Overexpression of Catalase in the Mitochondrial or Cytosolic Compartment Increases Sensitivity of HepG2 Cells to Tumor Necrosis Factor-α-induced Apoptosis*

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The sensitivity of HepG2 cells overexpressing catalase in either the cytosolic or mitochondrial compartment to tumor necrosis factor-α (TNF-α) and cycloheximide was studied. Cells overexpressing catalase in the cytosol (C33 cells) and especially in mitochondria (mC5 cells) were more sensitive to TNF-α-induced apoptosis than were control cells (Hp cells). The activities of caspase-3 and -9 were increased by TNF-α, with the highest activities found in mC5 cells. Sodium azide, an inhibitor of catalase, reduced the increased sensitivity of mC5 and C33 cells to TNF-α to the level of toxicity found with control Hp cells. Azide also decreased the elevated caspase-3 activity of mC5 cells. A pan-caspase inhibitor prevented the TNF-α-induced apoptosis and toxicity produced by catalase overexpression. Addition of H₂O₂ prevented TNF-α-induced apoptosis and caspase activation, an effect prevented by simultaneous addition of catalase. TNF-α plus cycloheximide increased ATP levels, with higher levels in C33 and mC5 cells compared with Hp cells. TNF-α did not produce apoptosis in mC5 cells maintained in a low energy state. TNF-α signaling was not altered by the overexpression of catalase, as activation of nuclear factor κB and AP-1 by TNF-α was similar in the three cell lines. These results suggest that catalase, overexpressed in the cytosolic or especially the mitochondrial compartment, potentiates TNF-α-induced apoptosis and activation of caspases by removal of H₂O₂.

Reactive oxygen species (ROS)† are thought to be involved in many forms of apoptosis. Increased levels of ROS have been detected in cells undergoing apoptosis (5, 6). Oxidative stress also affects the process of apoptosis. For example, treatment of cells in vitro with H₂O₂ causes either apoptosis or necrosis depending on the concentration of H₂O₂ employed and the type of cells being studied (7, 8). Recent reports indicated that oxidative stress inhibits apoptosis induced by the chemotherapeutic drug VP-16 (9) or by αCD95 (5).

TNF-α is a cytokine produced by a wide variety of cell types whose production is up-regulated in a number of stressful and pathological conditions (10, 11). TNF-α expression is increased in animal models of toxic liver injury (12, 13) and in humans during alcohol-induced liver disease (14, 15). TNF-α kills cancer cells in intact animals and a variety of cell lines in vitro by inducing these cells to undergo either apoptosis or necrosis. However, the biochemical basis for the cytotoxic action of TNF-α is still largely unknown. TNF-α has to been shown to increase production of ROS, and this appears to be an important step in its cytotoxic mechanism (16, 17). Although some studies reported that antioxidants could protect against TNF-α toxicity (18, 19), there are other reports that antioxidants including catalase did not prevent TNF-α-mediated cell death (20).

In a previous study (21), stable HepG2 cell lines overexpressing catalase in the cytosol or mitochondria were established by transfection with catalase cDNA or with a catalase cDNA with a manganese-superoxide dismutase mitochondrial leader sequence that could conduct catalase into mitochondria. We found that the cells overexpressing catalase in either cellular compartment were more resistant to H₂O₂-, menadione-, or antimycin A-induced toxicity and apoptosis compared with cells transfected with the empty plasmid vector. In view of the conflicting reports concerning the ability of antioxidants to prevent TNF-α toxicity, and since one major locus of TNF-α-induced oxidative stress appears to be the mitochondrial compartment, studies were carried out to investigate the sensitivity of HepG2 cells that overexpress catalase in the cytosol or mitochondria to apoptosis induced by TNF-α. To our surprise and in contrast to the decreased sensitivity to H₂O₂, menadione, or antimycin A, cells overexpressing catalase, especially in the mitochondrial compartment, displayed an increased sensitivity to TNF-α-induced apoptosis.

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† The abbreviations used are: ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; CHX, cycloheximide; MEM, minimal essential medium; PBS, phosphate-buffered saline; NF-κB, nuclear factor κB; Z-VAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone.
Catalase Increases Sensitivity to TNF-α-induced Apoptosis

MATERIALS AND METHODS

Reagents—Recombinant human TNF-α, cycloheximide (CHX), sodium azide, hydrogen peroxide (H₂O₂), horseradish peroxidase-conjugated goat anti-rabbit IgG, MEM, and fetal bovine serum were purchased from Sigma. Propidium iodide was purchased from Molecular Probes, Inc. (Eugene, OR). Bovine caspase, caspase inhibitor I, 5% CO₂ at 37 °C.

Cell Lines and Cell Culture—HepG2 cells overexpressing cytosolic catalase (C33 cells) and mitochondrial catalase (mC5 cells) as well as control cells (Hp cells) were established in our laboratory previously (21) by transfection with plasmid vector pZeoSV2 (+) containing human catalase cDNA (pZeoSV-CAT), plasmid vector pZeoSV2 (+) containing human catalase cDNA with a manganese-superoxide dismutase mitochondrial leader sequence (pZeoSV/MSP-CAT), and empty vector pZeoSV2 (+) into HepG2 cells. Cells were cultured in MEM containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml Zeocin, and 2 mM glutamine in a humidified atmosphere in 5% CO₂ at 37 °C.

DNA Fragmentation Assay—The DNA fragmentation pattern (DNA ladder) was carried out by agarose gel electrophoresis. Cells (1 × 10⁶) treated with various reagents 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-HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA, 100 μM proteinase K, and 0.5% SDS and incubated for 2 h at 50 °C. DNA was extracted with 1 ml of phenol/chloroform (1:1) and chloroform. The aqueous phase was air-dried and resuspended in 50 μl of Tris/EDTA buffer supplemented with 100 μg/ml RNase A. DNA was loaded onto a 1.5% agarose gel containing ethidium bromide, electrophoresed in Tris acetate/EDTA buffer for 2 h at 50 V, and photographed under UV illumination.

Densitometry—Protein and DNA analyses were used to quantify the percentage of apoptotic cells. Cells (5 × 10⁶) were seeded onto six-well plates and incubated with various reagents. At different time points, cells were harvested by trypsinization and washed twice with PBS. The pellet was resuspended in 80% ethanol and stored at 4 °C for 24 h. Cells were washed twice with ice-cold PBS, and 250 μl of ice-cold 2.5% (w/v) trichloroacetic acid was added to the six-well dishes. After scraping from the dishes, the cell extract was immediately centrifuged at 10,000 g for 5 min at 4 °C. The supernatant was diluted 10 times and neutralized with Tris acetate buffer, pH 7.5. 10-μl samples and 100 μl of luciferin-luciferase reagent were used for ATP measurements according to the instructions of the manufacturer (Promega). Mitochondria and lysates were used as an index of the intracellular ATP levels. The relative luminescence units were measured using a Model 1251 luminometer (LKB Wallace). Statistics—Results are expressed as means ± S.E. The numbers of experiments are indicated in the figure legends. Statistical evaluation was carried out using Student’s t test.

RESULTS

HepG2 cells were transfected with empty plasmid or plasmid containing human catalase cDNA or human catalase cDNA with a 80-base pair manganese-superoxide dismutase mitochondrial leader sequence, and stable cells were generated (21). Catalase activity was increased from values of 40 units/mg of total cell protein in the cells transfected with empty vector (Hp cells) to values of 100–120 units/mg of cell protein in the cells transfected with catalase cDNA (C33 cells) or catalase cDNA with the mitochondrial leader sequence (mC5 cells). Isolated mitochondria from Hp or C33 cells displayed low catalase activity (~10 units/mg of mitochondrial protein) compared with mitochondria from mC5 cells (~140 units/mg of mitochondrial protein). Western blot analyses showed similar results as the catalase activity assays, with levels of catalase protein increasing at least 2-fold in the cell extract from C33 or mC5 cells compared with Hp cells. Catalase was barely detectable in mitochondria isolated from Hp or C33 cells, whereas a prominent band with at least 10-fold increased staining intensity was found in mitochondria isolated from mC5 cells. The activity of the other major enzyme system for removal of H₂O₂, the glutathione peroxidase system, was found to be similar for all three cell lines; total cellular glutathione peroxidase activity was between 17.1 and 19.6 milliunits/mg of cell protein for the three cell lines, whereas mitochondrial glutathione peroxidase...
activity ranged between 6.1 and 6.7 milliunits/mg of mitochondrial protein for the three cell lines.

Overexpression of Catalase in the Cytosol or Mitochondria Increases Sensitivity to TNF-α-induced Apoptosis—HepG2 cells are known to be resistant to TNF-α-induced apoptosis; however, by combining with CHX or actinomycin D, TNF-α induces typical apoptosis at low concentrations and at short times (22). The appearance of apoptosis morphology, including cell shrinkage, membrane blebbing, and formation of apoptotic bodies, and biochemical changes such as DNA fragmentation and the activation of caspases occurs in the presence of TNF-α plus CHX. Hp, C33, and mC5 cells were treated with medium or with medium containing 15 ng/ml TNF-α and 40 μM CHX for 4, 8, and 12 h, and apoptosis was evaluated by a DNA fragmentation assay and by flow cytometry DNA analysis. Both assays showed that C33 cells and especially mC5 cells were more sensitive to TNF-α/CHX-induced apoptosis than were Hp cells (Fig. 1). DNA fragmentation assays showed clear DNA ladders in C33 cells (Fig. 1A, lane 7) and mC5 cells (lane 11) as early as 4 h in response to the TNF-α/CHX treatment, whereas no DNA ladder or a weak DNA ladder was seen in Hp cells at 4 h and even 8 h (lanes 3 and 4), respectively. A clear DNA ladder was observed in Hp cells 12 h after the TNF-α/CHX treatment (lane 5), and this was intensified in C33 cells (lane 9) and especially in mC5 cells (lane 13). The hypodiploidy of cellular DNA measured by flow cytometry after propidium iodide staining was used to indicate the percentage of apoptotic cells. These percentages were 9, 17, and 18% at 4, 8, and 12 h in Hp cells, respectively (Fig. 1B, white bars), and increased to 13, 33, and 35% in C33 cells (light-gray bars) and dramatically increased to 48, 57, and 73% in mC5 cells (dark-gray bars) at 4, 8, and 12 h, respectively. The histograms of Hp, C33, and mC5 cells treated with medium (control) or with TNF-α/CHX for 8 h are shown in Fig. 1C.

Overexpression of Catalase Increases TNF-α Activation of Caspase Activity—Activation of caspases, especially caspase-3, occurs in many models of apoptosis. In the absence of TNF-α/CHX, the activity of caspase-3 was very low and similar in the three cell lines (Fig. 2). Caspase-8 activity was higher, but also similar in the three cell lines. Addition of TNF-α/CHX increased caspase-3 activity in Hp cells, whereas only a small increase in caspase-8 activity was observed. The TNF-α/CHX activation of caspase activity was greater in C33 cells than in Hp cells and was highest in mC5 cells (Fig. 2). The increase in caspase-3 activity produced by TNF-α/CHX was >2-fold higher in mC5 cells than in Hp cells.

Inhibiting Catalase with Sodium Azide Decreases Apoptosis and Caspase Activation Produced by TNF-α—The only apparent difference between Hp cells and C33 or mC5 cells is the increased activity and content of catalase in the cytosol or mitochondria of the latter compared with the former. To validate that the increased sensitivity of C33 and mC5 cells to TNF-α is indeed due to overexpression of catalase, the effect of sodium azide on TNF-α/CHX-induced apoptosis and caspase activation was determined. Addition of sodium azide to a final concentration of 1 mM resulted in a 70% decrease in catalase activity in C33 and mC5 cells. As shown in Fig. 3A, pretreating the cells with 1 mM sodium azide for 6 h, followed by incubation with 15 ng/ml TNF-α and 40 μM CHX for 6 h, resulted in an inhibition of the TNF-α-induced DNA ladder formation in all three cell lines (compare lanes 4, 7, and 10 with lanes 3, 6, and 9). Similarly, sodium azide lowered the percentage of cells undergoing apoptosis in the presence of TNF-α/CHX (Fig. 3B). This prevention of apoptosis by sodium azide was observed in all three cell lines, and much of the increase in apoptosis found in C33 and mC5 cells was prevented by sodium azide (percent apoptosis induced by TNF-α/CHX was 16, 33, and 41% in Hp, C33, and mC5 cells in the absence of sodium azide and 8, 12, and 15% in its presence). The striking increase in caspase-3 activity induced by TNF-α/CHX in mC5 cells was also reduced by sodium azide (Fig. 3C).

H2O2 Inhibits Apoptosis Induced by TNF-α/CHX—The increased sensitivity of C33 and mC5 cells to TNF-α/CHX and the prevention of TNF-α toxicity by sodium azide suggest that H2O2 may be a key modulator of sensitivity to TNF-α. To evaluate this, Hp, C33, and mC5 cells were incubated with 15 ng/ml TNF-α and 40 μM CHX in the absence or presence of 200 μM H2O2; and after 6 h, apoptosis was determined by the DNA fragmentation assay. H2O2 completely inhibited apoptosis in Hp cells (Fig. 4A, lanes 3 and 4) and partially inhibited apoptosis in C33 and mC5 cells (lanes 8 and 9 and lanes 13 and 14, respectively). Adding catalase to the medium abolished this inhibitory effect of H2O2 and reestablished TNF-α-induced apoptosis (lanes 5, 10, and 15). At this dosage and time, cells did not show significant necrosis caused by H2O2. The increase in caspase-3 activity produced by TNF-α/CHX in all three cell lines was largely prevented by 200 μM H2O2 (Fig. 4B). H2O2 also produced inhibition of caspase-8 activity, which was slightly increased by addition of TNF-α/CHX, the inhibition of caspase-8 activity by H2O2 was less than the inhibition of caspase-3 activity (Fig. 4B). The inhibition of TNF-α-induced caspase activation by H2O2 was confirmed by Western blot analysis of the levels of procaspase-3. Addition of TNF-α/CHX to Hp cells and especially to mC5 cells decreased the levels of procaspase-3 (Fig. 4C, compare lanes 2 and 6 with lanes 1 and 5), consistent with the cleavage of the procaspase form to the active caspase-3 fragments. H2O2 prevented the TNF-α-induced cleavage of procaspase-3 especially in Hp cells, which show the least sensitivity to TNF-α-induced apoptosis (lane 3).

Effect of Z-VAD-fmk, a Pan-Caspase Inhibitor, on TNF-α/CHX Toxicity—The activation of caspases such as caspase-3 in all three cell lines upon addition of TNF-α/CHX, the increased activation of caspase-3 in mC5 cells, and the inhibition by H2O2 of TNF-α-induced apoptosis and caspase-3 activation suggest that caspases play an important role in the developing apoptosis and in the different sensitivity of Hp, C33, and mC5 cells to TNF-α. This was validated by studying the effect of Z-VAD-fmk, an inhibitor of caspase-3 as well as several other caspases, on the TNF-α toxicity. At a final concentration of 50 μM, Z-VAD-fmk prevented the induction of apoptosis by TNF-α/CHX in all three cell lines (Fig. 4A, compare lanes 6, 11, and 16 with lanes 3, 8, and 13), prevented the activation of caspase-3 by TNF-α/CHX in all three cell lines (Fig. 4B), and prevented the TNF-α/CHX-induced cleavage of procaspase-3 (Fig. 4C, compare lane 4 with lane 2 and lane 8 with lane 6). Caspase-8 activity was not inhibited by Z-VAD-fmk at a concentration that strongly inhibited caspase-3 activity (Fig. 4B). In summary, the results of Fig. 4 show that the TNF-α/CHX-induced apoptosis and activation of caspase-3 and the increased sensitivity of C33 cells and especially mC5 cells to TNF-α/CHX can be prevented by H2O2 and by the caspase inhibitor Z-VAD-fmk.

Release of Cytochrome c—Release of cytochrome c from the mitochondria to the cytosol occurs in certain systems undergoing apoptosis (23, 24). Cytochrome c in conjunction with caspase-9 can activate caspase-3 (25). The possible presence of cytochrome c in the post-mitochondrial supernatant fraction of Hp, C33, or mC5 cells was evaluated by Western blot analysis 4 and 6 h after addition of TNF-α plus CHX to the incubation system, a time frame when apoptosis is occurring. Very low levels of cytochrome c were detected in all three cell lines under these conditions (Fig. 5). Increased release of cytochrome c from the mitochondria does not appear to be responsible for the
increased caspase-3 activity and sensitivity to apoptosis of mC5 cells treated with TNF-α/CHX.

**Activation of NF-κB and AP-1 by TNF-α**—Activation of oxidant-sensitive transcription factors such as NF-κB by TNF-α has been observed in many experimental models (26–28). It is generally believed that activation of NF-κB and the subsequent activation of NF-κB-responsive genes are a cellular response to minimize the toxicity of TNF-α (29–31). Could the increased
sensitivity of C33 and mC5 cells to TNF-α reflects a failure to activate NF-κB in these cells (i.e. overexpression of catalase in C33 or mC5 cells minimizes TNF-α production of ROS and ROS activation of NF-κB (32))? We initially discounted differences in protective responses by NF-κB activation in the three cell lines because the presence of CHX, which was required for the TNF-α toxicity, would prevent synthesis of short-lived death antagonists; indeed, the requirement for CHX to observe TNF-α toxicity is likely due to the failure to synthesize protective factors. To study this further, the ability of TNF-α to activate NF-κB and AP-1 was evaluated by electrophoretic mobility gel shift assays. As shown in Fig. 6A, all three cell lines showed a very early response to TNF-α addition with respect to activation of NF-κB; increased binding to a NF-κB consensus sequence could be observed 15 min after addition of TNF-α. Similarly, all three cell lines showed a low activation of AP-1 binding, which increased 15 and 30 min after addition of TNF-α (Fig. 6B). In general, activation of NF-κB and AP-1 by TNF-α appeared to be similar in the three cell lines. Importantly, this suggests that TNF-α binding and signal transduction are not significantly altered by the overexpression of catalase.

Effect of TNF-α/CHX on Intracellular ATP Levels—ATP is necessary for cells to undergo apoptosis (33, 34), and ATP levels are changed during the process of apoptosis (35). Experiments were carried out to evaluate the ATP levels in the cells after incubation in the absence or presence of TNF-α/CHX. Under basal conditions, the intracellular ATP level in C33 cells was two times higher than in Hp or mC5 cells (Fig. 7); the ATP level in mC5 cells was the same as that in Hp cells. After 3 h of treatment with TNF-α/CHX, intracellular ATP levels in all

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**FIG. 2. Caspase activity.** Hp, C33, or mC5 cells were incubated with medium (control bars) or with medium containing 15 ng/ml TNF-α and 40 μM CHX (TNF+CHX bars) for 6 h. The activities of caspase-3 (upper panel) and caspase-8 (lower panel) were measured as described under “Materials and Methods.” Results are representative of at least three independent experiments.

Δ p < 0.05, * p < 0.01 ( compared to Hp, n = 4 )

**FIG. 3. Effect of sodium azide, an inhibitor of catalase, on apoptosis and caspase activation induced by TNF-α/CHX.** Hp, C33, and mC5 cells were pretreated with or without 1 mM sodium azide for 6 h, followed by incubation with 15 ng/ml TNF-α and 40 μM CHX for 6 h. A, DNA fragmentation. Lane 1, 100-base pair standard ladder; lanes 2–4, 5–7, and 8–10, Hp, C33, and mC5 cells, respectively, treated with medium (lanes 2, 5, and 8), TNF-α/CHX (lanes 3, 6, and 9), and sodium azide + TNF-α/CHX (lanes 4, 7, and 10). B, bar graph showing the percent of apoptotic cells as measured by flow cytometry. White bars, cells treated with medium (control); light-gray bars, cells incubated with sodium azide (NaAz) + TNF-α/CHX. Concentrations and incubation times are the same as described for A. C, caspase-3 activity in mC5 cells pretreated with or without sodium azide for 6 h, followed by incubation with 15 ng/ml TNF-α and 40 μM CHX for an additional 6 h. First bar, cells treated with medium as a control; second bar, cells treated with TNF-α/CHX; third and fourth bars, cells pretreated with 1 and 3 mM sodium azide followed by TNF-α/CHX (T/C), respectively.

Δ p < 0.05 * p < 0.01 ( compared to TNF + CHX, n = 3 )
cells increased, consistent with the need for ATP for apoptosis (Fig. 7). ATP levels in C33 and mC5 cells were 50% higher than in Hp cells, which may reflect the increase in apoptosis in these cells.

To further evaluate the possible role of energy state or ATP level in the TNF-α apoptosis in mC5 cells, the cells were incubated in MEM, in a medium lacking glucose/amino acids (phosphate-buffered saline), or in MEM plus an inhibitor of the mitochondrial respiratory chain (antimycin A). The cells were then challenged with TNF-α plus CHX for 6 h and assayed for apoptosis by DNA ladder formation or propidium iodide staining.

**FIG. 5.** Release of cytochrome c from mitochondria to the cytosol.Hp, C33, and mC5 cells were treated with 15 ng of TNF-α and 40 μM CHX for 0 h (lanes 3, 6, and 9), 4 h (lanes 4, 7, and 10), and 6 h (lanes 5, 8, and 11). The cytosolic fraction was then isolated by homogenization followed by differential centrifugation, and Western blot analysis for cytochrome c was carried out using anti-cytochrome c polyclonal antibody (1:1000). Pure cytochrome c (lane 1) and 10 μg of mitochondrial protein (lane 2) were used as positive controls.

**FIG. 6.** Analysis of NF-κB (A) and AP-1 (B) binding activities in Hp, C33, and mC5 cells. Nuclear extracts from Hp (lanes 1–3), C33 (lanes 4–6), and mC5 (lanes 7–9) cells treated with medium (lanes 1, 4, and 7) or with 15 ng/ml TNF-α for 15 min (lanes 2, 5, and 8) or 30 min (lanes 3, 6, and 9) were incubated with [32P]-labeled NF-κB (A) or AP-1 (B) oligonucleotide probe. Unlabeled excess oligonucleotide (1.75 pmol) was used as a specific competitor (lane 10). Results were examined by electrophoretic mobility shift assays and visualized by exposure to Kodak XAR5 film. NS, not specific.
in the presence of TNF-α. The percent apoptotic cells for the TNF-α/CHX incubation in PBS was only 11%, and that for cells incubated in MEM plus antimycin A was 10%. Energized conditions appear to be necessary for the TNF-α/CHX induction of apoptosis.

**DISCUSSION**

Mitochondria are a main source of ROS generation including H₂O₂ and an important target for interaction with H₂O₂; mitochondria are also a critical organelle for cells undergoing apoptosis since they can release cytochrome c or other apoptotic factors to the cytoplasm, which eventually leads to the activation of caspases (1, 23–25). Many apoptotic stimulators, similar to TNF-α, are able to induce the generation of ROS by interaction with the respiratory chain (6, 36), and ROS are thought to be mediators in the apoptotic pathway (37, 38). Low levels of H₂O₂ can cause apoptosis, whereas higher levels lead to necrosis (7, 8); and H₂O₂ plays a key role in the cytotoxicity and apoptosis induced by ceramide, antimycin A, arsenite, and TNF-α (38–40). H₂O₂ can induce a mitochondrial permeability transition and decrease the mitochondrial membrane potential (41–43). The primary enzymatic defense against H₂O₂ in mitochondria is the glutathione peroxidase system, as catalase is not present in mitochondria from most tissues. Because mitochondria are an important target for interaction with H₂O₂ and since they lack catalase, we developed a HepG2 cell line that expresses catalase in the mitochondrial compartment and compared the effectiveness of overexpression of catalase in mitochondria with that of overexpression of catalase in the cytosol in protecting HepG2 cells against toxin-induced injury. With respect to H₂O₂, menadione, or antimycin A, catalase overexpressed in the mitochondrial compartment was generally as effective as catalase overexpressed in the cytosolic compartment in protecting against the loss of cell viability produced by these agents (21).

In the present study, similar experiments were carried out with TNF-α as the toxic insult to HepG2 cells. In contrast to the previous results with H₂O₂, menadione, or antimycin A, the cells overexpressing catalase showed an increased sensitivity to TNF-α plus CHX. mC5 cells were the most sensitive to TNF-α-induced apoptosis, and these cells displayed the highest caspase-3 activity in response to TNF-α addition. The somewhat unique sensitivity to TNF-α when catalase is expressed in the mitochondrial compartment may be related to the propensity of TNF-α to increase ROS production in mitochondria. Hence, mitochondrial catalase may be more effective than cytosolic catalase in removal of H₂O₂ produced as a consequence of TNF-α interactions with the mitochondrial respiratory chain.

These considerations would suggest that H₂O₂ is preventing or limiting a full apoptotic response to TNF-α. To evaluate this, we first showed that azide, an inhibitor of catalase, protected against the TNF-α-induced apoptosis and activation of caspase-3 in all three cell lines and prevented the elevated apoptosis and caspase-3 activity in mC5 cells. Addition of H₂O₂ at concentrations and for incubation times that by themselves had no effect on cellular viability also protected against the TNF-α-induced apoptosis and activation of caspase-3 in all three cell lines and prevented the elevated apoptosis and caspase-3 activation in mC5 cells. These actions of azide and of added H₂O₂ are supportive of the concept that H₂O₂ limits a complete apoptotic response to TNF-α.

Why would H₂O₂ limit the ability of TNF-α to induce apoptosis? The observations that a pan-caspase inhibitor strongly prevented apoptosis in all three cell lines and that added H₂O₂ was a powerful inhibitor of caspase-3 activity suggested that the latter action of H₂O₂ was responsible for the negative effect of H₂O₂ on apoptosis. TNF-α/CHX activated caspase-3 by causing cleavage of procaspase-3 into catalytically active fragments. H₂O₂ and Z-VAD-fmk inhibit the cleavage of procaspase-3 induced by TNF-α/CHX. It is also likely that H₂O₂ could inhibit caspase activity directly by oxidizing cysteine residues required for the activity of these enzymes. In a cell-free system, we observed that H₂O₂ suppresses the activity of activated caspase-3 (data not show).

Recent studies have shown that the execution of apoptosis requires the maintenance of adequate intracellular ATP levels (44–46). Energy failure and concomitant ATP depletion affect one or more steps of the apoptotic program and preclude the activation of execution processes, which are required for apoptotic morphology changes (46). A decrease in ATP generation by inhibiting the mitochondrial respiratory chain with rotenone, antimycin A, or an inhibitor of ATP synthase prevents cells from undergoing apoptosis or switched cellular toxicity to necrosis (35). We therefore investigated the basal ATP level as well as the changes in ATP levels after treatment with TNF-α/CHX in the three cell lines. In the absence of TNF-α/CHX treatment, ATP levels were ~2-fold higher in C33 cells than in Hp or mC5 cells. Although the reason for this has not been studied, one possible explanation could involve the known sensitivity of glyceraldehyde-3-phosphate dehydrogenase, a key glycolytic enzyme, to oxidative stress and H₂O₂ (47). Since this enzyme is largely located in the cytosolic compartment, cytosolic catalase would probably be more effective in preventing endogenous H₂O₂-mediated loss of activity. Most of the ATP produced in HepG2 cells is via glucose metabolism, which requires glycolysis as the initial step. Treatment with TNF-α plus CHX increases intracellular ATP levels in all three cell lines; however, the levels of ATP were higher in C33 and mC5 cells than in Hp cells. These elevated ATP levels may be permissive for apoptosis (35, 44–46). We cultured mC5 cells in PBS rather than MEM or added antimycin A to the MEM system; under these conditions of lower energy production, TNF-α-induced apoptosis was decreased, indicating that an energized state or ATP was required for the TNF-α/CHX-induced apoptosis.

Based upon the above, the following model is proposed. TNF-α activates a signal transduction pathway ultimately resulting in cellular apoptosis. TNF-α increases mitochondrial production of H₂O₂; H₂O₂ may limit a full apoptotic response to TNF-α by two mechanisms. One mechanism involves inhibition of caspase-3 activity either via preventing cleavage of procaspase-3 or by direct inhibition of caspase-3; it is possible that

**Fig. 7. Effect of TNF-α plus CHX on intracellular ATP levels.** Hp, C33, and mC5 cells were treated with or without 15 ng/ml TNF-α and 40 μM CHX for 3 h. Intracellular ATP levels were measured using the luciferin-luciferase reagent as described under “Materials and Methods.” The bar graph shows the relative luminescence units as a reflection of the intracellular ATP levels in Hp (white bars), C33 (light-gray bars), and mC5 (dark-gray bars) cells.
the H2O2 is also modulating the activity of upstream caspases responsible for eventual cleavage of procaspase-3. A second mechanism may reflect the lower ATP levels in Hp cells compared with C33 and mC5 cells, as the TNF-α-increased production of ROS may limit the state 3 respiratory rate or may partially dissipate the transmembrane energy potential. Overexpression of catalase in the cytosol or especially in mitochondria efficiently removes the H2O2 produced by TNF-α, allowing increased activation of caspase-3 and maximal rates of ATP synthesis to occur. This sequence of events results in an enhanced response to TNF-α-induced apoptosis. The comparable activation of NF-κB and AP-1 by TNF-α in the three cell lines suggests that the overexpression of catalase did not alter TNF-α signal transduction pathways. Although further studies are necessary to evaluate the above model, the different sensitivities to toxins such as TNF-α versus menadione or antimycin A emphasize the complexity in attempting to predict the effectiveness of antioxidant enzymes such as catalase and their possible therapeutic effectiveness.

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**FIG. 8. Effect of PBS or antimycin A on apoptosis induced by TNF-α.** mC5 cells were incubated with PBS for 2 h, with MEM containing 15 μM antimycin A for 8 h, or with just MEM for 6 h, folowed by addition of 10 ng/ml TNF-α and 40 μM CHX for a further 6 h of incubation. A, DNA fragmentation. DNA was extracted and electrophoresed on a 1.5% agarose gel. Lane 1, 100-base pair standard ladder; lane 2, cells incubated with PBS alone for 8 h; lane 3, cells incubated with MEM and TNF-α/CHX for 8 h; lane 4, cells incubated with PBS for 2 h and then treated with PBS containing TNF-α/CHX for 6 h; lane 5, cells incubated with antimycin A for 8 h and then treated with MEM containing TNF-α/CHX for 6 h; lane 6, cells incubated with PBS for 8 h and then treated with MEM containing TNF-α/CHX for an additional 6 h. B, histograms of DNA analysis by flow cytometry. Panel a, mC5 cells incubated with PBS; panel b, cells incubated with MEM containing TNF-α/CHX for 6 h; panel c, cells incubated with PBS for 2 h and then treated with PBS containing TNF-α/CHX for 6 h; panel d, cells incubated with antimycin A for 8 h and then treated with MEM containing TNF-α/CHX for 6 h. The percentages of cells in the zone 1 hypodiploid area are depicted on the graphs.
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