Alcohol Induction of Liver Nuclear Ethanol and N-Nitrosodimethylamine Metabolism to Reactive Metabolites

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In previous studies from our laboratory we reported the presence in highly purified liver nuclei, free of contamination with other organelles, of an ethanol metabolizing system (NEMS) able to lead to acetaldehyde and 1-hydroxyethyl free radicals (1HEt). In the present study we tested whether this NEMS is inducible by chronic alcohol administration to rats and whether these nuclei also have increased ability to bioactivate N-nitrosodimethylamine (NDMA). Sprague Dawley male rats (125-150g) were fed with a nutritionally adequate liquid diet containing alcohol to provide 36% of total energy (standard Lieber-De Carli rat diet), for 28 days. Controls received an isocaloric diet without alcohol. Animals were sacrificed, livers were excised and microsomes and purified nuclear fractions were prepared. Both microsomes and nuclei from treated animals had significantly increased ability compared to controls, to biotransform ethanol to acetaldehyde using NADPH as cofactor under an air atmosphere. Both organelles also exhibited significantly increased capacity compared to controls, to bioactivate NDMA to formaldehyde and to reactive metabolites that bind covalently to proteins. Nuclear preparations from control animals were also able to metabolize NDMA to formaldehyde and reactive metabolites. Results indicate that liver nuclei may have a CYP2E1 able to bioactivate both NDMA and EtOH and that these processes are being induced by chronic alcohol drinking. The bioactivation of these xenobiotics to reactive metabolites in the neighborhood of nuclear proteins and DNA might have significant toxicological implications. *Teratogenesis Carcinog. Mutagen. 22:139–145, 2002.* © 2002 Wiley-Liss, Inc.

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INTRODUCTION

In the course of previous studies from our laboratory, the presence of a liver nuclear ethanol metabolizing system was reported (NEMS) [1]. During ethanol biotransformation by NEMS reactive metabolites such as acetaldehyde and hydroxyl and 1-hydroxyethyl free radicals (1HEt) were formed, which covalently bound to nuclear lipids and macromolecules [2]. Cytochrome P450 2E1 (CYP2E1) and cytochrome P450 reductase (P450 reductase) were found to be involved in the process [1,3]. Furthermore, P450 reductase metabolized ethanol to acetaldehyde and 1HEt in the presence of CYP2E1 but also in its absence and not only in the presence of oxygen but also under an atmosphere of oxygen free nitrogen [3].

In the present study, we report experiments directed to check whether chronic ethanol drinking is able to induce NEMS and whether besides ethanol metabolism, highly purified liver nuclei are able to biotransform N-nitrosodimethylamine (NDMA) to formaldehyde and to reactive methyl carbonium ions able to covalently bind to DNA and nuclear proteins. NDMA, like ethanol, requires CYP2E1 to generate the reactive metabolites [4–6] known to attack DNA and generate the alkylated bases considered to be involved in the NDMA cancer initiation process [7].

MATERIAL AND METHODS

Chemicals

N-nitrosodimethylamine (NDMA) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and [^{14}C]NDMA (55 mci/mmol, purity >99% by HPLC) from American Radiolabeled Chemicals, Inc. (St Louis, MO). All other chemicals were of the best available quality.

Animals and Treatments

Sprague Dawley male rats (125–150 g body weight) were fed with a nutritionally adequate liquid diet (Lieber-DeCarli standard rat diet) purchased from Dyets, Inc. (Pennsylvania, PA).

The rats were housed in individual cages and separated into two dietary groups: Ethanol group (EtOH) and Control group (Control), which were pair fed with the same diet, except that in the control group, ethanol was isocallorically replaced with carbohydrate.

The liquid diet used provided 1 kcal/ml where 35% of the total calories derived from fat, 47% from carbohydrate, and 18% from protein. In the EtOH-treated animals, EtOH provided 36% of the calories replacing isocalorically carbohydrate. Feeding of the Control and EtOH diet was continued for 28 days. The amount of ethanol was started with 30 g/liter of the liquid diet for the first 2 days, 40 g/liter for the subsequent 2 days followed by the final formula that contained 50g/liter [8,9]. In the case of the experiments on NDMA metabolism both groups were fed control diet the day prior to sacrifice. This is to allow sufficient time for ethanol disappearance from the body, thus avoiding its competition with NDMA [10].

Isolation of Highly Purified Nuclear Preparations and Microsomes

Highly purified nuclei were obtained as previously described [2,11]. Briefly, the liver homogenate in 0.25 M sucrose in TKM buffer (50 mM Tris-HCl, 5 mM

$MgCl_2$, and 2.5 mM KCl), pH 7.5, was passed through a 100-mesh nylon cloth and centrifuged at 1,000*g* for 20 min. After washing the pellet twice by resuspending with 0.25 M sucrose-TKM and centrifugation at 1,000*g* for 5 min, the crude preparation was resuspended in 2.2 M sucrose-TKM, layered on 2.3 M sucrose-TKM and centrifuged for 20 min at 80,000*g*. The pellet was gently rinsed with 0.25 M sucrose–TKM and resuspended in 2.2 M sucrose TKM and the ultracentrifugation step was repeated. Finally, the pellet was washed with 1.0 M sucrose-TKM (centrifuged for 5 min at 2,000*g*) and then with 0.25 M sucrose-TKM. The purity of nuclei was assessed on the basis of their lack of activity of marker enzymes for mitochondria (isocitric acid dehydrogenase), for cytosol (lactic dehydrogenase), and by electron microscopy in order to obtain a definitive assessment of the purity of nuclear preparations. These criteria were recently considered as the most suitable to assess that nuclei were free of detectable contamination from other organelles [12]. The hepatic microsomal fractions were isolated by the procedure already reported [13].

Metabolism of Ethanol to Acetaldehyde in Rat Liver Nuclei and Microsomes

Incubation mixtures that contained highly purified nuclei (5.0–5.5 mg protein/ml), or microsomes (2.0–2.5 mg protein/ml), NADPH generating system (0.45 mM NADP+, 4 mM dl-isocitric acid trisodium salt, and 0.25 units of isocitric dehydrogenase), and 0.21 M ethanol in STKM buffer (0.25 M sucrose, 50 mM Tris-HCl, 2.5 mM KCl, 5 mM $MgCl_2$), pH 7.5, for nuclei and PO_4H_2K 50 mM, pH 7.4, for microsomes, 3 ml final volume, were conducted for 1 h at 37°C under an air atmosphere. Incubations were performed in aluminum-sealed neoprene-septum stoppered glass vials (15 ml). Reaction was interrupted by placing the vials on ice for 20 min. After adding 1 ml of saturated NaCl solution, samples were kept at 40°C for 10 min and an aliquot (100 µl) of the head space was analyzed by GC-FID. Chromatographic conditions: column, Poraplot Q, 25 m × 0.53 mm i.d. (Chrompack, The Netherlands); temperature 140°C isothermal, injection port temperature: 150°C, FID: 200°C [1–3].

Metabolism of [^{14}C] NDMA to Formaldehyde and Covalent Binding to Proteins, in Rat Liver Nuclei and Microsomes

Incubation mixtures contained rat liver nuclei (4.0–4.5 mg protein/ml) in 0.25 M sucrose in TKM buffer, pH 7.4, or microsomes (2.5–3.0 mg protein/ml) in 0.17 M PO_4H_2K buffer, pH 7.2 [^{14}C]NDMA (0.1 mM, 0.6×10^6 dpm/ml) and NADPH generating system when indicated. Incubation was conducted for 1 h for the case of nuclei and 15 min for microsomes, both at 37°C and the reaction was stopped with 10% TCA.

[^{14}C] Formaldehyde produced from [^{14}C]NDMA was measured in the TCA-soluble fraction as previously described [14–16]. A known amount of carrier formaldehyde was added and precipitated with 5,5-dimethyl-1,3-cyclohexanedione. A weighed portion of the formaldemethone formed was counted for radioactivity in a toluene PPO mixture.

The covalent binding to nuclear proteins was determined in the acid insoluble precipitate as described [14–16]. Proteins were precipitated and washed with TCA, delipidated, and dried by successive washings with the use of a mixture of ethanol, ether, chloroform (2:2:1), acetone, and ether. Finally they were dissolved in concentrated formic acid and counted for radioactivity with a butyl cellosolve-PPO mixture.

Statistics

The significance of the difference between two mean values was assessed by the Student's *t* test [17].

RESULTS

Rats were fed with Lieber-De Carli diet during 28 days and were separated into two dietary groups as follows: ethanol and isocaloric maltose-dextrin pair-fed with ethanol (Control).

Ethanol Biotransformation to Acetaldehyde in Liver Nuclei and Microsomes From Rats Treated With Ethanol Liquid Diet

The metabolism of ethanol to acetaldehyde was measured in the presence and absence of NADPH in nuclei and microsomes from control and ethanol treated rats maintained on the Lieber-De Carli diet. Both subcellular fractions were able to biotransfrom ethanol to acetaldehyde and this metabolism was significantly higher in the presence of NADPH, in control as well as in ethanol treated rats, as it is shown in Table I, but the production of acetaldehyde was significantly increased in the presence of NADPH in ethanol treated rats in both fractions.

Formaldehyde Formation From NDMA by Liver Nuclei and Microsomes From Rats Treated With Ethanol Liquid Diet

In order to check whether rats chronically treated with ethanol liquid diet were able to induce the metabolism of NDMA, the biotransformation of NDMA to formaldehyde in rats untreated and those chronically treated with ethanol was measured. Results showed that rat liver nuclei and microsomes from control rats were able to biotransform NDMA to formaldehyde and the production of formaldehyde was significantly increased in the presence of NADPH (Table II). When the same parameter was measured in rats chronically treated with ethanol, it can be seen that formaldehyde formation in the presence of NADPH was significantly induced in the rats treated with ethanol with respect to controls (Table II).

TABLE I. Ethanol Biotransformation to Acetaldehyde in Liver Nuclei and Microsomes From Rats Treated With Ethanol Using a Liquid Diet

Experimental condition[a]	(Acetaldehyde ng/mg protein) Control	Ethanol liquid diet
Nuclei		
–NADPH	0.73 ± 0.04	0.71 ± 0.03
+NADPH	2.80 ± 0.12[b]	3.97 ± 0.13[b,c]
Microsomes		
–NADPH	28.88 ± 0.37	15.11 ± 0.80
+NADPH	756.36 ± 16.70[b]	880.68 ± 24.28[b,c]

[a]Incubation mixtures containing nuclear preparations (5.0–5.5 mg protein/ml) or microsomes (2.0–2.5 mg protein/ml), NADPH generating system, and 0.21 M ethanol were conducted for 1h at 37°C. Acetaldehyde was measured in the head space of each sample after adding 1 ml NaCl saturated solution. See Methods for details. Each result is the mean of three separate samples.

[b]$P < 0.05$ (+NADPH vs. –NADPH).

[c]$P < 0.05$ (Ethanol liquid diet vs. control).

TABLE II. Formaldehyde Formation From NDMA by Liver Nuclei and Microsomes From Rats Treated With Ethanol Using a Liquid Diet

Experimental condition[a]	(Formaldehyde formed, nmol/g liver) Control	Ethanol liquid diet
Nuclei		
–NADPH	5.10 ± 0.27	5.50 ± 0.32
+NADPH	7.16 ± 0.22[b]	10.29 ± 1.36[b,c]
Microsomes		
–NADPH	32.17 ± 2.53	25.53 ± 1.77
+NADPH	110.05 ± 4.99[b]	639.33 ± 11.26[b,c]

[a]Incubation mixtures contained liver nuclei (4.0–4.5 mg protein/ml) or microsomes (2.5–3.0 mg protein/ml), [^{14}C] NDMA (0.1 mM, 0.6 × 10^6 dpm/ml) and NADPH generating system when indicated. See Methods for details. Each result is the mean of three separate samples.
[b]$P < 0.05$ (+NADPH vs. –NADPH).
[c]$P < 0.05$ (Ethanol liquid diet vs. control).

Covalent Binding of [^{14}C] NDMA (CB) to Nuclei and Microsomes From Rats Treated With Ethanol Liquid Diet

CB was measured in nuclear and microsomal proteins from control and ethanol treated rats. Both nuclei and microsomes were able to bioactivate NDMA to metabolites that bound covalently to proteins and this metabolic transformation was significantly increased in the presence of NADPH (Table III). When CB was measured in proteins from nuclei and microsomes from ethanol treated rats, the ability for NDMA activation to reactive metabolites was enhanced in a very significant manner in the presence of NADPH (Table III).

DISCUSSION AND CONCLUSIONS

As is well known from literature [18], chronic administration of EtOH during 28 days through the standard Lieber-De Carli diet enhanced the liver microsomal NADPH-dependent biotransformation of EtOH to acetaldehyde (Table I). Interestingly the NEMS recently reported by our laboratory to be present in highly purified liver nuclei was also induced by the chronic EtOH feeding treatment (Table I). This is not unexpected in view of the fact that a significant part of the NEMS activity is also CYP2E1 depen-

TABLE III. Covalent Binding of ^{14}C From [^{14}] NDMA to Proteins by Liver Nuclei and Microsomes From Rats Treated With Ethanol Using a Liquid Diet

Experimental condition[a]	(Covalent binding, nmol × 10^{-1}/mg protein) Control	Ethanol liquid diet
Nuclei		
–NADPH	4.46 ± 0.05	5.13 ± 0.07
+NADPH	8.31 ± 0.48[b]	17.78 ± 0.70[b,c]
Microsomes		
–NADPH	12.81 ± 1.31	11.86 ± 3.10
+NADPH	27.79 ± 2.07[b]	134.05 ± 5.78[b,c]

[a]Incubation mixtures as in Table II. Each result is the mean of three separate samples. See Methods for details.
[b]$P < 0.05$ (+NADPH vs. –NADPH).
[c]$P < 0.05$ (Ethanol liquid diet vs. control).

dent as it is the liver microsomal counterpart [1]. These results are in agreement with the known fact that the outer membrane of the nuclei is an extension of the endoplasmic reticulum [19]. The increase in formation of reactive mutagenic-carcinogenic molecules such as acetaldehyde [20–22] as it occurs in the case of the chronic alcohol-treated animals might be important. In effect, we previously showed that acetaldehyde produced during liver nuclear metabolism by NEMS covalently binds to nuclear proteins and lipids [2]. Many critical nuclear proteins known to play a role in carcinogenic processes might thus be altered during these interactions and to a greater extent in the chronically treated animals. Some of those critical nuclear proteins include proto-oncogenes, cyclin-dependent kinases, and repair enzymes [23–28].

There is evidence that some of these proteins might be altered during ethanol poisoning. One clear example of this is the case of the O^6-methylguanine methyltransferase repair enzyme [29,30]. This enzyme repairs damage produced by some alkylating agents in DNA [29,30], the most studied case being the one of the O^6-guanine alkylation produced by the methyl group derived from the methyl carbonium ion resulting from NDMA biotransformation [7].

This NDMA biotransformation process is also known to be mediated by CYP2E1 [4–6] as in the case of EtOH [10,18]. In the present studies, we also report that liver nuclei from control rats were able to bioactivate NDMA to carbonium ions that covalently bind to nuclear proteins (Table III). Those interactions were more intense in chronically ethanol pretreated animals. However, we failed to detect interaction with DNA. The reason for this may rest in the lower intensity that the NDMA activation process has in the nuclei in relation to that in liver microsomes. This reinforces the idea that the microsomal metabolite that is the precursor of the carbonium ion has sufficient stability to travel from formation sites in the endoplasmic reticulum to the nuclear sites and decompose there to give the $^{+}CH_3$, which finally attacks DNA [31]. The production of that intermediate in the alcohol preinduced nuclei does not reach an equivalent intensity to that occurring in the endoplasmic reticulum and this might be the reason why the formation of $^{+}CH_3$ from NDMA at the liver nuclear membrane during nuclear activation studies in vitro might not be sufficient to reach DNA. Alternatively the formation of DNA adducts might be so low that it remained below our detection limit.

In summary, liver nuclei from alcohol-exposed animals have more ability to activate not only ethanol itself to harmful reactive metabolites but also enhances the possibility that they may bioactivate other CYP2E1 substrates of potent carcinogenic nature such as NDMA [7] or others [6] to increase the chances of cancer initiation in exposed individuals.

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