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Cytochrome P450 2E1 Expression Induces Hepatocyte Resistance to Cell Death from Oxidative Stress

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ABSTRACT

Increased expression of cytochrome P450 2E1 (CYP2E1) occurs in alcoholic liver disease, and leads to the hepatocellular generation of toxic reactive oxygen intermediates (ROI). Oxidative stress created by CYP2E1 overexpression may promote liver cell injury by sensitizing hepatocytes to oxidant-induced damage from Kupffer cell-produced ROI or cytokines. To determine the effect of CYP2E1 expression on the hepatocellular response to injury, stably transfected hepatocytes expressing increased (S-CYP15) and decreased (AN-CYP10) levels of CYP2E1 were generated from the rat hepatocyte line RALA255-10G. S-CYP15 cells had increased levels of CYP2E1 as demonstrated by Northern blot analysis, immunoblotting, catalytic activity, and increased cell sensitivity to death from acetaminophen. Death in S-CYP15 cells was significantly decreased relative to that in AN-CYP10 cells following treatment with hydrogen peroxide and the superoxide generator menadione. S-CYP15 cells underwent apoptosis in response to these ROI, whereas AN-CYP10 cells died by necrosis. This differential sensitivity to ROI-induced cell death was partly explained by markedly decreased levels of glutathione (GSH) in AN-CYP10 cells. However, chemically induced GSH depletion triggered cell death in S-CYP15 but not AN-CYP10 cells. Increased expression of CYP2E1 conferred hepatocyte resistance to ROI-induced cytotoxicity, which was mediated in part by GSH. However, CYP2E1 overexpression left cells vulnerable to death from GSH depletion. *Antioxid. Redox Signal.* 4: 701–709.

INTRODUCTION

THE CYTOCHROME P450s are a superfamily of hemoproteins that mediate the oxidative metabolism of numerous endogenous and foreign compounds (16). Although many of the individual cytochrome P450 enzymes have marked species differences in their expression and catalytic activities, the cytochrome P450 isoform 2E1 (CYP2E1) has been well conserved in evolution, suggesting that it may perform vital physiological functions (36). CYP2E1 serves a particularly important function in the liver, where this enzyme metabolizes a number of hepatotoxins, including ethanol (23, 29). As a component of the microsomal ethanol-oxidizing system, CYP2E1 oxidizes ethanol to the potentially toxic product acetaldehyde (28). In addition, CYP2E1 has uncoupled NADPH oxidase activity that, in the absence of substrate, results in increased production of superoxide and hydrogen peroxide (H_2O_2) (19). These activated oxygen species can react with and alter various cellular macromolecules, leading to cellular injury and death.

CYP2E1 is induced by ethanol in cultured hepatocytes (25), and CYP2E1 expression is increased in animals and humans with alcoholic liver disease (ALD) (28, 42). Chronic ethanol administration in rats also induces hepatic production of reactive oxygen intermediates (ROI) (1, 32). Microsomes isolated from ethanol-treated rats have increased superoxide, H_2O_2 and hydroxyl radical production, and enhanced lipid peroxidation (12). The presence of CYP2E1 induction in ALD, and this enzyme's ability to metabolize ethanol and generate ROI, have suggested that CYP2E1 may play a mechanistic role in the development of ALD (28). Support for this theory has come from animal studies in which CYP2E1 induction has

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been correlated to increased oxidative stress and hepatocellular injury (1, 15, 34, 39), and CYP2E1 inhibition has been demonstrated to prevent the development of ALD (35). However, studies in CYP2E1 knockout mice have suggested that induction of CYP2E1 may not be causal in the development of ALD (22). An alternative theory of the pathogenesis of ethanol-induced hepatocyte injury implicates the production of ROI and the cytokine tumor necrosis factor-α by Kupffer cells, the resident liver macrophage (18, 24). The effect of increased CYP2E1 expression in ethanol-induced injury to hepatocytes therefore remains unclear.

Although the cellular responses to acute oxidative stress have been well studied, little is known about adaptive changes that occur in cells in response to chronic oxidative stress. In the face of chronic ROI generation that occurs with sustained CYP2E1 overexpression, hepatocytes may up-regulate factors protective against oxidant-induced injury. Investigations in a rat hepatoma cell line transfected with a CYP2E1 expression vector have demonstrated that CYP2E1 expression sensitized these cells to an oxidant-induced apoptosis from ethanol (43). However, transformed cell lines may differ from normal cells in their antioxidant defenses (38), and the relevance of these findings to nontransformed hepatocytes remains unclear. As an alternative approach to determine the effects of CYP2E1 expression on the hepatocyte's response to injury, we established clones of a nontransformed, rat hepatocyte cell line with differential CYP2E1 expression. To test the hypothesis that CYP2E1 expression may modulate hepatocyte injury from exogenous oxidant stress, we examined the sensitivity of these cell lines to ROI-induced toxicity. Surprisingly, expression of CYP2E1 conveyed resistance to ROI-induced cell death. Rather than promoting cell injury and death, increased CYP2E1 expression may to some extent serve a beneficial effect by protecting hepatocytes from the damage of oxidative stress.

MATERIALS AND METHODS

Cells and culture conditions

The rat hepatocyte cell line RALA255-10G (5) was cultured in Dulbecco's modified Eagle medium (Life Technologies, Rockville, MD, U.S.A.) supplemented with 4% fetal bovine serum (Gemini BioProducts, Calabasas, CA, U.S.A.), 2 m*M* glutamine, and antibiotics (Life Technologies), as previously described (45). These cells are conditionally transformed with a temperature-sensitive T antigen. At the permissive temperature of 33°C, the cells express T antigen, remain undifferentiated, and proliferate. Culture at the restrictive temperature of 37°C suppresses T antigen expression, slows growth, and allows differentiated hepatocyte gene expression (5, 9).

To establish cells with differential CYP2E1 expression, RALA hepatocytes were transfected with pCI-neo expression vectors (Promega, Madison, WI, U.S.A.) containing the human CYP2E1 cDNA in either a sense or antisense orientation (the kind gift of Dr. A. Cederbaum) (37). Cells were transfected using Lipofectamine Plus (Life Technologies) according to the manufacturer's instructions. Clonal, stable transfectants were selected in medium containing 400 μg/ml G418 (Life Technologies).

For all experiments, the cells were cultured at 33°C until confluent, trypsinized, and replated at 0.65×10^6 cells/dish on 35-mm plastic dishes (Falcon, Becton–Dickinson, Lincoln Park, NJ, U.S.A.). After 24 h, the medium was changed to Dulbecco's modified Eagle medium supplemented with 2% fetal bovine serum, glutamine, antibiotics, and 1 μ*M* dexamethasone, and the cells were placed at 37°C. Following 3 days of culture at 37°C, the cells received fresh serum-free medium containing dexamethasone. Medium was supplemented with dexamethasone to optimize hepatocyte differentiation as previously described (5). Cells were treated 20 h after the addition of serum-free medium with acetaminophen (1 m*M*), H_2O_2 (1.25 μmol/10^6 cells), menadione (25 μ*M*), or diethyl maleate (300 μ*M*). To increase cellular glutathione (GSH) levels, cells were treated with 5 m*M* GSH ethyl ester (Sigma, St. Louis, MO, U.S.A.) 1 h prior to treatment with H_2O_2 or menadione.

RNA isolation and Northern blot hybridization

RNA isolation was performed as previously described (6). Steady-state mRNA levels were determined by Northern blot hybridizations using samples of 20 μg of total RNA (6). The membranes were hybridized with [^{32}P]dCTP (Perkin–Elmer Life Sciences)-labeled cDNA clones for CYP2E1 (23) and glyceraldehyde-3-phosphate dehydrogenase (41). The hybridized filters were washed under stringent conditions (6).

Protein isolation and Western blot analysis

Transfected clones were screened for their levels of CYP2E1 expression by Western blotting. For protein isolation, cells were scraped in medium and centrifuged. The cell pellet was resuspended in lysis buffer containing 10 m*M* HEPES (pH 7.4), 42 m*M* $MgCl_2$, 1% Triton, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* EDTA, 1 m*M* dithiothreitol, and 2 μg/ml pepstatin A, leupeptin, and aprotinin, and mixed at 4°C for 30 min. After centrifugation, the supernatant was collected and the protein concentration determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, U.S.A.).

Fifty micrograms of protein was resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels as previously described (45). Membranes were stained with Ponceau red to ensure equivalent amounts of protein loading and electrophoretic transfer among samples. Rabbit anti-CYP2E1 polyclonal IgG, kindly provided by Dr. A. Cederbaum, was used as a primary antibody at a 1:1,000 dilution. A goat anti-rabbit IgG conjugated with horseradish peroxidase (GibcoBRL) was used as a secondary antibody at a 1:10,000 dilution. Proteins were visualized by chemiluminescence (Supersignal West Dura Extended, Pierce, Rockford, IL, U.S.A.).

Chlorzoxazone assay

Cells were cultured for 24 h following the addition of 200 μ*M* chlorzoxazone (Sigma) alone, or together with 50 μ*M* 4-methylpyrazole (Sigma). Tissue culture medium (1.5 ml) was

diluted with 0.375 ml of water and incubated overnight at room temperature with 200 units of *Helix pomatia* type H-1 β-glucuronidase (Sigma) dissolved in 0.75 ml of 0.5 *M* sodium acetate buffer (pH 5.0). The release of 6-hydroxychlorzoxazone was determined by HPLC as previously described (4). Standards with 2.5 pmol of 6-hydroxychlorzoxazone were prepared in tissue culture media. After incubation overnight, 3.5 pmol of the internal standard, 7-hydroxycoumarin, was added to all tubes, and each was extracted with 7 ml of ethyl acetate. The organic layer was transferred to clean tubes and evaporated to dryness. Samples were resuspended in 200 μl of HPLC mobile phase (79% 0.25% acetic acid and 21% acetonitrile). Metabolite and internal standard were resolved on a C-18 Supelco column (150 × 4.6 mm) at a flow rate of 1.0 ml/min. The eluent was monitored at 296 nm.

MTT assay

The amount of cell death was determined by examining cell number with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (28), as previously described (44). The percentage of cell death was calculated by taking the optical density of cells given a particular treatment, dividing that number by the optical density for the untreated, control cells, subtracting the result from 1, and multiplying that number by 100.

Microscopic determination of apoptosis

The relative numbers of apoptotic and necrotic cells were determined by fluorescent microscopy following costaining with acridine orange and ethidium bromide (10), as previously described (45). The percentage of cells with apoptotic morphology (nuclear and cytoplasmic condensation, nuclear fragmentation, membrane blebbing, and apoptotic body formation) was determined by examining >400 cells/dish. Cells were counted as necrotic if they stained positive with ethidium bromide.

GSH assay

Total cellular GSH was determined by the 5,5´-dithiobis(2-nitrobenzoic acid)-GSH disulfide recycling assay (3), as previously described (46).

Statistical analysis

All numerical results are reported as means ± SE and represent data from a minimum of three independent experiments.

RESULTS

Establishment of RALA hepatocyte cell lines with differential CYP2E1 expression

To establish a hepatocyte model for the purpose of investigating the effects of chronic CYP2E1 overexpression, the adult, rat, hepatocyte cell line RALA255-10G was transfected with sense (S-CYP cells) and antisense (AN-CYP cells) CYP2E1 expression vectors. The use of this cell line allowed studies to be performed in a nontransformed, differentiated hepatocyte. Stable clonal transfectants were analyzed by Western blotting with an anti-CYP2E1 antibody, and a sense-CYP2E1 expressing clone S-CYP15 and an antisense clone AN-CYP10 were selected for further study because of their maximal differential CYP2E1 expression. By Northern blot analysis, S-CYP15 cells had a greater than fivefold increase in CYP2E1 mRNA relative to wild-type (WT) RALA hepatocytes (Fig. 1). Low levels of CYP2E1 mRNA were detected in AN-CYP10 cells that may represent sense and/or antisense CYP2E1 mRNA (Fig. 1). Levels of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene were similar in the three cell lines (Fig. 1). Similar to the mRNA findings, CYP2E1 protein levels were increased 10-fold in S-CYP15 cells as compared with WT cells, whereas CYP2E1 protein was essentially undetectable in AN-CYP10 cells (Fig. 2).

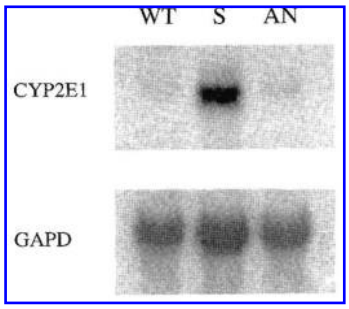

FIG. 1. CYP2E1 mRNA content is increased in S-CYP15 cells. Autoradiograms are of Northern blot hybridizations of 20 μg of total RNA isolated from WT, S-CYP15 (S), and AN-CYP10 (AN) cells hybridized with CYP2E1 and glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNAs as indicated.

To insure that elevated levels of CYP2E1 protein resulted in increased enzymatic activity, CYP2E1 catalytic activity was assayed by the 6-hydroxylation of chlorzoxazone. S-CYP15 cells had markedly increased chlorzoxazone metabolism compared with WT and AN-CYP10 cells (Table 1). This activity was decreased 65% by addition of the CYP2E1 inhibitor 4-methylpyrazole (Table 1). These data confirm that S-CYP15 cells express significantly higher levels of metabolically active CYP2E1 than WT or AN-CYP10 cells. No significant chlor-

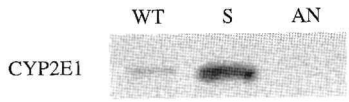

FIG. 2. CYP2E1 protein levels in WT, S-CYP15, and AN-CYP10 cells. Total protein was isolated from the WT, S-CYP15 (S), and AN-CYP10 (AN) cell lines. Aliquots were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting was performed using an anti-CYP2E1 antibody as described in Materials and Methods.

TABLE 1. CYP2E1 CATALYTIC ACTIVITY ASSAYED BY THE 6-HYDROXYLATION OF CHLORZOXAZONE

	6-OH-CZ
WT cells	0.04 ± 0.03
S-CYP15 cells	0.97 ± 0.12
AN-CYP10 cells	0.11 ± 0.05
S-CYP15 cells + 4-MP	0.33 ± 0.05

Release of 6-hydroxychlorzoxazone (6-OH-CZ) was determined as described in Materials and Methods. Levels of 6-OH-CZ are expressed as nmol/24 h/60-mm dish for the indicated cell lines. Some S-CYP15 cells were cotreated with 50 μ*M* 4-methylpyrazole (4-MP). Data are the means ± SE of three independent experiments done in duplicate.

zoxazone metabolism was detectable in WT or AN-CYP10 cells (Table 1), despite the detection of CYP2E1 protein in WT cells by Western immunoblotting (Fig. 1).

Acetaminophen toxicity is affected by CYP2E1 expression

In addition to the demonstration of increased CYP2E1 expression by Northern analysis, Western blotting, and chlorzoxazone assay, CYP2E1 function was assessed by an examination of toxicity from the CYP2E1-metabolized toxin, acetaminophen. Studies in CYP2E1 knockout mice have demonstrated that acetaminophen toxicity in hepatocytes is mediated predominantly by CYP2E1-dependent metabolism of acetaminophen to a toxic product (36). Treatment of the three cell lines with 1 m*M* acetaminophen for 24 h demonstrated that S-CYP15 cells were significantly more sensitive to acetaminophen toxicity with an almost threefold increase in cell death in these cells relative to AN-CYP10 cells (Fig. 3). Despite the failure to find differences in chlorzoxazone metabolism, but in keeping with the Western immunoblot evidence of increased CYP2E1 expression in WT cells as compared with AN-CYP10 cells, AN-CYP10 cells had a significant 36% decrease in cell death from acetaminophen when compared with WT cells (Fig. 3).

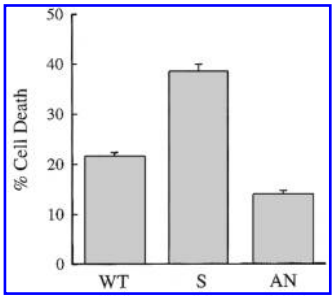

FIG. 3. Levels of acetaminophen-induced cell death correlate with CYP2E1 expression. WT, S-CYP15 (S), and AN-CYP10 (AN) cells were treated with 1 m*M* acetaminophen, and the percentage of cell death relative to untreated controls was determined at 24 h by MTT assay. Data are from three independent experiments, each done in duplicate.

CYP2E1 expression reduces hepatocyte sensitivity to ROI-induced toxicity

Increased CYP2E1 expression may promote liver cell injury and death not through the generation of an oxidative stress sufficient by itself to cause cell injury, but by weakening antioxidant defenses and making the cell susceptible to exogenously produced ROI. To examine the effects of CYP2E1 expression on hepatocyte sensitivity to ROI-induced cytotoxicity, cell survival was determined in WT, S-CYP15, and AN-CYP10 cells after 24 h of treatment with H_2O_2 or the superoxide generator menadione. WT and S-CYP15 cells exhibited similar toxicity to H_2O_2, whereas the amount of cell death in AN-CYP10 cells was increased approximately threefold (Fig. 4). S-CYP15 cells were completely resistant to a menadione concentration that led to virtually total AN-CYP10 cell death and 41% cell loss in WT cells (Fig. 4). Cell death from both H_2O_2- and superoxide-induced oxidative stress was therefore inhibited by increased CYP2E1 expression.

Previous studies have established that H_2O_2 and menadione induce an apoptotic cell death in WT RALA hepatocytes (20). To determine whether increased AN-CYP10 cell sensitivity to ROI-induced death was the result of apoptosis, S-CYP15 and AN-CYP10 cells were costained with acridine orange and ethidium bromide and examined under fluorescent microscopy for the presence of apoptosis and necrosis. In untreated cells, overexpression of CYP2E1 was associated with an increase in the number of apoptotic cells (Fig. 5). Six hours after treatment with H_2O_2, the percentage of S-CYP15 cells undergoing apoptosis had increased threefold (Fig. 5), consistent with previous findings that H_2O_2 induced an apoptotic cell death in WT cells (20). In contrast, AN-CYP10 cells had a decreased number of apoptotic cells after H_2O_2 treatment, but a fourfold increase in the percentage of necrotic cells (Fig. 5). Menadione treatment led to no increase in the numbers of apoptotic or necrotic S-CYP15 cells (Fig. 5), consistent with the absence of cell death in these cells by MTT assay (Fig. 4). In AN-CYP10 cells, menadione actually de-

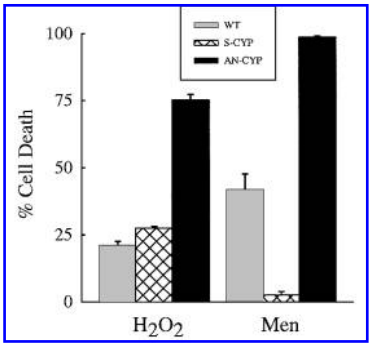

FIG. 4. CYP2E1 expression reduces hepatocyte cell death from ROI. WT, S-CYP15 (S-CYP), and AN-CYP10 (AN-CYP) cells were treated with H_2O_2 or menadione (Men) as described in Materials and Methods. The percentage of cell death was determined at 24 h relative to untreated controls by MTT assay. Results are from three independent experiments, with duplicate dishes for each data point.

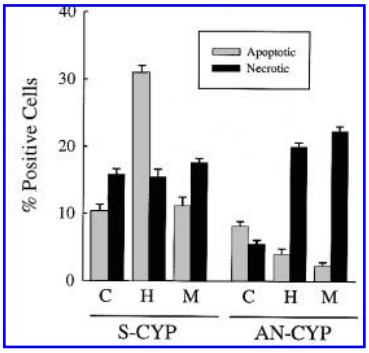

FIG. 5. Decreased CYP2E1 expression in hepatocytes converts ROI-induced cell death from apoptosis to necrosis. S-CYP15 (S-CYP) and AN-CYP10 (AN-CYP) cells were untreated (C), or treated with H_2O_2 (H) or menadione (M). The percentages of apoptotic and necrotic cells were determined at 8 h by fluorescent costaining with acridine orange and ethidium bromide as described in Materials and Methods. Data are from three independent experiments, each with duplicate dishes for every data point.

creased the numbers of apoptotic cells by 70%, whereas a fourfold increase in the percentage of necrotic cells occurred (Fig. 5). This induction of necrosis in AN-CYP10 cells by menadione contrasted with prior findings of a menadione-induced apoptosis in WT cells (20). AN-CYP10 cells therefore had not only an increased sensitivity to H_2O_2 and menadione toxicity, but also a conversion of ROI-induced cell death from apoptosis to necrosis.

CYP2E1 expression regulates hepatocyte levels of GSH

Cellular defenses against ROI-induced toxicity include antioxidant enzymes and nonenzymatic antioxidants, particularly GSH. The increased sensitivity of AN-CYP10 cells to H_2O_2- and menadione-induced cytotoxicity implied the existence of a relative deficiency in antioxidant defenses in AN-CYP10 cells as compared with WT and S-CYP15 cells. Levels of GSH, the principal nonenzymatic antioxidant in hepatocytes, were determined in the three cell lines. WT and S-CYP15 cells had equivalent levels of GSH, whereas GSH levels in AN-CYP10 cells were decreased 65% relative to WT and S-CYP15 cells (Fig. 6).

Effects of GSH supplementation and depletion on cell death

To evaluate the mechanistic involvement of decreased GSH levels in sensitizing AN-CYP10 cells to ROI-induced toxicity, the effect of GSH supplementation on ROI-induced AN-CYP10 cell death was determined. AN-CYP10 cells were pretreated with GSH ethyl ester, a cell-permeable form of GSH (2), 1 h prior to H_2O_2 or menadione administration. The percentage of cell death at 24 h measured by MTT assay was similar in H_2O_2-treated cells with and without GSH supplementation (Fig. 7). However, GSH pretreatment caused a ninefold reduction in cell death from menadione (Fig. 7). The

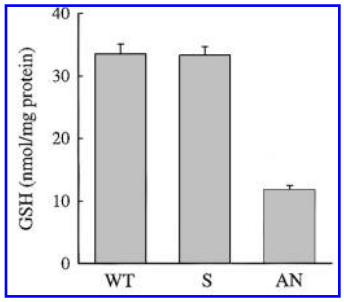

FIG. 6. AN-CYP10 cells have decreased GSH levels. Total cellular GSH levels were determined in untreated WT, S-CYP15 (S), and AN-CYP10 (AN) cells as described in Materials and Methods. Data are from three independent experiments, each with duplicate dishes for every condition.

reduction in cellular GSH levels in AN-CYP10 cells therefore played a role in their sensitization to menadione, but not H_2O_2-induced cytotoxicity.

Although S-CYP15 cells maintained cellular GSH levels equivalent to those in WT cells, overexpression of this prooxidant enzyme could leave S-CYP15 cells vulnerable to death upon depletion of their antioxidant defenses. To evaluate this possibility, WT, S-CYP15, and AN-CYP10 cells were treated with 300 μM diethyl maleate, a concentration of this GSH depleter previously demonstrated to result in a 90% decrease in RALA hepatocyte levels of GSH (46). This reduction in GSH induced 95 ± 3.5% cell death in S-CYP15 cells as determined at 24 h by MTT assay. In contrast, WT and AN-CYP10 cells tolerated this decrease in GSH with no induction of cell death. These data demonstrate that survival of CYP2E1-overexpressing cells was dependent on the maintenance of adequate stores of GSH.

S-CYP15 cell resistance to ROI-induced toxicity is not due to clonal variation

To insure that the findings in S-CYP15 and AN-CYP10 cells were due to their differential levels of CYP2E1 expres-

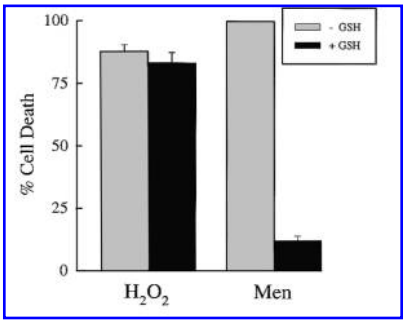

FIG. 7. GSH supplementation prevents menadione- but not H_2O_2-induced cell death in AN-CYP10 cells. AN-CYP10 cells were untreated (− GSH) or pretreated for 1 h with 5 mM GSH (+ GSH) prior to the addition of H_2O_2 or menadione (Men). The percentage of cell death relative to untreated controls was determined at 24 h by MTT assay. Data are from three independent experiments, with duplicate dishes for each condition.

sion, and not the result of nonspecific clonal differences, two additional sets of S-CYP and AN-CYP clones were investigated. Experiments were performed in S-CYP7 and AN-CYP9 cells, which by Western immunoblotting had increased and decreased CYP2E1 expression, respectively, relative to WT cells, and S-CYP10 and AN-CYP7 cells, which despite transfection and antibiotic selection had CYP2E1 protein levels equal to WT cells (data not shown). Identical to findings in S-CYP15 and AN-CYP10 cells, AN-CYP9 cells were significantly more sensitive to H_2O_2- and menadione-induced death than WT and S-CYP7 cells (Table 2). S-CYP7 cells also had markedly increased resistance to menadione toxicity compared with WT cells (Table 2). S-CYP7 cells were more sensitive, and AN-CYP9 cells more resistant, to acetaminophen toxicity relative to WT cells (Table 2). Finally, S-CYP7 cells had GSH levels equivalent to WT cells, whereas GSH content in AN-CYP9 cells was decreased 64% (Table 2). In contrast, there was no differential resistance to H_2O_2, menadione, or acetaminophen toxicity in S-CYP10 and AN-CYP7 cells, which have levels of CYP2E1 unchanged from WT cells (Table 2). Thus, the critical findings relating CYP2E1 expression to sensitivity to cell death and GSH content described in the S-CYP15 and AN-CYP10 cells lines were substantiated by similar results in two additional cell lines, S-CYP7 and AN-CYP9. In addition, the fact that these results specifically relate to CYP2E1 expression and not clonal artifact was further demonstrated by the finding that two cell lines, S-CYP10 and AN-CYP7, which have CYP2E1 levels equivalent to WT cells, underwent death responses similar to WT cells.

DISCUSSION

CYP2E1's capacity to metabolize ethanol (29), ability to generate toxic ROI (12), and increased expression in ALD (28, 42) have suggested that ethanol's induction of CYP2E1 expression may produce an oxidative stress leading to hepatocyte injury and ultimately ALD (28). This theory has been supported by *in vivo* studies in which chemical inhibition of CYP2E1 activity has prevented ALD in rats (35), but contradicted by studies that demonstrated that WT and CYP2E1 knockout mice were equally susceptible to the development of ALD (22). These divergent findings may be explained by species differences, the nonspecific activity of the CYP2E1 inhibitor, or the induction of other compensatory cytochrome P450 enzymes in CYP2E1 knockout mice. Although CYP2E1 overexpression has been studied in a hepatoma cell line (7, 33, 43), a full understanding of the hepatic effects of increased CYP2E1 levels has required an *in vitro* model of stable, increased CYP2E1 expression in a nontransformed, differentiated hepatocyte. Using RALA255-10G cells, we have established such cell lines with differential CYP2E1 expression. Of note is that WT cells had low levels of CYP2E1 expression by western blot analysis. Although these cells had no detectable CYP2E1 activity by chlorzoxazone assay, this failure to demonstrate catalytic activity may reflect insufficient sensitivity of this assay. Alternatively, a significant proportion of the chlorzoxazone metabolite may have remained intracellular and was therefore unmeasured. Supportive of the western blot findings of increased CYP2E1 expression in WT as compared with AN-CYP cells was the fact that WT cells did display increased susceptibility to the CYP2E1-dependent toxin acetaminophen as compared with AN-CYP cells.

The effect of CYP2E1 expression on cell injury from an exogenous oxidative stress was examined. In ALD, CYP2E1-expressing hepatocytes are exposed to such oxidants from activated Kupffer cells and neutrophils that constitute the accompanying inflammatory response (40). Expression of CYP2E1 in RALA hepatocytes decreased rather than increased sensitivity to ROI-induced cell death. Resistance to menadione-induced cell toxicity in particular strongly correlated with increased CYP2E1 expression. Not only were AN-CYP10 cells more sensitive to menadione, but also S-CYP15 cells were significantly more resistant than WT cells. Prior investigations of CYP2E1 expression in HeLa cells had demonstrated a twofold reduction in menadione toxicity in cells expressing CYP2E1 (17). Recent studies in CYP2E1-expressing hepatoma cells have also shown that CYP2E1 increased cellular resistance to oxidant-induced death (33). Thus, studies in several cell models all support the concept that chronic CYP2E1 expression protects against exogenous oxidants.

Studies of the mechanism by which CYP2E1 induced resistance to ROI toxicity revealed that inhibition of CYP2E1 expression led to a marked reduction in levels of the antioxidant

TABLE 2. PERCENTAGE OF CELL DEATH AFTER INJURY, AND GSH CONTENT IN WT RALA AND ADDITIONAL SENSE AND ANTISENSE CYP2E1 CLONES

	Percentage of cell death			
	H_2O_2	*Men*	*APAP*	*[GSH]*
WT	26.5 ± 5.7	33.0 ± 8.3	24.8 ± 1.9	30.1 ± 2.4
S-CYP7	28.8 ± 1.8	2.8 ± 1.1	38.1 ± 2.2	28.1 ± 1.8
AN-CYP9	51.6 ± 3.7	97.6 ± 1.6	12.3 ± 0.4	10.8 ± 1.0
S-CYP10	34.3 ± 3.9	45.0 ± 3.4	27.0 ± 1.7	ND
AN-CYP7	29.8 ± 6.0	35.2 ± 4.1	25.5 ± 2.6	ND

The percentage of cell death was determined by MTT assay 24 h after treatment with H_2O_2, menadione (Men), or acetaminophen (APAP). The GSH concentration ([GSH]) is in nmol/mg of protein. Results are means ± SE from three independent experiments each with duplicate dishes. ND, not determined.

GSH. Surprisingly, rather than causing a continuous oxidative stress that resulted in GSH depletion, CYP2E1 expression actually helped maintain cellular GSH levels. AN-CYP cells had reduced GSH levels, whereas WT and S-CYP cells had equivalent amounts. Although it is well known that an acute oxidative stress induces increased GSH production (30), the present studies are novel in their demonstration that constitutive cellular expression of a prooxidant enzyme can be essential for the maintenance of hepatocyte GSH content. Additional evidence of the ability of CYP2E1 to regulate GSH comes from recent investigations in the hepatocellular carcinoma cell line HepG2, where expression of CYP2E1 stimulated GSH synthesis (31).

The reduction in GSH in AN-CYP cells partially explained their increased sensitivity to ROI-induced cell death. GSH supplementation prevented AN-CYP10 cell death from menadione, but was ineffective against H_2O_2-induced cell death. Although H_2O_2 and superoxide are both ROI, we have previously demonstrated that H_2O_2 and menadione induce apoptosis in WT RALA hepatocytes through different cell death pathways (20). The failure of GSH supplementation to prevent H_2O_2-induced death implies that cytoprotective factors other than GSH must be up-regulated in S-CYP cells. Additional evidence of this fact is that S-CYP15 cells were significantly more resistant to menadione toxicity than WT cells despite their virtually identical GSH levels. These data indicate that some CYP2E1 expression is necessary to maintain normal GSH content in RALA hepatocytes, but increased CYP2E1 levels up-regulate other antioxidant factors. One such factor may be heat shock protein 70, which is overexpressed in S-CYP15 cells as compared with WT and AN-CYP10 cells (M.J. Czaja, unpublished data).

In addition to their increased sensitivity to ROI-induced cell death, AN-CYP10 cells underwent a death that was necrotic rather than apoptotic. Prior studies in nonhepatic cells have demonstrated that cell death can be converted from apoptosis to necrosis by reductions in cellular ATP (27), increases in the amount of oxidative stress (11), or caspase inhibition (21). It is possible that the low levels of GSH in AN-CYP10 cells in effect raised their relative level of oxidative stress and allowed it to interfere with ATP generation, thereby converting cell death from apoptosis to necrosis.

Despite the increased resistance of S-CYP15 cells to ROI-induced cell death, overexpression of CYP2E1 clearly had detrimental effects on RALA hepatocytes as well. As discussed previously, S-CYP15 cells were unable to survive an acute and marked depletion of their GSH. Presumably their higher constitutive level of oxidative stress became toxic when they were rapidly deprived of their principal, nonenzymatic antioxidant. Long-term ethanol treatment in rats leads to selective depletion of mitochondrial GSH in addition to the induction of CYP2E1 (13, 14). The combination of increased CYP2E1 expression and GSH depletion may result in oxidant-induced hepatocyte death in ALD. In addition, untreated S-CYP15 cells had higher levels of apoptotic and especially necrotic cells on fluorescent microscopy than did AN-CYP10 cells. At this time point, the cells had been in culture in serum-free medium for slightly more than 24 h. This high basal level of apoptosis/necrosis may reflect increased sensitivity to the lack of serum because these levels can be decreased by continuing to culture the cells in serum (M.J., Czaja, unpublished data). CYP2E1 overexpression may therefore be harmful to the cell at times of profound GSH depletion or nutrient deprivation as occur in ALD.

These investigations suggest that the effects of CYP2E1 expression on hepatocellular injury and cell death pathways are complex. Stable expression of this enzyme increases hepatocellular antioxidant defenses such as GSH presumably from the stimulation provided by an increased level of oxidant stress. CYP2E1 overexpression may then be an adaptive response that is initially protective to the cell. CYP2E1 overexpression by itself is unlikely to sensitize hepatocytes to Kupffer cell-produced ROI. Only when CYP2E1 overexpression is compounded by other factors, such as GSH depletion or nutrient deprivation, does this enzyme promote hepatocyte death. Overexpression of this prooxidant enzyme may therefore either inhibit or promote cell death depending on the physiological setting.

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ABBREVIATIONS

ALD, alcoholic liver disease; CYP2E1, cytochrome P450 2E1; GSH, glutathione; H_2O_2, hydrogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROI, reactive oxygen intermediates; WT, wild-type.

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