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Ethanol Cytotoxicity to a Transfected HepG2 Cell Line Expressing Human Cytochrome P4502E1*

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Defeng Wu and Arthur I. Cederbaum‡

From the Department of Biochemistry, Mount Sinai School of Medicine, New York, New York 10029

The effect of ethanol on the viability of a HepG2 cell model which was developed to constitutively express human CYP2E1 was studied in an attempt to establish a linkage between CYP2E1, reactive oxygen intermediates, and ethanol toxicity. Assays of toxicity included leakage of lactate dehydrogenase, trypan blue uptake, morphology, and formazan production. Ethanol was toxic to HepG2 E9 cells, which express CYP2E1, but not to HepG2 MV5 cells, which do not express CYP2E1. The ethanol toxicity was dependent on the concentration of ethanol, starting with 10 mM ethanol, and on the time of incubation with ethanol. Phorbol 12-myristate 13-acetate, which increases the expression of CYP2E1 in this model, increased the toxicity by ethanol. Ethanol toxicity was prevented by 4-methylpyrazole and by diallyl sulfide, inhibitors of CYP2E1. The ethanol toxicity was also prevented by radical trapping agents such as *N*-acetylcysteine and *N*-*t*-butyl-α-phenylnitrone, antioxidative agents such as catalase, superoxide dismutase, thiourea, and uric acid, and inhibitors of lipid peroxidation, such as vitamin E phosphate, Trolox, and diphenylphenylenediamine. Besides ethanol, other substrates such as Me_2SO, CCl_4, isoniazid, and *N*,*N*-dimethylnitrosamine were cytotoxic to cells expressing CYP2E1 but not to control cells. These results indicate that ethanol was toxic to HepG2 cells which express human CYP2E1 by a pathway sensitive to inhibitors of CYP2E1 and to a variety of antioxidative agents. This model appears to be useful in efforts to establish a CYP2E1-dependent ethanol hepatotoxicity system and to evaluate the role of oxidative stress and reactive radical species in the toxicity by ethanol.

A variety of mechanisms have been suggested to play important roles in pathways of ethanol toxicity to the liver. Some of these include effects of ethanol on mitochondrial functions and bioenergetics, membrane fluidity, and altered signal transduction, redox state changes as a consequence of ethanol oxidation, effects on the immune system or on eicosanoid metabolism, formation of acetaldehyde adducts, ethanol-induced hypoxia and reperfusion injury, ethanol-induced activation of Kupffer or stellate cells, and induction of CYP2E1. CYP2E1 oxidizes ethanol to acetaldehyde and oxidizes many agents to reactive metabolites that are hepatotoxic (1–3). CYP2E1 also displays high NADPH oxidase activity and is very reactive in production of O_2^- and H_2O_2 during NADPH oxidation (4, 5). Microsomes from ethanol-treated rats, in which CYP2E1 is induced, are more reactive than controls in catalyzing lipid peroxidation, generating ·OH and ·OH-like species, and forming hydroxyethyl radicals from ethanol (5–13). These increases in formation of reactive oxygen species are prevented by anti-CYP2E1 IgG, thus linking them to induction of CYP2E1 (5, 10). There is considerable interest in the role of oxidative stress and ethanol generation of reactive oxygen species in the mechanisms by which ethanol is hepatotoxic (14–16).

It has been difficult to establish direct linkage between CYP2E1, ethanol induction of reactive oxygen species, and ethanol hepatotoxicity largely because of the lack of suitable models which display alcoholic liver disease. A major advance has been the development of the intragastric model of ethanol feeding in which prominent induction of CYP2E1 occurs and in which significant alcohol liver injury occurs with rats consuming diets containing polyunsaturated fatty acids, but not saturated fatty acids (17–24). In these models, large increases in microsomal lipid peroxidation have been observed and the ethanol-induced liver pathology has been shown to correlate with CYP2E1 levels and elevated lipid peroxidation. The ethanol-induced liver injury was decreased by an oral iron chelator (25) and intensified by increasing the nonheme iron content of the diet (26). DAS,[1] an inhibitor of CYP2E1, prevented the elevation of lipid peroxidation and partially blocked the ethanol-induced liver pathology (27).

Our laboratory has recently established a HepG2 cell line which constitutively expresses the human CYP2E1 (28). The expressed CYP2E1 was catalytically active with typical CYP2E1 substrates, including ethanol, and was reactive in formation of reactive oxygen species, such as O_2^- and H_2O_2, and in catalyzing lipid peroxidation (28). Acetaminophen, an effective substrate for CYP2E1, was found to be cytotoxic to the HepG2 cells which expressed the CYP2E1 (E9 cells) but not to the control cells which were infected with retrovirus lacking the CYP2E1 cDNA (MV5 cells) (29). The goal of the current study was to evaluate the cytotoxic effects of ethanol to HepG2 cells expressing CYP2E1 and compare these effects to control cells which do not express CYP2E1 and to evaluate the effect of inhibitors of CYP2E1 and antioxidants on the ethanol toxicity. It was hoped that this model might prove useful in attempts to establish a CYP2E1-dependent ethanol hepatotoxicity system, and to evaluate the effect of antioxidants on the ethanol toxic-

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‡ To whom correspondence should be addressed: Box 1020, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Tel.: 212-241-7285; Fax: 212-996-7214.

[1] The abbreviations used are: DAS, diallyl sulfide; E9 cells, HepG2 cells infected with retrovirus containing cDNA for human CYP2E1; MV5 cells, HepG2 cells infected with retrovirus lacking the cDNA for human CYP2E1; MEM, minimal essential medium; 4MP, 4-methylpyrazole; PBN, *N*-*t*-butyl-α-phenylnitrone; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate; DPPD, diphenylphenylenediamine; NAC, *N*-acetylcysteine; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; FBS, fetal bovine serum; LTR, long terminal repeat.

TABLE I
Toxicity of CYP2E1 substrates and ligands to E9 cells

E9 and MV5 cells were seeded onto 24-well plates at a concentration of 4×10^4 per well. After 24 h of culture in 2 ml of MEM containing 10% FBS, fresh MEM containing 3% FBS plus 2% serum replacement TCH plus 0.025 μg of PMA/ml was added along with the indicated additions. After 2 days of treatment, formazan production from MTT was assayed using the Promega G4100 Kit. Viability is expressed as $A_{570\ nm}$ (peak formazan) minus $A_{630\ nm}$ (background). The plates containing ethanol (and their controls) were wrapped in Parafilm in order to minimize evaporation, and the medium and reagents were changed every 24 h.

Addition	Cell viability ($A_{570} - A_{630\ nm}$)	
	E9 cells	MV5 cells
None	0.40 ± 0.01	0.66 ± 0.03
Ethanol, 100 mM	0.24 ± 0.03 (−40)[a]	0.64 ± 0.03 (−3)
Me_2SO, 20 mM	0.20 ± 0.03 (−50)	0.60 ± 0.03 (−9)
N,*N*-Dimethylnitrosamine, 2 mM	0.24 ± 0.03 (−40)	0.67 ± 0.04 (+2)
Isoniazid, 1 mM	0.16 ± 0.02 (−60)	0.63 ± 0.03 (−5)

[a] The effect of the addition is shown in parentheses (%).

ity, in order to establish linkage between CYP2E1, generation of reactive species and ethanol-induced hepatotoxicity.

MATERIALS AND METHODS

Chemicals—Fetal bovine serum, MEM, antibiotics, and G418 were from Life Technologies, Inc. Ethanol (95%) was from Fisher. Trypan blue, LDH kit, Me_2SO, NAC, DAS, PBN, vitamin E phosphate, DPPD, uric acid, desferrioxamine, and thiourea were from Sigma. Trolox was from Aldrich. Catalase and superoxide dismutase were from Boehringer Mannheim. Cell culture flasks and plates were from Corning Inc. (Corning, NY).

In Vitro Model and Cell Culture Conditions—Two human hepatoma HepG2 sublines, which were established previously in our laboratory (28, 29), were used as models in this study. MVh2E1–9, contains a copy of human CYP2E1 cDNA under the control of the Moloney murine leukemia virus LTR promoter in the PMV-7 retroviral shuttle vector. This human CYP2E1 copy was from a full- length human cytochrome P4502E1 complementary DNA which was digested with Eco*RI* and subcloned into the Eco*RI* site of PMV-7. This cell line stably expresses human CYP2E1. The *p*-nitrophenol oxidation activity is usually at the level of 50 pmol/min/mg microsomal protein to 100 pmol/min/mg. In the presence of 5 mM 4-methylpyrazole or 0.025 to 0.1 μg/ml phorbol myristate acetate, this activity can be induced to levels 3–4-fold higher. PMA was usually present in the incubation medium for most experiments to increase the content of CYP2E1. MV5 is the control HepG2 cell line that has the PMV-7 vector alone. This cell line does not express CYP2E1 or only contains a trace amount of CYP2E1 on Western blot analysis using chemiluminescence detection. These cell lines were grown in MEM containing 10% fetal bovine serum and 0.1 mg/ml G418, supplemented with 1% penicillin-streptomycin-neomycin antibiotics. Cells were subcultured at a 1:5 ratio once a week. During the actual experiments with ethanol, the fetal bovine serum concentration was reduced to 3%, and 2% TCH, a commercial serum replacement was added. The TCH does not contain animal proteins but supports the growth of human cells very well. No significant LDH leakage was found in cell culture when the 3% fetal bovine serum plus 2% TCH mixture was used.

The Cell Titer[96] assay (Promega kit G4100) determines the conversion of a tetrazolium salt to a blue formazan product and is widely used to assess cell viability (30, 31). E9 or MV5 cells were seeded onto 24-well plates at a cell concentration of 4×10^4 in MEM containing 10% FBS for 24 h. The medium was then replaced with 3% FBS plus 2% TCH and treated with ethanol and/or various agents for 1–3 days. Most experiments involved a 2-day incubation period with ethanol. All plates (including controls) were wrapped in Parafilm to minimize evaporation of ethanol; medium and reagents were changed every 24 h. No change in pH was noted under these conditions in any of the samples. The medium was removed, the cells washed, and 1 ml of fresh medium containing MTT was added for a 1-h incubation at 37 °C. Stop solution was added (1 ml/well), and after 30–60 min, the absorbance at 570 and 630 nm was measured. The net difference $A_{570} - A_{630}$ was used to express the viability of the cells.

To assay for LDH leakage, E9 and MV5 cells were seeded onto six-well culture plates in the amount of 8×10^5 cells/well. After 24 h of culture in MEM medium containing 10% FBS, the medium was replaced with 3 ml of MEM containing 3% FBS plus 2% TCH, and ethanol and various agents were added, and the cells were incubated for varying times, usually for 2 days. At this point, the culture medium was collected and assayed for LDH using the Sigma LDH-20 diagnostic kit. Fresh medium (2 ml) was added to the cells, and the cells were scrapped with a cell scraper and collected in MEM. The cells were sonicated for 30 s using the microtip of an ultrasound sonicator, and aliquots were assayed for LDH. LDH leakage was expressed as the percentage of LDH in the medium to the sum of LDH in the medium plus LDH in the sonicated cell extract.

TABLE II
Assays of ethanol toxicity to E9 cells

E9 or MV5 cells were seeded onto either six- or 24-well plates and treated as described under "Materials and Methods" and the legend to Table I. The cells were treated with the indicated additions for 48 h. The medium and cells were collected from the six-well plates for assays of LDH leakage, while trypan blue exclusion and formazan production was assayed on cells in the 24-well plates. Results are from either two or four experiments.

Assay	Addition	Cell viability[a]	
		E9 cells	MV5 cells
LDH leakage	None	9 ± 1	17 ± 1
	Ethanol, 100 mM	41 ± 10	22 ± 3
	CCl_4, 300 μg/ml	41 ± 4	16 ± 1
Trypan blue uptake	None	2.5	2.4
	Ethanol, 100 mM	20.4	4.4
	CCl_4, 300 μg/ml	18.3	6.0
Formazan production	None	0.22	0.25
	Ethanol, 100 mM	0.13	0.27

[a] For LDH or trypan blue, assay is % leakage or % uptake. For formazan production, assay is $A_{570} - A_{630\ nm}$.

To assay for trypan blue exclusion, 1×10^4 E9 or MV5 cells were seeded onto 24-well culture plates and incubated for 24 h with MEM containing 10% FBS. Fresh MEM containing 3% FBS plus 2% TCH was added, followed by the addition of ethanol. After 2 days of incubation, a 0.1-ml solution of 0.05% trypan blue in 0.81% NaCl, 0.06% dibasic potassium phosphate was added to each well. After 5–10 min of incubation, the numbers of cells excluding or staining positively for uptake of trypan blue were counted under a light microscope. Cell morphology was also visualized under the light microscope.

Results refer to mean ± S.D., and most results are from four experiments.

RESULTS

Initial experiments evaluated the effect of several substrates and ligands for CYP2E1 on the viability of cells expressing CYP2E1 (E9 cells) and control HepG2 cells, which were infected with retrovirus lacking the cDNA for CYP2E1 (MV5 cells). Ethanol, Me_2SO, *N*,*N*-dimethylnitrosamine, and isoniazid all proved to be toxic to E9 cells at concentrations which had no effect on the viability of MV5 cells (Table I). Previous experiments reported on the toxicity of acetaminophen to E9 cells (29). The toxicity by ethanol was evaluated in further detail by a variety of techniques. Leakage of LDH was increased about 4-fold by ethanol in the E9 cells, whereas no effect was found with MV5 cells (Table II). Uptake of trypan blue was also increased by ethanol with the E9 cells, whereas vital dye staining (formazan production from MTT) was decreased by ethanol with E9 cells. No significant effects by ethanol were found with MV5 cells (Table II). Ethanol caused morphological changes in the E9 cells, as many cells were swollen, with condensed cytoplasm and nucleus, accumulation of intracellular vesicles, and irregular membrane shape with plasma membrane blebbing (Fig. 1). Ethanol had little or no effect on morphology of MV5 cells, as the cells maintained a regular plasma membrane, without blebbing, with few intracellular granules or vesicles and little condensation of the cytoplasm (Fig. 1).

Transcription of the CYP2E1 cDNA is driven by the LTRs of the viral promoter in this system (28). PMA was previously shown to increase the content of CYP2E1 about 3-fold by acti-

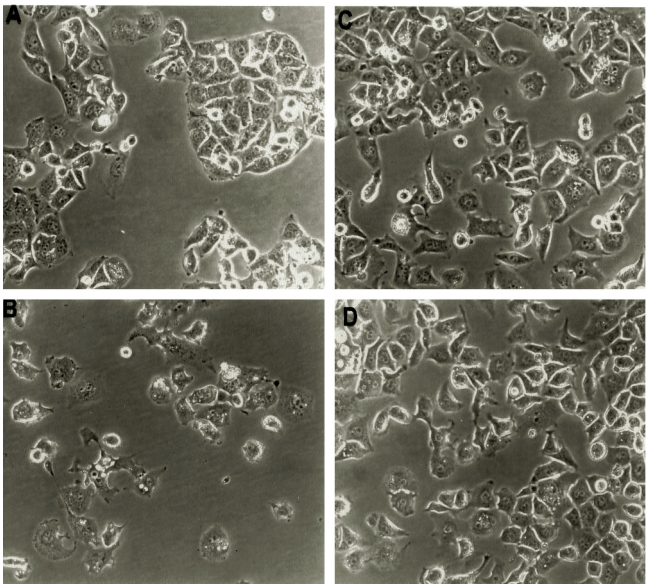

FIG. 1. **The morphology of E9 cells and MV5 cells exposed to ethanol was determined under the light microscope.** Cells were exposed to 100 mM ethanol for 48 h in the presence of 0.05 μg of PMA/ml (magnification, 10 × 20). *A*, control E9 cells; *B*, E9 cells plus ethanol; *C*, MV5 cells; *D*, MV5 cells plus ethanol.

vating the retroviral promoter (32). If ethanol toxicity in the E9 cells involved CYP2E1, increasing the expression of CYP2E1 by PMA treatment would be expected to enhance the toxicity of ethanol. This proved to be the case, as the increase in LDH leakage by E9 cells produced by 50 or 100 mM ethanol was increased from values of about 50 to 100% in the absence of PMA, to values of about 100 to 200% in the presence of PMA (Table III). Ethanol had no effect on LDH leakage by MV5 cells in the absence or presence of PMA (Table III).

The toxicity by ethanol increased as a function of time of incubation of the E9 cells with ethanol over a 1–3-day time period (Fig. 2). PMA increased the ethanol toxicity at all time periods. Significant toxicity could be observed at an ethanol concentration as low as 10 mM, with further increases as the ethanol concentration was elevated (Fig. 3). Ethanol concentrations as high as 200 mM had no significant toxic effect on MV5 cells, nor did incubating these cells with 100 mM ethanol for up to three days (Fig. 3).

4MP, besides being an inducer of CYP2E1, has been shown to be a ligand for CYP2E1, and an effective inhibitor of CYP2E1 catalytic activity including the oxidation of ethanol (33, 34). If ethanol toxicity to the E9 cells involved oxidation of ethanol by CYP2E1, 4MP would be expected to prevent this toxicity. 4MP proved to be completely protective against ethanol toxicity as assessed by either LDH leakage or formazan production (Table IV). 4MP also prevented the enhanced toxicity by ethanol in the presence of PMA (Table IV). In the absence of ethanol, 4MP alone had no effect on the viability of the E9 (or MV5) cells.

DAS is an inhibitor of CYP2E1 (35) and exerted some protective effect against alcoholic liver disease in the intragastric infusion model of liver injury (27). NAC reacts with a variety of nucleophiles and has been shown to protect cells against a variety of toxins and reactive oxygen intermediates (36). PBN is a spin-trapping agent and was shown to protect against toxicity in models of stroke and free radical generation (37). These three agents all proved to be effective in protecting E9 cells against acetaminophen toxicity (29). All three agents also proved to be effective in protecting the E9 cells against ethanol toxicity (Table V).

The effect of a variety of antioxidative agents on the ethanol

TABLE III
Effect of PMA on ethanol toxicity to E9 cells

E9 or MV5 cells were seeded onto six-well plates at a concentration of 8×10^5 cells/well and treated as described under "Materials and Methods." PMA was used at a concentration of 0.025 μg/ml of MEM + 3% FBS + 2% TCH. Experiments were carried out in the presence of the indicated concentrations of ethanol for 48 h prior to assays of LDH leakage.

Addition	Concentration of ethanol	LDH leakage	
		E9 cells	MV5 cells
	mM	%	
Control	0	9.5 ± 2	6.6 ± 3.6
	50	14.5 ± 3.3	5.9 ± 3.9
	100	19.7 ± 1.9	8.3 ± 1.3
PMA	0	9.8 ± 4.3	8.5 ± 0.5
	50	22.7 ± 8.4	3.5 ± 2
	100	37.4 ± 9.2	6.8 ± 2.3

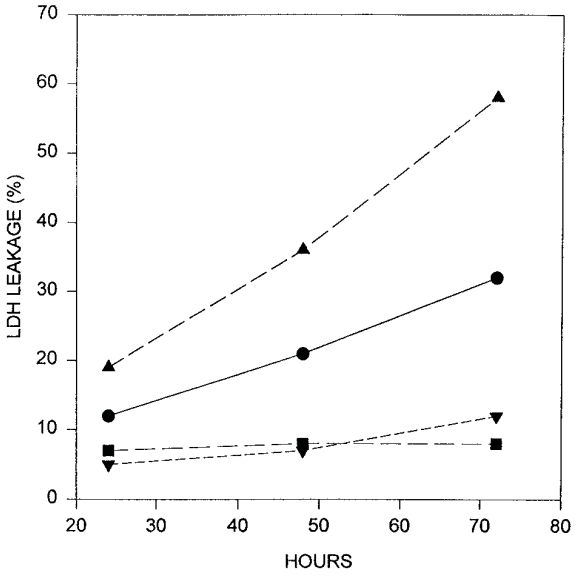

FIG. 2. **Time course curve for ethanol toxicity to E9 cells.** E9 or MV5 cells were seeded onto six-well plates at a concentration of 8×10^5 cells/well and incubated in 3 ml of MEM, 10% FBS for 24 h. Fresh MEM plus 3% FBS plus 2% TCH were added with or without 0.025 μg of PMA/ml, and the cells were incubated with 100 mM ethanol for 24, 48, or 72 h. Initial LDH leakage (zero time values) were between 3 and 7% for all treatments. Results are from four experiments. E9 (●——●), MV5 (■——■), E9 + PMA (▲——▲), MV5 + PMA (▼——▼).

toxicity to E9 cells was evaluated (Table V). The iron-chelating agent desferrioxamine proved to be very toxic to the E9 (and MV5) cells during the typical 2-day experimental period, most likely reflecting the need for iron for cell growth and viability. Superoxide dismutase and catalase were protective against the ethanol toxicity (Table V), suggesting a role for O_2^- and H_2O_2 in the overall mechanism. Uric acid and thiourea, antioxidants which were shown to protect HepG2 cells against H_2O_2-mediated cytotoxicity (38), were very powerful protectants against the ethanol toxicity to E9 cells as assayed by formazan production (Table V) or by morphology (Fig. 4). The cell swelling, the formation of intracellular vesicles, and the membrane blebbing caused by ethanol were largely prevented by thiourea and uric acid (Fig. 4).

The protection by superoxide dismutase, catalase, thiourea, uric acid, PBN, and NAC against the ethanol toxicity is consistent with a role for reactive radical intermediates in the pathway responsible for the ethanol-induced injury. Lipid peroxidation is one of the possible toxic consequences to cells as a result of elevated formation of reactive radical species. The effect of several powerful inhibitors of lipid peroxidation on the

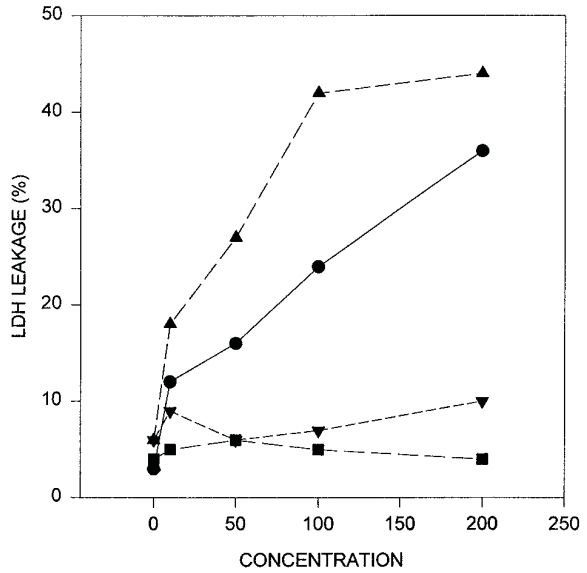

FIG. 3. **Concentration curve for ethanol toxicity to E9 cells.** Reaction conditions were the same as described in the legend to Fig. 2. The cells were incubated with 0, 10, 50, 100, or 200 mM ethanol for 48 h, followed by assays of LDH leakage. Results are from four experiments. E9 (●——●), MV5 (■——■), E9 + PMA (▲——▲), MV5 + PMA (▼——▼).

ethanol toxicity to the E9 cells was determined. Vitamin E phosphate, DPPD, and Trolox (a synthetic vitamin E analogue) all proved to be protective against the ethanol toxicity (Table V).

DISCUSSION

The toxicity of ethanol to the liver can probably be accounted for by a variety of mechanisms which are due to the metabolism of ethanol by alcohol dehydrogenase and cytochrome P450, and the direct interaction of ethanol with numerous biochemical pathways. Ethanol hepatotoxicity is likely to be multifactorial. Induction of CYP2E1 and the formation of reactive intermediates, including reactive metabolites, reactive oxygen species, and reactive ethanol radicals such as the 1-hydroxyethyl radical, appears to be one of the mechanisms that is receiving much current interest in studies evaluating how ethanol is hepatotoxic. It has been demonstrated that relative to several other P450 isozymes, CYP2E1 displays high NADPH oxidase activity, loose coupling and to be more reactive in oxidizing ethanol to the 1-hydroxyethyl radical (4, 5, 11–13). Microsomes from ethanol-treated rats are more reactive than controls in producing a variety of reactive oxygen intermediates by reactions sensitive to anti-CYP2E1 IgG and to chemical inhibitors of CYP2E1 (5–10). Correlations between induction of CYP2E1, lipid peroxidation and ethanol-induced liver injury have been reported with the continuous intragastric infusion model of ethanol feeding (18, 19, 22, 24, 27).

The presence of a stable cell line which constitutively expresses the human CYP2E1 appears to be an interesting model in which to assess a pathway of ethanol toxicity dependent on CYP2E1 and/or generation of reactive radical intermediates. HepG2 cells infected with retrovirus lacking the CYP2E1 cDNA would serve as the control for cells expressing CYP2E1. While it is recognized that the HepG2 cells are a transformed cell line, these human neoplastic liver cells express several liver-specific cell functions and share phenotypic similarities to hepatocytes (39, 40), catalyze oxidation of certain drug substrates (41), and contain NADPH-cytochrome P450 reductase and cytochrome b_5 (42–44), which are necessary for CYP2E1 catalytic activity. We have previously shown that CYP2E1 expressed in this model resembles its authentic human liver microsomal counterpart in molecular weight, oxidation of a variety of metabolic substrates, activation of hepatotoxins such as acetaminophen, and CCl_4, and generation of reactive oxygen species (28, 29, 32). The content of CYP2E1 in human liver microsomes varies considerably, due to the complex regulation of this P450. The CYP2E1 content in E9 cells was about 10–15 pmol/mg of microsomal protein; levels could be increased to about 30–40 pmol/mg of microsomal protein after "induction" (stabilization by 4MP or ethanol). Alcohol dehydrogenase activity could not be observed in these cells. Low K_m aldehyde dehydrogenase was present as the E9 cells oxidized 0.2 mM acetaldehyde at a rate of about 1–2 nmol/min/mg of protein (about 10–20% of that found with rat hepatocytes). We could not measure significant levels of acetaldehyde when ethanol was added to the E9 cells, presumably because the rate of acetaldehyde generation (ethanol oxidation by CYP2E1) was considerably lower than the rate of acetaldehyde removal by aldehyde dehydrogenase. Isolated microsomes from the E9 cells oxidized ethanol to acetaldehyde (28). Ethanol was found to cause toxicity to E9 cells, which express CYP2E1, at concentrations and reaction conditions in which no toxicity was observed with MV5 cells, which do not express CYP2E1. Toxicity was assessed by morphology, LDH leakage, trypan blue uptake, and formazan production. Some toxicity by ethanol could be observed after 1 day of culturing the E9 cells with ethanol, although more significant toxicity required more prolonged exposure to ethanol. Some toxicity could be observed at an ethanol concentration of 10 mM, a concentration which is the same as the K_m for ethanol oxidation by CYP2E1 (45); increased toxicity was found as the concentration of ethanol was elevated.

The most obvious factor indicating that the ethanol toxicity is dependent upon CYP2E1 is the observation that toxicity is observed in cells which express CYP2E1, but not in the cells which do not express CYP2E1. Increasing the expression of CYP2E1 by treatment with PMA also results in an increase in the ethanol toxicity. Ethanol toxicity is prevented by 4MP and DAS, inhibitors of CYP2E1 catalytic activity. 4MP, a potent inhibitor of alcohol dehydrogenase which has been used to treat methanol and ethylene glycol toxicity (46, 47), is an inducer of CYP2E1, binds to CYP2E1, and inhibits oxidation of ethanol and other substrates by CYP2E1 (33, 34, 48). This agent, unlike pyrazole, has been found to be relatively nontoxic upon prolonged administration (49). 4MP was effective in preventing the toxicity of acetaminophen to E9 cells as well as the labilization and degradation of CYP2E1 in E9 cells caused by CCl_4 (29, 32). 4MP proved to be very effective in preventing the toxicity by ethanol to the E9 cells and thus appears to be a valuable agent to prevent CYP2E1-dependent ethanol oxidation and toxicity.

The toxicity produced by ethanol appears to involve a mechanism involving reactive radical intermediates, since a variety of agents which interact with such species, *e.g.* NAC, PBN, thiourea, and uric acid, prevent the toxicity. The partial prevention by superoxide dismutase and catalase indicates roles for O_2^- and H_2O_2 in the ethanol toxicity; CYP2E1 is very reactive in producing these reactive oxygen species (5, 9). Since, in this model, ethanol increases the content of CYP2E1 by stabilizing the protein against degradation (50), ethanol toxicity may be due to an increased level of CYP2E1 leading to elevated production of O_2^- and H_2O_2. This may also contribute to the toxicity produced by Me_2SO, another ligand which can stabilize CYP2E1. Formation of ethanol radicals such as the 1-hydroxyethyl radical has been observed *in vitro* and *in vivo*, and is elevated after induction of CYP2E1 by ethanol (11–13, 51, 52).

TABLE IV
Prevention of ethanol toxicity to E9 cells by 4-methylpyrazole

E9 cells were seeded onto six- or 24-well culture plates at a concentration of either 8×10^5 or 4×10^4, respectively, and incubated in MEM, 10% FBS for 24 h. Fresh MEM containing 3% FBS, 2% TCH, and where indicated 0.025 μg/ml PMA was added, along with the indicated concentrations of ethanol and/or 4MP, and the cells were incubated for 48 h. A, LDH leakage was determined using the six-well plates. B, formazan production was assayed on the 24-well plates. No effect by ethanol was found, in the absence or presence of 4MP, with MV5 cells.

	Concentration of ethanol	Concentration of 4MP	LDH leakage or $A_{570} - A_{630\ nm}$	
			E9 cells	PMA E9 cells
	mM			
A	0	0	9 ± 2	11 ± 2
	0	5	11 ± 3	9 ± 5
	50	0	19 ± 1	28 ± 6
	50	5	10 ± 6	6 ± 3
	100	0	25 ± 1	38 ± 5
	100	5	5 ± 2	10 ± 2
B	0	0	0.77 ± 0.04	0.64 ± 0.02
	0	5	0.71 ± 0.06	0.59 ± 0.07
	50	0	0.54 ± 0.04 (30)[a]	0.36 ± 0.08 (43)
	50	5	0.71 ± 0.05 (0)	0.55 ± 0.01 (7)
	100	0	0.45 ± 0.05 (41)	0.25 ± 0.01 (61)
	100	5	0.75 ± 0.05 (+1)	0.59 ± 0.02 (0)

[a] The percent inhibition of vital dye reduction by ethanol is shown in parentheses (%).

TABLE V
Effect of antioxidants on ethanol toxicity to E9 cells

Experiments were carried out using 4×10^4 E9 cells seeded onto 24-well plates and treated as described under "Materials and Methods." The cells were cultured in the absence or presence of 100 mM ethanol and the indicated additions for 48 h and formazan production determined.

Addition	Cell viability ($A_{570} - A_{630\ nm}$) of E9 cells	
	−Ethanol	+Ethanol
None	0.56 ± 0.02	0.38 ± 0.01 (−32)[a]
NAC, 5 mM	0.45 ± 0.01	0.48 ± 0.01 (+7)
PBN, 1 mM	0.52 ± 0.04	0.52 ± 0.05 (0)
DAS, 0.01 mM	0.61 ± 0.04	0.54 ± 0.01 (−11)
4MP, 5 mM	0.54 ± 0.03	0.56 ± 0.02 (+4)
None	0.29 ± 0.01	0.19 ± 0.01 (−34)
Superoxide dismutase, 1000 μ/ml	0.27 ± 0.01	0.23 ± 0.01 (−15)
Catalase, 2000 μ/ml	0.27 ± 0.02	0.22 ± 0.01 (−18)
Desferrioxamine, 0.1 mM	0.09 ± 0.01	0.09 ± 0.01 (0)
Thiourea, 5 mM	0.27 ± 0.01	0.31 ± 0.03 (+15)
Uric acid, 1 mM	0.29 ± 0.02	0.33 ± 0.05 (+14)
None	0.32 ± 0.04	0.18 ± 0.06 (−44)
Vitamin E phosphate		
0.01 mM	0.28 ± 0.06	0.26 ± 0.04 (−7)
0.025 mM	0.28 ± 0.04	0.24 ± 0.06 (−14)
DPPD		
0.01 mM	0.28 ± 0.04	0.22 ± 0.04 (−21)
0.025 mM	0.22 ± 0.06	0.20 ± 0.01 (−9)
None	0.44 ± 0.04	0.28 ± 0.04 (−36)
Trolox		
0.025 mM	0.38 ± 0.02	0.44 ± 0.06 (+16)
0.05 mM	0.38 ± 0.02	0.42 ± 0.04 (+10)

[a] The effect of ethanol is shown in parentheses (%).

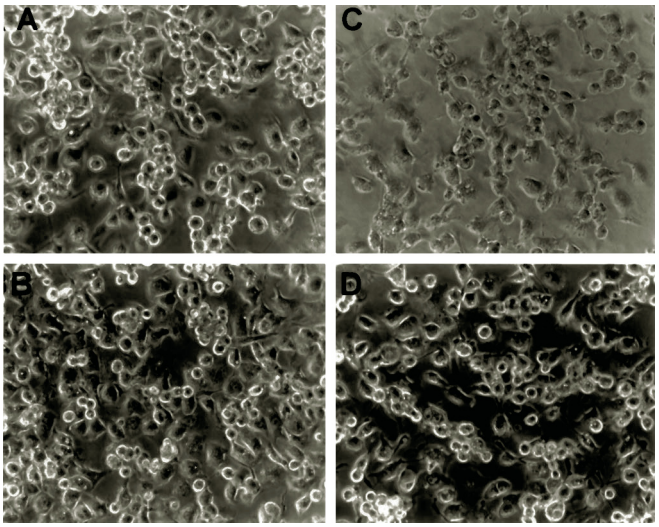

FIG. 4. **The effect of thiourea and uric acid on the morphology of E9 cells.** Experiments were carried out as described in Table V, incubating E9 cells with or without 100 mM ethanol for 48 h in the presence of either 5 mM thiourea or 1 mM uric acid (magnification, 10 × 20). *A*, E9 cells plus thiourea; *B*, E9 cells plus thiourea plus ethanol; *C*, E9 cells plus uric acid; *D*, E9 cells plus uric acid plus ethanol. Note that thiourea and uric acid prevent changes in cell morphology produced by ethanol (*e.g.* as shown in Fig. 1, *A* and *B*).

Oxidation of ethanol to the 1-hydroxyethyl radical may play a role in the ethanol-induced toxicity; in this respect, PBN, which reacts with such radicals, protected against the ethanol damage. Formation of 1-hydroxyethyl radical in E9 cells exposed to ethanol or formation of hydroxyethyl radical-protein adducts are planned in future studies. 1-Hydroxyethyl radicals can be produced from the oxidation of ethanol by oxidizing species generated from the interaction of non-heme iron with $O_2^{\cdot-}$ and/or H_2O_2 (11–13, 51, 52); hence superoxide dismutase and catalase will also prevent formation of hydroxyethyl radical, $^{\cdot}$OH-like species, and ferryl or perferryl-type oxidants. The question as to where superoxide dismutase and catalase are operative, intracellularly or extracellularly, is difficult to answer, since these enzymes can be taken up by liver cells and have been shown to be effective against hepatocyte damage produced by H_2O_2 by an endocytosis-dependent mechanism (53, 54). Since at least H_2O_2 is diffusable, extracellular catalase may function as an H_2O_2 trap, helping to remove H_2O_2 that is generated intracellularly.

Results shown in Table V suggest that lipid peroxidation appears to play a key role in the overall pathway of ethanol toxicity, since three antioxidants, which are classic inhibitors of lipid peroxidation, are all effective in preventing the injury by ethanol to the E9 cells. The key role of a lipid peroxidation type of mechanism in ethanol toxicity to the E9 cells is in agreement with an important contribution by lipid peroxidation to ethanol toxicity in the intragastric model of ethanol feeding (17–27) and a micropig model of alcohol-induced liver disease (55). Studies are currently in progress to provide evidence for oxidative stress including lipid peroxidation and protein carbonyl formation in the E9 cells as a consequence of ethanol addition. The role of lipid peroxidation in ethanol toxicity to the E9 cells is in contrast to the lack of a role of lipid peroxidation in acetaminophen toxicity to the E9 cells (29); in the latter case, reactive

protein adducts appeared to be responsible for the toxicity. The possible role of acetaldehyde or acetaldehyde protein adducts in the ethanol toxicity remains to be evaluated, however, preliminary studies with cyanamide, which nearly totally inhibits acetaldehyde oxidation by the HepG2 cells, indicated no effect on the ethanol toxicity. Since lipid peroxidation generally requires catalysis by iron, we studied the ability of desferrioxamine to prevent the ethanol toxicity. Unfortunately, desferrioxamine itself proved to be very toxic to the HepG2 cells; studies with other iron chelators are currently in progress. Preliminary studies indicate that addition of low concentrations of nonheme iron (ferric nitriloacetate) resulted in toxicity to E9 cells but not to the MV5 cells.

Recently, Neuman *et al.* (56, 57) reported that ethanol could induce hepatotoxicity to HepG2 cells. An approximate 30–40% loss of viability was observed after culturing HepG2 cells for 1 day with 60–80 mM ethanol as assessed by LDH release, and formazan production (56). Evidence employing electron microscopy and release of aspartate transaminase, glutamate dehydrogenase, and γ-glutamyltranspeptidase further validated the ethanol toxicity to HepG2 cells (56, 57). Alcohol dehydrogenase activity could not be detected in HepG2 cells; however, incubation with ethanol resulted in the appearance of such activity (57), which could explain the ethanol toxicity. We did not observe any significant toxicity to MV5 cells even after culturing them with 100 mM ethanol for up to 3 days. Whether these differences reflect varying culture conditions and media used, or differences between the parental HepG2 cell lines utilized, is not apparent at the present time; the latter may be an important factor for consideration when developing an "appropriate" HepG2 cell model to study ethanol metabolism, toxicity, or interaction with metabolic pathways.

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