Mechanisms of Acetaminophen-Induced Hepatotoxicity: Role of Oxidative Stress and Mitochondrial Permeability Transition in Freshly Isolated Mouse Hepatocytes

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ABSTRACT

Freshly isolated mouse hepatocytes were used to determine the role of mitochondrial permeability transition (MPT) in acetaminophen (APAP) toxicity. Incubation of APAP (1 mM) with hepatocytes resulted in cell death as indicated by increased alanine aminotransferase in the media and propidium iodide fluorescence. To separate metabolic events from later events in toxicity, hepatocytes were preincubated with APAP for 2 h followed by centrifugation of the cells and resuspension of the pellet to remove the drug and reincubating the cells in media alone. At 2 h, toxicity was not significantly different between control and APAP-incubated cells; however, preincubation with APAP followed by reincubation with media alone resulted in a marked increase in toxicity at 3 to 5 h that was not different from incubation with APAP for the entire time. Inclusion of cyclosporine A, trifluoperazine, dithiothreitol (DTT), or N-acetyl-cysteine (NAC) in the reincubation phase prevented hepatocyte toxicity. Dichlorofluorescein fluorescence increased during the reincubation phase, indicating increased oxidative stress. Tetramethylrhodamine methyl ester perchlorate fluorescence decreased during the reincubation phase indicating a loss of mitochondrial membrane potential. Inclusion of cyclosporine A, DTT, or NAC decreased oxidative stress and loss of mitochondrial membrane potential. Confocal microscopy studies with the dye calcein acetoxymethyl ester indicated that MPT had occurred. These data are consistent with a hypothesis where APAP-induced cell death occurs by two phases, a metabolic phase and an oxidative phase. The metabolic phase occurs with GSH depletion and APAP-protein binding. The oxidative phase occurs with increased oxidative stress, loss of mitochondrial membrane potential, MPT, and toxicity.

Acetaminophen is a commonly used analgesic/antipyretic that produces necrosis of the centrilobular cells of the liver when taken in overdose (Bessems and Vermeulen, 2001; James et al., 2003a). The initial step in toxicity is cytochrome P-450 metabolism to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). At therapeutic doses, NAPQI is efficiently detoxified by glutathione (GSH). In overdose, conjugation of the reactive metabolite with GSH leads to GSH depletion, and NAPQI covalently binds to cysteine residues on proteins to form acetaminophen adducts. Covalent binding of NAPQI to proteins is an excellent correlate of acetaminophen toxicity (Cohen et al., 1997; James et al., 2003a). These adducts occur only in the hepatic centrilobular cells that develop necrosis (Roberts et al., 1991), and toxicity has not been reported to occur in the absence of their formation or by GSH depletion alone (Mitchell et al., 1974). Necrosis is believed to be the principal mechanism for toxicity. Since acetaminophen toxicity does not occur with either an activation of caspasas or a substantial increase in apoptotic hepatocytes, it has been concluded that its toxicity is not mediated by an apoptotic mechanism (Lawson et al., 1999; Gujral et al., 2002). Even though much is known about the importance of acetaminophen metabolism leading to toxicity, the cellular events producing necrosis are unknown. We have previously shown that acetaminophen toxicity in mice is accompanied by increased NO synthesis and by the formation of nitrotyrosine-protein adducts (Hinson et al.,

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and Trypan Blue 0.4% solution were obtained from Sigma-Aldrich 2003a,b). Transition (MPT) (Jaeschke et al., 2003; James et al.,
oxide generation resulted from mitochondrial permeability 1995; Laskin et al., 1995; Michael et al., 1999). However, we origi-
source of the superoxide has not yet been defined. We origi-
following exposure to toxic doses of acetaminophen. The
endothelial cells) may participate in NO synthesis in the liver from inducible nitric oxide synthase (Michael et al., 2001),
acetaminophen. It has been shown that the NO is primarily
by GSH, but GSH is depleted by the reactive metabolite of
protein adducts are believed to be formed by nitration of
nitration of tyrosine by peroxynitrite, a highly reactive species generated from superoxide and NO. Peroxynitrite is normally detoxified by GSH, but GSH is depleted by the reactive metabolite of acetaminophen. It has been shown that the NO is primarily from superoxide and NO. Peroxynitrite is normally detoxified by GSH, but GSH is depleted by the reactive metabolite of acetaminophen. It has been shown that the NO is primarily from superoxide and NO. Peroxynitrite is normally detoxified by GSH, but GSH is depleted by the reactive metabolite of acetaminophen. It has been shown that the NO is primarily from superoxide and NO. Peroxynitrite is normally detoxified by GSH, but GSH is depleted by the reactive metabolite of acetaminophen.

MPT is an abrupt increase in the permeability of the inner mitochondrial membrane to ions and small molecular weight solutes. Oxidants such as peroxides and peroxynitrite, Ca2+, and Pi promote MPT, whereas Mg2+, ADP, low pH, and high membrane potential oppose the onset of MPT. Associated with the permeability change is inner mitochondrial mem-
membrane depolarization, uncoupling of oxidative phosphoryla-
tion, release of intramitochondrial ions and metabolic inter-
mediates, and mitochondrial swelling. These changes result in decreased ATP synthesis. MPT occurs with release of superoxide from the mitochondria and is a lethal event for the cell (Lemasters, 1999; Zorov et al., 2000). In this study, we have investigated the role of MPT in acetaminophen-induced hepatotoxicity in freshly isolated mouse hepatocytes and the relationship of MPT to increased oxidative stress.

Materials and Methods

Reagents. Acetaminophen (APAP; 4-acetamidophenol), Hepes, heparin sodium salt grade 1-A from porcine intestinal mucosa, pen-
cillin G sodium salt, Waymouth MB 752/1 media with glutamine and without sodium bicarbonate, propidium iodide solution of 1 mg/ml in water, N-acetylcysteine (NAC), trifluoperazine, Percoll, and Trypan Blue 0.4% solution were obtained from Sigma-Aldrich (St. Louis, MO). Collagenase A from Clostridium histolyticum was acquired from Roche Diagnostics (Indianapolis, IN). Cyclosporine A was obtained from Bedford Laboratories (Bedford, OH). Calcein AM of high-purity grade as well as tetramethylrhodamine methyl ester perchlorate (TMRM), 2',7'-dichlorodihydrofluorescein (DCF) diac-
ate, and 5,5',6',7'-tetrachloro-1',3',3'-tetratetraethylbenzimidazocarbocyanine iodide (JC-1) were purchased from Molecular Probes (Eugene, OR). Dithiothreitol (DTT)-Cleland's reagent of electrophoresis purity (DTT) was acquired from Bio-Rad (Hercules, CA). Alamine aminotransferase (ALT) (SGPT) colorimetric was obtained from TECO Diagnostics (Anaheim, CA). All chemicals were of the highest grade commercially available.

Animals. Six-week-old male B6C3F1 mice were obtained from Harlan (Indianapolis, IN). All animal experimentation and animal protocols were approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee. Animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health. Mice were acclimatized 1 week prior to the experiments and fed ad libitum until the time of sacrifice.

Hepatocyte Isolation and Incubations. Freshly isolated hepa-
tocytes were prepared from male B6C3F1 mice by collagenase per-
fusion following a modification of the method of Grewal and Racz (Grewal and Racz, 1993; Rafeiro et al., 1994). Briefly, for each indi-
vidual experiment, hepatocytes were isolated from a single mouse as previously described followed by centrifugation at 140g for 8 min in a 90% Percoll gradient to purify the cells followed by a wash in media and a 3-min centrifugation at 140g to wash the Percoll from cells. Preparations yielding >40 million cells and cell viability >90% as determined by Trypan blue exclusion were used for the experiments. The hepatocytes were incubated at a concentration of 1,000,000 cells/ml in Waymouth's media (supplemented with 25 mM Hepes, 10 IU heparin/ml, and 500 IU penicillin G/ml in 125-ml Erlenmeyer flasks at 37°C under an atmosphere of 95% O2-5% CO2. APAP (1 mM) was added to experimental hepatocytes, but no APAP was added to control flasks. At 2 h following drug addition, the hepa-
tocytes were centrifuged for 2 min at 140g. Supernatants were dis-
carded, and fresh media were added to the cells. This procedure was repeated to remove the APAP not covalently bound and has been reported to remove greater than 99% of the acetaminophen (Tee et al., 1986). Following the final wash, some cells were resuspended in fresh media containing 1 mM NAC, 10 μM cyclosporine A, 10 μM trifluoperazine, or 0.5 mM DTT. The toxicity data were obtained from three to four separate incubations that were performed on separate mice on different days.

Toxicity Assays. Toxicity was quantified by the presence of ALT in the media, which occurred as a result of lysis of or leakage from hepatocytes. ALT in the media was detected with a colorimetric endpoint method using a commercial kit (TECO Diagnostics). Briefly, the hepatocytes were separated from the media by centrifu-
gation. ALT substrate (50 μl) was added to each sample of media (10 μl) followed by incubation in a heating bath at 37°C for 30 min. ALT Color Reagent (50 μl) was subsequently added to each sample and reinubated for 10 min. ALT Color Developer (200 μl) was added to each sample, and the mixture was placed in heating bath for 5 min. The absorbance of samples was determined spectrophotometrically in a Bio-Rad 550 plate reader at a wavelength of 490 nm. The relative absorbance was used to calculate the ALT values as de-
scribed in the kit. Cell death was also determined by addition of propidium iodide to the cell suspension. Upon lysis of hepatocytes, propidium iodide enters the cell and binds DNA resulting in an increase in fluorescence. The increased fluorescence can be quanti-
fied as a relative index of cell death. Using a modified method of Niemenen et al. (1995), aliquots of hepatocytes (1 ml) were placed in 12-well plates, and the cells were incubated with 30 μM propidium iodide solution at room temperature for 30 min. Fluorescence in each well was measured using a Millipore CytoFluor 2350 fluorescence scanner (Millipore Corporation, Billerica, MA) using 560-nm excitation and 645-nm emission filters.

Fluorescence Assays. The relative mitochondrial membrane po-
tential was determined by a mitochondrial membrane specific cat-
iconic dye JC-1 (Reers et al., 1995). JC-1 enters the mitochondria
based on high negative membrane potential. JC-1 emits fluorescence as a monomer at 535 nm or an aggregate at 590 nm. The monomer indicates a low membrane potential, whereas the aggregate indi-
cates a high membrane potential. Briefly, hepatocytes of 2-ml ali-
quots were centrifuged at 140g for 2 min and supernatants dis-
carded. Cells were resuspended with 6.5 μM JC-1 in 3 ml of JC-1 buffer (containing 137 mM NaCl, 3.6 mM KCl, 0.5 mM MgCl2, 1.8 mM CaCl2, and 10 mM Hepes) and incubated for 25 min at 37°C in atmosphere of 95% O2-5% CO2. Following incubation, cells were centrifuged and washed to remove excess dye, resuspended in 2 ml of JC-1 buffer, and excited at 490 nm in a Hitachi F-2500 Fluorescence Spectrophotometer. Membrane potential was calculated using Hita-
chi Fluorescence Spectrophotometer FL Solutions software to calculate the area under the curve of the monomer and aggregate peak, and a 590-535 nm ratio of the peaks was determined using DataQ-Windaq software.

The production of ROS was determined in isolated hepatocytes using the dye DCF diacetate. In the hepatocytes, endogenous esterases hydrolyze the acetyl ester, trapping free DCF inside the cells. ROS convert the nonfluorescent dichlorofluorescein to the highly impermeable fluorescent dichlorofluorescein. Briefly, hepatocytes of 2-ml aliquots were centrifuged at 140g for 2 min and supernatants discarded. Cells were resuspended with DCF (100 μM) in 3 ml of phosphate-buffered saline and incubated for 25 min at 37°C in atmosphere of 95% O₂-5% CO₂. Following incubation, cells were centrifuged and washed free of excess dye, resuspended in 2 ml of phosphate-buffered saline, and excited at 488 nm in a Hitachi F-2500 Fluorescence Spectrophotometer. Oxidative stress was indicated by Hitachi Fluorescence Spectrophotometer FL Solutions software used to measure the 53- nm peak to determine DCF production.

Confocal Microscopy Assays. MPT was observed on a Zeiss LSM 410 confocal microscope following the methods of Nieminen et al. (1995) with modifications. Briefly, freshly isolated hepatocytes were placed in media on collagen/fibronectin-coated dishes containing acetaminophen (1 mM) for treated cells and media alone for control cells and allowed to adhere for approximately 2 h. Following cell adhesion, the cells were subsequently washed free of acetaminophen and reincubated in media alone, media plus cyclosporine A (10 μM), or media plus trifluoperazine (10 μM). Hepatocytes were subsequently labeled as previously described (Nieminen et al., 1995) with 500 nM TMRM for 15 min followed by TMRM plus 1 μM Calcein AM for 15 min. Following cell labeling, media were washed from cells to remove fluorescent tracers, and fresh media were placed back on the cells. The plated cells were then placed on the confocal microscope and mitochondria observed with images taken at h 4. The color intensity was adjusted in Adobe Photoshop using the auto contrast and auto level functions. Some experiments used APAP-treated and control cells that were labeled with 2',7'-dichlorodihydrofluorescein diacetate as an oxidative stress determinant in the confocal microscopy studies. The color intensity of the auto contrast and auto levels was adjusted in Adobe Photoshop.

Statistical Analyses. Analysis of variance was performed with a Tukey post hoc test using the SPSS 9.0 program (SPSS, Chicago, IL). Statistical significance was defined as experimental being p < 0.05 from control.

Results

Acetaminophen Toxicity in Freshly Isolated Mouse Hepatocytes. Davies, Boobis, and coworkers (Boobis et al., 1986; Tee et al., 1986) and Racz and coworkers (Grewal and Racz, 1993; Rafeiro et al., 1994) previously showed that incubation of acetaminophen with freshly isolated hepatocytes followed by washing the hepatocytes to remove acetaminophen and subsequent reincubation of the hepatocytes with media alone resulted in significant toxicity during the reincubation phase. We have used this as one approach to study the role of MPT, alteration of mitochondrial membrane potential, and production of oxidative stress in acetaminophen toxicity.

In initial experiments, acetaminophen (1 mM) was incubated with freshly isolated hepatocytes (Fig. 1). At the end of 1 and 2 h, hepatocytes were centrifuged and washed twice to remove acetaminophen followed by resuspension in media alone. The hepatocytes were subsequently reincubated. Acetaminophen was added back to other hepatocyte incubations for the full length of the incubation. Control hepatocytes were incubated with media alone for 2 h, washed twice, and resuspended in media alone. Cell death was determined by measuring increased propidium iodide fluorescence, an event that occurs as a result of membrane damage and permits propidium iodide to bind DNA (Nieminen et al., 1992). Toxicity was also determined by measuring the presence of ALT in the media that occurs as a result of hepatocyte lysis or leakage. As shown in Fig. 1, incubation of acetaminophen for 2 h followed by washing the cells resulted in toxicity at 5 h, and the relative amount of toxicity was not significantly different from the amount of toxicity when acetaminophen was added back to the hepatocytes. However, when the cells were washed at 1 h and reincubated, toxicity did not significantly increase. Incubation in media alone did not result in a significant increase in toxicity. We have previously shown in these mice that 2 h is a critical window for in vivo toxicity. GSH depletion and covalent binding to proteins are maximal at this time, and this time is immediately before toxicity occurs (James et al., 2003c). The effect of acetaminophen on GSH depletion and covalent binding to proteins was determined in the hepatocytes. In the isolated hepatocytes, we found that GSH was depleted by approximately 50% at 0.5 h and maximally (93%) at 1 h. Covalent binding had occurred by 2 h (data not shown). From these data, we chose 2 h as the optimal time to wash the hepatocytes free of acetaminophen and determine the effect of potential inhibitors of toxicity.

Acetaminophen-Induced Mitochondrial Permeability Transition in Mouse Hepatocytes. To determine the role of MPT in acetaminophen-induced toxicity in mouse hepatocytes, cyclosporine A and trifluoperazine were utilized. Cyclosporine A and trifluoperazine are known inhibitors of MPT (Nieminen et al., 1995, 1997; Crompton et al., 1999; Lemasters, 1999). Acetaminophen was incubated with hepatocytes, and at 2 h, the hepatocytes were washed free of acetaminophen. The hepatocytes were subsequently resuspended in media containing the MPT inhibitors. As shown in

Fig. 1. Time course for development of acetaminophen toxicity. Freshly isolated mouse hepatocytes were incubated with acetaminophen (1 mM) throughout the course of the experiment (APAP) for 1 h (W1H) or 2 h (W2H). At the indicated time, the hepatocytes were washed twice by centrifugation followed by resuspension in media containing 1 mM APAP or in media alone (arrow) (Control, W1H, W2H). Toxicity was determined at the designated time. Controls were incubated with media alone, washed twice at 2 h, and resuspended in media alone. A, toxicity determined by measurement of ALT into the media. B, toxicity determined by increased fluorescence following addition of propidium iodide. *, samples significantly different from control (p < 0.05).
Fig. 2, A through D, both cyclosporine A (10 μM) and trifluoperazine (10 μM) inhibited further development of toxicity in the hepatocytes as determined by ALT release (Fig. 2, A and C) and by propidium iodide fluorescence (Fig. 2, B and D). Likewise, addition of the dithiol reagent, DTT (0.5 mM), completely eliminated toxicity (Fig. 2, E and F). Dithiothreitol has been previously reported to reduce dithiols at the MPT pore and eliminate MPT (Petronilli et al., 1994).

To further evaluate the role of MPT in acetaminophen toxicity, confocal microscopy studies were pursued using the dye calcein AM. This dye enters the hepatocytes and is hydrolyzed by esterases to yield a nondiffusible ionized species. The green dye labels the cytosol green and is normally excluded from mitochondria, but following MPT, the dye enters the mitochondria. TMRM associates with mitochondria possessing high membrane potential, thus, labeling the mitochondria. Figure 3A shows mitochondria (dark red spheres) in the control hepatocytes, incubated with media alone, excluding the calcein. Figure 3B shows hepatocytes that have been incubated with acetaminophen for 4 h. As shown, the dye has entered the mitochondria, and the mitochondria are no longer visible, nor are they compart-
membrane potential remained high for the 5-h incubation. Figure 4 shows that mitochondrial cytochrome c indicated by a fluorescence emission shift from green (535 nm) to red (590 nm). Mitochondrial depolarization decreases green fluorescence in the cytoplasm and mitochondria that have low membrane potential. JC-1 exists as a monomer that produces green fluorescence in the cytoplasm and mitochondria that have low membrane potential. JC-1 was added to an aliquot of cells, and the relative fluorescence was determined as described under Materials and Methods. Figure 4 shows that mitochondrial membrane potential remained high for the 5-h incubation.

Acetaminophen-Induced Oxidative Stress. To determine the role of oxidative stress in acetaminophen toxicity, mouse hepatocytes were incubated with acetaminophen for 2 h followed by washing the cells to remove acetaminophen. The antioxidant NAC (1 mM) was then added in the late incubation phase (after 2 h). NAC completely eliminated toxicity (Fig. 2, G and H) and also eliminated the loss of mitochondrial membrane potential (Fig. 4).

The role of oxidative stress in acetaminophen toxicity was further evaluated using the dye DCF. This dye is converted by esterases in the cell to an impermeable product. Upon oxidation, it is converted to the green fluorescent product dichlorofluorescein (Myhre et al., 2003). In Fig. 5A, confocal microscopy was utilized to show that incubation of hepatocytes with acetaminophen resulted in increased fluorescence, whereas fluorescence was not observed in control hepatocytes. Thus, acetaminophen toxicity is accompanied by increased oxidative stress. In Fig. 5B, the relative increase in fluorescence in the hepatocytes was quantified using fluorometric analysis. Hepatocytes were incubated with acetaminophen for 2 h followed by washing to remove acetaminophen. Reincubation of hepatocytes in the presence of the dye DCF for 0.5 h resulted in an increase in fluorescence over time. Addition of cyclosporine A (10 μM) or N-acetylcysteine (1 mM) to hepatocytes in this reincubation phase significantly attenuated the large increase in fluorescence. There was no increase in fluorescence in control hepatocytes that were not incubated with acetaminophen.

Discussion

This study examines the hypothesis that the toxicity of acetaminophen occurs as a result of MPT. MPT is an important mechanism in various hepatotoxicities. MPT is an abrupt increase in the permeability of the inner mitochondrial membrane to small molecular weight solutes. It occurs with membrane depolarization, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, and mitochondrial swelling. MPT is a lethal event for the cell (Crompton et al., 1999; Lemasters, 1999; Kim et al., 2003) and occurs with release of superoxide. Thus, MPT can be mediated by oxidant stress and cause increased oxidant stress. It was previously postulated to be the source of superoxide leading to peroxynitrite and tyrosine nitration in acetaminophen-induced hepatotoxicity (Jaeschke et al., 2003; James et al., 2003a,b).

In this study, we utilized freshly isolated mouse hepatocytes in suspension to determine the role of MPT in acetaminophen toxicity because these hepatocytes have high levels of cytochrome P-450 enzymes necessary for acetaminophen metabolism and subsequent toxicity (James et al., 2003a). Cultured hepatocytes (cells that have been isolated and plated for more...
than 18–24 h) have significantly less cytochrome P-450 (Steward et al., 1985; Wu et al., 1990) and are not highly sensitive to the toxic effects of acetaminophen (Harman et al., 1991). We followed the approach of Davies and Boobis (Boobis et al., 1986; Tee et al., 1986), in which hepatocytes were incubated with acetaminophen for 2 h, subsequently washed free of acetaminophen, and reincubated in media alone. The washing step removes greater than 99% of the acetaminophen (Tee et al., 1986), thereby permitting an analysis of events occurring after the metabolic phase. As shown in Fig. 1, incubation of the hepatocytes with acetaminophen for 2 h produced an insignificant increase in toxicity. Subsequent reincubation of the washed hepatocytes with media alone resulted in significant toxicity during the reincubation phase. Addition of acetaminophen in the reincubation did not alter toxicity (Fig. 1). Incubating hepatocytes with acetaminophen for only 1 h did not result in increased toxicity (Fig. 1). These data indicate acetaminophen toxicity occurs in two phases (Fig. 6). The first phase depends upon the presence of acetaminophen for at least 2 h and involves acetaminophen metabolism to the reactive metabolite NAPQI leading to GSH depletion and covalent binding of NAPQI to proteins. The second phase does not depend upon the presence of acetaminophen. Consistent with previous reports (Boobis et al., 1986; Tee et al., 1986; Grewal and Racz, 1993; Rafeiro et al., 1994), we found that GSH was depleted maximally (93%) by 1 h, and Western blot studies indicated covalent binding by 2 h (data not shown).

To determine the role of MPT in the second phase, the effects of inhibitors and the fluorescence of specific dyes were monitored. The addition of inhibitors at the beginning of the reincubation phase eliminated the possibility that the effects were related to inhibition of cytochrome P-450 metabolism of acetaminophen. Elimination of toxicity by both cyclosporine A (Fig. 2, A and B) and trifluoperazine (Fig. 2, C and D) strongly suggests involvement of MPT in acetaminophen hepatotoxicity. Cyclosporine A associates with cyclophilin D in the MPT pore and has been shown to be a specific and potent inhibitor of MPT (Petronilli et al., 1994; Crompton et al., 1999; Lemasters, 1999). Furthermore, dithiothreitol also completely eliminate acetaminophen toxicity (Fig. 2, E and F). Oxidation of critical thiols at the pore results in MPT, and dithiothreitol reduces these disulfides thereby preventing MPT (Petronilli et al., 1994; Nieminen et al., 1997). Thus, the inhibitor data are consistent with the second phase of acetaminophen toxicity being mediated by MPT.

Confocal microscopy studies were performed using the dye calcine AM to visualize MPT during acetaminophen toxicity (Fig. 3). This dye is normally excluded from mitochondria and results in the appearance of a fluorescent background. We used TMRM, a dark red dye that fluoresces in mitochondria with a high membrane potential, to confirm that these spheres were in fact mitochondria. Figure 3A shows mitochondria as red

![Fig. 4](image-url) **Fig. 4.** Time course for effect of acetaminophen on mitochondrial membrane potential in hepatocyte incubations. Freshly isolated mouse hepatocytes were incubated with media alone or with 1 mM acetaminophen. At 2 h, hepatocytes were washed twice and resuspended in media alone. At the indicated time, 6.5 μM JC-1 was added, and relative fluorescence was determined on an aliquot of hepatocytes. To some incubations, 10 μM cyclosporine A (APAP + CSP) was added, and 1 mM N-acetylcysteine (APAP+NAC) was added to other incubations. *, samples significantly decreased from the same 2-h incubation (p ≤ 0.05).

![Fig. 5](image-url) **Fig. 5.** Effect of acetaminophen on oxidative stress in individual hepatocytes. A, freshly isolated mouse hepatocytes incubated with 100 μM DCF for 30 min followed by washing to remove excess dye. Hepatocytes were subsequently incubated with 1 mM APAP or media alone (Control). Individual hepatocytes were visualized for relative fluorescence by confocal microscopy at 3 h with a 40× objective. B, hepatocytes incubated with 1 mM acetaminophen for 2 h and subsequently washed and reincubated in media alone. To some hepatocytes, 10 μM cyclosporine A or 1 mM NAC was added. At the indicated time, DCF was added, and fluorescence was determined 0.5 h later. *, samples that significantly increased from the same 2-h incubation (p ≤ 0.05).

![Fig. 6](image-url) **Fig. 6.** Postulated mechanism of acetaminophen toxicity.
spheres and calcine being excluded from the mitochondria. Following MPT, mitochondrial membrane potential is lost, and the mitochondria no longer exclude calcine (Lemasters, 1999). Figure 3B shows that in acetaminophen-treated hepatocytes, the red spheres are no longer present indicating that calcine has entered the mitochondria. Figure 3, C and D, show that inhibitors of MPT blocked this effect and maintained compartmentalization between mitochondria and cytosol. These data further suggest that acetaminophen toxicity occurs with MPT.

Since MPT occurs with loss of mitochondrial membrane potential, a time course for loss of mitochondrial membrane potential was performed using JC-1. JC-1 is a cationic dye that exhibits potential-dependent accumulation and formation of red fluorescent J-aggregates in mitochondria that have high ΔΨm. There was a loss of mitochondrial membrane potential in the acetaminophen-treated hepatocytes (2–5 h) but not in the control untreated hepatocytes (Fig. 4). Inclusion of cyclosporine A, an inhibitor of MPT, in the reincubation phase prevented loss of mitochondrial membrane potential (Fig. 4). Thus, the finding that cyclosporine A inhibits not only APAP toxicity but also the accompanying loss of mitochondrial membrane potential supports the hypothesis that MPT is a critical event in toxicity.

MPT may occur as a result of oxidative stress and leads to additional oxidative stress (Lemasters, 1999). Zorov et al. (2000) have called this ROS-induced ROS release. The role of oxidative stress in acetaminophen toxicity was determined using DCF, a dye converted to a fluorescent derivative by oxidative stress. Confocal microscopy analysis indicated increased hepatocyte fluorescence (Fig. 5A). Other hepatocytes were incubated with acetaminophen to show a time-dependent increase in fluorescence (Fig. 5B). Addition of cyclosporine A eliminated the increased fluorescence (Fig. 5B). These data are consistent with MPT being the source of the large increase in oxidative stress that occurs in acetaminophen toxicity. These data, coupled with the finding that cyclosporine A inhibits toxicity and loss of mitochondrial membrane potential, support the hypothesis that oxidative stress leading to MPT is the cause of toxicity and that this occurs in a distinct phase following APAP metabolism to NAPQI.

Further support for oxidative stress and MPT being critical events in acetaminophen toxicity was determined using NAC. Addition of NAC in the reincubation phase eliminated toxicity (Fig. 2, G and H), eliminated loss of mitochondrial membrane potential (Fig. 4), and eliminated increased oxidative stress (Fig. 5). This compound is the primary antidote given to acetaminophen overdose victims. It is believed to prevent toxicity by increasing detoxification of the reactive metabolite NAPQI by a direct reaction or through increase in GSH (Bessems and Vermeulen, 2001; James et al., 2003a). The finding that NAC completely eliminated acetaminophen toxicity, the increased oxidative stress, and the loss of mitochondrial membrane potential suggests that NAC may play a major role in preventing acetaminophen toxicity in humans not only by detoxifying NAPQI but also by preventing MPT.

The mechanism of how the metabolic phase leads to the oxidative phase and the nature of the oxidizing species in MPT is unclear. MPT is believed to occur with release of superoxide, and this species may react with nitric oxide to form peroxynitrite or may be reduced to form peroxide. Both peroxynitrite and/or peroxide may be important in the initiation and/or propagation of MPT. Both are detoxified by GSH (Sies et al., 1997), and GSH is depleted in hepatocytes by NAPQI in the metabolic phase. We reported previously that nitrotyrosine occurred in the necrotic centrilobular hepatocytes of mice treated with acetaminophen (Hinson et al., 1998), which suggested involvement of peroxynitrite. Peroxynitrite is not only a nitrating agent, but it is also an oxidizing agent (Radi et al., 1991) and has been reported to produce MPT (Packer et al., 1997). Moreover, peroxynitrite oxidizes DCF to a fluorescent product (Myhre et al., 2003). Thus, peroxynitrite is an excellent candidate as the toxicant. Alternatively, peroxide may be important. Peroxide plus ferrous iron leads to the hydroxyl radical (Fenton mechanism), a potent oxidant. Iron chelators have been reported to decrease acetaminophen toxicity in cultured cells and delay toxicity in mice (Harman et al., 1991; Schnellmann et al., 1999). Covalent binding to an iron-containing protein in the metabolic phase could conceivably lead to the release and accumulation of free iron. Moreover, Fenton mechanisms will also oxidize DCF to a fluorescent product (Myhre et al., 2003). Thus, iron may be mechanistically important. Also, calcium has been implicated in acetaminophen toxicity (Ray et al., 1993), and calcium causes MPT (Lemasters, 1999). Boobis et al. (1990) found that the calcium chelator Quin 2 decreased acetaminophen toxicity in hamster hepatocytes when added in the reincubation phase. Thus, even though the data indicate MPT in acetaminophen toxicity, understanding the complex scenario of events by which the metabolic phase leads to the oxidative phase and toxicity will require further investigation.

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