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DIFFERENTIAL EFFECTS OF CHRONIC ETHANOL FEEDING ON CYTOCHROME P-448- AND P-450-MEDIATED DRUG METABOLISM IN THE RAT*†

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Abstract—The effects of chronic ethanol feeding on cytochrome P-448- and P-450-mediated drug metabolism have been studied both *in vivo* and *in vitro* in the rat, using caffeine, phenacetin, antipyrine and aminopyrine as test substrates. *N*-Demethylation of aminopyrine (P-450 mediated) was increased both *in vivo* and *in vitro* in rats after chronic ethanol feeding ($P < 0.05$) whereas *in vivo* *N*-demethylation of caffeine and *O*-dealkylation of phenacetin (P-448 mediated) were unchanged in the same animals. *N*-Demethylation of antipyrine was increased by both phenobarbital and 3-methylcholanthrene pretreatment and by chronic ethanol feeding ($P < 0.05$), possibly due to cytochrome P-450 induction. Furthermore, the Michaelis affinity constants, K_m, for hepatic microsomal aminopyrine *N*-demethylase and antipyrine *N*-demethylase were lower in chronic ethanol-fed animals ($P < 0.05$), suggesting a qualitative change in the enzymes resulting in greater substrate affinity. These findings suggest a differential effect of chronic ethanol feeding on the induction of cytochrome P-450- and cytochrome P-448-mediated drug metabolism, with a greater effect on the former microsomal system.

Chronic ethanol administration has been shown to increase hepatic microsomal metabolism of many drugs [1–3]. However, much heterogeneity exists among the hepatic mixed function oxidases [4]. This heterogeneity has been demonstrated through selective induction of enzyme activity for various substrates, as well as by difference in the electrophoretic mobility of purified microsomal proteins in polyacrylamide gels after solubilization and distribution within the liver lobule [5–7]. One component of this complex is induced by pretreatment with phenobarbital (PB) and is usually referred to as cytochrome P-450, P-450$_b$ or PB-P-450, while another component is induced by pretreatment with 3-methylcholanthrene (3-MC) and is called cytochrome P-448, P$_1$-450, P-450$_c$ or MC-P-450 [4, 5]. Cytochrome P-448 catalyzes the metabolism of substrates such as benzopyrene, 2-aminoanthracene and other carcinogens [4, 8]. The *N*-demethylation of caffeine and antipyrine and the *O*-dealkylation of phenacetin have also been shown to be increased after pretreatment with 3-MC and therefore are thought to be cytochrome P-448 mediated [9–11]. Even within the P-450 and P-448 cytochrome classes, multiple species of these hemoproteins are thought to be present.

Most studies on the effects of ethanol on microsomal oxidation have focused on those substrates that are cytochrome P-450 dependent, yet few have examined the effects of ethanol on cytochrome P-448-mediated metabolism. The available data are conflicting. Benzopyrene hydroxylation has been reported to be increased in microsomal preparations after chronic ethanol feeding in some studies [12], but decreased in others [13]. Hydroxylation of zoxazolamine, also cytochrome P-448 mediated [4, 14], is increased both *in vivo* and *in vitro* after a brief ethanol exposure, but these results may not accurately reflect long-term effects of chronic ethanol administration. Therefore, we have examined the effects of chronic ethanol feeding on the metabolism of cytochrome P-448-mediated substrates in female Wistar rats both *in vivo* and *in vitro*. These results were compared to those obtained for the metabolism of aminopyrine, a known substrate for cytochrome P-450, in the same animals.

MATERIALS AND METHODS

Female Wistar rats (Harlan Industries, Indianapolis, IN) weighing 150–175 g were divided into two groups, housed in individual cages and pair-fed liquid diets containing either 36% ethanol or an isocaloric diet substituting maltose-dextrins for ethanol as previously described [15]. Dietary fat was constant in both groups (30%) as was dietary protein (18%). Previous studies from this laboratory have shown that animals fed in this manner consume an average of 3.70 g of absolute ethanol/day yielding an average

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blood ethanol concentration of approximately 125 mg/dl [16].

In vivo *studies. In vivo N*-demethylation of aminopyrine, caffeine and antipyrine, and *O*-dealkylation of phenacetin were measured after 8–12 weeks of chronic ethanol feeding. Ethanol-fed animals were withdrawn from ethanol and given control diet 18 hr before study to eliminate the possible interaction of continued metabolism of ethanol on test substrate metabolism. Breath tests utilizing [^{14}C-1-methyl]-caffeine (ICN, Irvine, CA; sp. act. 2.5 mCi/mmole), [^{14}C-4-dimethyl]aminopyrine (Amersham/Searle, Arlington Heights, IL; sp. act. 108 mCi/mmole), [^{14}C-ethyl]phenacetin (gift of Dr. Ian Calder, sp. act. 0.9 mCi/mmole) or [^{14}C-3-N-methyl]antipyrine (New England Nuclear, Corp., Boston, MA; sp. act. 54.1 mCi/mmole) were carried out as previously described [17]. Animals were housed in individual air-tight cages after intraperitoneal injection of tracer doses of caffeine (2.5 μCi), aminopyrine (1.0 μCi), phenacetin (1.0 μCi), or antipyrine (2.,5 μCi). Total exhaled $^{14}CO_2$ (15-min aliquots over 3 hr) was collected in 10 ml of methanol/monoethanolamine solution (2:1, v:v) after passage through concentrated sulfuric acid to remove water vapor. Ten ml of ACS II scintillation fluor (Amersham/Searle) was added to each vial and counted in an Isocap 300 liquid scintillation counter (Searle Analytic Inc., Des Plaines, IL). Counts per minute were converted to disintegrations per minute using an automatic external standard, and the breath elimination rate (k_{el}) for the beta phase of $^{14}CO_2$ disappearance was determined.

In a separate series of experiments, female Wistar rats were pretreated with either phenobarbital (80 mg/kg, i.p., daily for 3 days), 3-MC (20 mg/kg in corn oil, i.p., daily for 2 days), saline alone, or corn oil alone (i.p. daily for the same time) in equal volumes. Antipyrine breath tests were performed as described above, 24 hr after the last dose of pretreatment, in an effort to establish whether antipyrine *N*-demethylation is mediated by cytochrome P-448.

In vitro *studies*. Microsomal aminopyrine *N*-demethylase and antipyrine *N*-demethylase kinetics were determined *in vitro* in the same animals after sacrifice at 13 weeks of chronic ethanol feeding. Ethanol-fed animals were switched to control diet 18 hr prior to being killed and all animals received equal calories in the 24 hr before sacrifice. Microsomes were prepared by differential centrifugation after the animals were decapitated. The livers were perfused *in situ* with 4 vol. of ice-cold saline and homogenized in 4 vol. of ice-cold 0.25 M sucrose. The homogenate was centrifuged at 10,000 *g* for 15 min at 4° and the supernatant fraction was recentrifuged at 18,000 *g* for 15 min to remove mitochondria. The remaining supernatant fraction was then centrifuged at 105,000 *g* for 60 min in a Beckman model L5-65 ultracentrifuge using a 50.2 Ti rotor at 4°. The pellet was washed once with 1.15% KCl and resuspended in 50 mM Tris–HCl (pH 7.4). The final reaction mixture contained an NADPH-generating system consisting of final concentrations of 1.0 mM $NADP^+$, 10 mM glucose-6-phosphate, 1.0 unit of glucose-6-phosphate dehydrogenase, and 5.0 mM $MgCl_2$. Aminopyrine substrate concentration was varied between 0.66 and 10 mM, and antipyrine concentration was varied between 0.5 and 20 mM with approximately 1.0 mg microsomal protein/ml in a final volume of 3.0 ml of 100 mM Tris–HCl (pH 7.4). Protein was determined by the method of Lowry *et al.* [18] using bovine serum albumin as the standard. Generation of formaldehyde was measured by the method of Nash [19] after incubation in a Dubnoff metabolic incubator for 15 min for aminopyrine and 30 min for antipyrine. Reaction velocity was expressed as nmoles formaldehyde formed $\cdot hr^{-1} \cdot$ (mg microsomal protein)$^{-1}$. Michaelis constants (K_m and V_{max}) were calculated from the Hanes–Woolf transformation of the results expressing $[S]/v$ vs $[S]$ [20].

Statistics. Student's *t*-test (two-tailed) was used to compare the mean values in all groups using paired data with $P < 0.05$ accepted as the minimum level of significance. Results are expressed as mean ± S.E.M. unless otherwise stated.

RESULTS

All animals gained weight over the period of study. There was no significant difference in weight gain between the ethanol-fed animals (275 ± 8.6 g) and the pair-fed controls (271 ± 3.8 g) at the time they were killed. Calculated total microsomal protein was similar in both groups, 131 ± 3.8 mg for chronic ethanol and 121 ± 5.8 for pair-fed controls ($P > 0.3$). Liver weights were slightly higher in chronic ethanol-fed animals (11.1 ± 0.48 g) compared to pair-fed controls (10.2 ± 0.58 g) ($P < 0.05$).

In vivo *studies*. Pretreatment of rats with either phenobarbital or 3-MC increased the breath elimination rate of antipyrine *N*-demethylation above control values. Phenobarbital pretreatment resulted in a 123% increase above control (saline), whereas 3-MC pretreatment increased breath elimination rate 277% above control (corn oil) (Fig. 1A and B).

Chronic ethanol feeding increased the aminopyrine breath elimination rate 32% when compared to pair-fed controls ($P < 0.001$). Antipyrine *N*-demethylation was also increased by 20% compared to pair-fed controls ($P < 0.05$). There was no difference between chronic ethanol-fed animals and pair-fed controls, however, for either caffeine or phenacetin breath elimination rates (Table 1).

In vitro *studies*. *In vitro* determinations of *N*-demethylase activity for aminopyrine and antipyrine both confirmed that the increase in *in vivo* elimin-

Table 1. Breath elimination rate constants*

	Chronic ethanol	Pair-fed control	P value
Aminopyrine	13.48 ± 0.649	10.23 ± 0.533	< 0.001
Antipyrine	5.30 ± 0.357	4.42 ± 0.439	< 0.05
Phenacetin	23.48 ± 0.750	22.43 ± 1.35	NS†
Caffeine	5.20 ± 0.644	5.08 ± 0.781	NS

* $K_{el} \times 10^{-3}$ min^{-1}. Each value is the mean ± S.E.M. of six pairs of animals.

† NS, not significant.

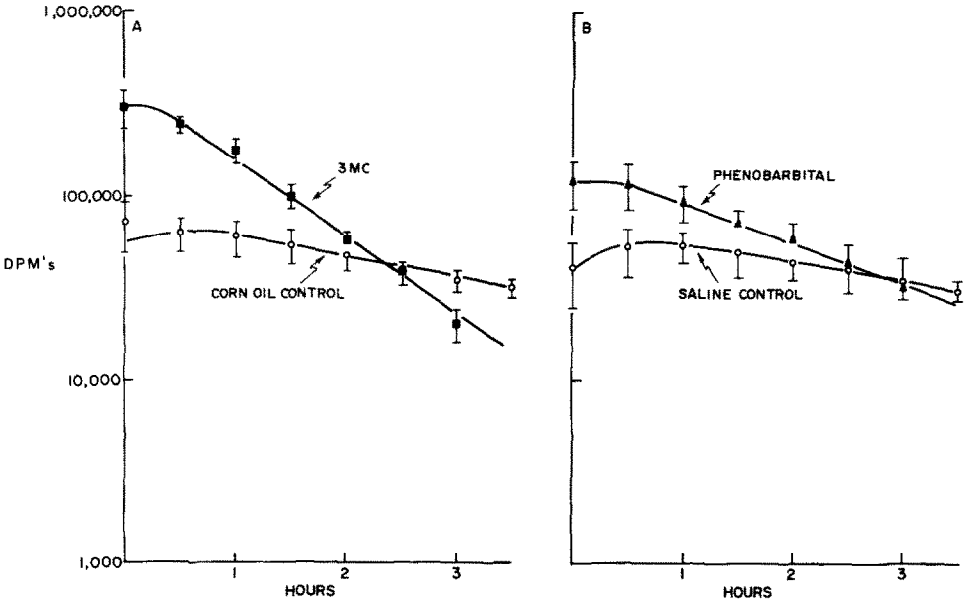

Fig. 1. Effects of pretreatment with 3-MC or PB on the [^{14}C]antipyrine breath test. (A) Rats were pretreated with 3-methylcholanthrene (20 mg/kg in corn oil) or an equal volume of corn oil, i.p., for 2 days. [^{14}C]Antipyrine breath tests were performed 24 hr after the last dose of 3-MC. Results are shown as mean dpm ± S.E.M. for six animals. The mean k_{el} for animals pretreated with 3-MC was 16.29×10^{-3} min^{-1} compared to 4.32×10^{-3} min^{-1} for controls. (B) Rats were pretreated with phenobarbital (80 mg/kg) in saline or an equal volume of saline, i.p., for 3 days. [^{14}C]Antipyrine breath tests were performed 24 hr after the last dose of phenobarbital. Results are shown as mean dpm ± S.E.M. for six animals. The k_{el} for animals pretreated with phenobarbital was 8.22×10^{-3} min^{-1} compared to 3.68×10^{-3} min^{-1} for controls.

ation was due to an increase in enzyme activity for the substrates. V_{max} was increased in chronic ethanol-fed animals 30% above pair-fed controls for aminopyrine *N*-demethylase and 27% above pair-fed controls for antipyrine *N*-demethylase ($P < 0.05$). In addition, the Michaelis affinity constant, K_m, was lower in chronic ethanol-fed animals compared to pair-fed controls for both aminopyrine and antipyrine *N*-demethylases, suggesting some qualitative change in the enzymes as well as an increase in amount of enzyme present per mg of microsomal protein (Fig. 2A and B, and Table 2).

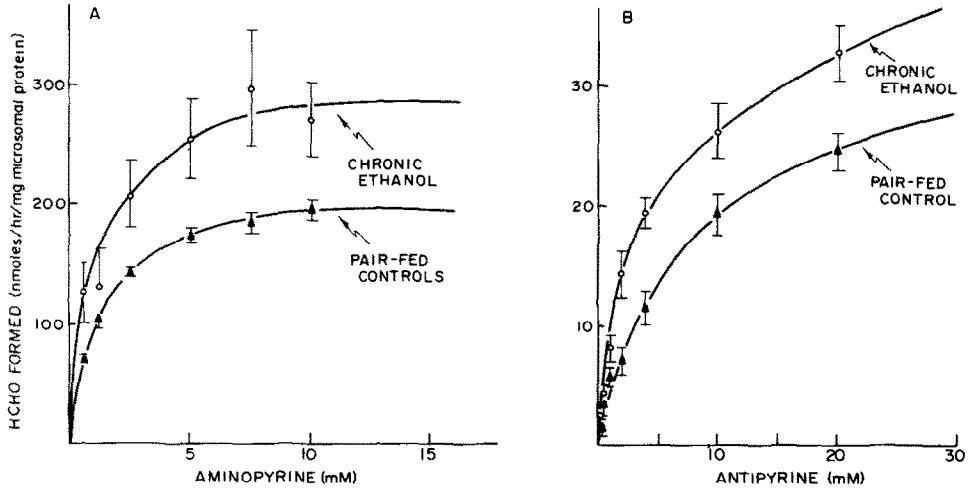

Fig. 2. Microsomal enzyme activities after chronic ethanol feeding. (A) Microsomal aminopyrine *N*-demethylase activity was measured in animals chronically fed ethanol and their pair-fed controls. Results shown are the mean nmoles formaldehyde formed · hr^{-1} · (mg microsomal protein)$^{-1}$ ± S.E.M. for five pairs of animals. (B) Microsomal antipyrine *N*-demethylase was measured in the same animals as above. Results shown are the mean nmoles formaldehyde formed · hr^{-1} · (mg microsomal protein)$^{-1}$ ± S.E.M. for five pairs of animals.

Table 2. Kinetic constants*

	Chronic ethanol	Pair-fed control	P value
Aminopyrine N-demethylase			
V_{max}†	289 ± 22.2	222 ± 7.2	< 0.05
K_m‡	1.07 ± 0.10	1.36 ± 0.09	< 0.02
Antipyrine N-demethylase			
V_{max}†	39.5 ± 3.23	31.0 ± 2.11	< 0.05
K_m‡	4.22 ± 0.74	6.06 ± 0.59	< 0.05

* Each result is the mean ± S.E.M. for five pairs of animals.

† Results are expressed as nmoles formaldehyde formed · hr^{-1} · (mg microsomal protein)$^{-1}$.

‡ Results are expressed as mM.

DISCUSSION

Aminopyrine, caffeine, phenacetin and antipyrine are all substrates for hepatic microsomal mixed function oxidases [9–11, 17]. All substrates, with the exception of phenacetin [21], are low extraction drugs whose clearance from blood is dependent on the activity of hepatic microsomal enzymes and is independent of liver blood flow [22]. Previous studies using the [^{14}C]aminopyrine breath test have shown that the clearance of aminopyrine from blood is closely correlated with the breath elimination rate (k_{el}) for disappearance of $^{14}CO_2$ from the breath of laboratory animals and man [23, 24]. This finding has led to increasing use of $^{14}CO_2$ breath tests as a relatively noninvasive measure of *in vivo* hepatic drug metabolism in the rat. The assumption that the breath elimination rate parallels plasma drug clearance has been further verified by us for aminopyrine in chronic ethanol-fed rats [25].

The increase in aminopyrine demethylation shown here both *in vivo* and *in vitro* is consistent with the results of previous studies which demonstrate that chronic ethanol feeding induces cytochrome P-450-mediated drug metabolism in the rat [1–3]. In contrast to these results is the finding that chronic ethanol feeding did not increase either caffeine demethylation or phenacetin dealkylation *in vivo*. Both caffeine and phenacetin metabolism are thought to be mediated by cytochrome P-448 (P-450$_c$) [9, 11].

Antipyrine elimination from blood is mediated by several apparently different pathways, all of which involve hepatic microsomal oxidation in the rat [10, 26]. The *in vivo* demethylation of antipyrine to norantipyrine has been reported to be induced by 3-MC in the rat as demonstrated by a greater production of $^{14}CO_2$ from [^{14}C]antipyrine labeled in the 3-*N*-methyl group [10]. However, other studies have not found a greater amount of norantipyrine appearing in 24-hr urine collections from rats pretreated with 3-MC [26]. Our data would suggest that antipyrine *N*-demethylation is induced by both phenobarbital and 3-MC, although 3-MC appeared to be a better inducer than phenobarbital. These data, therefore, suggest that the conversion of antipyrine to norantipyrine may not be mediated exclusively by cytochrome P-448.

Chronic ethanol feeding resulted in an increase in the breath elimination rate of antipyrine *in vivo* when compared to pair-fed controls. In addition, antipyrine *N*-demethylase activity was also increased in chronically ethanol-fed animals. The increase in *N*-demethylation of antipyrine by chronic ethanol feeding is consistent with an increase in cytochrome P-450-mediated *N*-demethylation of antipyrine which also occurs after pretreatment of rats with phenobarbital. Considering the effects of chronic ethanol feeding on the substrates tested, we conclude that the induction of cytochrome P-450-mediated metabolism by ethanol is quantitatively more significant than the induction by cytochrome P-448-mediated metabolism. These findings appear to differ from those of Rubin *et al.* [12] who reported an increase in benzopyrene hydroxylation after chronic ethanol feeding. In that study, however, rats were fed diets which were deficient in both protein (5%) and choline, as well as receiving chronic ethanol. Previous work has shown that the effects of chronic ethanol feeding on induction of microsomal metabolism are enhanced in animals fed protein-deficient diets [24]. The report of Capel *et al.* [13], on the other hand, shows that benzopyrene hydroxylation was actually decreased in microsomes from mice fed chronic ethanol diets. Since we have not measured benzopyrene hydroxylation, our results cannot be compared directly to these previous studies. In addition to differences in experimental design, there may be substrate heterogeneity within the cytochrome P-448 monooxygenases, which may also overlap partially with the cytochrome P-450 system. Ethanol may induce only some of these enzymes without affecting all of them.

The decreases in the Michaelis affinity constant (K_m) for aminopyrine *N*-demethylase and antipyrine *N*-demethylase are of note since these changes suggest that a qualitative change in the enzyme may have resulted from chronic ethanol feeding. Changes in the K_m of aminopyrine demethylase have also been noted after 3-MC pretreatment [27]. It has been suggested that chronic ethanol feeding induces a form of cytochrome P-450 which is distinct from those induced by phenobarbital and 3-MC [28, 29]. The decreases in the K_m of these enzymes indicate a greater affinity of the enzymes for the substrate and are consistent with the hypothesis that chronic ethanol exposure induces a qualitatively distinct form of cytochrome P-450 with differences in substrate specificity. Previous studies have shown that, using monospecific antibodies in untreated rats, quantitatively only 7% of total cytochrome P-450 is immunochemically identical with purified PB-P-450 and 6% with MC-P-450. Arochlor 1254 treatment increases the percentage of both PB-P-450 and MC-P-450 to 47 and 48%, respectively [30]. It is possible that chronic ethanol feeding may also change the percentage of other forms of P-450 as well as inducing a new form of the enzyme. Further studies using monospecific antibody techniques are needed to determine the overall spectrum of induction by chronic ethanol feeding.

Chronic ethanol feeding may also influence microsomal membrane fluidity leading to alterations in the affinity of cytochrome monooxygenases for sub-

strates; however, no data are currently available regarding this hypothesis. The fluidity of microsomal membranes has been shown to affect drug binding and metabolism in other situations [31, 32] and chronic ethanol feeding results in changes in the fluidity of enterocytes basolateral membranes in the rat [33]. However, no studies of the effects of chronic ethanol feeding on microsomal membrane fluidity and drug metabolism are available to assess any such interrelationship.

Decrease in the K_m of an enzyme may result in an increase in the intrinsic clearance (Cl_{int}) of a drug by the liver even if the maximum velocity of the enzyme remains unchanged, since intrinsic clearance may be defined as follows: $Cl_{int} = V_{max}/K_m$ [21, 34, 35]. Accordingly, both a decrease in the K_m of aminopyrine and antipyrine demethylases and a corresponding increase in V_{max} for these same enzymes should increase the intrinsic clearance of these drugs. Although kinetic constants derived from *in vitro* studies of metabolism in subcellular components may not accurately reflect the kinetic constants which might apply *in vivo*, our data suggest that these values change in the correct direction, as predicted by *in vivo* breath tests. However, it is not possible to establish the relative contribution to the overall *in vivo* effect of changes in either parameter (K_m or V_{max}) independently.

In conclusion, we have shown that the induction of microsomal drug oxidation which occurs after chronic ethanol feeding in the rat exhibits substrate heterogeneity. Chronic ethanol feeding also appears to result in a qualitative change in the microsomal mixed function oxidases. Furthermore, there appears to be a differential effect of ethanol on the induction of cytochrome P-448- and cytochrome P-450-mediated substrates, as those substrates which were metabolized by cytochrome P-448 seemed to be relatively unaffected by chronic ethanol feeding, whereas cytochrome P-450 substrates were induced. This differential effect may be important in further consideration of the relationship between ethanol intake and chemical carcinogenesis which may be induced by metabolism of potential carcinogens by cytochrome P-448-mediated enzymes.

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