Overexpression of Catalase in Cytosolic or Mitochondrial Compartment Protects HepG2 Cells against Oxidative Injury*

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HepG2 cells were transfected with vectors containing human catalase cDNA and catalase cDNA with a mitochondrial leader sequence to allow comparison of the effectiveness of catalase overexpressed in the cytosolic or mitochondrial compartments to protect against oxidant-induced injury. Overexpression of catalase in cytosol and in mitochondria was confirmed by Western blot, and activity measurement and stable cell lines were established. The intracellular level of H2O2 induced by exogenously added H2O2 or antimycin A was lower in C33 cell lines overexpressing catalase in the cytosol and mC5 cell lines overexpressing catalase in the mitochondria as compared with Hep cell lines transfected with empty vector. Cell death caused by H2O2, antimycin A, and menadione was considerably suppressed in both the mC5 and C33 cell lines, C33 and mC5 cells were also more resistant to apoptosis induced by H2O2 and to the loss of mitochondrial membrane potential induced by H2O2 and antimycin A. In view of the comparable protection by catalase overexpressed in the cytosol versus the mitochondria, catalase produced in both cellular compartments might act as a sink to decompose H2O2 and move diffusible H2O2 down its concentration gradient. The present study suggests that catalase in cytosol and catalase in mitochondria are capable of protecting HepG2 cells against cytotoxicity or apoptosis induced by oxidative stress.

Hydrogen peroxide (H2O2), one of the major reactive oxygen species (ROS), is produced at a relatively high rate as a product of aerobic metabolism. Under normal conditions, 1–2% of the oxygen reduced by mitochondria may be converted to O2 at the NADH dehydrogenase and ubiquinonemine intermediate steps of the mitochondrial respiratory chain (1, 2). Superoxide can be readily converted by mitochondrial superoxide dismutase (MnSOD) into H2O2 (3). The primary cellular enzymatic defense systems against hydrogen peroxide are the glutathione redox cycle and catalase. GSH is a cofactor for glutathione peroxidase, which converts H2O2 to H2O at the expense of oxidizing GSH to its disulfide form (GSSG). Glutathione reductase regenerates GSH from GSSG, using reducing equivalents from NADPH (4). Catalase also protects cells from the accumulation of H2O2 by converting it to H2O and O2 (5). The glutathione redox cycle system exists in both the cytosol and mitochondrial compartments of the cell. However, catalase is present only or primarily in the peroxisome fraction and is absent in mitochondria of mammalian cells, except rat heart mitochondria (6). Therefore, the only enzymatic defense system against hydrogen peroxide in mitochondria is the glutathione redox cycle system.

Pathological conditions, which increase the rate of H2O2 production or deplete components of the anti-oxidant system, e.g. GSH, will lead to the accumulation of H2O2 in the cytosol or mitochondria. In biological systems, H2O2 could readily diffuse across cellular membranes and lead to depletion of ATP, GSH, and NADPH. It could also induce a rise in free cytosolic Ca++ and activate poly(ADP-ribose) polymerase activity and cause DNA damage (7). By scission of the peroxide bond through the Fenton reaction, hydrogen peroxide could generate hydroxyl radical, an extremely potent oxidant (5, 8). The hydroxyl radical is able to cause the degradation of most biological macromolecules, e.g. peroxidation of lipids, oxidation of sugars and of protein thiols, DNA base damage, and strand breakage of nucleic acids (9).

Low levels of H2O2 have been shown to trigger apoptosis, while high levels lead to necrosis (10, 11). Many reports indicate that hydrogen peroxide plays a very crucial role in cytotoxicity and apoptosis induced by stimuli such as ceramide (12), Antimycin A (AA) (13), arsenite (14), and tumor necrosis factor-α (15). It might also contribute to human diseases such as Alzheimer’s disease, diabetes, stroke, and AIDS dementia complex (16–18). Studies in our laboratory indicate that CYP 2E1-dependent cytotoxicity or apoptosis by ethanol to HepG2 cells was due to the generation of ROS such as H2O2 (19, 20).

Mitochondria are a main target for damage by ROS. Hydrogen peroxide is known to induce a mitochondrial permeability transition and disrupt the mitochondrial membrane potential (Δφ). Such conditions can cause the release of cytochrome c from the mitochondria to the cytosol, thereby triggering cells to undergo apoptosis by activating caspase 3 (21). There are no reports on the possible protective actions caused by expression of functionally active catalase in the mitochondrial compartment against toxicity by ROS. In this study, stable HepG2 cell lines that constitutively express catalase in the mitochondrial compartment were developed. The main goal of the present study was to compare the effect of overexpression of human catalase in the cell cytosol to that in the mitochondria on the ability to protect cells from cytotoxicity or apoptosis induced by hydrogen peroxide and AA.
MATERIALS AND METHODS

Reagents—Rhodamine 123 (Rh123), propidium iodide (PI), and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were purchased from Molecular Probes (Eugene, OR). Polyclonal antibody raised in rabbit against human catalase was obtained from Calbiochem. Zeocin for cloning selection was from Invitrogen. Hydrogen peroxide, AA, horseradish peroxidase conjugated to goat anti-rabbit IgG, MEM, fetal bovine serum, and paraformaldehyde were purchased from Sigma.

Plasmid Construction—The pCAT10 plasmid containing the human catalase cDNA was obtained from American Type Culture Collection. pZeoSV2(+) mammalian expression vector was obtained from Invitrogen Corporation (San Diego, CA). A 900-base pair fragment containing the MnSOD mitochondrial signal peptide was PCR-amplified with HindIII-SalI ends from pCMVMnSOD (22). The resulting product was inserted into the pCDR1 TA cloning vector (Invitrogen Corp.). A 1-kilobase pair fragment containing the mitochondrial signal peptide and the CMV promoter region of pCR/CMV plasmid was then removed from the resulting plasmid by digestion with HindIII, which cleaves at the newly introduced HindIII site and the naturally occurring HindIII 3′ site in the MnSOD cDNA. This fragment was ligated into the pZeoSV2(+), precloned with HindIII. The new pZeoSV-MSP was digested with BstI, removing the CMV promoter region, and religated. The final pZeoSV-MSP was then digested with HindIII and NotI. The human fibroblast catalase cDNA from pCAT10 was equipped with a HindIII site at the 5′ end and a NotI site at the 3′ end with oligonucleotide linkers using PCR. The resulted 1.6-kilobase pair PCR product was ligated in frame into the pZeoSV-MSP to obtain the final recombinant plasmid pZeoSV/MSP-CAT. The mammalian expression vector pZeoSV-CAT, containing the 1.6-kilobase pair catalase cDNA insert, was ligated into pZeoSV2(+) precloned with HindIII and NotI. Each DNA insert was sequenced bidirectionally using the Taq DyeDeoxy terminator cycle sequencing kit and an ABI model 310 DNA sequencer (Applied Biosystems).

Cell Culture and Transfection—HepG2 cells (from ATCC) were cultured in MEM containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere, in 5% CO2, at 37 °C. Before transfection, 5 × 105 HepG2 cells were seeded onto 10-cm culture dishes and grown to 80% confluence. The cell pellets were washed twice with PBS, scraped, and resuspended in 3 ml of PBS, and the cell density was determined using a cell titer 96 nonradioactive cell proliferation assay kit (Promega) as described by Chen et al. (25). 1 × 105 cells were seeded onto 24-well plates and incubated with medium containing different concentrations of menadione for 18 h. After addition of the “stop” solution, the absorbance of the reaction solution at 570 nm was recorded. The absorbance at 630 nm was subtracted from each reading to correct for background. The net absorbance at 570 nm was taken as the index of cell viability. The viability of cells cultured with control medium was taken as the 100% viability value. The percentage of viability of the treated cells was calculated by the formula (A570 - A630 control)/A570control × 100.

For experiments involving toxicity of H2O2 and AA, cell viability was detected by the trypan blue exclusion assay. 1 × 105 cells were seeded onto 24-well plates and first incubated with medium containing 0.3 mM BSO for 16 h, followed by incubation with medium containing 100 μM H2O2 or 15 μM AA at different times. The cell pellets were resuspended and diluted by following staining with 0.2% trypan blue. The number of cells excluding trypan blue staining was taken as the index of cell viability. Cell morphology was also visualized under the light microscope, and pictures were taken.

Apoptosis Assay—The DNA fragmentation pattern (DNA ladder) was carried out by agarose gel electrophoresis. Cells (1 × 105) were scraped and centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in 1 ml of lysis buffer consisting of 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4. Cells were homogenized using a glass homogenizer. The homogenate was centrifuged at 13300 × g for 10 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 1 ml of lysis buffer, and then centrifuged for 10 min at 16,000 × g at 4 °C. The pellet was resuspended in 2 ml of isolation buffer, centrifuged again for 10 min at 6900 × g at 4 °C. The mitochrondrial pellet was suspended in 100 μl of PBS and used for Western blot analysis or catalase activity assay.

Western Blot—To assay for total cellular catalase protein levels, cells were grown to 80% confluence in 10-cm dishes, washed twice with PBS, harvested by scraping, and subsequently sonicated at duty cycle 50% and output control 4 for 29 s. To assay for mitochondrial catalase protein levels, the intact mitochondria prepared as described above were sonicated (Heat Systems-Ultrasonics, Inc.) at duty cycle 30 and output control 3 for 10 s. The sonicated suspensions were centrifuged at 8000 × g for 10 min at 4 °C. The supernatant was transferred to a new tube, and the protein concentration was measured (DC protein assay reagent, Bio-Rad). Ten μg of denatured protein were resolved on 10% acrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Bio-Rad). The membrane was incubated with rabbit anti-human catalase polyclonal antibody as the primary antibody (1:1000), followed by incubation with horseradish peroxidase conjugated to goat anti-rabbit IgG (Sigma) as the second antibody (1:5000). Detection by the chemiluminescence reaction was carried out for 1 min using the ECL kit (Amer sham Pharma Biotech), followed by exposure to Kodak X-Omat x-ray film (Eastman Kodak Co.).

Catalase Activity Assay—Fresh sonicated extracts from cells and mitochondria were used. Catalase activity was determined at 25 °C according to Clai broid and Fridovich (24). The decomposition of hydrog en 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 10 μg of total cellular or mitochondrial protein in a final volume of 1 ml. Specific activity of catalase was calculated from the equation: specific activity (units/mg of protein/min) = ΔA240 nm (1 min) × 1000/43.6 × mg protein.

Intracellular H2O2 Measurement—Fluorescence spectrophotometry and confocal microscopy were used to measure intracellular H2O2 with the H2O2-sensitive indicator DCF-DA as described by Plobova et al. (3). The new PZeoSV-MSP was digested with HindIII and NotI ends from pRCMV/MnSOD (22). The resulting product was ligated into the pZeoSV2(-) mammalian expression vector (Invitrogen Corp.). A 1-kilobase pair fragment containing the mitochondrial signal peptide and the CMV promoter region of pCR/CMV plasmid was then removed from the resulting plasmid by digestion with HindIII, which cleaves at the newly introduced HindIII site and the naturally occurring HindIII 3′ site in the MnSOD cDNA. This fragment was ligated into the pZeoSV2(+), precloned with HindIII. The new PZeoSV-MSP was digested with HindIII and NotI. Each DNA insert was sequenced bidirectionally using the Taq DyeDeoxy terminator cycle sequencing kit and an ABI model 310 DNA sequencer (Applied Biosystems)

Apoptosis Assay—The DNA fragmentation pattern (DNA ladder) was carried out by agarose gel electrophoresis. Cells (1 × 105) were scraped and centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in 1 ml of lysis buffer consisting of 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 100 μg/ml proteinase K, 0.5% SDS and incubated for 2 h at 50 °C. DNA was extracted with 1 ml of phenol, pH 8.0, followed by extraction with 1 ml of phenol/chloroform (1:1) and chloroform. The aqueous phase was precipitated with 2.5 volumes of ice-cold ethanol and 0.1 volume of 3 x sodium acetate, pH 5.2, at −20 °C overnight. The precipitates were collected by centrifugation at 13000 × g for 10 min. The pellets were air-dried, resuspended with 50 μl of TE buffer supplement, and the DNA was quantified using a spectrophotometer and electrophoresed in TAE buffer for 2 h at 50 V, and photographed under UV illumination.

DNA analysis by flow cytometry was used to quantify the percentage of apoptotic cells. 5 × 105 cells were seeded on six-well plates and incubated with medium containing 0.3 mM BSO for 16 h, followed by treatment with 100 μM H2O2. At different time points, cells were harvested, and samples were analyzed using a FACScan (Becton Dickinson, Mountain View, CA) with CellQuest software.
RESULTS

Overexpression of Catalase in Cytosol and in Mitochondria—Surviving clones of HepG2 cells transfected with the empty plasmid, or plasmid containing human catalase cDNA, or plasmid containing catalase cDNA with a 80-base pair MnSOD mitochondrial leader sequence were assayed for catalase expression by Western blot and catalase catalytic activity. Two high expression cytosol catalase clones (C1 and C33) and two high expression mitochondrial catalase clones (mC5 and mC26) were selected for detailed evaluation. Fig. 1 shows the expression of catalase in the total cell extract (panel a) and in the mitochondrial extract (panel b) from these cells and cells transfected with empty vector (Hp) as well as the parental HepG2 cells as determined by Western blot. Results from densitometric analyses of the intensity of the various bands indicated that the expression of catalase in total cell extracts of cell lines C1, C33, mC5, and mC26 was about 2-fold higher than that in HepG2 cells and the parental HepG2 cells. A high amount of catalase was found in the mitochondrial extracts from mC5 and mC26 cells, but very low levels of catalase were present in the mitochondrial extracts from C1, C33, Hp, and parental HepG2 cells. The catalase content in mitochondrial extracts from mC5 and mC26 cells was about 20-fold higher than that in HepG2 cells and at least 5–10-fold higher than the other transfected cell lines. The same results were obtained for the catalase activity assay (Fig. 2). The catalase activity in total extracts of cell lines C1, C33, mC5, and mC26 was 2–3-fold higher than that of the control cell line Hp and parental HepG2 cells. With respect to catalase activity in the mitochondria, high activity was found only in the mitochondrial extracts of cell lines mC5 and mC26. For the experiments shown below, results were compared between C33, mC5, and Hp cell lines, in order to assess the effectiveness of mitochondrially expressed catalase with that of cytosolically expressed catalase in protecting against oxidative stress and cellular toxicity.

Intracellular H$_2$O$_2$—Fig. 3A shows the result of intracellular H$_2$O$_2$ levels in cell lines C33, mC5, Hp, and parental HepG2 cells as determined by fluorescence spectrophotometry using the oxidant-sensitive dye 2,7'-DCF-DA. Higher H$_2$O$_2$ levels were detected in Hp cells and HepG2 cells than that found in C33 and mC5 cells after treatment with either 500 μM H$_2$O$_2$ or 15 μM AA for 2 h followed by incubation with 5 μM 2',7'-DCF-DA for 30 min. Cytosolic and mitochondrial expressed catalase were equally effective in lowering the fluorescence associated with oxidation of DCFH after treatment with either H$_2$O$_2$ or AA. We also analyzed the intracellular level of H$_2$O$_2$ by confocal microscopy after treating cells with 500 μM H$_2$O$_2$ for 2 h, followed by incubation with 2',7'-DCF-DA (Fig. 3B). The intensity of fluorescence in Hp cells was much stronger than that observed for the C33 and mC5 cells.

Suppression of Menadione-, H$_2$O$_2$-, and Antimycin A-induced Cytotoxicity—The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay was used to determine the cytotoxicity induced by the redox cycling agent, menadione. HepG2, Hp, C33, and
mC5 cells were treated with different concentrations (0–20 μM) of menadione for 18 h, and the viability of the cells was determined. The percentage of surviving cells decreased in a dose-dependent manner, and the decrease was similar for the parental HepG2 cells and the Hp cell line (Fig. 4). mC5 cells were more resistant to the cytotoxic effects of menadione than Hp and wild HepG2 cells, and C33 cells were even more resistant. The resistance against menadione toxicity by the mC5 cells was maintained up to menadione concentrations of 15 μM but then decreased at the menadione concentration of 20 μM; C33 cells still showed strong resistance even at 20 μM menadione.

Fig. 3. Determination of intracellular H₂O₂. A, cells were treated with buffer (first set of bars) or 15 μM AA (second set) or 500 μM H₂O₂ (third set) for 2 h, followed by incubation with 5 μM 2',7'-DCF-DA for 30 min. Cells were washed in PBS, trypsinized, resuspended in PBS, and the intensity of fluorescence was immediately read in a fluorescence spectrophotometer at wavelengths of 503 nm for excitation and at 529 nm for emission. The results were expressed as relative units with the fluorescence intensity of control Hp cells assigned a value of 1. Data are mean ± S.E. of triplicate experiments. B, cells were grown on slides. After treatment with 500 μM H₂O₂ for 2 h followed by 5 μM 2',7'-DCF-DA for 30 min, cells were washed in PBS, observed under the confocal microscope, and pictures taken. a, Hp cells; b, C33 cells; c, mC5 cells.

A trypan blue exclusion assay was used to detect the cytotoxicity induced by H₂O₂ and AA. In order to show the maximum protective effects of catalase and to lower the influence of the GSH system, BSO, an inhibitor of the synthesis of glutathione, was used to lower the level of GSH in the cells. Hp, C33, and mC5 cells were pretreated with 0.3 mM BSO for 16 h, followed by incubation with 100 μM H₂O₂ or 15 μM AA for different times. The number of surviving Hp cells rapidly decreased after 8 h of incubation with 100 μM H₂O₂, and almost none of the Hp cells were viable after 48 h (Fig. 5a). Both C33 and mC5 cells showed a comparable resistance to the cytotoxic effects of H₂O₂ as compared with the Hp cells; 60–70% of cells were still viable even after 48 h of treatment with H₂O₂ (Fig. 5a). Hp cells were also quite sensitive to toxicity by 15 μM AA, as only 50% of cells were viable after 4 h of treatment while few cells were alive after 12 h (Fig. 5b). Both C33 and mC5 cells were significantly resistant to the toxicity of AA, with about
The percentage of viability of the treated cells was calculated by the formula 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium method. The percentage of viability of the treated cells was calculated by the formula \((A_{570} - A_{490})\) sample/(\(A_{570} - A_{490}\)) control \times 100.

FIG. 4. Cytotoxicity of menadione to different cell lines. HepG2 (●), Hp (■), C33 (▲), and mC5 (●) cells were exposed to different concentrations of menadione for 18 h. Cell viability was detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium method. The percentage of viability of the treated cells was calculated by the formula \((A_{570} - A_{490})\) sample/(\(A_{570} - A_{490}\)) control \times 100.

FIG. 5. Cytotoxicity of \(\text{H}_2\text{O}_2\) and AA to different cell lines. Hp (■), C33 (▲), and mC5 (●) cells were seeded onto 24-well plates and incubated with medium containing 0.3 mM BSO for 16 h, followed by incubation with medium containing 100 \(\mu\text{M }\text{H}_2\text{O}_2\) (a) and 15 \(\mu\text{M AA}\) (b). At different time points, cells were trypsinized, diluted, and stained with 0.2% trypan blue. The number of cells excluding or staining for trypan blue were counted under the light microscope. Cell viability was expressed as the percentage of the cells excluding trypan blue out of the total number of cells. Data are the mean ± S.E. of triplicate experiments.

85% and 50% of cells viable after 4 and 12 h treatment, respectively (Fig. 5b). There is no significant difference between C33 and mC5 cells in protecting against the toxicity induced by 100 \(\mu\text{M }\text{H}_2\text{O}_2\) or 15 \(\mu\text{M AA}\). Essentially similar results were obtained after morphological visualization of the cells under the light microscope (data not shown). Most of the Hp cells lost normal morphology when treated with \(\text{H}_2\text{O}_2\) for 24 h or AA for 12 h, whereas the C33 cells and the mC5 cells retained their shape and structure.

DNA Fragmentation Induced by \(\text{H}_2\text{O}_2\)—DNA ladders are believed to be a biochemical marker for apoptosis. The cells were pretreated with 0.3 mM BSO for 16 h, followed by incubation with 100 \(\mu\text{M }\text{H}_2\text{O}_2\) for 48 h. DNA was isolated and electrophoresed on a 1.5% agarose gel. Hp and parental HepG2 cells showed a clear DNA ladder in response to the \(\text{H}_2\text{O}_2\) treatment, whereas no DNA ladder was seen in either the C33 or the mC5 cells (Fig. 6).

DNA analysis by flow cytometry after staining with PI was used to measure the percentage of apoptotic cells. Hp, C33, and mC5 cells were pretreated with 0.3 mM BSO for 16 h, followed by incubation with 100 \(\mu\text{M }\text{H}_2\text{O}_2\) for 0, 4, 8, 12, and 24 h. DNA analysis was carried out as described under “Materials and Methods.” There were less than 5% apoptotic cells after the BSO treatment for all three cell lines not incubated with the \(\text{H}_2\text{O}_2\) (0 h samples) (Fig. 7, A, panels a–c, and B, bar graph 2). In the Hp cells, the percentage of apoptotic cells increased to 17%, 40%, and 48% after 8, 12, and 24 h of treatment with \(\text{H}_2\text{O}_2\), respectively (Fig. 7B, bar graphs 4–6; A, panels d and g show the 8- and 24-h data). The C33 and mC5 cells were resistant to the \(\text{H}_2\text{O}_2\)-induced apoptosis, e.g., after 24-h treatment, the percentage of apoptotic cells was 48% in Hp cells and only 17% and 6% in C33 and mC5, cells respectively (Fig. 7, A, panels g–i; B, bar graph 6).

Mitochondrial Membrane Potential (\(\Delta\psi\)) and the Integrity of the Plasma Membrane—Rh123, a lipophilic cation, is selectively taken up by mitochondria, and uptake is directly proportional to mitochondrial \(\Delta\psi\) (26, 27). PI is imported into cells and binds to cellular DNA when the integrity of the plasma membranes is lost. After incubation with BSO, those Hp, C33, and mC5 cells not treated with \(\text{H}_2\text{O}_2\) or AA were predominantly located in the PI-negative and high \(\Delta\psi\) (strong Rh123 fluorescence) field (PI(−)–\(\Delta\psi\) high) reflective of viable, intact cells (Fig. 8, a–c, and insets a–c). A small percentage of cells were located in the (PI(+)−\(\Delta\psi\) low) field, reflective of damaged cells (Fig. 8, a–c). Hp cells that were pretreated with 0.3 mM BSO for 16 h followed by incubation with 100 \(\mu\text{M }\text{H}_2\text{O}_2\) for 24 h moved to the (PI(−)−\(\Delta\psi\) low) and (PI(+)−\(\Delta\psi\) low) field, and 67% of the cells displayed low Rh123 intensity (Fig. 8d, M1 population, compared with 27% of Hp control cells in panel a). However, only 42% and 30% of C33 and mC5 cells treated with \(\text{H}_2\text{O}_2\) were present in the \(\Delta\psi\) low field, respectively (Fig. 8, e and f). Hp cells incubated with 15 \(\mu\text{M AA}\) for 8 h also moved to the
Because mitochondria are an important target for interaction with H₂O₂ and since these organelles lack catalase, we developed a HepG2 cell line that expresses catalase in mitochondria by transferring a plasmid containing catalase cDNA with the peptide leader sequence of MnSOD into HepG2 cells. Western blot and catalase activity assay showed that higher amounts of catalase were present in mitochondria of these cells, compared with mitochondria from cells transfected with plasmid containing only catalase cDNA or cells transfected with empty vector or parental HepG2 cells. Thus, the MnSOD leader sequence could be used to successfully import catalase into mitochondria. Most of the increase in cellular catalase content and activity in the mC5 (and in mC26) clones is due to the expression of catalase in the mitochondrial fraction of these cells with just a small increase in cytosolic catalase.

Intracellular hydrogen peroxide generation induced by exogenous H₂O₂ or AA was suppressed by cytosolic catalase and by mitochondrial catalase. In general, the overexpression of cytosolic and mitochondrial catalase was equally effective in lowering DCF-DA fluorescence induced by exogenous H₂O₂ or by AA.

The overexpression of catalase in the cytosol and mitochondria protected the cells from cytotoxicity caused by menadione. Comparing C33 and mC5 cells, the cytosolic catalase showed stronger protective effect than the mitochondria catalase at higher menadione concentrations. However, comparable protection against menadione toxicity was observed in the C1 and mC26 clones (data not shown). Cytosolic catalase and mitochondrial catalase equally protected cells from cytotoxicity induced by H₂O₂ and AA. Mitochondrial catalase was protective against exogenously added H₂O₂, which suggests that some of the added H₂O₂ diffuses into the mitochondria and perhaps damage to the mitochondria is an important factor contributing to H₂O₂ toxicity. Similarly, cytosolic catalase was protective against AA-induced H₂O₂ production and toxicity, which suggests that mitochondrial catalase produced H₂O₂ diffuses into the cytosol where it may exert cytotoxic action. It would appear that catalase production in any cellular compartment might act as a sink for H₂O₂ and promote H₂O₂ movement down its concentration gradient. There are reports that catalase added to the culture medium might protect cells against oxidant-induced injury; while this may reflect some uptake of catalase into the cells with just a small increase in cytosolic catalase.

DISCUSSION

Under physiological conditions, H₂O₂ may play an important role in signal transduction pathways (28), and activation of the transcription factor NF-κB (29). However, under pathogenic conditions, H₂O₂ can produce apoptosis or necrosis (30, 31). HepG2 cells pretreated with BSO followed by exposure to 100 μM H₂O₂ could undergo apoptosis as a DNA ladder was detected in parental HepG2 and Hp cells after 48 h of incubation. Consistent with the protection afforded by cytosolic or mitochondrial catalase, a DNA ladder was not detected in the C33 and mC5 cells under the same conditions. Flow cytometry DNA analysis showed that 48% of Hp cells were in the apoptotic zone after incubation with BSO and 100 μM H₂O₂ for 24 h. However, only 17% and 6% percent apoptotic cells were detected in C33 and mC5 cells, respectively, under the same conditions. Thus both mitochondrial catalase and cytosolic catalase protect HepG2 cells from apoptosis induced by H₂O₂. It is also likely that some of the H₂O₂ toxicity may be necrotic in nature; for example, a DNA ladder was observed at 48 h but not at 24 h after treatment with 100 μM H₂O₂, yet 75% of Hp cells lost their viability at 24 h (Fig. 5). It would appear that cytosolic and mitochondrial catalase can protect against the apoptotic and the necrotic effects of H₂O₂.

Mitochondria permeability transition (MPT) and mitochondrial membrane potential (Δψ) are markers for mitochondrial...
Protection against Oxidant Injury by Mitochondrial Catalase

FIG. 8. Flow cytometry analysis of the mitochondrial membrane potential. Hp, C33, and mC5 cells were seeded onto six-well plates and incubated with medium containing 0.3 mM BSO for 16 h, followed by incubation with 100 μM H₂O₂ for 24 h or 15 μM AA for 8 h. The cells were then incubated with medium containing 5 μg/ml Rh123 for 1 h. Cells were harvested by trypsinization and resuspended in 1 ml of MEM medium containing 5 μg of PI. The intensity of fluorescence from PI and Rh123 was analyzed by flow cytometry. Panels a, b, and c refer to Hp, C33, and mC5 cells, respectively, incubated just with culture medium. Cells not treated with H₂O₂ or AA were predominantly located in the PI-negative and high Δψ (Rh123-positive) field (PI⁻Δψ high) while a smaller percentage of cells were in the (PI⁺Δψ low) field (insets of a–c). Panels d, e, and f refer to Hp, C33, and mC5 cells incubated with 15 μM AA for 8 h. M1 and M2 are two populations of cells with low and high Rh123 fluorescence intensity, and the percentage of cells with low Rh123 fluorescence is shown in each panel. The figure is one representative experiment out of three.

damage and dysfunction (32–34). Mitochondrial dysfunction caused by ROS, especially H₂O₂, can lead to necrosis and apoptosis (22, 35–38). We determined the mitochondrial membrane potential in Hp, C33, and mC5 cells pretreated with 0.3 mM BSO followed by incubation with either 100 μM H₂O₂ or 15 μM AA. The decline in Δψ caused by these agents was much less in C33 and mC5 cells than the Hp cells. These results suggest that both cytosolic catalase and mitochondrial catalase protected cells from oxidant-induced loss of mitochondrial potential, which may play an important role in the overall protection against oxidant-induced cytotoxicity.

Both bcl-2 and bcl-xL prevent cell death by inhibiting the loss of mitochondrial membrane potential and by inhibiting the release of cytochrome C or other apoptotic-inducing factors from mitochondria to cytosol (39–42). bcl-2 prevents cells from undergoing necrosis caused by inhibitors of the respiratory chain (chemical hypoxia) (43). Heat shock protein 70 also can prevent changes in mitochondrial membrane potential induced by H₂O₂ (44). These experiments demonstrate that protection of mitochondria might be an important target for prevention against oxidative injury. Overexpression of phospholipid hydroperoxide glutathione peroxidase in mitochondria was much more effective than overexpression in the cytosol of RBL-2H3 cells in protecting from oxidative injury (45). We found that cytosolic and mitochondrial catalase were equally effective in preventing loss of Δψ and loss of cellular viability. Perhaps one advantage of catalase over the GSH-glutathione peroxidase system in these actions is the lack of requirement for recycling of GSH and consumption of NADPH and perhaps avoiding the accumulation of GSSG and mixed protein disulfides.

In summary, the present study suggests that mitochondria are an important target for oxidative damage and that both catalase in cytosol and catalase in mitochondria are capable of protecting HepG2 cells against cytotoxicity or apoptosis induced by oxidative stress.

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