Non-transferrin-bound iron reaches mitochondria by a chelator-inaccessible mechanism: biological and clinical implications

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Submitted 7 February 2007; accepted in final form 26 July 2007

MITOCOHRIDNA PLAY a central metabolic role in cell iron processing, both in physiological and pathological conditions (24, 27), primarily in the synthesis of heme and FeS clusters (24, 33). The metal is acquired by cells via regulated receptor-mediated endocytosis of transferrin-iron complexes (2, 8, 14, 24). With the pathological appearance in plasma of nontransferrin labile iron (NTI) (6), there is a major ingress of iron into cells by unregulated routes (13), leading to mitochondrial iron accumulation and ensuing oxidative damage (8, 16).

At present, the physiological mechanisms of iron delivery to mitochondria have not been resolved. In reticulocytes, but not in nonerythroid cells, iron delivery from transferrin-iron-containing endosomes to mitochondria has been claimed to proceed rapidly and efficiently by direct interorganellar transfer (24, 41, 44). Mitochondrial transporters that have been implicated in iron import are members of the recently discovered mitochondrial carrier protein [MCP or SLC25 (26, 37)] family. The high-affinity carriers MRS3/4 were shown to mediate high-affinity iron import into yeast mitochondria (23), and their homolog mitoferrin (40), detected in zebrafish and mice, has been shown to be essential for heme synthesis in zebrafish embryos (40). In terms of mitochondrial iron export, the ATP-binding cassette (ABC) transporter ABCB7 has been implicated in export of FeS clusters from mitochondria to cytosol (32).

Because mitochondria are the major sites of cell iron metabolism, but also of reactive oxygen species (ROS) production, it is important to explore how these organelles cope with a rise in labile iron in various pathological states. This might be caused by 1) unregulated uptake of labile plasma iron found in systemic iron overload (13) or 2) faulty handling of iron due to mutations in mitochondrial iron-handling proteins such as frataxin in Friedreich ataxia (24, 27) or ABCB7 in X-linked sideroblastic anemia with ataxia (XLSA-A) (24, 32, 42). Iron-mediated mitochondrial damage is a common feature in all these pathologies, as exemplified by cardiomyopathy in hemosiderosis (16) and Friedreich ataxia (24, 27).

In this work, we explored the mechanism of iron acquisition by mitochondria of cardiac cells, using NTI as a pathological cell substrate. Murine H9c2 cells or rat primary cardiomyocyte cells (RPC) were used as a model for heart cells, with various NTI forms as iron sources and online fluorescence monitoring of labile iron for tracing the mobility of the metal from medium to cell cytosol and mitochondria (12). The results indicate that mitochondria rapidly acquire labile iron supplied to cells as NTI and that the cytosolic iron traffic to the organelle is not abolished by chelators of iron(II or III) targeted to the cytosol. This alleged traffic of iron to mitochondria in seemingly occluded chemical forms can be interpreted in terms of a vesicular transport mechanism as proposed for transepithelial iron delivery (19, 22), but more likely as a cytosolic chaperone-based mechanism as proposed for copper (11, 18).

MATERIALS AND METHODS

Cells and Cell Culture

Murine H9c2 cardiomyocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Biological Industries, Kibbutz Beit ha-Emek, Israel) supplemented with antibiotics (penicillin, streptomycin), glutamine, and 10% fetal calf serum (FCS). A day before experimentation, the cells were plated onto 96-well plates or onto microscopic slides attached to perforated tissue culture 3-cm plates and placed in culture conditions. RPC were obtained from the heart of newborn rats and handled as described previously (13). The animals used for the preparation of cardiomyocyte cells were bred and grown at the Hadassah Animal Facility in Ein Kerem, Jerusalem, and all the animal

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protocols used in this study were reviewed and approved by the Institute Review Board animal ethics committee (permit PE12123).

Chemicals

Acetoxymethyl esters (AM) of calcein green (CalG) and calcein blue (CalB) and their free acids, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA), and 5,5'-dimethyl-BAPTA (DMB) were purchased from Molecular Probes (Eugene, OR). Dihydrodiamine 123 (DHR123) was purchased from Biotium (Jerusalem, Israel). The mitochondrial metal sensor red rhodamine B-[[(1,10-phenanthroin-5-yl)-amino]carbonyl benzyl ester [RPA (29)] was a kind gift of U. Rauen (Institut für Physiologische Chemie, Universität Duisburg-Essen, Essen, Germany). All other materials were purchased as best available grade reagents.

The AM of (5'-methyl-[2,2']bipyridinyl-5-yl)-acetic acid (AM-AC-BIP), AC-BIP precursor, was synthesized as described in Fig. 1. 5-Bromomethyl-5'-methyl-2,2'-bipyridine (39) (bromo-BIP; Fig. 1, step a) was synthesized by 1) dissolving 5,5'-dimethyl-2,2'-bipyridine (2 g, 10.8 mmol), N-bromosuccinimide (2.8 g, 15.9 mmol), and benzoyl peroxide (2.6 g, 10.8 mmol) in carbon tetrachloride (100 ml) and refluxing for 20 min; 2) filtering the hot reaction mixture and washing with hot carbon tetrachloride solution; and 3) concentrating the crude material and purifying by flash chromatography with CHCl3-MeOH (1–3%; 12 mg, 34% yield): 1H-NMR (300 MHz, CDCl3) δ 3.03 (s, 2 H, CN-CH2-BIP), 7.6 (dd, J 8 H z ,1H ,4 H, 3.8 Hz, 1 H, 4-CH), 8.33 (d, J = 8 Hz, 1 H, 3-CH), 8.5 (s, 1 H, 5-CH), 8.58 (s, 1 H, 5'-CH).

5-Cyanomethyl-5'-methyl-2,2'-bipyridine (nitrito-BIP; Fig. 1, step b) was prepared by dissolving 200 mg (0.6 mmol) of bromo-BIP in a mixture of ethanol (10 ml) and concentrated hydrochloric acid (0.5 ml) and adding it dropwise to a refluxing solution of sodium cyanide (166 mg, 2.6 mmol) in methanol (10 ml) and refluxing for an additional 2 h and concentrated under reduced pressure. The brown-colored precipitate was dissolved in 30 ml of acetone and filtered out, and the filtrate was concentrated to obtain crude nitrite-substituted compound. The compound was further purified by flash column chromatography with CHCl3-MeOH (5–6%) (76 mg, 47% yield): 1H-NMR (300 MHz, CDCl3) δ 3.5 (s, 2 H, Br-CH2-BIP), 7.6 (dd, J = 8 Hz, 1 H, 3-CH), 7.7 (d, J = 8 Hz, 1 H, 4-CH), 8.26 (d, J = 8 Hz, 1 H, 4-CH), 8.33 (d, J = 8 Hz, 1 H, 3-CH), 8.5 (s, 1 H, 5-CH), 8.58 (s, 1 H, 5'-CH).

The ethyl ester of AC-BIP (Fig. 1, step c) was prepared by dissolving 70 mg (0.3 mmol) of nitrito-BIP in concentrated HCl (7 ml) and refluxing for 6–7 h; the mixture was concentrated, dissolved in ethanol (30 ml), and refluxed for 24 h, and the solvent was evaporated. The product was purified by flash chromatography using CHCl3-MeOH (4–6%) (30 mg, 35% yield): 1H-NMR (300 MHz, CDCl3) δ 1.2 (t, J = 7 Hz, 3 H, CH3-CH2-OOC-CH2-BIP), 2.4 (s, 3 H, 5'-CH3-BIP), 3.6 (s, 2 H, CH3-CH2-OOC-CH2-BIP), 4.2 (q, J = 7 Hz, 4 H, CH3-CH2-OOC-CH2-BIP), 7.6 (dd, J = 8 Hz, 1 H, 3-CH), 7.7 (d, J = 8 Hz, 1 H, 3'-CH), 8.23 (d, J = 8 Hz, 1 H, 4-CH), 8.31 (d, J = 8 Hz, 1 H, 4'-CH), 8.48 (s, 1 H, 5-CH), 8.55 (s, 1 H, 5'-CH).

Synthesis of the 5-AM of AC-BIP (Fig. 1, step d) was done by stirring ethyl ester-AC-BIP (30 mg, 0.09 mmol) in methanol (10 ml) with 1 N NaOH (1 ml) for 2 h and neutralizing it with 1 N HCl to obtain a white precipitate of the AC-BIP, which was dried and used for alkylation with bromomethyl acetate as described elsewhere (21). The crude monooxadode was dissolved in dry dimethylformamide (DMF; 2–3 ml) and basified with disopropylthethylamine (25 μl, 0.2 mmol). Bromomethyl acetate (34 mg, 0.8 mmol) was added, and the solution was stirred overnight under argon atmosphere. The solvent was evaporated under high vacuum. The product was purified by flash chromatography with CHCl3-MeOH (1–3%) (12 mg, 34% yield): 1H-NMR (300 MHz, CDCl3) δ 3.5 (s, 2 H, CH3-CO-O-CH2-O-CO-CH2-BIP), 7.6 (dd, J = 8 Hz, 1 H, 3-CH), 7.9 (d, J = 8 Hz, 1 H, 3'-CH), 8.3 (d, J = 8 Hz, 1 H, 4-CH), 8.35 (d, J = 8 Hz, 1 H, 4'-CH), 8.5 (s, 1 H, 5-CH), 8.65 (s, 1 H, 5'-CH); MS-ESI: 301.54 [M + H]+ 323.5 [M + Na]+.

Fluorescence Measurements

Iron ingress into mitochondria and cytosol of H9c2 or RPC was monitored by iron-evoked quenching of fluorescent metal sensors RPA [excitation wavelength (λex) 560 nm, emission wavelength (λem) 610 nm] (29) and CalG (λex 808 nm, λem 520 nm) (10, 13) or CalB (λex 390 nm, λem 430 nm) or by iron-evoked oxidation of the redox-sensitive mitochondrial or cellular probes DHR123 and CDCF-DA. These principles of monitoring iron ingress are described elsewhere (10, 12, 13). Briefly, fluorescence measurements were carried out either with a fluorescence plate reader (Tecan-Safire, Neotec, Männedorf, Austria) for simultaneous screening of multiple experimental conditions or by quantitative epifluorescence [Zeiss Axiovert 35 coupled to a Till Photonics system and PCO Sensicam charge-coupled device (CCD) camera or a Nikon microscope coupled to an Improvision-Velocity system (Improvision, Coventry, UK) coupled to a Hamamatsu Orca-Era CCD camera for high-resolution localization of the image processes]. Fluorescence measurements were carried out in HBS buffer (150 mM NaCl, 20 mM HEPES, pH 7.3), unless stated otherwise. When cells were labeled with CalB-AM or DCFDA, we supplemented the medium with 0.5 or 1 mM probenecid so as to prevent leakage of the anionic probes (12).

For intracellular metal sensor quenching, iron was added as either iron(III) [FeCl3-8-hydroxyquinoline 1:1 complexes (FHQ)] or iron(II) [from freshly prepared aqueous solution of ferrous ammonium sulfate (FAS)]. Iron ingress was stopped by adding the impermeant chelator diethylentriaminepentaacetic acid (DTPA, 50 μM), and identification of intracellular labile iron was obtained by reversing the calcium-Fe complexes by addition of the highly permeant chelator salicylaldehyde isonicotinyl hydrazone (SIH, 50 μM) (10).

For online monitoring of cellular or mitochondrial ROS formation, iron(III) (5 μM FHQ) and H2O2 (50 μM) were added to DCFDA-labeled (λex 488 nm, λem 520 nm) or DHR123-labeled (λex 488 nm, λem 520 nm) cells after acquisition of a steady baseline signal (12, 13).

Labeling Cells With Fluorescent Metal Sensors and Fluorogenic Redox Sensors

RPA loading into cells was done in DMEM-20 mM HEPES pH 7.3, in the presence of 200 or 400 μM DTPA (Sigma), to prevent quenching of the probe by contaminant iron from the medium (29). The loading medium did not contain phenol red. After 15-min loading at 37°C (0.1 μM RPA for plate reader and 1 μM RPA for fluorescence microscopy measurements), cells were washed with DMEM-HEPES.
and then incubated in either DMEM-HEPES alone or DMEM-HEPES containing calcein-AM (0.25 μM CalG-AM or 5 μM CalB-AM). CDCF-D A loading was carried out in HBS-10 mM glucose for 15 min at 37°C at 10 μM final concentration. CDCF oxidation was monitored in HBS-10 mM glucose-0.5 mM probenecid (Sigma). DHR123 (50 mM stock in DMSO) was administered to cells so as to attain 50 μM in HBS-10 mM glucose.

**Preloading of Chelators into Cell Cytosol**

Cells in DMEM-HEPES medium were incubated with 50 μM DMB-AM, or 25 μM bipyridyl derivative AM-AC-BIP (see Fig. 4A) for 10 min at 37°C. Desferrioxamine (DFO) treatment at 25 mM was done overnight in the regular sterilized DMEM growth medium, including 10% FCS.

**Quantification of Intracellular Chelator Concentration**

Quantification of total intracellular chelator concentration in H9c2 cells was done by growing cells to confluence in 75-cm² flasks, incubating them with chelators as described above, and washing and suspending them in 2–3 ml of HBS supplemented with n-octyl-β-glucopyranoside (final concentration 1%). For quantifying cellular AC-BIP, cell lysate was tested for its ability to recover the fluorescence of 0.5 μM CalG-Co (1:1) complexes in HBS. For quantifying cell DFO, similar tests were performed with 0.5 μM CalG-Fe (1:1) complexes. For quantification of BAPTA, the lysate was tested for its ability to inhibit quenching of 0.5 μM CalG by fixed concentrations of CoCl₂. For calibration we used lysates from untreated cells supplemented with either 0.5 μM CalG or the appropriate CalG-metal complexes and mixed with known concentrations of bipyridyl, DFO, or BAPTA.

**Monitoring of Chelator Access into Mitochondria of Permeabilized Cells**

H9c2 plates grown on microscopic dishes were treated with 1 mM succinyl-acetone (SA) for 3 h in DMEM-HEPES at 37°C, to induce mitochondrial iron accumulation. Cells were then washed, loaded with 1 μM CalG-AM for 10 min (37°C), and permeabilized in HBS buffer for 90 s with 25 μM digitonin. The permeabilized cells were washed with permeabilization buffer (100 mM KCl, 5 mM Na₂HPO₄, Eagle’s MEM-amino acids mix diluted 1:500, 10 mM HEPES, 1 μM CaCl₂, 1 mM MgSO₄, pH 7.2) and taken to fluorescence microscopy measurements in permeabilization buffer containing 1 mM succinate and 4 mM ATP. After the “zero time” snapshot, SIH dissolved in permeabilization buffer to 50 μM was added, and fluorescence recovery was monitored.

**Visualization of Iron Ingress to Mitochondria in Permeabilized Cells in Presence and Absence of Excess BAPTA**

H9c2 cells were labeled with 1 μM RPA and 0.1 μM CalG-AM as described above, permeabilized by digitonin, and taken to fluorescence microscopy measurements. DFO (1 μM) was present in all solutions during permeabilization and fluorescence measurements, to prevent RPA quenching by contaminant iron. After a 15-min baseline was recorded, iron was added as 10 μM FAS (with 1 mM ascorbate) or as 5 μM FHQ and RPA quenching was monitored for 20 min. In BAPTA-containing experimental systems, fluorescence measurements were done in the presence of 250 or 500 μM free BAPTA. Fluorescence measurements were done in the following buffer: 120 mM KCl, 5 mM Na₂HPO₄, 10 mM HEPES, 1 μM CaCl₂, 1 mM MgSO₄, pH 7.2.

**Comparing Iron Ingress to Mitochondria of Intact and Permeabilized Cells**

H9c2 cells were loaded with 0.5 μM RPA and 0.1 μM CalG-AM, permeabilized or not with digitonin (25 μM, 30 s, 37°C), and taken to measurements. Fluorescence measurements were carried out in HBS buffer for intact cells and in permeabilization buffer (in mM: 120 KCl, 5 Na₂HPO₄, 10 HEPES, 1 MgSO₄, and 2 succinate, pH 7.2, with 1 μM CaCl₂). Iron was added as FAS or ferric citrate, in the presence or absence of 2 mM ascorbic acid.

**Data Processing and Analysis**

The images acquired by microscopy were analyzed by the ImageJ program (National Institutes of Health) (1). Background was subtracted from 16-bit stacks, and fluorescence intensity values of cell areas were obtained by defining a single region of interest for each single cell in the field.

In plate reader experiments, we used three to eight replicate systems for each experimental set. The fluorescence intensity in each well was normalized to the zero time fluorescence intensity value, and the mean fluorescence values at any given time were calculated for each group of replicates. Where indicated, we determined the relative quenched fluorescence signal following 30-min exposure to an iron source (ΔQ, in relative units) and the first-order quenching rate constant (RC, in min⁻¹). The statistical significance of differences between treatments was determined by one-way ANOVA and Tukey’s test (Origin version 7.5, Originlab, Northampton, MA; at significance levels of 0.01 and 0.05).

**RESULTS**

**Localization of Fluorescent Metal Sensors in Cell Compartments**

The basic properties of the iron sources used as substrates and the fluorescent metal sensors and chelators applied for monitoring changes in labile iron levels in cell compartments are summarized in Table 1.

To assess the rate of labile iron ingress into cytosol and mitochondria, we labeled H9c2 or primary cardiomyocytes with the fluorescent phenanthroline-based mitochondrial metal sensor RPA (29) and/or with calcein-type cytosolic metal sensors (12, 13). CalG was generally used for detailed follow-up of iron ingress into cytosol with a fluorescence plate reader, whereas the more photostable CalB was used in similar studies using fluorescence microscopy imaging (12, 13). All of the metal sensors used here undergo fluorescence quenching on interaction with iron. Their stoichiometries of binding are RPA with iron(II) at 3:1, CalG with iron(II and III) at 1:1, and CalB with iron(II and III) at 3:1 (10, 13). Iron(II) oxidizes promptly to iron(III) on complexation to both calceins in an oxygen environment. As shown in Fig. 2, RPA accumulates in mitochondria, while the fluorescent CalG and CalB were generated in situ primarily by esterases (10), with >80% of the fluorescence associated with the cytosolic compartment (12). RPA is assumed (29) to be distributed across the inner mitochondrial membrane in a manner analogous to other potentiometric dyes of similar chemical features.

The patterns of cell and organellar labeling by metal sensors were similar in H9c2 and RPC (Fig. 2A vs. Fig. 2D), although in syncytium-forming RPC, the cell boundaries were not clearly delineated.

As revealed by fluorescence microscopy studies, both probes undergo swift intracellular quenching when cells are supplemented with 5 μM ferric iron (supplied as highly permeant FHQ). The degree of quenching after 10- or 20-min exposure to the metal provides a measure of the amount of metal reaching the respective compartment, the cytosol as both iron(II) and (III)
and the mitochondria as iron(II). Since the phenanthrolinonic RPA selectively binds iron(II), the iron(III) in FHQ must be reduced in the cell interior before or upon interaction with the probe (29). To ascertain that quenching resulted from the actual binding of the metal to the fluorescent sensor rather than from photochemical damage of the latter, we competed out the metal with a strong chelator added at relatively high concentrations also the mitochondria (revealed after cell permeabilization).

As shown in Fig. 2, both types of cells used in this study show similar patterns of labeling by metal sensors and sensitivity to extracellular addition of iron. However, time-dependent measurements of fluorescence in defined regions of interest could not be performed on RPC, because these cells are growing in beating aggregates or syncytia that preclude the maintenance of steady focus and precise delineation of individual cell boundaries. Therefore, in RPC, the major studies of iron ingress to cell compartments were followed by front-face fluorimetry in a fluorescence plate reader, whereas with H9c2 we used both fluorescence microscopy and fluorimetry.

**Monitoring the Access of Labile Iron to Cellular Compartments**

To carry out a comparative kinetic study of iron ingress into cytosol and mitochondria we followed the cell fluorescence intensity changes with a fluorescence plate reader that allowed high-throughput data acquisition of multiple samples in parallel. The fluorescence profiles of cardiomyocytes loaded with both CalG and RPA and exposed to different iron forms are depicted in Fig. 3 (H9c2 cells in Fig. 3, A and B; RPC in Fig. 3, C and D). Replicate samples of cells were exposed to permeant iron sources, FAS for iron(II) and FHQ for iron(III), in equivalent conditions.

As shown in Fig. 3, both cell types used in this study exhibited similar responses in terms of iron-induced metal sensor quenching and iron chelation. Although the two cell types showed quantitative differences in their responses, those can be attributed to intrinsic physiological properties as well as to physical factors related to surface/volume differences between cells.

Addition of the high-affinity permeant chelator at the end of the experiment provided a measure for the concentration of labile iron in a given compartment: SIH swiftly restored CalG...
fluorescence (Fig. 3, B and D) but restored RPA fluorescence at a relatively slow rate and only to a partial extent (Fig. 3, A and C). The small fraction of SIH-restorable CalG fluorescence in cells not exposed to iron (Fig. 3, B and D) corresponds to the basal labile iron pool (LIP) of cells, previously determined to be 0.8–1.2 μM in the resting state (8). Supplementation of iron(II) to H9c2 cells as FAS (5 and 10 μM) produced, after a 1.5- to 3.0-min lag period, a time-dependent drop in CalG fluorescence (Fig. 3B) that corresponded to a 2.5- and 5.5-fold increase in the labile pool within 20–30 min. On the other hand, the iron(II) quenching of RPA (Fig. 3A) in these cells was more robust and faster and occurred with no apparent lag. Supplementation of iron(III) as permeant complexes [2.5–5 μM iron(III) in the form of FHQ; Fig. 3, A and B] also led to fluorescence decreases in the cytosol and mitochondria, but with no apparent lag times. There was an equally fast quenching of both CalG and RPA by added FHQ, but the extent of CalG quenching by FHQ was relatively greater, possibly be-

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**Fig. 2. Intracellular localization of fluorescent metal sensors calcein blue (CalB) and rhodamine B-[(1,10-phenanthrolin-5-yl)aminocarbonyl]benzyl ester (RPA) in H9c2 and rat primary cardiomyocyte cells (RPC) and their susceptibility to iron.** H9c2 (A–A′, B1–B3, and C1–C3) and RPC (D1–D3) were labeled with both RPA and CalB, and the respective probes were examined microscopically with appropriate blue or red fluorescence settings. A–A′: H9c2 cells depicting CalB in cytosol (A), RPA in mitochondria (A′), and both merged (A′′). B1–B3: RPA in H9c2 cells before addition of iron (B1) and 10 min after addition of FHQ (B2), followed (after 10 min) by addition of the iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) (B3). C1–C3: same as B1–B3 but for CalB. D1–D3: merged images of RPC before and 20 min after addition of FeCl3-8-hydroxyquinoline complex (FHQ) (D1 and D2, respectively) followed by addition of SIH (D3).
cause of its relatively higher sensitivity for iron(III). Figure 3B shows that the extent of cytosolic CalG quenching by 5 μM FHQ in H9c2 cells is relatively larger than that by 5 μM FAS, whereas the opposite is true for mitochondrial RPA (Fig. 3A). Similar trends are observed for RPC (Fig. 3, C and D). We conclude that the ingress of iron into both cytosolic and mitochondrial compartments of both cell types is relatively fast, but the actual rate and extent of quenching of a specific probe vary with the type of iron source supplied. Nonetheless, the relatively rapid quenching of mitochondrial RPA fluores-

Fig. 3. Fluorescence quenching of calcine- and RPA-loaded H9c2 and RPC by permeant forms of iron as monitored with a fluorescence plate reader. Cells grown overnight to confluence in a 96-well plate were labeled with both calcine green (CalG) and RPA and monitored for fluorescence with time in a fluorescence plate reader using fluorescein settings for CalG and rhodamine for RPA. Iron was added as ferrous [ferrous ammonium sulfate (FAS), 5 and 10 μM] or ferric (FHQ, 2.5 or 5 μM) form at the indicated time (arrows) to H9c2 (A, B) or RPC (C, D). The impermeant chelator diethylenetriaminepentaacetic acid (DTPA, 50 μM) was added to chelate all extracellular iron after quenching reached a plateau, followed by the permeant chelator SIH (50 μM) for recovering the original cell fluorescence. Data shown are representative of 3 experiments with similar results, for each curve normalized fluorescence signals (F/F0) averaged from 8 replicate wells with SD <5%. Fluorescence graphs from cells that received no iron are marked as control. Fluorescence tracings from cells that received iron(II) are presented as dashed lines. ru, Relative units.

Fig. 4. Chelator access to labile iron in mitochondria and cytosol. A: fluorescence micrograph snapshot of CalG-AM-labeled mitochondria in H9c2 cells after succinyl-acetone treatment and digitonin permeabilization. B: fluorescence recovery in mitochondria after 20-min incubation with 50 μM SIH, as visualized by fluorescence microscopy. C: effect of chelators 5,5′-dimethyl-BAPTA (DMB), AC-BIP, and desferrioxamine (DFO) on succinyl-acetone-induced mitochondrial labile iron pool (LIP). Cells were loaded with the chelators (50 μM DMB for 10 min, 25 μM AC-BIP for 10 min, and 25 mM DFO overnight) and then subjected to succinyl-acetone treatment and digitonin permeabilization (A), followed by addition of SIH. Relative mitochondrial LIP levels are given in fluorescence units (f.u.). **Significant at P < 0.01. D: H9c2 cells were grown overnight to confluence in 96-well plates and treated with chelators (50 μM DMB for 10 min, 25 μM AC-BIP for 10 min, and 0.1 mM DFO overnight). Relative LIP levels (f.u.) were determined as described in Fig. 3A. **Significant at P < 0.01.
cence by added iron(II or III) indicates that, once in the cell interior, iron swiftly accesses mitochondria as iron(II).

We attributed the changes in mitochondrial fluorescence to incoming iron on the basis of the quantitative relationship between the two parameters. Although less plausible, there is also the theoretical possibility that incoming iron might induce, at least in part, a redistribution of resident mitochondrial iron without physically entering the organelles. Such an effect could occur by metal-evoked oxidative stress that in turn could lead to mitochondrial transition pore (MTP) opening and/or changes in mitochondrial membrane potential (ΔΨ) (see, e.g., Ref. 36). However, in studies carried out with H9c2 cells we observed that the presence of the MTP blocker cyclosporin A did not affect iron-evoked changes in mitochondria RPA, nor was the mitochondrial ΔΨ (measured with potentiometric dyes) affected by added iron (Shvartsman and Cabantchik, unpublished observations).

**Effect of Chelators in the Cytosol on Accumulation of Labile Iron(II and III) in Cytosol and Mitochondria**

**Intracellular loading of chelators.** Previous observations (12) indicated that iron entry into cells is in a form that is redox active and chelatable, i.e., labile. To assess whether the component of imported iron that reaches mitochondria is also labile, we established an experimental system whereby H9c2 cells or RPC were preloaded with chelators of various affinities for iron(II and III). We reasoned that if the access of iron to mitochondria is in labile forms it should be impeded by cytosol-loaded chelators. If, on the other hand, only the access to cytosol is impeded, it would mean that iron accesses mitochondria in forms that are shielded from chelators. Preliminary work indicated that the cytosolic presence of CalG, itself a relatively weak iron chelator, did not affect iron-evoked mitochondrial quenching of RPA (Shvartsman and Cabantchik, unpublished observations), even though it attains cytosolic concentrations in the range of 10–20 μM (10).

Cells were loaded with the following chelators: DMB (5, 15) for binding both iron(II) and iron (III), the bipyridyl derivative AC-BIP for iron(II), and the hydroxamate derivative DFO for iron(III) (see Table 1). Both DMB and AC-BIP were loaded rapidly, via their AM precursors, while DFO required prolonged (18 h) incubations. The intracellular concentrations attained following different loading procedures (determined as described in MATERIALS AND METHODS) were 2.5–3.0 mM for...
DMB, 10–12 mM for AC-BIP, and 0.3–0.5 mM for DFO. Because the aim of our chelator-loading procedure was to fill the cytosol with chelators while sparing the mitochondria, we assessed the intracellular chelator localization after loading (Fig. 4). For that purpose we measured the chelator-induced reduction of cytosolic and mitochondrial LIPs.

The capacity of chelators to bind mitochondrial labile iron was assessed by a method utilizing CalG loaded into mitochondria. CalG was used because RPA-iron complexes, unlike CalG-iron complexes, were found to be poorly reversible by excess chelators. The method is based on loading cells with excess CalG-AM followed by treatment with the detergent digitonin, which complexes with membrane cholesterol and selectively permeabilizes cell membranes according to their cholesterol content; plasma membrane > endoplasmic reticulum > mitochondria (25). This treatment revealed CalG accumulated principally in mitochondria. CalG-bound iron was detected by the increase in fluorescence after addition of SIH, providing a qualitative estimate of the mitochondrial LIP. To increase the mitochondrial LIP levels, cells were pretreated with SA, an inhibitor of heme synthesis that causes mitochondrial fluorogenic sensor DHR123.

**Ingress of iron into chelator-loaded cells.** The time-dependent follow-up of iron ingress into cells preloaded with DMB and labeled with both CalG and RPA is shown in Fig. 5. The results are depicted as normalized fluorescence traces (Fig. 5, A–C) and in terms of iron quenching parameters (Fig. 5, A′–C'); ΔQ is the maximum quenching fraction of initial fluorescence and RC is the quenching rate constant). Intracellular DMB demonstrably reduced the rate and extent of cytosolic CalG quenching by iron presented to cells as iron(II) and iron(III) (Fig. 5) but failed to prevent mitochondrial fluorescence quenching. Essentially identical results were obtained in H9c2 cells and in RPC (Fig. 5A vs. Fig. 5C).

A similar approach was used with the novel bipyridyl-iron(II) chelator AC-BIP, which was added to H9c2 cells as the permeant AM (Fig. 1) and generated intracellularly by in situ hydrolysis. As with DMB, AC-BIP markedly reduced the RC and ΔQ of cytosolic CalG by incoming iron (Fig. 6A) but had no statistically significant effect on mitochondrial RPA quenching (Fig. 6B). Similar results were obtained by fluoro-
cence microscopy experiments of RPA quenching in the presence and absence of AC-BIP (data not shown).

Thus, despite the access of AC-BIP to cytosolic and even mitochondrial LIPs, it failed to a large extent to prevent iron(II) delivery to mitochondria labeled with RPA, possibly because of the inability of AC-BIP to compete for iron binding with either a putative cytosolic iron carrier or mitochondrial RPA.

As revealed by fluorescence microscopy studies, the presence of >0.3 mM DFO in H9c2 cells significantly inhibited both cytosolic and mitochondrial fluorescence quenching by iron supplied as FHQ (Fig. 6, C and D), but not as FAS.

We assessed the possibility that in our experimental system agents such as CalG or BAPTA might actually donate their bound iron to a putative mitochondrial iron importer. The entry of iron(II) into mitochondria in the presence and absence of BAPTA was assayed with digitonin-permeabilized, RPA-loaded H9c2 cells. In this system, lacking a substantial portion of the cytosolic components, BAPTA (0.5 mM) caused marked inhibition of mitochondrial iron uptake. We conclude from this that if transfer of iron from BAPTA to mitochondrial RPA occurs in intact cells, its contribution to the total uptake is minor (Fig. 7).

Ingress of Iron into Mitochondria: Effect of Metal Valence and Anion

Because in aqueous solutions both iron(II) and iron(III) are mostly complexed to anions, it was important to assess the

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**Fig. 7.** Effect of BAPTA on the uptake of iron in the form of FAS into mitochondria of permeabilized H9c2 cells. H9c2 cells pre-loaded with RPA were permeabilized with digitonin and monitored by fluorescence microscopy. RPA quenching on addition of 10 μM FAS was monitored with time in the absence or presence of 500 μM BAPTA. Top: snapshots at 0 and 20 min after FAS addition in the absence (A) and presence (B) of 500 μM BAPTA. Bottom: kinetics of iron ingress into mitochondria, as visualized by fluorescence microscopy. □, No BAPTA; ■, 500 μM BAPTA. Plots represent an average of 5 or 6 cells, with SE.
extent to which the anions affect metal availability to mitochondrial uptake systems when offered to intact vs. permeabilized H9c2 cells. As shown in Fig. 8 we made the following observations. 1) The most permeant form of iron is iron(II), irrespective of the counterion (citrate or sulfate), provided ascorbate is present as a reducing agent. This was the case both in intact cells and in permeabilized cells (Fig. 8, A and C vs. B). 2) Iron(II) uptake into mitochondria in intact cells was relatively faster than in permeabilized cells, despite the presence of ascorbate in the medium, and, as seen before, relatively faster into mitochondria than into the cytosol of the same intact cells (Fig. 8, A and C). 3) On the other hand, although iron(III) ingress to mitochondria was markedly slower than that of iron(II) (Fig. 8B), it was still faster in intact vs. permeabilized cells, and faster into mitochondria than into the cytosol.

A possible interpretation of these results is a putative cytosolic facilitation of iron ingress into mitochondria. This holds provided mitochondria of permeabilized cells maintain their structural and functional integrity, which was in fact the case, as judged by the maintenance of their inner membrane potential (Shvartsman and Cabantchik, unpublished observations).

**DISCUSSION**

Although mammalian cells acquire iron primarily via the regulated transferrin-transferrin receptor-mediated mechanism, they can also take up NTI when it appears in the plasma of patients with systemic iron overload (6). NTI is apparently the major source of pathological tissue iron accumulation observed in transfusional hemosiderosis (6) affecting primarily cardiac and endocrine functions. The major part of organ damage has been associated with mitochondrial iron deposits. In inherited neuronal diseases like Friedreich ataxia (24, 27), XLSA-A

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**Fig. 8.** Permeant iron forms: effect of anions and iron valence. Intact H9c2 cells were loaded with CalG-AM and RPA as described in MATERIALS AND METHODS, and fluorescence was monitored by a fluorescence plate reader in HBS buffer at 37°C. In the case of permeabilized cells, H9c2 cells were loaded with RPA and CalG-AM, permeabilized with digitonin, and monitored in succinate-containing permeabilization buffer as described in MATERIALS AND METHODS. Iron was added at the moment indicated by arrow as iron salts [Fe(II)asc (A) or ferric citrate (B, C)] and, where indicated, 2 mM ascorbic acid (asc). Experiments (n = 3) were carried out in quadruplicate samples, and time data points of a representative experiment were averaged and depicted as means ± SD.

**Fig. 9.** Schematic model of possible routes of iron delivery to mitochondria. Two possibilities for non-transferrin-bound iron (NTI) entry into cells are presented. a: Uptake into cytosolic LIP via a ferrrireductase-coupled transporter. Subsequent import into mitochondria occurs either as labile metal (dashed arrow) or as a nonlabile ligand-iron complex. b: Uptake by nonspecific endocytosis. In this case, iron could reach mitochondria in a nonlabile form, via direct vesicular-mitochondrial contact. c: Transferrinic iron (Tf-Fe) could reach mitochondria by direct vesicle-mitochondrion apposition, as proposed by Zhang and colleagues (44). Irrespective of the mechanism of delivery, entry into mitochondria is mediated by the high-affinity transporter mitoferrin (MF). TfR, transferrin receptor; om, outer membrane; im, inner membrane; m, matrix.
(24), and neurodegeneration with brain iron accumulation (NBIA) (20), mitochondrial damage has also been linked to mitochondrial iron accumulation and the overburdening of cellular defense mechanisms. In all these conditions, the mitochondrial vulnerability to iron-mediated oxidative damage might reflect an inherently limited capacity of mitochondria to cope with massive influx of iron or, in the case of mutations, deficient processing of iron by the organellar biochemical machinery.

The chemical forms of iron that comprise the plasma NTI pool have not been defined in the different pathological conditions of iron overload, except for a component that is generally detected in plasma of iron-overloaded patients and is operationally defined as redox active, chelatable, and permeant to cells (6, 13). Previous studies indicated that NTI can transiently raise cell labile iron, but the distribution of iron into subcellular organelles has not been followed except in particular cases (13, 29). In this work we used cardiomyocytes in culture (from the cell line H9c2 and from isolated RPE) to trace the time-dependent rise in cytosolic and mitochondrial labile iron levels. It is noteworthy that the cumulative ingress of NTI into mitochondria is almost as rapid as into the cytosol, within the detection limits of our system. Unexpectedly, cytosolic chelators prevented iron access to the cytosol but not to the mitochondria. It is noteworthy that the cumulative ingress of NTI into mitochondria, as revealed in this study by in situ changes in mitochondrial RPA fluorescence, are robust (>80%), indicating a seemingly rapid and massive cytosolic-mitochondrial transfer system. On the other hand, with equivalent concentrations of the physiological transferrin-iron, changes in RPA fluorescence were minor (<5%) over an hour period and could not unequivocally be attributed to iron ingress into mitochondria (Shvartsman and Cabantchik, unpublished observations).

In that respect, it is interesting to note that in a recent study a quantitatively similar fluorescence change elicited in reticulocytes was attributed to direct endosomal-mitochondrial transorganellar delivery of iron derived from endocytosed transferrin (41). We hypothesize that mitochondria can acquire iron derived from NTI sources that in the cytosol might be “nonlabile” or partially solvent occluded. The role of such a hypothetical mechanism might be to provide cells with a “safe and efficient corridor” for expedient delivery of iron to mitochondria while evading exposure of transitory iron to cytosolic factors. The putative mechanism is analogous to the chaperone system proposed for the cytosolic transfer of copper to various acceptors residing in different cell compartments (11). Possible candidates for cytosolic traffic of iron are FeS cluster proteins such as IRP1 (iron responsive proteins) or ferritins that might carry a small fraction of labile and exchangeable iron. The operation of a “mediated” transfer of iron destined for mitochondria does not exclude the operation of other means of iron access to mitochondria, whether physiological or opportunistic. The “kiss and run” mechanism proposed for mitochondrial acquisition of transferrin-iron in erythroid cells and based on work done in reticulocytes (41, 44) was originally hypothesized to endow cells with a safe and efficient delivery of transferrin-derived iron from endosomes to mitochondria via transient appositions of the two organelles (41, 44).

This vesicle-borne delivery mechanism is assumed to be needed in reticulocytes (41, 44), and especially in erythroid cells, for swift and safe provision of large amounts of iron for hemoglobin synthesis. However, it remains to be identified in nonerythroid cells such as those used in the present study and also in erythroid cells. The various mechanisms of iron delivery to mitochondria are depicted schematically in Fig. 9. After entering the cell via a reductase-coupled transporter, or endocytic vesicle, the NTI can be released into the cytosolic LIP or intercepted by a cytosolic ligand that renders it nonlabile. The nonlabile form could be comprised of an iron complex of relatively high affinity [a siderophore/chelator (3), a metallochaperone (11, 18), or iron enclosed in vesicles (19, 41)]. The present results do not exclude the possibility that mitochondria can also import iron from the cytosolic LIP, possibly via the putative high-affinity mitochondrial membrane transporter mitoferrin (40). Since the form of iron that serves as a substrate for mitoferrin is unknown, it could conceivably be a high-affinity ligand complex, which releases the iron on entry into the mitochondria.

At present it is not clear whether mitochondrial iron utilization is the major factor that dictates the rate of iron uptake into these organelles. In physiological conditions, the balance between the regulated uptake of transferrin-iron and sequestration by cytosolic ferritin might be sufficient for averting potentially toxic labile iron from building up in mitochondria. However, under conditions of excessive iron supply, as it prevails in systemic iron overload, or during inefficient metal processing by mitochondria, as found in diseases of NBIA, there is a persistent rise in mitochondrial labile iron levels. The apparently limited ability of mitochondria to relieve themselves from labile iron accumulation results eventually in oxidative stress and ensuing damage.

**ACKNOWLEDGMENTS**

We thank Dr. U. Rauen and Prof. R. Sustmann (Essen, Germany) for the initial supply of RPA, Dr. W. Breuer (Jerusalem, Israel) for critical reading of the manuscript, and Prof. Paolo Arosio (Brescia, Italy) for sharing ideas and reagents.

**GRANTS**

This work was carried out in part at the C. E. Smith-Joel Elkes Institute of Psychobiology, Hebrew University, Jerusalem, Israel with the partial support of the Israel Science Foundation (ISF), the Association Française contre les Myopathies (AFM), and EEC Framework 6 (LSHM-CT-2006-037296 Euroiron1).