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INHIBITION OF NADPH OXIDATION AND RELATED DRUG OXIDATION IN LIVER MICROSOMES BY ZINC

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Abstract—Rat liver microsomes were incubated in the presence of zinc and the rate of NADPH oxidation and related metabolism of aniline and ethylmorphine by appropriate oxidases were studied. A competitive mechanism of the inhibition of NADPH oxidation by zinc was found, with $V_{max} = 10.3$ nmoles NADP/min/mg of protein and K_i amounting to 7.22 μM zinc. In microsomes dialyzed against EDTA, addition of Mn^{2+} but not of Mg^{2+} enhanced the rate of NADPH oxidation. A complex relation of Zn^{2+} and Mn^{2+} in liver microsomes was found, the data not obeying the rigorous treatment for enzyme kinetics. The activity of aniline hydroxylase and ethylmorphine-*N*-demethylase was inhibited by zinc; 50 per cent inhibition was reached at 60 and 55 μM Zn^{2+} respectively. Another microsomal enzyme, glucose 6-phosphatase, independent of NADPH, was not affected by zinc. The content and spectral characteristics of cytochrome P-450 were not affected by zinc. It is concluded that Zn^{2+} inhibits oxidation of NADPH and prevents this pyridine nucleotide from functioning in the microsomal electron transport system. The possibility that Zn^{2+} may interfere with other ions or enzymes involved in microsomal electron transport cannot be excluded.

In a recent study, we showed that the administration of Zn^{2+} to rats protected the liver from CCl_4-induced hepatotoxicity [1]. Our data indicated a decrease in the peroxidation of unsaturated fatty acids, since the rate of malondialdehyde formation was significantly slower in liver microsomes from Zn^{2+}-treated animals. It is generally assumed that CCl_4 initiates lipid peroxidation after it is converted to a trichloromethyl free radical ($\cdot CCl_3$) by microsomal drug-metabolizing enzymes and that $\cdot CCl_3$ is the actual hepatotoxic species [2, 3]. Two possible mechanisms could explain these findings: (1) that Zn^{2+} was preventing the microsomal conversion of CCl_4 to $\cdot CCl_3$ and/or (2) that Zn^{2+} was interacting with the polyunsaturated fatty acids of the biomembranes, thus rendering them resistant to peroxidative deterioration.

In this paper we explore the first hypothesis, namely, that zinc interferes with mixed-function oxidases residing in smooth endoplasmic reticulum of liver microsomes. It has been well established that the mixed-function oxidation consumes equal amounts of NADPH, oxygen and drug as a substrate. Thus, the initial step in liver microsomal electron transport involves transfer of electrons from NADPH to reduce the oxidized heme P-450-substrate complex by way of NADPH-cytochrome-*c* reductase. The reduced cytochrome P-450 substrate complex then reacts with molecular oxygen to form the oxygenated complex [4]. The oxidation of NADPH in the initiation of microsomal electron transport resulting in oxidation of a drug was assumed to be the likely target for zinc effect. The preliminary report indicating such an effect has already been published [5].

The microsomes will also catalyze an NADPH-dependent peroxidation of endogenous lipid [6]. This reaction involves the transient formation of lipid peroxides, leading to deterioration of polyunsaturated fatty acids and producing a variety of degradation products, including malondialdehyde. Assuming that zinc inhibits microsomal NADPH oxidation [5], we suggest that by this mechanism both oxidation of drugs and NADPH-oxidation dependent lipid peroxidation in the endoplasmic reticulum should be inhibited. The experimental data supporting the view that zinc ions inhibit enzymatic lipid peroxidation were published elsewhere [7].

METHODS

All experiments reported in this study were performed with microsomes isolated from adult Sprague–Dawley rats of both sexes. After exsanguination of the animal, the liver was thoroughly perfused with at least 20 ml of ice-cold saline through either the protal vein or the inferior vena cava.

Liver was homogenized in phosphate buffer (0.01 M), pH 7.0) or Tris–KCl buffer (0.05 M Tris HCl and 0.153 M KCl buffer, pH 7.4) and is described for each study. Tris–KCl buffer had a tendency to decrease enzyme activity as compared to phosphate buffer but phosphate buffer precipitated Zn^{2+} at higher levels so that the buffer was chosen according to the parameter being studied. Homogenization was performed in an all-glass homogenizer and microsomes were isolated from the supernatant of 15,000 *g* centrifuged for 20 min as described in our previous paper [8].

In experiments with dialyzed microsomes, an aliquot of microsomal fraction homogenized in 0.01 M phosphate buffer, pH 7.0, was dialyzed for 2 hr at 4° against 5 mM EDTA, 1 mM 1,10-phenanthroline,

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pH 7.0, and then against 0.01 M phosphate buffer for 24 hr. The pH of the microsomal fraction after dialysis was adjusted to pH 7.0.

NADPH oxidation. The rate of NADPH oxidation was recorded at 340 nm at 32° on a Beckman Acta III spectrophotometer in quartz cuvettes. The reaction vessel contained 141.7 mM sucrose and 96 μM NADPH (Sigma) in either Tris–KCl or phosphate buffer. Enzyme activity was initiated by adding 1–6 mg of microsomal protein in a final volume of 3 ml. $MnCl_2$, $MgCl_2$ or $ZnCl_2$ was added as indicated in Results. The initial velocity of the oxidation was obtained in min 1 of the reaction, since the rate tended to decrease with time.

Microsomal drug oxidases. The microsomal fraction for the determination of microsomal drug-metabolizing activity and glucose 6-phosphatase activity was prepared as follows: livers were perfused *in vitro* as described, homogenized in 1:3 (w/v) of Tris–KCl buffer (50 mM Tris·HCl–154 mM KCl, pH 7.4), and the homogenate was centrifuged at 15,000 *g* for 20 min. The resulting supernatant was then centrifuged at 105,000 *g* for 60 min to obtain the microsomal pellet. This pellet was gently re-homogenized in 1.5 vol of the same buffer. All the above operations were carried out at 2°. The protein content of the microsomal suspension was determined by the method of Lowry *et al.* [9].

The microsomal metabolism of ethylmorphine and aniline was determined in 3.0-ml mixture consisting of: 5 mM $MgCl_2$, 12 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase (Sigma), 0.33 mM NADP, 50 mM Tris–KCl buffer (pH 7.4), 6 mg of microsomal protein and 10 mM ethylmorphine or 1 mM aniline. The mixture was incubated at 37° for 10 min with shaking, after which the reactions were terminated with 1 ml of 15% $ZnSO_4$ (ethylmorphine) or with 0.8 g NaCl and 25 ml ether (aniline). The degree of *N*-demethylation was estimated by measuring the amount of formaldehyde formed according to the method of Nash [10]. Aniline hydroxylase was assayed according to Smuckler *et al.* [11].

In order to insure that Zn^{2+} was not selectively precipitating the microsomal enzymes, the activity of microsomal glucose 6-phosphatase was determined by incubating 8 μmoles glucose 6-phosphate with 1 mg of microsomal protein in 0.2 ml of 50 ml Tris–KCl buffer (pH 7.4). The procedure employed was essentially that described by Harper [12] with a microsomal suspension being substituted for filtered liver homogenate. The phosphate content was determined by the colorimetric method of Fiske and Subbarow [13] utilizing the Fisher Gram-Pac (A0974).

Cytochrome P-450. Cytochrome P-450 was determined spectrophotometrically [11] and calculated using the extinct coefficient of 91 mM^{-1} cm^{-1} [14].

Metal analysis. This analysis was done on an atomic absorption spectrophotometer, Perkin Elmer model 305, after digesting the liver sample initially with concentrated nitric acid followed by digestion in equal parts of nitric acid and 30% hydrogen peroxide. The digest was evaporated to dryness, the residue was diluted in deionized distilled water, and appropriate aliquots were taken for the analysis of zinc, iron, manganese and magnesium.

RESULTS

A linear relation was found between the rate of formation of NADP and the protein content of liver microsomes within the concentration of 1–6 mg proteins in 3 ml medium. Further work reported was carried on within this concentration range of microsomal fraction.

Effect of zinc on NADPH oxidation. This effect was studied in a microsomal fraction of the liver at various concentrations of zinc and at 7.2, 14.4 and 28.8 mM NADPH added to the microsomes, suspended in 0.04 M phosphate buffer. The rate of NADP formation in the system was related to the amount of NADPH added. At any concentration of NADPH its oxidation was inhibited by zinc in a concentration-related manner. The K_s value of this reaction was 6.45 mM NADPH. These data presented in Lineweaver–Burk double-reciprocal plots (Fig. 1) show a competitive mechanism for Zn^{2+} inhibition. With a $1/V$ intercept of 0.097, the V_{max} for the reaction was 10.3 nmoles NADP/min/mg of proteins. The K_i was calculated to be 7.22 μM Zn^{2+}.

There are several possible mechanisms by which Zn^{2+} could inhibit NADPH oxidation. Direct interaction with NADPH may render this pyridine nucleotide more resistant to oxidation. Another possibility is the interference of Zn^{2+} with certain metals involved in the microsomal electron transport system. Finally, the inhibition of NADPH oxidation by Zn^{2+} may reflect the interference of this metal with enzymes at any step of the microsomal electron transport chain. In the next section, the results referring to the last two mentioned hypotheses will be presented. The first hypothesis is under investigation.

Interaction of Zn^{2+} with other metals. We tested the possibility that zinc displaces some metals involved in the microsomal electron transport system. It was shown that the granular fraction of polymorphonuclear leucocytes [15, 16] and macrophages [17, 18] contains an NADPH oxidase which is

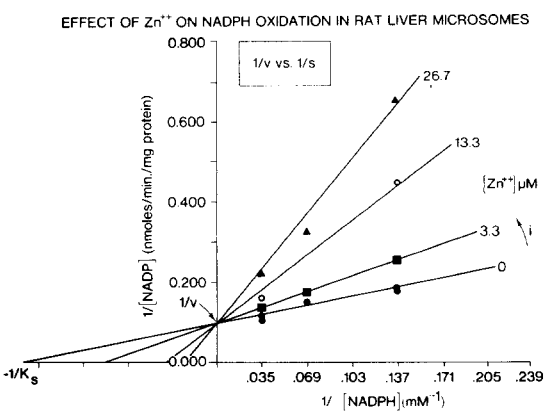

Fig. 1. Lineweaver–Burk curve of 1/substrate vs 1/velocity based on the initial rate of enzyme activity at four different Zn^{2+} concentrations. NADPH oxidation was measured at 340 nm in a cuvette containing 22.5 mM KH_2PO_4, 13.3 mM Na_2HPO_4, 141.7 mM sucrose, and the amounts of NADPH and Zn^{2+} given in the figure; 2.0 mg of microsomal proteins was added to initiate the reaction at 32°. The $1/V_{max}$ value is 0.097 (nmole NADPH/min/mg of protein)$^{-1}$. The $1/K_s$ value is 0.155 (mM NADPH)$^{-1}$.

Table 1. Content of some metals in intact and $CaNa_2EDTA$ dialyzed rat liver microsomes*

Sample	Me^{2+} (μg/g protein)			
	Fe	Zn	Mn	Mg
Microsomes				
Nondialyzed	878	95	3.2	452
Dialyzed	800	63	1.6	167
% Dialyzed out	9	30	50	63

* Liver microsomes (250 mg protein) were dialyzed for 2 hr against 5 mM EDTA and 1 mM phenanthroline, pH 7.0, at 4° and then 0.01 M PO_4 buffer, pH 7.0, for 24 hr at 4°. Metal content was determined by atomic absorption. Data are presented/g protein of microsomal fraction.

strongly activated by Mn^{2+} ions [16, 18]. In a recent paper, it was reported that Zn^{2+} inhibited NADPH oxidase from pulmonary alveolar macrophages in a competitive manner [18]. In liver microsomes, it is the activity of cytochrome *c* reductase which functions as NADPH oxidase.

The studies on the role of Mn^{2+} in microsomal NADPH oxidation were done with a microsomal fraction dialyzed for 2 hr against 1 mM 1,10-phenanthroline and 5 mM EDTA at pH 7.0 and then for another 24 hr at 4° against 0.01 mM phosphate buffer, pH 7.0. The change in the content of zinc, iron, magnesium and manganese in microsomal fraction due to dialysis is shown in Table 1. Only a minimal amount of iron (less than 9 per cent of total iron) was removed by dialysis. After the second dialysis the content of Mn^{2+} decreased from the original 3.2 to 1.6 μg/g of microsomal protein. Thus, only 50 per cent of manganese was removed by dialysis. Further addition of Mn^{2+} up to 0.43 mM final concentration resulted in a linear increase of the rate of NADPH oxidation (Table 2). No effect of added Mg^{2+} within the same concentration range was observed. Surprisingly, addition of small amounts of Zn^{2+} (up to 5 μM final concentration) to the EDTA-treated and extensively dialyzed microsomal fraction stimulated the oxidation of NADPH (Fig. 2). Within a range of concentrations of 5–30 μM Zn^{2+}, an inhibition of NADPH oxidation was found in two independent experiments. The pertinent data from our experiments are shown in Fig. 2.

Table 2. Rate of oxidation of NADPH in EDTA-treated and dialyzed liver microsomes in the presence of various divalent cations*

Mn^{2+} or Mg^{2+} concn (mM)	Zn^{2+} concn (μm)	NADP (nmoles/min/mg protein)		
		Mn^{2+}	Mg^{2+}	Zn^{2+}
0.0	0	29	29	19.7
0.10	3.33	50	29	37.5
0.33	10.00	157	30	24.0
0.43	13.33	169	29	20.5

* Liver microsomes were dialyzed as is described in Methods. NADPH oxidation was initiated by 2.0 mg of microsomal proteins being added to a cuvette containing 22.5 mM KH_2PO_4, 13.3 mM Na_2HPO_4, 141.7 mM sucrose, 96 μM NADPH and the above metals at 32°. The pH value of the incubation medium was 7.4. Indicated metal concentration refers to exogenous amount of single metal added to the microsomal sample.

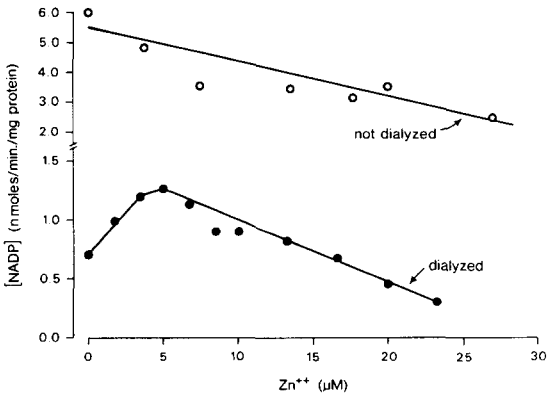

Fig. 2. Effect of Zn^{2+} additions on dialyzed and nondialyzed liver microsomal fractions in phosphate buffer. NADPH oxidation was initiated by 1.5 mg protein of microsomal preparation being added to a cuvette containing 22.5 mM KH_2PO_4, 13.3 mM Na_2HPO_4, 141.7 mM sucrose, 96 μM NADPH and the given concentrations of Zn^{2+}. The nondialyzed liver microsomes contained 3.5 μg Zn^{2+}/g protein and the dialyzed sample contained 2.5 μg Zn^{2+}/g protein as determined by atomic absorption.

The differing effect of increasing concentrations of zinc ions on NADPH oxidation in nondialyzed and dialyzed microsomes (Fig. 2) was suggestive of possible interaction between Zn^{2+} and Mn^{2+}. Further analysis of this effect indicated rather complex relations between both metals. Table 3 represents the results of one typical experiment, which has been reproduced in three similar experiments. The data show that increasing Zn^{2+} concentration in a dialyzed sample from 3.3 to 13.3 μM without exogenous Mn^{2+} resulted in inhibition of NADPH oxidation. When 0.1 mM Mn^{2+} was added, the effect was just the opposite; nevertheless, at 0.3 mM Mn^{2+}, the oxidation of NADPH was again inhibited with increasing Zn^{2+}. The analysis of the kinetic data suggests

Table 3. Rate of oxidation of NADPH in dialyzed microsomes at various concentrations of Zn^{2+} or Mn^{2+}*

Zn^{2+} (μM)	NADP (nmoles/min/mg protein)			
	Mn^{2+} (mM)			
	0	0.1	0.2	0.3
0.0	19.7	20.8	35.1	26.3
3.33	37.5	17.5	34.0	59.2
10.00	24.0	28.5	35.1	24.1
13.33	20.5	42.8	25.6	15.4

* Microsomal fraction was dialyzed as described in Methods. NADPH oxidation was measured in a cuvette containing 22.5 mM KH_2PO_4, 13.3 mM Na_2HPO_4, 141.7 mM sucrose, 96 μM NADPH, the above amounts of metals and 1–6 mg of microsomal proteins, initiating the reaction at 32°. The pH value of the medium was 7.4. The indicated concentrations of individual metals refer to amounts added to the sample in excess of their content left after dialysis. The oxidation rate was based on min 1 of the reaction.

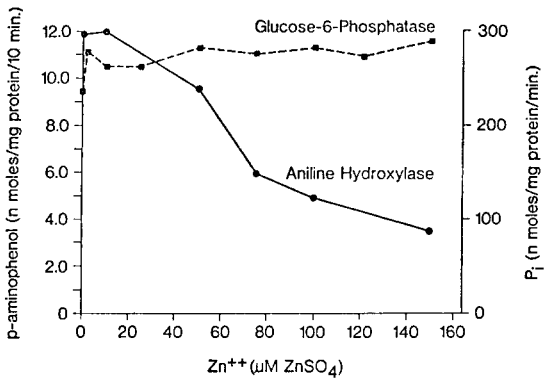

Fig. 3. Effect of Zn^{2+} on the activity of aniline hydroxylase and glucose 6-phosphatase in liver microsomes. Aniline (1 mM) was incubated for 10 min in a 3-ml incubation medium (pH 7.4) consisting of 5 mM $MgCl_2$, 12 mM glucose 6-phosphate, 1 unit glucose 6-phosphate dehydrogenase, 0.33 mM NADP, 6 mg of microsomal protein, 50 mM Tris and 154 mM KCl. Glucose 6-phosphatase activity was determined by incubating glucose 6-phosphate with microsomes suspended in Tris–KCl and the Pi determined by the colorimetric procedure of Fiske and Subbarow [13]. Each point represents the mean of two incubations.

that the mechanism involving various metal ions in NADPH oxidation is too complex for rigorous treatment at the present time.

Effect of zinc on some enzymes of microsomal electron transport. To test the hypothesis that zinc interacts directly with some enzyme components of liver microsomal electron transport, we studied the effect of this metal cation on cytochrome P-450. Supplementation of Zn^{2+} at two different concentrations (1 and 100 μM) to isolated microsomal fraction incubated under slight shaking in Tris–KCl buffer for 10 or 40 min at 25° did not result in any change of spectral characteristics of cytochrome P-450. The characteristic maximum at 450 nm as well as the shoulder at 420 nm was identical in all samples analyzed.

Effect of zinc on the activity of some drug oxidases in liver microsomes. Inhibition of NADPH oxidation in liver microsomes by zinc, as documented above, should result in inhibition of drug oxidation. To experimentally verify this statement, we studied the effect *in vitro* of Zn^{2+} in a final concentration of 5–150 μM on the NADPH-dependent microsomal oxidation of aniline and *N*-demethylation of ethylmorphine. The data in Fig. 3 indicate the inhibition of aniline hydroxylase activity by zinc, 50 per cent inhibition being obtained at 60–70 μM zinc concentrations. These data are similar to the previously published data for ethylmorphine *N*-demethylation [5]. Under similar experimental conditions, the activity of glucose 6-phosphatase, an enzyme independent of NADPH oxidation, was not affected by the presence of Zn^{2+} (Fig. 3).

Figure 4 presents data on the effect of zinc on the activity of aniline hydroxylase as well as ethylmorphine *N*-demethylase by plotting the reciprocal rates for velocity of product formation as a function of zinc concentration. The slopes of linearly transformed data clearly indicate the inhibitory effect of zinc on both enzymes dependent on NADPH.

To ascertain if various anions of zinc compounds play any role in the inhibitory effect of Zn^{2+}, we studied the effect of four zinc salts at 0.1 mM concentration on the activity of ethylmorphine *N*-demethylase in liver microsomes. Our results indicate that zinc gluconate was the least inhibitory (50 per cent inhibition) and zinc acetate the most inhibitory (63 per cent) when initial rates of product formation were followed. Zinc chloride and zinc sulfate inhibited by 50 and 56 per cent respectively.

DISCUSSION

The aim of this study was to explain the possible mechanism(s) by which zinc administered *in vivo* protects the liver against CCl_4 hepatotoxicity [1, 19]. The results of this study *in vitro* indicate that zinc inhibits the oxidation of NADPH in liver microsomes by a competitive mechanism. A logical consequence of blocking NADPH oxidation in the presence of Zn^{2+} would be inhibition of all microsomal reactions dependent on the transfer of electrons from NADPH. The activity of two NADPH-dependent microsomal enzymes, i.e. aniline hydroxylase and ethylmorphine *N*-demethylase, was inhibited by the addition of Zn^{2+} to the incubation medium. Another enzyme, bound also to endoplasmic reticulum, but independent of NADPH oxidation, i.e. glucose 6-phosphatase, was not affected by zinc ions. This finding indicates that the described effect of Zn^{2+} on NADPH oxidation or the metabolism of two drugs was not related to eventual microprecipitation of microsomal proteins

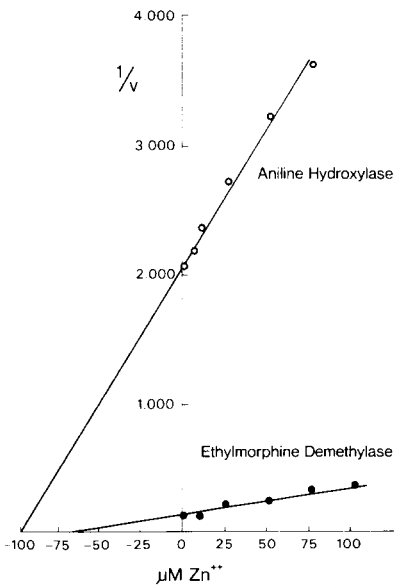

Fig. 4. Reciprocal plot of specific activity of aniline hydroxylase and ethylmorphine *N*-demethylase as a function of Zn^{2+} concentration. Aniline (1 mM) or ethylmorphine (10 mM) was incubated for 10 min in 3 ml medium (pH 7.4) consisting of 5 mM $MgCl_2$, 12 mM glucose 6-phosphate dehydrogenase, 0.33 mM NADP, 6 mg of microsomal protein, 50 mM Tris and 154 mM KCl. The rate of reaction was determined by measuring the product of each reaction: HCHO for ethylmorphine *N*-demethylase and *p*-aminophenol for aniline hydroxylase. Each point is the mean of two incubations.

by Zn^{2+}, but to specific interaction of the metal with some components of the drug-oxidizing system. Thus, the decreased oxidation of some drugs metabolized to a hepatotoxic product might explain the protective effect of zinc on carbon tetrachloride-induced liver damage [1, 19].

Zinc is obviously not the only metal inhibiting microsomal electron flow. Mn^{2+} and Co^{2+} were shown to inhibit peroxidation of phospholipids in liver microsomes [20] possibly by competing with Fe^{3+} for binding sites on the microsomes [21]. It has to be stressed, however, that contrary to the Zn^{2+} effect, both Mn^{2+} and Co^{2+} were effective at mM concentrations, while Zn^{2+} was inhibitory at μM concentrations. While this study suggests that increase in zinc content in liver microsomes may reduce hepatic drug metabolism, a similar effect was reported by an opposite situation, i.e. by zinc deficiency [22]. Furthermore, despite the complexity of Zn^{2+} and Mn^{2+} interaction in the tested system, the data do not suggest an additive or synergistic effect.

It would be premature to speculate on the possible biological implications of this finding. A direct proof should be presented first that such an inhibition of drug metabolism and eventually lipid peroxidative deterioration of polyunsaturated fatty acids by zinc occur also after administration *in vivo* of this metal. The already mentioned protection of CCl_4 hepatotoxicity by zinc in rats supports such an assumption [1, 19].

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