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ROLE OF CYP2E1 IN THE PATHOGENESIS OF ALCOHOLIC LIVER DISEASE:MODIFICATIONS BY cAMP AND UBIQUITIN-PROTEASOME PATHWAY

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1. ABSTRACT

The ethanol inducible isoform of cytochrome P450, CYP2E1, may play a role in ethanol-induced liver injury. Therefore, the factors which govern CYP2E1 degradation and turnover were investigated. These factors include cAMP, ubiquitin, proteasomal enzymes and CYP2E1 mRNA. Rats fed ethanol or pair-fed isocaloric dextrose were pair-fed with rats fed ethanol or dextrose treated with cAMP for 2 months. The liver pathology, regenerative activity, fatty acid composition, NFkappaB activation, ubiquitin conjugates and proteasomal enzymes were measured as were the apoprotein levels of CYP2E1, CYP3A, CYP4A and mRNA levels for CYP2E1 and ubiquitin expression. The results showed, that the cAMP treatment ameliorated the increase liver fat storage and changes in the fatty acid composition in the livers of ethanol fed rats. Other histologic features of alcoholic liver disease were not changed. Western blot quantitation showed that the amount of ubiquitin and ubiquitin conjugates were markedly reduced by ethanol treatment. Similarly, ethanol decreased the level of ubiquitin mRNA. cAMP ameliorated the inhibition of the proteasomal enzyme proteolysis caused by ethanol feeding. The ethanol-induced increase in the CYP2E1 protein was partially inhibited by cAMP treatment. cAMP treatment decreased CYP2E1 mRNA levels in both ethanol-fed and pair fed control rats. Likewise NFkappaB activation was not increased by ethanol but cAMP reduced the level of NFkappaB activation. CAMP treatment also reduced CYP4A but not CYP3A. The results support the concept that cAMP treatment partially protects the liver from ethanol-induced fatty liver by reducing CYP2E1 induction through cAMP's effects on CYP2E1 synthesis.

2. INTRODUCTION

Rats fed intragastrically to maintain high blood alcohol levels (BAL) develop changes which resemble those seen in alcoholic liver disease (ALD) including fatty

change, focal inflammation, necrosis, and fibrosis after two months of feeding (13). The severity of the liver pathology can be reduced in this model by a variety of methods including: feeding a diet low in linoleic acid (30, 28), feeding a diet containing lactobacillus to reduce gut endotoxin production (29), blocking Kupffer response to ethanol-induced endotoxemia by gadolinium treatment (1) or feeding a diet rich in glycine (19). Another model of ALD, where the diet was rich in carbohydrate and was fed by intragastric cannula, also prevented liver pathology (40). In the latter case, the liver protection provided by carbohydrate probably resulted because a high carbohydrate diet reduces the induction of cytochrome P450 by ethanol (27) specifically CYP2E1 (41). Inhibitors of CYP2E1 partially protected the liver from ethanol-induced injury in proportion to the reduction in liver CYP2E1 when the inhibitors were fed with ethanol using the intragastric tube feeding rat model (24, 25, 26). The mechanism of CYP2E1 induction by ethanol is due to enzyme stabilization (34, 31) increased synthesis (39) and at high blood levels, increased mRNA levels (36).

The mechanism of turnover of CYP2E1 and other isozymes of cytochrome P450 in the liver involves phosphorylation which has been shown *in vitro* to be regulated by cyclic AMP and protein kinase A (18, 9, 20) followed by proteolysis by ATP dependent proteasomes (33, 34, 35). cAMP generation in tissue culture depresses the phenobarbital-induction of CYP2B1, 2B2 and 3A1 gene expression *in vitro* (37). The question then arises, does cAMP treatment *in vivo* reduce ethanol-induction of CYP2E1.

In this report we asked the question, will cAMP treatment reduce the ethanol-increased CYP2E1 levels and if so would this ameliorate the effect of ethanol on liver pathology? The possible role of ubiquitination in CYP2E1 degradation was also studied since steps in the ubiquitin-proteasomal pathway such as proteasomal proteolytic enzyme activity were measured even though ubiquitination of 2E1 has not been shown to be involved in the degradation of CYP2E1 in *in vitro* studies (35). Liver pathology was assessed histologically, biochemically and by immunoperoxidase measurement of proliferating cell nuclear antigen (PCNA) labeling of nuclei in the liver as an indication of regeneration. We found that cAMP treatment ameliorated the fatty liver and fatty acid changes induced by ethanol and decreased the induction of CYP2E1 by ethanol associated with a decreased in ubiquitin conjugates, proteasome proteolytic activity, ubiquitin mRNA expression and CYP2E1 mRNA. The results have been reported in part in abstract form (42).

3. METHODS

3.1. Animals

Male Wistar rats weighing ~200 g were purchased from Charles River Laboratories (Hollister, CA). Animals were pair-fed ethanol or isocaloric dextrose, continuously infused via a permanent intragastric cannula for 2 months (13). Half of the ethanol and dextrose fed rats received dibutyryl-cAMP (10 mg/kg I.P., daily except Saturday and Sunday) which was provided as a gift from Daiichi Pharmaceuticals in Japan. The intragastric cannula was implanted under pentobarbital sodium (30-50 mg/kg, I.P.) anesthesia when a liver biopsy was performed for baseline morphology. Blood sampling was done via the tail vein at the initial operation to establish the baseline blood chemistries (see below). The animals were then allowed ~6-10 days of recovery from the surgery before starting ethanol infusion. Urine alcohol level (UAL, Q.E.D. test, STC Diagnostics Inc., PA) and body weights were measured weekly, and blood alcohol levels (BAL) was measured monthly so as to determine the ethanol dose needed to maintain the BAL over 200 mg/ld. The rats were maintained according to the Guidelines of Animal Care as described by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985).

3.2. Biochemical and liver morphological analysis

Blood chemistries including serum glucose, total bilirubin, BAL, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were done on a clinical analyzer at 0 time, 1 month and 2 months. At 2 months, rats were sacrificed and the livers were weighed and fast frozen in liquid nitrogen for future studies including cytochrome P450 isozymes in the microsomal fraction by Western blot. The liver cAMP level was measured with an ELISA kit (Cayman, Upstate, NY). Liver tissue was studied by H&E for morphology using a previously described scoring method (13). The livers were examined blinded as to the treatment given. Tissue slices from zinc formalin fixed tissue were stained with an antibody towards proliferating cell nuclear antigen (PCNA) (mouse monoclonal from DAKO Corp., CA). PCNA staining was analyzed by counting 1000 hepatocyte nuclei and reported as % positive. The percent positive nuclei in non-parenchymal cells and the percent positive nuclei of bile duct epithelium were determined by counting 100 cells. All counting was done blinded as to treatment by a pathologist not otherwise involved in the study. Livers were also processed for electron microscopic examination with an Hitachi 600 microscope to document the liver morphology. Collagen was stained using Sirius Red and reticulim stain.

3.3. Hepatic lipid analysis

Lipid was extracted (3) from weighed, homogenized liver as previously described (Morimoto *et al*., 1995). Protein in the homogenate was determined by the Bradford method (4), and an internal standard of 19:0 was added to the triacylglycerol and phospholipid TLC fractions before methylation by the procedure of Metcalf *et al*., (1966). Gas chromatographic analysis of fatty acid composition of these two lipid fractions was done as described (26).

3.4. Western blot analysis

Cytochrome P450 isozymes and ubiquitin conjugates were measured by Western blot after 12% polyacrylamide gel electrophoresis using identical amounts of protein (5 μg) from either the liver homogenates or microsomes. For the whole liver preparation, immunoblots

were exposed to specific primary polyclonal antibodies to CYP2E1 and ubiquitin (DAKO Corp., CA) at 4°C overnight. The membrane was then exposed for 2 hours to the second antibody conjugated to alkaline phosphatase (Bio-Rad Lab, Richmond, CA). For the microsomal preparations, rabbit antisera raised against rat CYP2E1 (9), or rat CYP3A1 (gift from Dr. James R. Halpert, Tucson, AZ) and rat CYP4A1 (gift from Dr. Gordon Gibson, University of Surren, UK) were used at 1:400 to 1:200 dilutions. Linearity was established in all cases with respect to chemiluminescense detected by using the enhanced chemiluminescense method (Amersham). Protein was measured by BioRad based on Bradford method (4). The CYP2E1 and/or ubiquitin bands were quantified using a BioRad software program and a video densitometer (Bio-Rad, CA).

3.5. Northern blot analysis

The method used for RNA extraction from snap-frozen liver tissue was described in detail previously (27). Identical amounts (20 μg) of RNA were then fractionated by electrophoresis on 1.2% agarose gels, transferred to Zetabind membranes (AMF, Meriden, CN) and immobilized to the membranes by UV linker (UV stratalinker 1800); Stratagene cloning systems, La Jolla, CA). Thereafter, ^{32}P-labeled cDNA probes (1 x 10^6 cpm/mL) prepared by random priming were hybridized to the membranes which were then autoradiographed at -70° C using Kodak XAR-2 film. The complementary DNA clone in pUC9 of rat CYP2E1 was provided as a gift from Dr. Frank Gonzales (Laboratory of Metabolism, National Cancer Institute, Bethesda, MD). The complimentary DNA clone of maize ubiquitin was obtained as a gift from Dr. Monique Cadrin (University of Quebec, Trois Rivieres, Quebec, Canada). Human 18S ribosomal RNA was used to rehybridize the membranes for quantitation purposes. The autoradiographic bands were quantified using a BioRad video densitometer. The ratio of densitometric units of the mRNA signals divided by the 18S ribosomal RNA signals was calculated to normalize the results. Results are expressed as a percentage of arbitrary units.

3.6. Electrophoretic mobility shift analysis (EMSA) of NFkappaB

Nuclear isolates were prepared (11). Liver samples were homogenized in buffer A (300 mM sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Hepes (pH 7.5), 2 mM EDTA, and 14 mM Beta-mercaptoethanol (beta-ME) with the following protease inhibitors; 0.7 μg/ml pepstatin (Sigma Chemical Co., St Louis MO), 2 μg/ml aprotinin (Sigma), 50 μg/ml soybean trypsin inhibitor (Sigma), 10 mM benzamidine (Sigma), 0.5 mM AEBSF (Calbiochem, CA), 0.5 mM phenylmethyl sulfonyl fluoride (PMSF), 0.05 mM Spermidine (Sigma), 0.15 mM spermine (Sigma), 0.5 mM N,N,N^1,N^1-tetraacetic acid (Sigma), 2 μg/ml antipain (Sigma), 0.7 μg/ml leupeptin (Sigma).

A glass-teflon homogenizer at 0_C was used. The homogenate was filtered through cotton gauze, and centrifuged 5 min at 800 g at 4° C. The resulting pellet was resuspended in buffer A with 0.5% NP-10 and examined by light microscopy. Insufficiently lysed homogenates were doused two to three times, layered over buffer A with 30x sucrose (buffer B), centrifuged 5 min at 1,600 g, resuspended in buffer A, and recentrifuged as 1,600 g at 4° C. Pelleted nuclei were briefly washed in buffer A with 10% glycerol and then frozen in liquid nitrogen.

The nuclear pellet was resuspended in low salt solution: 20 mM Hepes (pH 7.9, 4° C), 0.02 mM KCl, 1.5 mM $MgCl_2$, 2.5% glycerol, 1% NP-40, 0.5 mM DTT, 0.2 mM PMSF, and 0.2 mM EDTA. High-salt solution (1.2 M KCl) was added drop-wise over 30 min on ice with gentle mixing and then it was centrifuged at 12,500 rpm with a Beckman type 16 rotor for 30 min. The nuclear extract was dialyzed at 4° C for 1hr twice against a 100 x vol solution: 2 mM Hepes, 50 mM KCl, 20% glycerol, 0.1% NP-40, 1 mM betaME, 0.2 mM PMSF, 0.5 mM DTT, and then centrifuged at 12,500 rpm in a Beckman type 16 rotor for 20 min. The protein concentration was then measured (4) for gel electrophoresis.

The EMSA was performed essentially as described (5). All assays were performed with an excess of probe. Four micrograms of protein extract was incubated with the radiolabeled probe (2 x 10^6 cpm) for 15 min at room temperature in binding buffer (10 mM Hepes), 50 mM KCl, 5 mM $MgCl_2$, 8% glycerol, 1% mM DTT, 0.2 mM PMSF, 0.2 mM EDTA with 2 μg of poly (dI-dC). In supershift experiments, antibody (2 μl) was incubated with nuclear extract for 45 min at room temperature before or after adding NFkappaB probe. NFkappaB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was labeled with (gamma-^{32}P) ATP. In cold competitive experiments, unlabeled oligonucleotide (30X excess) was incubated with the extracts for 15 min at room temperature for 4° C prior to the addition of the radiolabeled probe. Binding reactions were electrophoresed on nondenaturing 5% polyacrylamide gels in 0.5 X TBE buffer (44 mM Tris, 44 mM boric acid, and 1 mM EDTA). The gels were dried and exposed to X-ray film.

3.7. Assay of proteasomal proteolytic enzymes

The activities of the proteasomal enzymes chymotrypsin-like (Cht-L), trypsin-like (T-L) and peptidyl-glutamyl peptide hydroxylase (PGPH) were measured in the liver cytosol fraction after the method of Donohue *et al*., (8) using the respective substrates for hydrolysis: LLUY-AMC, LSTR-AMC and LLE-NA.

3.8. Statistical Analysis

Results are presented as mean $\pm$ SE. ANOVA one way analysis of variance, Student's t test and multiple group comparisons by Bonferroni *t* test were used (Sigma Stat software, San Francisco, CA) or the Student-Newman-Keuls method. Linear regression analysis was used for correlations. $p<0.05$ was considered statistically significant.

4. RESULTS

4.1. Animals and liver pathology

Effects of ethanol and cAMP treatment on the parameters of liver damage are summarized in table 1. The body weight gain was similar in all groups indicating a

Table 1. Effects of Ethanol and cAMP Treatment on liver damage

Treatment	cAMP+E	cAMP+D	E	D
Number of rats	5	6	7	6
E-fed (g/kg/D)	13.7±1.3*		13.3±0.4	
BAL mg/dl	171±92		164±81	
Body wt (g)	357±50	325±42	368±33	346±24
Liver wt (g)	17.4±2.5[a]	11.0±2.0	18.8±2.8[a]	12.0±1.8
ALT (IU/L)	50.6±14	73±20	78±33	58±18
Necrosis (0-2+)	0.5±0.5	0.9±0.8	1±1	0.8±0.8
Inflam (0-2+)	1.5±0.5	0.7±0.7	1.6±0.5	0.5±0.5
Fibrosis (0-2+)	0.4±0.8	0±0	0.6±0.9	0±0

*: Mean ± S.D. E= ethanol, D+ dextrose a: P= < 0.05 when compared with their controls (Bonferroni t test).

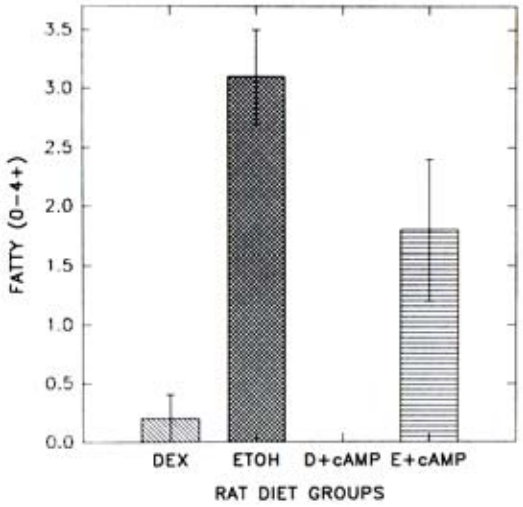

Figure 1. The histology score of liver fat (0-4+) for each experimental group is shown. DEX= dextrose pair-fed control for ethanol-fed group (ETOH). D= dextrose and E= ethanol for the cAMP treated groups, E compared with DEX, D+cAMP and E+cAMP= 0.05 (Bonferroni t test).

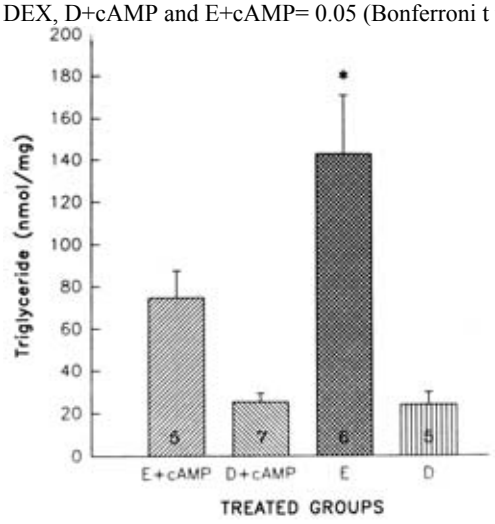

Figure 2. Effects of ethanol and cAMP on the liver triglyceride levels in the four treatment groups. E= ethanol and D= dextrose. Note that the ethanol-induced increase in triglycerides was significantly reduced by cAMP treatment (E+cAMP). * S.E.M., n= 5-7 as indicated, E= compared with E=cAMP D+ cAMP, D= p<0.05 (Bonferroni t test).

comparable nutrition. The average 2 months dose of ethanol fed was the same for Ethanol (E) and E+cAMP groups. The average BAL results obtained at the moment of sacrifice were identical with or without cAMP treatment. However, the mean UAL was significantly lowered by cAMP. In general, peak alcohol levels reached in two weeks were maintained high thereafter depending on the rate of ethanol elimination by the individual rats (data not shown). The liver weights were increased by ethanol with or without cAMP when compared to controls. By contrast, the ALT, bilirubin and glucose (not shown) levels were not significantly different between groups indicating that neither ethanol nor cAMP altered these parameters and, therefore, did not cause severe loss of liver function. The pathology scores were the same for E and cAMP+E except for the liver fat score which was significantly reduced by cAMP treatment (figure 1). When triglycerides were quantitated a significant reduction of fat in the E-cAMP livers was documented (figure 2).

4.2. Fatty acid composition

The levels of the fatty acid composition of the triglycerides in the four groups of rats are presented in table 2. The triglycerides and phospholipids were affected differently by ethanol feeding as reported previously (26). In the present experiment, ethanol did not affect the phospholipid levels (data not shown). Individual fatty acids in the triglyceride fraction such as 16:1, 18:2n-6 and 20:2 were increased, and 20:4n-6 and 22:6n-3 were decreased by ethanol (table 2). Likewise, the 18:1/18:0 and 20:4/18:2 ratios were increased and reduced by alcohol, respectively (figures 3 and 4). cAMP treatment tended to ameliorate the effect of ethanol on these fatty acids and the 18:1/18:0 and 20:4/18:2 ratios but these changes didn't reach significant levels. These results establish that cAMP reduced the effect of ethanol on the triglyceride fraction by half and tended to normalize the conversion of 18:2n-6 to 20:4n-6, a function of δ^5 and δ^6 desaturase. Likewise, they tended to normalized the conversion of 18:0 to 18:1 by δ^9 desaturase.

4.3. Liver regeneration

The PCNA staining of hepatocyte nuclei tended to be increased by ethanol, D+cAMP or E+cAMP treatment, when compared with dextrose controls (table 3 and figure 5). A tendency to increase the PCNA nuclear staining of non-parenchymal cells was also found with ethanol and D+cAMP treatment. This increase became

Table 2. Fatty acid composition of the triglyceride fraction of liver lipids in rats given ethanol and cAMP and their controls

	DIET GROUPS			
	Dextrose (D) (n=5)	**Ethanol (E) (n=6)**	**D+cAMP (n=7)**	**E+cAMP (n=5)**
Fatty acids %	**(mol/100 mol)**			
16:0	25.1±0.5	25.8±1.3	25.0±1.7	21.9±1.1
16:1	0.68±0.11	1.25±0.08[a]	0.85±0.14	1.06±0.38[a]
18:0	12.5±3.1	3.1±0.1	8.1±1.3	4.1±0.5
18:1n-9	13.7±1.7	17.1±0.6	15.1±0.5	16.3±0.8
18:2n-6	27.8±4.9	38.9±0.7[a]	35.5±1.6[a]	40.0±1.8[a]
20:0	0.58±0.10	0.83±0.12	0.53±0.20	0.60±0.15
20:2	0.44±0.02	0.70±0.08[b]	0.33±0.10	0.80±0.10
20:4n-6	13.2±2.3	7.1±0.1[a]	10.2±1.0	9.1±1.5
22:5n3	0.30±0.08[c]	0.18±0.04	0.03±0.03	0.16±0.07
22:6n3	1.45±0.37	0.33±0.02[a]	0.74±0.13[a]	0.38±0.15[a]

Data are expressed by means ± S.E.M. $p<0.05$, a: when compared with Dextrose;b: compared with dextrose and D+cAMP (Student - Newman - Keuls method).

Table 3. Effect of ethanol and cAMP treatment on liver cell proliferation (percent PCNA positive nuclei)

Treatment Groups	**cAMP+E**	**cAMP+D**	**E**	**D**
Number	6	6	4	5
Hepatocytes	14.6±13.3[+]	16.3±12.8	18.2±15.3	1.7±1.5[a]
Non-parenchymal cells	2.1±1.1	8.1±6.7[c]	8.6±4.2[b]	0.03±0.06
Bile ducts	0.5±0.6[c]	0.4±0.4[c]	0.14±0.15	0.01±0.01

+: Mean ± S.D. Student T test: a: $p<0.05$ when compared with E and cAMP+D, b: $p<0.003$ when compared with D, c: $p<0.04$ when compared with D

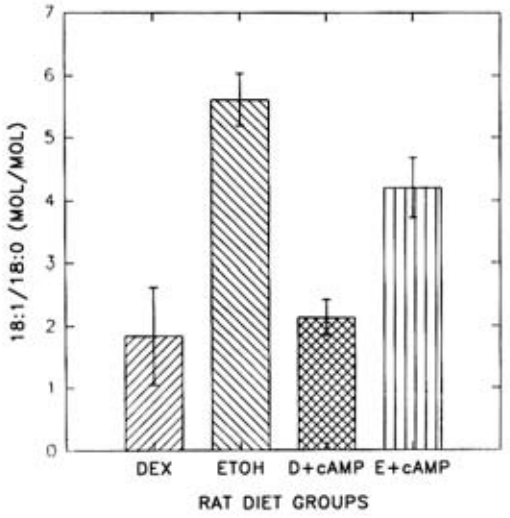

Figure 3. Effect of ethanol and cAMP on the liver fatty acid ratio 18:1/18:0. There was a significant increase in the ratio when ETOH (ethanol) and E+cAMP (ethanol+cAMP) were compared with their pair-fed controls ($p<0.05$, Student-Newman-Keuls method).

significant in ethanol animals treated with cAMP (table 3**)**. cAMP treatment tended to increase PCNA positive bile duct nuclei with or without ethanol **(**table 3). Linear regression analysis of the data from the 4 groups of rats revealed that there was a positive correlation between the PCNA positive bile duct nuclei and the pathology score ($p=0.011$, $R=0.545$). A positive correlation between the PCNA positive hepatocytes and the pathology score was $R=0.467$, $p<0.033$; The positive correlation between PCNA positive non parenchymal cells and the pathology score was $R=0.548$, $p<0.01$. Increased mitoses was also apparent microscopically in the liver cells from the rats fed ethanol (figure 6).

4.4. Cyclic AMP levels

The cAMP levels in the livers of rats given cAMP intraperitoneally were significantly increased compared with the rats not treated with cAMP (E and D groups) **(**figure 7). Thus, the liver cAMP levels were documented to be doubled by the cAMP treatment.

4.5. CYP2E1 levels

Ethanol increased the microsomal CYP2E1 levels 14 fold over the control levels **(**figure 8). The cAMP treatment (E+cAMP) significantly prevented the increase in liver microsomal CYP2E1 induced by ethanol ($p<0.05$). The other levels of CYP3A were not significantly different between groups, whereas cAMP reduced the CYP4A levels seen with ethanol alone (table 4).

4.6. CYP2E1 expression

CYP2E1 mRNA levels were decreased by cAMP treatment in both ethanol and dextrose fed rats ($p<0.05$) which correlated with the decrease in CYP2E1 protein levels in these groups (figure 9). Ethanol and control rats not treated with cAMP had the same CYP2E1 mRNA levels probably because the BAL was below 200 mg/dl at the time of sacrifice.

Table 4. Cytochrome P-450 isozyme levels in liver microsomes: Effects of ethanol and cAMP

ISOZYME	DIET GROUPS			
	Dextrose (D) (n=6)	Ethanol (E) (n=7)	D+cAMP (n=7)	E+cAMP (n=5)
CYP3A	93.7±27.4	94.6±38.0	130.4±28.3	63.5±42.8
CYP4A	111.5±36.3	137.9±30.1	75.0±35.6	42.1±13.4

The results are given as arbitrary units/mg protein. *n=4, Mean ± S.E.

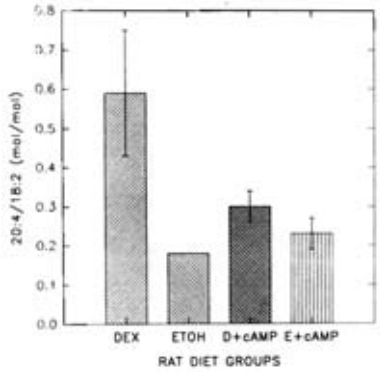

Figure 4. Effect of ethanol and cAMP on the liver fatty acid ratio 20:4/18:2. There was a significant decrease in the ratio between DEX (dextrose) and ETOH (ethanol), D+cAMP and E+cAMP (ethanol + cAMP) p=<0.05.

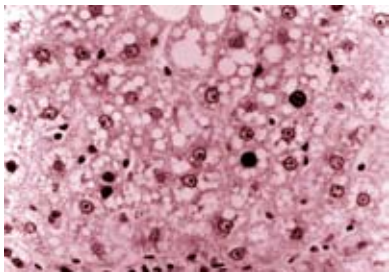

Figure 5. Rat liver from a rat treated with cAMP stained with an antibody to PCNA. Note the positively stained hepatocytic nuclei (arrows). Immunoperoxidase stain X 624.

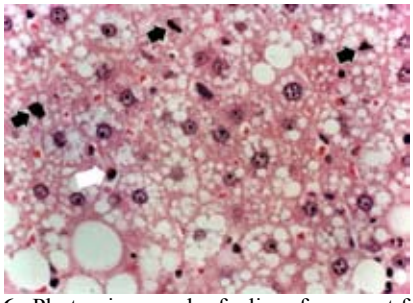

Figure 6. Photomicrograph of a liver from a rat fed ethanol showing fatty liver, apoptosis (white arrow) and several mitoses (black arrows), hematoxylin and eosin. X 624.

4.7. Ubiquitin levels

The Western blots of whole liver homogenates stained for ubiquitin showed a significant decrease ($p<0.05$) in the ubiquitin conjugated proteins in the ethanol fed rats compared with the dextrose controls (figure 10). This was also true after cAMP treatment (E+cAMP vs D+cAMP). The same pattern was seen when the mRNA for ubiquitin was measured by Northern blot (figure 11) suggesting that the reason that the ubiquitin conjugates were decreased was because ethanol inhibited expression at least at the level of the message. Ubiquitin levels measured by Western blot were as follows: E+cAMP= 133±28 (n=5), D+cAMP= 221±35 (N=7), E= 112±37 (n=7) and D=185±61 (n=5) (S.D.). Ethanol with or without cAMP significantly reduced ubiquitin levels ($p<0.05$).

4.8. Proteasome peptidase activity

The proteasomal proteolytic enzymes activities in the four experimental groups did not differ significantly except for chymotrypsin like (ChT-L) and tyrosine-like (T-L) activity (n=4). Ethanol decreased the ChT-L and T-L activity when compared to the controls ($p<0.05$) (figures 12 and 13). The PGPA activity was not effected by ethanol or cAMP (results not shown). cAMP treatment did not significantly ameliorate this effect of ethanol on the enzyme.

4.9. NFkappaB activation

The effect of ethanol and cAMP on NFkappaB activation is given in table 5. There was no difference between the ethanol-fed and dextrose control rats, however, cAMP significantly reduced the NFkappaB activation in both the ethanol fed and dextrose fed controls ($p<0.05$, n=3). Since activation of NFkappaB is initiated by phosphorylation of IkappaBalpha followed by digestion of IkappaBalpha by the proteasome to allow NFkappaB to go to the nucleus, and since ethanol inhibits the proteasomal peptidases, this inhibition could account for the decrease in NFkappaB activation observed. This raised the question whether NFkappaB is involved in regulating the expression of CYP2E1.

5. DISCUSSION

5.1. Proteolytic and synthetic pathways of CYP2E1 regulation

Cytosolic proteins are destined for degradation by proteasomal enzymes after being tagged by ubiquitin (14). In the case of ethanol-induced CYP2E1, which is rapidly decreased to constitutive levels by 24 hrs of ethanol withdrawal (33, 34), ubiquitination and proteolysis appears to be initially signaled by phosphorylation by a cAMP dependent protein kinase (PKA) (18, 9). We report here for the first time that cAMP diminished CYP2E1 expression selectively *in vivo*. After phosphorylation, the CYP2E1 which is stabilized by ethanol is probably degraded by the ubiquitin-proteasome pathway of intracytoplasmic proteolysis although this has not been conclusively proven (36,37). The mechanism of substrate (ethanol) stabilization

Table 5. The effect of ethanol and cAMP on NFkappaB activation

TREATMENT	NFkappaB (OD x mm^2)
Ethanol	55.2±8.3*
Ethanol + cAMP	25.0±13.8
Dextrose	55.7±17.7
Dextrose + cAMP	16.8±2.3

*: Mean ± S.D. n=3, $p < 0.05$ when Ethanol + cAMP was compared with Ethanol and Dextrose and when Dextrose + cAMP was compared with dextrose and ethanol.

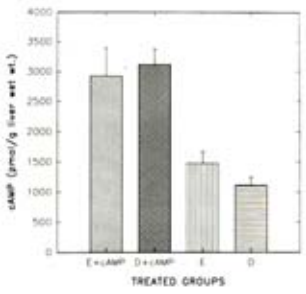

Figure 7. The liver levels of cAMP are shown for each of the 4 groups, E= ethanol, D= dextrose. There was a significant increase in cAMP levels in the livers from the rats treated with cAMP with or without ethanol ($P < 0.05$). (n=E+cAMP=5, D+cAMP=6, E= 7, D=6).

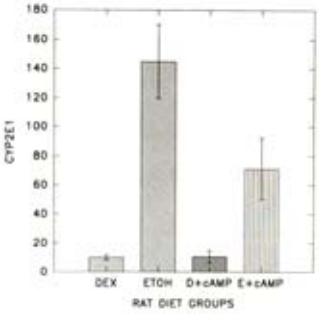

Figure 8. Effect of ethanol and cAMP on liver CYP2E1 levels. The ETOH (ethanol) levels are 14 fold higher than DEX (dextrose) and significantly different from D+cAMP, DEX, and E+cAMP ($p<0.05$, Bonferroni + test). n= DEX= 6, ETOH= 7, D+cAMP=7, E+cAMP= 5.

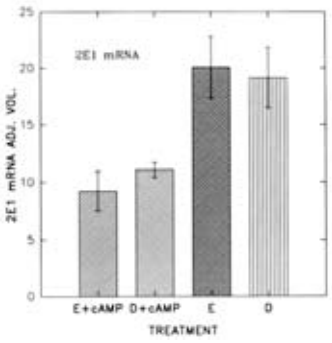

Figure 9. CYP2E1 mRNA levels (n=5-7) measured by Northern blot and correlated by 18S rRNA levels is shown. The cAMP treatment reduced the CYP2E1 mRNA levels in both E+cAMP and D+cAMP groups ($p<0.05$).

of CYP2E1 is mediated through protection from phosphorylation (9), thus preventing degradation by the ubiquitin triggered, ATP dependent, proteolysis by proteasomes (Figure 14). cAMP also may regulate CYP2E1 at the transcriptional level, although no specific cAMP-responsive element is present in the 5'- regulatory region (31).

The loss of CYP2E1 modified by the metabolism of substrates such as CCl_4 is associated with increased formation of high molecular weight microsomal ubiquitin conjugates (38). The data reported here indicates that ethanol feeding diminished ubiquitin, ubiquitin conjugate levels and proteasome enzyme activity whether the cAMP treatment was given or not while induced CYP2E1 levels were reduced only in the cAMP treated rats. Therefore, the mechanism by which the cAMP treatment prevented the induction of CYP2E1 by ethanol *in vivo* is probably by inhibiting the increase in CYP2E1 message rather than by accelerating the degradation of CYP2E1 protein. However, it is not clear that, at the mRNA level, cAMP can inhibit the transcription or induce degradation of the CYP2E1 mRNA. Examining the nucleotide sequence of DNase I protected regions in the CYP2E1 promoter, there are several potential transcription factor binding sites including the binding site for C/EBP, NF-1, HNF1, HNF-5 etc. No cAMP response element was noted (21). Therefore, if cAMP has an effect on the CYP2E1 gene itself, it is probably indirect.

The question is, how did the cAMP treatment reduce the ethanol-induced levels of CYP2E1. The ubiquitin levels and proteasomal enzyme activities were decreased, this may be the mechanism for CYP2E1 induction by ethanol. However, the cAMP treatment could have destabilized the CYP2E1 by favoring CYP2E1 phosphorylation. Ingelman-Sundberg has shown that CYP2E1 destabilization by phosphorylation initiated its degradation (9). To establish this mechanism in the rat model used we need to immunoprecipitate CYP2E1 and measure the level of phosphorylation of the CYP2E1 band using antibodies to phosphothreonine, serine and tyrosine in the rats fed ethanol with or without cAMP treatment. Similarly, the ubiquitination of CYP2E1 should be measured. Although ubiquitin levels were reduced by ethanol and the fact that ubiquitin is not involved in degradation of CYP2E1 *in vitro* (35) by the proteasome, it is still possible that ubiquitin is involved *in vivo* in the ethanol-induced CYP2E1 proteolytic pathway.

5.2. Influence of cAMP on ethanol-induced liver injury

Since cAMP treatment inhibits the induction of CYP2E1 by ethanol makes it possible to determine the role that CYP2E1 plays in the pathogenesis of alcoholic liver pathology. In fact, the ethanol-induced fatty liver was ameliorated in the cAMP treated rats fed ethanol when compared with the untreated ethanol treated rats. This was due to a reduction in the fatty liver caused by ethanol as documented morphologically and biochemically. However, the ameliorating effects of ethanol may be the result of multiple factors in addition to the reduction of CYP2E1, since other types of damage were not prevented

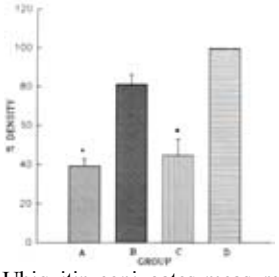

Figure 10. Ubiquitin conjugates measured by western blot (% density using control group D as 100%). Group A= cAMP+E (n=6), B= cAMP+D (n=7), C=E (n=6), D= D (n=5). A (cAMP+E) is significantly less than control B ($p<0.05$). Likewise C (E) is significantly less than the control D ($p<0.05$).

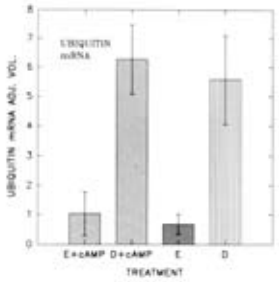

Figure 11. Ubiquitin mRNA levels measured by Northern blot and corrected by 18S rRNA levels are shown. The E+cAMP and E groups (n=4) were significantly different from the D+cAMP and D groups ($p<0.05$). Therefore the ethanol induced decreased ubiquitin mRNA was not effected by the cAMP treatment.

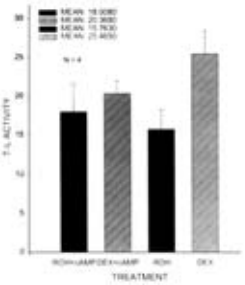

Figure 12. The effect of ethanol and cAMP on the proteasome proteolytic enzyme trypsin-like (T-L) activity. Ethanol significantly reduced the activity compared to the pair-fed dextrose controls ($p<0.05$, n=4).

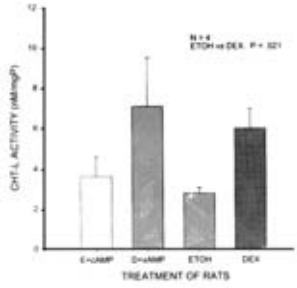

Figure 13. The effect of ethanol and cAMP on the proteasome peptidase chymotrypsin-like (CHT-L) activity. Ethanol significantly reduced the activity compared to the pair-fed dextrose controls (n=4, $p<0.03$).

by reducing CYP2E1 levels stimulated by ethanol in this model (24, 25, 26).

Ethanol has been shown to partly increase the liver weight due to an increase in liver cell protein levels (2) and by decreasing the rate of protein catabolism by the liver (7, 32). Since ethanol has been shown in this report to markedly reduce ubiquitin protein conjugates and proteasomal enzyme activity in the liver, the latter may, in part, explain the former, since ubiquitination is a major pathway by which cytosolic proteins are catabolized. This decrease in ubiquitin conjugates was found in both ethanol + cAMP and ethanol + dextrose treated rats, which correlated with the increased liver weights in both of these groups.

In the present report it was found that both cAMP and ethanol-feeding tended to cause an increase in nuclear PCNA labeling of different cell types. These changes correlated positively with the severity of the liver pathology. It is possible that the facilitation of the cell cycle by the elevated levels of cAMP in liver (15) augmented the regenerative response to injury, which may have had a beneficial effect on the liver damage by ethanol. Additionally, cAMP may have improved hepatic blood flow and in this way, countered the ethanol induced hypoxic damage to the liver (23). Ishii *et al* (16) reported that cAMP treatment reduced the necrosis seen with CCl_4 poisoning and at the same time increased hepatic blood flow.

The combination of the ethanol effect and the cAMP effect on the stimulation of liver cell proliferation probably involves multiple pathways and "cross talking" (6, 17) which could explain why the stimulation by ethanol and cAMP was not additive. Another explanation for the effect of cAMP on the growth stimulus of ethanol is that extracellular cAMP down regulates and intracellular cAMP up regulates gene transcription (10). This is complicated by the fact that extracellular cAMP increases intracellular cAMP by G-protein-mediated signal transduction. Liver damage may be the signal for the increased liver cell and stromal cell replication caused by ethanol-feeding since the percent liver positive nuclei correlated positively with the severity of the liver pathology score. However, the exact mechanism of how ethanol stimulates regeneration of hepatocytes, bile duct cells and non-parenchymal cell is not known. Similar findings are found in human ALD (12).

6. ACKNOWLEDGEMENTS

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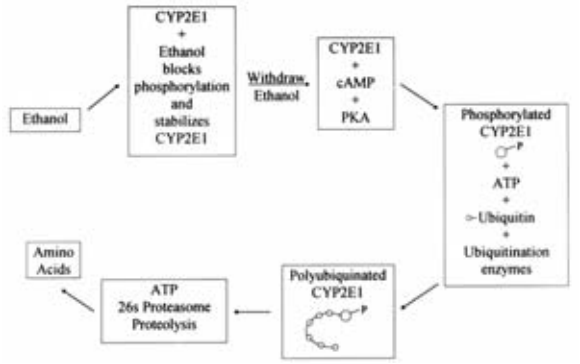

Figure 14. Diagram showing how stabilization of CYP2E1 by ethanol is followed by phosphorylation, ubiquitinization and proteolysis when ethanol is withdrawn.

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Key words: Ubiquitin mRNA, CYP2E1 mRNA, 26S Proteasome, Peptidases, Alcohol, Hyperplasia, cAMP

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