at 37° C for 15 min. (b) PBN was initially omitted and then added at the end of the incubation period. The free radical trapped was indicated by asterisks.

Table 3 The ESR signal of rats challenged with CCl_4

Diets	Intensity of ESR signal (total signal area)
ZnAL	10.1 ± 2.7[a]
ZnPF	8.2 ± 1.4[a]
ZnDF	4.5 ± 1.1[a]

Values are means ± SEM, n = 5 liver samples. Each sample contained one liver (ZnAL) or was pooled from three livers (ZnPF and ZnDF). Means with the same superscripts are not significantly different ($P < 0.05$).
ZnAL: zinc-deficient with free access group; ZnPF: zinc-adequate pair-fed group; ZnDF: zinc-deficient group.

or FeNTA-challenged rats regardless of dietary treatment *(Table 2)*.

Discussion

The ability of zinc to function as an antioxidant is clearly demonstrated in vitro using tissue fractions and in vivo using pharmacologic doses of zinc.[15] The physiological role of zinc as an antioxidant has yet to be uncovered. Dietary zinc deficiency causes an accumulation of prooxidants.[7,8] To cause an observable increase of oxidative damage in animals, dietary zinc deficiency would have to compromise the overall free radical defense capability. However, the primary free radical defense system, both enzymatic and nonenzymatic, was found to be essentially intact in dietary zinc-deficient rats.[16] Therefore, it is important to examine the effects of dietary zinc deficiency on free radical production in livers under acute oxidative stress in vitro and in vivo.

CCl_4, EtOH, and FeNTA are well-known oxidants used to impose acute oxidant stress. In the present study, CCl_3·, a free radical metabolite of CCl_4, was produced in vitro (whole-liver homogenates) and in vivo. Similarly, 1-hydroxylethyl radical, a free radical metabolite of EtOH, was detected in the whole-liver homogenates, but not in vivo. It is important to note that oxidative stress imposed by these two oxidants only resulted in the detection of the free radical metabolites of these oxidants without detection of other free radicals. In contrast, Bray et al.[8] reported that dietary zinc deficiency causes an increased production of an endogenous carbon-centered free radical in lung microsomes. The cellular free radical defense systems, both cellular antioxidants and antioxidant enzymes, are primarily present in cytosol and in subcellular organelles.[10,11] The stimulating effect of zinc deficiency on the production of this carbon-centered free radical could have been attributed to the lack of free radical defense in the microsomes, which would possibly have been attenuated in the whole cell. The importance of the free radical defense systems to prevent oxidative damage is further demonstrated in the study in vivo, which only resulted in the detection of CCl_3· *(Table 2)*, and no effect of dietary zinc deficiency was observed. Thus, it seems that the free radical defense system in liver is essentially intact[16] and functionally adequate to protect dietary zinc-deficient rats from oxidative damage under an acute oxidative stress.

Fe is well known for its capacity to promote free radical chain reactions and induce free radical-mediated tissue damage.[10] In this study, generation of a free radical in whole-liver homogenate appeared to be Fe-dependent because exclusion of Fe from the reaction mixture resulted in no detection of the radical *(Figure 2a)*. Spin trapping agents, such as PBN, are capable of functioning as free radical scavengers via formation of the spin adduct. Consequently, the free radicals trapped are unavailable to initiate free radical-chain reactions. When PBN was initially omitted from the reaction mixture, the ESR signal was greatly enhanced *(Figure 2b)*. These results suggest that this Fe-dependent free radical may be a product of Fe-stimulated free radical reactions. However, more in-depth examination would be required to characterize the reactions and the chemical structure of this Fe-dependent free radical.

A peculiar finding concerning elevated plasma Zn concentrations during exposure to CCl_4 was observed in ZnPF and ZnDF rats *(Table 1)*. There was no effect on the plasma Zn concentration in CCl_4-treated ZnAL rats. This elevated plasma zinc concentration seems to result from reduced energy intake, rather than zinc deficiency per se. The mechanism by which CCl_4 induced an increase in plasma zinc concentration in CCl_4-treated ZnPF and ZnPF rats was not determined.

In summary, free radical metabolites (CCl_3· and 1-hydroxyethyl radical) and an Fe-dependent free radical (α_N = 14.9 G, α_β^H = 4.8 G) were generated in whole-liver homogenate. Oxidative stress in vivo resulted in the detection of CCl_3· radical in livers. Furthermore, dietary zinc deficiency neither resulted in a detection of free radicals nor increased production of free radical metabolites and Fe-dependent free radicals. It seems that dietary zinc-deficient rats are capable of preventing an increased free radical production in liver, both in vitro and in vivo, under acute oxidative stresses.

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