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See related article, "Fetuin-A Protects against Atherosclerotic Calcification in CKD," on pages 1264–1274.

Illuminating Mitochondrial Function and Dysfunction using Multiphoton Technology

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J Am Soc Nephrol 20: 1164–1166, 2009.
doi: 10.1681/ASN.2009040419

Studying differential cell function or dysfunction within the kidney is made difficult by cellular heterogeneity, alterations in regional blood flow, oxygen tension, and interstitial tonicity, resulting in extremely complex anatomic and physiologic arrangements. Creative investigators have developed techniques to reduce this heterogeneity but often with loss or alteration of three-dimensional cellular associations, anatomic and physiologic cell-to-cell interactions, and harsh isolation and experimental conditions. These limitations have made "absolutes" difficult to determine, especially in relation to pathologic processes such as cell type involvement in renal ischemia. In this issue of *JASN*, Hall *et al.*[1] use multiphoton microscopy of kidney slices to compare mitochondrial parameters and differential cellular responses to stress. This approach allows them to quantify several key parameters of mitochondrial function and dysfunction in proximal tubular (PT) cells and directly compare different PT segments with each other and with distal tubular (DT) cells of the thick ascending limb (TAL). Because mitochondrial alterations play a central role in normal cell function and in response to injury, the article adds to our previous knowledge about an important area.

To understand the significance and limitations of their contribution, one must first understand what is known about differences between PT and TAL cells. Abundant mitochondria in cortical and outer medullary renal epithelial cells are necessary to meet the ATP demands of sodium transport by high-capacity aerobic metabolism. They compose 33% of the volume of proximal convoluted tubular S1 cells, 39% of the cells in the S2 segment, and 22% of the volume of cells in the proximal straight S3 segment; in the medullary and cortical TAL cells, mitochondria account for 30 to 44% of cell volume.[2] Differential tubular cell metabolism is also notable for the absence of aerobic and anaerobic glycolysis in the proximal convoluted tubule (S1),[3,4] which removes an important mechanism for preserving cell ATP and viability during injury.[5] In contrast, DT segments, including the TAL, have well-developed glycolytic pathways.[3,4,6,7] Glycolysis occurs in the S3 segment of the proximal tubule, albeit to a lesser extent than in distal tubules, and contributes to maintaining ATP there when mitochondrial function is impaired.[3,8]

Mitochondrial ATP production depends on substrate-supported, electron transport–mediated proton extrusion from the mitochondrial matrix that generates a proton gradient across the inner mitochondrial membrane. In turn, this is used to drive phosphorylation of ADP to ATP by proton movement down the gradient back into the matrix through the inner membrane F_1F_O-ATPase. Electron transport–driven proton extrusion is also responsible for the net potential across the inner membrane ($\Delta\Psi_m$), and $\Delta\Psi_m$ in combination with the pH gradient accounts for the net proton electrochemical gradient across the membrane, also called the proton motive force. $\Delta\Psi_m$ is the larger of the two components of proton motive force under both normal and pathologic conditions[9]; therefore, it serves as a valuable index of the state of the entire system, as shown in recent studies documenting a major role for nonesterified fatty acids in the persistent mitochondrial

Published online ahead of print. Publication date available at www.jasn.org.

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dysfunction seen in re-oxygenated proximal tubules.[9,10] As discussed by Hall *et al.*,[1] when $\Delta\Psi_m$ is low and ATP is available, the F_1F_O-ATPase reverses the flow of protons by hydrolyzing ATP to extrude protons and restore $\Delta\Psi_m$.

Fluorescence approaches, using both chemical probes and autofluorescence of components of the mitochondrial electron transport chain, provide powerful tools for dynamically following determinants of mitochondrial function and have been widely applied to studies of isolated mitochondria and whole cells. Parameters related to mitochondrial function including $\Delta\Psi_m$, superoxide production, cellular GSH levels, and the redox state of NADH and FAD^{2+} can be quantified in individual cells and $\Delta\Psi_m$ within individual mitochondria.[11] Hall *et al.*[1] show that mitochondrial uptake of two different lipophilic cationic probes (tetramethyl rhodamine methyl ester and rhodamine 123), as a measure of $\Delta\Psi_m$, follows the pattern TAL>proximal convoluted tubule>proximal straight tubule. This pattern is not changed by inhibition of the multidrug resistance transporter with verapamil, suggesting it is due to differences in $\Delta\Psi_m$ between tubular segments rather than in transport of the probe across the plasma membrane of individual cells; however, during chemical anoxia, $\Delta\Psi_m$ is well maintained in TAL cells but not in PT cells. Inhibition of the F_1F_O-ATPase with oligomycin abolishes this difference, indicating that differences between PT and TAL cells are due to support of $\Delta\Psi_m$ by F_1F_O-ATPase–mediated ATP hydrolysis. Proximal tubule cells had a lower ratio of ATPase to IF1, an endogenous low pH–activated inhibitor of the ATPase.[12] Less inhibition of the ATPase by IF1 can adaptively conserve residual ATP under conditions such as ischemia, in which mitochondrial aerobic metabolism is absent,[12] but can also be deleterious insofar as a low $\Delta\Psi_m$ favors opening of the mitochondrial permeability transition pore, which leads to necrosis or apoptosis.[13] The maintenance of $\Delta\Psi_m$ in the TALs also indicates persistence of significant glycolytic ATP production in those cells despite omission of glucose from the medium during the period of chemical hypoxia.

Additional biochemical questions that could be addressed with this approach come to mind. Would keeping pH 7.4 during chemical hypoxia to limit activation of IF1 allow for better preservation of $\Delta\Psi_m$ in the PTs? Are there differences between the S2 and S3 segments of the PT in that regard that reveal differences in glycolytic capacity? Would perfusion of glucose during chemical hypoxia help compensate for their lack of glycogen stores relative to the TAL cells[7] and produce better preservation of $\Delta\Psi_m$? The observations on superoxide production and redox state could also be expanded in informative ways in future studies.

Fluorescence techniques are rapidly becoming the approach of choice for studying mitochondrial function and individual mitochondria within a cell can now be studied using membrane-permeant cations and fluorescence resonance energy transfer techniques.[11] In addition, calcein entry can be used to measure the mitochondrial permeability transition.[13] This has important implications regarding understanding disease pathophysiology and therapy and can be used *in vivo*.[14,15] Multiphoton microscopy increases the depth of tissue penetration, reduces phototoxicity, and can follow three different fluorescence probes at once. This allows for direct correlation of multiple dynamic processes in the kidney.[16] Recent advances in the use of infrared-multiphoton microscopy now offer doubling of imaging depth and further reduction of phototoxicity.[17] Enhanced speed of image capture rates is now available through line-screening confocal microscopy, enhancing temporal resolution to real-time speeds.[18]

Certain limitations of the Hall *et al.* studies must be kept in mind. First, the studies use multiphoton techniques in tissue sections. Tissue sections allow for enhanced labeling with fluorescent probes and increased control over the environment and experimental conditions. They also allow for elucidating new information about complex pathways in fully differentiated cells in their normal anatomic tissue environment; however, tubules are not being perfused with glomerular filtrate, O_2 and substrate delivery occurs by diffusion, transport across cells is markedly decreased, and certain limitations of multiphoton microscopy must be considered. Previous qualitative intravital studies showed that PT cells had a greater Ψ_m than DT cells under physiologic conditions when using rhodamine 123 as the probe.[15] After intravenous delivery, rhodamine 123 enters cells from the basolateral aspect, indicating adequate access of the dye to all cells (unpublished observations). The reason for this apparent difference in $\Delta\Psi_m$ between tissue sections and intravital measurement is unclear. In addition, because mitochondrial diameter is approximately 1 μm and multiphoton optical sections are slightly less than 1 μm, $\Delta\Psi_m$ in some mitochondria is underestimated; therefore, creation of three-dimensional volumes would help to minimize this inherent concern.

In summary, the new multiphoton data provided by Hall *et al.*[1] focus our understanding of mitochondrial function to individual cells with direct comparisons between adjacent PT and TAL cells. The data provide additional information as to why PT cells are more sensitive to ischemia. Application and extension to individual mitochondria within cells, quantifying their response to stress and toxins, and use of this knowledge to understand therapies further await future studies.[13,14] Indeed, the future is bright for multiphoton microscopy's advancing our mechanistic understanding to individual cells and organelles with enhanced spatial and temporal resolution and increased imaging depth.

ACKNOWLEDGMENTS

J.M.W. is funded by National Institutes of Health grant DK034275, and B.A.M. is funded from National Institutes of Health grants P30-DK079312 and R01-DK069408, a VA Merit Review, and an INGEN (Indiana Genomics Initiative) grant from the Lilly Foundation to Indiana University School of Medicine.

DISCLOSURES

None.

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See related article, "Multiphoton Imaging Reveals Differences in Mitochondrial Function between Nephron Segments," on pages 1293–1302.

Splicing a Kinase and the Regulation of Salt Transport

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J Am Soc Nephrol 20: 1166–1168, 2009.
doi: 10.1681/ASN.2009040410

Thiazide-sensitive Na^+-Cl^- co-transport by the aldosterone-sensitive distal convoluted tubule (DCT) is responsible for reabsorption of approximately 10% of filtered Na^+ and Cl^-.[1] The considerable clinical importance of Na^+ and Cl^- reabsorption by the DCT is illustrated by the therapeutic efficacy of thiazide diuretics and the clinical phenotype of Gitelman syndrome,[2] caused almost exclusively by loss-of-function mutations in the thiazide-sensitive Na^+-Cl^- co-transporter (NCC). A gain-in-function of NCC occurs in pseudohypoaldosteronism type II (PHA-II; also known as Gordon syndrome or hereditary hypertension with hyperkalemia); however, this disorder is caused by mutations not in NCC but in two of the four WNK (with no K/lysine) kinases, so-named for the absence of a conserved catalytic lysine.[3,4]

Since the seminal genetic study implicating WNK1 and WNK4 in PHA-II,[5] an increasingly populous signaling complex has been uncovered, encompassing WNK1, WNK3, WNK4 and the downstream STE20/SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress-responsive kinase 1 (OSR1).[3,4] WNK1,[5] WNK3,[6] WNK4,[5] and SPAK[7] all are coexpressed with NCC in the DCT. Elements of this pathway also regulate the Cl^- and volume sensitivity of all functional members of the cation-chloride co-transporter gene family, including NCC, the two Na^+-K^+-$2Cl^-$ co-transporters (NKCC1 and NKCC2), and the four K^+-Cl^- co-transporters (KCC1 through 4).[3,4] More recently, there are outlines of the interactions of this pathway with aldosterone[8] and angiotensin-dependent[9] regulation of NCC. Variation in the human

Published online ahead of print. Publication date available at www.jasn.org.

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