Adenovirus-mediated Overexpression of Catalase in the Cytosolic or Mitochondrial Compartment Protects against Cytochrome P450 2E1-dependent Toxicity in HepG2 Cells*

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Cytochrome P450 2E1 (CYP2E1) is an effective producer of reactive oxygen species such as superoxide radical and hydrogen peroxide, which may contribute to the development of alcohol liver disease or cytotoxicity. To investigate the protective role of catalase against CYP2E1-dependent cytotoxicity, E47 cells, a transfected HepG2 cell line overexpressing CYP2E1, were infected with adenoviral vectors containing human catalase cDNA (AdCat) and catalase cDNA with a mitochondrial leader sequence (AdmCat). Forty-eight hours after infection with AdCat or AdmCat at a multiplicity of infection of 100, intracellular catalase protein was increased >2-fold compared with uninfected E47 cells and E47 cells infected with empty adenoviral vector (AdNull) as determined by Western blotting and catalase activity measurements. Overexpression of catalase in the cytosol (AdCat) and in mitochondria (AdmCat) was confirmed by confocal microscopy. Cell death caused by arachidonic acid plus iron was considerably suppressed in both AdCat- and AdmCat-infected E47 cells as determined by assays of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide absorbance, lactate dehydrogenase release, and morphology changes. AdCat- and AdmCat-infected cells were also more resistant to the loss of mitochondrial membrane potential and to the increase in lipid peroxidation induced by arachidonic acid and iron. This study indicates that catalase in the cytosol and catalase in mitochondria are capable of protecting HepG2 cells expressing CYP2E1 against cytotoxicity induced by oxidants that promote lipid peroxidation and suggests the possibility that such agents may be useful in protecting against the development of alcohol liver injury.

Reactive oxygen species (ROS)* such as hydrogen peroxide may contribute to several diseases (1–5) and play a role in the cytotoxicity of agents such as ceramide, arsenite, and tumor necrosis factor-α (6–8). In the presence of transition metals such as ferric and copper, hydrogen peroxide and superoxide generate hydroxyl radical by the Fenton reaction or the metal-catalyzed Haber-Weiss reaction (9, 10). Hydroxyl radical is able to cause the degradation of most biological macromolecules, e.g. peroxidation of lipids, oxidation of sugars and protein thiols, DNA base damage, and strand breakage of nucleic acids (11).

Cytochrome P450 2E1 (CYP2E1), the ethanol-inducible form, is of interest because of its ability to metabolize and activate many toxicologically important substrates to more toxic products (12, 13). There is considerable interest in the mechanism by which ethanol is hepatotoxic (14, 15). In the intragastric model of ethanol feeding, prominent induction of CYP2E1 occurs, and significant alcohol liver injury occurs only in the presence of polyunsaturated fatty acids (16–18). In these models, large increases in lipid peroxidation have been observed, and the ethanol-induced liver pathology has been shown to correlate with CYP2E1 levels and elevated lipid peroxidation. In our laboratory, stable HepG2 cell lines that express human CYP2E1 were established; ethanol, arachidonic acid (AA), or iron was toxic to cells expressing CYP2E1, but not to control cells lacking CYP2E1 (19–21). Induction of a state of oxidative stress appears to play a central role in this CYP2E1-dependent cytotoxicity.

Mitochondria are an important organelle in the cell and are not only a major source of generation of ROS from the mitochondrial respiratory chain (22), but also a critical target of ROS that are generated either in the mitochondria or derived from outside of the mitochondria. ROS, including superoxide radical or hydrogen peroxide and especially hydroxyl radical, attack a variety of molecules present within the mitochondria that subsequently cause loss of mitochondrial membrane potential, decrease in ATP generation, and release of cytochrome c, events that lead cells to undergo necrosis or apoptosis (23–25).

Catalase is a very efficient enzyme to protect cells from the accumulation of H₂O₂, catalyzing the conversion of H₂O₂ to H₂O and O₂; catalase is effective at high levels of H₂O₂ (9). Catalase is mainly located in the cytoplasm (erythrocyte) and peroxisomes (most cells, especially liver). With the exception of rat heart (26), catalase is not normally present in the mitochondria. Another major system that catalyzes the reduction of H₂O₂ is glutathione peroxidase, which is localized in both the cytosolic and mitochondrial compartments. This system also requires NADPH plus glutathione reductase to catalyze the reduction of GSSG back to reduced GSH.

In a previous study, we established HepG2 cells with overexpressed catalase in the cytosolic and mitochondrial compart-

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† The abbreviations used are: ROS, reactive oxygen species; CYP2E1, cytochrome P450 2E1; AA, arachidonic acid; PI, propidium iodide; Ad5, adenovirus type 5; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; Fe-NTA, ferric nitrilotriacetate; LDH, lactate dehydrogenase; MDA, malondialdehyde.

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mements by transfection with plasmids containing catalase cDNA and catalase cDNA linked to the leader sequence of manganese-superoxide dismutase (27). The HepG2 cell lines that constitutively expressed catalase in the mitochondrial as well as cytosolic compartments were protected from cytotoxicity or apoptosis induced by hydrogen peroxide and antimycin A (27).

In the present study, adenovirus-mediated gene transfer was used to compare the effect of overexpression of human catalase in the cell cytosol with that in the mitochondria on the ability to protect HepG2 cells from CYP2E1-dependent cytotoxicity induced by arachidonic acid and iron. It was hoped that studies with these adenoviral constructs and cell culture would be useful for eventual in vivo studies employing catalase localized in different cellular compartments.

MATERIALS AND METHODS

Reagents—Rhodamine 123, propidium iodide (PI), and MitoTracker® Red were purchased from Molecular Probes, Inc. (Eugene, OR). Polyclonal antibody raised in rabbit against human catalase was obtained from Calbiochem. Arachidonic acid, horseradish peroxidase-conjugated goat anti-rabbit IgG, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, minimal essential medium, and fetal bovine serum were purchased from Sigma.

Recombinant Adenovirus Production—Ad5 adenoviral vector with compensating deletions in the early region 1 (E1), as developed by Graham and co-workers (28, 29), was purchased from Microbix Systems Inc. Human catalase cDNA and catalase cDNA with a manganese-superoxide dismutase mitochondria leader sequence were obtained 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 JM17 were transfected into human embryonic kidney 293 cells, which provide the EIA gene product necessary for viral replication during transfection. After transfection, plates were overlaid with agar, and initial plaques were harvested, amplified, and screened for enzymatic activity. Adenovirus containing no cDNA (AdNull) was used as a control.

Virus possessing cytosolic catalase (AdCat) and mitochondrial catalase (AdmCat) as well as AdNull virus were purified twice 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 using C34 cells. Viral particles were consistently 1×109 particles/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 Culture and Infection—E47 cells, a HepG2 cell line overexpressing CYP2E1, and C34 cells, a HepG2 cell line transfected with empty plasmid (pCI-neo) (30), were cultured in minimal essential medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere at 5% CO2 at 37°C. Cells were fixed at room temperature for 10 min with freshly prepared 2.5% paraformaldehyde in PBS. Cells were washed three times for 10 min in 0.3% glycine prepared in PBS and permeabilized for 1 min with 0.5% Triton X-100 at room temperature, followed by three washes in PBS. Cells were incubated for 1 h with a 1:500 dilution of a rabbit anti-human erythrocyte catalase polyclonal antibody, followed by three washes in PBS, and incubated with a 1:160 dilution of a goat anti-rabbit antibody labeled with fluorescein isothiocyanate. After three washes in PBS, cells were incubated with 0.1% Triton X-100 for 5 min, followed by an additional three washes in PBS. The coverslips were mounted on slides and examined by confocal microscopy.

LDH Release Assay—LDH activity was measured as another index of cytotoxicity. E47 cells were plated on 96-well plates and infected with adenovirus, followed by treatment with different concentrations of AA (0, 10, 20, and 30 μM) plus 30 μM Fe-NTA. Similarly treated uninfected E47 cells were used as a control. The medium was removed, and cells viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (27).

LDH Release Assay—LDH activity was measured as another index of cytotoxicity. E47 cells were plated on 96-well plates and infected with AdNull, AdCat, or AdmCat adenovirus, followed by treatment with different concentrations of AA (0, 10, 20, and 30 μM) plus 30 μM Fe-NTA. Similarly treated uninfected E47 cells were used as a control. The culture medium was collected to measure LDH activity as LDHmean. Cells

FIG. 1. Expression of catalase in E47 cells infected with adenovirus. E47 cells were infected with AdCat, AdmCat, or AdNull virus at m.o.i. = 100. Forty-eight hours later, a cell extract was prepared. Cell extracts from C34 cells and uninfected E47 cells were also prepared as controls. Ten micrograms of protein was loaded onto each lane for 10% SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis with polyclonal rabbit anti-human catalase antibody as described under "Materials and Methods." Lane 1, C34 cells; lane 2, E47 cells; lane 3, E47 cells infected with AdNull virus; lane 4, E47 cells infected with AdCat virus; lane 5, E47 cells infected with AdmCat virus. Ten micrograms of protein from cell extracts was used to measure the catalytic activity of catalase (B). Specific catalase units (units/mg of protein/min) were calculated as described under "Materials and Methods." First bar, C34 cells; second bar, E47 cells; third bar, E47 cells infected with AdNull virus; fourth bar, E47 cells infected with AdCat virus; fifth bar, E47 cells infected with AdmCat virus. Data are the means ± S.D. of triplicate experiments.
were harvested by scraping, washed in PBS, suspended in 1 ml of PBS, and sonicated for 10 s. The lactate dehydrogenase assay kit (Sigma) was used for the quantitative kinetic determination of LDH activity. The LDH activity of the cell suspension was measured as LDH$_{in}$. The percentage of LDH release was calculated by the formula $\frac{LDH_{out}}{LDH_{in}} \times 100\%$ and used to express the cytotoxicity.

**Flow Cytometry Analysis of the Mitochondrial Membrane Potential**—Changes in the integrity of the plasma membrane and in the mitochondrial membrane potential were examined by monitoring the cells after double staining with PI and rhodamine 123. E47 cells ($5 \times 10^5$) were seeded onto 6-well plates and infected with adenovirus. Forty-eight hours after infection, cells were incubated with 20 $\mu$M AA for 16 h, followed by incubation with 30 $\mu$M Fe-NTA for an additional 6 h. The cells were then incubated with medium containing 5 $\mu$g/ml rhodamine 123 for 1 h. Cells were harvested by trypsinization and resuspended in 1 ml of minimal essential medium containing 5 $\mu$g of PI. The intensity of fluorescence from PI and rhodamine 123 was analyzed by flow cytometry.

**Lipid Peroxidation Assay**—Lipid peroxidation of the cells was measured with the thiobarbituric acid assay using minor modifications of the method described by Niehaus and Samuelsson (32). Briefly, C34 and uninfected E47 cells as well as E47 cells infected with AdNull, AdCat, or AdmCat adenovirus were incubated in the absence of any addition or treated with 20 $\mu$M AA for 16 h, followed by incubation with 30 $\mu$M Fe-NTA for an additional 6 h. Cells were then incubated with medium containing 5 $\mu$g/ml rhodamine 123 for 1 h. Cells were harvested by trypsinization and resuspended in 1 ml of PBS containing 0.5 mM trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Cells were centrifuged at 2000 rpm for 5 min. The supernatant was discarded, and the cells were resuspended in 50 $\mu$l of PBS containing 0.5 mM trolox. Fifty microliters of cell suspension and 100 $\mu$l of 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N hydrochloric acid were mixed in a Vortex mixer and incubated for 15 min at 100 °C, followed by centrifugation for 5 min at 13,000 rpm. The absorbance of the supernatant was measured at 535 nm. The concentration of malondialdehyde (MDA) equivalents was calculated using an extinction coefficient of $1.56 \times 10^5$ M$^{-1}$ cm$^{-1}$.

**RESULTS**

**Overexpression of Catalase in the Cytosol and Mitochondria**—To examine the capacity of AdCat and AdmCat to enhance the expression and activity of catalase in cells, E47 cells were infected with AdCat and AdmCat as well as the empty adenovirus AdNull at m.o.i. = 100. Using an adenovirus containing the lacZ gene to infect E47 cells followed by 5-bromo-4-chloro-3-indolyl $\beta$-D-galactopyranoside (X-gal) assay, it was determined that >98% of the E47 cells were infected with the adenovirus under these reaction conditions. After 48 h, cell lysates were prepared and subjected to Western blot analysis. Fig. 1A shows the expression of catalase in the total cell extract from these cells. Results from densitometric analyses of the intensity of the various bands indicated that the expression of catalase in total cell extracts of cells infected with AdCat and AdmCat was 2-fold higher than that in uninfected E47 cells or E47 cells infected with AdNull. Catalase levels in the uninfected E47 cells were 2-fold higher than those in C34 cells; this may reflect an up-regulation of catalase expression to remove ROS generated from CYP2E1. Similar results were obtained by...
localized to the mitochondria, whereas catalase in E47 cells infected with AdCat was localized to the cytosol.

Suppression of Arachidonic Acid-plus Iron-induced Cytotoxicity—Arachidonic acid plus iron can induce cytotoxicity in HepG2 cells expressing CYP2E1 by a process involving increased lipid peroxidation (20, 21). To determine whether mitochondrial or cytosolic catalase can protect against AA- plus iron-induced cytotoxicity, E47 cells were infected with AdCat, AdmCat, and AdNull adenovirus at m.o.i. = 100 for 48 h, followed by incubation with different concentrations of AA (0, 10, 20, and 30 μM) for 16 h and 30 μM Fe-NTA for an additional 8 h. The viability of the cells was determined by the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and by LDH release. The percentage of surviving cells decreased in an AA concentration-dependent manner, and this decrease was similar for uninfected E47 cells and E47 cells infected with AdNull virus (Fig. 3A). E47 cells infected with AdCat or AdmCat were more resistant to the cytotoxic effects of AA plus iron than were uninfected E47 cells or E47 cells infected with AdNull virus (Fig. 3A). There was no significant difference between E47 cells infected with AdCat or AdmCat in protecting against the toxicity induced by AA plus Fe-NTA. Essentially similar results were obtained using the LDH release assay to determine the cell viability in the same treatment protocol as described above (Fig. 3B). Large amounts of LDH were released from untreated E47 cells or E47 cells infected with AdNull virus into the medium when cells were treated with different concentrations of AA plus 30 μM Fe-NTA. However, little LDH was detected in the medium of E47 cells infected with AdCat or AdmCat. The morphology of uninfected E47 cells and E47 cells infected with AdNull, AdCat, or AdmCat adenovirus in the absence and presence of treatment with different concentrations of AA (0, 10, 20, and 30 μM) for 16 h, followed by incubation with 30 μM Fe-NTA for 8 h and 24 h, was recorded by visualizing cells under a light microscope. Fig. 4 shows the results obtained with control cells (a, c, e, and g) and cells treated with 30 μM AA plus Fe-NTA for 24 h (b, d, f, and h). Almost all of the uninfected E47 cells and E47 cells infected with AdNull lost normal morphology when treated with AA plus Fe-NTA, whereas most of the E47 cells infected with AdCat or AdmCat retained their shape and structure (Fig. 4).

Mitochondrial Membrane Potential (ΔΨ) and Integrity of the Plasma Membrane—Forty-eight hours after infection with adenovirus, E47 cells were incubated with or without 20 μM AA for 16 h and then incubated with 30 μM Fe-NTA for an additional 6 h. Cells not treated with AA plus Fe-NTA were predominantly located in the PI-negative and high ΔΨ (strong rhodamine 123 fluorescence) field (PF−/ΔΨ high), reflective of viable, intact cells (Fig. 5, a, c, e, and g). A small percentage of cells were located in the PI−/ΔΨ low field, reflective of damaged cells (Fig. 5, a, c, e, and g). Uninfected E47 cells or E47 cells infected with AdNull that were treated with AA plus Fe-NTA moved to the PI−/ΔΨ low and PI−/ΔΨ low fields, and 67.3 and 82.5% of the cells displayed low rhodamine 123 intensity, respectively (Fig. 5, b, d, f, and M1), compared with 30.8% of uninfected E47 cells and 40.4% of E47 cells infected with AdNull, but not treated with AA plus Fe-NTA (Fig. 5, a and c). However, after treatment with AA plus Fe-NTA, less cells were in the PI− field, and only 40.2 and 32.4% of E47 cells infected with AdCat and AdmCat were present in the ΔΨ low field, respectively (Fig. 5, f and h). These results indicate that both cytosolic catalase and mitochondrial catalase protected the cells from loss of mitochondrial membrane potential and loss of membrane integrity caused by AA plus iron.

Suppression of Lipid Peroxidation—Uninfected E47 cells and
E47 cells infected with AdCat, AdmCat, and AdNull virus were treated with 20 \( \mu \)M AA for 16 h, followed by incubation with 30 \( \mu \)M Fe-NTA for 6 h, and lipid peroxidation was measured by detecting the concentration of MDA. C34 cells without CYP2E1 expression were used as a control. There was no significant difference in MDA production among all these cells in the absence of AA plus Fe-NTA treatment. The MDA concentration in E47 cells infected with AdCat or AdmCat was significantly lower than that in uninfected E47 cells or E47 cells infected with AdNull virus when cells were treated with AA plus Fe-NTA (\( p, 0.01, \) AdCat compared with E47 or AdNull; \( p, 0.05, \) AdmCat compared with E47 or AdNull). Lipid peroxidation in C34 cells was lower than that in E47 cells (\( p, 0.05 \)) or E47 cells infected with AdNull (\( p, 0.01 \)) due to the lack of CYP2E1. There was no significant difference in MDA production between E47 cells infected with AdCat or AdmCat (Fig. 6).

**DISCUSSION**

Using adenovirus-mediated gene transfer, high levels of catalase could be expressed in E47 cells as determined by Western blot and catalase activity assays. Immunofluorescence studies confirmed that catalase with a manganese-superoxide dismutase mitochondrial signal peptide, which was encoded by the recombinant adenovirus AdmCat, localized to the mitochondria. Thus, the manganese-superoxide dismutase leader sequence could be used to successfully import catalase into mitochondria. Catalase in AdCat-infected E47 cells was localized in the cytosol in a more diffused manner. Why the cytosolic catalase distributes in the whole cytosol, but not the peroxisome as the endogenous catalase does, is not clear, but might be due to accumulation of catalase in the cytosol in excess of available peroxisomal content.

Alcohol liver disease has been associated with oxidative stress (14, 15). Although CYP2E1 is not the only primary source of ROS, CYP2E1 is a major producer of ROS, including superoxide and hydrogen peroxide. HepG2 cell lines overexpressing CYP2E1 were developed in our laboratory by stably inducing or transfecting human CYP2E1 cDNA into HepG2 cells (30, 33). Compared with the HepG2 cells line transfected with empty plasmid vector, HepG2 cells overexpressing CYP2E1 are more sensitive to arachidonic acid-, \( \beta \)-lactamase sulfoximine (which lowers cellular GSH levels)-, iron-, and ethanol-induced toxicity and apoptosis (19–21, 30). Ascorbic acid, the iron chelator desferrioxamine, and several typical inhibitors of lipid peroxidation produce efficient protection against arachidonic acid toxicity in CYP2E1-expressing HepG2 cells (20) probably by quenching or preventing formation of powerful oxidants such as hydroxyl radical or other initiators of lipid peroxidation. These studies indicate that ROS are responsible for the CYP2E1-dependent cytotoxicity.

The overexpression of catalase in the cytosol or mitochondria protected the E47 cells from cytotoxicity caused by AA and iron. The cytosolic catalase and mitochondrial catalase appear to be equally effective in protecting the cells from cytotoxicity induced by AA plus iron. Because CYP2E1 is present in the endoplasmic reticulum, CYP2E1-derived \( \text{H}_2\text{O}_2 \) would be generated in the endoplasmic reticulum and diffuse into the cytosol.
Therefore, cytosolic catalase might be anticipated to readily perform its function to reduce H$_2$O$_2$. Since catalase in mitochondria was equally protective against CYP2E1-dependent AA- plus iron-induced toxicity, it is likely that some of the H$_2$O$_2$ diffuses into the mitochondria, and perhaps damage to the mitochondria may be an important factor contributing to AA- plus iron-induced toxicity. Catalase in mitochondria might act as a sink for H$_2$O$_2$ and promote H$_2$O$_2$ movement down its concentration gradient, thereby decreasing lipid peroxidation in the cells and protecting mitochondria from damage.

Mitochondrial permeability transition and mitochondrial membrane potential are markers for mitochondrial damage and dysfunction (34–36). Mitochondrial dysfunction caused by ROS, especially H$_2$O$_2$, can lead not only to necrosis by depleting ATP, but also to apoptosis by inducing the release of cytochrome c, which activates caspases together with other mitochondrial factors such as Apaf-1 (37–39). We determined the mitochondrial membrane potential in cells treated with 20 mM AA and incubated with 30 mM Fe-NTA. The decline in ΔΨ caused by these agents was much less in E47 cells infected with AdCat or AdmCat adenovirus than in uninfected E47 cells or E47 cells infected with AdNull. These results suggest that both cytosolic catalase and mitochondrial catalase protected cells from oxidant-induced loss of mitochondrial membrane potential, which may play an important role in the overall protection against oxidant-induced cytotoxicity.
arachidonic acid at concentrations that prevented toxicity of ethanol, CCl₄, and acetaminophen (19, 20, 42), and CYP2E1 substrates such as ethanol did not effectively inhibit the arachidonic acid toxicity (20). These results indicate that the direct metabolism of arachidonic acid to potentially toxic products by CYP2E1 does not contribute significantly to the polyunsaturated fatty acid toxicity, but rather metabolism of AA by CYP2E1-derived oxidants is likely to be responsible for the ensuing lipid peroxidation and loss of cell viability. This explains the effectiveness of H₂O₂-degrading enzymes such as catalase in protecting the E47 cells from the AA- plus Fe-NTA-induced toxicity. Indeed, the lipid peroxidation assays showed that, after treatment with AA plus iron, the concentration of MDA in E47 cells infected with AdCat or AdmCat was significantly lower than that in uninfected E47 cells or E47 cells infected with AdNull. The catalase expression in E47 cells lowered MDA levels to those in C34 cells.

In summary, this study suggests that mitochondria are an important target for oxidative damage and that both catalase in the cytosol and catalase in mitochondria are capable of protecting HepG2 cells against CYP2E1-dependent cytotoxicity induced by arachidonic acid plus iron. It is hoped that these adenoviral constructs may prove to be effective in preventing oxidative damage caused by alcohol or other xenobiotics under in vivo conditions, and future studies evaluating the in vivo expression of these constructs have been initiated.

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