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INFLUENCE OF CHRONIC ETHANOL CONSUMPTION ON HAMSTER LIVER MICROSOMAL *O*-DEALKYLASE ACTIVITIES AND CYTOCHROME b_5 CONTENT

G. DAVID MCCOY,* GEORGE J. DEMARCO and JOSEPH A. BIAGLOW
Department of Environmental Health Sciences, Case Western Reserve University, School of Medicine, Cleveland, OH 44106, U.S.A.

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Abstract—The effects of two different methods of administering ethanol to hamsters on liver microsomal cytochrome levels and the activities of ethoxyresorufin *O*-deethylase and *p*-nitroanisole *O*-demethylase have been examined. Administration of ethanol in liquid diets resulted in enhanced levels of cytochrome P-450, NADPH-supported aniline hydroxylase (Form I), and both NADPH- and NADH-supported *p*-nitroanisole *O*-demethylase. NADH–ferricyanide reductase was also increased. No change in NADPH–cytochrome *c* reductase or in the NADPH-supported rate of ethoxyresorufin *O*-deethylase was observed. In contrast, both NADH-supported ethoxyresorufin *O*-deethylase and cytochrome b_5 levels were decreased. Administration of ethanol in the drinking water to chow-fed animals had no effect on total cytochrome P-450 levels; however, the rates of NADPH-supported aniline hydroxylase (Form I) and *p*-nitroanisole *O*-demethylase activity were increased. No changes in NADPH–cytochrome *c* reductase, NADH–ferricyanide reductase, or NADH-supported *p*-nitroanisole *O*-demethylase activity were noted. Cytochrome b_5 levels were decreased as were both the NADPH- and NADH-supported rates of ethoxyresorufin *O*-deethylase. These data suggest that chronic consumption of ethanol by hamsters either in liquid diet form or as ethanol–water solutions to chow-fed animals lowers cytochrome b_5 levels. When cytochrome b_5 levels are lowered and total chromosome P-450 levels remain unchanged, the NADPH-supported rate of microsomal O-dealkylation of ethoxyresorufin is decreased. These data suggest that cytochrome b_5 participates in the NADPH-supported microsomal O-dealkylation of ethoxyresorufin.

The hamster is an ethanol-preferring species in that it will preferentially consume unsweetened ethanol/water mixtures with no apparent adverse effects on growth [1, 2]. Previous studies from our laboratories have shown that chronic consumption of ethanol in liquid diets by hamsters increases the content of cytochrome P-450, the specific activities of aniline hydroxylase, *N*-nitrosopyrrolidine α-hydroxylase, and *p*-nitroanisole *O*-demethylase, and the content of microsomal protein [3–5]. In addition, we have shown that both the specific content of cytochrome b_5 and the stearoyl CoA-desaturase activity are lowered by chronic ethanol consumption [5].

In view of the studies of Kamataki and Kitagawa [6] and Sugiyama *et al.* [7, 8] demonstrating that cytochrome b_5 was required for reconstitution of *p*-nitroanisole *O*-demethylase activity, we were surprised by the lack of effect of decreased cytochrome b_5 levels on the rates of NADH-supported *p*-nitroanisole *O*-demethylase activity in our preparations. Since consumption of ethanol in liquid diets resulted in opposite effects on the two microsomal cytochromes, it is possible that the increases in cytochrome P-450 may compensate for the decrease in cytochrome b_5. To examine the role of cytochrome b_5 in the microsomal metabolism of xenobiotics, a system in which cytochrome P-450 remains unchanged was needed. The purpose of this paper is to present evidence that administration to hamsters of ethanol in the drinking water lowered cytochrome b_5 levels without altering total cytochrome P-450 levels and to report that the NADPH-supported rates of O-dealkylation of ethoxyresorufin were also decreased.

MATERIALS AND METHODS

Chemicals. Aniline, cytochrome *c* (Type III), nicotinamide adenine dinucleotide phosphate (monosodium salt), nicotamide adenine dinucleotide phosphate (reduced form, Type X), nicotinamide adenine dinucleotide (reduced form, Grade III), Tricine (*N*-tris-[hydroxymethyl]-methyl glycine), Tris (tris-[hydroxymethyl]-amino-methane), glucose-6-phosphate (monosodium salt), and glucose-6-phosphate dehydrogenase (Type XII) were obtained from the Sigma Chemical Co., St. Louis, MO; potassium ferricyanide, *p*-aminophenol and *p*-nitroanisole from Fisher Scientific, Pittsburgh, PA; and 7-ethoxyresorufin and resorufin from the Pierce Chemical Co., Rockford, IL. Sucrose (special enzyme grade) was from Schwarz/Mann, Orangburg, NY.

Animals and treament. Twenty-four 6-week-old male LVG Syrian golden hamsters (*Mesocricetus auratus*) were obtained from the Charles River Breeding Laboratories, Lakeview Colony, Newfield, NJ. Animals were housed three per cage in regulation stainless steel cages and given free access to

* Author to whom all correspondence should be addressed.

NIH-07 lab chow (Ziegler Brothers, Gardners, PA) and water. At 8 weeks of age, animals were randomized by weight, and twelve were continued on NIH-07 lab chow and water while the other twelve were given control liquid diet (711 + added fiber obtained from Bio-Serv Inc., Frenchtown, NJ). At 9 weeks of age, six animals in each diet group were given ethanol either as an 18.5% ethanol–water mixture or an ethanol-containing liquid diet where ethanol isocalorically replaced carbohydrate. Animals in the chow-fed group had free access to chow and liquid. Liquid consumption in the chow-fed group was measured twice weekly over a 24-hr interval. Chow consumption was measured once weekly over a 48-hr interval. Liquid diet intake per cage was monitored daily and the amount given to control animals was adjusted to match the intake of ethanol-consuming animals. All animals were killed by decapitation after 4 weeks of ethanol consumption.

Subcellular fractionation and biochemical analysis. Livers were rapidly removed, weighed, minced, rinsed three times with 10 vol. of ice-cold SET (0.3 M sucrose, 0.5 mM EDTA, and 5 mM Tricine, pH 7.4), and suspended in 5 vol. of ice-cold SET. Homogenization of livers and microsome isolation were performed as described previously [4]. Microsomal fractions were assayed as follows: aniline hydroxylase as previously described [4], NADPH–cytochrome *c* reductase by the method of Phillips and Langdon [9]; NADH-ferricyanide reductase as described by Comai and Gaylor [10]; *p*-nitroanisole *O*-demethylase as described by Kato and Gillette [11]; ethoxyresorufin *O*-deethylase as described by Burke and Mayer [12], except that the final substrate concentration was 243 nM; total cytochrome P-450 according to the method of McLean and Day [13], using an extinction coefficient of 91 mM^{-1} for $A_{450-490}$; and cytochrome b_5 as described by Omura and Sato [14] using an extinction coefficient of 124 mM^{-1} for $A_{425-409}$. Protein was determined by the method of Lowry *et al.* [15]. Statistical significance was evaluated using Student's two-tailed *t*-test.

RESULTS

The effects of 28-day consumption of ethanol in either liquid diet form or as ethanol–water mixtures to chow-fed animals are presented in Table 1. Little change in body weight or liver weight was observed in chow-fed animals. Ethanol-consuming animals on liquid diets gained less weight than controls even though their total caloric intake was slightly higher. Total caloric intake of control and ethanol-consuming animals was similar within groups. The lowered total caloric intake observed in animals on liquid diets was due to the fact that intake of liquid diet by control animals was restricted to match the intake of ethanol-consuming animals. As observed previously [16], although total caloric intake was similar for both control and ethanol-consuming chow-fed animals, caloric intake from chow was lower in ethanol-consuming animals. The percentage of total caloric intake as ethanol was similar for animals on the two different diets.

The effects of ethanol consumption on the specific content of microsomal cytochromes and the associated reductases are presented in Table 2. No significant differences in microsomal protein content were observed in chow-fed animals; however, these values were elevated in ethanol-consuming animals on the liquid diet. No change in the specific content of total cytochrome P-450 was seen in chow-fed animals, whereas ethanol-consuming animals on liquid diets had elevated levels of cytochrome P-450. In contrast, microsomes isolated from ethanol-consuming animals in both groups had decreased content of cytochrome b_5. No differences in specific activity of NADPH–cytochrome *c* reductase were observed in animals consuming ethanol in either group. Ethanol-consuming animals on liquid diet had increased rates of NADH-ferricyanide reductase activity while this activity remained unchanged in ethanol-consuming animals on chow.

The effects of ethanol consumption on both the high and low affinity forms of aniline hydroxylase activity are shown in Table 3. As reported previously

Table 1. Effect of chronic ethanol consumption on weight gain and food consumption

	Liquid diet		Chow diet	
	Control	Ethanol	Control	Ethanol
Initial weight (g)	102 ± 7 (6)	98 ± 9 (6)	102 ± 8 (6)	102 ± 9 (6)
Final weight (g)	132 ± 9 (6)	119 ± 15 (6)	129 ± 12 (6)	130 ± 13 (6)
Liver weight (g/100 body weight)	4.1 ± 0.3 (6)	4.0 ± 0.2 (6)	4.2 ± 0.4 (6)	4.0 ± 0.2 (6)
Intakes				
Liquid diet* (ml or kcal/animal/day)	32.8 ± 0.7 (4)	36.4 ± 1.3† (4)		
Chow (g/animal/day)			10.3 ± 1.3 (8)	7.2 ± 0.5† (8)
Chow (kcal/animal/day)			42.2 ± 5.3 (8)	29.5 ± 1.9† (8)
Liquid (ml/animal/day)			9.0 ± 0.9 (16)	10.7 ± 1.5† (16)
Liquid (kcal/animal/day)			0	14.0 ± 2.0
Total caloric intake (kcal/animal/day)	32.8 ± 0.7 (4)	36.4 ± 1.3† (4)	42.2 ± 5.3 (8)	43.2 ± 3.2 (8)
% Caloric intake as ethanol	35		32	

All values are means ± S.D. Numbers in parentheses are the number of observations.

* Liquid diet consumption was measured daily. Values are means S.D. of weekly group averages. Chow and liquid consumption was measured as described in Materials and Methods.

† $P \leq 0.01$.

Table 2. Effect of chronic ethanol consumption on hamster microsomal cytochrome and associated reductases

	Microsomal protein content (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)	Cytochrome b_5 (nmoles/mg protein)	NADPH–cytochrome *c* reductase (μmoles/min/mg protein)	NADH–ferricyanide reductase (μmoles/min/mg protein)
Liquid diet					
Control	15.4 ± 1.4	1.15 ± 0.16	0.47 ± 0.04	0.27 ± 0.04	3.95 ± 0.31
Ethanol	20.4 ± 2.7*	1.75 ± 0.31*	0.34 ± 0.05*	0.28 ± 0.02	5.06 ± 0.63*
Chow diet					
Control	11.9 ± 1.6	1.11 ± 0.15	0.48 ± 0.08	0.21 ± 0.02	3.58 ± 0.24
Ethanol	13.1 ± 1.5	1.11 ± 0.13	0.26 ± 0.05*	0.20 ± 0.02	3.30 ± 0.47

All values are means ± S.D. for six separate preparations.
* $P \leq 0.01$.

[2], the specific activity of high affinity activity (Form I) was enhanced in ethanol-consuming animals on liquid diets, while the low affinity activity (Form II) was decreased slightly. Similar changes were observed in ethanol-consuming chow-fed animals.

The effects of ethanol consumption on the rates of O-dealkylation of ethoxyresorufin and *p*-nitroanisole are shown in Table 4. The NADPH-supported rates of ethoxyresorufin O-deethylation were not changed in animals on liquid diet while the rates in chow-fed animals were decreased as a result of chronic ethanol consumption. Small decreases in the NADH-supported rates of ethoxyresorufin *O*-deethylase were observed in ethanol-consuming animals on liquid diets and larger decreases were seen in ethanol-consuming animals on chow diets. The rates of NADPH-supported *p*-nitroanisole *O*-demethylase activity were enhanced in ethanol-consuming animals in both groups, while the NADH-supported rates slightly increased in animals on the liquid diet. Ethanol consumption did not alter the K_m values for ethoxyresorufin *O*-deethylase in either group; however, slightly higher affinities were observed in chow-fed animals.

Table 3. Effect of chronic ethanol consumption on the two forms of hamster liver microsomal aniline hydroxylase

	Aniline hydroxylase (nmoles/min/mg protein)		
	Form I	Form II	Total
Liquid diet			
Control	0.75 ± 0.08	0.74 ± 0.12	1.49 ± 0.20
Ethanol	1.60 ± 0.18*	0.54 ± 0.14†	2.20 ± 0.25*
Chow diet			
Control	0.49 ± 0.04	0.44 ± 0.06	0.92 ± 0.09
Ethanol	1.10 ± 0.23*	0.38 ± 0.09	1.47 ± 0.35*

Assays were performed in the presence of 0.1 or 20 mM aniline. Form I activity was determined with 0.1 mM aniline, and total activity was determined with 20 mM aniline. Form II activity then was the difference between total activity and Form I activity [4]. Values are means ± S.D. for six separate preparations.
* $P \leq 0.01$.
† $P \leq 0.05$.

DISCUSSION

The investigations of Rubin *et al.* as well as others have shown that chronic ethanol consumption increases hepatic smooth endoplasmic reticulum, cytochrome P-450 and associated enzymatic activities [17, 18]. Microsomal cytochrome P-450 consists of a family of distinct hemoproteins each capable of being differentially induced by a wide variety of xenobiotics including ethanol [19]. Ethanol-associated increases in liver cytochrome P-450 levels and aniline hydroxylase activity have been consistently observed in rats, mice and hamsters consuming liquid diets [5, 20].

Cytochrome b_5 is an integral component of the microsomal NADH-dependent fatty acyl CoA desaturase system, functioning as an intermediate electron carrier between the flavoprotein reductase and the terminal desaturase [21]. Recent evidence suggests that cytochrome b_5 may also participate directly in NADPH-cytochrome P-450 associated metabolic activation of the liver carcinogen aflatoxin B_1 [22] and may act as the donor for the second electron in several P-450 linked oxidations [23, 24]. In contrast to cytochrome P-450, the level of cytochrome b_5 is only modestly increased following induction with phenobarbital, 3-methylcholanthrene or Aroclor [25, 26]. Recent studies have shown that chronic ethanol consumption by both rats and hamsters results in decreased microsomal stearoyl CoA-desaturase activity and that, in hamsters, this decrease is accompanied by lowered cytochrome b_5 levels [20, 27, 28].

Chronic ethanol consumption by hamsters results in several changes in microsomal metabolism which are not dependent on the type of diet. Enhanced specific activities of the high affinity form of aniline hydroxylase, and of the NADPH-supported rate of *p*-nitroanisole *O*-demethylase, with decreased specific content of cytochrome b_5 and NADH-supported ethoxyresorufin *O*-deethylase were observed consistently. Animals consuming ethanol in liquid diet exhibited increases in both the amount of microsomal protein as well as the specific content of total cytochrome P-450, whereas these variables were unchanged in animals on chow diet receiving ethanol. In contrast, no change in the NADPH-supported rate of ethoxyresorufin *O*-deethylase was seen in ethanol-consuming animals on liquid diets, while this

Table 4. Effect of chronic ethanol consumption on hamster liver microsomal *O*-dealkylase activities

	Ethoxyresorufin *O*-deethylase (pmoles/min/mg protein)		Ethoxyresorufin *O*-deethylase (nM)		*p*-Nitroanisole *O*-demethylase (nmoles/min/mg protein)	
	NADPH supported	NADH supported	K_m NADPH supported	K_m NADH supported	NADPH supported	NADH supported
Liquid diet						
Control	97 ± 14	35 ±6	61 ± 25 (3)	45 (2)	1.30 ± 0.23	0.42 ± 0.08
Ethanol	102 ± 14	27 ± 4*	72 ± 12 (4)	33 (2)	1.94 ± 0.28†	0.58 ± 0.04
Chow diet						
Control	112 ± 18	26 ± 7	43 ± 14 (5)	31 (2)	0.37 ± 0.15	0.16 ± 0.04
Ethanol	74 ± 15†	5 ± 3†	45 ± 11 (5)	24 (2)	1.22 ± 0.17†	0.17 ± 0.05

Enzymatic activities are means ± S.D. for six separate preparations. Numbers in parentheses are the number of observations.

* $P \leq 0.05$.

† $P \leq 0.01$.

activity was decreased in ethanol-consuming animals on chow diets.

Although there are numerous small differences in the specific micro and macro nutrient levels of the two diets, one major difference is the level of dietary fat. The fat content of the chow diet is 5% and represents approximately 11% of the total caloric intake [29], while the liquid diet contains 19% fat which represents 35% of the total caloric intake. The studies of Joly and Hetu have clearly shown that both the quantity and quality of the dietary fat are crucial for ethanol-associated increases in the total cytochrome P-450 content in rats [30], and it is not unreasonable to assume that a similar phenomon occurred in our experiments. Less easily explainable are the increases in enzymatic activity that were observed in both the absence and presence of changes in the specific content of total cytochrome P-450. Our observation that both aniline hydroxylase (Form I) and NADPH-supported *p*-nitroanisole-*O*-demethylase were enhanced in microsomes from chow and liquid diet fed hamsters clearly demonstrates that changes in cytochrome P-450 as usually measured are a poor predictor of ethanol-associated changes in microsomal metabolism. Studies are underway to determine if there are changes in one or more of the minor isozymes of cytochrome P-450 which could account for the alterations in enzymatic activity which we have observed. It is possible that such changes would be undetectable when the total amount of cytochrome P-450 is determined spectrally.

The decrease in cytochrome b_5 levels seen in both chow-fed and liquid diet-fed animals may be due to an adaptive response to alcohol consumption. Several recent studies have shown that chronic ethanol consumption in both rats and hamsters results in lowered rates of microsomal stearoyl CoA-desaturase [5, 27, 28]. Since it has been shown that purified cyrochrome b_5 is an absolute requirement for the reconstitution of *p*-nitroanisole *O*-demethylase activity [6–8], we attributed our earlier observation that ethanol consumption by hamsters on liquid diets increased demethylase activity as being due to increased levels of P-450, or to the possibility that cytochrome b_5 may not be rate-limiting in whole microsomal preparations.

In our search to find a readily assayable substrate for microsomal mixed-function oxygenase activity which could be used to study the consequences of lowered microsomal cytochrome b_5, we observed that both the NADPH- and NADH-supported O-dealkylation of ethoxyresorufin were decreased in microsomes isolated from ethanol-consuming chow-fed animals. That these observed decreases in ethoxyresorufin O-dealkylation are associated with lowered levels of cytochrome b_5 is further supported by the fact that neither of the activities of the associated reductase was altered by ethanol. In addition, although there were small differences in the K_m for ethoxyresorufin between chow-fed and liquid diet-fed hamsters, no ethanol-associated changes within groups were observed.

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