USING 2-PHOTON MICROSCOPY TO UNDERSTAND ALBUMINURIA

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ABSTRACT

Intravital 2-photon microscopy, along with the development of fluorescent probes and innovative software, has rapidly advanced the study of intracellular and intercellular processes at the organ level. Researchers can quantify the distribution, behavior, and dynamic interactions of up to four labeled chemical probes and proteins simultaneously and repeatedly in four dimensions (3D + time) with subcellular resolution in real time. Transgenic fluorescently labeled proteins, delivery of plasmids, and photoactivatable probes enhance these possibilities. Thus, multi-photon microscopy has greatly extended our ability to understand cell biology intravitally at cellular and subcellular levels. For example, evaluation of rat surface glomeruli and accompanying proximal tubules has shown the long held paradigm regarding limited albumin filtration under physiologic conditions is to be questioned. Furthermore, the role of proximal tubules in determining albuminuria under physiologic and disease conditions was supported by direct visualization and quantitative analysis.

INTRODUCTION

Multiphoton microscopy (MPM) has equipped investigators with novel techniques to uniquely address biologically important questions that can only be accomplished at the organ level (1–6). In parallel, advances in fluorophores with increased quantum yields and ease of labeling (7–9), molecular and transgenic approaches, and new delivery techniques have allowed for the development of intravital studies with remarkable spatial and temporal resolution and sensitivity at subcelluar levels (5, 10, 11). MPM is uniquely positioned to complement other *in vivo* biochemical and molecular techniques. However, MPM lacks deep tissue penetration, limiting its use in clinical situations. Exponential developments in computer software and hardware have re-

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Potential Conflicts of Interest: None disclosed

moved many obstacles previously limiting the use of MPM to observe and quantify dynamic cellular and subcellular processes (12, 13). In particular, developments in hardware, software, bandwidth, and data storage now provide systems that possess the necessary speed to effectively and efficiently approach data intensive processes using digital imaging analysis. These imaging technologies enable the dynamic measurement of four-dimensional (3-D plus time) using fluorescently labeled molecular agents including drugs and proteins. Of paramount importance, it allows for simultaneous quantification of the rates of multiple physiological processes and correlation with structural events. This disruptive technology has already brought into question many previously held paradigms and many more will follow.

THE GLOMERULAR-TUBULAR UNIT

Figure 1 shows the glomerulus and proximal tubule (PT) portion of the nephron. Although universally considered two distinct functional units, the function of each component is dependent on the other. For instance, blood flow to peritubular capillaries must first go through the glomerulus. Furthermore, interruption of function of either component results in paralysis of the other, and fine tuning of each component's function is determined by the other. Renal physiologists have prided themselves in understanding these interactions and dependencies, and yet the assignment of pathologic processes to one or the other component has been the dogma.

Proteinuria is an excellent example of this approach. Proteinuria has been defined as glomerular or tubular depending on the size of the proteins involved. If a protein is greater than say 40 kDa, then by definition it must have leaked through the glomerular filter otherwise known as the glomerular filtration barrier. By definition, only proteins in the 20-kDa range end up in the urine following minimally restricted filtration and subsequent proximal tubule dysfunction and lack of endocytic uptake. The idea that albumin (69 kDa) would end up in the urine due to tubular dysfunction is still considered heresy by many. Yet, isolated proximal tubule studies, dating back to the 1970s, documented proximal tubule uptake and transcytosis of albumin (14). Furthermore, early studies using selective pharmacologic-induced PT injury documented nephrotic range proteinuria (15). Now, in the past year, using molecular approaches to selective injury to the PT, three different investigative groups have shown that selective injury to the PT results in nephrotic range proteinuria that increases as PT cell (PTC) dysfunction occurs and resolves with PT repair (16–18). Finally,

recent data using enhanced scanning electron microscopy have shown that podocyte slit diaphragm pore size is much larger than previously thought, and is sufficiently large enough to allow for albumin filtration (19).

ALBUMIN FILTRATION ACROSS THE GLOMERULUS: QUANTITATION

Numerous techniques and experimental approaches have been used to determine the quantitative role of glomerular albumin permeability and PTC reabsorption/catabolism in albuminuria. Values for the glomerular sieving coefficient of albumin have ranged from 0.000077 to 0.07 under various physiologic and pathologic conditions (20). Of particular importance has been the use of Munich-Wistar (MW) rats that have surface glomeruli allowing for direct dynamic visualization, instrumentation, and manipulation. Munich-Wistar Fromter (MWF) rats have a large number of surface glomeruli, have been used in micropuncture studies, and spontaneously develop hypertension and progressive albuminuria beginning by week 8 and increasing to >300 mg/24h urinary albumin excretion by week 32. By week 40, 50% of glomeruli are sclerotic (21–23). Munich-Wistar Simonsen (MWS) rats have fewer surface glomeruli and do not develop spontaneous kidney failure or albuminuria under physiologic conditions. These rat models are useful in helping to identify structural and functional changes in disease states in which excess protein is found in the urine. Mice and other strains of rats unfortunately lack surface glomeruli; therefore, direct visualization methods cannot be utilized, unless pathologic processes, such as ureteral obstruction for several days, are used (24).

Prior micropuncture studies in Sprague-Dawley or MWF rats with surface glomeruli measured low glomerular filtration of albumin in fasting states, with a glomerular sieving coefficient (GSC) of 0.00057–0.00062, consistent with low amounts of measured albumin observed in excreted urine (<30 mg/d) (25–27). This has been attributed to the charge barrier and size selectivity at the glomerular filtration barrier. Previous *in vivo* rat filtration studies and noninvasive studies by one group using isolated perfused rat kidneys showed a much higher GSC of albumin using [3H]-albumin. By measuring total radioactivity in urine, and by inhibiting protein uptake in the PTC, it was shown that the GSC of albumin may actually be approximately 0.074 — more than 120-fold greater than previously thought (28). High GSCs for albumin were also observed by another group using glomerular volumetric analysis in rat glomeruli (0.02 ± 0.01) (29). This finding was strength-

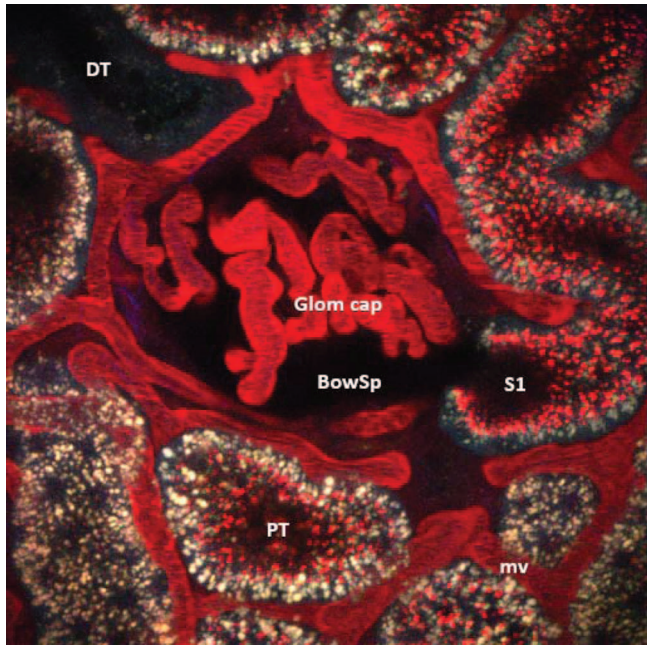

FIG. 1. Texas Red Rat Serum Albumin uptake by proximal tubule cells. A 12-micron volume of a superficial glomerulus given a single bolus of labeled albumin (red) ~20 minutes post-infusion shows avid uptake in the early S1 segment and other proximal tubules (PT). Note the absence of labeled albumin in distal tubules (DT), corroborating the high capacity of PTs to internalize and transcytose filtered albumin. (Abbreviations: mv, microvasculature; Glom cap, glomerular capillary loops; BowSp, Bowman's space; Bar = 20 μm.)

ened by other investigators showing that high–molecular weight dextrans, with similar radii and molecular weights as albumin (3.6 nm, 66 kDa) and not reabsorbed through receptor-mediated endocytosis, had

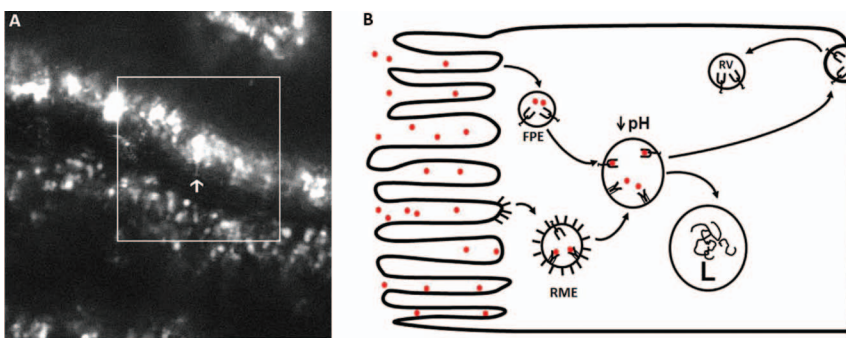

FIG. 2.

comparable high GSCs in normal kidneys: pancreatic isoamylase (3.4 nm, 45 kDa, GSC 0.03) (30); horseradish peroxidase (3.0 nm, 40 kDa, GSC 0.06); Bence-Jones protein (2.8 nm, 44 kDa, GSC 0.09) (31). Furthermore, large single-wall carbon nanotubes, which are 100–500 nm and ~350–500 kDa in size, were shown to be filtered through the normal glomerulus and remain intact before being taken up partially by PTCs (32).

Intravital 2-photon microscopy allows direct visualization and quantification of GSCs of albumin (GSC_A) and proximal tubule endocytosis of the filtered albumin. It has also allowed direct visualization of subcellular trafficking, transcytosis, catabolism, and reclamation of proteins and other molecules from glomerular filtrate by PTCs (3, 33). It provides real-time imaging and is minimally invasive (the kidney is exteriorized yet remains itself completely intact and fully functional), is non-toxic, non-disruptive, and mirrors normal physiology most closely, compared to other techniques. MWS rats, which do not develop spontaneous albuminuria, have a GSC_A of 0.034 under physiologic fed states, while simultaneously measuring a GSC of 1.0 for inulin and approximately a 500-fold lower GSC for high–molecular weight dextrans (34, 35). The GSC_A for MWS rats in fasting states is considerably lower at 0.016 (36). MWF rats, which develop albuminuria spontaneously with aging, have a lower fed GSC_A of 0.010 and also display a GSC_A reduction in fasting states to 0.007. These studies indicate that the GSC for albumin is higher than previously thought, that feeding has significant impact on glomerular albumin filtration (36), and albumin filtration differs markedly in two genetically related rat strains.

FIG. 2. Transcytosis of albumin across proximal tubule cells in vivo using 2-photon microscopy. **(A)** Two-photon intra-vital time image taken in a Simonsen Munich Wistar rat given 2 mg of Alexa 568-RSA intravenously 24 hours before imaging shows vesicular and tubular structures containing albumin. A single frame with a large accumulation of albumin is indicated by the arrow; note the orientation of the apical membrane is opposite the arrow. The formation of a tubular structure extending from an intracellular compartment toward the basal pole of the PTC is shown at the end of the arrow. **(B)** Schematic of albumin entering into a PTC either via unbound in a fluid phase vesicle/endosome (FPE) or bound to megalin-cubulin as a receptor mediated endosome (RME) at the apical surface via a clatherin coated pit. With acidification to a pH of less than or equal to 6, the megalin-cubulin binding of albumin diminishes while that of FcRn increases dramatically. As such, there is an exchange of albumin from megalin-cubulin to binding to FcRn and this carrier then mediates transcytosis. When the transcytotic vesicle unites with the basolateral membrane, the increase in pH of the interstitial compartment releases albumin to diffuse into the interstitium and be transcytosed across endothelial cells again using FcRn as the carrier. FcRn then recycles to the apical membrane area via the recycling vesicle (RV). L stands for lysosome.

The MPM data documenting a higher than anticipated GSC for albumin immediately caused great skepticism within the nephrology community (26, 37, 38). Indeed, conflicting MPM data were generated indicating that the GSC for albumin is lower than we have reported (39–41). However, these data were subsequently shown to be inaccurate primarily based on using a background cut-off that removed important fluorescent data, thus reducing sensitivity and resulting in an erroneously low GSC (42). However, the controversy remains and one must proceed with caution.

THE PROXIMAL TUBULE: ALBUMIN REABSORPTION AND TRANSCYTOSIS

Classically, proximal tubule uptake of proteins and other molecules by endocytic pathways has been attributed to receptor-mediated endocytosis by apical membrane-bound receptors, such as megalin and cubilin clustering into clathrin-coated pits. These pathways have been studied extensively and numerous reviews exist (43, 44). In addition, other mechanisms of protein internalization have also been described, including caveolin-dependent internalization and fluid-phase endocytosis (Figure 2). Molecules endocytosed in this manner are similarly routed to the sorting endosomal compartment and are either degraded through lysosomal pathways or undergo transcytosis back into circulation (45). Albumin uptake by nonselective fluid-phase endocytosis is likely a quantitatively important process in PTCs as shown by the rapid cellular uptake of molecules not having receptors on the apical membrane such as neutral fluorescent dextrans — markers of fluid-phase endocytosis (46, 47).

The endocytic apparatus is found throughout the proximal tubule, although clathrin-coated pits and vesicles are notably less in the S3 segment (48). Protein reabsorption and degradation are greatest in the S1 segment of the PTC and least in the S3 segment (49). Kinetic studies of the rat PT have shown that internalization of cargo at the brush border is highly active. The amount of membrane contained in the apical membrane invaginations is internalized within 78 seconds (50). This rapid rate of turnover means that a great deal of luminal fluid is internalized via endocytic vesicles and likely indicates an important role for fluid phase endocytosis. However, there has been great difficulty in quantifying the overall importance of fluid phase endocytosis as all endocytic vesicles contain fluid and thus luminal contents.

The megalin-cubilin receptor complex is well studied and recent reviews to describe its function and role in protein absorption and

metabolism (43, 51). The dissociation coefficient (K_d) of albumin to cubilin is very low and is estimated at 0.63 μM at pH 7.0 (52), resulting in a high-affinity, low-capacity pathway of endocytosis that primarily targets proteins to the lysosome for degradation. Megalin and cubilin work in concert to reabsorb more than 40 filtered molecules (51). In megalin knockout models, the internalization of endogenous ligands bound to apical cubilin, especially cubilin-albumin complexes, is markedly reduced. Urinary albumin excretion is increased 6-fold in cubilin knockout mouse models (53) and in humans (54), although neither reaches nephrotic range; this suggests that an additional mechanism(s) for albumin reabsorption exists. In Dab2 knockout mice — a protein involved in coated pit formation — mild proteinuria was found (55). Type I diabetic patients with albuminuria were found to have significantly elevated urinary levels of megalin and cubilin, suggesting possible proximal tubule shedding of these proteins as a contributing factor to albuminuria (56). Also, in early streptozoticin-induced diabetes in rats, the GSC_A was unchanged but PTC uptake of albumin was markedly reduced (34).

The Neonatal Fc Receptor and Its Role in Albumin Transcytosis

The neonatal Fc receptor (FcRn) is a heterodimer with class I major histocompatibility (MHC)–like properties, containing a membrane-bound heavy chain and a β2-microglobulin light chain. Wild-type FcRn has two separate and distinct binding sites for albumin and immunoglobulin G (IgG) (57), with binding being low-affinity and high-capacity at physiologic pH with increasing affinity occurring dramatically at lower pH. It is known to be resident on vascular endothelium; epithelial cells of the proximal small intestine, liver, spleen, and lung; placental syncytiotrophoblasts; polymorphonuclear neutrophils, monocytes, and phagocytes; dendritic cells; and in the kidney (58, 59). Within the kidney, FcRn is found in the vascular endothelia, podocytes, cortical collecting duct, and PT epithelial cells (60). In human kidney sections, it is found at the brush border of PTCs and in endosomes (60).

FcRn is known to transport albumin across membranes, preserving albumin's function and life span as a carrier protein, colloid, buffer, and one that maintains oncotic hemostasis. Overexpression of FcRn in transgenic mice and rabbits increases serum albumin concentrations and generates a 3- to 10-fold increase in IgM and IgG concentrations in serum (61). FcRn is believed to mediate transcytosis and recycling of

IgG by PTCs back into circulation (62). The mechanism of FcRn-mediated transcytosis has been well studied in the small intestine and its role in IgG endocytosis via clathrin-coated pits at low luminal pH is known (63, 64).

The FcRn receptor mediates intracellular selection, sorting, and preservation of reabsorbed albumin and IgG. FcRn is concentrated into the apical area in the PT. Whether it participates in luminal albumin binding is not known, but this would not be favored by luminal pH. However, at the low pH found in endosomes, albumin dissociates from megalin-cubilin, whereas FcRn's albumin-binding affinity increases from 34–408 μM at pH 7 to 0.2–0.7 μM at pH 5 (65). Thus, albumin is likely capable of moving from a low-capacity lysosomal degradation pathway (66) to enter a high-capacity pathway of transcytosis and recycling mediated by FcRn based on inherent binding properties of the receptors (67, 68). When the trancytotic vesicle fuses with the plasma membrane and encounters neutral physiological pH, a rapid dissociation of albumin from FcRn would occur releasing it to the interstitium.

The first direct evidence for transcytosis of albumin came from PT microperfusion studies (14). Subsequent studies using transmission electronic microscopy immunogold studies revealed albumin uptake across the apical membrane and release across the basolateral membrane of PTC (35). Multiphoton studies showed actual intracellular vesicles and tubules uniting with the basolateral membrane and releasing fluorescently labeled albumin into the interstitium (36). Finally, Tenten (69) showed a definitive role for FcRn mediating albumin transcytosis in PTC (Figure 2). However, the magnitude of this process remains to be determined. Finally, additional information highlighting the role of FcRn in albumin reabsorption by PTC comes from FcRn knockout mice lacking the neonatal Fc receptor. In these mice, the plasma albumin half-lives were reduced to 75% of wild-type and plasma concentrations were reduced by ~50% (70–72). This new equilibrium state probably results from the lack of transcytosis by PTC after filtration and a new set point for serum albumin concentration and glomerular filtration is achieved.

CLINICAL SIGNIFICANCE

These data imply that both glomerular permeability and PT reabsorption and transcytosis play significant and likely interactive roles in determining albuminuria. Under physiologic states, the amount of albumin filtered by the glomerulus can be effectively and efficiently

reabsorbed by the PTC. Whether the PTC process is saturated or inducible remains to be determined. We have shown that an increase in albumin filtration mediated by endothelin infusion can be compensated for by increased PTC endocytosis (73). Also, data indicate that feeding and genetics influence the GSC_A (36). This could be playing a role in diabetic kidney disease and variability in GSC_A could influence progression in chronic kidney disease. Finally, that selective injury to PTCs has been shown by three investigative teams to lead to massive proteinuria and albuminuria in a time course consistent with PTC dysfunction highlights the potential importance of the PTC in albumin homeostasis (16, 17).

ACKNOWLEDGMENTS

The authors acknowledge grant support to BAM from the National Institutes of Health, DK 091623, 088934, and 079312, and support from the Veterans Administration through a Merit Review award.

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DISCUSSION

Zeidel, Boston: Bruce, absolutely wonderful work. I know how long it took to get the technical aspects of this to work. When you can make new measurements and look at things in new ways, you come up with new information, which is what's really terrific. So, there are a lot of models of kidney injury that have been studied over the years — including things like crush injury with injection of glycerol into the muscle and puromycin and other things — which have been touted as glomerular injuries but, in fact, probably aren't. So, if you take your work and what Joe was showing us, it's sounding more and more like often the first injury we are talking about is something in the tubule, which then results in glomerular damage. Are you beginning to use this to look at specific injury models? That's the first question; and the second question: is there any way that you can get a mouse into your machine and watch this going on in mice, because obviously the ability to do mutagenesis and other studies would be really powerful?

Molitoris, Indianapolis: So first question — and thank you, Mark, for those questions — is are we looking at other models. Two of the models we've studied pretty extensively include the albumin overload, that may reduce neonatal Fc receptor (FcRn), and in CKD we find both an increased glomerular sieving coefficient and reduced proximal tubule uptake. We also have Roger Wiggin's rats where he has put diphtheria toxin into the podocytes and we can give small, medium, and large doses, and we can regulate the sieving coefficient across the glomerulus and then look at the proximal tubular cell response. So, yes, we are trying to rip this apart. Also, mice can be studied.

DuBose, Winston Salem: Thank you very much, Bruce, for a spectacular presentation. Something of a modification of Mark's question, but in the initial uptake step at the

apical membrane endocytosis, the H^+ ATPase is playing a major role in regulating that pH that is so critical to the transcytosis of albumin. I wonder if you have considered examining some of the models of knockouts of various subunits of the H^+ ATPase to see if it modifies or impedes transcytosis.

Molitoris, Indianapolis: You could evaluate those animals. We're still trying to determine the disease-specific aspects of this before we dive off into the molecular mechanisms.