Current perspective

Multiphoton imaging of kidney pathophysiology

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ABSTRACT

The number of people being diagnosed with end-stage renal disease is increasing globally. Therapeutic options to slow or halt the progression of kidney disease are limited and are not always successful, despite the increasing body of research and number of basic scientific reports in this field. Further studies are required to investigate new approaches to renal pathophysiology. State of the art optical imaging is a powerful tool used to non-invasively observe the pathophysiology of small animals and has the potential to elucidate the unknown mechanisms of renal disease and aid in our understanding of the disease. This paper is a brief summary of the current usefulness of intravital imaging using multiphoton microscopy and discusses possible future applications of the technique.

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1. Introduction

Multiphoton microscopy is a powerful tool that can be used to analyze physiology in vivo. Although there were initial technical difficulties in observing peripheral organs, especially by using an upright microscope, several supporting devices have been developed and it is now easier to access the target organs. Additionally, developments in biotechnology have allowed for targeted cell fluorescence in mice and rats. While studies using these genetically modified mice and rats have shown the usefulness of intravital imaging to address a number of physiological questions, there are alternative techniques to visualize the physiology of animals. This article reviews the current availability of intravital imaging using multiphoton microscopy for the analysis of renal pathophysiology, such as acute kidney injury and glomerular permeability, especially focusing on techniques that do not use genetically modified mice, and also examines pharmacological experiments and discusses the future application of such methods for the treatment of kidney disease.

2. Acute kidney injury

Acute kidney injury (AKI) is a common clinical syndrome defined as the sudden onset of reduced renal function and characterized by a reduction in the glomerular filtration rate (GFR) and oliguria (1,2). Although basic research has identified several pathways involved in the mechanisms of AKI (3–6) which has enabled the development of new therapeutic strategies, specific treatments that can improve AKI outcome are not yet clinically available (7–9). Molitoris et al. from Indianapolis University are the first to introduce multiphoton in vivo imaging (10) as a useful tool to detect dynamic changes in the renal environment during AKI. Even though there are limits in accessibility, with observation being restricted to superficial areas (<100 μm from the surface), this technique, combined with the genetic engineering of mice, holds promise in exploring the hidden mechanisms behind AKI pathology and for evaluation of the efficacy of new therapeutic strategies (11).

Acute tubular necrosis, cell sloughing, and cast formation are typical histopathological changes observed in ischemic AKI. These pathological changes are often seen in the medullary region, but also in the cortical region using in vivo multiphoton imaging (Figs. 1 and 2, and Supplementary Movie 1, 2 and 3).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2016.08.001.

As seen in Fig. 1 and Supplementary Movie 1 and 2, we were able to observe both tubular cell sloughing and the subsequent migration of tubular cells in the wound healing process in vivo. It is classically hypothesized that the ‘hole’ in the proximal tubular monolayer after cell detachment closes with the migration of surrounding cells that have survived the trauma. To the best of our knowledge, our data are the first to confirm this hypothesis.
Surprisingly, cell sloughing quickly triggered the observed migration of cells, and the wound created by the loss of the two cells in the image healed within 300 s (Fig. 1B). This quick healing response may preserve the tubular monolayer after massive numbers of cell death even though it results in a shortening of the nephron length (12). We also observed that cellular cast formation could occur within 1 min (Fig. 2, and Supplementary Movie 3) but failed to observe other events such as clearance of debris (13,14) and repair of tubules with the proliferation of cells that survived (15). These events may take a longer time to occur in comparison with our observation time (24–25 h after ischemia/reperfusion injury), or occur deeper within the nephron, such as in the medullary region.
which is known to show the most severe damage after ischemia/reperfusion injury.

AKI induces irregular capillary blood flow and a reduction/cessation in the supply of oxygen from red blood cells (16–18). Prolonged hypoxia, which potentially accelerates atherosclerotic changes of vessels (19), limits mitochondrial activity and ATP production. As tubular cells require large amounts of energy not only for cell survival but also for reabsorption of electrolytes and other substances, hypoxia further exacerbates the injury.

Mitochondria play an important role in recovery from damage induced by AKI (20,21), and are now a therapeutic target (22). It would be very useful if multiphoton imaging enabled the measurement of mitochondrial activity and ATP production in vivo. Hall et al. (23) have reported that changes in mitochondrial membrane potential were able to be visualized using NADH-derived autofluorescence or the fluorescence dye tetramethylrhodamine methyl ester. We found large heterogeneity in mitochondrial membrane potential measured using tetramethylrhodamine methyl ester at the kidney surface, and significant co-localization between a low tetramethylrhodamine methyl ester signal and disrupted blood cell flow in adjacent capillaries in the endotoxicemic kidney (18). Visualization of ATP at high resolution using multiphoton microscopy is still being developed.

Autophagy has been implicated as a compensatory phenomenon against the development of tubular damage (24–27). There are several challenges with transgenic models aimed at visualizing autophagic flux in vivo (28–30), as it is recognized that simply measuring the amount of one molecule, such as LC-3, does not reflect autophagy (31). To the best of the authors’ knowledge, there is little information on the involvement of dynamic functional changes in AKI, such as hemodynamic changes and autophagy.

Analysis of ischemic AKI can be performed as either a histopathological or a physiological (functional) examination. Notably, only modest histological changes in the kidney have been observed in septic AKI (32,33), one of the major causes of AKI with a high mortality rate. Such limited observations using a histopathological approach suggest the need for further studies of septic AKI using a physiological approach. Oliguria is one of the major functional changes seen with AKI and is a criterion for AKI (34–36). Reduction in urine output is thought to depend on a reduced GFR induced by hypoperfusion and/or intravascular depletion. In the intensive care unit, maintaining hemodynamics by fluid resuscitation and treatment with additional vasoactive agents frequently fails to restore urine output. The mechanism by which urine output decreases or is halted, even when renal circulation appears to be normally maintained, has not yet been fully clarified. Furthermore, there is no consensus on the use of diuretics for oliguric patients. Consideration of these problems led us to examine the physiology of septic AKI using multiphoton microscopy in vivo (37–39). Intravital imaging enables real-time in vivo analysis of intra-tubular urine flow rate after a bolus injection of freely filtered dye (such as conjugated inulin with fluorescein isothiocyanate), and this simple technique can directly measure the post-glomerular urine flow rate in the kidneys of experimental animals. Analysis of the intra-tubular urine flow rate using multiphoton microscopy revealed that lipopolysaccharide signaling through a toll-like receptor 4-dependent mechanism is associated with a reduced intra-proximal tubular urine flow rate (18). Neither hypotension nor reduction in GFR were likely the essential factor initiating oliguria, suggesting the potential need for a therapy targeting proximal tubular injury in addition to hemodynamic maintenance in AKI patients.

Ascending urinary tract infection in pyelonephritis causes acute renal failure as well as AKI. Richter-Dahlors’ group (40–42) visualized an Escherichia coli infection by microinjection of green fluorescent protein-expressing E. coli into either the bladder or tubules. The studies revealed processes of colonization, infection of the closed circulation system, tubular obstruction by the colony, and microcirculation failure in the peritubular capillaries. Although the studies used adult rats, this technique is applicable to infant rats or mice and could aid research on pediatric pyelonephritis.

3. Glomerular filtration barrier

Multiphoton microscopy is now used for the imaging of glomerular function in Munich Wistar or Munich Wistar Frömter rats, with these strains having glomeruli through the juxtamedullary to just beneath the kidney capsule, and young mice. An alternative method is to transplant glomeruli into the anterior chamber of the eye, which enables non-invasive long-term serial analysis (43).

Measuring the glomerular sieving coefficient (GSC) has drawn increasing interest from researchers. GSC used to be measured using micro-puncture (44), which involves a relatively difficult technique and careful handling of equipment and samples. It was thought that multiphoton microscopy would enable the quick and easy analysis of protein filtration from glomeruli by comparing the fluorescence intensity of the fluorophore-conjugated target protein in plasma and Bowman’s space. As it turns out, multiphoton analysis of GSC is influenced by the many possible experimental settings (45), resulting in international debate on the analysis technique.

Limitations of multiphoton analysis of GSC include the following: 1) part of the protein conjugated to the dye might be nonspecifically catalyzed into small fragments in the blood and filtered by glomeruli, which gain the fluorescent intensity in the Bowman’s space independent of the filtration of full-length protein; 2) detector settings could artificially overestimate fluorescence intensity in the Bowan’s space; and 3) as we could analyze GSC only from a small number of glomeruli in the kidney (<20 superficial glomeruli from a rat and a few from a mouse) in vivo even by using multiphoton microscopy, it is not possible to propose that GSC values measured by multiphoton images are representative of GSC of all nephrons, although GSC values measured by multiphoton microscopy are strongly correlated with albuminuria in Munich Wistar Frömter rats (39). Furthermore, pathological changes in glomeruli are observed relatively early in juxtamedullary glomeruli, which are inaccessible by multiphoton imaging, compared with superficial glomeruli (46); therefore, GSC values of juxtamedullary glomeruli might be higher than values reported in the study using multiphoton microscopy. The ‘true’ GSC value would be even smaller than that which the researchers recorded using a two-photon microscopy technique (39). Indeed, in a recent study by Mori et al., the whole kidney GSC using mice absent for megalin, a protein responsible for clathrin-dependent endocytosis in renal proximal tubules, was reported to be lower than 0.00002 (47).

GSC analysis using multiphoton microscopy would be useful for comparing inter-group differences (11,39,48–50) rather than analyzing the absolute value of GSC itself (though there are limitations in terms of glomeruli number and region at present). Comparing the GSC at the same experimental setting could reveal the protective/exaggerative effects of drugs/gene modification on the glomerular filtration barrier. In addition, multiphoton analysis of glomeruli could aid in examining cell–cell interactions and subsequent changes in GSC in the glomerular filtration barrier (51,52). Studies are, to date, mainly focusing on podocyte–podocyte or podocyte–parietal cell interactions. Peti-Peterdi’s group used genetically modified podocyte-specific...
calcium indicator mice to demonstrate a rapid gain in the transfer of intracellular Ca\textsuperscript{2+} through a podocyte to adjacent podocytes via the ATP/purine receptor-dependent pathway \((51)\). It is theoretically possible to examine the interaction of each cell in glomeruli; for example, between endothelial cells and podocytes, podocytes and parietal cells \((52)\), and leukocytes and endothelial cells \((53)\).

Proximal tubules reabsorb protein leaked from the glomerular filtration barrier. There is a detectable intensity in fluorescence in proximal tubules of animals that have been administered fluorophore-conjugated proteins intravenously, indicating proximal tubular uptake of the filtered proteins. However, we do not recommend quantitative analysis of tubular uptake of dye-conjugated proteins for the following reasons. First, tubular fluorescence intensity will be influenced by the distance from the glomerulus (upstream tubules have an increased uptake). Therefore, proximal tubular fluorescence intensity should only be analyzed at the origin of the proximal tubule, this being the S1 segment. However, the depth of the S1 segment from the kidney surface is different for each glomerulus, which means that it is not possible to analyze tubular fluorescence intensity using the same laser power and detector settings. We cannot eliminate the possibility that both the capsule itself and the blood cells in stellate veins scatter the fluorescence even if glomeruli exist at the ‘surface’ of the kidney in Munich Wistar strains. Second, since there is a difference in the sieving coefficient for each glomerulus, it is necessary to analyze multiple S1 segments from one kidney, which is very difficult to do in mice. Third, few studies have clarified the basal metabolism of the target protein that is conjugated with the fluorophore in vivo, and it is hard to distinguish whether: 1) small fragmented proteins in plasma that are still conjugated to the dye passed through the glomerular filtration barrier and were taken up and accumulated in proximal tubules; or 2) dye-conjugated full length proteins passed through the glomerular filtration barrier and were taken up in proximal tubules and degraded in lysosomes, accumulating as small fragments in proximal tubules. We observed that the majority of fluorophore-conjugated proteins in the kidney parenchyma had been degraded into small fragments (>15 kDa) within 90 min after intravenous injection \((39)\). Therefore, it is difficult to assess what amount of the target protein (and not already fragmented metabolites) is actually taken up in proximal tubules. Taken together, it is suggested that using multiphoton microscopy for quantitative analysis of proximal tubular protein uptake is not suitable.

4. Conclusion

Intravital imaging of the kidney with multiphoton microscopy has the potential to help answer questions on renal physiology, even without the use of gene-modified fluorescent mice; for example, blood cell velocity, tubular flow rate, mitochondrial membrane potential, and glomerular permeability. The pathophysiological features explored and identified using intravital imaging could be targets for drug therapy and further pharmacological studies.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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