Adenovirus-Mediated Overexpression of Catalase in the Cytosolic or Mitochondrial Compartment Protects against Toxicity Caused by Glutathione Depletion in HepG2 Cells Expressing CYP2E1

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ABSTRACT
Induction of cytochrome P450 CYP2E1 by ethanol appears to be one of the mechanisms by which ethanol creates a state of oxidative stress. Glutathione (GSH) is a key cellular antioxidant that detoxifies reactive oxygen species. Depletion of GSH, especially mitochondrial GSH, is believed to play a role in the ethanol-induced liver injury. Previous results reported that depletion of GSH by buthionine-(S,R)-sulfoximine (BSO) treatment caused apoptosis and necrosis in HepG2 cells, which overexpress CYP2E1. In the current work, adenoviral infection with vectors that resulted in expression of catalase either in the cytosol or mitochondrial compartments was able to abolish the loss of mitochondrial membrane potential or damage to mitochondria observed in HepG2 cells overexpressing CYP2E1 that were treated with BSO. Loss of cell viability, either necrotic or apoptotic, was also prevented by the catalase overexpression after infection with the adenoviral vectors. The protective effects of catalase were associated with the suppression of the increase in the production of reactive oxygen species and of mitochondrial lipid peroxidation observed after GSH depletion. These results reveal a prominent role for H2O2 as a mediator in the cytotoxicity observed after depletion of GSH in HepG2 cells overexpressing CYP2E1. Damage to mitochondria may be a critical step for cellular toxicity by CYP2E1-derived reactive oxygen species.

One suggested mechanism by which ethanol can damage the liver involves the formation of reactive oxygen intermediates, lipid peroxidation, and oxidative stress (Nordmann et al., 1992). Ethanol can increase the content of CYP2E1, a cytochrome P450 that is active in oxidizing ethanol to acetaldehyde and in oxidizing many agents to reactive metabolites that are hepatotoxic (Lieber, 1997). CYP2E1 is also active in the production of O2- and H2O2 during microsomal mixed function oxidase activity (Gorsky et al., 1984; Ekstrom and Ingelman-Sundberg, 1989). Induction of CYP2E1 and formation of reactive intermediates is one of the mechanisms by which ethanol produces oxidative stress. Correlations between induction of CYP2E1, lipid peroxidation, and ethanol-induced liver injury have been found in the intragastric infusion model of ethanol-induced liver injury (Castillo et al., 1992; Ingelman-Sundberg et al., 1993; Morimoto et al., 1994; Nanji et al., 1994; Sadrrzadeh et al., 1994; Tsukamoto et al., 1995; Fang et al., 1998) and inhibitors of CYP2E1 partially prevent the injury (Morimoto et al., 1994; Fang et al., 1998). To study biochemical and toxicological effects of CYP2E1 a HepG2 cell line that overexpresses human CYP2E1 was established (E47 cells) (Chen and Cederbaum, 1998). The addition of ethanol, iron, or arachidonic acid to E47 cells decreased cell viability and caused apoptosis (Dui et al., 1993; Wu and Cederbaum, 1996; Chen et al., 1997); these effects were enhanced when cellular glutathione (GSH) levels were lowered by treatment with buthionine-(S,R)-sulfoximine (BSO). Moreover, the treatment of E47 cells with BSO, to deplete GSH, resulted in apoptosis as well as necrosis (Wu and Cederbaum, 2001), whereas no toxicity was found with control HepG2 cells (C34 cells) or HepG2 cells that expressed CYP3A4 instead of CYP2E1. The antioxidant Trolox partially prevented the apoptosis and necrosis after BSO treatment, whereas diallylsulfide, a CYP2E1 inhibitor, was fully protective.

As previously described, high levels of catalase can be expressed in HepG2 cells overexpressing CYP2E1 by using this study was supported by U.S. Public Health Service Grant AA 06610 from the National Institute on Alcohol Abuse and Alcoholism.

ABBREVIATIONS: GSH, glutathione; BSO, buthionine-(S,R)-sulfoximine; Ad, adenovirus; mCAT, mitochondrial catalase; MnSOD, manganese-superoxide dismutase; cCAT, cytosolic catalase; ROS, reactive oxygen species; DCF-DA, 2',7'-dichlorofluorescein diacetate; Ad-Null, adenovirus containing no cDNA; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide; MDA, malondialdehyde; PI, propidium iodide; AnnexV, Annexin V; FITC, fluorescein isothiocyanate; Rh123, rhodamine 123.
Virus possessing cytosolic catalase (Ad-cCAT) and mitochondrial catalase (Ad-mCAT) as well as Ad-Null were plaque-purified two times and amplified in 293 cells. Purified high-titer stocks of recombinant adenovirus were generated by two sequential rounds of CsCl density purification. The preparations were dialyzed and stored in dialysis buffer (10 mM Tris-Cl, pH 7.8, 15 mM NaCl, 10 mM MgCl₂, and 10% glycerol) and stored at −80°C. The titer of each viral stock was determined by plaque assay on 293 cells; titers were consistently $1 \times 10^{10}$ plaque-forming units/ml. The concentration of recombinant adenovirus was quantified also by absorbance, and the ratio of particles to plaque-forming units consistently ranged between 20 and 30.

**Cell Infection.** Before infection, C34 and E47 cells were seeded onto dishes or plates, grown to 60% confluence, and infected with Ad-cCAT, Ad-mCAT, and Ad-Null at multiplicity of infection = 100. Forty-eight hours after infection, cells were collected and assayed for catalase expression. Mitochondria were isolated by Percoll gradient centrifugation as previously described (Bai et al., 1999). The GSH content was assayed by following the increase in absorbance at 412 nm for 2 min in a cuvette containing: 0.1 M sodium phosphate, 5 μM EDTA buffer, pH 7.5, 0.6 mM M₅⁺-dithiobis(2-nitrobenzoic acid), 0.2 mM NADPH, 1 U/ml glutathione reductase, and 10 μl of sample (corresponding to ~100 μg of protein). The increment in absorbance at 412 nm was converted to GSH concentration by using a standard curve with known amounts of GSH.

**DCF Fluorescence As a Measure of Reactive Oxygen Production.** After treatment of the cells with or without BSO and the appropriate adenoviral vector, DCF-DA was added at a final concentration of 2 μg/ml, and plates were incubated for 30 min at 37°C in the dark. Cells were washed twice with 1× PBS, trypsinized, resuspended in 1 ml of 1× PBS, and fluorescence was immediately read in a PerkinElmer 650–105 fluorescence spectrometer at 490 nm for excitation and 525 nm for emission with a slit width of 5 nm for both excitation and emission monochromators. Background readings from cells incubated without DCF-DA were subtracted. Results are expressed as arbitrary units of fluorescence per milligram of protein.

**MTT Assay.** Cytotoxicity of BSO to C34 and E47 cells was determined by the MTT assay. Cells per milliliter per well (1.5 × 10⁴) were plated onto a 24-well plate and incubated in 5% CO₂ at 37°C. The MTT assay was performed using the Cell Titer 96 nonradioactiviss proliferation assay kit (Promega). Briefly, 15% volume of dye solution was added to each well after the appropriate incubation time. After 4 h of incubation at 37°C, an equal volume of solubilization/stop solution was added to each well for an additional 1-h incubation. The absorbance of the reaction solution at 570 nm was recorded. The absorbance at 630 nm was used as a reference. The net A₅₇₀ - A₆₃₀ was taken as the index of cell viability. The net absorbance change taken from the wells of untreated cultured cells was used as the 100% viability value. The percentage of viability was calculated by the formula $(A_{570} - A_{630})_{sample}/(A_{570} - A_{630})_{control} \times 100$.

**Lipid Peroxidation Measurement.** Malondialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acids, was used as an index of lipid peroxidation reactions according to the method of Niehaus and Samuelsson (1968). Briefly, 1 × 10⁴ cells were cultured onto 10-cm plates and after treatment, cells were scraped in PBS containing 0.5 mM Trolox. The pellets were collected by centrifugation and resuspended in PBS. An aliquot of the cell suspension or of the mitochondrial or microsomal suspension was incubated with trichloroacetic acid-thiobarbituric acid plus 0.5 mM Trolox for 15 min at 100°C. After centrifugation, the absorbance of the supernatant was measured at 535 nm.

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In Vitro Model and Cell Culture Conditions. Two human hepatoma HepG2 sublines, which were established previously in our laboratory (Chen and Cederbaum, 1998), were used as a model in this study. E47 cells contain the human CYP2E1 cDNA (kindly provided by Dr. F. Gonzalez, National Cancer Institute/National Institutes of Health, Bethesda, MD) inserted into the EcoRI restriction site of the pCI-neo expression vector (Promega, Madison, WI) in the sense orientation. C34 cells contain the pCI-neo vector alone. The HepG2-transduced clones C34 and E47 were cultured in minimal essential medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere in 5% CO₂ at 37°C. Cells were maintained in the presence of 0.5 mg/ml genetin.

Most reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The protein content of cell lysates or isolated mitochondria or microsomes was determined using the DC-20 protein assay kit (Bio-Rad, Hercules, CA). 2′,7′-Dichlorofluorescin diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR). Specific reagents are described below. CYP2E1 levels were routinely monitored by assaying the oxidation of p-nitrophenol to p-nitrophenol.

**Recombinant Adenovirus Production.** Ad5 adenoviral vector with compensating deletions in the early region 1, as developed by Graham and coworkers (McGorvy et al., 1988; Bett et al., 1994), was purchased from Microbix Biosystems, Inc. (Toronto, Canada). Human catalase cDNA and catalase cDNA with a manganese-superoxide dismutase mitochondria leader sequence were obtained as described previously (Bai et al., 1999) by digestion of plasmids pZeoSV-CAT and pZeoSV/MSP-CAT, respectively (kindly provided by Dr. J. Andres Melendez, Albany Medical College, Albany, NY). The plasmid shuttle vectors pAd5-CMV-Cat and pAd5-CMV-mCat were constructed by inserting catalase cDNA or catalase cDNA with a manganese-superoxide dismutase mitochondria leader sequence, respectively, into the Ad5 shuttle vector pCA13. These adenoviral shuttle plasmids together with the Ad5 genomic DNA JMI7 were transfected into human embryonic kidney 293 cells, which provide the E1A gene product necessary for viral replication during transfer. After transfection, plates were overlaid with agar, and initial plaques were harvested, amplified, and screened for enzymatic activity. Adenovirus containing no cDNA (Ad-Null) was used as a control.
Catalase Activity. Fresh sonicated extracts from cells or purified mitochondria were used. Catalase activity was determined at 25°C according to Claiborne and Fridovich (1979). The decomposition of hydrogen peroxide by catalase was followed by ultraviolet spectroscopy at 240 nm. The reaction was performed using a solution of 20 mM hydrogen peroxide in 50 mM KH2PO4 containing 20 μg of total cellular or purified mitochondrial protein in a final volume of 1 ml. Specific activity of catalase was calculated from the equation: specific activity (units/mg protein/min) = ΔA240 nm (1 min) × 1000/43.6 × milligrams of protein.

Western Blot. Cells isolated from 80% confluent 75-cm2 culture flasks were washed twice with 1× PBS and harvested by scraping and subsequent sonication for 30 s. Total cell extract or mitochondria prepared by discontinuous Percoll gradient centrifugation (Bai et al., 1999) were resuspended in 0.1 M potassium phosphate buffer, pH 7.4. After protein determination, 10 μg of either purified mitochondria or cell extract was resolved on 12% SDS-polyacrylamide gel electrophoresis and electrobotted onto nitrocellulose membranes (Bio-Rad). Membranes with transferred proteins were incubated with rabbit anti-human catalase antibody (1:1000) (Calbiochem, San Diego, CA) as primary antibody, followed by incubation with horse-radish peroxidase conjugated to goat anti-rabbit IgG (1:10,000; Sigma Chemical Co.) as the secondary antibody. Chemiluminescence reaction using the ECL kit (Amersham plc; Little Chalfont, Buckinghamshire, UK) was carried out for 1 min followed by exposure to Kodak X-Omat radiograph film (Eastman Kodak, Rochester, NY).

Flow Cytometry Analysis of the Mitochondrial Membrane Potential. Changes in the mitochondrial membrane potential were examined by monitoring the cells after double staining with PI and rhodamine 123 (Lemasters and Nieminen, 1997). E47 cells (5 × 105) were seeded onto six-well plates and infected with adenovirus. After 72 or 96 h with 0.1 mM BSO, the cells were then incubated with medium containing 5 μg/ml rhodamine 123 for 1 h. Cells were harvested by trypsinization and resuspended in 1 ml of minimal essential medium containing 5 μg of PI. The intensity of fluorescence from PI and rhodamine 123 was analyzed by flow cytometry.

Analysis of Apoptosis by Annexin V Staining. Apoptosis was determined using an apoptosis V-FITC apoptosis detection kit (Oncogene Science, Cambridge, MA). Briefly, after treatment with BSO and the appropriate adenoviral vector, C34 and E47 cells were collected, washed twice in cold PBS, and then resuspended in binding buffer at a density of 1 × 106 cells/ml. Fluorescein-labeled Annexin V and PI were added to the cells, followed by an incubation for 15 min before analysis with an FACSscan. Annexin V-FITC generated signals were detected with a FITC signal detector (FL1).

Statistics. Results refer to mean ± standard deviation and are average values from two to four values per experiment; experiments were repeated at least twice. Groups were compared among themselves by using Student’s t test for unpaired data. Differences at p < 0.05 were considered significant.

Results

Depletion of Glutathione in HepG2 Cells Expressing CYP2E1 Induces Cell Death: Protection by Adenoviral Catalase Infection. Previous studies (Wu and Cederbaum, 2001) have shown that treatment of HepG2 cells expressing CYP2E1 (E47 cells) with BSO resulted in apoptosis as well as necrosis but little or no toxicity was found with control HepG2 cells (C34 cells) or cells expressing CYP3A4 instead of CYP2E1. In the present work, the possibility that catalase could protect against this CYP2E1 plus BSO-dependent toxicity was evaluated by infection with adenovirus containing either empty vector (Ad-Null), cytosolic catalase (Ad-cCAT), or mitochondrial catalase (Ad-mCAT). The infection was performed 24 h before treatment with BSO. Previous experiments in non-BSO-treated C34 and E47 cells, by using confocal microscopy, Western blot analysis, and catalytic activity assays (Bai and Cederbaum, 2001), showed that infection with Ad-cCAT elevated the content and activity of catalase 2- to 3-fold (multiplicity of infection = 100) in the whole cell extract and the cytosolic compartment with little or no detectable catalase in the mitochondrial compartment. Infection with Ad-mCAT also elevated the content and activity of catalase in the whole cell extract about 2- to 3-fold with little effect over the basal content and activity in the cytosolic compartment. However the infection with Ad-mCAT resulted in the expression of, and activity of, catalase in the mitochondrial compartment. Table 1 shows the activity of catalase in the absence or presence of BSO treatment and after infection with Ad-cCAT or Ad-mCAT in the presence of BSO. In non-infected C34 or E47 cells, catalase activity in cell extracts was the same in the absence or presence of BSO (i.e., BSO treatment did not induce catalase activity). Mitochondrial catalase activity could not be, or was barely detectable in the absence or presence of BSO (Table 1). As described previously (Mari and Cederbaum, 2001) catalase activity was higher in E47 cells extracts. Infection with Ad-cCAT increased catalase activity about 2-fold in cell extracts of C34 and E47 cells, without any affect on catalase activity in mitochondria. However, infection with Ad-mCAT, which elevated catalase activity about 2.5-fold in C34 and E47 cell extracts, also resulted in catalase activity in the isolated mitochondria of both cell lines. Protein levels of catalase were determined in total homogenate (Fig. 1A) and in purified mitochondrial fraction (Fig. 1B) of C34 and E47 cells after treatment with BSO. Treatment with BSO did not affect the content of catalase in the C34 or E47 cell extracts (nor result in the presence of catalase in the mitochondrial fractions). Infection with both Ad-cCAT and Ad-mCAT increased catalase levels in total cell extract of C34 and E47 cells (Fig. 1A). As shown on Fig. 1B, Ad-mCAT (but not Ad-cCAT) infection induces an increase of catalase into the mitochondrial fraction of both C34 and E47 cells. The results of Table 1 and Fig. 1 demonstrate the effectiveness of the MnSOD mitochondrial leader peptide in driving catalase expression in the mitochondrial compartment.

Treatment of E47 cells with BSO for 72 or 96 h resulted in a loss of cell viability (Fig. 2, A and B). Toxicity by BSO treatment of E47 cells was dose-dependent (Fig. 2C). Treatment of E47 cells with BSO for 72 or 96 h resulted in a loss of cell viability (Fig. 2, A and B). Toxicity by BSO treatment of E47 cells was dose-dependent (Fig. 2C). Table 1 shows the activity of catalase in the absence or presence of BSO.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Addition or Infection</th>
<th>Activity of Catalase</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cell Extract</td>
</tr>
<tr>
<td>C34</td>
<td>Control</td>
<td>27.2 ± 3.2</td>
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<tr>
<td></td>
<td>BSO</td>
<td>30.1 ± 2.9</td>
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<tr>
<td></td>
<td>BSO, Ad-mCAT</td>
<td>73.7 ± 8.2</td>
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<tr>
<td></td>
<td>BSO, Ad-cCAT</td>
<td>65.6 ± 7.6</td>
</tr>
<tr>
<td>E47</td>
<td>Control</td>
<td>50.6 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>BSO</td>
<td>46.3 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>BSO, Ad-mCAT</td>
<td>112.1 ± 9.4</td>
</tr>
<tr>
<td></td>
<td>BSO, Ad-cCAT</td>
<td>98.4 ± 8.7</td>
</tr>
</tbody>
</table>

N.D., nondetectable.
treatment was considerably less with C34 cell, e.g., at 72 h after BSO treatment, 85% C34 cells remained viable, whereas only 50% E47 cells were viable; at 96 h after BSO treatment, 65 and 25% of C34 and E47 cells, respectively, were viable (p < 0.05 at 72 and 96 h for E47 viability versus C34 viability). Infection with Ad-Null vector afforded no protection against CYP2E1 plus BSO-dependent toxicity; however, infection with either Ad-cCAT or Ad-mCAT provided almost complete protection (Fig. 2A) or increased cell viability from less than 25% to about 60% (Fig. 2B). Even the lesser toxicity seen at 96 h of BSO treatment in C34 cells was prevented by the two catalase expression vectors. The cCAT and mCAT were equally effective in their protective actions.

**Infection with Catalase Prevents the Increase in ROS Observed after BSO Treatment in E47 Cells.** Twenty-four hours after treatment with BSO the glutathione levels in both control C34 cells and CYP2E1-expressing E47 cells were lowered by approximately 80% (p < 0.01); the infection with either Ad-cCAT or Ad-mCAT did not prevent this GSH depletion (data not shown). ROS production as detected by DCF fluorescence was 2-fold higher in E47 cells compared with C34 cells (Fig. 3, first columns C34 versus E47, p < 0.05). Treatment for 24 h with BSO resulted in an increase in ROS production by both cell lines; however, the increase in ROS after 24 h is greater in the CYP2E1-expressing E47 cells (2-fold increase versus a 50% increase for C34 cells treated with BSO, p < 0.05; Fig. 3, first columns all sets of graphs). The infection with either mitochondrial or cytosolic catalase prevented the initial burst of ROS caused by the combination of glutathione depletion and CYP2E1-derived generation of radicals (Fig. 3, last four columns). This suggests that the increased DCF fluorescence in the E47 plus BSO-treated cells is largely due to H₂O₂ as the major contributing ROS species. Interestingly, the infections with catalase did not lower the basal (non-BSO-treated) increase in DCF fluorescence found with the E47 cells compared with non-BSO-treated C34 cells (Fig. 3, first eight columns). This
suggests that other ROS, not H$_2$O$_2$, may be responsible for the increase in DCF fluorescence in the E47 cells. For example, superoxide radical is produced by CYP2E1 in addition to H$_2$O$_2$ during NADPH-dependent microsomal electron transport (Ekstrom and Ingelman-Sundberg, 1989; Dai et al., 1993). We previously found that, in contrast to catalase activity, activity of superoxide dismutase is not increased in the E47 cells (Marı́ and Cederbaum, 2001), hence superoxide-derived oxidants rather than H$_2$O$_2$ may be the major ROS responsible for the DCF fluorescence in the non-BSO-treated cells.

**Effect of Infection with Catalase on BSO-Induced Lipid Peroxidation.** Lipid peroxidation of C34 and E47 cells was assessed by measuring production of the lipid peroxidation end product MDA by the trichloroacetic acid-thiobarbituric assay. As shown in Fig. 4 (compare Ad-Null versus Ad-Null + BSO), depletion of GSH by BSO treatment induced lipid peroxidation at 72 h in E47 cells in the total cell extract and in the mitochondrial and microsomal fractions. Considerably less lipid peroxidation was observed in C34 cells, especially in the mitochondrial fraction. In fact, the increase in lipid peroxidation in E47 total cell extract compared with C34 total cell extract appears to be largely due to the enhanced peroxidation of the mitochondrial fraction of the E47 cells, because the increase in microsomal lipid peroxidation was equivalent with the C34 and E47 cells. The significant difference ($p < 0.05$) between C34 and E47 cells with respect to lipid peroxidation in whole homogenate and in isolated mitochondrial fractions suggests that the overexpression of CYP2E1 combined with glutathione depletion is responsible for the increased lipid peroxidation. Adenoviral infection with Ad-cCAT or Ad-mCAT completely blocked the increase in lipid peroxidation induced in both C34 and E47 cells by the treatment with BSO in all cellular fractions.

**Effect of BSO Treatment on Mitochondrial Membrane Potential.** Mitochondrial membrane potential was assayed by flow cytometry after double staining with Rh123 and PI. Rh123 uptake to the mitochondria is proportional to the mitochondrial membrane potential (Lemasters and Niemen, 1997). PI is imported into the cells and binds to cellular DNA when the integrity of the plasma membrane is lost. Figure 5 shows flow cytometry graphs of C34 and E47 cells infected with Ad-Null (control) (Fig. 5, A and B, E and F), Ad-cCAT (Fig. 5, C and G), or Ad-mCAT (Fig. 5, D and H) treated with BSO for 72 h (Fig. 5, A and E, are nontreated controls). Control or fully viable cells appear mostly on the Rh123 high-fluorescence (+) PI (–) (lower right quadrant) field (Fig. 5, A and E). Treatment of C34 cells with BSO had no effect on this distribution pattern (Fig. 5, A and B). However, BSO-treated E47 cells have an increasing population of

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**Fig. 4.** Effect of BSO on lipid peroxidation of C34 and E47 cells. C34 and E47 cells were infected with Ad-Null, Ad-cCAT, or Ad-mCAT 24 h before treatment with 0.1 mM BSO. After 72 h of treatment with BSO, cells were harvested by scrapping, cell extracts were prepared, and mitochondrial and microsomal fractions were isolated. The production of MDA, as a measure of lipid peroxidation, was assayed as described under Materials and Methods. Results are expressed as mean ± S.D. *p < 0.05 versus E47 Ad-Null; †, p < 0.05 versus C34 Ad-Null.

**Fig. 5.** Flow cytometry analysis of the mitochondrial membrane potential. C34 (A–D) or E47 cells (E–H) were infected with Ad-Null (A, B, E, and F), Ad-cCAT (C and G), or Ad-mCAT (D and H) and 24 h after infection were treated with BSO (B, C, D, F, G, and H) or buffer (A and E) for 72 h. At the end of this treatment, cells were incubated with medium containing 5 μg/ml Rh123 for 1 h and stained with PI afterward. The intensity of Rh123 fluorescence was analyzed by flow cytometry, as described under Materials and Methods. The figure is one representative experiment of three.
cells (20% compared with 1–6% in the other panels) in the Rh123 low-fluorescence (−) PI (−) (lower left quadrant) field (Fig. 5F). The population in this specific quadrant refers to cells that are still viable [i.e., PI (−)] but with damaged mitochondria, showing that GSH depletion affects the mitochondria of CYP2E1-expressing cells before the onset of overt toxicity in these cells. Moreover, in BSO-treated E47 cells (compare Fig. 5F with B or E) there is also an increase in the population of the Rh123 low-fluorescence (−) PI (+) (upper left quadrant), corresponding to the beginning of cell death. As shown in Fig. 5, G and H, infection with either cytosolic or mitochondrial catalase prevented the loss of membrane potential observed in BSO-treated E47 cells infected with Ad-Null, suggesting that H₂O₂ is an important mediator of the decline in mitochondrial membrane potential.

Mode of Cell Death by BSO in CYP2E1-Expressing HepG2 Cells and Protection by Catalase Adenoviral Infection. Neither significant cell death nor morphological changes were observed in C34 cells even after 72 h after BSO treatment (Fig. 6, A and B). However, at this time point, E47 cells lost normal morphology, and displayed a round shape with swelling (Fig. 6D). These morphological changes produced by BSO treatment in the E47 cells were prevented after infection with either cytosolic (Fig. 6F) or mitochondrial (Fig. 6H) catalase adenovirus. The cell death, apoptosis or necrosis, produced by BSO treatment was evaluated in C34 and E47 cells by flow cytometry after double staining with Annexin V and PI. When apoptosis occurs Annexin V is externalized in the plasma membrane, which can be detected by flow cytometry. As shown in Fig. 7, control and viable cells appear on the Annexin V (−) PI (−) (lower left quadrant) field as observed for C34 cells even after BSO treatment (Fig. 7A–D). However, BSO-treated E47 cells displayed a different pattern (compare Fig. 7F with B or E). There was an increase in fluorescence of Annexin V as evidenced by the population in the Annexin V (+) PI (−) (lower right quadrant) field, indicative of apoptosis; on the other hand, there was also an increase in the population of cells in the upper field, PI (+), indicative of cell death by necrosis. Thus, BSO treatment in CYP2E1-expressing cells caused a mixed type of cell death, involving apoptosis and necrosis. Infection with both cytosolic (Fig. 7G) or mitochondrial (Fig. 7H) catalase protected against both modes of toxicity exerted in E47 cells depleted of GSH, further validating the role of ROS, especially H₂O₂, in this toxicity.

Discussion

Induction of CYP2E1 by ethanol appears to be one of the central pathways by which ethanol generates a state of oxidative stress. It has been demonstrated that CYP2E1, when reduced by NADPH-cytochrome P450 reductase, is a loosely coupled enzyme that displays high NADPH oxidase activity (Gorsky et al., 1984; Ekstrom and Ingelman-Sundberg et al., 1989). Formation of reactive oxygen species can occur even in the absence of added substrates [e.g., formation of superoxide and H₂O₂ by microsomes from CYP2E1-expressing cells was not altered by addition of substrates and ligands of CYP2E1 (Dai et al., 1993)].

GSH, the most abundant nonprotein antioxidant in cells, is critical in preserving the proper cellular redox balance and for its role as a cellular protectant (Hall, 1999; Lu, 1999). There is considerable interest in the effects of ethanol on GSH homeostasis and the role that GSH depletion plays in ethanol-induced liver injury. Mitochondrial GSH levels are decreased after chronic ethanol treatment, and this decrease has been suggested to play a role in ethanol-induced liver injury (Fernández-Checa et al., 1991; Colell et al., 1998).

HepG2 cells overexpressing CYP2E1 are more sensitive to arachidonic acid-, iron-, and ethanol-induced toxicity and apoptosis (Dai et al., 1993; Wu and Cederbaum, 1996; Chen et al., 1997) than control cells and this toxicity is enhanced after removal of GSH by BSO treatment (Chen and Cederbaum, 1998; Wu and Cederbaum, 2001). Removal of GSH causes toxicity in E47 cells even in the absence of added toxin...
or pro-oxidant, suggesting that GSH is critical in protecting the E47 cells against CYP2E1-dependent toxicity. In fact, in the E47 cells GSH levels were 30% higher than the levels in the control C34 cells because of a 2-fold increase in activity and expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis (Mari and Cederbaum, 2000). We suggested that this up-regulation of GSH synthesis was an adaptive response to attenuate CYP2E1-dependent oxidative stress and toxicity.

Using adenovirus-mediated gene transfer, high levels of catalase can be expressed in E47 cells as determined by Western blot and catalase activity assays. Although CYP2E1 is not the only primary source of ROS, CYP2E1 is a major producer of ROS, including superoxide and hydrogen peroxide. Previous studies have shown that the overexpression of catalase in cytosol or mitochondria protected the E47 cells from cytotoxicity caused by arachidonic acid and iron (Bai and Cederbaum, 2001).

ROS such as O$_2^-$ and H$_2$O$_2$ generated by CYP2E1 would be at least partially detoxified by antioxidant systems present in the cytosol such as catalase, the GSH plus glutathione peroxidase and reductase system, copper-zinc superoxide dismutase. H$_2$O$_2$ diffusing into or generated within the mitochondria would be detoxified by the GSH plus glutathione peroxidase system, a system compromised after chronic ethanol treatment (Fernández-Checa et al., 1991; Colell et al., 1998). Damage to the respiratory chain as a result of chronic ethanol treatment (Gordon, 1984; Fernández-Checa et al., 1993; Fromenty et al., 1995; Cunningham and Bailey, 2001) and/or externally derived ROS (e.g., due to ethanol-induced elevated levels of CYP2E1) could result in a further increase in mitochondrial ROS production, which coupled to MnSOD-catalyzed dismutation of O$_2^-$ to H$_2$O$_2$ in the presence of a compromised GSH peroxidase system would exacerbate mitochondrial oxidant stress. With these considerations, it was felt that expressing catalase in the mitochondria, a compartment where this enzyme is not normally expressed (except for heart mitochondria; Radi et al., 1993), might be especially effective in reducing mitochondrial oxidative stress and damage. We therefore compared the effectiveness of catalase expressed in the cytosol with that in the mitochondria in protecting against CYP2E1 plus BSO-dependent oxidative stress, mitochondrial damage, and cell toxicity.

Adenoviral infection of cytosolic or mitochondrial catalase protects E47 cells from the cell death, apoptosis, and necrosis, observed after GSH depletion by BSO treatment. The finding that the infection with cytosolic catalase, and more importantly, mitochondrial catalase is able to rescue the E47 cells from injury caused by GSH depletion by lowering the amount of ROS generated and the consequent lipid peroxidation reveals a prominent role for H$_2$O$_2$, and damage to the mitochondrial compartment, as critical steps in the cytotoxicity exerted under these conditions. Indeed, most of the enhanced lipid peroxidation observed in the BSO-treated E47 cells was in the mitochondrial compartment. The results obtained by flow cytometry with either Rh123 or AnnV combined with PI and Annexin V conjugated with FITC, and then analyzed by flow cytometry, as described under Materials and Methods. The figure is one representative experiment of three.

**Fig. 7.** Assessment of cell death by flow cytometry. C34 (A–D) or E47 cells (E–H) were infected with Ad-Null (A, B, E, and F), Ad-cCAT (C and G), or Ad-mCAT (D and H) and 24 h after infection were treated with BSO (B, C, D, F, G, and H) or buffer (A and E) for 72 h. At the end of this treatment cells were collected, washed twice, stained with PI and Annexin V combined with PI and Annexin V conjugated with FITC, and then analyzed by flow cytometry, as described under Materials and Methods. The figure is one representative experiment of three.

References


