Assessment of mitochondrial membrane potential in proximal tubules after hypoxia-reoxygenation

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Feldkamp, Thorsten, Andreas Kribben, and Joel M. Weinberg. Assessment of mitochondrial membrane potential in proximal tubules after hypoxia-reoxygenation. Am J Physiol Renal Physiol 288: F1092–F1102, 2005. First published December 29, 2004; doi:10.1152/ajprenal.00443.2004.—Proximal tubules develop a severe energetic deficit during hypoxia-reoxygenation (H/R) that previous studies using fluorescent potentiometric probes have suggested is characterized by sustained, partial mitochondrial deenergization. To validate the primary occurrence of mitochondrial deenergization in the process, optimize approaches for estimating changes in mitochondrial membrane potential (∆Ψm) in the system, and clarify the mechanisms for the defect, we further investigated the behavior of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazocarboxyamine iodide (JC-1) in these cells and introduce a more dynamic and quantitative approach employing safranin O for use with the tubule system. Although use of JC-1 can be complicated by decreases in the plasma membrane potential that limit cellular uptake of JC-1 and such aggregates, fluorescence signal and the 595/535-nm ratio (12, 21, 27, 36, 37). Only the multimeric, red fluorescent form of JC-1 specifically measures membrane damage, generalized cell disruption, or sustained opening of the mitochondrial permeability transition pore. It is characterized during reoxygenation by impaired respiration utilizing substrates dependent on the function of complex I, persistence of mitochondrial matrix condensation (36, 37), and, based on measurements with fluorometric probes, partial recovery of mitochondrial membrane potential (∆Ψm). These changes can be substantially ameliorated and ATP recovery improved by providing succinate, which bypasses complex I, during reoxygenation (37). Although these characteristics suggest a central role for complex I dysfunction in the pathogenesis of the defect, recent studies have shown that complex I and electron transport in general are not limiting factors for mitochondrial function (12). Recovery of mitochondrial function can also be strongly enhanced via mechanisms separate from the provision of succinate by supplementing the tubules with citric acid metabolites separately and in combinations such as α-ketoglutarate (KG) plus aspartate or α-KG plus malate (α-KG/MAL) during either hypoxia or reoxygenation (36–38). These substrates appear to act in large part by virtue of their ability to support low-level but continuing anaerobic mitochondrial ATP production and energization that limit development of the lesion during hypoxia and promote recovery from it during reoxygenation (36, 37).

Mitochondrial energization assessed by either tetramethylrhodamine or 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazocarboxyamine iodide (JC-1) uptake was consistently decreased but not absent in affected tubules and was restored by the protective citric acid cycle metabolites (12, 36, 37). JC-1 appeared to be particularly useful for following changes in ∆Ψm during the model. JC-1 has the unique property among ∆Ψm-sensitive fluorophores of developing large shifts in its fluorescence signal at the levels of uptake induced by the high ∆Ψm, characteristic of energized mitochondria. This is due to the formation of red fluorescent (595 nm) J-aggregates of the molecule in the mitochondrial matrix. At the levels of JC-1 uptake seen during low ∆Ψm, the fluorophore remains as a green fluorescent (535 nm) monomer (9, 10, 15, 17). The reciprocal behavior at the two emission wavelengths, which can easily be measured simultaneously, results in large changes in the 595/535-nm ratio (12, 21, 27, 36, 37). Only the multimeric, red fluorescent form of JC-1 specifically measures ∆Ψm-dependent mitochondrial uptake of the probe (5, 10, 17). The green fluorescence of the monomeric form may be highly sensitive to its distribution in nonmitochondrial cell membranes (10, 21, 25). In our previous studies, the H/R-induced decreases in JC-1 uptake were evident in both the 595-nm signal and the 595/535-nm ratio (12, 36, 37).

Cellular entry of potentiometric fluorophores and other markers of ∆Ψm, like their mitochondrial uptake, can be...
plasma membrane potential ($\Delta \Psi_m$) dependent (11, 17, 22, 26), so that decreases in $\Delta \Psi_m$ as expected during ATP depletion due to lack of ATP for the Na$^+$-K$^+$-ATPase (30–32), could decrease mitochondrial concentration of the fluorophores independently of changes in $\Delta \Psi_m$ and exaggerate the differences between unprotected and substrate-protected tubules during reoxygenation. Although variability of uptake across the plasma membrane has been reported to not be a problem for JC-1 (24), the issue has not been extensively studied and behavior could vary among different cell types. In the original work describing JC-1 as a $\Delta \Psi_m$-sensitive agent in cells, the red J-aggregates were described as being reversible (27). This would allow the probe to provide readouts of dynamic changes in $\Delta \Psi_m$ and facilitate its use for further investigating the mechanisms of observed changes. The reversibility of aggregate formation in situ has been subsequently described, but in most reports it has not appeared to be complete (11, 15, 25), and there is evidence for $\Delta \Psi_m$-independent modification of J-aggregates by oxidants and pH changes (8, 11, 25).

Given the central role apparently played by changes in $\Delta \Psi_m$ in the energetic deficit after H/R and its importance to the involvement of mitochondria in a variety of other pathophysiological processes affecting tubules, including reactive oxygen species production (6, 18, 19) and as a determinant of susceptibility to opening of the permeability transition pore (5), we sought in the present studies to better define its behavior after H/R and the approaches to measuring it. We have clarified the characteristics of JC-1 in the system and developed for use with the tubules an alternative method based on safranin O uptake (1–3, 7, 14, 20, 29) that confirms the role of deenergization in the lesion and provides an approach to more dynamically follow the changes in $\Delta \Psi_m$ that occur and assess the mechanisms for them.

**MATERIALS AND METHODS**

**Materials**

Female New Zealand White rabbits (1.5–2.0 kg) were obtained from Harlan (Indianapolis, IN). Type I collagenase was from Worthington Biochemical (Lakewood, NJ). Percoll was purchased from Amersham Biosciences (Piscataway, NJ). HPLC-grade acetonitrile was from Fisher Scientific (Pittsburgh, PA). JC-1 was supplied by Molecular Probes (Eugene, OR). High-purity digitonin was purchased from Calbiochem (catalog no. 300411, San Diego, CA). All other reagents and chemicals were of the highest grade available from Sigma (St. Louis, MO). Aqueous stock solutions of experimental reagents were all pH adjusted so as not to alter the final pHs of the experimental medium. Regents that required solubilization in ethanol or dimethylsulfoxide were delivered from Jazz 1,000× stock solutions.

**Isolation of Tubules**

Proximal tubules were prepared from the kidney cortex of female New Zealand White rabbits by collagenase digestion and centrifugation on self-forming Percoll gradients as described (12, 34, 36–38). Incubation conditions were similar to our prior studies (12, 35–38). Tubes were suspended at 3.0–5.0 mg tube protein/ml in a 95% air-5% CO$_2$ gassed medium containing (in mM) 110 NaCl, 2.6 KCl, 25 NaHCO$_3$, 2.4 KH$_2$PO$_4$, 1.25 CaCl$_2$, 1.2 MgCl$_2$, 1.2 MgSO$_4$, 5 glucose, 4 sodium lactate, 0.3 alanine, 5 sodium butyrate, 2 glycine, and 0.5 mg/ml bovine gelatin (75 bloom; solution A). After 15- to 30-min preincubation at 37°C, tubules were resuspended in fresh medium with experimental agents and gassed with either 95% air-5% CO$_2$ (normoxic controls) or 95% N$_2$-5% CO$_2$ (hypoxia). Hypoxic tubules were kept at pH 6.9 to simulate tissue acidosis during ischemia in vivo (35). These incubation conditions result in near-anoxic conditions for the tubules, but it is not possible to confirm the presence of complete anoxia in the flasks, so we use the term “hypoxia” to describe it. After 60 min, samples were removed for analysis. The remaining tubules were pelleted and then resuspended in fresh 95% air-5% CO$_2$-gassed, pH 7.4 medium with experimental agents as needed. Sodium butyrate was replaced with 2 mM heptanoic acid and, to ensure availability of purine precursors for ATP resynthesis, 250 µM AMP was added at the start of reoxygenation (35, 37). After 60 min of reoxygenation, samples were removed again for analysis. When studied, α-KG/MAL was delivered from stock solutions of neutralized sodium salts of the two substrates to a final concentration of 4 mM each. Inclusion of glycine ensures that tubules do not develop lethal plasma membrane damage during either hypoxia or reoxygenation (35, 37). Details on variations of these protocols for individual experiments are provided with the data in RESULTS.

**Measurement of ATP Levels**

Samples of tubule cell suspension were immediately deproteinized in trichloroacetic acid, neutralized with trioctylamine/CFC 113, and stored at −20°C as previously described (35). Purine nucleotides and their metabolites in 20-µl aliquots of the neutralized extracts were separated and quantified using a reverse-phase ion-pairing, gradient HPLC method and factored for protein measured by the Lowry assay as previously described (12).

**JC-1 Fluorescence**

An aliquot of JC-1 from a frozen 2 mg/ml stock solution in DMSO was mixed 1:4 with calf serum, dispersed as an intermediate stock solution in phosphate-buffered saline, and then used at a final concentration of 9 µg/ml in the tubule suspension (21, 27).

**Measurements on unpermeabilized tubules.** At the end of the desired experimental period, JC-1 was added to the flask and the suspension was gassed with air-CO$_2$ and incubated in the dark for an additional 15 min at 37°C. Then, tubules were pelleted, washed three times in an ice-cold solution containing (in mM) 110 NaCl, 25 Na-HEPES, pH 7.2, 1.25 CaCl$_2$, 1.0 MgCl$_2$, 1.0 KH$_2$PO$_4$, 3.5 KCI, 5.0 glycine, and 5% polyethylene glycol (average molecular weight 8,000; solution B). Fluorescence was measured immediately on a 300-µl aliquot of the tubules containing 1.2–1.5 mg tubule cell solution protein brought up to 2.3 ml with additional ice-cold solution B and then scanned during continuous gentle stirring using a Photon Technology International (Monmouth Junction, NJ) Alphascan fluorometer at 488-nm excitation/500- to 625-nm emission collected using the right-angle mode of the fluorometer. After smoothing of the resulting curve, peak green fluorescence of the monomeric form of the dye was measured at 535 nm and peak red fluorescence of the J-aggregates was measured at 595 nm.

**Measurements on permeabilized tubules.** At the end of the desired experimental period, tubules were pelleted and resuspended in an intracellular buffer type solution containing 120 mM KCl, 1 mM KH$_2$PO$_4$, 2 mM EGTA, 100 µg digitonin/mg protein, and 10 mM K-HEPES, pH 7.2, at 37°C (solution C) supplemented with 4 mM potassium succinate and additional reagents as described for specific experiments. The flask was rescaled and incubated for an additional 15 min followed by processing and fluorescence measurements as described for unpermeabilized tubules. Digitonin permeabilization conditions for these studies were the same as used for earlier work with the tubules (12, 33, 36) that confirmed selective effects of the digitonin to increase plasma membrane permeability without impairing mitochondrial membrane function.
Measurements of total JC-1 uptake by extraction with DMSO. JC-1-loaded tubules from 300 μl of the suspension in solution A or solution C were pelleted and directly extracted into 2.0 ml of DMSO by mixing and then incubation at 4°C overnight. Fluorescence of the total extract volume was then measured as for unextracted tubules. The entire signal in the extracted tubules was at 535 nm, indicating complete dissolution of the JC-1 aggregates.

Viewing of cellular fluorescence. Aliquots (25 μl) of the suspension as used for the fluorometric measurements were placed between two coverslips and viewed on a Leica DM IRB fluorescence microscope (Leica, Bensheim, Germany) using a ×20 HC PL Fluotar lens and an I3 filter set consisting of a 450- to 490-nm band-pass excitation filter, a 510-nm dichroic mirror, and 520-nm-long pass emission filters. Images were captured using a SPOT Diagnostic Instruments RT Slider camera (Spot Diagnostics, Troy, MI).

Use of Safranin O to Assess ΔΨm

At the end of the experimental period, tubules were pelleted, washed three times in solution B, and then held in that solution on ice until use. For the safranin O uptake measurements, the tubules were resuspended at a final concentration of 0.1–0.15 mg/ml in solution C containing 5 μM safranin O and supplemented as needed for specific experiments with substrates and other experimental reagents that are described in RESULTS. Fluorescence was followed at 485-nm excitation, 586-nm emission using Photon Technology International (Lawrenceville, NJ) Deltascan and Alphascan fluorometers equipped with temperature-controlled, magnetically stirred cuvette holders as illustrated by the tracings shown in RESULTS. For studies done in control, normoxic tubules, all experiments used tubules from the same suspension, so variability from cuvette to cuvette was limited to pipetting differences and was under 1–2%. For studies comparing tubules subjected to different experimental conditions in separate flasks before sampling for safranin O, protein concentrations were targeted to be the same as for the normoxic control and were always within 10% of each other. For studies where changes in fluorescence between groups were compared, fluorescence changes were factored by protein.

The digitonin permeabilization conditions used for these studies were the same as in the JC-1 studies on permeabilized tubules (12, 33, 36). The digitonin permeabilization conditions used for these studies were the same as in the JC-1 studies on permeabilized tubules (12, 33, 36). The digitonin permeabilization conditions used for these studies were the same as in the JC-1 studies on permeabilized tubules (12, 33, 36). The digitonin permeabilization conditions used for these studies were the same as in the JC-1 studies on permeabilized tubules (12, 33, 36).

Statistics

Paired and unpaired t-tests were used as appropriate. Where experiments consisted of multiple groups, they were analyzed statistically by analysis of variance for repeated measures or independent group designs as needed. Individual group comparisons for the multigroup studies were then made using the Holm-Sidak test for multiple comparisons (SigmaStat 3, SPSS, Chicago, IL). P < 0.05 was considered to be statistically significant. Data shown are either means ± SE for no fewer than 3–5 experiments or are tracings representative of the behavior in that many experiments.

RESULTS

ATP Recovery and Mitochondrial Energization Measured with JC-1 After H/R

At the end of 60-min hypoxia and 60-min reoxygenation, ATP recovered to 15.6% of the normoxic control level with no extra substrate in the medium and to 54.3% of the control level when α-KG/MAL was present during reoxygenation (Fig. 1). The 595/535-nm emission ratio after JC-1 loading at the end of the H/R period was 33% of the normoxic control value in the tubules with no extra substrate during reoxygenation and increased to 69% in the tubules with α-KG/MAL (Fig. 1). The

Fig. 1. Effects of hypoxia-reoxygenation (H/R) on ATP and 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazocarbocyanine iodide (JC-1) uptake. Tubules were subjected to 60-min hypoxia followed by reoxygenation with either no extra substrates (NES) or 4 mM α-ketoglutarate plus malate (α-KG/MAL) present during only reoxygenation. Values are means ± SE for 6 experiments given as percentages of ATP levels and JC-1 595/535-nm emission ratios of tubules incubated continuously under oxygenated conditions for the same durations (normoxic controls). All values are significantly different from the normoxic control with P < 0.01, except for JC-1 ratio in the α-KG/MAL group, which was P < 0.05. The ATP level of the normoxic control group was 17.9 ± 1.4 nmol/mg protein, and its JC-1 ratio was 9.8 ± 0.6. *Significantly different from corresponding time point in NES group at P < 0.002 for ATP and P < 0.02 for JC-1.

Uptake of JC-1 by Unpermeabilized and Permeabilized Normoxic Tubules and After Treatment with Ouabain or FCCP

To assess the effect of ΔΨm on the use of JC-1 fluorescence to follow mitochondrial energization, Na+ -K+-ATPase was inhibited with ouabain, which decreases ΔΨm (32). In these studies, JC-1 uptake was measured both as usually done in unpermeabilized tubules and in tubules that were permeabilized with digitonin in an intracellular buffer during the JC-1 uptake period (Fig. 3). In untreated control tubules, the red (595 nm) signals from the high-ΔΨm-dependent aggregates of the fluorophore were the same in the unpermeabilized and permeabilized tubules. The green (535 nm) signal from the monomeric form of JC-1 that predominates at low ΔΨm was increased after digitonin permeabilization. This likely results from a change in fluorescence of the monomer due to altered membrane partitioning (10, 21, 25) rather than from an increase in the amount of monomer, because total JC-1 uptake measured in DMSO extracts of the whole cells was unaffected by permeabilization (Fig. 3). In the unpermeabilized tubules,
ouabain decreased the 595-nm signal by 37% compared with the untreated control tubules while minimally changing the 535-nm signal. In contrast, FCCP, which directly deenergizes the mitochondria, decreased the 595-nm signal by 97% and increased the 535-nm signal by 3.6-fold. Total JC-1 uptake measured in DMSO extracts was decreased 26% by ouabain and 42% by FCCP. Digitonin permeabilization during JC-1 uptake did not affect the 595-nm signal in the FCCP-treated tubules but significantly increased it in the ouabain-treated tubules to 84% of the control permeabilized tubules. The 535 nm signal was increased by digitonin permeabilization to similar degrees in control and ouabain-treated tubules, but was not affected in FCCP-treated tubules. Total JC-1 in DMSO extracts after ouabain was significantly increased by permeabilization to 83% of the control permeabilized tubules, but was not changed by permeabilization after FCCP.

Uptake of JC-1 by Unpermeabilized and Permeabilized Tubules After H/R

Given the evidence in the Fig. 3 studies with ouabain for at least some dependence of JC-1 uptake on ΔΨm, additional experiments were done to test JC-1 uptake by permeabilized tubules after H/R. Figures 4 and 5 summarize measurements of JC-1 fluorescence at the end of normoxic control incubation and after 60-min hypoxia followed by 60-min reoxygenation with either no extra substrate or 4 mM α-KG/MAL and compare uptake of JC-1 by permeabilized and unpermeabilized tubules and in DMSO extracts. After unprotected H/R, unpermeabilized tubules showed decreases in the 595-nm signal and increases in the 535-nm signal (Fig. 4) and a small decrease in total JC-1 in DMSO extracts (Fig. 5) relative to normoxic controls. These changes were prevented by supplementation with α-KG/MAL during reoxygenation. In contrast to ouabain-treated tubules (Fig. 3), permeabilization after H/R did not increase the 595-nm signal in the unprotected H/R tubules (Fig. 4). The 535-nm signals from the monomeric form of JC-1 showed parallel behavior during the experimental maneuvers under both unpermeabilized and permeabilized loading conditions, with a higher green signal in the unprotected tubules (Fig. 4).

Incomplete Reversal of JC-1 Aggregate Formation During Deenergization

To test the extent to which JC-1 aggregates formed in the tubules can dissociate if subsequent deenergization occurs, tubules were loaded with JC-1 and then treated with FCCP for 60 min. As shown in Fig. 6, these tubules still retained 52% of the red fluorescence signal of untreated tubules incubated for the same period after JC-1 loading and increased their green fluorescence to only 43% of the level seen in tubules that were preincubated with FCCP before JC-1 loading.

Tubule Safranin O Uptake Energized by Respiratory Substrates

After addition of tubules to intracellular buffer solution C, safranin O uptake indicated by a decrease in fluorescence began after a lag of ~60 s and was maximal at 600 s (Fig. 7A). Uptake was substrate dependent. In the absence of an exogenous substrate, a transient period of incomplete safranin O uptake was followed by release. Unlike the behavior of JC-1, safranin O was rapidly and completely released when mitochondria were deenergized with FCCP. The decreased fluorescence of safranin O resulting from quenching of safranin O after uptake by energized mitochondria means that low fluorescence corresponds to high ΔΨm. To make it easier to follow the tracings relative to high and low ΔΨm, the scale can be inverted (Fig. 7B). Subsequent safranin O figures are presented using the inverted format. The very rapid release of safranin O after deenergization with FCCP indicates that safranin O can
equilibrate very rapidly between the mitochondria and the medium in this system and contrasts to the initial lag and slow rate of safranin O uptake. Factors that can account for the initial slow safranin O uptake are the time taken for digitonin to fully permeabilize all tubules and the need for the cold tubules to be rewarmed in solution C. Figure 8 shows that preincubating the tubules for 10 min with digitonin and substrate in solution C at 37°C before the safranin uptake period eliminated the initial lag phase and greatly accelerated the initial rate. However, preincubation could obscure or change elements of tubule behavior after injury conditions and could complicate experimental manipulations, so we elected not to use it routinely.

**Tubule Safranin O Uptake Energized by Hydrolysis of ATP by the F1F0-ATPase**

In the absence of electron transport, mitochondrial energization can be supported by reverse operation of the F1F0-ATPase if ATP is available (10, 16, 23, 28). Figure 9 shows the results of studies using safranin O to follow this process in permeabilized tubules. Tubules were initially energized with α-KG/MAL as a substrate. They rapidly deenergized when the complex I inhibitor rotenone was added. Following rotenone with 2.0 mM ATP then produced strong reenergization. As was seen in the Fig. 7 study, tubules in the absence of a substrate displayed only incomplete and transient energization compared with the tubules with α-KG/MAL present during safranin O uptake. That this transient energization without added substrate is due to electron transport using residual endogenous substrate is shown by the additional study here demonstrating that it was completely blocked by rotenone. Inclusion of 2.0 mM ATP in the medium of the no-substrate/rotenone tubules resulted in energization. The necessary involvement of the F1F0-ATPase and of the adenine nucleotide translocase in ATP-driven energization is demonstrated by the Fig. 10 experiments showing essentially complete inhibition of the ATP effects by oligomy-
cin, an inhibitor of the F1F0-ATPase (16), and atractyloside, an inhibitor of the adenine nucleotide translocase (16).

Effects of H/R on Substrate- and ATP-Supported Safranin O Uptake

Figure 11 illustrates typical safranin O uptake tracings measured after 60-min hypoxia followed by 60-min reoxygenation. Figure 12 summarizes the results of a group of these experiments along with the behavior of ATP and JC-1 sampled at the end of reoxygenation. In the measurements using safranin O to follow energization (Fig. 11), safranin O uptake was first supported by \( \text{H}_9\text{Kg}/\text{Mal} \). Then, rotenone was added to deenergize the tubules, followed by 2.0 mM ATP to reenergize them, then succinate to test for any further energization and, finally, FCCP. During the periods of \( \text{H}_9\text{Kg}/\text{Mal} \) and ATP-supported energization, the normoxic tubules behaved similarly to the corresponding experiments in Figs. 9 and 10 in that ATP strongly reenergized the tubules after rotenone. As shown by the group data (Fig. 12), safranin O uptake after ATP reached a level only slightly, but significantly, lower than that reached during the initial uptake period supported by \( \text{H}_9\text{Kg}/\text{Mal} \). This lesser recovery of energization with ATP after rotenone was not, however, due to any deterioration of the preparation during the assay because subsequent addition of succinate led to further safranin O uptake that significantly exceeded the initial uptake supported by \( \text{H}_9\text{Kg}/\text{Mal} \).

Changes in ATP and JC-1 uptake after H/R were similar to those seen in the Figs. 1, 4, and 5 experiments, showing poor recovery of ATP and decreased 595/535-nm ratios and protein-factored, absolute 595-nm JC-1 signals after unprotected H/R with no extra substrates. These parameters were significantly
improved when α-KG/MAL was present during the 60 min of reoxygenation (Fig. 12). Despite the presence of α-KG/MAL during the initial phase of the safranin O protocol, uptake of safranin O by tubules at the end of unprotected H/R was strongly inhibited (Figs. 11 and 12). Addition of 10 μM cytochrome c did not significantly ameliorate this inhibition (data not shown). ATP-supported safranin O uptake by these tubules was also inhibited. Subsequent addition of succinate, however, substantially improved uptake, although it did not normalize it. Tubules that had been protected by α-KG/MAL during reoxygenation had substantially better energization than the unprotected tubules during the α-KG/MAL and ATP-supported safranin O uptake periods but were not different from the unprotected tubules in the presence of succinate+ATP.

The stronger energization during the final succinate period of the Fig. 11 and 12 experiments in both the normoxic control and the H/R tubules could derive from a primary benefit of succinate, or a combined effect of succinate+ATP. It could also have been influenced by the 25-min prior exposure of the permeabilized tubules to high levels of α-KG/MAL and ATP during the safranin O uptake period before addition of the succinate. To distinguish among these possibilities, safranin O uptake by normoxic control and unprotected H/R tubules was compared using either α-KG/MAL or succinate as the substrate, with and without supplemental ATP present from the start of permeabilization (Fig. 13). In both the normoxic control and H/R tubules, safranin O uptake with succinate alone was better than with α-KG/MAL alone. Both substrates in combination with ATP, however, supported the same amount of uptake.

DISCUSSION

Mitochondrial uptake of cationic dyes like JC-1 that accumulate in the matrix as a function of ΔΨm is also dependent on initial uptake of the probe across the plasma membrane, which can be affected by its potential (11, 17, 22). In ouabain-treated tubules, permeabilization with digitonin during JC-1 uptake increased formation of high ΔΨm-dependent red aggregates in the mitochondria and total JC-1 uptake measured in whole cell DMSO extracts (Fig. 3). This provides evidence for a ΔΨp-dependent component of JC-1 uptake in these cells. Under the conditions used, ouabain effectively inhibits Na+-K+-ATPase, as indicated by measurements of cell Na+ and K+ (32) and thereby decreases ΔΨp, while maintaining ATP levels (Ref. 32 and current data).

Protonophoric uncouplers such as FCCP collapse ΔΨm and deplete almost all cell ATP (Ref. 31 and current data). JC-1 uptake after FCCP was not affected by digitonin permeabilization. Since mitochondria are the only significant source of ATP production in the tubules (4, 39) and ΔΨp is dependent on ATP to support activity of Na+-K+-ATPase (31), severe mitochondrial deenergization will collapse the potential across both membranes. The FCCP studies (Fig. 3), therefore, provide a very useful estimate of the extent to which ΔΨp, as opposed to ΔΨm limits JC-1 uptake. Despite maximal collapse of both ΔΨm (595-nm signal 3% of untreated control tubules) and ΔΨp, FCCP-treated tubules still had 58% of the total JC-1 uptake of untreated controls, as measured in whole cell DMSO extracts. JC-1, however, was not concentrated by the mitochondria and remained entirely in the monomeric form.

JC-1 uptake after H/R was not increased by permeabilizing the tubules to bypass the plasma membrane (Fig. 5). Moreover, the 25% decrease in formation of red aggregates after H/R occurred despite a much smaller 10% decrease of total JC-1 uptake (Fig. 5). These data indicate that the decreased 595-nm signal (Figs. 4 and 5) and 595/535-nm ratios (Fig. 1) in unprotected tubules at the end of H/R reflect decreased ΔΨm and are not affected by ATP depletion-induced differences in ΔΨp.

Our studies also addressed the reversibility of the JC-1 aggregates during mitochondrial deenergization. Although these aggregates have been reported to dissociate back to monomers in situ (11, 15, 25, 27), we had observed that tubules left in the cold overnight without any special efforts to preserve them still retained virtually all their red signal (not shown). The experiments testing FCCP treatment after JC-1 uptake (Fig. 6)
confirm some dissociation of aggregates during deenergization at 37°C but indicate that this process is slow and incomplete.

Thus the present observations validate the use of JC-1 as an easily visualized end-point marker reflecting mitochondrial deenergization in the tubules after H/R and demonstrate that plasma membrane uptake of JC-1 is not significantly limiting for the mitochondrial signal in that setting. However, they indicate that JC-1 is not suitable for monitoring processes that require dissociation of the red aggregates once formed. These findings led us to investigate safranin O as an alternative to both better quantify the changes of energization and to allow a more dynamic assessment of the determinants of these changes.

Safranin O has been previously used to a limited extent with permeabilized (1, 14) and unpermeabilized (2, 13, 40) whole cells. Use of whole cells rather than isolated mitochondria avoids the problems of selection of subpopulations and further damage to already injured organelles that can occur during the isolation and requires much less cell material. We have previously defined conditions under which digitonin selectively permeabilizes the plasma membrane without impairing mitochondrial function in the preparation and used them to assess mitochondrial calcium uptake capacity (33), respiration (36), and intracellular compartmentation of metabolites (12). The same approaches proved to be applicable for use with safranin O in the present studies. Although initial safranin O uptake was relatively slow, once warming and permeabilization for the tubules was complete (Fig. 8) safranin O movements were very rapid and, unlike JC-1, could be used to follow repeated cycles of energization, deenergization, and reenergization (Fig. 11). The tubules were very stable during 30 min of safranin O fluorescence measurements in the longest multistep protocols used (Fig. 11). Importantly, normoxic control tubules could be held for 3–5 h in the cold after the end of the warm-flask incubation period, with highly reproducible behavior for the safranin O measurements. H/R tubules were never studied after such long delays but were easily usable for 1–2 h after the end of the experimental periods. The method was highly sensitive, requiring only 0.10–0.15 mg·ml tubule protein−1·ml intracellular reaction buffer−1 during the safranin O uptake period. Safranin O uptake by normoxic controls per milligram protein was so reproducible that the initial calibration done each day to confirm the amount of tubule material being used was in the linear range for safranin O uptake predicted the final tubule protein concentration as well as the Lowry protein assay that was done subsequently (data not shown). Safranin O uptake by isolated mitochondria is well documented to be a linear function of $\frac{m}{\Delta V_m}$ (3, 7, 14). Using valinomycin to induce $K^+$ diffusion potentials, we confirmed this to be true for the permeabilized tubules (data not shown). However, $K^+$ diffusion calibrations were not available for every experiment, so data are reported in terms of safranin uptake only.

Based on the incomplete and transient safranin O uptake seen in the absence of added substrate (Figs. 7 and 9), support of energization by endogenous substrate pools under the digitonin permeabilization conditions of these studies was minimal. Energization of normoxic control tubules was well sup-

![Fig. 10. Effects of oligomycin and atractyloside on ATP-supported safranin O uptake. Safranin O uptake was initially supported by 4 mM α-KG/MAL. Rotenone (5 μM), ATP (2 mM), oligomycin (15 μM), and atractyloside (500 μM) were added at the indicated points.](image)

![Fig. 11. Substrate and ATP-supported safranin O uptake after H/R. Tubules were subjected to 60 min of hypoxia followed by either 60 min of unprotected reoxygenation with no extra substrate or protected reoxygenation with 4 mM α-KG/MAL. Then, safranin O uptake was measured starting with 4 mM α-KG/MAL as the substrate, followed at the indicated points by rotenone (5 μM), then ATP (2 mM), then succinate (4 mM), and then FCCP (5 μM).](image)
ported by either complex I (α-KG/MAL) (Figs. 9–13) or complex II (succinate) (Figs. 7, 8, 11–13)-dependent substrates, and, when electron transport was inhibited, by ATP via reversal of the F1F0-ATPase (Fig. 10). Although the differences were small, succinate energized more strongly than α-KG/MAL, which was slightly stronger than ATP (Figs. 12 and 13).

Similar to the JC-1 results (Figs. 1, 2, 4, 5, 12), energization of unprotected H/R tubules measured with safranin O (Figs. 11 and 12) was decreased relative to normoxic control and protected H/R tubules. Decreases of safranin O uptake were irrespective of whether energization was supported by α-KG/MAL, succinate, or ATP. Thus neither optimal substrate availability to the mitochondria nor by the F1F0-ATPase hydrolysis of ATP corrects the failure of energization. We have previously shown that the electron transport chain itself is not limiting in the unprotected H/R tubules (12). Taken together, these results indicate that other processes, which could include decreased substrate and ATP delivery into the mitochondria, inhibition of the F1F0-ATPase, and uncoupling, contribute to the failure of energization after H/R. The safranin O system should provide a powerful approach for future studies to distinguish among these possibilities.

As is in our earlier work, supplementation of the tubule suspensions during reoxygenation with α-KG/MAL strongly promoted recovery of cell ATP and mitochondrial energization. In our previous studies, we have also described beneficial effects of other citric acid cycle metabolites during reoxygenation, including succinate (37). In the present studies, neither the α-KG/MAL nor the succinate used at high concentration to support safranin O uptake by the permeabilized tubules corrected the defect of energization during those measurements. This indicates that the processes set in motion by these metabolites that promote recovery in the intact cells take longer than the 15 min during which the safranin O uptake measurements are done or require additional events that can occur in the intact cells but not after permeabilization. The time element would be consistent with our previous observation that progressive recovery of the tubules can occur as the duration of reoxygenation is extended from 1 to 2 h (37). The present studies suggest that a factor in addition to time of recovery is involved because the combination of succinate+ATP delivered during
the safranin uptake protocol at the end of reoxygenation was just as effective for restoring energization as α-KG/MAL during the 60 min of reoxygenation (Fig. 12). It is possible that the addition of ATP to the permeabilized tubules better reproduces conditions during reoxygenation in the intact cells where ATP increases over time, even in the unprotected tubules (37). The effect of ATP to improve energization is likely due to a combination of factors. It could act by directly supporting energization via hydrolysis by the F1FO-ATPase (Figs. 9 and 10), but this is ultimately a futile and damaging process within the cell because it serves to consume ATP (37). True sustained benefit would result from the promotion by ATP of repair processes. We have provided evidence that anaerobic substrate-level ATP production in the citric acid cycle promoted by α-KG/MAL is a primary mechanism for its protective effects when provided during hypoxia and plays a critical role to initiate protection when it is present during reoxygenation (36).

The current studies also bear on another fundamental issue relevant to understanding the energetic deficit in the tubules after H/R. Our initial experiments suggested that complex I dysfunction played a major role (36, 37), but this conclusion was not supported by further work more directly measuring complex I function and the redox state of cellular NADH (H) (12). In the present studies, succinate produced more energization than α-KG/MAL, but the difference was relatively small and was similar in normoxic control tubules and after H/R (Fig. 13), thus supporting the more recent observations that complex I function is not limiting for the H/R tubules (12). True sustained benefit would result from the promotion by ATP of repair processes. We have provided evidence that anaerobic substrate-level ATP production in the citric acid cycle promoted by α-KG/MAL is a primary mechanism for its protective effects when provided during hypoxia and plays a critical role to initiate protection when it is present during reoxygenation (36).

In summary, the studies in this paper have clarified the basis for the mitochondrial deenergization observed after H/R in proximal tubules and have optimized the approaches to measuring it. Use of JC-1 as an index of $\Delta \Psi_m$ can be complicated by decreases in $\Delta \Psi_p$, that limit cellular uptake of JC-1, but this factor does not contribute to the behavior of JC-1 after H/R. Decreases in JC-1 red fluorescent aggregate formation and red/green fluorescence in the tubules after H/R result entirely from mitochondrial deenergization. The red JC-1 aggregates do not readily dissociate in situ, making the probe unsuitable for dynamic studies of energization. Safranin O uptake by digitonin-permeabilized tubules, as introduced in this work, provides a more versatile, dynamic, and quantitative measurement of changes in $\Delta \Psi_m$ that requires very small amounts of tubules and allows assessment of the tubules for prolonged periods after the end of the experimental maneuvers. The safranin O studies indicate that both substrates and ATP-supported mitochondrial energization are impared in unprotected tubules after H/R, but combining substrates and ATP can substantially restore $\Delta \Psi_m$. The safranin method will allow informative further studies to better define the mechanisms for these changes.

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