Free Radical Biology and Medicine 63 (2013) 243–253

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

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

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ARTICLE INFO

Article history:
Received 8 March 2013
Received in revised form
22 April 2013
Accepted 1 May 2013
Available online 9 May 2013

Keywords:
Cell death
Iron
Ischemia
Lysosome
Mitochondria
Free radicals

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, chloromethyldichlorofluorescein, 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.

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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 ($O_2^{\bullet -}$), and hydroxyl radical ($OH^{\bullet}$). $OH^{\bullet}$, 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 $OH^{\bullet}$ 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

Abbreviations: ΔΨ, mitochondrial membrane potential; cmDCF, chloromethyldichlorofluorescein; cmH_2DCF-DA, chloromethyldihydrodichlorofluorescein diacetate; IR, ischemia–reperfusion; KRH, Krebs–Ringer–Hepes; LTR, LysoTracker Red; MCU, mitochondrial Ca^{2+} uniporter; MPT, mitochondrial permeability transition; $OH^{\bullet}$, hydroxyl radical; PI, propidium iodide; RhDex, rhodamine–dextran; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester; V-ATPase, vacuolar proton-pumping ATPase.

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.05.004

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), chloromethyldihydrodichlorofluorescein diacetate (cmH_2DCF-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 $NaHCO_3$, 10% fetal calf serum, 100 nM insulin, and 100 nM dexamethasone in 5% CO_2/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 μM 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 μM PI, and 300 μM 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 μM calcein-AM was added 15 min before the end of ischemia, or 10 μM cmH_2DCF-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 μM 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 μM calcein-AM in complete culture medium for 1 h at 4 °C and washed. Hepatocytes were then incubated overnight in culture medium in 5% CO_2/air.

Confocal microscopy

Green and red fluorescence was imaged with an inverted Zeiss LSM510 laser scanning confocal microscope using a 63 × 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 crosshair 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 ± 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

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