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Effects of Diet and Ethanol on the Expression and Localization of Cytochromes P450 2E1 and P450 2C7 in the Colon of Male Rats

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ABSTRACT. Local activation of procarcinogens in target tissues such as the colon by cytochrome P450-dependent microsomal monooxygenases is considered to be an important factor in the etiology of cancer. Diet and alcohol consumption are considered risk factors in colon cancer, and the cytochrome P450 isozymes CYP2E1 and CYP2C7 have been implicated in the biochemical mechanisms underlying colon cancer. The current study was conducted to determine the effects of diet and ethanol consumption on colonic and hepatic expression of these two enzymes. Adult male rat Sprague-Dawley rats were fed rat chow *ad lib.* or were infused intragastrically with control or ethanol-containing diets. Our results indicate that CYP2E1 is present in colonic epithelial cells, and expression of colonic and hepatic microsomal CYP2E1 and CYP2C7 was increased by chronic ethanol intake. As compared with rats having *ad lib.* access to standard rat food, rats receiving total enteral nutrition had significant ($P < 0.01$) reductions of CYP2C7 and slight, but not statistically significant, reductions in the expression of CYP2E1 in colon. Diet and ethanol differentially regulated CYP2E1 and CYP2C7 in a tissue-specific manner such that ethanol induced CYP2E1 and CYP2C7 in the colon and liver, and the intragastric diet alone had a tendency to *induce* these isozymes in the liver and *reduce* them in the colon. These results may provide a partial explanation for the mechanisms underlying effects of diet and ethanol on colon cancer. BIOCHEM PHARMACOL 51;1:61–69, 1996.

KEY WORDS. nutrition; diet; ethanol; CYP2E1; CYP2C7; colon

The microsomal monooxygenase system consists of a short electron transport chain in the endoplasmic reticulum comprised of a flavoprotein, cytochrome P450 reductase, and a multigene super family of hemeproteins, the cytochromes P450. This system is involved in activation and detoxication of endogenous compounds such as steroids, fatty acids, vitamins, and eicosanoids and xenobiotics such as drugs, pesticides, chemical carcinogens, and pollutants [1, 2]. It is well established that cytochrome P450 isozymes are active in the metabolic conversion of many structurally diverse chemicals to electrophiles capable of reacting with cellular macromolecules. Thus, procarcinogens can be bioactivated to reactive species and can covalently modify chromosomal DNA causing irreversible cellular changes that may ultimately lead to cancer [3, 4].

Epidemiological and animal studies have demonstrated that excessive alcohol consumption increases the risk of large bowel cancer and suggest that diet is an important modulator of carcinogenesis in this tissue [5–13]. One possible explanation for how ethanol acts as a cocarcinogen at remote sites, as well as ethanol-contact sites, resides in the capacity of ethanol to act as an inducer or suppresser of the microsomal monooxygenase system [14–16].

Ethanol induction of the P450 isozyme CYP2E1 in the liver has received much attention, not only because of the capacity of this isozyme to metabolize ethanol to acetaldehyde, but also because of its potential to cause cellular damage. CYP2E1 is a loosely coupled enzyme capable of oxygen radical formation in the absence of substrate [17]. CYP2E1 is involved in the bioactivation of various xenobiotics to active carcinogens, especially nitrosamines such as *N*-nitrosopyrrolidine and *N*-nitrosodimethylamine [18–21]. The greatest concentrations of CYP2E1 are found in the perivenous region of the hepatic lobule in the three to four layers of hepatocytes surrounding the hepatic central vein [22]. CYP2E1 is also present in lower concentrations in the extrahepatic tissues such as kidney [23], lung [24], nasal mucosa [25], testis, ovaries [24], and small intestine [26].

CYP2C7 is a constitutive isozyme with high structural homology (69%) to the male and female specific rat isozymes

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CYP2C11 and CYP2C12, respectively. It catalyzes the 4-hydroxylation of retinal and retinoic acid [27, 28], and it has been reported to be slightly elevated (1.3-fold) by ethanol in liver [29].

Alterations in the expression of intestinal cytochromes P450 may have important consequences for first-pass metabolism of many orally active drugs in clinical use. Cytochrome P450 isozymes in the small intestine are thought to also activate and detoxify dietary procarcinogens. Our laboratory has demonstrated recently that purified diets similar to those used clinically decrease the expression of microsomal CYP1A1, CYP2B1 and CYP3A in the jejunal and ileal regions, and chronic ethanol treatment results in further reduction of CYP3A apoprotein levels in the jejunal region [30].

In the current study, we used a nutrient delivery system (TEN)‡ to provide adequate nutrition and high levels of ethanol intake to study the effects of diet, long-term ethanol exposure, and ethanol-diet interaction on expression of CYP2E1 and CYP2C7 in rat colon and liver.

MATERIALS AND METHODS

Materials

Virus-free adult male Sprague–Dawley rats (300 g) were purchased from Harlan Industries (Indianapolis, IN). Trypsin inhibitor was purchased from the Sigma Chemical Co. (St. Louis, MO). Heparin was purchased from ESI Inc. (Cherry Hill, NJ), and nitrocellulose filters were obtained from the Fisher Scientific Co. (St. Louis, MO). ECL Western Blot Kits were a gift from Amersham (Arlington Heights, IL), and horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio-Rad (Richmond, CA). Avidin-biotin-peroxidase complex was purchased from Vector Laboratories (Burlingame, CA), and DAB chromogen was obtained from Biogenex (San Roman, CA). Antibodies against CYP2E1 and rabbit polyclonal antibody 127, which recognizes a second ethanol-inducible rat liver P450 isozyme, P450 EtOH2, and cross-reacts with CYP2C7, were prepared as previously described [31, 32].

Animals and Diets

Male Sprague–Dawley rats were kept in an AAALAC-approved facility with lights on between 6:00 a.m. and 6:00 p.m. All rats were implanted with a single gastric cannula as previously described [33]. After a 14-day recovery, rats (340 g) were assigned randomly to three different groups (4–10 rats per group) according to diet. Two groups were infused with liquid diets at rates of 3 mL/hr and 160 kcal/kg$^{0.75}$/day. A third group had *ad lib.* access to standard rat food (Purina, St. Louis, MO) while being infused with water at a volume equal to that of the liquid diet infused into other groups.

The liquid diet was formulated to support normal body weight gains in rats according to the National Research Council [34]. The composition of the diet used was identical to that previously described, except that corn oil was used as source of fat and the level of fat decreased [35]. The basic diet had 16% protein, 59% carbohydrate, and 25% fat (Table 1). The ethanol dose started at 8 g/kg/day and was increased progressively to 13 g/kg/day, which corresponded to 41% of the total calories. The TEN control diet was maintained as isocaloric to the ethanol-containing diet by altering the carbohydrate content. All rats had *ad lib.* access to drinking water throughout the study.

Tissue Preparation

All rats were killed by decapitation, and a small portion of liver and proximal colon was fixed in 10% buffered formalin for histological examination. The rest of the liver was removed and immediately frozen in liquid nitrogen. The entire colonic segment, from distal cecum to 3 cm proximal to the anus, was separated and the lumen exposed, rinsed in ice-cold saline, frozen in liquid nitrogen, and stored at −70°. Liver and colon microsomes were prepared as previously reported [30].

Immunoquantitation of P450 Apoproteins

Microsomal protein was assayed with Bio-Rad protein assay reagent. The levels of cytochrome P450 apoproteins were measured by SDS-PAGE followed by western blot analysis. Liver microsomes were loaded at 5 μg protein/well, and colon microsomes from individual rats were loaded at 100 μg protein/well. Western blot analysis was conducted by separating microsomal proteins by SDS-PAGE using 8% polyacrylamide and then electroblotting overnight onto nitrocellulose filters. Rabbit polyclonal antisera against CYP2E1 (at 1:10,000) and CYP 127 (1:1,000) dilution were used as primary antibodies. The bound primary antibody was visualized by enhanced chemiluminescent detection using horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody according to manufacturer's procedures. Autoradiographs to several exposure periods were used to assure the presence or absence of immunoreactive bands. Immunoquantitation of the western blots was accomplished by densitometric (Molecular Dynamics, Sunnyvale, CA) scanning of the resulting autoradiographs.

TABLE 1. Diet composition

		% Total calories*		
			Ethanol	
Component	Source	TEN	8 g	13 g
Protein†	Whey	16	16	16
Carbohydrate	Maltodextrin and dextrose	59	31	18
Fat	Corn oil	25	25	25
Ethanol		0	28	41

* Diet contains the vitamins and minerals as previously reported [35].

† Protein was a partial hydrolysate (2% free amino acids, 60% peptides mol wt < 5000 and 40% mol wt > 5000).

‡ Abbreviations: TEN, total enteral nutrition; and pNP, *p*-nitrophenol.

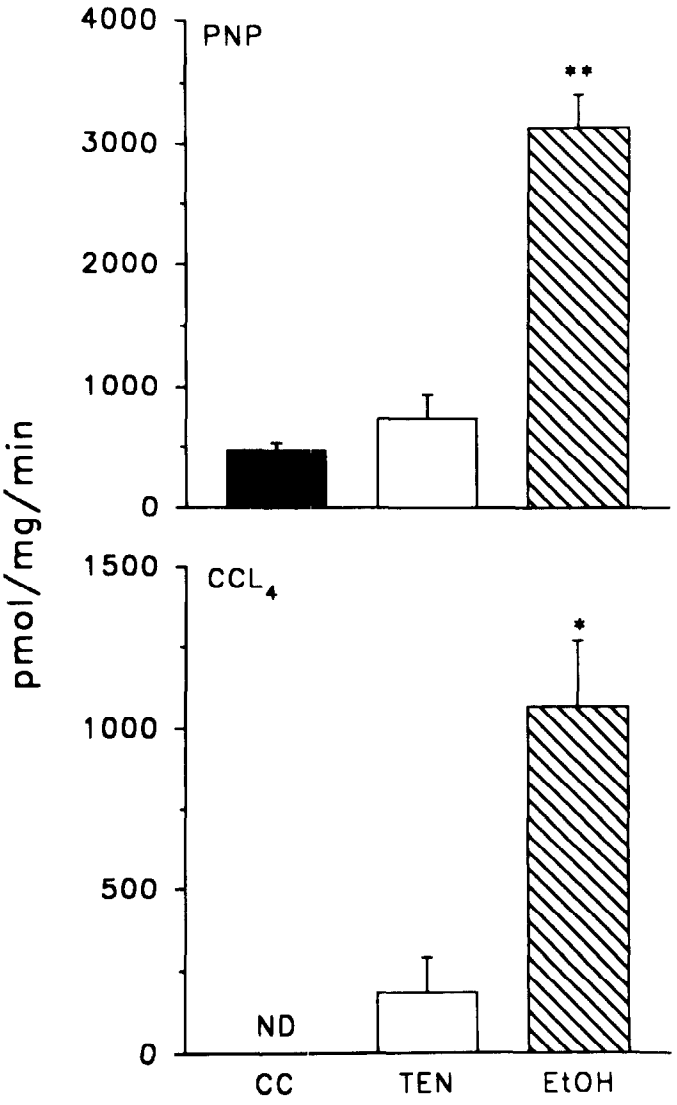

FIG. 1. Effects of chronic ethanol and diet on hepatic CYP2E1-dependent activities. The top panel represents the pNP-hydroxylase activity (mean ± SEM), and the bottom panel represents CCl_4-dependent lipid peroxidation of N = 4–8 rats per group. Abbreviations: ND, not detected; CC, rats having *ad lib.* access to standard rat food; and TEN, total enteral nutrition. Statistical significance was calculated as CC vs TEN and TEN vs EtOH: *$P < 0.05$; and **$P < 0.001$.

Monooxygenase Activities

The activity of hepatic CYP2E1 was assayed indirectly by measuring carbon tetrachloride-dependent lipid peroxidation [36] and directly measuring the hydroxylation of pNP [37]. The activities of colon microsomes were below the detection limits of these two procedures.

Immunohistochemical Procedures

Sections (3 mm thick) of proximal colon were fixed in 10% buffered formalin and embedded in paraffin. Four-micron sections from these blocks were deparaffinized, rehydrated, and trypsinized in 1% trypsin solution for 20 min at ambient temperature. Slides were washed four times with double-distilled water for 20 min to eliminate endogenous peroxidase activity, and the slides were incubated with 3% H_2O_2 for 15 min at room temperature and rinsed with double-distilled water. Nonspecific protein binding was blocked through incubation with 2% rat serum for 20 min at room temperature. The sections were incubated with rabbit polyclonal anti-CYP2E1 at 1:1000 dilution overnight in a humid chamber at room temperature. After washing sections with PBS for 10 min, slides were incubated with avidin-biotin complex (Vectastain ABC kit, Vector Laboratories) according to the manufacturer's instructions. All sections were washed in PBS for 20 min and developed with 3,3′-diaminobenzidine (Biogenex) for 5 min and counter-stained with Mayer's hematoxylin. The staining intensity and distributions were evaluated by a pathologist using light microscopy. The intensity of staining was semi-quantified using the SAMBA 4000 computerized microscopic image analysis system and its software (Dynatech Laboratories Imaging Products, Chantilly, VA).

Statistical Analysis

All data were analyzed by Student's *t*-test with $P < 0.05$ considered statistically significant. The results are expressed as means ± SEM.

RESULTS

Effects of Diet and Chronic Ethanol on Body Weight

The mean body weight at the beginning of the experiment was 340 g and at the end of the experiment (50 days) body weights of three groups of rats were as follows: CC, 424 ± 24 g; TEN, 536 ± 17 g; and ethanol, 444 ± 5 g. As we have reported previously [30, 35], rats on TEN gained more ($P \leq 0.05$) weight than rats that had *ad lib.* access to standard rat food, and ethanol-treated rats gained less weight ($P \leq 0.05$) than TEN rats.

Effects of Diet and Chronic Ethanol on Hepatic and Colonic CYP2E1 and CYP2C7

Figure 1 depicts hepatic microsomal CYP2E1 activity as determined from metabolism of pNP and CCl_4, both preferred substrates for CYP2E1 [33, 36]. Results from both assays demonstrated that enzyme activities associated with CYP2E1 were greater in rats fed the TEN diets than in those fed the standard rat food. Hepatic microsomal CCl_4-dependent lipid peroxidation (an indirect measure of CYP2E1 activity) was below the lower limits of assay sensitivity in rats fed standard rat food, but was clearly observable in TEN rats and increased ($P \leq 0.001$) in EtOH rats (lower panel, Fig. 1). A non-significant increase also was observed for pNP hydroxylase between the CC and TEN rats, and increases ($P \leq 0.001$) were observed in EtOH rats in CYP2E1-dependent activities (pNP hydoxylase and CCl_4 reduction) compared with TEN rats. Thus, chronic ethanol treatment increases pNP metabolism and CCl_4-dependent lipid peroxidation by 4- and 5-fold, respectively.

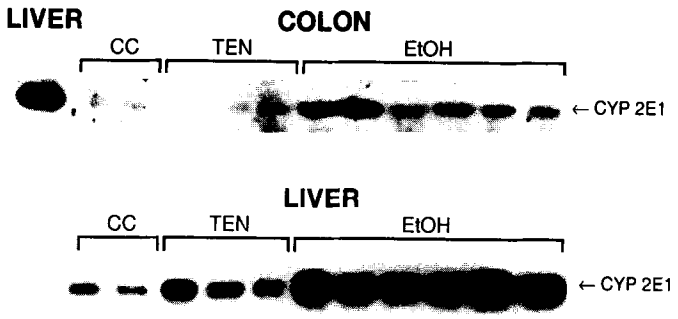

FIG. 2. Western blot analysis of CYP2E1 in the liver and colon. A representative immunoblot of CYP2E1 in the liver and colon is presented for 2, 3, and 6 individual rats having access to standard rat food (CC), total enteral nutrition (TEN), or TEN plus EtOH, respectively. The immunoquantitation of all rats in each group is presented in Fig. 4.

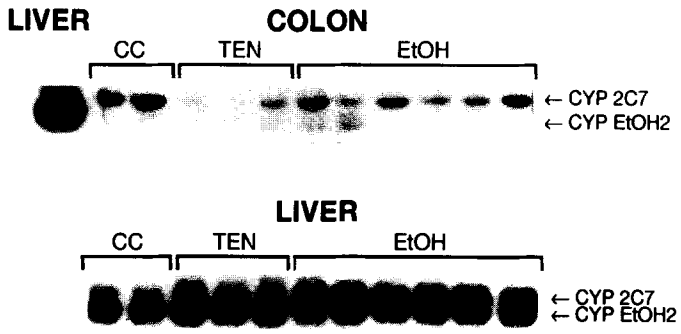

FIG. 3. Western blot analysis of CYP2C7 in the liver and colon. A representative immunoblot of CYP2C7 in the liver and colon is presented for 2, 3, and 6 individual rats having access to standard rat food (CC), total enteral nutrition (TEN), or TEN plus EtOH, respectively. Antibody 127, which recognizes both CYP2C7 and CYP EtOH2, was used for this analysis. The immunoquantitation of all rats in each group is presented in Fig. 4.

CYP2E1 was found to be present in the colon and induced by ethanol. Fig. 2 and 3 depict a representative western blot of the liver and the colon probed with antisera recognizing CYP2E1 and CYP2C7, respectively, from individual samples, and Fig. 4 illustrates the means ± SEM following immunoquantification. Hepatic CYP2E1 apoprotein levels were greater ($P \leq 0.001$) in samples from the TEN-treated rats as compared with rats on standard food (CC), and the colonic apoprotein of CC rats did not differ significantly from that of TEN rats. Chronic ethanol treatment increased expression of both hepatic ($P \leq 0.01$) and colonic ($P \leq 0.05$) CYP2E1 by 3-fold. Immunoquantitation of colon samples from rats treated with TEN demonstrated reduced ($P \leq 0.001$) CYP2C7 when compared with those of CC rats, and ethanol-treated rat colons had greater ($P \leq 0.05$) CYP2C7 when compared with TEN controls. While no differences in hepatic CYP2C7 apoprotein levels were observed between CC and TEN-treated rats, increases ($P \leq 0.05$) were observed in ethanol-treated rats.

Immunohistochemical staining demonstrated that CYP2E1 was present within epithelial cells of the proximal colon crypts, and ethanol-treated rats exhibited a very strong CYP2E1 immunostaining as compared with TEN-treated rats, indicating that expression of this isozyme is regulated by ethanol in these cells (Fig. 5). Our observations by light microscopy were confirmed by a semiquantitative assay using image analyzer "SAMBA 4000" (Figs. 6 and 7).

DISCUSSION

It has been postulated that ethanol acts as a cocarcinogen by induction of the microsomal cytochrome P450-dependent biotransformation system. For example, CYP2E1 has been reported to be involved in the metabolic activation of carcinogens such as benzene [38] and 1,2-dimethylhydrazine [39] and the conversion of azoxymethane to methylazoxymethanol [40] in the liver. Furthermore, it is possible that CYP2E1 may play a direct role in the activation of dietary promutagens within colonic tissue. Thus, CYP2E1 is a potentially important isozyme involved in alcohol metabolism and other adverse effects of alcohol intake, and it may be an important factor in alcohol potentiation of colon cancer incidence.

CYP2C7 may be involved in pathological conditions related to diet and alcohol intake. CYP2C7 participates in the catabolic metabolism of retinoic acid leading to the elimination of retinoids from the body [27, 28]. Thus, induction of this process by chronic ethanol consumption may increase the catabolism of retinoic acid in the liver and result in the decreased hepatic storage of vitamin A so commonly reported in human alcoholics [41]. Reduction of tissue vitamin A concentrations has potential consequences related to the pathophysiologic

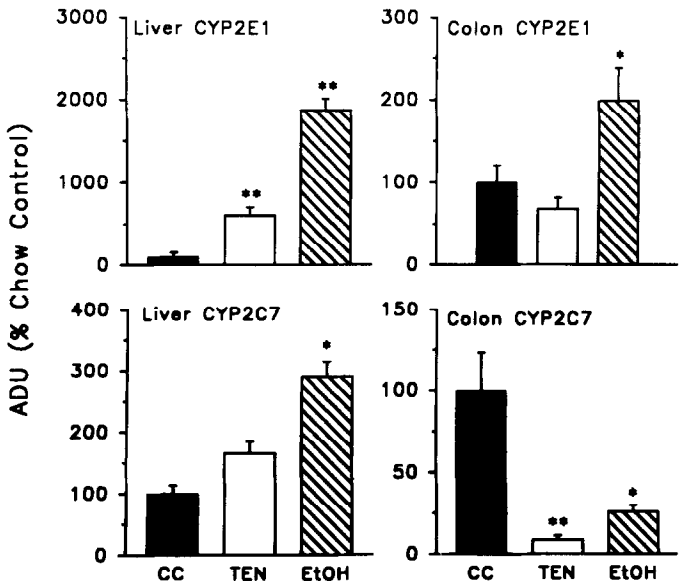

FIG. 4. Effects of diet and ethanol on CYP2E1 and CYP2C7 apoproteins in the colon and liver. Shown is the immunoquantitation of western blots of hepatic and colonic microsomes from N = 4–8 rats per group. ADU = arbitrary density units. Statistical significance was calculated as CC vs TEN and TEN vs EtOH: *$P < 0.05$ and **$P < 0.001$.

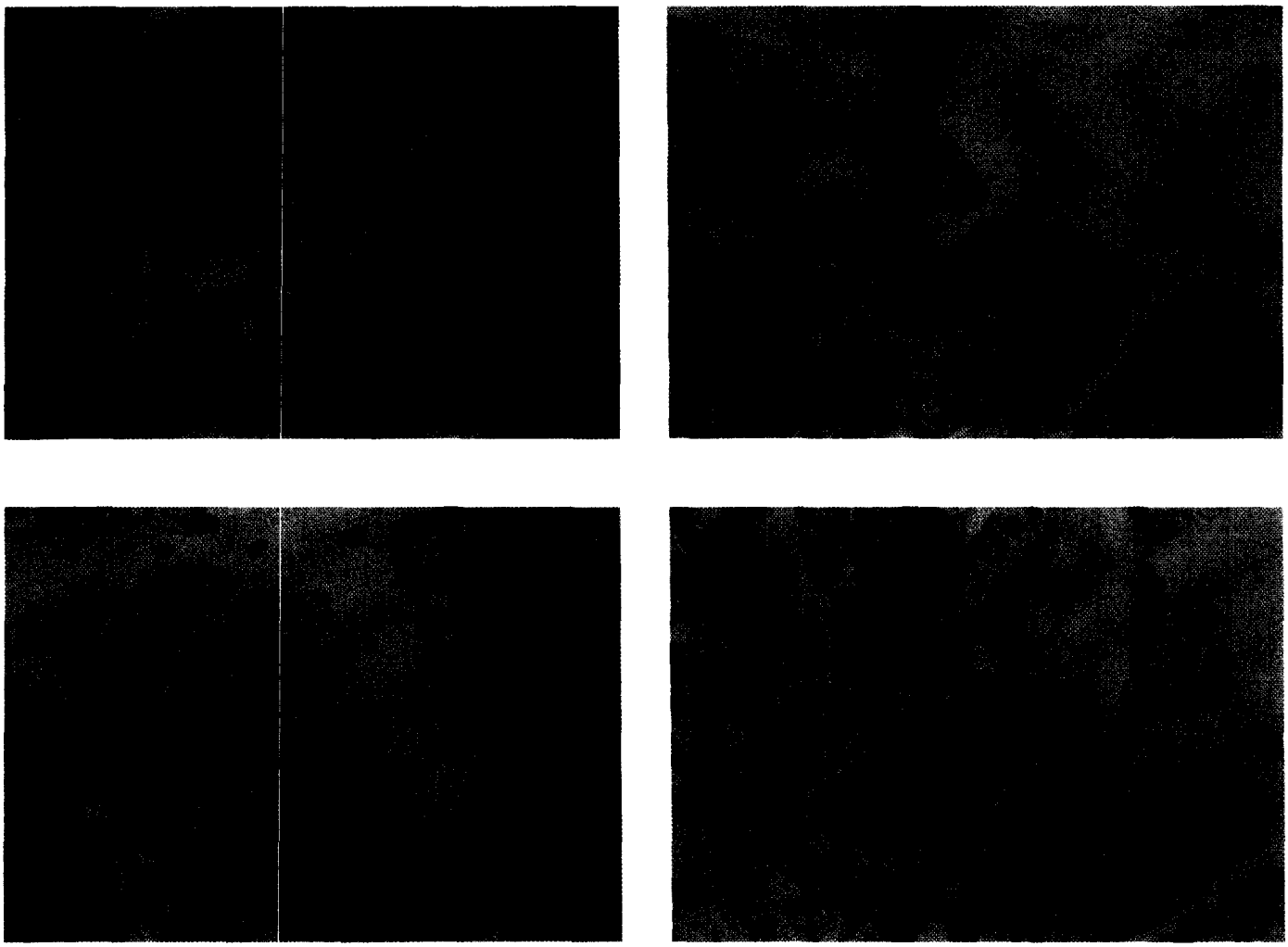

FIG. 5. Effects of alcohol and diet on immunohistochemical localization of CYP2E1 in the colon. Micrographs of representative rats treated with TEN or EtOH are shown. Panel A is a TEN colon section at 100× and panel B is the same section magnified to 400×. Panel C represents the colon of a rat treated with ethanol at 100× and panel D is the same section at 400×. The brown staining indicates immunoactivity of CYP2E1.

importance of retinoids. For example, retinol can apparently compete with dimethylnitrosamine for its activation in liver microsomes [42]. It is conceivable, therefore, that decreased vitamin A may indirectly favor formation of chemical carcinogens in target tissues. In addition, local alterations in retinoid levels or altered patterns of retinoid metabolism may produce abnormal cellular differentiation or excessive cellular proliferation in affected epithelia [43].

CYP2C7 was detected in western blot analysis in the present study using an antiserum that was produced against purified rat CYP2E1. This antiserum (called 127) recognized two P450 isozymes in Ouchterlony double diffusion analysis: CYP2E1 and a new P450 in gene family 2C that is referred to as P450 EtOH2 until molecular characterization is completed. In western blot analysis of liver microsomes, the 127 antiserum does not recognize CYP2E1, but does recognize P450 EtOH2 and a closely related isozyme CYP2C7. In addition, when screened with purified rat liver cytochrome P450 isozymes, including CYP2E1, antibody 127 recognized only CYP2C7 in western blot analysis ([32] and unpublished data). Thus, we used this antiserum in western blot analysis to study the effects of diet and ethanol in CYP2C7.

This report is the first to describe use of the TEN model to study the modulatory effects of diet and chronic ethanol treatment on induction of CYP2E1 and CYP2C7 in the colon of male rats. We found that CYP2E1 and CYP2C7 are: (1) present in the colon, the gastrointestinal segment with the greatest constitutive level of these two isozymes; (2) induced by ethanol; and (3) differentially expressed according to diet in a tissue-specific manner.

Induction of CYP2E1 in the rat colon has been reported [26], but only in a qualitative fashion. Interestingly, ethanol induction of CYP2E1 was not confirmed in the mouse [44]. The present study confirms that ethanol does induce colonic CYP2E1 in the rat, and this is the first study to quantify the induction of this important isozyme in the colon.

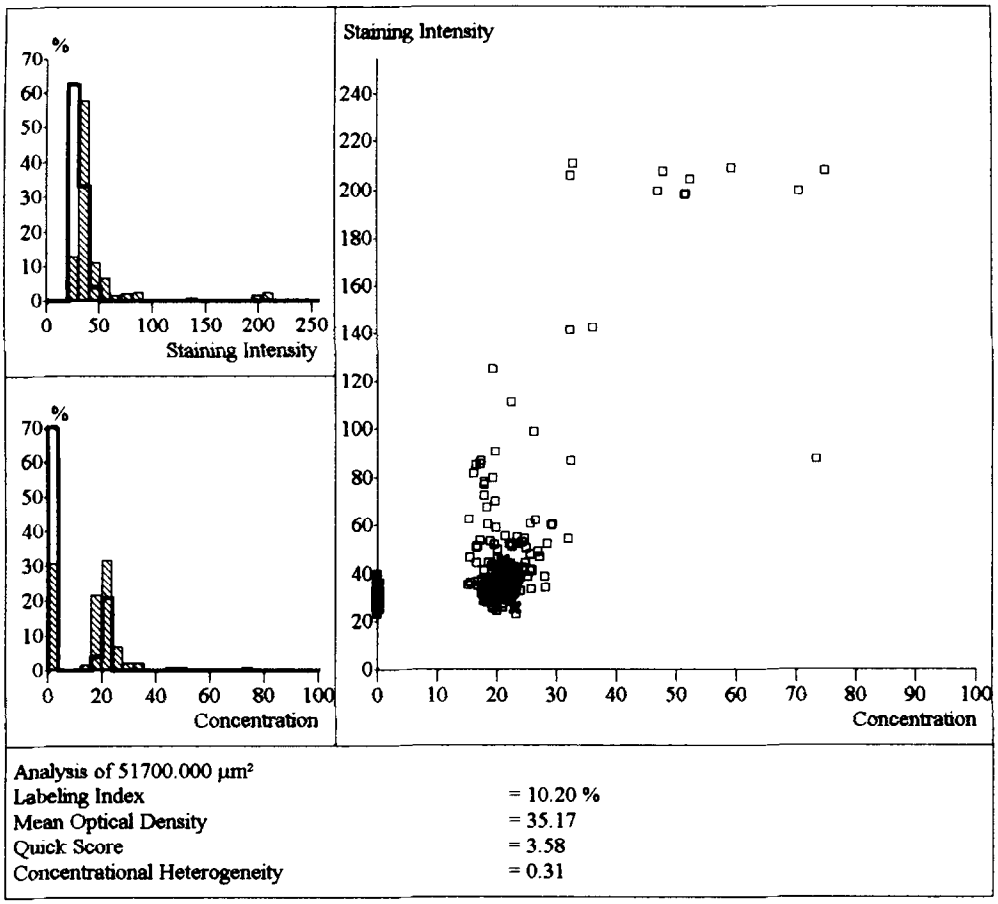

FIG. 6. Semiquantitative analysis of CYP2E1 in colon of the TEN rat. Concentration versus staining intensity are plotted, with the open squares representing the positive staining and the Xs (which appear as very dark points) representing background. These data were also plotted individually in bars graphs, where the open bars correspond to the open squares (positive staining) and the cross-hatched bars correspond to the Xs (background). The intensity of staining was semi-quantified using the SAMBA 4000 computerized microscopic image analysis system and its software (Dynatech Laboratories Imaging Products). Two different programs were used, "cytoplasm" and "area," the former involving assessment of individual cells and the latter program allowing evaluation of clusters of cells. In the latter program, appropriate thresholding allowed assessment of the cytoplasm in the clusters of cells. Two hundred cells were evaluated in each case for cytoplasmic programs, while 10 visual fields containing at least 200 cells (and most commonly >200 cells) were evaluated for the area program. Each program provided the following data values for each case: (1) a "labeling index" (LI), representing the percentage of cells showing positive immunostaining in the cytoplasm and area programs, respectively; (2) the "mean optical density" (MOD) corresponding to the mean intensity of the immunoreaction; and (3) the "quick score" (QS), corresponding to LI · MOD/100.

Ethanol may not have induced CYP2E1 in the mouse colon [44] because the ethanol concentrations may not have achieved sufficient levels, and the previously reported induction of CYP2E1 in the rat may have been due to a combination of reduced caloric intake and ethanol using the liquid diet system employed in that study. This latter possibility exists because of the well established induction of this isozyme by fasting [31]. In the present study, TEN was employed to control for nutritional intake, and blood alcohol concentrations were high. Thus, our data clearly demonstrate that ethanol, independent of caloric restriction, induces CYP2E1 in the colon. The demonstration of ethanol-inducible CYP2E1 in colon, provides a possible molecular basis for cocarcinogenic effects of long-term ethanol on colonic tissue.

Induction of hepatic CYP2E1 by chronic ethanol has been well documented [15, 31, 35, 45], but was included in this study to permit comparison with colonic CYP2E1. Immunohistochemical staining demonstrated that CYP2E1 was present predominantly within epithelial cells of the proximal colon crypts, and this histological localization agrees well with previous reports of CYP2E1 in the colon of the rat [26] and mouse [44].

There were significant effects of diet on the constitutive expression of CYP2E1. Rats fed the TEN diet had significantly

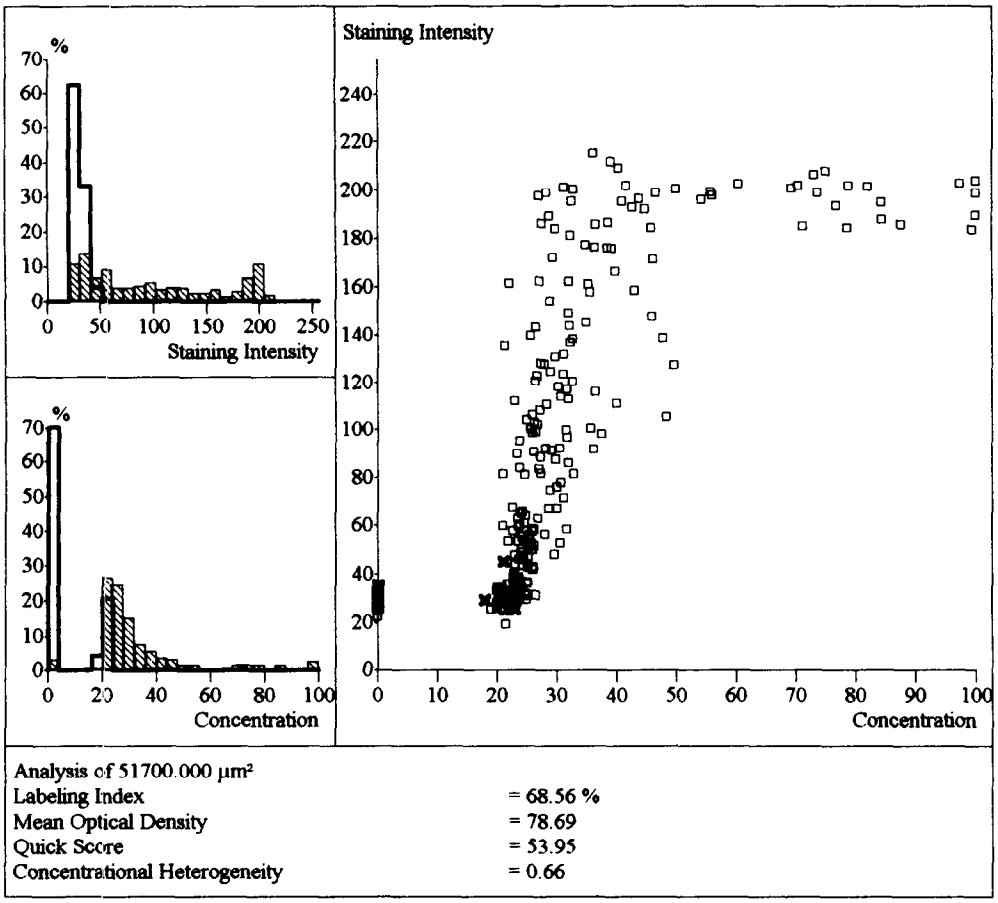

FIG. 7. Semiquantitative analysis of CYP2E1 in colon of the EtOH rat. Concentration versus staining intensity are plotted with the open squares representing the positive staining and the Xs (which appear as very dark points) represent background. These data were also plotted individually in bars graphs, where the open bars correspond to the open squares (positive staining) and the cross-hatched bars correspond to the Xs (background). The intensity of staining was semi-quantified using the SAMBA 4000 computerized microscopic image analysis system and its software (Dynatech Laboratories Imaging Products). Two different programs were used, "cytoplasm" and "area," the former involving assessment of individual cells and the latter program allowing evaluation of clusters of cells. In the latter program, appropriate thresholding allowed assessment of the cytoplasm in the clusters of cells. Two hundred cells were evaluated in each case for cytoplasmic programs, while 10 visual fields containing at least 200 cells (and most commonly >200 cells) were evaluated for the area program. Each program provided the following data values for each case: (1) a "labeling index" (LI), representing the percentage of cells showing positive immunostaining in the cytoplasm and area programs, respectively; (2) the "mean optical density" (MOD) corresponding to the mean intensity of the immunoreaction; and (3) the "quick score" (QS), corresponding to LI · MOD/100.

increased hepatic microsomal CYP2E1-dependent activities and CYP2E1 apoprotein levels as compared with rats consuming standard rat food. The level and degree of unsaturation of dietary fat are significant factors in the regulation of CYP2E1 expression in liver [46, 47]. The TEN diet contained greater levels of dietary fat (25%) composed of 57% unsaturated fatty acids such as linoleic acid. Thus, this may account in part for the nutritional regulation of hepatic CYP2E1 expression in liver of the present study. However, there are so many other differences between standard rat food and our TEN diet that it is difficult to determine which dietary factor(s) is actually responsible for regulation of either the hepatic or the colonic P450 systems. Interestingly, however, dietary regulation of CYP2E1 appears tissue specific, as indicated by the significant increase in hepatic apoprotein levels as compared with the downward trend in colonic CYP2E1 apoprotein levels.

CYP2C7 expression appears to be tissue specific and differentially regulated. For example, in the colon CYP2C7 apoprotein was reduced significantly in TEN rats as compared with rats fed standard rat food (CC group), while there was a tendency towards increased expression of this isozyme in liver of TEN rats. As far as we are aware, these are the first data to demonstrate that CYP2C7 is present in the colon, and modulated by diet. Interestingly, both hepatic and colonic

CYP2C7 were induced ($P \leq 0.05$) in rats chronically treated with ethanol. This is in agreement with the qualitative observation of increased CYP2C7 in liver microsomes from ethanol-treated rats reported by Leo *et al.* [28], but in contrast with the small and non-significant elevation reported by Bandiera *et al.* [29]. Since CYP2C7 has been demonstrated to catalyze the metabolism of retinoic acid and retinal [27, 28], the CYP2C7 induction observed in our rats may provide evidence for a mechanistic process by which to partially explain the reduced hepatic vitamin A storage in alcoholics and may result in locally altered retinoids in the colon.

Rats on TEN gained more weight than rats fed standard rat food (CC group) and rats receiving the ethanol diet gained less than TEN rats regardless of receiving the same amount of calories/per kilogram per day as the TEN control. This confirms previous data from our laboratory, and the reasons for those differences have been discussed previously [35, 48].

In conclusion, we have demonstrated that CYP2E1 and CYP2C7 are present in the colonic tissue and are inducible by ethanol. Since human hepatic P450 2E1 (CYP2E1) and P450 2C7 (CYP2C8 and CYP2C10) orthologues exist in the liver [2], it is reasonable to assume that the organ distributions of cytochrome P450 isozymes are similar, and it is possible that these isozymes may be induced by ethanol in human colonic tissue. Therefore, alterations in expression of these isozymes, especially CYP2E1, which is known to be involved in the activation of procarcinogens, may provide mechanistic insights into the effects of diet and ethanol on colorectal cancer in humans.

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