Effect of Zinc on Lipid Peroxidation in Liver Microsomes and Mitochondria[1] (36734)

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While studying the role of certain metals and chelating agents in controlling tissue injury, we found that zinc salts stabilize lysosomal membranes *in vitro* (1, 2): the rupture of these particles with the resulting release of their enzymes was decreased roughly 50% by low (millimolar) concentrations of zinc. The lysis of cells or subcellular structures is frequently attributed to peroxidation of the lipid components of their membranes (3–5). Thus, one possible explanation of this effect of zinc is that it interferes in some way with peroxidation of unsaturated fatty acids in the lysosomal membrane. This hypothesis was evaluated in *in vivo* and *in vitro* experiments using carbon tetrachloride to induce lipid peroxidation.

The toxic effects of CCl_4 *in vivo* are due to the metabolism of this agent by the liver microsomal drug oxidizing system to the trichloromethyl radical $\cdot CCl_3$ (6). This free radical attacks unsaturated lipids in intracellular membranes, oxidizing them and causing membrane distortion. This process terminates in cell necrosis (7). Lipid peroxidation can also be induced *in vitro* by incubating isolated liver microsomes with CCl_4 in the presence of NADPH or a NADPH-generating system (8). We have found that zinc prevents or significantly reduces the CCl_4-induced formation of lipid peroxides both *in vivo* and *in vitro*.

Methods. Male rats (Simonson, 200 ± 15 g) fed standard Purina lab chow diet were injected sc twice weekly with CCl_4 at the dose 0.1 ml/100 g. Zinc acetate (5 mg/100 g) was administered daily by intragastric gavage. Control rats were gavaged accordingly with saline. Rats were sacrificed by decapitation after 20 and 34 days. The livers were perfused *in situ* with 20 ml of ice cold saline and homogenized by hand with all-glass homogenizers in three volumes of Tris–KCl buffer (0.05 *M*, pH 7.4). The homogenates were fractionated into mitochondria and microsomes by centrifugation: material sedimenting between 800 and 15,000*g* (20 min—mitochondria—and between 15,000 and 105,000*g* (60 min)—microsomes—was collected and resuspended in Tris–KCl, 1.5 or 1.0 ml/g liver. The mitochondrial fraction thus obtained includes both mitochondria and lysosomes, although the latter are present in relatively minor amounts. However, for convenience we will refer to the fraction as a crude mitochondrial preparation. Lipid peroxides were measured in both fractions by the formation of malonaldehyde using the thiobarbituric acid (TBA) method (9). The activity of β-glucuronidase was determined (10) in aliquots of the mitochondrial pellet (bound) and supernatant (free enzyme) as a measure of lysosomal membrane stability. For *in vitro* studies, mitochondria and microsomes were prepared from rat liver and incubated under the conditions described in the Tables. The content of thiol groups was determined according to Elman (11), and the activity of cytochrome *c* oxidase by the method of Wharton and Tzagoloff (12).

Results. The *in vivo* administration of CCl_4 to rats for 20 days caused a 2- to 4-fold increase in the level of lipid peroxides in both the mitochondrial and microsomal fractions of the liver (Table I); these changes were even more pronounced after treatment for 34 days. The mitochondria showed a relatively much greater formation and/or accumulation of these products following CCl_4, even though in control rats the concentra-

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TABLE I. Effect of Zinc on Lipid Peroxides and β-Glucuronidase Distribution in Liver Injured by CCl_4.[a]

Group	Microsomes; lipid peroxides (μmoles/mg prot.)	Mitochondria and lysosomes	
		Lipid peroxides (μmoles/mg prot.)	β-Glucuronidase (free, % of total)
Control	50.2 ± 5.42	21.3 ± 0.81	20.1 ± 2.3
Control + Zn (34 days)	30.9 ± 5.82[c]	21.1 ± 3.1	—
CCl_4 (20 days)	111 ± 18.2[b]	76.3 ± 14.4[b]	—
CCl_4 + Zn (20 days)	—	40.0 ± 4.7[c]	—
CCl_4 (34 days)	197 ± 18.9[b]	204 ± 30.4[b]	43.0 ± 4.3[b]
CCl_4 + Zn (34 days)	126 ± 17.4[c]	127 ± 16.2[c]	30.4 ± 5.9

[a] The results are presented as mean ± SE for four rats in each group.
[b] Values significantly different from the controls at $p < .01$.
[c] Values significantly different after zinc treatment at $p < .05$.

tion of lipid peroxides was roughly twice as great in the microsomes as in the mitochondria.

Simultaneous treatment with zinc acetate caused a striking reduction in CCl_4-induced peroxidation. The concentration of malonaldehyde (TBA-reactive product) in both fractions was approximately half of that present in animals receiving CCl_4 alone (Table I). When control rats were given zinc for 34 days the lipid peroxide content of liver mitochrondria was unchanged while that of the microsomes was significantly lower than in the controls.

Changes in the distribution of the lysosomal enzyme β-glucuronidase paralleled the changes in the lipid peroxide content of the mitochondrial–lysosomal fraction. Carbon tetrachloride increased the proportion of free β-glucuronidase, while simultaneous treatment with zinc caused a considerable (although not statistically significant) decrease in the amount of free enzyme. Indeed, the amount of free β-glucuronidase showed a close and significant correlation to the lipid peroxide content of the liver ($r = 0.89$, $p > .01$). These results *in vivo* are reminiscent of the dramatic stabilization of lysosomes by zinc *in vitro* (1, 2).

This effect of zinc on lipid peroxidation was studied further using two different model systems: liver microsomes stimulated by CCl_4 *in vitro* and mitochondria exposed to high concentrations of oxygen. When microsomes were isolated from normal tissue and incubated at 37° in the presence of the cofactors and substances essential for activity of the drug oxidizing enzymes (NADPH, glucose-6-PO_4, Mg^{2+}, nicotinamide) there was a 50% increase in lipid peroxides (Table II). Carbon tetrachloride stimulated peroxidation 2-fold. Both the endogenous as well as the CCl_4-induced peroxidation of lipids were markedly inhibited by 0.01 to 0.1 m*M* zinc chloride. There was no apparent correlation between changes in lipid peroxides and thiol groups: incubation at 37° caused a slight decrease in the thiol content, but CCl_4 had no further effect. The highest concentration of zinc tested (1.0 m*M*) caused a slight but reproducible decrease in total thiol groups. Although lower concentrations of zinc (0.01 to 0.1 m*M*) were equally as effective in preventing CCl_4-induced peroxidation, they did not affect the sulfhydryl content.

Mitochondria prepared from the livers of normal rats contained significant amounts of endogenous lipid peroxides (Table III). Incubating these particles at 37° in the presence of O_2 caused a 100% increase in the peroxide content. In contrast to the results of the previous experiments, zinc (0.01 to 1.0 m*M*) did not decrease peroxidation of mitochondrial lipids, but instead enhanced this reaction in a manner that was concentration dependent. When the mitochondria were kept cold rather than incubated at 37°, zinc was without any effect. The increase in lipid peroxidation caused by 1 m*M* zinc parallels an inhibition in the activity of cytochrome *c*

TABLE II. Effect of Zinc Lipid Peroxidation and Thiol Groups Content in Liver Microsomes *in Vitro*.[a]

Sample	Lipid peroxides (nmoles/ml)	SH-groups (nequiv/ml)
Control, 0°	3.20	485
Control, 37°	4.69	425
Zn^{2+}, 1 m*M*	3.26	385
0.1 m*M*	3.60	410
0.01 m*M*	3.40	420
CCl_4, 5 μl/ml	6.80	420
Zn^{2+}, 1 m*M* + CCl_4, 5 μl/ml	5.21	380
0.1 m*M* + CCl_4, 5 μl/ml	5.51	403
0.01 m*M* + CCl_4, 5 μl/ml	5.45	420

[a] A microsomal fraction was prepared and resuspended 1:1 (w/v) in Tris–KCl buffer (0.05 *M*, pH 7.4). Samples containing 2 ml of suspension (18 mg protein/ml), 1 ml of the cofactors for the microsomal oxidizing enzymes (NADPH, 0.2 m*M*; glucose-6-phosphate, 2 m*M*; $MgSO_4$ 5 m*M*; and nicotinamide, 20 m*M*) plus 1 ml of zinc chloride in Tris–KCl buffer were incubated at 37° for 30 min. Samples containing both zinc and CCl_4 were pretreated with $ZnCl_2$ for 10 min at 20° before the addition of CCl_4.

oxidase. The level of thiol groups was decreased upon incubation at 37°, but was elevated by 1 m*M* zinc. Thus these two systems, *i.e.*, microsomes incubated with CCl_4 and mitochondria incubated with O_2, respond quite differently to zinc salts regarding the peroxidation of lipids and oxidation of thiol groups.

Discussion. We had previoulsy shown that zinc salts stabilize the membranes of isolated lysosomes at acid pH (1, 2). The present study extends this observation to show that administering zinc to intact rats reduces membrane damage caused by injecting CCl_4: the level of lipid peroxides in liver microsomes and mitochondria is significantly reduced, and the stability of lysosomes (judged by the distribution of β-glucuronidase) is increased. Furthermore, zinc decreases both endogenous as well as CCl_4-induced peroxidation in microsomes *in vitro*.

The failure of zinc to decrease lipid peroxidation in mitochondria *in vitro* was rather unexpected but may be explained in several ways. The system used (incubating mitochondria in the presence of oxygen) is not directly analogous to that used in the experiments described in Tables I and II. However, the fact that CCl_4 must be metabolized by microsomes to the actual toxic product precludes using this agent to induce membrane damage in isolated mitochondria. Molecular oxygen is known to oxidize membrane lipids (13); thus this sytem could provide a sensitive alternative to $\cdot CCl_3$ damage. The fact that zinc increased rather than decreased O_2-induced peroxidation suggests to us that zinc may protect mitochondria *in vivo* by an indirect method. Zinc might interfere with initiation or propagation of $\cdot CCl_3$-induced peroxidation in the microsomes, with the metabolism of CCl_4 to $\cdot CCl_3$, or with a secondary generation of peroxidation in mitochondria or other cellular components. In this connection it is interesting to note that 0.1 m*M* zinc inhibits reduction of dichlorophenolindophenol by liver microsomes *in vitro*, indicating that this metal may interfere with the normal flow of electrons (unpublished results).

Alternatively, the increased peroxidation caused by 1 m*M* zinc may reflect either a specific toxic effect on mitochondria, or may be due to too great a concentration of zinc. Low concentrations of zinc (10^{-6} *M*) inhibit respiratory activity (14) and induce rapid swelling (15) in mitochondria, suggesting a specific effect on functional activity rather than on structural integrity. It is possible that extremely low concentrations of zinc should be used in testing for increased stability of mitochondrial membranes or decreased

TABLE III. Effect of Zinc on Some Functions of Liver Mitochondria.[a]

Sample	Lipid peroxides (μmoles/ml)	SH-groups (nequiv/ml)	Cytochrome *c* oxidase (K/min/mg)
Control, 0°, no O_2	1.47	336	—
Control, 0°, O_2	1.69	342	—
+ 0.1 m*M* Zn^{2+}	1.70	350	—
Control, 37°, O_2	3.79	226	6.45
+ 1 m*M* Zn^{2+}	8.74	320	4.09
+ 0.1 m*M* Zn^{2+}	4.64	240	4.74
+ 0.01 m*M* Zn^{2+}	4.06	230	—

[a] The mitochondrial fraction was suspended 1:1.5 in 0.9% NaCl–0.01 *M* sodium acetate, pH 5.0, or for the determination of cytochrome *c* oxidase, in Tris–KCl, pH 7.4. Samples were treated with $ZnCl_2$, bubbled with oxygen (95% O_2, 5% CO_2) for 5 sec, adjusted to 4 ml and incubated at 37° for 30 min.

lipid peroxidation. We have observed a bimodal response to zinc *in vivo*: low doses protect rats but higher doses enhance liver injury and lipid peroxidation (16). Thus in this case micromolar rather than millimolar levels may prevent peroxidation.

Although zinc reduces peroxidation of microsomal lipids, it does not prevent oxidation of protein thiol groups. We are unable to explain the fact that 1 m*M* zinc decreases the number of detectable thiol groups in microsomes but increases them in mitochondria.

We conclude that one of various possible mechanisms by which zinc stabilizes a variety of biomembranes (excluding mitochondrial) both *in vivo* and *in vitro* is related to the inhibition of peroxidation of membrane lipids.

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