Preservation of complex I function during hypoxia-reoxygenation-induced mitochondrial injury in proximal tubules

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Feldkamp, Thorsten, Andreas Kribben, Nancy F. Roeser, Ruth A. Senter, Sarah Kenner, Manjeri A. Venkatachalam, Itzhak Nissim, and Joel M. Weinberg. Preservation of complex I function during hypoxia-reoxygenation-induced mitochondrial injury in proximal tubules. Am J Physiol Renal Physiol 286: F749–F759, 2004. First published December 9, 2003; 10.1152/ajprenal.00276.2003.—Inhibition of complex I has been considered to be an important contributor to mitochondrial dysfunction in tissues subjected to ischemia-reperfusion. We have investigated the role of complex I in a severe energetic deficit that develops in kidney proximal tubules subjected to hypoxia-reoxygenation and is strongly ameliorated by supplementation with specific citric acid cycle metabolites, including succinate and the combination of α-ketoglutarate plus malate. NADH:ubiquinone reductase activity in the tubules was decreased by only 26% during 60-min hypoxia and did not change further during 60-min reoxygenation. During titration of complex I activity with rotenone, progressive reduction of NAD+ to NADH was detected at >20% complex I inhibition, but substantial decreases in ATP levels and mitochondrial membrane potential did not occur until >70% inhibition. NAD+ was reduced to NADH during hypoxia, but the NADH formed was fully reoxidized during reoxygenation, consistent with the conclusion that complex I function was not limiting for recovery. Extensive degradation of cytosolic and mitochondrial NAD(H) pools occurred during either hypoxia or severe electron transport inhibition by rotenone, with patterns of metabolite accumulation consistent with catabolism by both NAD+ glycohydrolase and pyrophosphatase. This degradation was strongly blocked by α-ketoglutarate plus malate. The data demonstrate surprisingly little sensitivity of these cells to inhibition of complex I and high levels of resistance to development of complex I dysfunction during hypoxia-reoxygenation and indicate that events upstream of complex I are important for the energetic deficit. The work provides new insight into fundamental aspects of mitochondrial pathophysiology in proximal tubules during acute renal failure.

Acute renal failure; citric acid cycle; NADH; ATP

Kidney proximal tubules in vivo have minimal glycolytic capacity, making them dependent on mitochondrial metabolism for ATP synthesis (1, 48). Freshly isolated proximal tubules subjected to hypoxia-reoxygenation (H/R) develop a profound mitochondrial functional deficit (44, 45) that limits restoration of cell ATP levels during reoxygenation and, thereby, plays a pivotal role in overall cellular recovery (46). The deficit is not due to lethal plasma membrane damage, generalized cell disruption, or sustained opening of the permeability transition pore and is characterized during reoxygenation by partial recovery of mitochondrial membrane potential (ΔΨm), impaired respiration utilizing substrates dependent on the function of electron transport complex I, and persistence of mitochondrial matrix condensation (44, 45). The lesion can be substantially ameliorated by providing succinate, which bypasses complex I, during reoxygenation (45). Taken together, these characteristics suggest a central role for complex I dysfunction in its pathogenesis. Recovery of mitochondrial function can also be strongly enhanced via mechanisms separate from provision of succinate by supplementing the tubules with α-ketoglutarate plus aspartate or α-ketoglutarate plus malate (α-KG/MAL) during either hypoxia or reoxygenation (44–46). 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 (44, 45).

Complex I abnormalities have frequently been implicated in the mitochondrial dysfunction that contributes to organ damage during ischemia-reperfusion (9, 34, 39). In part, this may reflect the sensitivity of complex I to oxidant insults during reoxygenation (19, 28, 32, 49), but substantial inhibition has been reported during complete ischemia without any reperfusion (34). Inhibition of complex I can contribute to cell injury by both limiting electron transport required for oxidative phosphorylation and increasing production of reactive oxygen species (19). Moreover, in ischemic heart NAD+ is extensively degraded to nicotinamide and ADP-ribose (30), and it has recently been proposed that this process can be explained by loss of mitochondrial matrix NAD+ via the cyclosporin-sensitive permeability transition pore followed by catabolism by a NAD+ glycohydrolase localized outside the matrix space (14). Decreases in NAD+ could limit delivery of reducing equivalents to complex I and thus further impair complex I-dependent respiration and oxidative phosphorylation during the postischemic period. In the present studies, we have sought to determine whether complex I dysfunction can account for...

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the impairment of tubule cell energetics after H/R by directly measuring NADH:ubiquinone (Q) reductase activity and the redox state of NAD(H) during the H/R insult and testing the effect of titrating complex I activity with rotenone. The results indicate that most ATP depletion and deenergization occur only after >70% inhibition of NADH:Q reductase by rotenone. Complex I function is surprisingly well preserved during the H/R insult based on both the measurements of enzyme activity and assessment of the redox state of NAD(H) and is unlikely to be a major limiting factor for mitochondrial function or the target of the main protective effects of α-KG/MAL. The lesion is, however, accompanied by substantial depletion of both the mitochondrial and cytosolic NAD(H) pools during hypoxia that is ameliorated by α-KG/MAL.

**Materials and Methods**

*Materials.* Female New Zealand White rabbits (1.5–2.0 kg) were obtained from Harlan (Indianapolis, IN). Type A collagenase was from Roche Molecular Bioproducts (Indianapolis, IN). Percoll was purchased from Amersham Biosciences (Piscataway, NJ). HPLC-grade acetonitrile was from Fisher Scientific (Pittsburgh, PA). 5,5′,6,6′-Tetrafluoro-1,1′-3,3′-tetraethylbenzimidazocarbocyanine iodide (JC-1) was supplied by Molecular Probes (Eugene, OR). High-purity digitonin was purchased from Calbiochem (San Diego, CA). All other reagents and chemicals were from Sigma (St. Louis, MO) and were of the highest grade commercially available. Agents solubilized in ethanol or dimethyl sulfoxide were delivered from ≥1,000× stock solutions.

*Isolation of tubules.* Proximal tubules were prepared from the kidney cortex of female New Zealand White rabbits by digestion with combinations of type A and Sigma Blend type F collagenase and centrifugation on self-forming Percoll gradients as described (42, 44–46).

*Experimental procedure.* Incubation conditions were similar to our prior studies (43–46). Tubules were suspended at 3.0–5.0 mg tubule protein/ml in a 95% air-5% CO₂-gassed medium containing (in mM) 110 NaCl, 2.6 KCl, 25 NaHCO₃, 2.4 KH₂PO₄, 1.25 CaCl₂, 1.2 MgCl₂, 1.2 MgSO₄, 5 glucose, 4 sodium lactate, 0.3 alanine, 5 sodium butyrate, and 2 glycine as well as 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 regassed with either 95% air-5% CO₂ (normoxic controls) or 95% N₂-5% CO₂ (hypoxia). Hypoxic tubules were kept at pH 6.9 to simulate tissue acidosis during ischemia in vivo (43). 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 washed twice to remove any experimental substrates being tested for efficacy only during hypoxia and were then resuspended in fresh 95% air-5% CO₂-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 and then again at 60 min of reoxygenation (43, 45). After 60 and 120 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 or rotenone exposure (43, 45).

*HPLC determination of purine and pyrimidine nucleotides and their metabolites.* Samples of tubule cell suspension or subcellular fractions prepared from it were immediately deproteinized in trichloroacetic acid, neutralized with triethylamine:CFC 113, and stored at −20 °C as previously described (43). Nucleotides and their metabolites in 20-μl aliquots of the neutralized extracts were separated using a 150-mm C₁₈ reverse-phase ion-pairing column preceded by a 45-mm guard column (Beckman-Coulter, Fullerton, CA) with UV detection at 254 nm using a modified gradient method (5). Buffer A of the mobile phase consisted of 100 mM KH₂PO₄, 5 mM tetrabutylammonium dihydrogen phosphate, and 2.5% (vol/vol) acetonitrile, pH 6.0. Buffer B consisted of 100 mM KH₂PO₄, 5 mM tetrabutylammonium dihydrogen phosphate, and 25% acetonitrile, pH 5.5. After sample injection, the column was eluted at 25 °C for 1 min with 100% buffer A followed by 0.1 ml/min, then switched to 100% buffer B at 1.0 ml/min, then for 2 min with solution A plus buffer B increasing to 11%, and then for a further 25 min with buffer B progressively increasing to 100%. The column was then reequilibrated for 20 min with 100% buffer A before the next injection. For the purposes of the present studies, the procedure cleanly separated nicotinamide mononucleotide, hypoxanthine, inosine, NAD⁺, adenosine, AMP, GMP, GDP, ADP-ribose, ADP, GTP, and ATP. Concentrations in samples were determined by comparison with mixtures of pure standards of each compound. Although NADH is also separated by this procedure, coelution of other compounds with NADH in alkaline sample extracts needed to stably extract the NADH precluded use of the HPLC method for measuring NADH.

*Enzymatic assay of NADH.* Tubule samples were subjected to acidification extraction in an acidic potassium hydroxide. NADH was then oxidized enzymatically to NAD⁺ using glutamate dehydrogenase, and the NAD⁺ formed was quantitated using alcohol dehydrogenase (26). NAD⁺ was measured enzymatically in these samples rather than by HPLC because other peaks coeluted with and obscured NAD⁺ in the alkaline extracts. Because glucose in the medium substantially increased the background for this assay, it was omitted from the experimental solutions used for samples that would be taken for NADH assay. This did not affect the behavior of the model.

*JC-1 fluorescence.* JC-1 fluorescence was measured as follows (33, 36). An aliquot of JC-1 from a 1,000× stock solution of the probe in dimethylsulfoxide was mixed 1:4 with calf serum, dispersed as an intermediate 100× stock solution in phosphate-buffered saline, and then added at the end of the desired experimental period to a final concentration of 7 μg/ml in the tubule suspension. The suspension was resuspended with air-CO₂ and incubated in the dark for an additional 15 min at 37 °C. Then tubules were pelleted and washed three times in an ice-cold solution containing (in mM) 110 NaCl, 25 sodium HEPES, pH 7.2, 1.25 CaCl₂, 1.0 MgCl₂, 1.0 KH₂PO₄, 3.5 KCl, and 5 glycine as well as 5% polyethylene glycol (average molecular mass 8,000). Immediately after sampling and washing were completed, a 300-μl aliquot of the tubules containing 1.2–1.5 mg protein was brought up to 2.5 ml with additional ice-cold wash solution and then scanned during continuous gentle stirring using a Alphascan fluorometer (Photon Technology, Monmouth Junction, NJ) at 488-nm excitation/500- to 625-nm emission collected in 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.

*Tubule respiration.* Tubules preincubated at 37 °C for 45 min in 95% O₂-5% CO₂ in solution A were added to a sealed chamber with a Clark oxygen electrode (YSI, Yellow Springs, OH) (40). After measurement of basal respiration, rotenone or ethanol vehicle was added, followed, when the rotenone rate was stable, by 5 μM FCCP. *Measurement of NADH-Q reductase activity.* NADH-Q reductase activity was measured as follows (adapted from Ref. 16). After the desired experimental maneuver(s), tubules were pelleted, immediately resuspended in 10 mM KCl, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4, snap-frozen in liquid N₂, and stored at −80 °C. Immediately before assay, the samples were thawed and sonicated (ice for 40 s before each of 5 s on/5 s off). Aliquots of sonicate containing 0.5–1 mg tubule protein were added to 2 ml of a reaction medium containing 50 mM KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 1 mM potassium...
cyanide, 10 μM antimycin, 75 μM NADH, and 120 μM decylubiquinone at 37°C. NADH oxidation was followed fluorometrically at 360-nm excitation, 450-nm emission. After an initial rate was obtained, 20 μM rotenone was added to measure the rotenone-insensitive component (consistently <10% of the total under these conditions). Activity was calculated as the difference between total and rotenone-insensitive nanomoles NADH oxidized per minute per milligram total tubule cell protein. The use of sonicated, frozen-thawed aliquots of the whole tubule suspensions rather than isolated mitochondria for this assay avoids the problems of low yields of mitochondria from the small amounts of tubules available and the confounding effects of differential enrichment and isolation procedure-induced alterations of isolated mitochondria (see DISCUSSION). All experimental and control conditions started with a common tubule preparation and were fully paired each day, so adjusting for total tubule cell protein normalizes for small sample-to-sample differences in protein content (generally 5–10%).

Distribution of NAD+ and NADH between cytosol and mitochondria. Samples of the tubule suspension were quenched in 4 vol of ice-cold solution A containing glycine, but no substrates. The tubules were immediately pelleted and resuspended in ice-cold 120 mM KCl, ice-cold dria. Samples of the tubule suspension were quenched in 4 vol of H9262 pH 7.2, with 100 mM sampled for NADH and NAD+ incubation on ice with mixing, aliquots of the whole lysate were prepared and were fully paired each day, so adjusting for total tubule cell protein normalizes for small sample-to-sample differences in protein content (generally 5–10%).

Statistics. Paired and unpaired t-tests were used as appropriate in studies with two groups. Where experiments consisted of more than two groups, they were analyzed statistically by analysis of variance for repeated measure or independent group designs as needed. Individual group comparisons for the multigroup studies were then made using the Newman-Keuls test for multiple comparisons (SigmaStat, SPSS, Chicago, IL). P < 0.05 was considered to be statistically significant. The n given indicates the numbers of separate tubule preparations studied.

RESULTS

Energetic function during H/R. At the end of hypoxia, ATP was decreased to 2.2% of the level in normoxic control tubules (Fig. 1A). It recovered to only 22% of the normoxic control level during 60 min of reoxygenation and to 39% at 120 min of reoxygenation (Fig. 1A). Inclusion of α-KG/MAL in the medium only during reoxygenation nearly tripled the recovery of ATP during the first hour of reoxygenation. When α-KG/MAL was present during both hypoxia and reoxygenation, the end-hypoxia ATP level was 31% of the normoxic control, and almost full recovery of ATP was seen at the end of the first hour of reoxygenation. With no extra substrates, formation of red, high-∆Ψm-dependent JC-1 aggregates (595 nm) at 60-min reoxygenation was decreased to 62% of that in normoxic control tubules, whereas fluorescence of the green, monomeric,

![Fig. 1. Behavior of ATP and 5,5′,6′,6′-tetraethyl-1,1′,3′,3′-tetrachloro-1,1-diaminocarbocyanine iodide (JC-1) uptake during hypoxia-reoxygenation (H/R). A: cell ATP at end hypoxia and after 60- and 120-min reoxygenation. 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, A/M) present during either reoxygenation alone (A/M R) or hypoxia and reoxygenation (A/M HR). Values are means ± SE for 5 experiments given as percentages of ATP levels of tubules incubated continuously under oxygenated conditions for the same durations (normoxic time controls). Overall treatment effects: P < 0.01 for end-hypoxia values and P < 0.001 for reoxygenation values. *P < 0.001 vs. corresponding time point in normoxic time controls, †P < 0.001 vs. corresponding time point in both the NES (P < 0.001) and A/M R (P < 0.05) groups. All values are significantly less than the corresponding normoxic time controls, P < 0.01. The time control ATP levels were (nmol/mg protein) 7.3 ± 0.5 for end-hypoxia samples, 16.3 ± 1.3 for 60-min reoxygenation, and 25.2 ± 2.4 for 120-min reoxygenation. The higher normoxic time control ATP levels corresponding to reoxygenation time points reflect increases in cell ATP due to the successive additions of AMP to the medium during reoxygenation (43). B: JC-1 fluorescence after 60 min of reoxygenation for the same experimental conditions as described in A. The 595- and 535-nm emission intensities for tubule cell protein and the 595/535-nm ratios are shown as percentages of corresponding values for control tubules incubated under normoxic conditions for the same duration. Values are means ± SE for 8 experiments that included NES and A/M R conditions and 3 experiments that included all 3 reoxygenation conditions. *P < 0.001 vs. corresponding NES value, †P < 0.001 vs. corresponding time control value. In the time controls, the 595- and 535-nm signals were 276.0 ± 21.2 and 314 ± 32.7 cytoplasmic protein, respectively, and the 595/535-nm ratio was 9.5 ± 1.1.](http://ajprenal.physiology.org/)
ΔΨm-independent form of the probe (535 nm) doubled, resulting in a large decrease in the 595/535-nm ratio (Fig. 1B). As previously described (44, 45), fluorescence microscopy of the JC-1-loaded tubules showed that these changes were predominantly due to partial shifts from red to green in individual cells rather than to increased numbers of cells that were totally deenergized and entirely green (not shown). α-KG/MAL during reoxygenation alone substantially improved each of the three JC-1 parameters. When α-KG/MAL was present during both hypoxia and reoxygenation, all of the JC-1 parameters were nearly normalized (Fig. 1B).

NADH:Q reductase activity during H/R. The earliest and most severe abnormality of electron transport in mitochondria isolated from ischemic tissues is inhibition of NADH:Q reductase activity (34), which would be consistent with both the impaired respiration of the isolated tubules for complex I-dependent substrates after H/R and the improvement produced by succinate (44, 45). Figure 2 summarizes the results of NADH:Q reductase activity measurements on control tubules kept normoxic throughout and at the end of 60-min hypoxia and 60-min subsequent reoxygenation. NADH:Q reductase activity was stable during control, normoxic incubation of the isolated tubules (Fig. 2, inset). Activity was inhibited during 60-min hypoxia (END HYPOXIA values in Fig. 2), but only by 26%, and did not change further during subsequent reoxygenation (END REOX values in Fig. 2). When α-KG/MAL was present during hypoxia, activity tended to be slightly higher at the end of hypoxia, but this difference was not statistically significant. α-KG/MAL during reoxygenation significantly increased activity irrespective of whether it had been present during hypoxia.

Titration of NADH:Q reductase activity by rotenone and its effect on cellular energetics. The 26% inhibition of NADH:Q reductase activity in studies shown in Fig. 2 is relatively small, but the extent of complex I inhibition required to affect net energetic function has been reported to vary widely in mitochondria from different cell types (10, 11). To more directly assess the impact of inhibiting complex I in the intact tubules, we used rotenone to titrate NADH:Q activity (12). Figure 3 displays the relationship between rotenone concentration in nanomoles per milligram cell protein and percent inhibition of NADH:Q reductase. Maximal inhibition was seen at >0.8 nmol/mg protein, there was no inhibition at <0.002 nmol/mg protein, and the Ki was 0.06 nmol/mg protein. NADH:Q reductase inhibition began to affect cellular energetic parameters when it exceeded 20% (Fig. 4A). Peak respiratory capacity measured by stimulation with uncoupler dropped sharply and was maximally decreased at 40% inhibition of NADH:Q reductase. In contrast, basal respiration decreased gradually, reaching 40% of control when NADH:Q was 70% inhibited (Fig. 4A). NADH increased progressively at >20% NADH:Q inhibition (Fig. 4B), with the maximal increase reached at 65–70% inhibition. ATP dropped sharply and maximally only at >70% NADH:Q inhibition, at which

Fig. 2. NADHubiquinone (Q) reductase activity measurements. Tubules were subjected to 60-min hypoxia and 60-min reoxygenation with either NES or 4 mM A/M or to normoxic incubation for the same total duration (inset). NADH-Q reductase activity was measured at the end of hypoxia (END HYPOXIA) and then after reoxygenation (END REOX). Values are means ± SE; n = 3–4. Overall treatment effect: P < 0.001. *P < 0.05 vs. corresponding END HYPOXIA value. **P < 0.05 vs. normoxic control.
point the red/green JC-1 ratio was still down only moderately. Maximal decreases in the JC-1 ratio required >85% NADH:Q inhibition (Fig. 4B).

Figure 5 further elaborates on the ATP depletion and changes of pyridine nucleotide metabolism in these rotenone titration studies. As expected, NAD\(^+\) fell as NADH increased, but total NADH+NAD\(^+\) also decreased by 36% from 3.83 nmol/mg protein in untreated tubules to 2.44 nmol/mg protein at the highest level of rotenone, with most of the change occurring at rotenone concentrations that maximally depleted ATP. ADP-ribose, a product of NAD\(^+\) metabolism by NAD\(^+\)-glycohydrolase (15), increased in parallel to ATP depletion, with a net accumulation of 0.23 nmol/mg protein. Nicotinamide mononucleotide, a product of NAD\(^+\) metabolism by nucleotide pyrophosphatase (15), increased to a greater degree than ADP-ribose, with an accumulation of 0.50 nmol/mg protein, but only after maximal ATP depletion at the highest concentrations of rotenone. The combined accumulation of nicotinamide mononucleotide and ADP-ribose, 0.73 nmol/mg protein at the highest concentrations of rotenone tested, accounted for 52% of the 1.39 nmol/mg protein decrease in NADH+NAD\(^+\).

Changes in NAD\(^+\) and NADH during H/R. The studies shown in Fig. 4B suggest that substantially more complex I inhibition than that found in the tubules subjected to H/R is required to produce the degree of energetic compromise seen. To alternatively test whether complex I was limiting in the reoxygenated tubules, we assessed whether NADH levels remained increased during reoxygenation as would be expected if functionally significant complex I inhibition were present. During 60-min hypoxia, NADH doubled compared with tubules that were normoxic for the same period (Fig. 6). However, during reoxygenation, NADH then decreased to levels lower than those of normoxic tubules. During hypoxia, NAD\(^+\) decreased disproportionately to the increase in NADH so that total NADH+NAD\(^+\) decreased by 50% compared with normoxic tubules (Fig. 6). During reoxygenation, total NADH+NAD\(^+\) did not change significantly relative to the end-hypoxia values (Fig. 6).
When α-KG/MAL was added only during reoxygenation, NADH tended to be slightly higher than in untreated reoxygenated tubules, but the small differences were not statistically significant (Fig. 6). NADH+NAD⁺ was slightly higher during reoxygenation of α-KG/MAL-treated tubules than at the end of hypoxia, but the change was not statistically significant. Tubules that were supplemented with α-KG/MAL during hypoxia displayed notably different behavior. The decrease in NADH+NAD⁺ during hypoxia was substantially less than in the unsupplemented tubules. Higher levels of both NADH and NAD⁺ were maintained. During reoxygenation of these tubules in the continued presence of α-KG/MAL, NADH and NAD⁺ significantly increased and returned to levels identical to those of normoxic controls.

Subcellular pools of NAD⁺ and NADH during H/R and rotenone treatment. The data in Fig. 6 do not distinguish between mitochondrial and cytosolic pools of NADH and NAD⁺. To assess whether the two pools were being differentially affected during the insult, additional studies were done in which tubules were fractionated at the time of sampling into a digitonin-releasable supernatant that contains the cytosolic pool and a pellet that contains the mitochondrial pool (Fig. 7, A and B). Measurements at the end of hypoxia (Fig. 7A) were limited to NADH+NAD⁺ because some NADH was oxidized in the fractionation buffer despite the inclusion of rotenone. In normoxic control tubules, 63% of total NADH+NAD⁺ was in the pellet. Both the supernatant and pellet pools decreased during hypoxia (Fig. 7A), but the distribution of NADH+NAD⁺ between them did not. At the end of hypoxia, 68% of total NADH+NAD⁺ was in the pellet, which was not significantly different from the fraction in the pellet of the normoxic control tubules (Fig. 7A). The changes in both pools were ameliorated when α-KG/MAL was present.

Concentrations of rotenone that fully inhibit complex I decrease cell ATP and deplete NADH+NAD⁺ similarly to hypoxia (Figs. 5 and 6), and tubules preincubated with rotenone maintain constant levels of NADH during subcellular fractionation. Therefore, we used tubules treated with rotenone for 60 min to further investigate the subcellular patterns of NADH and NAD⁺ depletion and the effects on them of α-KG/MAL. In control tubules without rotenone, 87% of the NADH was mitochondrial (Fig. 7B), consistent with previous
inferences from the behavior of pyridine nucleotide fluorescence about the predominant mitochondrial localization of NADH (1). In the control tubules, 56% of NAD$^+$ was mitochondrial. During rotenone treatment without α-KG/MAL, 49% of mitochondrial NAD$^+$ was lost, and 28% was converted to NADH. The corresponding values in the presence of rotenone and α-KG/MAL were 19% lost and 58% converted (both significantly different from rotenone without α-KG/MAL). In the cytosol of tubules given rotenone without α-KG/MAL, 61% of NAD$^+$ was lost, and 5% was converted to NADH. In the rotenone plus α-KG/MAL-treated tubules, 49% of cytosolic NAD$^+$ was lost, and there was no measurable net conversion to NADH. These data confirm that mitochondrial pools account for the changes in NADH measured in whole cell extracts and that the effects of α-KG/MAL occur in both cytosol and mitochondria.

Time course of changes in NAD(H) and its metabolites during hypoxia. NAD$^+$ decreased sharply during the first 15 min of hypoxia (Fig. 8). Most of this initial decrease was accounted for by formation of NADH, which was maximal at 15 min of hypoxia. Thereafter, both NAD$^+$ and NADH decreased in parallel up until 60 min, after which the rate of decrease of both metabolites slowed (Fig. 8). The decrease of NADH+NAD$^+$ was progressive during hypoxia. Nicotinamide mononucleotide did not increase during hypoxia. However, ADP-ribose accumulated, peaking with 45-min hypoxia, then decreased at longer durations of hypoxia, but remained above normoxic control levels (Fig. 8). ADP-ribose returned to the low baseline levels measured in normoxic controls during the first 60 min of reoxygenation (not shown).

Effects on NAD(H) levels of nicotinamide, 3-aminobenzamide, and cyclosporine A. To further assess the striking depletion of NADH+NAD$^+$, we tested several agents that have been reported to modify NAD$^+$ catabolism during cell injury by inhibition of NAD$^+$ glycohydrolase (14, 29) and poly(ADP-ribose) polymerase (2) (5 mM nicotinamide, 3 mM 3-aminobenzamide) or antagonism of permeability transition pore opening (14) (5 μM cyclosporine A). None of the three agents significantly affected the changes of NADH or NAD$^+$ that occurred during hypoxia in this system or the accumulation of nicotinamide mononucleotide or ADP-ribose (data not shown).

Fig. 8. Time course of changes in NADH, NAD$^+$, and their metabolites during hypoxia. NADH, NAD$^+$, NMN, and ADP-ribose levels were measured in samples of the tubule suspension after the indicated durations of hypoxia. 0 min, Control normoxic tubules before start of hypoxia. Results are means from replicate flasks in a single experiment that is representative of 3 that were done. Statistical analysis of all 3 experiments indicated that the time-dependent changes in each parameter except for NMN were significant, $P < 0.001$. 
DISCUSSION

These studies provide a number of insights into the severe energetic deficit developed by kidney proximal tubule cells during H/R that is ameliorated by certain citric acid cycle metabolites. The model behaved similarly to our previous experience (44, 45) and nicely illustrates major characteristics of the lesion and its modification by α-KG/MAL. ATP levels of tubules that did not receive α-KG/MAL recovered to only 22% of similarly incubated normoxic control tubules during the first 60 min of reoxygenation despite the presence of ample amounts of other substrates that are favorable for metabolism of uninjured tubules and supplementation of the medium with AMP to provide precursors to enhance recovery of the purine nucleotide pool. Only moderate further recovery of ATP took place during an additional 60 min of reoxygenation. Tubules that were supplemented with α-KG/MAL only during reoxygenation had substantially stronger recovery of ATP, and most of this recovery, to ATP levels threefold greater than in unsupplemented tubules, occurred during the first hour. Tubules that received α-KG/MAL during hypoxia and reoxygenation had nearly complete recovery of ATP during the first hour. We have previously shown that the impaired ATP recovery in the affected, unsupplemented tubules under these study conditions is of a degree that prevents restoration of normal protein phosphorylation and cytoskeletal architecture during the reoxygenation period (46). Unprotected tubules also showed decreased formation of ΔΨm-dependent, red-fluorescing aggregates of JC-1, measured as both absolute red fluorescence and as red aggregate/green monomer ratios. These alterations were also substantially ameliorated by α-KG/MAL during reoxygenation and were largely eliminated by supplementing these substrates during both hypoxia and reoxygenation.

Complex I dysfunction has frequently been reported as a consequence of mitochondrial oxidant production and injury states (3, 9, 19, 28, 32, 35, 49), including studies of kidney mitochondria (28) and ischemia-reperfusion of the intact kidney (19). However, most of this work has been done using either in vitro insults to isolated mitochondria or measurements in mitochondria isolated from injured tissue, both of which take mitochondria out their normal intracellular environment and potentially increase the sensitivity of complex I to either oxidant damage during the in vitro insults or isolation of the mitochondria after damage in vivo. Other studies using surface fluorescence measurements showing persistent NADH reductase in intact tissues during ischemia-reperfusion (9) are complicated by reflow abnormalities that can result in persistent patchy ischemia, which would affect the measurements independently of primary complex I dysfunction. In the present work, H/R was studied using intact fully differentiated proximal tubules, and complex I activity measurements were done without the confounding artifacts of prior mitochondrial isolation. Under these conditions, inhibition of complex I measured as NADH:Q reductase activity did not exceed 26% during 60-min hypoxia and did not worsen during 60-min subsequent reoxygenation (Fig. 6). Although these are unavoidably average values measured in populations of cells, our previous work (44, 45) has shown that the energetic deficit involves most cells, so the results cannot be explained by the presence of a subpopulation of cells with especially severe inhibition while the rest are normal. Even after 120 min of hypoxia, NADH:Q reductase was inhibited by only 31%, and this also did not worsen during reoxygenation (data not shown).

Disruption of mitochondria by freeze-thaw and sonication to allow access of NADH to complex I on the matrix side of the inner mitochondrial membrane as used in our studies has been the standard approach for measuring NADH:Q reductase activity (8, 16). However, it has been reported that alamethicin can alternatively be used to permeabilize the membrane to NADH and allow measurement of NADH:Q reductase activity in a less disruptive, potentially more physiological context that could better reflect functional changes relevant to injury behavior (20, 21). Although the current studies were completed in large part before this methodology appeared, it was of considerable interest to us and, as part of the review process for this manuscript, we assessed whether permeabilization with alamethicin (in combination with digitonin to permeabilize the plasma membrane of the intact tubules) could be applied to our experimental system. Alamethicin worked well to deenergize the mitochondria in the intact tubules but did not permeabilize them to NADH sufficiently to allow its use to reliably assess NADH:Q reductase activity (data not shown).

The extent of complex I inhibition necessary to limit respiration and oxidative phosphorylation has been reported to vary depending on the type of mitochondria. Nonsynaptic brain mitochondria required 72% inhibition of complex I activity before respiration and ATP synthesis decreased (10), whereas synaptic mitochondria from the same region had a much lower threshold of 25% (11). We used rotenone titration of complex I (12) to address this question for the tubules. The concentration dependence of respiratory inhibition and ATP depletion by rotenone in intact tubules have been studied in detail previously (22), but not relative to the amount of complex I inhibition produced by the rotenone. The present studies provide this information (Fig. 3) and then use it to assess the complex I dependence of NAD(H) redox state, respiration, oxidative phosphorylation, and the changes in ΔΨm reflected by JC-1 uptake (Figs. 4–6). Although much of the decrease in FCCP-uncoupled respiration occurred between 20 and 30% inhibition of NADH:Q reductase, decreases in basal respiration became maximal only at >70% NADH:Q reductase inhibition, and substantial decreases in ATP and formation of red fluorescence, high-ΔΨm-dependent JC-1 aggregates were only seen after that point. Notably, the 60-min exposure time to rotenone in these concentration-dependence studies is the same duration used for hypoxia in the H/R experiments so that any cumulative indirect effects of continued complex I inhibition would be the same under both conditions. Thus inhibition of oxidative phosphorylation and substantial deenergization required far more complex I inhibition than was measured during H/R. Although α-KG/MAL ameliorated the inhibition of NADH:Q reductase, it is unlikely that the small changes produced by α-KG/MAL relative to the mild inhibition seen contributed to the benefit of α-KG/MAL.

Our assays of NAD+ and NADH under normoxic control conditions in whole cells and fractionated samples gave results for both protein-factoried absolute levels and intracellular distributions that were comparable, when adjusted for differing units, to available data from previous studies of isolated hepatocytes and kidney cortex (25, 38, 47). These measurements do not distinguish free and bound forms of the nucleotides and...
are, therefore, less precise about the changes in redox state and their compartmentation than are enzyme mass action ratios (38). However, the assays of NAD$^+$ and NADH provided information about the substantial decreases in absolute nucleotide levels that occurred during the injury conditions, which is not available from enzyme mass action determinations, and were more feasible for use with the amounts of cell material that we had available from the incubation conditions necessary to run the model. Dynamic monitoring of cellular autofluorescence as an index of pyridine nucleotide reduction (1) would not have provided information about compartmentation of changes, is not amenable to absolute quantitation, and is potentially confounded by the changes in the total NAD(H) pool size that occur.

The increase in NADH concentration was similar during hypoxia in the absence of protective substrates and maximal rotenone treatment as expected from our use of hypoxic conditions that approach complete anoxia (37). During reoxygenation of unprotected tubules after hypoxia, NADH levels decreased to less than those of normoxic controls. This extensive oxidation of NADH validates the conclusion from the NADH:Q reductase measurements that complex I function was not limiting during reoxygenation. That the main limitation, instead, is delivery of reducing equivalents to complex I (1, 27) is further supported by the observations that tubules maximally protected by α-KG/MAL had higher NADH levels during reoxygenation than the unprotected tubules. Effects upstream of complex I that could account for this behavior include dysfunction of citric acid cycle enzymes such as aconitase and the α-KG dehydrogenase complex that are known to be sensitive to oxidant insults (17, 31, 32), depletion of citric acid cycle metabolites, or impairment of their mitochondrial uptake. All of these processes could conceivably be improved by increasing metabolite levels with the supplemental substrates that ameliorate the lesion. Like electron transport inhibition, limitation of delivery of reducing equivalents to the electron transport chain is associated with matrix condensation (23), the characteristic ultrastructural change seen in the unprotected tubules during reoxygenation (44). Theoretically, increased delivery of reducing equivalents to a damaged electron transport chain could be toxic by virtue of increased generation of reactive oxygen species (19, 32). However, in the present studies and our prior work (44, 45), protective substrates that increased electron transport were consistently beneficial even when electron transport remained partially inhibited.

Substantial depletion of total NADH+NAD$^+$ with accumulation of NAD$^+$ metabolites was observed in both the rotenone titration experiments and during hypoxia (Figs. 5 and 6). In the rotenone titration experiments, pyridine nucleotide catabolism became pronounced only at concentrations of rotenone that maximally decreased ATP. Cytosolic, but not mitochondrial, NAD$^+$ in isolated hepatocytes varies as a function of moderate changes in ATP levels in the physiological range (13). In the present studies of kidney tubules, both cytosolic and mitochondrial pools of NADH+NAD$^+$ decreased and the changes only followed large decreases in ATP. Although all of the well-defined catabolic pathways utilize NAD$^+$ rather than NADH (15), reduction of NAD$^+$ to NADH did not preserve the pool. After peaking at 15 min of hypoxia, NADH declined largely in parallel with the remaining NAD$^+$ (Fig. 8). This NADH degradation was intramitochondrial, where nearly all NADH was localized (Fig. 7B). The NAD$^+$ degradation reflected mainly changes in the cytosol (Fig. 7B). The pattern of accumulation of NAD$^+$ metabolites during rotenone treatment is consistent with actions of both NAD$^+$ glycohydrolase, which produces ADP-ribose and nicotinamide, and pyrophosphatase, which produces nicotinamide mononucleotide (15). Together, these two metabolites accounted for 50% of the decrease in NADH+NAD$^+$ at the maximal concentrations of rotenone. This is an underestimate of the contribution of the two pathways to the extent that ADP-ribose and nicotinamide mononucleotide were further catabolized to products such as cADP-ribose during the 60-min experimental period (15, 18).

Our data for kidney tubules differ from a recent study of cardiac mitochondria and ischemia-reperfusion of the isolated heart, where NAD$^+$ degradation occurred to a large extent during reperfusion and could be largely accounted for by the action of a NAD$^+$ glycohydrolase on NAD$^+$ released from the matrix after opening of the cyclosporine-sensitive permeability transition pore (14). NAD$^+$ degradation in the tubules was insensitive to cyclosporine, although interpretation of the cyclosporine studies is somewhat limited by the long duration of the insult used. However, unlike myocyte behavior, degradation in the present studies occurred entirely during hypoxia (Fig. 6), and sustained opening of the permeability transition pore does not occur in the tubules during reoxygenation (44, 45). Despite using them in concentrations that have been effective in a variety of other systems (6, 14, 24) and documenting cellular uptake (not shown), we were unable to limit degradation of NAD$^+$ or ameliorate the energetic deficit (not shown) with nicotinamide or 3-aminobenzamide, agents that inhibit both mitochondrial NAD$^+$ glycohydrolase (14, 29) and poly(ADP-ribose) polymerase (2), another major route of NAD$^+$ catabolism during injury states (7). We also tested fluoride as an inhibitor of mitochondrial pyrophosphatase (4), but it was without effect (not shown).

In contrast to the lack of effect of the various inhibitors, supplementation with α-KG/MAL during hypoxia strongly blocked NAD$^+$ degradation. Although α-KG/MAL maintained higher levels of NADH, the decreased NAD$^+$ degradation was not simply due to a shift to NADH because NAD$^+$ levels of the α-KG/MAL-treated tubules were also increased. α-KG/MAL maintains slightly higher levels of ATP during the 60-min hypoxic period relative to untreated tubules by driving anaerobic substrate level phosphorylation during metabolism of the α-KG to succinate (44, 45). Although the increments of ATP produced by α-KG/MAL are small, differences in ATP levels of the same magnitude in the rotenone titration studies (Fig. 5) were associated with a large fraction of the NAD$^+$ degradation that occurred in them.

The beneficial effects of providing α-KG/MAL only during reoxygenation on overall energetics, as reflected by higher ATP levels and formation of high-ΔΨm-dependent JC-1 aggregates (Fig. 1, A and B), were not associated with significant increases in NADH+NAD$^+$ relative to untreated tubules (Fig. 6). Whether the large effect of α-KG/MAL during hypoxia to preserve the NAD(H) pool contributes to the greater functional benefit seen when α-KG/MAL is provided during both hypoxia and reoxygenation remains to be determined. Addressing this question requires alternate approaches for modifying the losses of NAD$^+$ and NADH during hypoxia, particularly the mitochondrial pool. In addition to testing the
NAD + glycohydrolase and poly(ADP-ribose) polymerase inhibitors, which were not effective for this purpose, we assessed whether we could increase cellular NAD(H) before hypoxia by supplementation of the tubules with exogenous NAD +, nicotinamide, or nicotinamide mononucleotide. NAD(H) supplementation of the tubules with exogenous NAD + therefore, do not help determine whether the size of the mitochondrial NAD(H) pool becomes limiting for respiration. In summary, this work provides new observations important for understanding the severe, but reversible, mitochondrial dysfunction in kidney proximal tubules that develops during H/R and its modification by protective substrates. They show that complex I must be inhibited by >70% before large decreases in ATP and deenergization occur in kidney proximal tubules. Complex I is relatively resistant to damage and dysfunction during H/R based on both direct measurement of its activity, which remains at a level sufficient to maintain nearly normal respiration and ATP production, as well as by assessment of changes in the redox state of NAD(H). This places the previously reported (44) defect for support of respiration by complex I-dependent substrates upstream of complex I. Both the cytosolic and mitochondrial NAD(H) pools are substantially depleted during hypoxia. Based on the pattern of metabolite accumulation, degradation occurs in part by NAD + glycohydrolase, but sustained opening of the permeability transition pore is not involved in these cells, and there is no further loss of NAD(H) during reoxygenation or as result of utilization by poly(ADP-ribose) polymerase. α-KG/MAL preserves the NAD(H) pool during hypoxia, which may contribute to enhanced recovery, but the strong benefit seen when α-KG/MAL is provided only during reoxygenation does not require increases in the NAD(H) pool.

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