Published in final edited form as:

Free Radic Biol Med. 2016 August ; 97: 418–426. doi:10.1016/j.freeradbiomed.2016.06.024.

Translocation of Iron from Lysosomes to Mitochondria during Acetaminophen-Induced Hepatocellular Injury: Protection by Starch-Desferal and Minocycline

Jiangting Hu^{1,2}, Andaleb Kholmukhamedov^{1,2}, Christopher C. Lindsey², Craig C. Beeson², Hartmut Jaeschke⁵, and John J. Lemasters^{1,2,3,4,6,*}

¹Center for Cell Death, Injury & Regeneration, Departments of Medical University of South Carolina, Charleston, SC 29425

²Drug Discovery & Biomedical Sciences and Medical University of South Carolina, Charleston, SC 29425

³Biochemistry & Molecular Biology, Medical University of South Carolina, Charleston, SC 29425

⁴Hollings Cancer Center, Medical University of South Carolina, Charleston, SC 29425

⁵Department of Pharmacology, Toxicology & Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160

⁶Institute of Theoretical & Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russian Federation

Abstract

Acetaminophen (APAP) overdose causes hepatotoxicity involving mitochondrial dysfunction and the mitochondrial permeability transition (MPT). Iron is a critical catalyst for ROS formation, and reactive oxygen species (ROS) play an important role in APAP-induced hepatotoxicity. Previous studies show that APAP disrupts lysosomes, which release ferrous iron (Fe^{2+}) into the cytosol to trigger the MPT and cell killing. Here, our aim was to investigate whether iron released from lysosomes after APAP is then taken up into mitochondria via the mitochondrial electrogenic Ca^{2+} , Fe^{2+} uniporter (MCFU) to cause mitochondrial dysfunction and cell death. Hepatocytes were isolated from fasted male C57BL/6 mice. Necrotic cell killing was assessed by propidium iodide fluorimetry. Mitochondrial membrane potential (ψ) was visualized by confocal microscopy of rhodamine 123 (Rh123) and tetramethylrhodamine methylester (TMRM). Chelatable Fe^{2+} was monitored by quenching of calcein (cytosol) and mitoferrofluor (MFF, mitochondria). ROS generation was monitored by confocal microscopy of MitoSox Red and plate reader fluorimetry of chloromethylchlorodichlorofluorescein diacetate (cmH₂DCF-DA). Administered 1 h before APAP (10 mM), the lysosomally targeted iron chelator, starch-desferal (1 mM), and the MCFU

*Corresponding author: Medical University of South Carolina. DD504 Drug Discovery Building. 70 President Street, MSC 140. Charleston, SC 29425. Fax: 843-876-2353. J.J.Lemasters@musc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

inhibitors, Ru360 (100 nM) and minocycline (4 μ M), decreased cell killing from 83% to 41%, 57% and 53%, respectively, after 10 h. Progressive quenching of calcein and MFF began after ~4 h, signifying increased cytosolic and mitochondrial chelatable Fe^{2+} . Mitochondria then depolarized after ~10 h. Dipyrityl, a membrane-permeable iron chelator, dequenched calcein and MFF fluorescence after APAP. Starch-desferal, but not Ru360 and minocycline, suppressed cytosolic calcein quenching, whereas starch-desferal, Ru360 and minocycline all suppressed mitochondrial MFF quenching and mitochondrial depolarization. Starch-desferal, Ru360 and minocycline also each decreased ROS formation. Moreover, minocycline 1 h after APAP decreased cell killing by half. In conclusion, release of Fe^{2+} from lysosomes followed by uptake into mitochondria via MCFU occurs during APAP hepatotoxicity. Mitochondrial iron then catalyzes toxic hydroxyl radical formation, which triggers the MPT and cell killing. The efficacy of minocycline post-treatment shows minocycline as a possible therapeutic agent against APAP hepatotoxicity.

Keywords

APAP; hepatocytes; iron; lysosome; mitochondria

Introduction

Acetaminophen (APAP) overdose produces severe hepatotoxicity and is the leading cause of acute liver failure in North America (1). Although extensively studied, the mechanisms of APAP-induced liver injury remain incompletely understood. APAP toxicity is initiated by cytochrome P450-mediated metabolism of APAP to produce the reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), which is in turn detoxified by adduction to cellular glutathione (GSH) (2, 3). However, as GSH becomes exhausted, NAPQI begins to bind covalently to cellular proteins and promote oxidative stress with onset of the mitochondrial permeability transition (MPT), resulting in mitochondrial dysfunction and hepatocellular death (4–6). Oxidative stress is an important mediator of toxicity and has been suggested as a major mechanism in APAP-induced hepatotoxicity. Formation of reactive oxygen species (ROS) increases after APAP exposure, and agents that augment antioxidant defenses and scavenge ROS protect against APAP toxicity in vitro and in vivo (7).

Iron is a catalyst for formation of hydroxyl radical ($\bullet\text{OH}$), a particularly toxic ROS. Iron has been identified to have a critical role in oxidative stress in many injuries, including hepatic injury, myocardial injury and neurological injury (8–10). Iron also appears to play an important role in APAP hepatotoxicity but does not promote formation of NAPQI protein adducts (11, 12). In liver and other tissues, two pools of iron exist. The first is “non-chelatable” iron that is sequestered in ferritin and as structural components of proteins (e.g., heme, iron–sulfur complexes [ISC]). Non-chelatable iron cannot be removed by conventional iron chelators like desferal. The second pool is “chelatable” iron, which includes free iron and iron loosely bound a wide variety of anionic intracellular molecules. Previous studies identified the lysosomal/endosomal compartment as a source of mobilizable chelatable iron (13–15). Disruption of lysosomes occurs after APAP, which is prevented by

the iron chelator, desferal. Desferal also prevents mitochondrial depolarization and protects hepatocytes against cell death after APAP (14, 16).

The mitochondrial Ca^{2+} and Fe^{2+} uniporter (MCFU [to be distinguished from the 40 kDa CCDC109A gene product called MCU, a core component of MCFU]) transports Fe^{2+} into mitochondria during oxidative injury to hepatocytes (13, 17). Ru360 and minocycline inhibit MCFU and protect cells from chemical hypoxia and I/R injury (18, 19). Since Ru360 and minocycline block Fe^{2+} uptake via MCFU, protection might be by preventing mitochondrial Fe^{2+} uptake (19, 20).

Accordingly, we hypothesized that iron released from lysosomes is taken up into mitochondria via mitochondrial MCFU to promote iron-dependent formation of $\bullet\text{OH}$, mitochondrial depolarization and cell death in APAP-induced toxicity. Therefore, our goals were to characterize pathways of this iron movement and to determine whether blockade of iron mobilization into mitochondria protects against APAP toxicity to mouse hepatocytes. The data indicate a key role of MCFU in iron mobilization into mitochondria and that the lysosomal iron chelator, starch-desferal, and the MCFU inhibitors, Ru360 and minocycline, prevent ROS production and APAP toxicity to cultured hepatocytes.

Materials and Methods

Materials

Calcein acetoxymethylester (calcein-AM), calcein free acid, chloromethyl-dihydrodichlorofluorescein diacetate (cmH₂DCF-DA) MitoSox Red, and tetramethylrhodamine methylester (TMRM) were purchased from Life Technologies (Grand Island, NY); RPMI 1640 from Gibco, Rockville, MD; Ru360 from Calbiochem, San Diego, CA; glass bottom Petri dishes from MatTek Corp. (Ashland, MA); and minocycline, propidium iodide (PI), rhodamine 123 (Rh123) and other reagent grade chemicals from Sigma-Aldrich (St. Louis, MO). Starch-desferal was the generous gift of Biomedical Frontiers (Minneapolis, MN). Mitoferrofluor was synthesized in the Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina (Charleston, SC). When used, calcein-AM, cmH₂DCF-DA, MitoSox Red and Mitoferrofluor were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1%. Vehicle for other agents was water or hormonally defined medium (HDM). Vehicle controls were performed in all experiments.

Isolation and Culture of Mouse Hepatocytes

All experiments were conducted using animal protocols approved by the Institutional Animal Care and Use Committee. Hepatocytes were isolated from 20 to 25 g overnight-fasted male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) via collagenase perfusion through the inferior vena cava, as described previously (13). Hepatocytes were resuspended in Waymouth's medium MB-752/1 supplemented with 2 mM L-glutamine, 10% fetal calf serum, 100 nM insulin, 100 nM dexamethasone, 100 U/ml penicillin and 100 µg/ml streptomycin before plating on 0.1% type 1 rat-tail collagen-coated 24-well microtiter plates (1.5×10^5 cells per well) or glass bottom Petri dishes (3.0×10^5 cells per dish) (21). Cell

viability was greater than 85% by trypan blue exclusion. After attaching for 3 h in humidified 5% CO₂, 95% air at 37°C, hepatocytes were washed once and placed in HDM consisting of RPMI 1640 supplemented with 240 nM insulin, 2 mM L-glutamine, 1 µg/ml transferrin, 0.3 nM selenium, 100 U/ml penicillin, and 100 µg/ml streptomycin at pH 7.4.

Fluorimetric Assay of Cell Viability and Reactive Oxygen Species

Cell death and ROS production were assessed using a NovoStar multiwell plate reader (BMG Lab Technologies, Offenburg, Germany), as previously described (22, 23). Briefly, after attachment to 24-well plates for 3 h, hepatocytes were washed once and replaced with HDM containing 30 µM PI or 10 µM cmH₂DCF-DA. In some experiments, hepatocytes were preincubated 1 h with 1 mM starch-desferal, 100 nM Ru360 or 4 µM minocycline. After pretreatment, hepatocytes were then incubated with 10 mM APAP.

PI fluorescence from each well was measured at excitation and emission wavelengths of 544 nm and 620 nm (40-nm bandpass), respectively. For each well, fluorescence was first measured at 20 min after addition of PI (Initial) and then at various times after treatment with APAP (X). Experiments were terminated by permeabilizing plasma membranes with 375 µM digitonin. After another 20 min, a final fluorescence measurement (Final) was collected. The percentage of nonviable cells (D) was calculated as $D = 100(X - \text{Initial})/(\text{Final} - \text{Initial})$. Cell killing assessed by PI fluorimetry correlates closely with trypan blue exclusion and enzyme release as indicators of oncotic necrosis (22).

Green fluorescence of cmDCF from each well was measured at excitation and emission wavelengths of 485 nm and 520 nm, respectively. Conversion of nonfluorescent cmH₂DCF-DA to green-fluorescing cmDCF indicates generation of a variety of cellular hydroperoxides after lipid peroxidation (24).

Loading of fluorophores

Hepatocytes plated on cover glasses were incubated in HDM with 50 mM Na-HEPES buffer (pH 7.4) to maintain pH. In some experiments to stabilize the plasma membrane after APAP exposure, hepatocytes were incubated with 20 mM fructose plus 5 mM glycine (5). After 1.5 h of treatment with 10 mM APAP or no addition, cells were loaded with 500 nM rhodamine 123 (Rh123) and 1 µM mitoferrofluor (MFF) for 20 min. Cells were then washed once and incubated in HDM at pH 7.4 containing 100 nM Rh123, but no MFF. In some experiments, hepatocytes were loaded with 300 nM tetramethylrhodamine methylester (TMRM) and 1 µM calcein acetoxymethyl (calcein-AM) for 20 min. Cells were then washed once and incubated in HDM-HEPES at pH 7.4 containing 100 nM TMRM, 300 µM calcein free acid and 3 µM PI. For assessing mitochondrial ROS formation in some experiments, hepatocytes were loaded with 1 µM MitoSox Red for 15 min. Cells were then washed three times and incubated in HDM at pH 7.4.

Laser Scanning Confocal Microscopy

Hepatocytes loaded with various combinations of fluorophores were placed in an environmental chamber in 5% CO₂, 95% air at 37°C on the stage of an Olympus FV10i laser scanning confocal microscope (Center Valley, PA). Green fluorescence (Rh123, calcein) was

excited at 473 nm, and emission was collected through a 490–540-nm band-pass filter. Red fluorescence (TMRM, PI, MFF) was excited at 559 nm, and emission was imaged through a 570–670-nm band-pass filter. MitoSox Red fluorescence was excited at 473 nm, and emission was collected through a 570–670-nm bandpass filter.

Image Analysis

Calcein, Rh123, MFF and MitoSox Red fluorescence was quantified using Adobe Photoshop CS4 (San Jose, CA, USA). Briefly, cells were outlined, and mean fluorescence intensity was determined by histogram analysis of the appropriate red and green channels (25). Background values were obtained from images collected while focusing within the coverslip were subtracted from mean fluorescence of each field.

Statistics

Data are presented as means $\pm$ SEM. Images shown are representative of three or more experiments. Differences between groups were analyzed by ANOVA followed by Tukey's multiple comparison procedure using $P < 0.05$ as the criterion of significance. Experiments were representative of at least three different hepatocyte isolations.

Results

Iron Chelation and Inhibitors of the Mitochondrial Ca^{2+} , Fe^{2+} -Uniporter Decrease Acetaminophen-induced Necrotic Cell Killing

APAP-induced cell killing was determined by PI fluorescence assay. When mouse hepatocytes were exposed to 10 mM APAP (toxic dose for mouse hepatocytes), loss of cell viability increased to 40% and 83% after 6 and 10 h (Fig. 1), as observed previously (5). After individual treatment with 1 mM starch-desferal, 100 nM Ru360 and 4 μM minocycline 1 h before APAP addition, cell killing after APAP decreased substantially at 6 to 10 h (Fig. 1). Because starch-desferal is a lysosomally targeted iron chelator and Ru360 and minocycline are MCFU inhibitors, these results implicated important roles for lysosomal iron and MCFU in APAP hepatotoxicity.

Acetaminophen Increases Cytosolic and Mitochondrial Chelatable Iron

To visualize iron mobilization into the cytosol of hepatocytes during APAP hepatotoxicity, hepatocytes were co-loaded with TMRM, calcein-AM and PI. TMRM is a cell-permeant, cationic, red-fluorescing dye that is sequestered by active mitochondria as an indicator of mitochondrial polarization (26, 27). De-esterification of calcein-AM releases calcein free acid in the cytosol. Quenching of calcein fluorescence signifies an increase of cytosolic chelatable Fe^{2+} (28–30). In these experiments, 20 mM fructose and 5 mM glycine were added to the medium to prevent plasma membrane permeabilization and necrotic cell death (5).

When hepatocytes were exposed to 10 mM APAP, green cytosolic calcein fluorescence began to decrease at 4 h and progressively decreased to intensities well below that of calcein free acid (300 μM) placed in the extracellular medium, as observed previously (14). Calcein quenching was nearly complete at 10 h (Fig. 2A). Mitochondria began to lose red TMRM

fluorescence at 6 h, indicating declining ψ , and were completely depolarized at ~10 h. The membrane-permeant iron chelator, dipyrindyl (DPD), partially restored intracellular calcein fluorescence (Fig. 2A). Subsequent plasma membrane permeabilization with digitonin led to nuclear labeling with PI, indicating cell death. These data confirm that cytosolic Fe^{2+} increases after APAP treatment.

To assess mobilization of chelatable iron into mitochondria after APAP, hepatocytes were co-loaded with Rh123 and MFF. PI was added after DPD to avoid any possible interference of PI fluorescence with MFF fluorescence. Rh123 like TMRM is an indicator of mitochondrial polarization but fluoresces green. MFF is a Fe^{2+} -quenched cationic red fluorophore that accumulates electrophoretically and binds covalently inside mitochondria (31). During control incubations, Rh123 and MFF fluorescence was relatively unchanged after an incubation of up to 12 h (Fig. 2B and not shown). At 2 h after APAP, mitochondrial MFF fluorescence was bright and comparable with control hepatocytes (Fig. 2C). Subsequently, mitochondrial MFF fluorescence progressively became quenched, beginning at 4 h and becoming complete within 10 h (Fig. 2C). Mitochondrial depolarization (loss of Rh123) again began to occur at 6 h and was complete at ~10 h. DPD partially restored quenched MFF fluorescence, and digitonin led to nuclear PI staining (Fig 2C). Together with Fig. 2A, these results indicate that APAP leads to iron release into the cytosol and that some of this iron enters mitochondria.

Starch-desferal, but not Ru360 and Minocycline, Suppresses Cytosolic Calcein Quenching

Previous studies indicate that lysosomes release chelatable iron into the cytosol after oxidative stress in hepatocytes (13, 14). To confirm the origin of cytosolic iron after APAP, mouse hepatocytes were preincubated with the lysosomally targeted iron chelator, starch-desferal (1 mM). Starch-desferal inhibited APAP-induced mitochondrial depolarization and cytosolic calcein quenching (Fig. 3A). Quantification revealed that calcein fluorescence decreased only 28% at 10 h in the starch-desferal group, compared to 73% in the APAP group (Fig. 3C). Cytosolic calcein quenching was not prevented by the MCFU inhibitors, Ru360 and minocycline (Fig. 3B). Starch-desferal is membrane impermeant and is taken up into the lysosomal/endosomal compartment via endocytosis to chelate iron but not other cations that quench calcein (32, 33). Thus, prevention of quenching of cytosolic calcein fluorescence by starch-desferal indicated that chelatable iron released into cytosol after APAP originated from endosomes/lysosomes. Lack of prevention of quenching by MCFU inhibitors signified that the source of increased cytosolic Fe^{2+} was not release through MCFU from depolarized mitochondria.

Starch-desferal, Ru360 and Minocycline Prevent Mitochondrial MFF quenching and Mitochondrial Depolarization

To assess further the role of MCFU in translocation of chelatable iron into mitochondria during APAP hepatotoxicity, mouse hepatocytes were pretreated with the MCFU inhibitors, Ru360 (100 nM) and minocycline (4 μM), 1 h before APAP. Ru360 and minocycline largely suppressed the quenching of MFF in mitochondria after APAP that otherwise began about 4 h after APAP (Fig. 4B and C, compare to Fig. 2). Starch-desferal also protected against MFF quenching after APAP (Fig. 4A). Fluorescence quantification revealed that MFF

fluorescence after APAP decreased 20% and 30% at 10 h, respectively, in the Ru360- and minocycline-treated groups compared to 68% in the APAP only group (Fig. 4D). Protection by starch-desferal against MFF quenching was slightly stronger than that by Ru360 and minocycline, although only the difference between starch-desferal and minocycline was statistically significant ($p < 0.01$) (Fig. 4D). Starch-desferal, Ru360 and minocycline each nearly fully protected against loss of Rh123 fluorescence after APAP (Fig. 4D). These data indicated that MCFU was responsible for iron uptake into mitochondria after APAP, which then induced mitochondrial depolarization.

APAP Induces ROS Formation that is Suppressed by Starch-desferal, Ru360 and Minocycline

Previous studies show that APAP leads to ROS formation (7, 34). To determine the role of chelatable iron in ROS formation after APAP, mouse hepatocytes were pretreated with starch-desferal, Ru360 and minocycline 1 h before APAP and then loaded with the mitochondrial superoxide ($O_2^{\bullet-}$) indicator, MitoSox Red, or the general oxidative stress indicator, cmH₂DCF-DA (35, 36). Confocal microscopy revealed that mitochondrial MitoSox Red fluorescence increased 116% of its value at 6 h compared to 4 h after APAP, indicating ROS generation in mitochondria (Fig. 5A). At later time points, MitoSox Red fluorescence increased by 163%, although the signal appeared to be approaching saturation. With starch-desferal, Ru360 and minocycline pretreatment, MitoSox Red fluorescence increased much more slowly after APAP (Fig. 5A).

Plate reader fluorimetry also showed a progressive increase of cmDCF fluorescence after APAP, confirming ROS generation in a larger cell population (Fig. 5B). Between 2 and 4 h after APAP, cmDCF fluorescence increased only slightly. By contrast, between 6 and 10 h after APAP, cmDCF fluorescence increased by 886% over its value at 2 h. Starch-desferal, Ru360 and minocycline inhibited this ROS generation by about half at all time points (Fig. 5B). Together with Fig. 2 and 4, these results indicate that iron taken up by mitochondria catalyzed toxic ROS formation, which then triggered MPT onset and subsequent cell death in APAP hepatotoxicity.

Minocycline Treatment after APAP Decreases Cell Killing

To test the therapeutic effect of minocycline against APAP hepatotoxicity, mouse hepatocytes were treated with minocycline at 1 h after APAP exposure, and cell killing was determined by PI fluorimetry. After APAP alone, hepatocytes progressively lost viability, leading to 73% and 83% cell death at 8 h and 10 h, respectively, which minocycline post-treatment attenuated to 31% and 50% at 8 h and 10 h, respectively (Fig. 6). These results extended the benefit of minocycline against APAP hepatotoxicity to a post-APAP treatment therapeutic effect.

Discussion

APAP overdose is one of the most common causes of acute liver failure in humans (1). Mouse hepatocytes are a proven and appropriate model to study APAP hepatotoxicity. To increase their susceptibility to APAP hepatotoxicity, hepatocytes were isolated from mice

fasted overnight to decrease hepatic GSH and ATP generation and expression of cytochrome P450 2E1, the enzyme responsible for producing toxic NAPQI (14, 37, 38). In agreement with our previous findings that APAP causes time-dependent necrotic cell killing (5), 10 mM APAP produced 40% and 83% killing of cultured mouse hepatocytes at 6 h and 10 h, respectively (Fig. 1). A role of iron in APAP hepatotoxicity was implied previously from the observation that the iron chelator, desferal, protects hepatocytes after APAP (12, 14). Likewise as shown previously, starch-desferal also decreased cell killing by half (Fig. 1) (14). Because membrane-impermeant starch-desferal is specifically taken up via endocytosis into the lysosomal/endosomal compartment, protection by starch-desferal against APAP-induced cell killing implied that endosomes/lysosomes are the source of mobilizable chelatable iron during APAP hepatotoxicity.

Ru360 and minocycline are MCFU inhibitors and protect cells from chemical hypoxia and I/R injury (18, 19). Ru360 and minocycline also prevented APAP-induced cell killing by half at 6 h, suggesting that uptake of Fe^{2+} into mitochondria via MCFU contributed to APAP hepatotoxicity (Fig 1). Accordingly, the goals of this study were to characterize pathways of chelatable iron movement and identify whether blockade of iron mobilization protects against APAP toxicity to mouse hepatocytes.

To visualize iron mobilization in hepatocytes during APAP hepatotoxicity, we used confocal microscopy of cytosolic calcein and a newly developed mitochondrial iron indicator, MFF (31, 32) (Fig 2). In our study, quenching of green calcein fluorescence was specific for cytosolic chelatable Fe^{2+} (28, 29). Transition metals rapidly and stoichiometrically quench calcein fluorescence with the following relative potencies: $\text{Cu} > \text{Ni} > \text{Co} > \text{Fe}^{2+} > \text{Mn} > \text{Zn} > \text{Pb} > \text{Fe}^{3+} > \text{Ca, Mg}$ (32). Although copper, nickel and cobalt quench calcein fluorescence, quenching in our experiments was blocked by the specific iron chelator, starch-desferal. Al^{3+} is the only other biologically relevant metal chelated by starch-desferal, but Al^{3+} does not quench calcein fluorescence (33). Ester-loaded calcein is well-retained in the cytoplasm of hepatocytes but does not distribute into mitochondria (39). Thus, calcein quenching is specific for cytosolic chelatable Fe^{2+} . In contrast, red-fluorescing cationic MFF accumulates electrophoretically into mitochondria and then binds covalently to proteins via a halomethyl residue. Hence, quenching of red MFF fluorescence is specific for mitochondrial chelatable Fe^{2+} (31). After oxidative stress and ischemia-reperfusion (IR) injury, lysosomes (and/or endosomes) release iron into the cytosol, which then translocates into mitochondria (13, 40). Here after APAP, cytosolic calcein and mitochondrial MFF fluorescence began to quench at 4 h, indicating increases of chelatable Fe^{2+} in both cytosol and mitochondria. This time point is also associated with maximal GSH depletion after APAP (4), consistent with the conclusion that GSH depletion precedes lysosomal Fe^{2+} release and translocation into mitochondria.

Accumulation of iron in the cytosol and iron uptake into mitochondria occurred progressively until the abrupt onset of mitochondrial depolarization after ~10 h (Fig. 2). Progressive lysosome breakdown or lysosomal release of iron by a controlled pathway might explain the kinetics of iron release. For example, divalent metal transporter 1 (DMT-1) and transient receptor potential mucolipin subfamily member 1 (TRPML1) are iron transporters

in lysosomal membranes (41, 42). Further studies will be needed to determine the role of DMT-1 and TRPML1 in lysosomal iron release during APAP hepatotoxicity.

MCFU is the principal transporter for iron entry into mitochondria during oxidative stress, chemical hypoxia and IR-induced hepatocellular injury (13, 19, 40). Here, MCFU inhibitors, Ru360 and minocycline, suppressed mitochondrial MFF quenching but not cytosolic calcein quenching after APAP, indicating that MCFU is also a principal pathway for chelatable iron uptake into mitochondria during APAP hepatotoxicity (Fig. 4). However MCFU also transports Ca^{2+} into mitochondria, which may also have an important role in chemical hypoxia and ischemia/reperfusion injury (19). Nonetheless, because starch-desferal chelates Fe^{2+} but not Ca^{2+} and because Fe^{2+} but not Ca^{2+} quenches calcein and MFF, mitochondrial iron rather than Ca^{2+} seems the likely primary instigator in APAP-induced mitochondrial dysfunction. However, a permissive role of Ca^{2+} in APAP-induced injury cannot be excluded.

After APAP, mitochondrial generation of ROS is a critical factor triggering the MPT, and agents that inhibit ROS formation protect against APAP hepatotoxicity (7, 34, 43). $\bullet\text{OH}$ whose formation is catalyzed by iron via the Fenton reaction is a particularly toxic ROS that damages DNA, protein and membranes (44, 45). However, evidence for lipid peroxidation is limited after APAP overdose, and mitochondrial loading of the lipid soluble antioxidant tocopherol fails to protect (46). In this study, APAP induced a robust increase of MitoSox Red fluorescence, an indicator of mitochondrial generation of $\text{O}_2^{\bullet-}$ and other more strongly oxidizing radicals, but not H_2O_2 (Fig. 5A) (35, 47). Starch-desferal, Ru360 and minocycline inhibited mitochondrial ROS generation substantially, indicating a critical role of mitochondrial iron loading in APAP-induced ROS formation (Fig. 5A). Since MitoSox Red specifically targets mitochondria, we also used cmDCF to detect ROS. Whole cell cmDCF fluorescence increased to a greater extent than mitochondrial MitoSox Red fluorescence after APAP exposure, suggesting that some ROS generation may also occur in the cytosol (Fig. 5B). However, the nearly exclusive formation of glutathione disulfide and nitrotyrosine inside of mitochondria supports the conclusion that the oxidant stress is confined mostly to mitochondria and not the cytosol (48, 49). Alternatively, the MitoSox Red signal may have become saturated due to consumption of the probe and saturation of fluorescence-enhancing mitochondrial DNA binding sites. APAP-induced increases of cytosolic iron may also enhance DCF-based detection of ROS, since Fe^{2+} activates hydroperoxide-dependent oxidation of H_2DCF (24, 50). However, increased MitoSox Red fluorescence was independent confirmation of increased ROS generation after APAP.

Our findings support a “two hit” hypothesis of the role of oxidative stress and iron in APAP hepatotoxicity (Fig. 7). In the first hit, NAPQI generated by APAP metabolism induces mitochondrial protein adduct formation, disrupting mitochondrial respiration and leading to generation of $\text{O}_2^{\bullet-}$ and H_2O_2 . In the second hit, toxic NAPQI causes lysosomal damage and release of chelatable Fe^{2+} into the cytosol. Fe^{2+} is then taken up into mitochondria via MCFU. In the presence of $\text{O}_2^{\bullet-}$ and H_2O_2 , such mitochondrial Fe^{2+} loading induces $\bullet\text{OH}$ formation via the Fenton reaction, which in turn causes MPT onset and mitochondria depolarization. The results indicate that blocking this specific route of Fe^{2+} mobilization into mitochondria with the iron chelator, starch-desferal, and the MCFU inhibitors, Ru360 and

minocycline, protect against APAP-induced hepatic injury. Interestingly, minocycline treatment 1 h after APAP also protects, suggesting its potential therapeutic benefit in the clinic (Fig. 7). Future studies will be needed to place other hits, such as activation of JNK (6, 51, 52), in this overall chain of causation in APAP hepatotoxicity.

In conclusion, iron mobilization from damaged lysosomes to the cytosol and then into mitochondria via MCFU mediates APAP-induced hepatocellular injury. Increased mitochondrial $O_2^{\bullet-}/H_2O_2$ generation and chelatable iron together promote highly toxic $\bullet OH$ generation to cause MPT onset, mitochondrial depolarization and cell death. Chelating lysosomal iron with starch-desferal and blocking iron uptake into mitochondria with Ru360 and minocycline protect against injury. Since minocycline is an FDA-approved drug, future studies will focus on optimizing conditions for protection against APAP injury by minocycline in vivo.

Acknowledgments

This work was supported, in part, by Grants DK073336 and DK037034 to JJJ and DK102142 to HJ from the National Institutes of Health. Imaging facilities were supported, in part, by P30 CA138313 and P20 GM103542 with animal facility support from Grant C06 RR015455.

Abbreviations

OH	hydroxyl radical
APAP	acetaminophen
calcein-AM	calcein acetoxymethylester
cmDCF	chloromethyldihydrodichlorofluorescein
cmH₂DCF-DA	chloromethyldihydrodichlorofluorescein diacetate
DPD	dipyridyl
GSH	glutathione
HDM	hormonally defined medium
MCFU	mitochondrial Ca^{2+} Fe^{2+} uniporter
MFF	mitoferrofluor
MPT	mitochondrial permeability transition
ψ	membrane potential
NAPQI	N-acetyl-p-benzoquinone imine
$O_2^{\bullet-}$	superoxide anion
PI	propidium iodide
PT	permeability transition

Rh123	rhodamine 123
ROS	reactive oxygen species
TMRM	tetramethylrhodamine methylester

References

1. Fontana RJ. Acute liver failure including acetaminophen overdose. *Med Clin North Am.* 2008 Jul; 92(4):761–94. viii. [PubMed: 18570942]
2. Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J Pharmacol Exp Ther.* 1973 Oct; 187(1):211–217. [PubMed: 4746329]
3. James LP, Mayeux PR, Hinson JA. Acetaminophen-induced hepatotoxicity. *Drug Metab Dispos.* 2003 Dec; 31(12):1499–1506. [PubMed: 14625346]
4. Bajt ML, Knight TR, Lemasters JJ, Jaeschke H. Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetyl cysteine. *Toxicol Sci.* 2004 Aug; 80(2):343–349. [PubMed: 15115886]
5. Kon K, Kim JS, Jaeschke H, Lemasters JJ. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. *Hepatology.* 2004 Nov; 40(5):1170–1179. [PubMed: 15486922]
6. Hanawa N, Shinohara M, Saberi B, Gaarde WA, Han D, Kaplowitz N. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. *J Biol Chem.* 2008 May 16; 283(20):13565–13577. [PubMed: 18337250]
7. Jaeschke H, Bajt ML. Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol Sci.* 2006 Jan; 89(1):31–41. [PubMed: 16177235]
8. Pucheu S, Coudray C, Tresallet N, Favier A, de LJ. Effect of iron overload in the isolated ischemic and reperfused rat heart. *Cardiovasc Drugs Ther.* 1993 Aug; 7(4):701–711. [PubMed: 8241014]
9. Wang YQ, Wang MY, Fu XR, Yu P, Gao GF, Fan YM, et al. Neuroprotective effects of ginkgetin against neuro-injury in Parkinson's disease model induced by MPTP via chelating iron. *Free Radic Res.* 2015 May; 12:1–39.
10. Ghate NB, Chaudhuri D, Das A, Panja S, Mandal N. An Antioxidant Extract of the Insectivorous Plant *Drosera burmannii* Vahl. Alleviates Iron-Induced Oxidative Stress and Hepatic Injury in Mice. *PLoS One.* 2015; 10(5):e0128221. [PubMed: 26010614]
11. Gibson JD, Pumford NR, Samokyszyn VM, Hinson JA. Mechanism of acetaminophen-induced hepatotoxicity: covalent binding versus oxidative stress. *Chem Res Toxicol.* 1996 Apr; 9(3):580–585. [PubMed: 8728501]
12. Schnellmann JG, Pumford NR, Kusewitt DF, Bucci TJ, Hinson JA. Deferoxamine delays the development of the hepatotoxicity of acetaminophen in mice. *Toxicol Lett.* 1999 May 20; 106(1):79–88. [PubMed: 10378453]
13. Uchiyama A, Kim JS, Kon K, Jaeschke H, Ikejima K, Watanabe S, et al. Translocation of iron from lysosomes into mitochondria is a key event during oxidative stress-induced hepatocellular injury. *Hepatology.* 2008 Nov; 48(5):1644–1654. [PubMed: 18846543]
14. Kon K, Kim JS, Uchiyama A, Jaeschke H, Lemasters JJ. Lysosomal iron mobilization and induction of the mitochondrial permeability transition in acetaminophen-induced toxicity to mouse hepatocytes. *Toxicol Sci.* 2010 Sep; 117(1):101–108. [PubMed: 20584761]
15. Kurz T, Terman A, Brunk UT. Autophagy, ageing and apoptosis: the role of oxidative stress and lysosomal iron. *Arch Biochem Biophys.* 2007 Jun 15; 462(2):220–230. [PubMed: 17306211]
16. Woolbright BL, Ramachandran A, McGill MR, Yan HM, Bajt ML, Sharpe MR, et al. Lysosomal instability and cathepsin B release during acetaminophen hepatotoxicity. *Basic Clin Pharmacol Toxicol.* 2012 Dec; 111(6):417–425. [PubMed: 22900545]
17. Flatmark T, Romslo I. Energy-dependent accumulation of iron by isolated rat liver mitochondria. Requirement of reducing equivalents and evidence for a unidirectional flux of Fe(II) across the inner membrane. *J Biol Chem.* 1975 Aug 25; 250(16):6433–6438. [PubMed: 808543]

18. Moore CL. Specific inhibition of mitochondrial Ca^{++} transport by ruthenium red. *Biochem Biophys Res Commun*. 1971 Jan 22; 42(2):298–305. [PubMed: 4250976]
19. Schwartz J, Holmuhamedov E, Zhang X, Lovelace GL, Smith CD, Lemasters JJ. Minocycline and doxycycline, but not other tetracycline-derived compounds, protect liver cells from chemical hypoxia and ischemia/reperfusion injury by inhibition of the mitochondrial calcium uniporter. *Toxicol Appl Pharmacol*. 2013 Nov 15; 273(1):172–179. [PubMed: 24012766]
20. Hung HI, Schwartz JM, Maldonado EN, Lemasters JJ, Nieminen AL. Mitoferrin-2-dependent mitochondrial iron uptake sensitizes human head and neck squamous carcinoma cells to photodynamic therapy. *J Biol Chem*. 2013 Jan 4; 288(1):677–686. [PubMed: 23135267]
21. Qian T, Nieminen AL, Herman B, Lemasters JJ. Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. *Am J Physiol*. 1997 Dec; 273(6 Pt 1):C1783–C1792. [PubMed: 9435481]
22. Nieminen AL, Gores GJ, Bond JM, Imberti R, Herman B, Lemasters JJ. A novel cytotoxicity screening assay using a multiwell fluorescence scanner. *Toxicol Appl Pharmacol*. 1992 Aug; 115(2):147–155. [PubMed: 1641848]
23. Nieminen AL, Byrne AM, Herman B, Lemasters JJ. Mitochondrial permeability transition in hepatocytes induced by t-BuOOH: NAD(P)H and reactive oxygen species. *Am J Physiol*. 1997 Apr; 272(4 Pt 1):C1286–C1294. [PubMed: 9142854]
24. Cathcart R, Schwieters E, Ames BN. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal Biochem*. 1983; 134:111–116. [PubMed: 6660480]
25. Zinchuk V, Zinchuk O. Quantitative colocalization analysis of confocal fluorescence microscopy images. *Curr Protoc Cell Biol*. 2008 Jun. Chapter 4 Unit.
26. Ehrenberg B, Montana V, Wei M-D, Wuskell JP, Loew LM. Membrane potential can be determined in individual cells from the Nernstian distribution of cationic dyes. *Biophys J*. 1988; 53:785–794. [PubMed: 3390520]
27. Chacon E, Reece JM, Nieminen AL, Zahrebelski G, Herman B, Lemasters JJ. Distribution of electrical potential, pH, free Ca^{2+} , and volume inside cultured adult rabbit cardiac myocytes during chemical hypoxia: a multiparameter digitized confocal microscopic study. *Biophys J*. 1994 Apr; 66(4):942–952. [PubMed: 8038398]
28. Epsztejn S, Kakhlon O, Glickstein H, Breuer W, Cabantchik I. Fluorescence analysis of the labile iron pool of mammalian cells. *Anal Biochem*. 1997 May 15; 248(1):31–40. [PubMed: 9177722]
29. Uchiyama A, Kim JS, Kon K, Jaeschke H, Ikejima K, Watanabe S, et al. Translocation of iron from lysosomes into mitochondria is a key event during oxidative stress-induced hepatocellular injury. *Hepatology*. 2008 Nov; 48(5):1644–1654. [PubMed: 18846543]
30. Rauen U, Petrat F, Sustmann R, de GH. Iron-induced mitochondrial permeability transition in cultured hepatocytes. *J Hepatol*. 2004 Apr; 40(4):607–615. [PubMed: 15030976]
31. Kholmukhamedov A, Lindsey C, Beeson C, Lemasters JJ. Mitoferrofluor-A New Approach to Determine Mitochondrial Chelatable Iron [Abstract]. *Hepatology*. 2015; 60:812A.
32. Breuer W, Epsztejn S, Millgram P, Cabantchik IZ. Transport of iron and other transition metals into cells as revealed by a fluorescent probe. *Am J Physiol*. 1995 Jun; 268(6 Pt 1):C1354–C1361. [PubMed: 7611353]
33. Liu ZD, Hider RC. Design of clinically useful iron(III)-selective chelators. *Med Res Rev*. 2002 Jan; 22(1):26–64. [PubMed: 11746175]
34. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. *Toxicol Sci*. 2002 Feb; 65(2):166–176. [PubMed: 11812920]
35. Mukhopadhyay P, Rajesh M, Yoshihiro K, Hasko G, Pacher P. Simple quantitative detection of mitochondrial superoxide production in live cells. *Biochem Biophys Res Commun*. 2007 Jun 22; 358(1):203–208. [PubMed: 17475217]
36. Yan HM, Ramachandran A, Bajt ML, Lemasters JJ, Jaeschke H. The oxygen tension modulates acetaminophen-induced mitochondrial oxidant stress and cell injury in cultured hepatocytes. *Toxicol Sci*. 2010 Oct; 117(2):515–523. [PubMed: 20616211]
37. McClain CJ, Price S, Barve S, Devalarja R, Shedlofsky S. Acetaminophen hepatotoxicity: An update. *Curr Gastroenterol Rep*. 1999 Feb; 1(1):42–49. [PubMed: 10980926]

38. Hu Y, Ingelman-Sundberg M, Lindros KO. Induction mechanisms of cytochrome P450 2E1 in liver: interplay between ethanol treatment and starvation. *Biochem Pharmacol*. 1995 Jul 17; 50(2): 155–161. [PubMed: 7632158]
39. Nieminen AL, Saylor AK, Tesfai SA, Herman B, Lemasters JJ. Contribution of the mitochondrial permeability transition to lethal injury after exposure of hepatocytes to t-butylhydroperoxide. *Biochem J*. 1995 Apr 1; 307(Pt 1):99–106. [PubMed: 7718000]
40. Zhang X, Lemasters JJ. Translocation of iron from lysosomes to mitochondria during ischemia predisposes to injury after reperfusion in rat hepatocytes. *Free Radic Biol Med*. 2013 Oct.63:243–253. [PubMed: 23665427]
41. Dong XP, Cheng X, Mills E, Delling M, Wang F, Kurz T, et al. The type IV mucopolipidosis-associated protein TRPML1 is an endolysosomal iron release channel. *Nature*. 2008 Oct 16; 455(7215):992–996. [PubMed: 18794901]
42. Gunshin H, Fujiwara Y, Custodio AO, Drenth C, Robine S, Andrews NC. Slc11a2 is required for intestinal iron absorption and erythropoiesis but dispensable in placenta and liver. *J Clin Invest*. 2005 May; 115(5):1258–1266. [PubMed: 15849611]
43. Takemoto K, Hatano E, Iwaisako K, Takeiri M, Noma N, Ohmae S, et al. Necrostatin-1 protects against reactive oxygen species (ROS)-induced hepatotoxicity in acetaminophen-induced acute liver failure. *FEBS Open Bio*. 2014; 4:777–787.
44. Winterbourn CC. Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol Lett*. 1995 Dec.82–83:969–974.
45. Kehrer JP. The Haber-Weiss reaction and mechanisms of toxicity. *Toxicology*. 2000 Aug 14; 149(1):43–50. [PubMed: 10963860]
46. Knight TR, Fariss MW, Farhood A, Jaeschke H. Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. *Toxicol Sci*. 2003 Nov; 76(1):229–236. [PubMed: 12944590]
47. Kalyanaraman B. Oxidative chemistry of fluorescent dyes: implications in the detection of reactive oxygen and nitrogen species. *Biochem Soc Trans*. 2011 Oct; 39(5):1221–1225. [PubMed: 21936793]
48. Jaeschke H. Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice in vivo: the protective effect of allopurinol. *J Pharmacol Exp Ther*. 1990 Dec; 255(3):935–941. [PubMed: 2262912]
49. Cover C, Mansouri A, Knight TR, Bajt ML, Lemasters JJ, Pessayre D, et al. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. *J Pharmacol Exp Ther*. 2005 Nov; 315(2):879–887. [PubMed: 16081675]
50. Rothe G, Valet G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J Leukoc Biol*. 1990 May; 47(5):440–448. [PubMed: 2159514]
51. Gunawan BK, Liu ZX, Han D, Hanawa N, Gaarde WA, Kaplowitz N. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology*. 2006 Jul; 131(1): 165–178. [PubMed: 16831600]
52. Saito C, Lemasters JJ, Jaeschke H. c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. *Toxicol Appl Pharmacol*. 2010 Jul; 246(1–2):8–17. [PubMed: 20423716]

Highlights

- Damaged lysosomes release iron during acetaminophen toxicity to mouse hepatocytes
- Mitochondria take up released Fe_{2+} via the mitochondrial Ca_{2+} , Fe_{2+} -uniporter
- Increased iron catalyzes $\bullet\text{OH}$ formation to cause mitochondrial injury
- Chelation of lysosomal iron with starch-desferal protects against injury
- Blocking Fe_{2+} uptake into mitochondria with minocycline also protects

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.