Published in final edited form as:

Free Radic Biol Med. 2013 October ; 63: 243–253. doi:10.1016/j.freeradbiomed.2013.05.004.

Translocation of iron from lysosomes to mitochondria during ischemia predisposes to injury after reperfusion in rat hepatocytes

Xun Zhang and John J. Lemasters*

Center for Cell Death, Injury & Regeneration, Departments of Drug Discovery & Biomedical Sciences and Biochemistry & Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, USA

Abstract

The mitochondrial permeability transition (MPT) initiated by reactive oxygen species (ROS) plays an essential role in ischemia–reperfusion (IR) injury. Iron is a critical catalyst for ROS formation, and intracellular chelatable iron promotes oxidative injury-induced and MPT-dependent cell death in hepatocytes. Accordingly, our aim was to investigate the role of chelatable iron in IR-induced ROS generation, MPT formation, and cell death in primary rat hepatocytes. To simulate IR, overnight-cultured hepatocytes were incubated anoxically at pH 6.2 for 4 h and reoxygenated at pH 7.4. Chelatable Fe^{2+} , ROS, and mitochondrial membrane potential were monitored by confocal fluorescence microscopy of calcein, chloromethylchlorofluorescein, and tetramethylrhodamine methyl ester, respectively. Cell killing was assessed by propidium iodide fluorimetry. Ischemia caused progressive quenching of cytosolic calcein by more than 90%, signifying increased chelatable Fe^{2+} . Desferal and starch–desferal 1 h before ischemia suppressed calcein quenching. Ischemia also induced quenching and dequenching of calcein loaded into mitochondria and lysosomes, respectively. Desferal, starch–desferal, and the inhibitor of the mitochondrial Ca^{2+} uniporter (MCU), Ru360, suppressed mitochondrial calcein quenching during ischemia. Desferal, starch–desferal, and Ru360 before ischemia also decreased mitochondrial ROS formation, MPT opening, and cell killing after reperfusion. These results indicate that lysosomes release chelatable Fe^{2+} during ischemia, which is taken up into mitochondria by MCU. Increased mitochondrial iron then predisposes to ROS-dependent MPT opening and cell killing after reperfusion.

Keywords

Cell death; Iron; Ischemia; Lysosome; Mitochondria; Free radicals

Hepatic ischemia–reperfusion (IR)¹ injury can develop after liver transplantation, hepatic surgery, and circulatory shock [1]. During ischemia, acidosis due to anaerobic metabolism protects against necrotic cell death. By contrast, the return of oxygen and normal extracellular pH after reperfusion precipitates lethal cell injury [2]. Much evidence indicates that the mitochondrial permeability transition (MPT) is the penultimate event leading to cell death after reperfusion [1,3–5]. Opening of MPT pores in the mitochondrial inner membrane causes the MPT and induces mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large amplitude swelling, which lead to apoptosis, necrosis, or both

modes of cell death. Cyclosporin A and NIM-811 specifically block the MPT and protect against IR injury of liver [5,6].

After reperfusion, mitochondrial generation of reactive oxygen species (ROS) is a critical factor initiating the MPT [7]. ROS include hydrogen peroxide (H_2O_2), superoxide ($\text{O}_2^{\bullet-}$), and hydroxyl radical ($\text{OH}^\bullet$). $\text{OH}^\bullet$, especially, damages DNA, protein, and membranes [8]. Antioxidants inhibit the MPT and protect against IR-induced cell injury, consistent with a cytotoxic role of ROS in the pathogenesis of IR injury [9].

In IR, cell killing after reperfusion increases with longer times of ischemia. Changes occurring during ischemia that predispose to damage from reperfusion remain poorly understood. Anoxia, ATP depletion, acidosis, and reduction of pyridine nucleotides and other redox components become maximal within seconds or a few minutes of ischemia, but vulnerability to reperfusion injury continues to increase with increasing duration of ischemia. Iron, which catalyzes $\text{OH}^\bullet$ formation via the Fenton reaction [10], may be one factor that affects vulnerability to reperfusion injury.

Two pools of iron exist in mammalian cells: nonchelatable iron, which includes iron tightly bound to ferritin and other iron-containing proteins, and chelatable iron, which is free iron and iron loosely bound to anionic molecules such as citrate and ATP. Chelatable, but not nonchelatable, iron catalyzes ROS generation and is removed by iron chelators [11]. Previous studies show that chelatable iron contributes to oxidative stress and toxicant-induced killing of hepatocytes and other cell types, because iron chelation is protective [9,12–14]. Chelatable iron also contributes to MPT onset after IR, which is strongly suppressed by iron chelators [15]. However, little is known concerning the sources of chelatable iron promoting hepatotoxicity after IR.

Endosomes and lysosomes are the principal reservoirs of chelatable iron in hepatocytes, because iron uptake is chiefly via endocytosis of transferrin [16]. Bafilomycin, an inhibitor of the vacuolar proton-pumping ATPase (V-ATPase), releases chelatable iron from lysosomes [14]. Iron released by lysosomes enters the cytosol and is then taken up by mitochondria [14]. Inside mitochondria, chelatable iron promotes ROS formation, the MPT, and cell killing during oxidative stress. Increased chelatable iron from lysosomes also triggers MPT onset and cell death after acetaminophen [12]. Accordingly, we hypothesized that mobilization of lysosomal iron to mitochondria during ischemia contributes to ROS-dependent MPT onset and cell death after reperfusion. Therefore, our goals were to characterize the redistribution of chelatable iron during ischemia and determine how such redistribution predisposes hepatocytes to reperfusion injury.

Materials and methods

Materials

Calcein-AM, tetramethylrhodamine methyl ester (TMRM), LysoTracker Red (LTR), chloromethylchlorodichlorofluorescein diacetate (cmH₂DCF-DA), and calcein free acid were purchased from Life Technologies (Grand Island, NY, USA). Ru360 was purchased from Calbiochem (San Diego, CA, USA). Starch–desferal was purchased from Biomedical Frontiers (Minneapolis, MN, USA). Other chemicals of analytical grade were obtained from Sigma.

Hepatocyte isolation and culture

Hepatocytes isolated from Sprague–Dawley rats were plated onto collagen-coated microtiter plates or 40-mm round glass coverslips and incubated overnight in Waymouth's medium MB-752/1 containing 2 mM L-glutamine, 27 mM NaHCO₃, 10% fetal calf serum, 100 nM

insulin, and 100 nM dexamethasone in 5% CO₂/air, as described [5]. Experiments were performed in Krebs–Ringer–hydroxyethylpiperazine-*N*-2 ethanesulfonic acid buffer (KRH) [5].

Ischemia–reperfusion

To simulate IR, hepatocytes were incubated in KRH at pH 6.2 in an anaerobic chamber for up to 4 h followed by reoxygenation with KRH at pH 7.4, as described [5].

Assay for ROS generation

Hydroperoxides generated after lipid peroxidation, a measure of ROS, were assessed using a plate reader, as described [13].

Cell viability

Cell viability was assessed by propidium iodide (PI) fluorimetry, as described [17].

Loading of fluorophores

Hepatocytes were loaded with 1 μ M calcein-AM and 200 nM TMRM or 200 nM LTR for 20 min. Cells were then incubated in anaerobic KRH at pH 6.2 containing 50 nM TMRM or 70 nM LTR, 3 μ M PI, and 300 μ M calcein free acid. For confocal imaging of ischemia, anoxic hepatocytes were additionally incubated with oxygen-consuming Oxyrase (3.3%; Mansfield, OH, USA) to prevent back diffusion of oxygen [18]. In some experiments, 0.5 μ M calcein-AM was added 15 min before the end of ischemia, or 10 μ M cmH₂DCF-DA was included during both ischemia and reperfusion. Reperfusion was induced by infusion of aerobic KRH at pH 7.4 containing 200 nM TMRM and 3 μ M PI.

In some experiments, 70-kDa rhodamine–dextran (RhDex, 5 mg/100 g body wt, ip) was injected into rats 12 h before hepatocyte isolation to label lysosomes, as described [19]. Cold loading/warm incubation was used to localize calcein to both mitochondria and lysosomes but not the cytosol [20]. Briefly, 6-h-cultured hepatocytes loaded with RhDex were cold ester loaded with 1 μ M calcein-AM in complete culture medium for 1 h at 4 °C and washed. Hepatocytes were then incubated overnight in culture medium in 5% CO₂/air.

Confocal microscopy

Green and red fluorescence was imaged with an inverted Zeiss LSM510 laser scanning confocal microscope using a 63 $\times$ NA 1.4 oil-immersion planapochromat lens [5].

Image analysis

Calcein and TMRM fluorescence was quantified using Adobe Photoshop CS4 (San Jose, CA, USA) and Zeiss LSM 510 software (Thornwood, NY, USA). To quantify TMRM and cytosolic calcein fluorescence, cells were outlined, and mean fluorescence intensity was determined by histogram analysis of the appropriate red and green channels [21]. Background values were obtained from images collected while focusing within the coverslip and were subtracted from mean fluorescence of each field. To quantify calcein in the mitochondrial and lysosomal compartments, the colocalization tool of Zeiss LSM software was used. Each pixel was plotted in a scatter diagram in which the two axes represented the intensities of calcein and RhDex staining, respectively. The cross-hair function mode was used to produce four quadrants representing pixels with (1) both calcein and RhDex fluorescence, (2) RhDex fluorescence only, (3) calcein fluorescence only, and (4) background fluorescence (low calcein and RhDex). The calcein intensity (minus background) of pixels in the calcein-only quadrant represented mitochondrial calcein

fluorescence, whereas calcein intensity (minus background) of RhDex-containing pixels represented lysosomal calcein intensity.

Statistics

Means were compared by the Student *t* test using a criterion of significance of $p < 0.05$. Data were expressed as means $\pm$ SEM. Experiments were representative of at least three different cell isolations.

Results

Increase in chelatable iron in the cytosol during ischemia

To investigate the effect of ischemia on cytosolic chelatable iron, hepatocytes were coloaded with calcein-AM, TMRM, and PI. When hepatocytes were subjected to ischemia, cytosolic calcein fluorescence decreased progressively, beginning as soon as 30 min after ischemia, and quenching was nearly complete after 4 h (Fig. 1A). Calcein fluorescence was quantified for individual hepatocytes after background subtraction and averaged. After ischemia, calcein fluorescence decreased 91% compared to that at the

beginning of ischemia (Fig. 1D). Calcein fluorescence decreased to intensities well below that of calcein free acid (300 μ M) placed in the extracellular medium. Plasma membrane blebbing and cellular swelling occurred during ischemia (Fig. 1A). Mitochondrial depolarization (loss of TMRM) also occurred within 30 min and was virtually complete within 1 h (Fig. 1A and E). However, no nuclear labeling with PI occurred even after 4 h of ischemia, signifying no loss of viability.

To determine whether quenching of cytosolic calcein was due to an increase in chelatable iron, hepatocytes were preincubated with the iron chelators desferal (1 mM) and starch–desferal (1 mM desferal equivalent). Desferal and starch–desferal did not prevent ischemia-induced blebbing, cellular swelling, or mitochondrial depolarization (Fig. 1B and C). However, desferal and starch–desferal each strongly inhibited calcein quenching, and calcein fluorescence decreased only 21 and 29%, respectively (Fig. 1D). Desferal chelates iron but not other biologically relevant cations that quench calcein [22,23]. Thus, calcein quenching during ischemia signified an increase in chelatable Fe^{2+} in the cytosol. Moreover, because membrane-impermeative starch–desferal is taken up via endocytosis into the lysosomal/endosomal compartment, prevention of cytosolic calcein quenching by starch–desferal indicated that chelatable iron was probably released by lysosomes/endosomes.

Chelatable iron in mitochondria and lysosomes during ischemia

To assess chelatable iron in mitochondria and lysosomes during ischemia, RhDex-labeled hepatocytes were cold ester loaded to localize calcein selectively to mitochondria and lysosomes. During ischemia, mitochondrial calcein fluorescence progressively became quenched, beginning within 1 h and becoming virtually complete within 4 h (Fig. 2A). Quantitation revealed that mitochondrial calcein fluorescence decreased 95% during ischemia (Fig. 2D). As mitochondrial calcein fluorescence decreased, fluorescence in RhDex-labeled lysosomes increased twofold, suggesting a decrease in lysosomal chelatable iron (Fig. 2A, arrows, and 2E). Labeling of lysosomes with RhDex was stable with no indication of lysosomal swelling or rupture.

Although lysosomal integrity was maintained during ischemia, acidic lysosomal pH gradients monitored with LTR progressively collapsed, and virtually all LTR fluorescence was lost after 1 h (Supplementary Fig. 1). To determine if increased chelatable iron during ischemia was due to ATP depletion, hepatocytes were subjected to ischemia in the presence

of fructose, a substrate for glycolytic ATP generation during ischemia–anoxia [24]. With fructose, calcein quenching was substantially diminished (Supplementary Fig. 2), indicating that lysosomal iron release during ischemia was caused by lysosomal alkalization from ATP depletion-dependent inhibition of V-ATPase.

Desferal and starch–desferal each suppressed ischemia-induced quenching of mitochondrial calcein, whereas dequenching of lysosomal calcein was not prevented (Fig. 2B–E). Together with Fig. 1, these results suggest that lysosomes release iron into the cytosol during ischemia and that some of this iron enters mitochondria.

Electrogenic Ca^{2+} uniporter in ischemic mitochondrial iron uptake

The mitochondrial Ca^{2+} uniporter (MCU), which transports Fe^{2+} but not Fe^{3+} , has been implicated in mitochondrial Fe^{2+} accumulation during oxidative injury to hepatocytes [12,14,25]. To determine the role of MCU in mitochondrial iron uptake during ischemia, hepatocytes were pretreated 1 h before ischemia with Ru360, a specific MCU inhibitor. In the presence of Ru360, initial loading of calcein and subsequent quenching of cytosolic calcein during ischemia were unchanged (Fig. 3A). By contrast, Ru360 largely suppressed quenching of calcein localized to mitochondria by cold ester loading followed by overnight incubation, which indicated that MCU was responsible for chelatable Fe^{2+} uptake into mitochondria during ischemia (Fig. 3B).

Suppression of generation of reactive oxygen species by iron chelation and Ru360

To determine the role of chelatable iron in ROS formation after IR, hepatocytes were coloaded with cmH₂DCF-DA, TMRM, and PI to monitor ROS, $\Delta\Psi$, and loss of cell viability, respectively. During ischemia, little cmDCF fluorescence was evident (Fig. 4A). By contrast, cmDCF fluorescence increased substantially within 10 min of reperfusion, predominantly in mitochondria (Fig. 4A). After 20 min, cmDCF fluorescence appeared in the cytosol as the mitochondria lost TMRM fluorescence. Subsequently, fluorescence of cmDCF was lost virtually completely as hepatocytes lost viability (Fig. 4A, arrows). With desferal or starch–desferal pretreatment, cmDCF fluorescence both in mitochondria and in cytosol increased much more slowly after reperfusion (Fig. 4B and C). Additionally, mitochondria underwent sustained repolarization, and cell death did not occur. Ru360 also promoted mitochondrial repolarization and suppressed the increase in mitochondrial cmDCF fluorescence after reperfusion (Fig. 4D). However, somewhat in contrast to desferal and starch–desferal, a late increase in cytosolic cmDCF fluorescence occurred after reperfusion with Ru360.

Protection against the mitochondrial permeability transition by iron chelation and Ru360

To overcome calcein quenching after ischemia, hepatocytes were reloaded with calcein-AM 15 min before the end of ischemia [5]. As shown previously [5], dark voids in the green fluorescence represented intact mitochondria that excluded calcein entry. After 20 min of reperfusion, voids relatively abruptly filled with green fluorescence, indicating onset of the MPT (Fig. 5A). Coincident with permeabilization, mitochondria depolarized and hepatocytes subsequently lost all calcein fluorescence, and cell death occurred (Fig. 5A, arrow). In hepatocytes pretreated with desferal or starch–desferal, voids of calcein and mitochondrial TMRM persisted, and loss of viability did not occur (Fig. 5B and C). Thus, desferal and starch–desferal protected against the MPT after reperfusion. Ru360 also suppressed mitochondrial depolarization and cell killing after reperfusion but to a lesser extent (Fig. 5D). With Ru360, some cell killing occurred after 40–60 min (arrows) but apparently without preceding mitochondrial permeabilization to calcein.

Fluorimetric determination of reperfusion-induced generation of reactive oxygen species and loss of cell viability: protection by iron chelation and Ru360

To confirm our findings in a larger cell population, hepatocytes in multiwell plates were incubated with cmH₂DCF-DA, and cmDCF formation was measured using a plate reader. After reperfusion, cmDCF increased progressively, signifying ROS generation (Fig. 6A). After 2 h, cmDCF was 400% of its value immediately after reperfusion. Desferal and starch–desferal blocked this ROS generation by two-thirds at all time points, whereas Ru360 inhibited ROS formation to about half this extent (Fig. 6A).

In parallel, cell killing was determined by PI fluorimetry. During ischemia, little cell killing occurred (Fig. 6B). By contrast after reperfusion, hepatocytes progressively lost viability, leading to 80% cell death after 2 h. Desferal and starch–desferal attenuated cell killing by about two-thirds, whereas Ru360 protected to about half this extent (Fig. 6B). These results confirmed our confocal findings that desferal and starch–desferal protected against ROS generation and cell death after reperfusion and that Ru360 protected to a lesser extent.

Discussion

During ischemia, lysosomes released chelatable iron into the cytosol, which was taken up by mitochondria. Upon reperfusion, mitochondrial iron promoted ROS generation, the MPT, and cell death. Chelation of iron within lysosomes with starch–desferal or throughout cells with desferal suppressed ROS generation, MPT onset, and cell killing after reperfusion. The pathway for mitochondrial entry of iron was MCU, because MCU inhibition with Ru360 decreased mitochondrial iron uptake during ischemia and attenuated iron-dependent mitochondrial ROS generation, MPT onset, and cell death after reperfusion.

High serum iron is associated with more severe ischemic heart disease, diabetes, and alcoholic liver disease [26–28], and iron chelation prevents oxidative and toxicant-induced injury to liver cells [12–14]. Here, calcein visualized changes in chelatable iron in hepatocytes during IR. Ischemia caused progressive quenching of cytosolic calcein, signifying increased chelatable iron. Initially, cytosolic and nuclear calcein fluorescence was greater than that of calcein in the extracellular space, indicating that intracellular calcein concentration was greater than 300 μ M (Fig. 1A). During ischemia, calcein fluorescence decreased virtually completely. Assuming one-to-one binding of calcein with iron, the rise in chelatable iron in the cytosol was >300 μ M. In rat hepatocytes incubated aerobically, chelatable Fe²⁺ is estimated to be 5–10 μ M [11]. Compared to intracellular calcein loading in the range of 300 μ M, chelation of endogenous chelatable iron by iron chelators did not have a measurable effect on initial calcein fluorescence, because we never observed differences with or without iron chelators.

For this study, imaging of chelatable iron using an iron indicator such as calcein had distinct advantages over conventional analysis of chelatable iron in cellular extracts. Imaging uniquely permitted iron measurement in single cells and revealed intracellular iron mobilization that could not have been determined in whole-cell extracts or subcellular fractions that leak their chelatable iron during fractionation. Desferal-sensitive calcein quenching is a well-established assay for chelatable Fe²⁺ [11], and previous work from our laboratory showed that Fe²⁺ but not Fe³⁺ specifically quenches calcein fluorescence [12]. Transition metals rapidly and stoichiometrically quench calcein fluorescence with the following relative potencies: Cu > Ni > Co > Fe²⁺ > Mn > Zn > Pb > Fe³⁺ > Ca, Mg [22]. At physiological pH, Ca²⁺ has little effect on calcein fluorescence, especially at the submicromolar concentrations of free Ca²⁺ that exist in the cytosol and mitochondria. Although Cu²⁺ also quenches calcein fluorescence, calcein quenching in our experiments was blocked by the iron chelators, desferal and starch–desferal. The only other

physiologically relevant metal chelated by desferal is Al^{3+} , but Al^{3+} does not quench calcein fluorescence [23]. Thus, quenching of calcein fluorescence is specific for chelatable Fe^{2+} [11,14].

Previous reports indicate that chelatable iron increases in ischemic heart and kidney, but the source of this iron was not known [29,30]. Lysosomes are a reservoir of chelatable iron, and lysosomal iron mobilization plays a key role in oxidative injury to hepatocytes and other cells [12,14,31]. In this study, cytosolic calcein fluorescence decreased and lysosomal fluorescence increased during ischemia. Moreover starch–desferal, an iron chelator taken up into lysosomes/endosomes, blocked cytosolic calcein quenching. These findings indicate that ischemia induces release of chelatable iron from lysosomes into the cytosol.

Lysosomal disruption and alkalization are independent mechanisms mobilizing lysosomal iron to the cytosol in oxidative and acetaminophen-induced hepatocellular injury [12,14]. Previously, the integrity of lysosomes in hepatocytes was unchanged after up to 4 h of ischemia [32]. Similarly, in this work, ischemia did not cause lysosomal breakdown, as indicated by RhDex retention (Fig. 2A). Instead, lysosomal iron release was probably due to ATP depletion causing lysosomal alkalization, because pharmacological inhibition of V-ATPase leads to similar quenching of cytosolic calcein in parallel with collapse of the lysosomal pH gradient [14]. In this study, lysosomal pH gradients also collapsed during ischemia, as shown by release of lysosomal LTR (Supplementary Fig. 1). Moreover fructose, a substrate for glycolytic ATP generation by hepatocytes [24,33], blocked ischemia-induced quenching of cytosolic calcein (Supplementary Fig. 2). Thus, release of lysosomal iron during ischemia was probably due to lysosomal alkalization caused by ATP depletion-mediated inhibition of V-ATPase. The pathway for iron movement out of lysosomes remains to be characterized. Divalent metal transporter 1 (DMT-1) and transient receptor potential mucolipin subfamily member 1 (TRPML1) are cation transporters implicated in iron transport out of lysosomes [34,35]. Future studies will be needed to determine the roles of DMT-1 and TRPML1 in lysosomal iron release.

After bafilomycin and during acetaminophen hepatotoxicity, lysosomal iron releases into the cytosol and translocates into mitochondria [12,14]. Here, during ischemia, iron from lysosomes also moved into mitochondria, as shown by mitochondrial calcein quenching. The principal pathway for iron entry into mitochondria was MCU, because MCU inhibition with Ru360 suppressed mitochondrial calcein quenching in confirmation of previous studies showing Fe^{2+} uptake via MCU in isolated mitochondria and intact hepatocytes after bafilomycin and hepatotoxic stress [12,14,25].

Mitochondrial uptake of iron during ischemia promoted mitochondrial ROS formation, inner membrane permeabilization, and cell death after reperfusion (Fig. 4A and B). Such toxic effects of iron are consistent with the previous finding that addition of a membrane-permeative iron complex to hepatocytes induces MPT onset and cell killing [36]. Iron overload and oxidative stress are implicated in a variety of liver diseases, including iron-overdose-induced hepatotoxicity and alcoholic and nonalcoholic steatohepatitis [37–39]. In this study, ROS formed primarily within mitochondria after reperfusion (Fig. 4A). Desferal, starch–desferal, and Ru360 suppressed mitochondrial ROS generation, the MPT, and loss of cell viability after reperfusion, which indicates that mitochondrial iron loading during ischemia predisposes to these events after reperfusion.

Although to a lesser extent than in mitochondria, cmDCF fluorescence also increased in the cytosol after reperfusion. Desferal and starch–desferal prevented this increase in cytosolic cmDCF, indicating that cytosolic ROS formation was iron-dependent. Ru360 was less effective than desferals at blocking cytosolic ROS after reperfusion (Fig. 4D). Ru360 was

also less effective at preventing reperfusion-induced cell death, and cell death in the presence of Ru360 did not appear to be preceded by mitochondrial inner membrane permeabilization (Fig. 5D). These observations suggest that when mitochondrial iron uptake is blocked, iron-dependent ROS formation occurs instead in the cytosol to contribute to reperfusion injury by mechanisms not directly involving the MPT.

Injury after reperfusion is proportional to time of ischemia [2]. After short times, reperfusion leads to recovery without loss of cell viability. By contrast, with longer times of ischemia, cell death occurs minutes to hours after reperfusion [40]. In organ transplantation, extended cold storage of organs is associated with graft injury, dysfunction, and failure [41]. Previous studies focused on loss of high-energy compounds and lactic acidosis to explain vulnerability to reperfusion injury, but such events occur early during ischemia and before the development of vulnerability to reperfusion injury [42,43]. This study provides new insight into how longer times of ischemia predispose to reperfusion injury. As ischemia progresses, the V-ATPase became inhibited and acidic lysosomal pH gradients collapsed (Supplementary Fig. 1; see also Ref. [44]). Consequently, lysosomes released chelatable iron into the cytosol, which then moved into mitochondria via MCU. Although $\Delta\Psi$ is required for mitochondrial accumulation of Fe^{2+} against a concentration gradient, MCU can still equilibrate Fe^{2+} between the cytosol and the mitochondrial matrix at low or zero $\Delta\Psi$. In addition, the mitochondrial inner membrane may maintain a small negative inside Donnan potential even during anoxic ischemia, which can also drive uptake of divalent cations by MCU. Patch-clamp studies show that MCU transports divalent cations such as Ca^{2+} even when mitochondrial $\Delta\Psi$ is low [45]. Thus, high concentrations of chelatable iron (300 μM) released from lysosomes/endosomes into the cytosol can pass into mitochondria via MCU in the absence of $\Delta\Psi$. This iron movement into mitochondria will be further augmented by any persisting Donnan potential during ischemia.

Blebbing, cellular swelling, and mitochondrial depolarization are common features of respiratory inhibition and ATP depletion after ischemia, hypoxia, and other stresses in hepatocytes, which can be reversible [5]. In this study, iron chelation by desferal and starch–desferal did not prevent blebbing, cellular swelling, or mitochondrial depolarization caused by respiratory inhibition and ATP depletion during ischemia (Figs. 1 and 2). By contrast, after reperfusion, desferal and starch–desferal pretreatment suppressed mitochondrial ROS generation, onset of the MPT, and loss of cell viability (Figs. 4–6). Because desferal and starch–desferal chelate Fe^{2+} but not Ca^{2+} , we conclude that mitochondrial iron is the primary instigator of the MPT. Nonetheless, Ca^{2+} may still have a permissive role, because many MPT inducers act to decrease the threshold amount of Ca^{2+} for MPT induction.

Although desferal is highly polar and poorly permeative through cellular membranes, at high doses (0.5–1 mM) enough desferal may enter the cytosol and other cellular compartments to chelate iron and protect cells. Previous studies from our laboratory showed that desferal at such high doses blocks cell killing after IR and oxidative stress [9,13]. For this reason, we chose to use desferal at 1 mM in this study. Alternatively, like starch–desferal, desferal may be taken up by endocytosis to chelate iron selectively in the endosomal/lysosomal compartment. Indeed, the effects of desferal and starch–desferal on cell death, calcein fluorescence, and other measures were always very similar. Desferal entry by either route is fully consistent with our conclusion that endosomes/lysosomes release iron into the cytosol during ischemia, which is then accumulated in part into mitochondria to predispose to reperfusion injury.

Lysosomal iron release and uptake into mitochondria were gradual rather than abrupt and occurred progressively during the first few hours of ischemia. Mitochondrial iron loading during ischemia then predisposed to mitochondrial ROS generation after reperfusion,

presumably by iron-catalyzed Fenton chemistry with generation of highly reactive $\text{OH}^\bullet$ [10]. During reperfusion, H_2O_2 forms as a by-product of respiration, but until chelatable iron increases, such H_2O_2 formation causes little harm. However, once mitochondrial Fe^{2+} loading occurs, H_2O_2 formation after reperfusion causes $\text{OH}^\bullet$ generation, leading ultimately to the MPT and cell death. Because H_2O_2 generated in mitochondria diffuses across membranes, cytosolic iron can also contribute to injury, which would explain why iron chelators such as desferal and starch–desferal that decrease iron in both cytosol and mitochondria are more protective than Ru360, which blocks mitochondrial iron loading but not iron release into the cytosol. Iron also catalyzes other reactions, particularly lipid peroxidation chain reactions, and we do not rule out that iron exerts toxicity by these other radical-generating pathways as well.

A role for iron in predisposing cells to reperfusion injury is consistent with previous studies. For example, iron overload by dietary iron sensitizes myocardium to IR injury [46]. Moreover, the greater sensitivity of old hearts to IR injury compared to young is substantially reversed by iron chelators [47]. These reports together with the present findings are consistent with the conclusion that translocation of chelatable iron into the cytosol and then mitochondria during ischemia predisposes to reperfusion injury. Our findings extend to IR the “two-hit” hypothesis of iron-catalyzed $\text{OH}^\bullet$ formation in the pathogenesis of oxidative injury [14]. During ischemia, lysosomes release chelatable iron into the cytosol, which mitochondria then accumulate (first hit). However, during the anoxia of ischemia, H_2O_2 cannot form and $\text{OH}^\bullet$ formation does not occur. After reperfusion in the presence of increased mitochondrial iron, H_2O_2 from respiratory activity becomes a second hit leading to $\text{OH}^\bullet$ formation, MPT onset, and cell death (Fig. 7). Although either single hit alone is insufficient to cause injury, the combined hits have lethal consequences.

In summary, lysosomes release chelatable iron into the cytosol during ischemia, which is taken up by mitochondria. Mitochondrial iron then predisposes to $\text{OH}^\bullet$ formation after reperfusion, causing the MPT and cell death. Iron chelation and inhibition of mitochondrial iron uptake by Ru360 attenuate iron-dependent mitochondrial ROS generation, inner membrane permeabilization, and loss of cell viability in IR. Iron translocation from lysosomes to mitochondria provides a mechanistic basis for why prolonged ischemia predisposes to reperfusion injury. Future studies will be needed to determine whether lysosomal iron mobilization occurs in vivo during IR injury to liver and other organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported, in part, by Grants DK073336 and DK37034 from the National Institutes of Health. Imaging facilities were supported, in part, by P30 CA138313 to the Hollings Cancer Center with animal facility support from Grant C06 RR015455.

Abbreviations

$\Delta\Psi$	mitochondrial membrane potential
cmDCF	chloromethyldichlorofluorescein
cmH ₂ DCF-DA	chloromethyldihydrodichlorofluorescein diacetate
IR	ischemia–reperfusion

KRH	Krebs–Ringer–Hepes
LTR	LysoTracker Red
MCU	mitochondrial Ca^{2+} uniporter
MPT	mitochondrial permeability transition
$\text{OH}^{\bullet}$	hydroxyl radical
PI	propidium iodide
RhDex	rhodamine–dextran
ROS	reactive oxygen species
TMRM	tetramethylrhodamine methyl ester
V-ATPase	vacuolar proton-pumping ATPase

References

1. Jaeschke H, Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. *Gastroenterology*. 2003; 125:1246–1257. [PubMed: 14517806]
2. Currin RT, Gores GJ, Thurman RG, Lemasters JJ. Protection by acidotic pH against anoxic cell killing in perfused rat liver: evidence for a pH paradox. *FASEB J*. 1991; 5:207–210. [PubMed: 2004664]
3. Halestrap AP. The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. *Biochem Soc Symp*. 1999; 66:181–203. [PubMed: 10989667]
4. Kim JS, He L, Qian T, Lemasters JJ. Role of the mitochondrial permeability transition in apoptotic and necrotic death after ischemia/reperfusion injury to hepatocytes. *Curr Mol Med*. 2003; 3:527–535. [PubMed: 14527084]
5. Qian T, Nieminen AL, Herman B, Lemasters JJ. Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. *Am J Physiol*. 1997; 273:C1783–C1792. [PubMed: 9435481]
6. Theruvath TP, Zhong Z, Pediaditakis P, Ramshesh VK, Currin RT, Tikunov A, et al. Minocycline and N-methyl-4-isoleucine cyclosporin (NIM811) mitigate storage/reperfusion injury after rat liver transplantation through suppression of the mitochondrial permeability transition. *Hepatology*. 2008; 47:236–246. [PubMed: 18023036]
7. Kim JS, Wang JH, Lemasters JJ. Mitochondrial permeability transition in rat hepatocytes after anoxia/reoxygenation: role of Ca^{2+} -dependent mitochondrial formation of reactive oxygen species. *Am J Physiol Gastrointest Liver Physiol*. 2012; 302:G723–G731. [PubMed: 22241863]
8. Kehrer JP. The Haber–Weiss reaction and mechanisms of toxicity. *Toxicology*. 2000; 149:43–50. [PubMed: 10963860]
9. Kim JS, Jin Y, Lemasters JJ. Reactive oxygen species, but not Ca^{2+} overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia–reperfusion. *Am J Physiol Heart Circ Physiol*. 2006; 290:H2024–H2034. [PubMed: 16399872]
10. Winterbourn CC. Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol Lett*. 1995; 82–83:969–974.
11. Petrat F, de Groot H, Rauen U. Subcellular distribution of chelatable iron: a laser scanning microscopic study in isolated hepatocytes and liver endothelial cells. *Biochem J*. 2001; 356:61–69. [PubMed: 11336636]
12. 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; 117:101–108. [PubMed: 20584761]
13. 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; 272:C1286–C1294. [PubMed: 9142854]

14. 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; 48:1644–1654. [PubMed: 18846543]
15. Rauen U, Kerkweg U, Weisheit D, Petrat F, Sustmann R, de Groot H. Cold-induced apoptosis of hepatocytes: mitochondrial permeability transition triggered by nonmitochondrial chelatable iron. *Free Radic Biol Med*. 2003; 35:1664–1678. [PubMed: 14680689]
16. Richardson DR, Ponka P. The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. *Biochim Biophys Acta*. 1997; 1331:1–40. [PubMed: 9325434]
17. 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; 115:147–155. [PubMed: 1641848]
18. Herman B, Nieminen AL, Gores GJ, Lemasters JJ. Irreversible injury in anoxic hepatocytes precipitated by an abrupt increase in plasma membrane permeability. *FASEB J*. 1988; 2:146–151. [PubMed: 3342967]
19. Zahrebeliski G, Nieminen AL, al Ghouli K, Qian T, Herman B, Lemasters JJ. Progression of subcellular changes during chemical hypoxia to cultured rat hepatocytes: a laser scanning confocal microscopic study. *Hepatology*. 1995; 21:1361–1372. [PubMed: 7737643]
20. Lemasters JJ, Trollinger DR, Qian T, Cascio WE, Ohata H. Confocal imaging of Ca^{2+} , pH, electrical potential, and membrane permeability in single living cells. *Methods Enzymol*. 1999; 302:341–358. [PubMed: 12876784]
21. Zinchuk, V.; Zinchuk, O. *Current Protocols in Cell Biology*. Vol. Chap. 4. New York: Wiley; 2008. Quantitative colocalization analysis of confocal fluorescence microscopy images.
22. 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; 268:C1354–C1361. [PubMed: 7611353]
23. Liu ZD, Hider RC. Design of clinically useful iron(III)-selective chelators. *Med Res Rev*. 2002; 22:26–64. [PubMed: 11746175]
24. Anundi I, King J, Owen DA, Schneider H, Lemasters JJ, Thurman RG. Fructose prevents hypoxic cell death in liver. *Am J Physiol*. 1987; 253:G390–G396. [PubMed: 3631273]
25. 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; 250:6433–6438. [PubMed: 808543]
26. Lele S, Shah S, McCullough PA, Rajapurkar M. Serum catalytic iron as a novel biomarker of vascular injury in acute coronary syndromes. *EuroIntervention*. 2009; 5:336–342. [PubMed: 19736158]
27. Leoncini S, Rossi V, Signorini C, Tanganelli I, Comporti M, Ciccoli L. Oxidative stress, erythrocyte ageing and plasma non-protein-bound iron in diabetic patients. *Free Radic Res*. 2008; 42:716–724. [PubMed: 18668391]
28. Tavill AS, Qadri AM. Alcohol and iron. *Semin Liver Dis*. 2004; 24:317–325. [PubMed: 15349808]
29. Healing G, Gower J, Fuller B, Green C. Intracellular iron redistribution: an important determinant of reperfusion damage to rabbit kidneys. *Biochem Pharmacol*. 1990; 39:1239–1245. [PubMed: 2322308]
30. Voogd A, Sluiter W, van Eijk HG, Koster JF. Low molecular weight iron and the oxygen paradox in isolated rat hearts. *J Clin Invest*. 1992; 90:2050–2055. [PubMed: 1430227]
31. Kurz T, Gustafsson B, Brunk UT. Intralysosomal iron chelation protects against oxidative stress-induced cellular damage. *FEBS J*. 2006; 273:3106–3117. [PubMed: 16762036]
32. Kim JS, Nitta T, Mohuczy D, O'Malley KA, Moldawer LL, Dunn WA Jr, et al. Impaired autophagy: a mechanism of mitochondrial dysfunction in anoxic rat hepatocytes. *Hepatology*. 2008; 47:1725–1736. [PubMed: 18311843]
33. Nieminen AL, Dawson TL, Gores GJ, Kawanishi T, Herman B, Lemasters JJ. Protection by acidotic pH and fructose against lethal injury to rat hepatocytes from mitochondrial inhibitors, ionophores and oxidant chemicals. *Biochem Biophys Res Commun*. 1990; 167:600–606. [PubMed: 2322245]

34. 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; 455:992–996. [PubMed: 18794901]
35. 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; 115:1258–1266. [PubMed: 15849611]
36. Rauen U, Petrat F, Sustmann R, de Groot H. Iron-induced mitochondrial permeability transition in cultured hepatocytes. *J Hepatol*. 2004; 40:607–615. [PubMed: 15030976]
37. Imeryuz N, Tahan V, Sonsuz A, Eren F, Uraz S, Yuksel M, et al. Iron preloading aggravates nutritional steatohepatitis in rats by increasing apoptotic cell death. *J Hepatol*. 2007; 47:851–859. [PubMed: 17825453]
38. Petersen DR. Alcohol, iron-associated oxidative stress, and cancer. *Alcohol*. 2005; 35:243–249. [PubMed: 16054986]
39. Swanson CA. Iron intake and regulation: implications for iron deficiency and iron overload. *Alcohol*. 2003; 30:99–102. [PubMed: 12957292]
40. Ferdinandy P, Schulz R, Baxter GF. Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacol Rev*. 2007; 59:418–458. [PubMed: 18048761]
41. Ojo AO, Wolfe RA, Held PJ, Port FK, Schumouder RL. Delayed graft function: risk factors and implications for renal allograft survival. *Transplantation*. 1997; 63:968–974. [PubMed: 9112349]
42. Rehnkrone S. Molecular mechanisms for ischemic brain damage and aspects on protection. *Acta Neurochir Suppl (Wien)*. 1986; 36:125–128. [PubMed: 3467540]
43. Reichelt KL. The chemical basis for the intolerance of the brain to anoxia. *Acta Anaesthesiol Scand Suppl*. 1968; 29:35–46. [PubMed: 4233924]
44. Bronk SF, Gores GJ. Efflux of protons from acidic vesicles contributes to cytosolic acidification of hepatocytes during ATP depletion. *Hepatology*. 1991; 14:626–633. [PubMed: 1717357]
45. Kirichok Y, Krapivinsky G, Clapham DE. The mitochondrial calcium uniporter is a highly selective ion channel. *Nature*. 2004; 427:360–364. [PubMed: 14737170]
46. Pucheu S, Coudray C, Tresallet N, Favier A, de Leiris J. Effect of iron overload in the isolated ischemic and reperfused rat heart. *Cardiovasc Drugs Ther*. 1993; 7:701–711. [PubMed: 8241014]
47. Tanguy S, de Leiris J, Besse S, Boucher F. Ageing exacerbates the cardiotoxicity of hydrogen peroxide through the Fenton reaction in rats. *Mech Ageing Dev*. 2003; 124:229–235. [PubMed: 12633943]

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2013.05.004>.

Fig. 1. Inhibition of ischemia-induced quenching of cytosolic calcein by desferal and starch-desferal. Hepatocytes were coloaded with calcein-AM, TMRM, and PI and subjected to ischemia. In (A), confocal images were collected after 3–240 min. In (B) and (C), hepatocytes were preincubated with desferal or starch-desferal 1 h before ischemia. In (D) and (E), average calcein and mitochondrial TMRM fluorescence of individual hepatocytes after background subtraction was quantified at 3–240 min as the percentage of fluorescence after 3 min. * $p < 0.01$ vs other groups ($n = 3$ –5 hepatocytes from at least three independent experiments per group).

Fig. 2. Prevention of ischemia-induced quenching of mitochondrial calcein by desferal and starch-desferal. RhDex-labeled hepatocytes were loaded with calcein-AM by cold ester loading/warm incubation and subjected to ischemia. Hepatocytes were preincubated with (A) vehicle, (B) desferal, or (C) starch-desferal. In (D) and (E), average mitochondrial and lysosomal calcein fluorescence was determined. * $p < 0.01$ vs other groups ($n = 3-5$ hepatocytes from at least three independent experiments per group).

Fig. 3. Suppression of mitochondrial calcein quenching during ischemia by Ru360. In (A) and (B), hepatocytes were loaded with fluorophores as described for Figs. 1 and 2. Cells were treated with Ru360 1 h before ischemia.

Fig. 4.

Inhibition of mitochondrial ROS generation and cell death after reperfusion by desferal, starch–desferal, and Ru360. Hepatocytes were subjected to IR as described for Fig. 1 except in the absence of calcein. CmH₂-DCF-DA was added 30 min before and continuously after reperfusion. Hepatocytes were pretreated with (A) vehicle, (B) desferal, (C) starch–desferal, or (D) Ru360. Arrows, PI-labeled nuclei; 3 ×, fluorescence intensity increased by factor of 3.

Fig. 5. Protection by desferal, starch-desferal, and Ru360 against reperfusion injury. Hepatocytes were loaded with fluorophores and subjected to ischemia. During the last 15 min of ischemia, cells were reloaded with calcein-AM (0.5 μ M). Images were collected after reperfusion (A) without or (B–D) with pretreatment with (B) desferal, (C) starch-desferal, and (D) Ru360. Arrows, PI-labeled nuclei.

Fig. 6.

Protection by desferal, starch-desferal, and Ru360 against reactive oxygen species generation and cell killing after ischemia-reperfusion. Hepatocytes were subjected to IR. Some cells were pretreated with desferal, starch-desferal, or Ru360. In (A), cmH₂DCF-DA (10 μ M) was added 30 min before and continuously after reperfusion, and cmDCF formation was measured with a plate reader. In (B), hepatocytes were incubated with PI (30 μ M), and cell killing was measured with a plate reader. * $p < 0.01$ vs other groups.

Fig. 7.

Two-hit model of vulnerability to ischemia–reperfusion injury. During ischemia, ATP depletion inhibits V-ATPase of lysosomes/endosomes. Consequently, lysosomal pH increases, leading to Fe^{2+} release into the cytosol. Mitochondria then accumulate some this iron via MCU. Translocation of iron from lysosomes into mitochondria represents a first hit predisposing to injury. The second hit occurs after reperfusion when mitochondrial respiration leads to formation of H_2O_2 , which reacts with Fe^{2+} to form toxic $\text{OH}^\bullet$, leading to the MPT, bioenergetic failure, and cell death. In liver, fructose supports glycolytic ATP formation to sustain V-ATPase activity during anoxia. Desferal (Desf) and starch–desferal (St-Desf) chelate iron and prevent $\text{OH}^\bullet$ formation, with starch–desferal acting specifically within lysosomes. Ru360 blocks mitochondrial iron uptake via MCU. Thus, Ru360 suppresses iron-catalyzed Fenton chemistry in the mitochondrial matrix but not the cytosol.