Alleviation of fatty acid and hypoxia-reoxygenation-induced proximal tubule deenergization by ADP/ATP carrier inhibition and glutamate

Thorsten Feldkamp,1,2 Andreas Kribben,2 Nancy F. Roeser,1 Tiffany Ostrowski,1 and Joel M. Weinberg1

1Division of Nephrology, Department of Internal Medicine, Veterans Affairs Ann Arbor Healthcare System and University of Michigan, Ann Arbor, Michigan; and 2Division of Nephrology and Hypertension, Department of Internal Medicine, University Duisburg-Essen, Essen, Germany

Submitted 30 November 2006; accepted in final form 15 January 2007

Feldkamp T, Kribben A, Roesser NF, Ostrowski T, Weinberg JM. Alleviation of fatty acid and hypoxia-reoxygenation-induced proximal tubule deenergization by ADP/ATP carrier inhibition and glutamate. Am J Physiol Renal Physiol 292: F1606–F1616, 2007. First published January 23, 2007; doi:10.1152/ajprenal.00476.2006.—Kidney proximal tubules develop a severe but highly reversible energetic deficit due to nonesterified fatty acid (NEFA)-induced dissipation of mitochondrial membrane potential (ΔΨm) during reoxygenation after severe hypoxia. To assess the mechanism for this behavior, we have compared the efficacies of different NEFA for inducing mitochondrial deenergization in permeabilized tubules measured using safranin O uptake and studied the modification of NEFA-induced deenergization by inhibitors of the ADP/ATP carrier and glutamate using both normoxic tubules treated with exogenous NEFA and tubules deenergized during hypoxia-reoxygenation (H/R). Among the long-chain NEFA that accumulate during H/R of isolated tubules and ischemia-reperfusion of the kidney in vivo, oleate, linoleate, and arachidonate had strong effects to dissipate ΔΨm that were slightly greater than palmitate, while stearate was inactive at concentrations reached in the cells. This behavior correlates well with the protonophoric effects of each NEFA. Inhibition of the ADP/ATP carrier with either carboxyatractyloside or bongkrekic acid or addition of glutamate to compete for the aspartate/glutamate carrier improved ΔΨm in the presence of exogenous oleate and after H/R. Effects on the two carriers were additive and restored safranin O uptake to as much as 80% of normal under both conditions. The data strongly support NEFA cycling across the mitochondrial membrane using anion carriers as the main mechanism for NEFA-induced deenergization in this system and provide the first evidence for a contribution of this process to pathophysiological events that impact importantly on energetics of intact cells.

Acute renal failure; kidney; membrane potential; mitochondria

Nonesterified fatty acids (NEFA) accumulate progressively during renal ischemia in vivo (31, 79) and during hypoxia and metabolic inhibition of isolated tubules in vitro (21, 65, 69, 77) via both calcium-dependent and -independent mechanisms (21, 41, 42, 65, 69, 77). Studies attempting to elucidate their role with phospholipase inhibitors have been conflicting or inconclusive (reviewed in Ref. 11). More informatively, treatment of isolated tubules subjected to hypoxia-reoxygenation (H/R) with exogenous phospholipase A2 to increase NEFA levels or with arachidonate has been shown to impair recovery of ATP during reoxygenation despite at the same time decreasing plasma membrane damage (77, 78). Recent work further emphasizes the importance of NEFA has shown that defects of both mitochondrial and peroxisomal β-oxidation of fatty acids persist for prolonged periods, are aggravated by downregulation of enzyme gene transcription controlled by peroxisome proliferator-activated receptor-α (PPARα), and can be alleviated by PPARα ligands in both ischemia-reperfusion and toxic models with improvement of renal function and prevention of necrotic and apoptotic tubule cell death (29, 40).

Isolated proximal tubules subjected to H/R under conditions relevant to ischemia-reperfusion in vivo develop persistent mitochondrial dysfunction that plays a pivotal role in overall cellular recovery (64, 68). The energetic deficit is characterized by preserved function of the electron transport chain (10), absence of cytochrome c release (67), preserved activity of the mitochondrial F1F0-ATPase and ADP/ATP carrier (13), and partial, but incomplete recovery of mitochondrial membrane potential (ΔΨm) (12, 66, 67). Mitochondrial function can be improved by supplementing tubules with specific citric acid cycle metabolites such as α-ketoglutarate and malate individually and in combination (α-KG/MAL) during hypoxia or reoxygenation (66).

We have recently optimized approaches for quantitative and dynamic measurements of ΔΨm in permeabilized isolated tubules using safranin O (12) and used this system to demonstrate a critical role for NEFA in the persistent deenergization that develops during H/R (11). Addition of the long-chain NEFA oleate to permeabilized, normoxic control tubules strongly dissipated ΔΨm at levels corresponding to the total NEFA content measured after H/R. Delipidated bovine serum albumin (dBSA) prevented the effects of exogenous NEFA and nearly fully restored ΔΨm in permeabilized tubules studied during 60 min of reoxygenation after 60 or 120 min of hypoxia. In unpermeabilized tubules, the protective substrate combination of α-KG/MAL limited NEFA accumulation during hypoxia by 50% and lowered NEFA during reoxygenation. dBSA improved ATP recovery when added to unpermeabilized tubules during reoxygenation and was additive to the effect of α-KG/MAL (11). These observations provide strong evidence that NEFA overload is the primary cause of energetic failure of reoxygenated proximal tubules and indicate that lowering NEFA substantially contributes to the benefit from supplementation with protective substrates such as α-KG/MAL.

Studies of isolated mitochondria have shown that long-chain NEFA can act as rapidly reversible protonophoric uncouplers by entering the mitochondrial matrix in their protonated, nonpolar form followed by dissociation of the proton and transport of the deprotonated fatty acid ion out of the matrix by anion...
carriers, including the ADP/ATP carrier and the aspartate/glutamate, dicarboxylate, and citrate carriers. (60, 73). This results in ΔΨm-dissipating, sustained proton entry into the matrix with resultant decreased ATP production. Whether these processes are involved in any physiological- or pathophysiological-regulatory mechanisms in intact cells has not been established. However, they provide an attractive explanation for the highly reversible NEFA effects seen in the proximal tubules. To further address the mechanism for the NEFA tubule effects, in the present studies we have compared the efficacies of different NEFA for inducing deenergization, studied concentration dependence of the behavior in more detail, and assessed modification of the process by inhibitors of the ADP/ATP carrier and by glutamate using both normoxic tubules treated with exogenous NEFA and tubules deenergized as a result of H/R. The data strongly support a major role for shuttling of long-chain NEFA by mitochondrial anion carriers in the pathogenesis of the important NEFA-induced mitochondrial dysfunction in proximal tubules after H/R.

MATERIALS AND METHODS

Materials. Female New Zealand White rabbits (1.5–2.0 kg) were obtained from Harlan, (Indianapolis, IN). Animal use protocols for the studies in this manuscript adhered to the APS Guiding Principles in the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee of the University of Michigan. 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). High-purity digitonin (catalog no. 300411) and carboxyatractyloside were purchased from Calbiochem (San Diego, CA). Bongkrekic acid was kindly provided by Dr. J. A. Duine, Delft University of Technology, Delft, The Netherlands. All other reagents and chemicals, including dBSA (catalog no. A6003), were of the highest grade available from Sigma-Aldrich, 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 were delivered from greater than ×300 stock solutions in volumes of ethanol that did not by themselves affect the measured functions.

Isolation of tubules. Proximal tubules were prepared from kidney cortex of female New Zealand White rabbits by collagenase digestion and centrifugation on self-forming Percoll gradients as described (10, 63, 66–68).

Experimental procedure. Incubation conditions were similar to our previous studies (10, 12, 13, 64, 66–68). 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 1.0 mg/ml bovine gelatin (75 bloom; solution A). After 15- to 30-min preincubation at 37°C, tubules were resuspended in fresh solution A with experimental agents and reoxygenated with either 95% air-5% CO2 (normoxic controls) or 95% N2-5% CO2 (hypoxia). During hypoxia, solution A was kept at pH 6.9 to simulate tissue acidosis during ischemia in vivo (64) and omitted glucose, lactate, alanine, and butyrate. These incubation conditions result in near-anoxic conditions. It is not possible to confirm the presence of complete anoxia in the flasks, so we use the term hypoxia to describe the oxygen deprivation. After 60 min, samples were removed for analysis. The remaining tubules were pelleted and then resuspended in fresh 95% air-5% CO2-gassed, pH 7.4 solution A with experimental agents as needed. Sodium butyrate in solution A was replaced with 2 mM heptanoic acid during reoxygenation, and, to ensure availability of purine precursors for ATP resynthesis, 250 μM AMP was included (64, 67). After 60 min of reoxygenation, samples were again removed for analysis.

Measurement of ATP levels. Samples of tubule cell suspension were immediately deproteinized in trichloroacetic acid, neutralized with triethylamine-CFC 113, and stored at −20°C as previously described (64). 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 as previously described (10).

Measurement of ΔΨm with safranin O. At the end of either normoxic control incubation or H/R, samples of tubule suspension were diluted into an ice-cold solution containing (in mM) 110 NaCl, 25 Na-HEPES, pH 7.2, 1.25 CaCl2, 1.0 MgCl2, 1.0 KH2PO4, and 3.5 KCl as well as 5.0 glycine, 5% polyethylene glycol (average MW 8,000), and 2.0 mg/ml bovine gelatin, washed once in the same solution, and then held in it at 4°C until use. For the safranin O uptake measurements (12, 13), the tubules were resuspended at a final concentration of 0.10–0.15 mg/ml in an intracellular buffer type solution containing 120 mM KCl, 1 mM KH2PO4, 2 mM EGTA, 5 μM safranin O, 100 μg digitonin/mg protein, 10 mM K-HEPES, pH 7.2 at 37°C (solution B) supplemented with other experimental agents that are described with the data. Potassium succinate (4 mM) was included as a respiratory substrate during safranin O uptake in all studies unless otherwise indicated. 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. For studies done using normoxic control tubules, all experiments used tubules from the same suspension, so variability between cuvettes 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 average changes in net safranin O uptake between groups were compared, values were calculated as differences of fluorescence between time 0 and the point of maximal uptake during the period of observation and were factored for the amount of tubule protein in the sample.

ΔΨm-dependent ATP production by permeabilized tubules. Tubules were suspended in solution B as for safranin O uptake measurements with further addition of reagents to couple ATP production to conversion of NADP to highly fluorescent NADPH: 10 mM glucose, 10 U/ml hexokinase, 0.2 mM NADP, and 5 U/ml glucose-6-phosphate dehydrogenase along with 30 μM diadenosine-5′-pentaphosphate to inhibit adenylate kinase (11). ATP hydrolysis by Na+–K+–ATPase was prevented by the high-K+ content of the buffer, so ouabain was not included. ATP production was followed as formation of NADPH at 360-nm excitation/450-nm emission. Experiments were ended with addition of 5 μM ATP as an internal standard. Safranin O fluorescence was followed simultaneously at 485-nm excitation/586-nm emission.

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 measure 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 mean ± SE of no less than 3–5 experiments or are tracings representative of the behavior in that many experiments.

RESULTS

Comparison of NEFA effects on ΔΨm in permeabilized normoxic tubules. Figure 1 illustrates the typical effects of NEFA addition on ΔΨm of mitochondria in permeabilized...
normoxic tubules measured using safranin O uptake. For these determinations, tubules are suspended in an intracellular buffer with digitonin, succinate as respiratory substrate, and safranin O. Uptake of safranin O into the mitochondrial matrix occurs as a function of $\Delta \Psi_m$ where it is quenched, decreasing its fluorescence (12). Data in this figure and all of the others are shown as inverted “Relative Fluorescence” values (1.0 = fluorescence before any uptake), so that higher $\Delta \Psi_m$ corresponds to increases in the height of the tracings. The slow initial uptake of safranin O reflects the time to fully permeabilize all cells (12). Subsequent safranin O movements and associated fluorescence changes are rapid. Uptake was measured either with no added NEFA in the presence of 0.5 mg/ml delipidated BSA (dBSA), with 5 $\mu$M of the indicated NEFA in the presence of 0.5 mg/ml dBSA, with no added NEFA, with 0.5 $\mu$M NEFA, or with 5 $\mu$M NEFA. Arrows indicate late additions of 0.5 mg/ml dBSA in those experiments that did not have it from the start.

Fig. 1. Nonesterified fatty acid (NEFA) effects on mitochondrial membrane potential ($\Delta \Psi_m$) in permeabilized normoxic tubules. Safranin O uptake shown as inverted fluorescence relative to the initial value before uptake was measured either with no added NEFA in the presence of 0.5 mg/ml delipidated BSA (dBSA), with 5 $\mu$M of the indicated NEFA in the presence of 0.5 mg/ml dBSA, with no added NEFA, with 0.5 $\mu$M NEFA, or with 5 $\mu$M NEFA. Arrows indicate late additions of 0.5 mg/ml dBSA that did not have it from the start.

Fig. 2. Comparative effects of long- and short-chain NEFA. Arachidonate (20:4), arachidic acid (20:0), linoleate (18:2), oleate (18:1), stearate (18:0), palmitate (16:0), myristate (14:0), and laurate (12:0) were studied at 0.5 and 5 $\mu$M NEFA as in Fig. 1. Heptanoate (7:0) and butyrate (4:0) were tested at 100 $\mu$M after initial studies showed no effects at 5 $\mu$M (not illustrated). Effects of arachidic acid, stearate, and laurate at 100 $\mu$M are discussed in the text. Values are means ± SE; $n$ = 3–7 given as percentages of uptake by tubules from the same preparations without added NEFA. *$P < 0.001$ vs. tubules without NEFA.
Concentration dependence of oleate-induced deenergization. Detailed concentration-dependence studies are shown in Fig. 3 for oleate. Deenergization was consistently detected at 0.2 μM (~1.6 nmol/mg protein). Figure 3, B and C, display simultaneous measurements of ΔΨm and ATP production as a function of the amount of oleate added. ATP production was assessed by including glucose, NADP, hexokinase, and glucose-6-phosphate dehydrogenase in the medium and following formation of NADPH as described in MATERIALS AND METHODS. In B and C, ADP was added at 600 s to initiate ATP synthesis.

Efficacy of ADP/ATP carrier inhibitors in the system. In the studies in Fig. 4, tubules were energized with α-KG/MAL rather than the usual succinate. Addition of the complex I inhibitor rotenone at 500 s collapses ΔΨm and allows subsequent addition of ATP to produce reenergization that is completely dependent on delivery of ATP to the mitochondrial matrix by the ADP/ATP carrier and proton extrusion by the F1F0-ATPase driven by hydrolysis of the ATP (13). Addition of either of the ADP/ATP carrier inhibitors carboxyatractysolid (2 μM) or bongkrekic acid (1.5 μM) (20) completely blocked ATP-induced reenergization (Fig. 2). The same concentrations of these inhibitors did not affect succinate or α-KG/MAL-supported energization (not shown).

Antagonism of oleate-induced deenergization by glutamate and ADP/ATP carrier inhibitors. Inclusion of glutamate or either carboxyatractysolid or bongkrekic acid significantly ameliorated the dissipation of ΔΨm produced by oleate (Fig. 5). Carboxyatractysolid and bongkrekic acid in combination with glutamate were additive. These observations support involvement of the ADP/ATP carrier and aspartate/glutamate carrier in the oleate-induced deenergization. ATP (2 mM) also strongly increased ΔΨm to the same extent as carboxyatractysolid + glutamate (Fig. 5, B and C). The effect of ATP was mediated from the matrix, since blocking entry of ATP into the matrix with carboxyatractyloside decreased ΔΨm to the same level as that seen with carboxyatractyloside alone. Uncoupling protein 2 (UCP2) is another candidate mediator for dissipation of ΔΨm by NEFA. Its uncoupling effects are blocked by adenine nucleotides and guanine nucleotides (9, 27). However, 2 mM GDP did not increase ΔΨm in the presence of oleate (Fig. 5, B and C).

Figure 6 shows additional related studies testing the effects of carboxyatractyloside and glutamate on oleate-induced dissipation of ΔΨm (Fig. 6A) along with simultaneous measurements of ATP production (Fig. 6B). ATP production was assessed by following the conversion of NADP to NADPH as in Fig. 3. Carboxyatractyloside did not affect ΔΨm in the absence of oleate, but blocked ATP production from the added ADP as expected from its effect to irreversibly bind and inhibit the ADP/ATP carrier (Fig. 4) (20). Oleate decreased ΔΨm and blocked ATP production as in Fig. 3. Glutamate in the presence of oleate increased ΔΨm and moderately increased ATP production. Carboxyatractyloside in the presence of oleate increased ΔΨm without increasing ATP production. Carboxyatractyloside added to the effect of glutamate to increase ΔΨm, while still blocking ATP production. Addition of...
ADP in these Fig. 6 studies as well as in the Fig. 3 experiments produced a slight amount of reenergization seen as small upward deflections at the points of ADP addition in the “OLE” tracing in Fig. 6 and in the tracings for 2.0 and 5.0 μM oleate in Fig. 3. This effect of ADP was blocked by carboxyatractylloside (Fig. 6).

Concentration dependence of the effect of glutamate is shown in Fig. 7. As little as 0.25 mM increased \( \Delta \Psi_m \). The effect was maximal at 4 mM. Antagonism of H/R-induced deenergization by glutamate and ADP/ATP carrier inhibitors. In our previous studies (11), deenergization after H/R was completely reversed by dBSA, just like the deenergization seen with the addition of exogenous NEFA to permeabilized normoxic tubules (Fig. 1) (11). If the same mechanisms account for deenergization under both types of conditions, inhibition of the ADP/ATP carrier and addition of glutamate should be beneficial after H/R as they are for the normoxic tubules treated with oleate during safranin O uptake. ADP in these Fig. 6 studies as well as in the Fig. 3 experiments produced a slight amount of reenergization seen as small upward deflections at the points of ADP addition in the “OLE” tracing in Fig. 6 and in the tracings for 2.0 and 5.0 μM oleate in Fig. 3. This effect of ADP was blocked by carboxyatractylloside (Fig. 6).

Concentration dependence of the effect of glutamate is shown in Fig. 7. As little as 0.25 mM increased \( \Delta \Psi_m \). The effect was maximal at 4 mM.

Antagonism of H/R-induced deenergization by glutamate and ADP/ATP carrier inhibitors. In our previous studies (11), deenergization after H/R was completely reversed by dBSA, just like the deenergization seen with the addition of exogenous NEFA to permeabilized normoxic tubules (Fig. 1) (11). If the same mechanisms account for deenergization under both types of conditions, inhibition of the ADP/ATP carrier and addition of glutamate should be beneficial after H/R as they are for the normoxic tubules treated with oleate during safranin O uptake (Figs. 5–7). Figure 8 summarizes results of studies addressing this question and also illustrates the effects of the reagents on control normoxic tubules in the absence of added oleate. dBSA and ATP were also studied to provide direct comparison with the magnitude of the beneficial effects of these agents after H/R reported previously (11).

In normoxic tubules, dBSA during safranin O uptake slightly increased \( \Delta \Psi_m \) relative to no addition as expected from our earlier work (11). None of the other maneuvers individually significantly increased \( \Delta \Psi_m \) in the normoxic controls, although the average safranin O uptakes for carboxyatractylloside and glutamate tended to be slightly higher. The combination of carboxyatractylloside+glutamate significantly increased \( \Delta \Psi_m \) relative to no addition, and GDP worsened it.
Discrimination or classification of mitochondrial function by NEFA has been recognized for over 50 years (71). However, distinctions among the various mechanisms by which this can occur have only been clarified over the past 10–15 years (60, 73), and the physiological and pathophysiological relevance of the processes that have been defined has remained uncertain, even as new evidence suggesting important roles for them in common disease processes mediated by lipotoxicity has emerged (62).

Our recent studies have documented a critical role for NEFA-mediated dissipation of ΔΨm in energetic failure of isolated proximal tubules subjected to H/R under conditions relevant to ischemia-reperfusion injury in vivo (11). The levels of NEFA required to deenergize normal tubules were similar to those present in tubules subjected to H/R and in the kidney during ischemia-reperfusion in vivo (11). Most circulating NEFA are bound to albumin (43), while intracellular NEFA are bound to fatty acid-binding proteins (FABP) (16, 28, 59). Levels of unbound, circulating, and intracellular free fatty acids have been estimated to be as low as 5-10 nM in serum and cytosol (43, 59). Given the availability of intracellular FABP and low free fatty acid levels, it has been questioned whether free fatty acid levels ever reach concentrations sufficient to uncouple mitochondria in cells (23, 59). However, the binding capacity of endogenous FABP is likely well under NEFA levels reached during ATP depletion. There is limited information about absolute FABP levels in proximal tubules, and this issue is further complicated by the presence of endocytosed, liver-derived L-FABP (24, 36). In liver, which is considered to have abundant L-FABP with a NEFA-binding stoichiometry of 2, the measured L-FABP level of 0.86 nmol/mg protein (16) would only bind a total of 1.7 nmol NEFA/mg protein, which is well under the 17 nmol/mg present in hypoxic tubules (11). MDCK cells overexpressing L-FABP only reached 0.3 nmol/mg protein and were not protected against injury from cyanide+deoxyglucose (82). Interestingly,
however, in a preliminary report (35) transgenic mice overexpressing L-FABP were protected against renal ischemia-reperfusion injury in vivo. In our studies comparing the effects of exogenous NEFA to dissipate $\Delta \Psi_m$ in permeabilized normoxic control tubules with the deenergization seen after H/R, the H/R tubules had total NEFA levels similar to the levels required to decrease $\Delta \Psi_m$ by addition to permeabilized normoxic control tubules (11), which indicates insufficient buffering by the native FABP to suppress mitochondrial effects of NEFA during H/R.

In this paper, we provide evidence that the NEFA effects are mediated by at least two of the major mitochondrial inner membrane anion carriers, the ADP/ATP carrier and the aspartate/glutamate carrier. Involvement of these proteins in NEFA effects has been previously studied in isolated mitochondria but has not been documented to play a regulatory role in any physiological or pathophysiological setting. The present data provide strong evidence that they can account for a substantial component of the NEFA-induced deenergization in tubules after H/R.

Classic protonophoric uncoupling is characterized by concurrent stimulation of mitochondrial respiration, inhibition of ATP synthesis, and stimulation of ATP hydrolysis (7, 23, 38). These effects are now recognized to all result from a decrease in $\Delta \Psi_m$ secondary to availability of abnormal pathways for reentry of protons into the mitochondrial matrix that are alternative to their normal movement through the mitochondrial F$_{1}$/F$_{0}$-ATPase that phosphorylates ADP (38, 44, 72). The role of NEFA as protonophoric uncouplers for mitochondria is well established (23, 30, 38, 44, 55, 58, 60, 74). The most abundant NEFA released in rabbit proximal tubules during H/R are palmitate, stearate, oleate, linoleate, and arachidonate in a ratio of 1.0/2.0/1.0/1.5/0.5, respectively (65). All but stearate strongly dissipated $\Delta \Psi_m$ at low concentrations (Fig. 2) similar to those present in tubule cells after H/R and ischemia-reperfusion in vivo (11). The ability of long-chain NEFA to flip-flop across the membrane in their protonated form and, therefore, their protonophoric activity depend on the presence of an unsubstituted carboxylic acid and absence of changes to the hydrocarbon chain that alter its hydrophobicity (38, 74). For saturated NEFA, it is consistently maximal at chain lengths of 14 (myristate) and 16 (palmitate) as assessed by a variety of different end points reflecting NEFA entry into the matrix (5, 18, 45, 51, 56, 74, 80). Although there is some variability in reported efficacy of laurate (C12) depending on the parameter assessed, substantially lower efficacy for both laurate and stearate compared with myristate and palmitate as found in the tubules (Fig. 2) has been commonly described (18, 56, 74). Cis-unsaturation decreases the distance between the two ends of the molecule and confers substantial additional protonophoric activity (e.g., linoleate and oleate vs. stearate, and arachidonate vs. arachidonic acid in Fig. 2) (5, 18, 45, 51, 56, 61, 74, 80). The transbilayer mobility of undisassociated NEFA is greatest when the hydrodynamic length of the molecule is close to half the thickness of the hydrodynamic core of the membrane (38, 72). Short- and medium-chain NEFA (e.g., butyrate and heptanoate in Fig. 2) do not penetrate the membrane in their protonated forms and therefore do not deenergize (72).

The simple flip-flop of undisassociated NEFA across the membrane is not sufficient to maintain a sustained proton flux that will dissipate $\Delta \Psi_m$, unless the NEFA anion can leave the mitochondrial matrix and then reenter with another proton. The recognition, initially by Starkov et al. (1, 2), that coupled transmembrane movement of dissociated NEFA can occur on normal membrane anion carriers, provided a mechanism for such sustained proton flux occurring in the absence of mitochondrial structural disruption. The ADP/ATP carrier is highly abundant, comprising up to 10% of inner mitochondrial membrane protein (19, 20). Carboxyatractyloside is an impermeant, tightly binding inhibitor of the ADP/ATP carrier that stabilizes the transporter in the so-called “C-state,” in which the nucleotide binding site faces the cytoplasm. Bongkrekic acid is an uncompetitive inhibitor of the ADP/ATP carrier that stabilizes it in the “M-state,” where the nucleotide-binding site faces the matrix (20). Both agents improved NEFA-induced deenergization in studies of isolated mitochondria with stronger effects for carboxyatractyloside (1, 2, 7). Consistent with these sites of action, carboxyatractyloside did not inhibit NEFA effects on inside-out submitochondrial particles, but bongkrekic acid did (7). Multiple other lines of evidence support involvement of the ADP/ATP carrier in NEFA-induced deenergization. Both the uncoupling effects of NEFA and the inhibition of these effects by carboxyatractyloside are a function of the ADP/ATP carrier content of different types of mitochondria, with renal cortical mitochondria showing levels intermediate between those in myocytes and hepatocytes (7, 50). Hypothyroidism, which decreases ADP/ATP carrier expression in hepatocyte mitochondria, decreases their sensitivity to NEFA uncoupling and to its modification by carboxyatractyloside, and both effects are restored by thyroid hormone treatment (57). Yeast mutants lacking the ADP/ATP carrier show weaker mitochondrial uncoupling effects of NEFA (39). Bongkrekic acid itself is a polyunsaturated, long-chain fatty acid derivative (20) and common long-chain NEFA inhibit nucleotide transport on the ADP/ATP carrier (74, 75). Azido derivatives of long-chain NEFA inhibit nucleotide transport by the ADP/ATP carrier, induce carboxyatractyloside-sensitive uncoupling, and photolabel the protein (53). ADP/ATP carrier-mediated NEFA extrusion from the matrix may normally serve as a mechanism for removing peroxidized NEFA (8, 17).

Both of the ADP/ATP carrier inhibitors, carboxyatractyloside and bongkrekic acid, ameliorated the dissipation of $\Delta \Psi_m$ produced by olate in permeabilized tubules (Figs. 5 and 6). The question arises whether the normal substrates for the carrier would compete with NEFA for it. ATP strongly ameliorates deenergization (Fig. 5) but can act by mechanisms in addition to modifying fatty acid anion transport on the ADP/ATP carrier. In particular, ATP can promote utilization of fatty acids as respiratory substrates by supporting their esterification in the matrix with coenzyme A (61) and it can drive proton extrusion from the matrix by the F$_{1}$/F$_{0}$-ATPase to antagonize NEFA-mediated proton entry (13). A contribution of matrix actions of ATP is indicated by the effect of carboxyatractyloside to decrease the benefit of ATP (Fig. 5). ADP has been reported to have strong effects to recouple isolated skeletal muscle mitochondria treated with palmitate (1), but it had only weak and variable effects on liver mitochondria that were dependent on the timing of its addition (26, 49). These published studies were done in the presence of oligomycin to prevent ATP production in the matrix. However, unlike the present work with ADP (Figs. 3 and 6), they did not incorporate inhibition of ATP production by adenylate kinase, so some
of the observed effects could have been ATP mediated. In our studies, ADP had a very small effect to increase $\Delta \Psi_m$ in the presence of oleate (Figs. 3 and 6). Our experiments were done using diadenosine-5'-pentaphosphate to inhibit adenylate kinase, but did not include oligomycin because we were interested in measuring electron transport-driven ATP production. We have also tested ADP in the presence of both diadenosine-5'-pentaphosphate and oligomycin. Under this condition, ADP had a very small effect to increase $\Delta \Psi_m$ in the presence of oleate (Figs. 3 and 6). Our experiments were done using diadenosine-5'-pentaphosphate to inhibit adenylate kinase, but did not include oligomycin because we were interested in measuring electron transport-driven ATP production. We have also tested ADP in the presence of both diadenosine-5'-pentaphosphate and oligomycin. Under this condition, ADP failed to improve oleate-induced deenergization (data not shown), indicating that the small effects of ADP in Figs. 3 and 6 were mediated by ATP generated in the matrix rather than by actions of the nucleotide on fatty acid anion movement on the ADP/ATP carrier.

The ADP/ATP carrier is a member of the SLC25 family of mitochondrial anion carriers that includes the aspartate/glutamate carrier, the dicarboxylate carrier, the citrate carrier, the phosphate carrier, and the uncoupling proteins (37). In isolated liver mitochondria, glutamate, aspartate, and diethylpyrocarbonate (a histidine-reactive reagent that inhibits the glutamate aspartate carrier) (4, 47, 49, 70); malonate and phenylsuccinate (inhibitors of the dicarboxylate carrier) (58, 70); and benzenetricarboxylic acid, an inhibitor of the citrate carrier (58), all antagonized NEFA effects, implicating those additional carriers in NEFA anion cycling during protonophoric uncoupling. The phosphate carrier also appears to mediate fatty acid anion movements, but only under relatively nonphysiological conditions (46). In liver mitochondria, glutamate effects to ameliorate NEFA-induced deenergization were maximal in physiological high-ionic-strength medium (47). Glutamate is normally present at millimolar levels in proximal tubule cells (63). In the present studies, the effects of glutamate were evident at concentrations as low as 0.25 mM and were maximal at 4 mM (Fig. 7), indicating that it can serve as a natural inhibitor of NEFA-induced deenergization in tubules and that its effects will be maximal at levels normally present in vivo. The effect of glutamate was greater than that of carboxyatractyloside or bongkrekic acid. Metabolism of the glutamate was not responsible for its behavior because its effects were not reproduced by $\alpha$-KG or accompanied by any changes of NAD(P)H redox state in our tubule studies (not shown) and were not prevented by rotenone (4, 47, 49, 70).

UCP2 mRNA is expressed in the kidney and is among the genes most prominently upregulated during the first day after ischemia-reperfusion (76), although much of this change may be secondary to influx of inflammatory cells, where it is also highly expressed (3, 15, 33). There was initial uncertainty about the extent of UCP2 message translation in the kidney (6), but recent studies of isolated renal mitochondria employing Western blotting (27), high-affinity GTP binding (22), and tests of functional effects (9, 27) are all consistent with significant protein expression in tubules. In this regard, renal cortical mitochondria have been the main system used to provide evidence that NEFA-dependent dissipation of $\Delta \Psi_m$ promoted by superoxide-induced activation of UCP2 may be an important feedback regulatory mechanism for limiting mitochondrial reactive oxygen species production (9). Lipid peroxidation products such as 4-hydroxynonenal also activate UCP2, which, along with the ADP/ATP carrier, may have a role in removing these toxic compounds from the mitochondrial matrix (8, 17, 32). The proton leak mediated by UCP2 is inhibited by purine nucleotides, among which GDP has been most informative because it does not have the multiple other effects of adenine nucleotides (22, 33, 59). GDP did not increase $\Delta \Psi_m$ in mitochondria of NEFA-treated, normoxic control tubules (Fig. 5) and did not increase $\Delta \Psi_m$ after H/R (Fig. 8), which could indicate that UCP2 is not involved in NEFA effects in the tubules. However, GDP does not inhibit ucp2 under all conditions (14), can be antagonized by excess NEFA (59), and may inhibit the ADP/ATP carrier as well as ucp2 (25), so the question of UCP2 involvement in the tubule NEFA effects will require additional studies with alternate approaches.

Glutamate + carboxyatractyloside and glutamate + bongkrekic acid restored safranin O uptake to as much as 80% of normal in both normoxic tubules treated with exogenous oleate (Fig. 5) and after H/R (Fig. 8). These data are similar to those reported for isolated liver mitochondria (48) and suggest that

![Diagram of mitochondrial ion carriers](image-url)

Fig. 9. Role of anion carriers in dissipation of $\Delta \Psi_m$. NEFA (FA) flip-flop across the inner mitochondrial membrane in their protonated form. The proton is released into the matrix and the NEFA anion exits the matrix on the ADP/ATP carrier (AAC), the aspartate/glutamate carrier (AGC), or uncoupled protein 2 (UCP2) to pick up another proton to continue the cycle. The present studies provide support for involvement of the AAC and AGC but are inconclusive about UCP2.
the ADP/ATP carrier and aspartate/glutamate carrier in combination account for all but 20% of NEFA-mediated decrease of safranin O uptake under these study conditions. The remainder could be due to involvement of the other anion carriers or to promotion by NEFA of fluxes of ions other than H+. NEFA have direct ionophoric effects on monovalent cations in unilamellar vesicles that increase with their unsaturation and are dependent on phospholipid composition of the vesicle, with the strongest effects by NEFA corresponding to those in the vesicle’s phospholipids. They can increase inner membrane K+ cycling and chloride permeability via effects on complex matrix Mg2+. NEFA are also well documented to promote the mitochondrial permeability transition. However, development of the permeability transition in the tubules cannot account for these NEFA effects because the low-Ca2+ conditions used for the studies prevent development of the transition and, when the transition is allowed to occur by providing Ca2+, it is not reversed by dBSA (unpublished data).

In conclusion, we have shown that the potency of NEFA for dissipating ΔΨm in permeabilized proximal tubules correlates with their efficacy as protonophores and that the decreases in ΔΨm can be substantially ameliorated by either inhibiting the ADP/ATP carrier or providing glutamate. These observations support the scheme for NEFA cycling on anion carriers diagrammed in Fig. 9 as the main initial mechanism for impairment of mitochondrial energetics by the NEFA in proximal tubules. Antagonism of NEFA cycling on anion carriers in this fashion strongly improved ΔΨm to the same extent after tubules were subjected to H/R, showing for the first time the operation of these pathways in a commonly occurring pathophysiological setting that importantly impacts on overall cellular energetics.

ACKNOWLEDGMENTS

Some of these studies have been reported in abstract form (J Am Soc Nephrol 17: 712A, 2006).

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-34275 and the Research Service of the Department of Veterans Affairs.

REFERENCES


ALLEVIATION OF FATTY ACID-INDUCED MITOCHONDRIAL DYSFUNCTION


