Effects of dietary zinc deficiency on the expression of hepatic microsomal cytochrome P450 2E1 in rats

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The objective of this study was to investigate if the altered 2E1 drug metabolism capacity in rats is due to effects of dietary zinc deficiency on the expression of hepatic microsomal P450 2E1 in rats. 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. At the end of the feeding trial, each dietary group was further divided into the drug control and ethanol treatment group. Ethanol (EtOH, 8 mL/kg body weight, intubation) was given once a day for 3 consecutive days. Within the drug control group, zinc deficiency reduced total P450 concentration but had no effect on the level of NADPH-P450 reductase, 2E1 protein, and 2E1 mRNA. 2E1 activity, which was indicated by N-nitrosodimethylamine demethylase (NDMA), aniline hydroxylase (AH), and p-nitrophenol hydroxylase (NH) activity, was also not affected by dietary zinc deficiency. The NDMA demethylase activity and 2E1 mRNA level were higher in zinc pair-fed rats than in zinc-adequate rats. EtOH treatment induced total P450 concentration in zinc-adequate and zinc-deficient rats and NADPH-P450 reductase level in zinc-adequate rats. 2E1 activity was induced by EtOH, while the level of 2E1 protein and 2E1 mRNA was unchanged. In conclusion, the constitutive expression of hepatic microsomal 2E1 was not affected by zinc deficiency per se; however, the prolonged severe depression of feed intake caused by zinc deficiency increased the level of 2E1 mRNA in rats. An acute EtOH treatment markedly induced 2E1 activity without affecting the level of 2E1 protein and mRNA. (J. Nutr. Biochem. 5:308–313, 1994.)

Keywords: zinc deficiency; 2E1 mRNA; 2E1 protein; 2E1 activity; ethanol

Introduction

Zinc is an essential trace element for all species studied to date including humans. It has been previously shown that dietary zinc deficiency in rats apparently results in a reduced metabolism of CCl_4 measured by in vivo electron spin resonance (ESR) spin-trapping techniques.[1] In contrast, the metabolism of methylbenzylnitrosamine is elevated in esophageal and hepatic microsomes prepared from zinc-deficient rats.[2] Both CCl_4 and methylbenzylnitrosamine are mainly metabolized by microsomal cytochrome P450 2E1 (2E1) in rats.[3,4] Thus, it appears that the drug metabolism capacity of microsomal 2E1 is altered in zinc-deficient animals. However, the biochemical basis for the altered 2E1 function in rats is unclear.

The microsomal cytochrome P450 (P450) enzyme system plays a critical role in metabolism and detoxification of numerous compounds in animals including humans.[5] P450 is a gene superfamily consisting of more than 100 isoforms.[6] P450 is characterized by the broad and overlapping substrate specificity and inducibility of various isoforms. 2E1 is one of the main constitutively expressed P450 isoforms.[7] The tissue concentration of 2E1 can be induced in animals by chemicals such as ethanol[8] or by nutritional related conditions such as fasting[8] and diabetes.[9]

The objective of this study was to investigate whether the altered 2E1 drug metabolism capacity in rats is due to effects of dietary zinc deficiency on the expression of hepatic microsomal 2E1. In this study, the expression of 2E1 was assessed by measuring the constitutively expressed 2E1 activity, protein, and mRNA. 2E1 activity was assessed by its characteristic reactions: N-nitrosodimethylamine (NDMA) demethylase, aniline hydroxylase (AH), and p-nitrophenol

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hydroxylase (NH) activity. To further assess the effects of zinc deficiency on the inductive expression of 2E1, the expression of 2E1 in ethanol (EtOH)-treated rats was also determined.

Methods and materials

Chemicals

Diethylpyrocarbonate (DEPC), N-lauroylsarcosine (Sarkosyl), prestained sodium dodecyl sulfate (SDS) molecular weight markers (27,000 to 180,000 Kd) and Sigacolte were obtained from Sigma (St. Louis, MO USA). Guanidinium thiocyanate was obtained from Fisher (Fair Lawn, NJ USA). Polyvinylidene difluoride (PVDF) membranes, Zeta-probe GT blotting membranes, and alkaline phosphatase conjugate goat anti-rabbit IgG were purchased from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ontario, Canada). Rabbit anti-rat cytochrome 2E1 Western Blot Kit containing 2E1 standard and polyclonal rabbit anti-rat 2E1 IgG was from Oxygen Dallas (Dallas, TX USA).

Animals and diets

Three-week-old, male Wistar rats with an average body weight of 59 g (Charles River, St. Constant, Quebec, Canada) were randomly assigned to the zinc adequate control (ZnAL) or to the zinc deficient (ZnDF) group. The rats in these two groups had free access to their assigned diet. Voluntary feed refusal is a characteristic zinc deficiency sign, and it is therefore necessary to include a secondary control, the pair-fed control group (ZnPF). The rats in this group were individually paired to ZnDF rats and fed the zinc adequate diet at the amount that ZnDF rats consumed the day before. The diet composition[10] and procedures of animal care[11] were the same as previously reported.

After 3 weeks of consuming these diets, each dietary group was further divided into two drug treatment groups, the drug control and the EtOH treatment group (EtOH, 8 mL/kg body weight, intubation). EtOH was given once a day for 3 consecutive days. At the end of the drug treatment period, livers were removed for biochemical analyses.

SDS-polyacrylamide gel electrophoresis

Liver microsomes were prepared by differential centrifugation in a Tris-HCl buffer.[11] Both liver microsomes and 2E1 standard were diluted in Laemmli buffer system[12] and placed in a boiling water bath for 2 min. Microsomal samples were loaded at 100 μg microsomal protein/well and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for both stacking gel (4% acrylamide) and separating gel (12% acrylamide, 1.5 mm). The electrode buffer was as previously reported.[13]

Western blot analysis

After microsomal proteins were resolved by SDS-PAGE, the proteins were then analyzed by Western blotting based on the procedures described by Towbin et al.[14] with modifications. Microsomal proteins were electrophoretically transferred to PVDF membranes at 10° C for 1 hr at 100 V in a buffer mixture containing 20% methanol, 35 mmol/L Tris, 192 mmol/L glycine, and 0.01% SDS (pH 8.3). The completion of transfer was confirmed by Coomassie blue R250 (Bio-Rad) staining of the polyacrylamide gels after transfer. The membranes were subsequently incubated with 10% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.05% sodium azide for 2 hrs. The membranes were then incubated with polyclonal rabbit anti-rat 2E1 IgG (1:100 dilution) for 3 hrs, washed with PBS three times for a total of 20 min, and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3,000 dilution) for 2 hrs. After washing three times with PBS, the alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate as substrate as described by the manufacturer. All blocking, washing, and detecting procedures were carried at room temperature. The intensity of the band was quantified with a Shimadzu scanning densitometer (CS9000U, Shimadzu Scientific Instruments, Inc., Columbia, MD USA).

Purification of total RNA

Livers (~1 g liver/rat and three rats/treatment) were removed immediately after euthanasia and placed in liquid nitrogen. They were then pulverized in a mortar and homogenized with a glass-glass tissue grinder in 10 mL denaturing solution containing guanidinium thiocyanate as described by Chomczynski and Sacchi.[15] The RNA pellets were precipitated with isopropanol at −20° C and resuspended with LiCl (4 mmol/L) to wash out glycogen present in livers. After purification, the total RNA was stored in DEPC-treated water under 2 volumes of 95% ethanol containing 2% sodium acetate at −80° C until used for Northern blot analysis.

Northern blot analysis

Total RNA (~ 40 μg/sample) was dissolved in DEPC-treated water, heated at 55° C, and fractionated by electrophoresis in a 1.2% denaturing formaldehyde-agarose gel according to the procedures described by Ausubel et al.[16] After capillary transfer to Zeta-probe GT membrane overnight at 4° C, RNAs were cross-linked to the membranes with UV Stratalinker 2400 (Stratagene, La Jolla, CA USA) following a 1 hr bake at 80° C. The membranes were prehybridized with 0.25 mmol/L sodium phosphate, pH 7.2, 7% SDS at 65° C for 4 hrs. The 2E1 cDNA probe (provided by F.J. Gonzalez, Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD USA) was labeled with α^{32}P-dCTP (~ 3,000 mCi/mmol) by nick-translation and hybridized with the membranes at 65° C for 20 hrs. Following hybridization, the membranes were washed at 65° C with 20 mmol/L sodium phosphate, pH 7.2, 5% SDS twice for 1 hr and then with 20 mmol/L sodium phosphate, pH 7.2, 1% SDS twice for another hour. The membranes were then exposed to Kodak X-Omat XAR (Kodak, Rochester, NY) film with intensifying screens for 14 hrs at −80° C. The level of 2E1 mRNA was quantitated by scanning the intensity of bands on the x-ray film with a Shimadzu scanning densitometer. The amount of total RNA per lane was confirmed by rehybridizing the blots with ^{32}P-labeled yeast cDNA probe pTZR19U for rRNA (L. Goode, Department of Molecular Biology, University of Guelph, Guelph, Ontario, Canada).

Enzyme assays

NADPH-cytochrome P450 reductase (NADPH-P450 reductase) level was quantitated by determining the absorption at 550 nm with cytochrome c as electron acceptor.[17] Aniline hydroxylase activity was determined by quantitation of p-aminophenol formed.[18] N-nitrosodimethylamine demethylase activity[19] and p-nitrophenol hydroxylase[20] activity were determined as previously described.

Other assays

The plasma zinc concentration was determined by flame atomic absorption spectroscopy as previously described.[11] Total P450 concentration was determined by difference spectrophotometry with some modifications.[11] Microsomal protein concentration was quantitated by Lowry's method.[21]

Statistical analysis

The means between dietary treatments or drug treatments were subjected to analysis of variance followed by least significant difference test ($P < 0.05$) according to preplanned comparisons.[22]

Results

The development of severe zinc deficiency in ZnDF rats resulted in severe growth depression and significantly lower plasma zinc concentration as compared with ZnAL and ZnPF rats, regardless of drug treatment *(Table 1)*. The liver weight and liver to body weight ratio were not significantly affected by zinc deficiency. When compared with the drug control group, EtOH treatment increased the final body weight in ZnPF rats. This increase in the final body weight in ZnPF rats is possibly due to the EtOH that was administered providing an additional energy source to these rats. In contrast, EtOH-treated ZnAL rats had a lower final body weight, and the final body weight of ZnDF rats was not affected by EtOH treatment *(Table 1)*. The lack of the negative effect of EtOH treatment on the final body weight in ZnDF rats is probably attributed to the acute EtOH treatment. EtOH treatment resulted in a higher liver weight in ZnPF rats. EtOH-treated ZnDF rats had a lower liver weight and liver to body weight ratio than the ZnDF rats in the drug control group. Plasma zinc concentration in both ZnAL and ZnPF rats were significantly lowered by EtOH treatment when compared with their controls *(Table 1)*.

Total liver microsomal protein concentrations were largely unaffected by either dietary treatments or drug treatments, except that the concentrations were lower in EtOH-treated ZnAL rats *(Figure 1A)*. Within the drug control group, both feed restriction and dietary zinc deficiency caused a decrease in total P450 concentration *(Figure 1B)*. EtOH-treated ZnAL and ZnDF rats, but not ZnPF rats, showed a 32% and 46% increase in total P450 concentration, respectively *(Figure 1B)*. The microsomal NADPH-P450 reductase level did not differ between ZnPF and ZnDF rats, indicating no effect of zinc deficiency on the NADPH-P450 reductase in rats. EtOH treatment increased NADPH-P450 reductase level in ZnAL rats, but not in ZnPF and ZnDF rats, as compared with the drug control group *(Figure 1C)*.

The activities of NDMA demethylase *(Figure 2A)*, AH *(Figure 2B)*, and NH *(Figure 2C)* were measured to assess the effect of dietary zinc deficiency and drug treatment on 2E1 activity in rats. Within the drug control group, there was an increase in NDMA demethylase activity in ZnPF rats

Table 1 Effects of zinc deficiency and ethanol treatment on the final body weight, liver weight, liver to body weight ratio, and plasma zinc concentration

	Control	Ethanol
Final body weight (g)		
ZnAL	199 ± 6[a]	171 ± 4[a,*]
ZnPF	81 ± 1[b]	88 ± 2[b,*]
ZnDF	69 ± 1[c]	69 ± 1[c]
Liver weight (g)		
ZnAL	8.3 ± 0.6[a]	7.4 ± 0.7[a]
ZnPF	2.4 ± 0.1[b]	2.7 ± 0.1[b,*]
ZnDF	2.8 ± 0.6[b]	2.2 ± 0.1[b,*]
Liver/body weight ratio (g/100 g)		
ZnAL	4.3 ± 0.2[a]	4.4 ± 0.4[a]
ZnPF	3.0 ± 0.1[c]	3.1 ± 0.1[b]
ZnDF	4.0 ± 0.1[a]	3.2 ± 0.1[b,*]
Plasma zinc (μmol/L)		
ZnAL	24.5 ± 1.6[a]	18.4 ± 1.6[a,*]
ZnPF	24.5 ± 1.6[a]	18.4 ± 1.6[a,*]
ZnDF	7.6 ± 0.6[b]	7.6 ± 1.6[b]

Values are means ± SEM, $n = 12$ rats for ZnDF, and ZnPF, and six rats for ZnAL. The significant difference ($P < 0.05$) among the means of dietary treatment groups receiving the same drug treatment and between the means of the drug treatment groups receiving the same dietary treatment are indicated by lower-case letters and *, respectively. ZnAL, zinc adequate ad libitum; ZnPF, zinc adequate pair-fed; ZnDF, zinc deficiency.

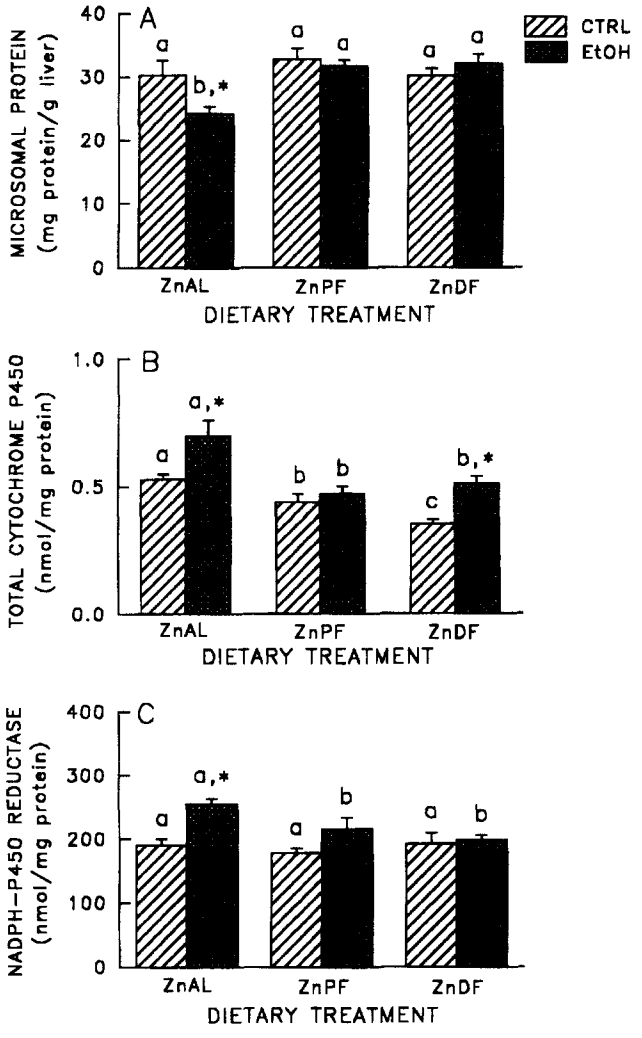

Figure 1 Effects of dietary zinc deficiency and ethanol treatment on the total microsomal protein concentration (A), total P450 concentration (B), and NADPH-P450 reductase level (C) in rat livers. Values are means ± SEM, $n = 6$ rats. The significant difference ($P < 0.05$) among the means of dietary groups receiving the same drug treatment and between the means of the drug treatment groups receiving the same dietary treatment are indicated by lower-case letters and *, respectively. Ctrl, the drug control group; EtOH, the ethanol treatment group.

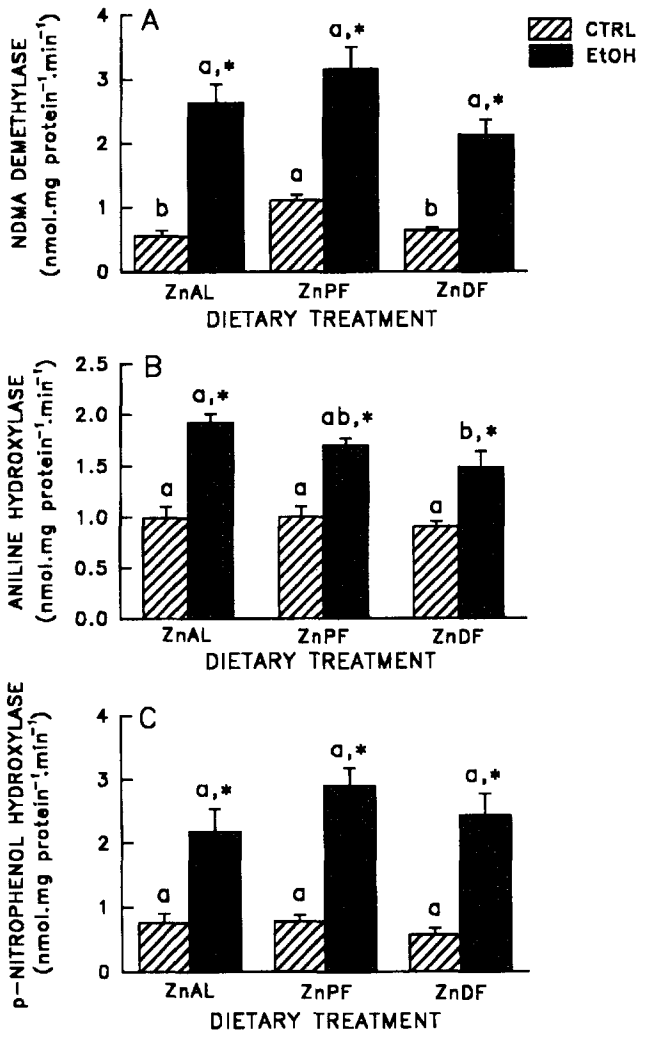

Figure 2 Effects of dietary zinc deficiency and ethanol treatment on the N-nitrosodimethylamine (NDMAD) demethylase activity (A), aniline hydroxylase activity (B), and p-nitrophenol hydroxylase activity (C) in rat livers. Values are means ± SEM, n = 6 rats. The significant difference ($P < 0.05$) among the means of dietary groups receiving the same drug treatment and between the means of the drug treatment groups receiving the same dietary treatment are indicated by lower-case letters and *, respectively. Ctrl, the drug control group; EtOH, the ethanol treatment group.

when compared with ZnAL and ZnDF rats. EtOH treatment resulted in a 2.6- to 4.8-fold increase in NDMA demethylase activity in all dietary treatments when compared with the drug control group *(Figure 2A)*. Within the drug control group, the AH activity was not affected by dietary treatment *(Figure 2B)*. EtOH treatment generally induced the AH activity regardless of dietary treatment *(Figure 2B)*. The NH activity was also not affected by dietary treatment, and EtOH treatment significantly induced the NH activity in all dietary treatment groups *(Figure 2C)*. Overall, dietary zinc deficiency had little effect on 2E1 catalytic activity in rats. EtOH treatment markedly induced 2E1 catalytic activity in all dietary treatment groups. When the activity of NDMA, AH, and NH activity was expressed per nmol P450, the effects of dietary and EtOH treatment on the 2E1 activity were similar as expressed per mg protein (the data are not shown).

Effects of dietary zinc deficiency and drug treatment on the 2E1 protein level in rats were assessed using Western blot analysis *(Figure 3)*. The 2E1 protein level was the same in ZnAL, ZnPF, and ZnDF rats in the drug control group, indicating that the constitutive 2E1 protein level was affected by neither dietary zinc deficiency nor severe depression of feed intake. After a 3-day EtOH treatment, the 2E1 protein level was 20% to 50% higher than the level in the drug control group, but the differences were not statistically significant. Within the EtOH group, zinc deficiency significantly reduced the 2E1 protein level to about 60% of that in ZnAL and ZnPF rats.

The level of mRNA encoding hepatic microsomal 2E1 was determined by Northern blot analysis *(Figure 4)*. The constitutive 2E1 mRNA level in ZnPF rats was significantly increased (threefold) as compared with the level in ZnAL and ZnDF rats. There was no statistically significant effect of EtOH treatment on 2E1 mRNA level in all dietary treatment groups, although EtOH-treated ZnPF rats had a 40% decrease in 2E1 mRNA levels as compared with the ZnPF rats in the drug control group.

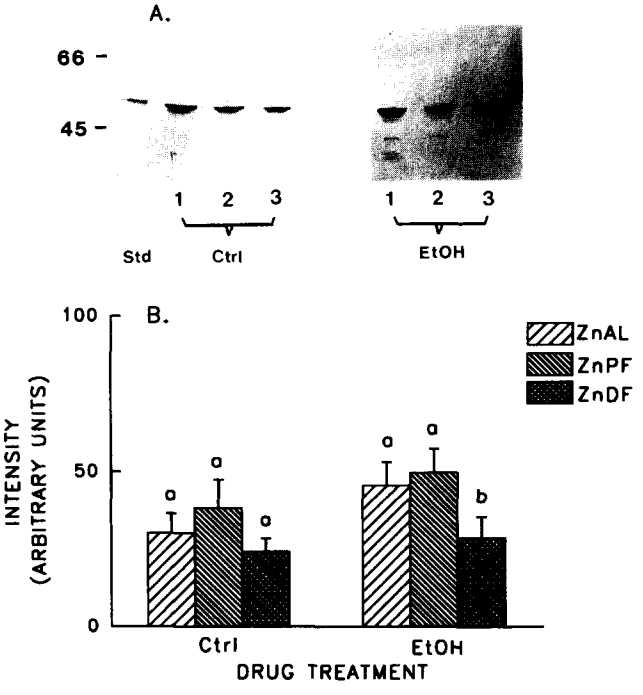

Figure 3 Immunoblot analysis of rat liver microsomes for P450 2E1 protein. (A): each lane was loaded with hepatic microsome (100 μg protein) isolated from individual rat that was fed zinc-adequate diet ad libitum (1), zinc-adequate diet pair-fed (2), or zinc-deficient diet (3) receiving no drug (Ctrl) or ethanol (EtOH) treatment. The standard (Std) for P450 2E1 was supplied by the manufacturer. Microsomal protein from SDS-PAGE was electrophoretically transferred to PVDF membrane and immunochemically stained with anti-P450 2E1 IgG as described in Methods and materials. (B): the average of relative intensity of bands (n = 5 rat livers). The significant difference ($P < 0.05$) among the means of dietary groups receiving the same drug treatment and between the means of the drug treatment groups receiving the same dietary treatment are indicated by lower-case letters and *, respectively.

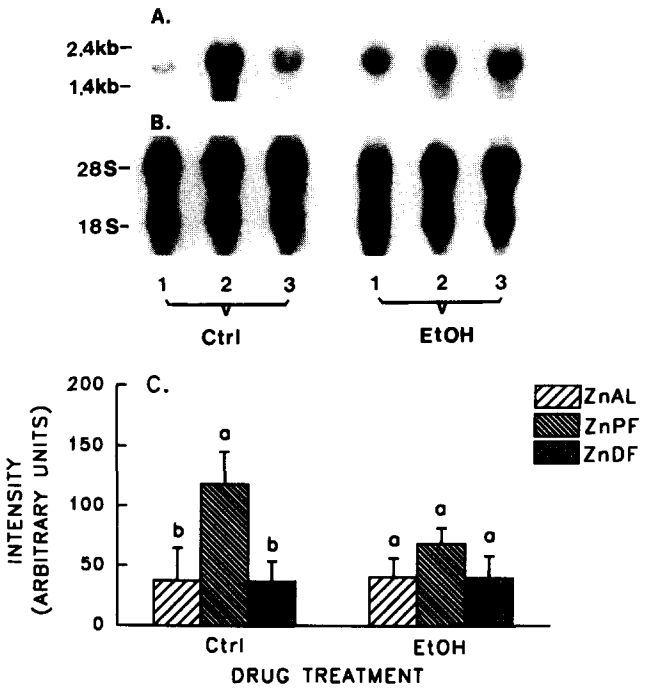

Figure 4 Northern analysis of total RNA in rat liver for P450 2E1 mRNA. (A): each lane was loaded with ~ 40 μg total RNA purified from individual liver of rat that was fed zinc adequate diet ad libitum (1), zinc-adequate diet pair-fed (2), or zinc-deficient diet (3) receiving no drug (Ctrl) or ethanol (EtOH) treatment. Autoradiography was conducted for 14 hr at −80° C as described in Methods and materials. Total RNA from denaturing formaldehyde-agarose gel was capillary transfered to Zetaprobe GT (Bio-Rad) membrane, hybridized with rat cDNA probe for P450 2E1, stripped, and rehybridized with yeast cDNA probe for rRNA to indicate total RNA in each lane (B). Autoradiography was conducted for 40 min at −80° C. (C): the average of relative intensity of bands (n = 3 rat livers). The significant difference ($P < 0.05$) among the means of dietary groups receiving the same drug treatment and between the means of the drug treatment groups receiving the same dietary treatment are indicated by lower-case letters and *, respectively.

Discussion

Although the microsomal protein concentration and NADPH-P450 reductase concentration were not affected by dietary treatments, both zinc deficiency (by comparing the ZnDF rats with the ZnPF rats) and severe feed restriction (by comparing the ZnPF rats with the ZnAL rats) caused a significant decrease in total P450 concentration. However, the activity of the constitutively expressed 2E1 was not affected by either feed restriction or dietary zinc deficiency except the NDMA demethylase activity, which was significantly higher in ZnPF rats in the drug control group. The immunoblot analysis showed that the level of 2E1 protein was also the same across the dietary treatments, while the 2E1 mRNA level was significantly increased in ZnPF rats. Clearly, the decreased concentration of total microsomal P450 in dietary zinc-deficient rats is unrelated to the constitutive expression of 2E1 in rats.

In the present study, the ZnPF rats showed a significantly higher constitutive level of 2E1 mRNA and NDMA demethylase activity when compared with ZnAL and ZnDF rats. Both ZnAL and ZnPF rats received the same diet, except that ZnPF rats had a restricted feed intake. During the last week of the feeding trial, the daily feed intake of ZnPF rats is about one-quarter of the amount consumed by ZnAL rats.[23] The microsomal 2E1 can be induced by fasting through posttranscriptional stabilization, resulting in a marked increase in 2E1 mRNA.[24] The postulated mechanism for fasting induction of 2E1 is due to an increased production of ketone bodies that is either directly or indirectly involved in the induction of 2E1.[25] In this study, 2E1 mRNA level was the same in EtOH-treated ZnAL, ZnPF, and ZnDF rats after a 3-day EtOH treatment. Energy provided from EtOH consumption may reduce the ketone body production. Consequently, the inductive effect of prolonged severe depression of feed intake on 2E1 mRNA was abolished in EtOH-treated ZnPF rats *(Figure 4)*. Thus, it seems that the prolonged severe depression of feed intake mimicked the inductive effect of fasting on 2E1 and resulted in a significant increase in 2E1 mRNA in ZnPF rats.

At present, it is generally agreed that EtOH induction of microsomal 2E1 activity in rats is due to stabilization of 2E1 protein. This ligand-mediated stabilization protects 2E1 protein from cAMP-dependent phosphorylation, which results in denaturation and degradation of 2E1.[26] In this study, EtOH treatment clearly increased 2E1 catalytic activity, as indicated by twofold to fourfold induction of NDMA demethylase, AD, and NH activity when compared with the drug control group. However, the 2E1 protein level was not affected by the 3-day EtOH treatment in all dietary treatment groups. In contrast, the inductive effect of EtOH on 2E1 protein is clearly demonstrated using a chronic model (20-day to 28-day EtOH treatment).[8,27-29] Thus, the lack of inductive effect of EtOH treatment on 2E1 protein observed in the present study may result from the short period of EtOH treatment. This induction of 2E1 catalytic activity without a concomitant increase in 2E1 protein in EtOH-treated rats may indicate the presence of catalytically inactive 2E1, which becomes active upon EtOH treatment.

In the EtOH treatment group, the microsomal 2E1 protein level was significantly lower in ZnDF rats than in ZnAL and ZnPF rats. This reduction of 2E1 protein was unrelated to transcription because the 2E1 mRNA level was the same in all dietary treatment groups. It is possible that EtOH treatment caused an increased degradation of 2E1 protein in ZnDF rats. In a series of ESR spin trapping studies, Reinke et al.[30,31] directly detected a 1-hydroxyethyl radical in heart and liver of rats administered EtOH. The 1-hydroxyethyl radical appears to be localized in the endoplasmic reticulum.[30] In addition, the microsomal P450 is a "leaky" enzyme system and produces reactive oxygen species.[32] Moreover, dietary zinc deficiency in rats causes an accumulation of iron in microsomal membranes.[10] The accumulation of these prooxidants may facilitate the oxidative destruction of microsomal P450, as seen in CCl_4-stimulated P450 degradation,[33] resulting in a lower 2E1 protein level in EtOH-treated ZnDF rats.

In summary, the constitutively expressed hepatic microsomal 2E1 activity, protein level, and mRNA level were not affected by dietary zinc deficiency in rats. A short-term EtOH treatment markedly induced 2E1 activity without a measurable increase in the level of 2E1 protein and mRNA

and dietary zinc deficiency had no effect on the inductively expressed 2E1 activity in rats. EtOH treatment may have stimulated oxidative stress in ZnDF rats resulting in an increased oxidative destruction of microsomal 2E1 protein. The prolonged severe depression of feed intake caused by dietary zinc deficiency resulted in an increased 2E1 mRNA level in rats.

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