Pergamon

Biochemical Pharmacology, Vol. 49, No. 11, pp. 1665–1673, 1995.
Elsevier Science Ltd
Printed in Great Britain.

0006-2952(95)00098-4

RAPID CHANGES IN CYTOCHROME P4502E1 (CYP2E1) ACTIVITY AND OTHER P450 ISOZYMES FOLLOWING ETHANOL WITHDRAWAL IN RATS

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(*Received* 7 *May* 1994; *accepted* 28 *December* 1994)

Abstract—This study describes the effects of chronic ethanol (ETOH) treatment and withdrawal on the rat hepatic mixed-function mono-oxygenase system. Male Sprague–Dawley rats (150–200 g, 10 per group) were administered ETOH as part of the Lieber–deCarli liquid diet for 3 weeks. Ethanol was removed, and the animals were euthanized at 0, 24, 48, 72 and 168 hr post-withdrawal. Microsomes were prepared, and ethanol-inducible cytochrome P4502E1 (CYP2E1) activity was measured using the enzyme markers *N*-nitrosodimethylamine demethylase (NDMAd), *p*-nitrophenol hydroxylase (PNPH) and aniline hydroxylase (AH). Activities were found to be induced significantly after chronic ETOH feeding using all three assays (NDMAd, 5-fold; PNPH, 3.5-fold; AH, 9-fold). Upon ETOH withdrawal, all three activities dropped markedly, with NDMAd and PNPH at control values at 24 hr and all subsequent time points. AH activity remained 3-fold higher than controls at 24, 48 and 72 hr. Western blot analyses showed that immunoreactive CYP2E1 returned to control at 24 hr, consonant with NDMAd and PNPH activities. The prolonged induction of AH activity following ETOH withdrawal indicates that it is not a specific marker of CYP2E1-catalyzed reactions. Collectively, these data are suggestive of a rapid mechanism of CYP2E1 degradation in the rat liver. Of the other parameters investigated in this study, total cytochrome P450 content was increased 2.5-fold after ETOH feeding, with levels dropping markedly 24 hr post-withdrawal. NADPH-dependent cytochrome *c* reductase activity was unchanged throughout the course of the study. CYP1A1, CYP2B1 and CYP3A activities were assessed by the substrate probes ethoxyresorufin *O*-dealkylase (EROD), pentoxyresorufin *O*-dealkylase (PROD) and erythromycin *N*-demethylase (ERNd). EROD and PROD were induced significantly by ETOH administration (2-fold) at 0 hr, with EROD remaining elevated over controls 24 hr post-withdrawal. Quantitative western blot analysis of CYP1A1 and CYP2B1 revealed a pattern of immunostaining generally consistent with but less variable than levels predicted by the respective substrate markers. Both proteins were induced significantly by chronic ethanol administration (CYP1A1, 1.9-fold; CYP2B1, 4-fold). Induction of these P450 isoforms persisted for several days following withdrawal. In contrast, immunoreactive CYP1A2 was found to decrease significantly (by 30–40%) during ethanol withdrawal (24, 48, 72, 168 hr). ERNd activity was induced significantly by chronic ETOH feeding (2.5-fold) and remained so for 24 hr into the withdrawal period (2-fold). Immunoreactive CYP3A1 was also induced significantly following ETOH administration (0 hr) and 24 hr following withdrawal. Collectively, the data presented suggest that chronic ethanol feeding induces *in vivo* at least four distinct P450 isoforms: CYP2E1, CYP1A1, CYP2B1 and CYP3A1. Ethanol withdrawal resulted in a 30–40% loss of CYP1A2. CYP1A1, CYP2B1 and CYP3A1 were elevated during ethanol withdrawal, unlike CYP2E1, a protein that undergoes rapid degradation following the removal of ethanol. Such findings are consistent with several distinct mechanisms of P450 isoform induction/degradation by ethanol.

Key words: ethanol; withdrawal; CYP1A; CYP2E1; CYP2B1; CYP3A

The oxidation of ETOH† is accomplished via the activities of ADH, MFO, and catalase. Although all three pathways have been studied extensively over the past 10–20 years, the relative contributions of one or all of these enzyme pathways to overall ETOH oxidation are still not clear. The difficulty arises from the fact that the contribution of MFO may shift according to drug administration and

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† Abbreviations: ETOH, ethanol; CYP2E1, cytochrome P4502E1; 2E1, the principal ethanol-inducible cytochrome P450 form; CYP1A1 and CYP1A2, the major benzo[*a*]-pyrene/3-methylcholanthrene-inducible rat cytochromes P450; CYP2B1, the primary phenobarbital-inducible cytochrome P450; CYP3A1, the major rat cytochrome P450 inducible by dexamethasone catalyzing the *N*-demethylation of erythromycin; ADH, alcohol dehydrogenase; AH, aniline hydroxylase; NDMA, *N*-nitrosodimethylamine; NDMAd, *N*-nitrosodimethylamine demethylase; PNPH, *p*-nitrophenol hydroxylase; EROD, ethoxyresorufin *O*-dealkylase; PROD, pentoxyresorufin *O*-dealkylase; ERNd, erythromycin *N*-demethylase; and MFO, mixed-function mono-oxygenase system.

various pathophysiological states. A specific isozyme of the cytochrome P450 gene family, CYP2E1, is largely responsible for the NADPH-dependent oxidation of ETOH to acetaldehyde in rats and humans, and is preferentially induced by its substrate. In humans, CYP2E1 oxidizes many low M_r cancer suspects such as NDMA [1] to their bioactive forms, with epidemiological evidence supporting a causal relationship between exposure to nitrosamines and the development of human cancer [2]. Others have proposed that CYP2E1 is pivotal in alcohol-induced liver damage, possibly by causing perturbations in lipid peroxidation [3]. The amount and activity of CYP2E1 are increased by ETOH, fasting, high fat feeding, diabetes and other low M_r organics, each of which may invoke one or more regulatory mechanisms (see Refs. 4 and 5 for reviews).

The present study, conducted on male rats, describes the use of three enzyme markers (AH, PNPH and NDMAd) to detect CYP2E1 activity during ETOH withdrawal. CYP2E1 levels were also assessed using a polyclonal antibody specific to the rat antigen, as there is evidence that catalytic deactivation of CYP2E1 precedes a drop in immunoreactive protein [6]. Alcohol withdrawal has been investigated previously using human [7, 8] and rodent [9, 10] models; however, the absence of isozyme specific probes in earlier studies clouds the interpretation of these data. Considering the potential of ETOH to modulate the activities of other P450 isozymes such as CYP1A1 [10], CYP2B1 [10, 11] and to a lesser extent CYP3A [11], the activities of these three isozymes were also determined using enzyme markers and quantitative western blotting. From this study, an animal model was chosen using the Lieber–deCarli liquid diet in a free access feeding paradigm. The purpose of this animal model is to remove the ambiguities presented by pharmacological and cigarette coadministration on P450 isozyme content (two factors frequently encountered in alcoholism). Data concerning the effects of ETOH withdrawal on the male rat hepatic MFO are presented herein.

MATERIALS AND METHODS

Materials

Lieber–deCarli shake and pour liquid diet (LD'82) and isocaloric control were obtained from Bio-Serv (Frenchtown, NJ), as were all feeding tubes and accessories. Ethanol (190 proof) was purchased from the Midwest Grain Products Co. (Atchison, KS). Ethoxyresorufin, pentoxyresorufin, resorufin, erythromycin, cytochrome *c* (horse heart), NADPH (tetra sodium salt), isocitric dehydrogenase, NADP (sodium salt) and isocitric acid were obtained from the Sigma Chemical Co. (St. Louis, MO). NDMA, *p*-nitrocatechol and *p*-nitrophenol were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Sodium dithionite, aniline hydrochloride and *p*-aminophenol were obtained from Kodak-Eastman Fine Chemicals (Rochester, NY). Glacial acetic acid was purchased from the Mallinkrodt Specialty Chemical Co. (Paris, KY). DMSO and potassium cyanide were obtained from the Fisher Scientific Co. (Fair Lawn, NJ). NAD–ADH ETOH determination kits were purchased from the Sigma Chemical Co. Goat anti-rabbit secondary antibodies conjugating with alkaline phosphatase were obtained as a kit from Kirkegaard & Perry Laboratories (Gaithersburg, MD). All other reagents and laboratory consumables were of the highest grade commercially available.

Animals and treatment

Outbred male Sprague–Dawley rats (150-200 g) were obtained from Taconic Farms (Germantown, NY) and kept in temperature-controlled rooms with a 12-hr light–dark cycle. Throughout the study, animals were housed, maintained and treated in accordance with the guidelines of the National Institutes of Health. Rats were administered ETOH (35% of total calories) as part of the Lieber-de Carli liquid diet. Two additional groups were pair fed isocaloric maltose dextrin liquid control. Animals were allowed access to ETOH for 3 weeks, after which time the ETOH was immediately withdrawn (time 0 hr), and substituted with liquid control. At this time, tail veins from each animal in the study were nicked, 50 μL of blood was extracted, and ETOH levels were determined using an NAD–ADH reagent kit. Animals (10 per group) were decapitated at 0, 24, 48, 72 and 168 hr, with pair-fed controls (10 per group) euthanized at 0 and 168 hr. Throughout the study "0 hr" is used to describe chronic ETOH administration. Following decapitation, livers were removed immediately, and hepatic microsomes were prepared.

Liver sub-fractionation

Liver microsomes were prepared as described previously [12]. Aliquots (2 mL) of the microsomal preparation were stored at −70° in 0.1 M Tris–HCl, pH 7.4, 20% (w/v) glycerol, at a protein concentration of approximately 5 mg/mL. All samples were thawed once only for enzymatic analyses.

Enzyme assays

Total P450 and NADPH-dependent cytochrome c reductase. Total hepatic microsomal cytochrome P450 content was measured according to the carbon monoxide (CO) difference spectrum procedure originally described by Omura and Sato [13]. The assay was further optimized according to the specifications of Rutten *et al.* [14] using a molar extinction coefficient of 91 $mM^{-1}\,cm^{-1}$. NADPH cytochrome *c* reductase activity was determined according to Williams and Kamin [15] with modifications. The final assay volume (1.1 mL) contained the following: 25 mM NADPH, 31 mM cytochrome *c*, 100 mM KCN, 87 mM Tris–HCl, pH 7.4, and 100–150 mg of microsomal protein. The reaction was started in the cuvette by the addition of NADPH, and the rate was measured on a Beckman model DU-65 spectrophotometer at 550 nm at 25° for 5 min. Blanks contained no NADPH. Activity was calculated using an extinction coefficient of 21 $mM^{-1}\,cm^{-1}$.

Enzyme markers of CYP2E1 activity. AH activity was assessed using the method of Imai and Sato [16] with the following minor modifications. The reaction mixture consisted of 6 mM $MgCl_2$, 1.2 mM NADP,

9.8 mM isocitric acid, 0.2 U isocitric dehydrogenase, 8 mM aniline hydrochloride, 2% glycerol, 0.46% KCl, 18 mM Tris–HCl, pH 7.4, and 1 mg microsomal protein in a total volume of 1.0 mL. Blanks and standards contained no NADPH-generating system and standards contained 10–100 nmol *p*-aminophenol. The reaction, carried out at 37° for 20 min, was commenced by the addition of isocitric dehydrogenase and terminated by the addition of 1.0 mL of 10% trichloroacetic acid. The incubate was centrifuged at 1000 *g* for 15 min, and 1.0 mL of supernatant was added to 0.5 mL of 10% Na_2CO_3 and 1 mL of 1% phenol in 0.1 N NaOH. The change in absorbance was measured at 629 nm after 30 min on a Beckman model DU-65 spectrophotometer. NDMAd activity was determined by the Hantzsch reaction [17], as per the modified method described by Peng *et al.* [18]. Blanks contained no NADPH-generating system. An NDMA substrate concentration of 0.05 mM was used in this study. PNPH activity was measured according to the protocol described by Carlson [19] with a minor modification: 0.5 mM NADPH was used in the final incubation mixture instead of 1 mM. The assay procedure was otherwise identical.

*Enzyme markers of CYP*1A1, *CYP*2B1 *and CYP*3A *activity*. EROD and PROD activities were determined essentially according to the method of Burke *et al.* [20] with modifications and optimization of assay procedures as described by Rutten *et al.* [21]. The rate of resorufin formation was quantitated using a Perkin–Elmer model LS 50B spectrofluorimeter at an excitation wavelength of 530 nm and an emission wavelength of 585 nm. Resorufin standard was used to calibrate activity. ERNd activity was measured using the formation of formaldehyde via the Hantzsch reaction [17]. Briefly, the incubation mixture consisted of 0.4 mM NADPH, 10 mM $MgCl_2$, 1 mM erythromycin (made as a 200 mM stock in DMSO), 450 mM $NaPO_4$ buffer, pH 7.4, and 250 μg of microsomal protein in a total volume of 2 mL. The reaction was started with the addition of NADPH and allowed to proceed for 30 min at 37° before termination with 0.1 mL of 25% (w/v) $ZnSO_4$ and 0.1 mL of saturated $BaOH_2$. Tubes were centrifuged at 1000 *g* for 20 min, and 1 mL of supernatant was added to 0.45 mL of concentrated Nash reagent prepared as described previously [18]. Tubes were mixed thoroughly, incubated for 1 hr at 60°, and centrifuged at 1000 *g* for 25 min. Aliquots (1 mL) of supernatant were extracted, and the absorbance was read at 412 nm. Activity was calibrated against a formaldehyde standard curve, with NADPH blanks included for each sample. All protein measurements were carried out using the Pierce BCA protein assay reagent kit (Rockford, IL).

Immunoblot analyses

The preparation and properties of the polyclonal antibodies raised to CYP2E1 and CYP3A1 have been described previously [22–24]. These antibodies have been reported to be highly specific to their rat antigens [22–24]. Polyclonal antibodies to CYP1A1, CYP1A2 and CYP2B1 were the gifts of Dr. James Hardwick (Department of Biochemistry and Molecular Biology, Ohio Northeastern University, Kent, OH). Immunoblot analyses following gel electrophoresis were performed as outlined by Gonzalez *et al.* [22] with minor modifications. After the microsomal protein was resolved by gel electrophoresis [10% (w/v) SDS–polyacrylamide] and the proteins were transferred, nitrocellulose filters were incubated with 3% (w/v) non-fat dry milk in 15 mM PBS, pH 7.4, for 3 hr. After washing the membrane three times with PBS (10 min each), the membranes were incubated overnight with primary polyclonal antisera. After further washing with PBS (3 × 10 min), they were incubated with secondary antibody (goat anti-rabbit IgG) for 3 hr

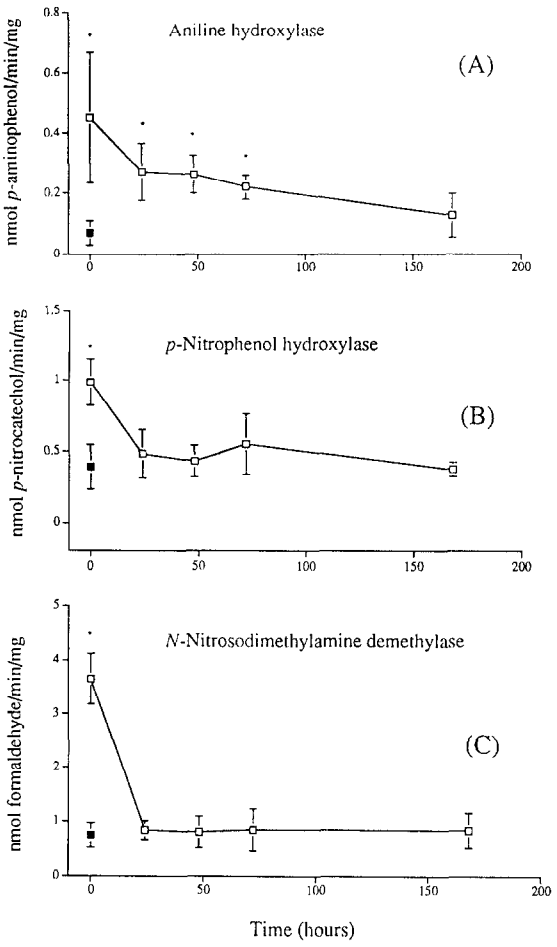

Fig. 1. Effect of ETOH administration and withdrawal enzyme markers of CYP2E1 activity. Control (■) and ETOH (□) fed rats were euthanized at the time points indicated (0, 24, 48, 72 and 168 hr), with microsomal preparation and enzyme assays performed as described in Materials and Methods. Results are expressed as means ± SD of groups of 6–9 animals. Catalysis rates of each reaction are displayed on the Y axis as nanomoles of reaction product formed per minute per milligram of microsomal protein. The X axis denotes the number of hours after ETOH removal from the diet. Control and ETOH treatment data were compared using ANOVA, and levels of statistical significance were determined by Student's *t*-test, as described in Materials and Methods. Key: (*) $P < 0.05$ vs control.

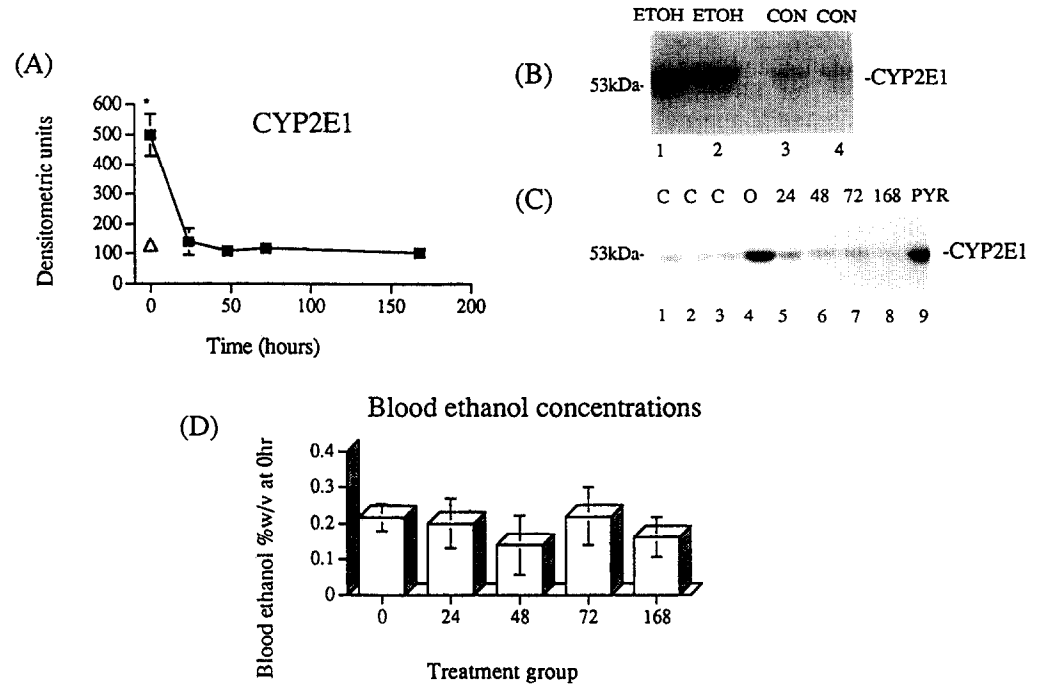

Fig. 2. Immunoblot analyses of microsomal CYP2E1 from control and ETOH fed rats. In panels A, B, and C, western blots of rat hepatic microsomal protein (5 μg/well) were performed on ETOH-treated (■) and control (△) animals using a polyclonal antibody specific to the rat CYP2E1 antigen. Panel A shows the mean ± SD scanned immunoreactive CYP2E1 from 6 animals in each treatment group (randomly selected). Microsomal preparation, immunoblotting and densitometric scanning were carried out as described in Materials and Methods. Key: (*) $P < 0.05$ vs control. Panel B shows the relative quantity of immunoreactive CYP2E1 from ETOH rats (lanes 1 and 2) compared with controls (lanes 3 and 4) after 3 weeks of pair feeding with the Lieber–deCarli ethanol diet (0 hr). Panel C is a representative blot of immunoreactive CYP2E1 levels following ETOH administration and withdrawal. Lanes 1–3 correspond to control; lane 4, 0 hr; lane 5, 24 hr; lane 6, 48 hr; lane 7, 72 hr; lane 8, 168 hr; and lane 9, pyridine treated. The M_r of the band is shown on the left. Panel D shows the mean ± SD blood ethanol concentration of every animal used in this study at time 0 hr (9–10 per group, 48 in all). Blood ethanol determination was performed as described in Materials and Methods. Control and ETOH treatment data were compared using ANOVA, and levels of statistical significance were determined by Student's *t*-test as described in Materials and Methods.

and visualized as previously indicated [24]. All immunoblotting was performed on microsomal protein prepared from individual rats, and was repeated in different animals from the same treatment groups. Western blots were quantitated using a scanning laser densitometer, using a single microsomal sample as an internal standard from blot to blot. Several concentrations of protein were chosen to ensure a linear detection range of immunostained protein. Results are expressed as relative densitometric units.

Statistical analyses

All data were analyzed using an ANOVA factorial measures design. Statistical significance was determined using Student's unpaired *t*-test with $P \leq 0.05$ considered a significant effect. Results are expressed throughout the study as means ± SD.

RESULTS

Animal behavior following ETOH withdrawal and blood ETOH concentrations

Rats exhibited activity typically associated with ETOH withdrawal symptoms, including increased irritability upon handling and in 2 animals, clonic seizures subsequently resulting in death. To ensure that differences in enzyme activity were not due to variable ETOH concentrations at 0 hr, blood concentrations were determined in each animal. No significant differences were observed in any of the groups, with the average blood ETOH concentration 0.2 mg/mL (see Fig. 2D).

Immunochemical and enzymatic determination of CYP2E1 activity following ETOH withdrawal

CYP2E1 activity was assessed using three enzyme markers: PNPH, NDMAd and AH (Fig. 1). At time 0 hr, all markers of CYP2E1 were elevated significantly over controls ($P < 0.05$). Mean AH, NDMAd and PNPH activities were 9-, 5- and 3.5-fold higher than control means after 3 weeks of ETOH administration. Twenty-four hours after ETOH removal, CYP2E1 activities had returned to control levels according to the enzyme markers NDMAd and PNPH. Furthermore, these activities were significantly different from their 0-hr counterparts ($P < 0.05$). Although AH activity dropped to

1/3 of its 0-hr value, this was still significantly higher than controls and remained so at 24, 48 and 72 hr ($P < 0.05$), returning to control levels 168 hr post-withdrawal. NDMAd and PNPH activities showed no significant changes relative to controls at any of the other time points.

Densitometric scanning of six animals randomly selected from the treatment groups revealed a 4.5-fold induction of CYP2E1 in comparison with controls ($P < 0.05$, Fig. 2A). At 24 hr there was no significant induction of CYP2E1 compared with controls, or at any subsequent time points. Immunoblotting of hepatic microsomes revealed the presence of an intensely stained band at 53 kDa, corresponding to the M_r of rat hepatic CYP2E1 (Fig 2, B and C). Figure 2B shows the effects of ETOH administration on hepatic CYP2E1 protein. Lanes 1 and 2 correspond to ETOH-fed animals and lanes 3 and 4 to controls. Figure 2C is a representative blot, showing the effect of ETOH withdrawal on CYP2E1 protein levels. At 0 hr (lane 4), immunoreactive CYP2E1 protein was induced, dropping markedly 24 hr following ETOH withdrawal (lane 5). Lanes 6, 7 and 8 represent immunostained protein from microsomes prepared 48, 72 and 168 hr post-withdrawal. Lane 9 denotes rats treated with pyridine, a positive control for CYP2E1 induction. No immunostaining at lower M_r, indicative of CYP2E1 degradation products, was observed. Blood ethanol determination performed at 0 hr on all rats in this study (Fig. 2D) showed a mean concentration of approximately 0.2% (w/v) in all groups. No significant differences were observed between any of the treatment groups.

Total hepatic cytochrome P450, NADPH-dependent cytochrome c reductase, EROD, PROD, immunoreactive CYP1A1/2 and CYP2B1 following ETOH withdrawal

Total hepatic cytochrome P450 content was increased significantly (2.5-fold, $P < 0.05$) after ETOH administration at time 0 hr (Fig. 3A). Mean P450 values continued to be elevated through the withdrawal period; however, none of these changes was statistically significant. Hepatic NADPH-dependent cytochrome *c* reductase activity was not altered significantly from controls at any of the time points (Fig. 3D). EROD and PROD activities were increased significantly ($P < 0.05$) by ETOH treatment at 0 hr (2-fold higher, Fig. 3, B and C). Mean EROD activity (CYP1A1 catalyzed) remained elevated over controls at 24, 48 and 72 hr post ETOH withdrawal with significant increases observed at 24 hr ($P < 0.05$). This pattern was also observed with mean PROD activity (CYP2B1 catalyzed); however, none of these changes was statistically significant. Western blot analysis of microsomal protein using a CYP1A1 specific antibody (Fig. 4A) revealed a small but significant induction after chronic ethanol administration (1.9-fold, $P < 0.05$), which persisted up to 72 hr post-withdrawal. Immunoblotting with a CYP1A2 specific antibody (Fig. 4B) showed no significant induction of this P450 isoform by ethanol. Instead, a significant loss (30–40%, $P < 0.05$) in CYP1A2 was observed at all withdrawal time points. Immunoblotting of CYP2B1 (Fig. 4C) showed the appearance of two bands, with closely related molecular weights. The apical band corresponds to CYP2B1 (M_r 52 kDa). Densitometric scanning revealed marked induction of CYP2B1 by ethanol (4-fold, $P < 0.05$), maximal at 0 hr, but significantly higher than controls for up to 1 week following ethanol withdrawal (1.5-fold at 168 hr, $P < 0.05$).

ERNd activity following ETOH withdrawal and immunoreactive CYP2A1

Ethanol administration significantly elevated CYP3A-catalyzed ERNd activity (2.5-fold, $P < 0.05$, Fig. 3E). Mean activity remained higher than controls at 24, 48, 72 and 168 hr post-withdrawal, with a statistically significant effect observed only at 24 hr (2-fold, $P < 0.05$). Immunoblotting with a polyclonal antibody specific to rat glucocorticoid inducible CYP3A1 (Fig. 4D) revealed the presence of a dark staining band at 52 kDa and a lighter staining band at 49 kDa. The higher M_r protein corresponded to CYP3A1, and showed increased amounts at 0 and 24 hr. Densitometric analysis showed CYP3A1 to be induced significantly at 0 and 24 hr (2-fold, $P < 0.05$). At other withdrawal time points, the staining was variable from animal to animal and was not found to be significantly higher than controls.

DISCUSSION

Enzyme marker and immunoblot analyses showed a significant elevation in CYP2E1 following chronic ETOH administration. The substrate probes PNPH and NDMAd exhibited 3.5- and 5-fold changes over controls in their respective CYP2E1-catalyzed reactions. These changes were consistent with those previously reported in the literature following treatment with the Lieber–deCarli diet [10, 25, 26]. Observations from rat studies suggest that hepatic microsomal AH activity is elevated after high caloric ETOH feeding [9, 10, 25], and in the case of Ref. 9 remained so for several days after ETOH clearance returned to control levels. In humans, reports conflict. Clark and Senior [27] found enhanced ETOH clearance 14 days after ETOH abstinence, as opposed to Mezey and Tobon [8], who found ETOH clearance at control levels 7 days post-withdrawal. In the latter study, MFO activity was still elevated for at least 7 days after the cessation of elevated ETOH clearance. Apparent discrepancies in correlating ETOH clearance with MFO may be explained by cross-reactivity of the P450 isozyme system. For example, Koop *et al.* [28] have suggested that only 40% of PNPH is CYP2E1 catalyzed in control microsomes. Upon ETOH administration this percentage shifts, with PNPH activity predominantly mediated by CYP2E1 (85%, [28]). The increases in AH activity reported during withdrawal in this and previous studies are therefore likely to be due to the induction of other P450s. As our data show, the rapid turnover of CYP2E1 *in vivo* questions the use of measuring CYP2E1 several hours after the removal of ETOH. Using a CYP2E1 specific antibody, immunochemical detection of microsomal CYP2E1 content was found to be most accurately reflected by NDMAd activity. Induction was 4.5 to 5-fold higher than that of the pair-fed controls.

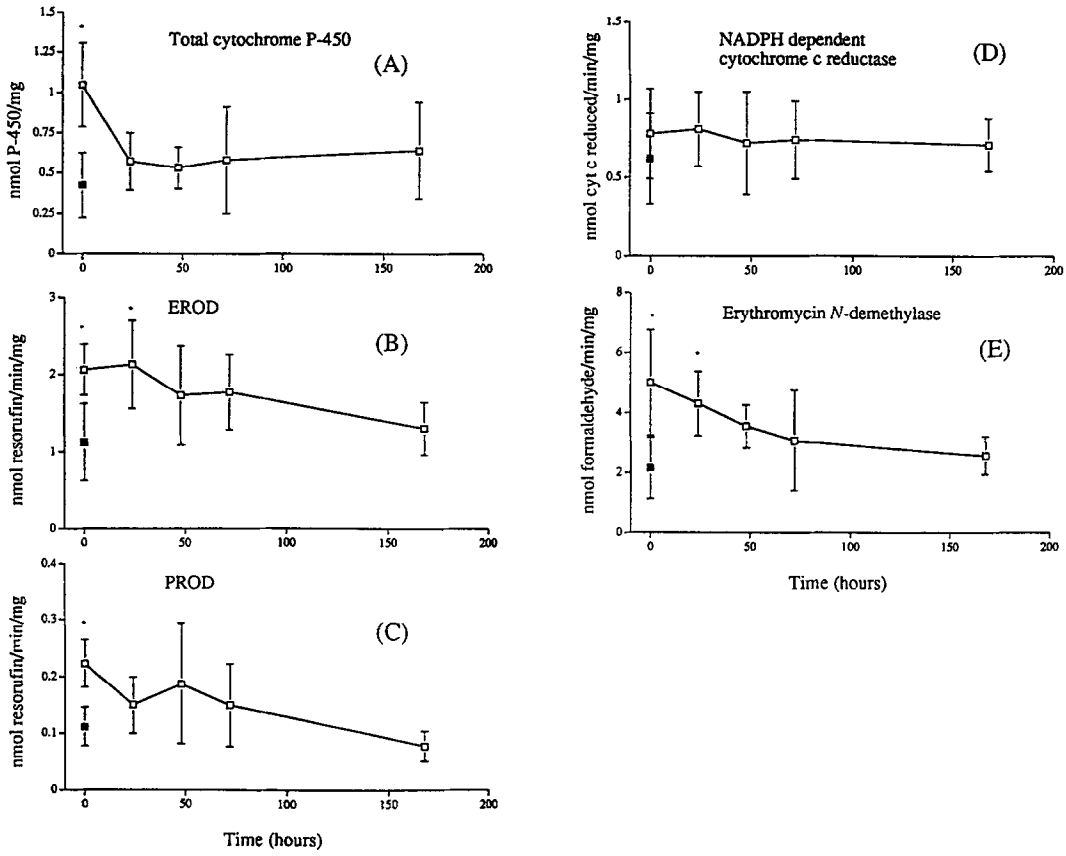

Fig. 3. The effects of ETOH treatment and withdrawal on P450 enzyme marker activities. Control (■) and ETOH (□) treated rats (6–9 per group) were euthanized at the time points indicated (0, 24, 48, 72, 168 hr). Microsomes were prepared and enzyme assays were performed as described in Materials and Methods. Results are expressed as means ± SD of groups of 6–9 animals. In each case, the X axis denotes time after ETOH withdrawal. Panel A represents total nanomoles of microsomal P450 content per milligram of microsomal protein (Y axis). Panels B and C show the rate of formation of resorufin from ethoxyresorufin *O*-dealkylase (EROD) and pentoxyresorufin *O*-dealkylase (PROD), respectively. Activity is displayed on the Y axis as nanomoles of resorufin formed per minute per milligram of microsomal protein. Panel D shows NADPH-dependent cytochrome *c* reductase activity, with the reaction rate calculated (Y axis) as nanomoles of cytochrome *c* reduced per minute per milligram of microsomal protein. Panel E represents erythromycin *N*-demethylase activity, calculated as nanomoles of formaldehyde formed per minute per milligram of microsomal protein (Y axis). Control and ETOH treatment data were compared using ANOVA, and levels of statistical significance were determined by Student's *t*-test as described in Materials and Methods. Key: (*) $P < 0.05$ vs control.

Conversely, the level of induced PNPH activity was lower than that of the immunoreactive protein, confirming that NDMAd is the most suitable enzyme marker to determine CYP2E1 induction/degradation in rats by ethanol.

The regulatory mechanism responsible for the marked elevation of hepatic CYP2E1 content and its subsequent return to control 24 hr post-withdrawal is still debated. In general, CYP2E1 induction elicited by chronic ETOH administration, diabetes, fasting, and chemical treatment may invoke several pre- and post-translational pathways (see Refs. 4 and 5 for reviews). In this study, blood ethanol concentrations [approximately 0.15 to 0.2% (w/v)] were lower than those reported to induce CYP2E1 mRNA increases using the Tsukumoto-French model [29]. At blood ETOH levels below 0.25% (w/v), protein stabilization is hypothesized to account for the majority of CYP2E1 induction. If the 4- to 5-fold drop in CYP2E1 following ETOH withdrawal after 24 hr is not due to a loss of protein stabilization, then the post-translational, translational, or transcriptional events involved must be strikingly sensitive to changes in tissue ETOH concentrations.

EROD and PROD activities were demonstrated to increase significantly following ETOH administration (approximately 2-fold in each case). Several studies [10, 11, 30, 31] support these findings, collectively suggesting that ETOH differentially induces several other P450 isozymes in addition to CYP2E1. In contrast with CYP2E1, EROD exhibited significant increases over controls after 24 hr withdrawal and was unchanged from its 0-hr induced value. Data from western blotting of CYP1A1, CYP1A2 and

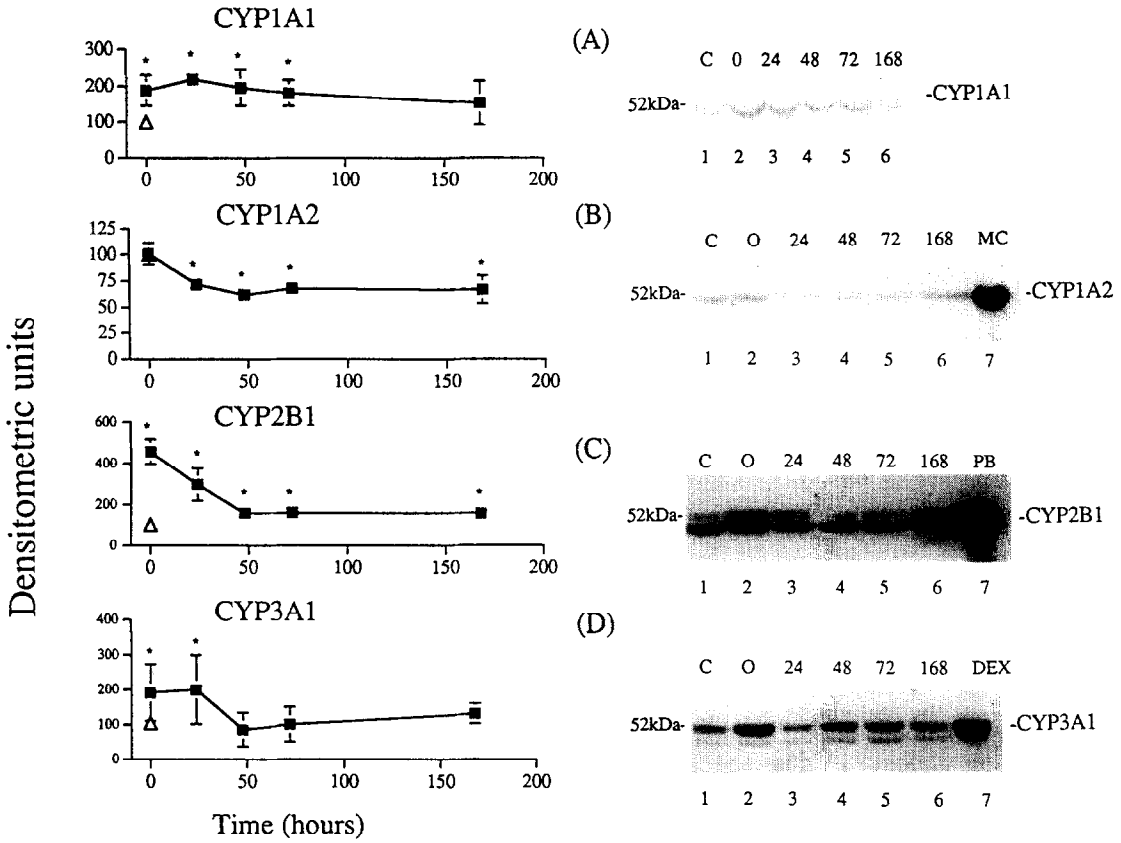

Fig. 4. Immunoblot analyses of microsomal CYP1A1/2, CYP2B1 and CYP3A1. Western blots of microsomal protein and subsequent densitometric scanning were performed on ethanol-treated (■) and control (△) animals as described in Materials and Methods. In panel A, microsomal protein from 5 animals in each group (12.5 μg of microsomal protein/well) was stained with an antibody specific for CYP1A1, and immunoreactive levels were measured. Results represent means ± SD, with a sample blot shown on the right. Lane 1 represents control; lane 2, 0 hr; lane 3, 24 hr; lane 4, 48 ; lane 5, 72 hr; and lane 6, 168 hr. This process was repeated using an antibody specific for CYP1A2 (panel B), with the addition of 3-methylcholanthrene-induced microsomes (lane 7). In panel C, microsomes from 5 animals (12.5 μg/well) in each treatment group were stained with an antibody specific for CYP2B1, and band densities were determined. A representative blot is shown on the right. Lane numbers denote treatment groups identical to those of panel A except for lane 7, which contained phenobarbitone-induced microsomes. Panel D shows the effect of ethanol feeding and withdrawal on levels of immunoreactive CYP3A1 from 5–6 animals in each treatment group (12.5 μg/well). A representative blot is shown on the right, with the lanes containing microsomal samples as described in panel A. Lane 7 contained dexamethasone-induced microsomes. The M_r of each P450 is shown on the left of the blot. Control and ETOH treatment data were compared using ANOVA, and levels of statistical significance were determined by Student's *t*-test as described in Materials and Methods. Key: (*) $P < 0.05$ vs control.

CYP2B1 showed some inconsistencies between the reported enzyme marker activities and immunoreactive protein. As has been pointed out previously [20], EROD and PROD are only specific for their respective P450s when the isoforms are selectively induced. The magnitude of induction observed in this study was probably insufficient to remove the contribution of other constitutively expressed P450s. Immunoblotting of CYP2B1 showed significant induction (4-fold) over controls that persisted for 7 days following withdrawal. The level of induction was higher and less variable than the data obtained using the marker enzyme, PROD. Immunoreactive CYP1A1 showed less variability than the marker enzyme EROD, although in this instance the magnitude of induction was approximately equal. CYP1A1 was elevated significantly up to 72 hr after ethanol withdrawal. Rather surprising was the significant loss of CYP1A2 observed during ethanol withdrawal. It is important to point out that the loss was not observed after chronic ethanol administration (0 hr), but only during the withdrawal period itself (24, 48, 72 and 168 hr). Although CYP1A1 and CYP1A2 are members of the same gene subfamily, it is clear that ethanol is able to cause differential expression of these isoforms. This is particularly apparent during ethanol withdrawal. The method by which ethanol

feeding and withdrawal effects these changes is not clear; however, it is known that CYP1A1 contains an intronic glucocorticoid response element (see Ref. 32 for review). Perturbations in glucocorticoid secretion are observed frequently during ethanol intake, conceivably providing one mechanism by which CYP1A1 could be induced. In contrast, CYP1A2 does not appear to contain the same response elements as CYP1A1 and is thought, for the most part, to be induced post transcriptionally [32]. The differential effects of ethanol on these P450s warrant further investigation.

The immunochemical and enzymatic findings suggest that the induction of CYP2B1 may be regulated differently from that of CYP2E1. Previous reports show that chemically induced increases in the activity of this isozyme by phenobarbitone are mediated by elevated transcription/mRNA levels, not protein stabilization (for reviews see Refs. 32 and 33). Moreover, Johansson *et al.* [34] have shown that acetone (a stabilizer of CYP2E1) induces CYP2B1 by an increase in gene transcription. In a study by Eliasson *et al.* [35], it was shown *in vitro* that CYP2B1 is degraded via a mechanism different from that of CYP2E1, effectively resulting in a longer half-life. Our data generated *in vivo* support these findings, by demonstrating that although CYP2E1 and CYP2B1 are induced to a similar extent, their patterns of degradation are quite different.

CYP3A-catalyzed ERNd activity was also induced following ETOH administration (2.5-fold, $P < 0.05$). Induction persisted for 24 hr post-withdrawal (2-fold at 24 hr, $P < 0.05$). Immunochemical data using an antibody specific to CYP3A1 confirmed that variable but significant induction occurred at 0 and 24 hr. Ethanol has been shown to increase CYP3A1 in culture, although the degree of induction is relatively small [11] in comparison with that of dexamethasone, an established inducer of CYP3A1. Considering the role of CYP3A in oxidizing a variety of steroids [36, 37] and its inducibility by glucocorticoids, it is conceivable that stress- or ETOH-induced release of either cortisol (humans) or corticosterone (rats and mice) following ETOH administration and withdrawal stimulates the induction of CYP3A1. Elevations in plasma glucocorticoids following ETOH consumption and withdrawal are documented in humans and rodents [38–40]. Our own unpublished animal data also suggest that plasma corticosterone levels are elevated during ETOH feeding and 24 hr following withdrawal. Ma *et al.* [41] concluded that the induction of CYP3A1 observed during fasting may be indirectly due to the release of glucocorticoids. Conversely, the cell culture data of Sinclair *et al.* [11] suggest that CYP3A1 induction in the rat liver is mediated directly by ETOH. It is still unclear whether glucocorticoids and ETOH act independently in provoking CYP3A1 induction, as the findings of de Waziers *et al.* [30] indicate synergism between the two. In view of our findings in the rat, it may be of clinical interest in male alcoholics to determine if cycles of episodic drinking/withdrawal behavior affect drug metabolism mediated by this P450 isoform.

Summarizing, it is clear from the present study that ethanol is capable of inducing several different P450s *in vivo*. The pattern of induction takes several forms, both in magnitude and duration, with CYP2E1 the only induced enzyme to be degraded rapidly. Whether the induction of CYP1A1, CYP2B1 and CYP3A1 is mediated directly or indirectly by ethanol awaits confirmation.

Acknowledgements—We are grateful to Ms. Toni Calzone and the animal care staff of the NIAAA for their assistance throughout the animal feeding phase of these studies.

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