Fluid flow in the juxtaglomerular interstitium visualized in vivo

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The Juxtaglomerular Apparatus (JGA) is a major structural component of the renin-angiotensin system and is an important regulatory site of renal salt and water conservation and systemic blood pressure homeostasis. Major functions of the JGA include the feedback regulation of glomerular filtration rate (tubuloglomerular feedback, TGF) and renin release from JG granular cells. These mechanisms are based on the salt composition and balance in the JGA (and consequently the extraglomerular mesangial interstitium (25), and the demonstration of high endothelial permeability in the JGA using ferritin particles (26, 27). These studies suggested that the fluid composition and balance in the JGA (and consequently the TGF and renin release mechanisms) do not depend solely on the reabsorptive transport of the MD, but also on the rate of bulk fluid flow directed to the JGA interstitium (25, 27, 30). The existence of significant fluid flow, however, has been difficult to study due to the small size and inaccessibility of the JGA.

Multiphoton excitation fluorescence microscopy offers a state-of-the-art imaging technique for the visualization of dynamic renal processes in real-time (7, 10, 14, 19–21). Noninvasive, quantitative imaging of basic functions in the intact kidney has been recently reported using this technology (10, 39). The processes of fluid filtration through the glomerular barrier, glomerular permeability, tubular fluid flow, and capillary blood flow have been visualized and quantified with high optical and temporal resolution (10, 14, 39).

The aim of the present study was to directly visualize the putative fluid flow in the JGA using in vivo multiphoton imaging. Both morphological and functional signs of a significant and dynamic interstitial fluid flow are presented here, challenging the existing paradigm of the stable and isolated JGA environment.

MATERIALS AND METHODS

Multiphoton excitation laser-scanning fluorescence microscopy. The multiphoton microscope used in these studies was a Leica TCS SP2 AOBS MP confocal microscope system (Leica Microsystems, Wetzlar, Germany).

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Heidelberg, Germany) with the following components: a Leica DM IRE2 inverted microscope powered by a wideband, fully automated, infrared (710–920 nm) combined photo-diode pump laser and mode-locked titanium: sapphire laser (Mai-Tai, Spectra-Physics, Mountain View, CA). Images were collected in time (xyt) series (0.5–5 Hz) with the Leica Confocal Software (LCS 2.61.1537) and analyzed with the LCS 3D, Process, and Quantify packages. The principles and advantages of conventional confocal and two-photon microscopy and their application in imaging renal tissues have been recently reviewed (14, 21).

Nontoxic, water-soluble fluorophores were injected intravenously to label specific structures in the living kidney as described before (10). Briefly, a 70-kDa dextran-rhodamine B conjugate (Invitrogen) was used to label the circulating plasma or intravascular space. Tubular segments and more specifically the content of individual renin granules were visualized using quinacrine (1, 10, 20, 21). The extracellular fluid marker lucifer yellow (LY, Invitrogen) was used to visualize interstitial fluid movements (6), since LY is impermeable to cell membranes but freely filtered through basement membranes (10) due to its small size (0.45 kDa). All three of the fluorescent probes were excited using the same, single excitation wavelength of 860 nm (Mai-Tai, Spectra-Physics), and the emitted, nondescanned fluorescent light was detected by two external photomultipliers (green and red channels) with the help of a FITC/TRITC filter block (Leica).

Animals. All animal protocols have been approved by the Institutional Animal Care and Use Committee at the University of Southern California. Munich-Wistar male rats (200 g, Harlan, Madison, WI) were maintained on standard diet, while C57/B6 mice (20 g, inbred) were fed either a standard or a low-fat diet (TD 90228: 0.01% NaCl, Harlan) rodent diet for up to 1 wk. Animals were anesthetized with thiobutabarbital (Inactin, 130 mg/kg body wt) alone (for rats) or in combination with 50 mg/kg body wt ketamine (for mice). After assuring adequate anesthesia, the trachea was cannulated to facilitate breathing. The left femoral vein and artery were cannulated for dye infusion and blood pressure measurements, respectively. Subsequently, a 10– to 15-mm dorsal incision was made under sterile conditions and the kidney was exteriorized. To image the JGA in the mouse kidney, a less than 1-mm-thin cortical slice was removed (parenchymal window) to allow imaging of the most superficial AA and glomeruli. Bleeding was minimal and stopped spontaneously within 2 min. Imaging through the thin blood clot did not cause any apparent changes in image quality or in the characteristics of fluorescent probes. This procedure was not required for rats, since Munich-Wistar rats have superficial glomeruli. The animal was placed on the stage of an inverted microscope with the exposed kidney placed in a coverslip-bottomed heating chamber bathed in normal saline and the kidney was visualized from below as described by Dunn et al. (7) using a HCX PL APO 63X/1.4NA oil CS objective (Leica). During all procedures and imaging, core body temperature was maintained with a homeothermic table.

During in vivo imaging, systemic blood pressure of animals was monitored through a cannula inserted into the left femoral artery and using an analog single-channel transducer signal conditioner model BP-1 (World Precision Instruments, Sarasota, FL), transducer model BLPR. Calibration was performed using a Pressure manometer model PM-015 and data were collected using data-acquisition system QUAD-161.

In vitro isolated and microperfused AA-JGA-glomerulus. A superficial AA with its glomerulus and attached distal tubule containing the MD was microdissected from kidneys of New Zealand white female rabbits (500 g, Irish Farms, Norco, CA) and microperfused as described before (21). Briefly, the dissection medium was prepared from DMEM (DME mixture F-12; Sigma) with the addition of 1.2 g/l NaHCO3, and 3% fetal bovine serum (Hyclone). Before use, this solution was aerated with 95% O2-5% CO2 for 45 min, and the pH was adjusted to 7.4. The arteriole was perfused with a modified Krebs-Ringer-HCO3 buffer containing (in mM) 115 NaCl, 5 KCl, 25 NaHCO3, 0.96 NaH2PO4, 0.24 Na2HPO4, 1.2 MgSO4, 2 CaCl2, 5.5 d-glucose, 100 μM l-arginine, and perfusion pressure was maintained at around 50 mmHg (1 psi) throughout the experiment. The tubular perfusate was an isosmotic, low NaCl containing Ringer solution consisting of (in mM): 10 NaCl, 135 N-methyl-D-glucamine (NMDG) cyclamate, 5 KCl, 1 MgSO4, 1.6 Na2HPO4, 0.4 NaH2PO4, 1.5 CaCl2, 5 d-glucose, and 10 HEPES. The tubule segment was cannulated and perfused at a baseline rate of ~2 nl/min. The bath was identical to the arteriolar perfusate and was continuously aerated with 95% O2-5% CO2 and exchanged at a rate of 1 ml/min. The preparation was kept in the dissection solution and temperature was kept at 4°C until cannulation of the arteriole and tubule was completed then gradually raised to 37°C for the remainder of the experiment. Fluorescence imaging of MD cells was performed using Fluo-4 and Fura-Red, and a Leica TCS SP2 AOBS MP confocal microscope as described before (23). Chemicals, if not indicated, were purchased from Sigma (St. Louis, MO).

Data analysis. Data are expressed as means ± SE. Statistical significance was tested using paired t-test and linear regression. Significance was accepted at P < 0.05.

RESULTS

Endothelial fenestration. High-resolution images of the AA endothelium and JG renin cells were obtained from the rat and mouse kidney in vivo. The content of renin granules in JG cells was labeled using quinacrine, a selective marker of acidic and secretory granules, and the endothelium was stained using LY
endocytosed from the plasma (Fig. 1). Quinacrine staining of individual renin granules was highly specific and localized to the JG segment of the AA. As expected, the length of the quinacrine (renin)-labeled AA segment increased with respect to the time on salt-deficient diet (from around 20 up to 90 μm, Fig. 2 x-axis). Interestingly, LY stained the proximal and JG AA endothelium segments differently. In the proximal portion, the endothelium appeared as a solid and intensely fluorescent line, but it was weakly fluorescent with a discontinuous line pattern in the JG segment (Fig. 1A). High magnification revealed a porous, fenestrated structure of AA endothelium adjacent to renin granules, but a thick, and smooth endothelium in renin-negative segments (Fig. 1B). In fact, there was a highly significant correlation ($r = 0.91$, $n = 15$) between the length of quinacrine-labeled, renin-positive AA (stimulated by salt-deficient diet) and that of the weakly LY fluorescent, fenestrated endothelium (Fig. 2).

**JGA fluid flow in vivo.** A single intravenous bolus of the fluid marker LY was injected into the femoral vein, which appeared in the AA and glomerulus within 5 s and then filtered into the Bowman’s space and early proximal tubule. A supplementary video file showing the appearance of LY in the JGA area is available in the online version of this article. Using high temporal resolution, fluorescence intensity changes of LY were measured in different regions of interest in the JGA as shown in Fig. 3. Simultaneously with the appearance of LY in the lumen of the AA, fluorescence of LY was detected in the JGA interstitium as well, around the AA (Fig. 3A). The increase in LY fluorescence in the JGA interstitium preceded the appearance of LY in the Bowman’s capsule and early proximal tubule (Fig. 3, A and B). A wave of interstitial LY fluorescence was observed moving away from the JGA in all directions (see the representative video supplement) with a speed of $27.9 \pm 7.2$ μm/s ($n = 6$). Parallel with its plasma and glomerular clearance, LY quickly washed out of the JGA interstitium within 5 s (Fig. 3, A-D). The maximal fluorescence intensity of LY in the JGA interstitium was $17.9 \pm 3.5\%$ of that in the AA plasma ($n = 6$; Fig. 3D).

![Fig. 2. Correlation between the lengths of renin-positive and fenestrated endothelial segments of the mouse AA. To stimulate renin content, animals were fed a salt-deficient diet for various times, up to 1 wk.](http://ajprenal.physiology.org/)

![Fig. 3. In vivo imaging of the JG AA in the intact rat kidney. Circulating plasma is labeled with rhodamine B-conjugated 70-kDa dextran (red), content of individual renin granules with quinacrine (green). Note the significant renin content around the JG portion of the afferent (AA), but not around the proximal AA segment (P) or the efferent arteriole (EA). G, glomerulus. A-C: fluorescence of iv injected LY (yellow) was visualized in the AA and surrounding interstitium in real time. Panels show the time it first appeared in the AA and JGA (A), 2 s later (B) when it filtered into the early proximal tubule (PT), and another 4 s later (C) when it almost completely cleared from the JGA area. Scale is 20 μm. Note a typical thin-walled peritubular capillary in the top left corner surrounded by interstitial cells around the PT. For comparison, the AA and EA are at least twice the diameter and have thick vascular smooth muscle (or JG) cells in the vessel wall. The “dark structures” excluding LY are the many cell types in the particular optical section. Since the cell membranes are impermeable to LY, only the extracellular fluid is labeled, but cells are unstained. D: representative time-lapse changes in LY fluorescence in the AA lumen, JGA interstitium, and the PT. E: profile of LY fluorescence along a line segment in the JGA interstitium, parallel with the AA and upstream from JG toward P segments (as depicted in A). The quinacrine-labeled JG AA portion is labeled as renin-segment. F: correlation between the length of quinacrine (renin)-labeled JG AA and that of the intensely LY-stained (LY-rich) segment of the JGA interstitium.)
The distribution of LY fluorescence in the JGA interstitium was heterogeneous along the AA (Fig. 3A). Only the JG portion closest to the glomerular vascular pole showed high LY intensity, while fluorescence around proximal AA segments was minimal (Fig. 3, A and B). The length of this intensely LY fluorescent segment of JGA interstitium showed significant correlation ($r = 0.85, n = 9$) with the length of the quinacrine-labeled, renin-positive AA (Fig. 3, E and F).

In addition to LY, the presence of a high molecular weight dextran-rhodamine conjugate, originating from the circulating plasma, was also detected in the JGA interstitium (Figs. 3–5). Interestingly, the fluorescence intensity of dextran-rhodamine in the JGA was oscillatory, similar to that in the Bowman’s capsule (Fig. 4). The oscillations showed the same phase and cycle time at these two locations, and had two components: a slow (one every 45–50 s) and a fast (one every 5–10 s) oscillation (Fig. 4).

Following iv bolus injections, LY fluorescence was detected not only in the JGA interstitium, but in the tubular lumen of the MD as well (Fig. 5). Luminal LY fluorescence appeared to be specific for the MD area and was not visible in slightly upstream segments of the thick ascending limb (Fig. 5A). Maximal LY intensity in the MD lumen was observed about 2–5 s later than that in the AA and JGA (Fig. 5B). The intratubular transit time of LY filtered in the glomerulus and transported to the MD area along the nephron was much longer, usually between 30–50 s (not shown). Systemic blood pressure was normal in all animals studied; the average mean arterial blood pressure was $98.6 \pm 8.0$ mmHg ($n = 6$).

**JGA fluid flow in vitro.** Further experiments were performed to study fluid flow in the JGA, particularly in the MD area using the isolated, simultaneously microperfused AA-thick ascending limb with attached glomerulus preparation. The effect of increased Bowman’s capsule hydrostatic pressure was visualized by blocking the outflow of glomerular filtrate with a holding pipette (Fig. 6, and a supplementary video file is available at http://ajprenal.physiology.org/cgi/content/full/). In response to compressing the early proximal tubule with a micropipette (during constant AA perfusion with 50 mmHg), significant increases in the volumes of the Bowman’s capsule, JGA interstitium, and the MD were observed (Fig. 6, A and B). MD cells increased their volume by $62 \pm 4\%$ ($n = 4$), as measured by changes in intracellular fluorescence of fluo-4 (Fig. 6C). Similar data were also obtained by using fura red (not shown). In addition to MD cell volume changes, the extraglomerular mesangial and MD intercellular lateral spaces also distended significantly, particularly in the center of the MD plaque (Fig. 6B). All these responses were fully reversible upon removing the proximal tubule block (Fig. 6C, and video supplement).

**DISCUSSION**

In the present study, fluid flow in the JGA interstitium was directly visualized for the first time in the intact rat kidney using intravital multiphoton imaging. Both morphological prerequisites and functional signs of significant JGA interstitial fluid flow were observed. The length of the porous, fenestrated AA endothelium that is unique to JG segments correlated highly with the length of the quinacrine-labeled, renin-positive AA. Real-time imaging of the iv injected low molecular weight fluid marker LY revealed that a significant portion of plasma is transported to the MD area along the nephron was much longer, usually between 30–50 s. Systemic blood pressure was normal in all animals studied; the average mean arterial blood pressure was $98.6 \pm 8.0$ mmHg ($n = 6$).

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region. Overall, these findings challenge the existing view of the JGA interstitium as a sequestered, stable environment.

The morphological features of the AA visualized in vivo in the present experiments are consistent with earlier works (25–30, 37) describing the heterogeneity of the AA using electron microscopy. In vivo labeling of the vascular endothelium by endocytosed LY (Fig. 1) confirmed that the AA is not a vessel with uniform characteristics along its entire length. The two structurally and functionally different portions include a renin-negative proximal portion lined by continuous, nonpermeable endothelium and a renin-positive JG portion lined by fenestrated, highly permeable endothelium. It has been demonstrated that the lengths of these two segments are highly variable, tracking the actual status and activity of the renin-angiotensin system (27). Consistent with this, we found that salt-deficient diet increased the length of both the renin-containing and fenestrated endothelial AA segments (Figs. 1 and 2). Also, there was a strong correlation between fenestration and LY permeability (Fig. 3F).

Only a limited number of vascular beds feature fenestrated endothelium without a diaphragm, such as those in the glomerulus and endocrine tissues (8, 36). The presence of fenestrations in the JG portion of AA endothelium which was first described by Rosivall and Taugner (25) is very peculiar since intravascular pressure in this region is high (around 50–60 mmHg). This high pressure most likely favors significant fluid filtration into the interstitium. Fenestration of the JG AA was found in various species from amphibians to mammals (25–29), suggesting it has an important, general physiological function. It is very likely that fenestration is an integral part of the endocrine function of the JGA. It may help the diffusion of released renin directly into the circulating plasma through the endothelial fenestrae (12) and it could also help to filter renin into the cortical interstitium (20) along with the fluid it generated. Although it is a very active area of research, it is unclear at present how the renin content signals the formation of fenestrae, how these pores are formed, or how their size is controlled. Several studies suggest that vascular endothelial growth factor (VEGF), a major regulator of vascular permeability, is one of the key players in this phenomenon (8, 24, 31, 36).

It should be noted that the fenestrations appear to be larger in the intact living kidney when fluid actually moves through the pores, compared with that measured by TEM on fixed, dehydrated tissue sections. We believe that this is due to the different techniques used. On a similar note, the volume of the renal interstitium also seems to be larger in vivo. Histology textbooks describe the extraglomerular mesangium as “densely packed mesangial cells separated by long, narrow interstitial space filled with basal laminar material” (2, 15). This is not the case in the intact living kidney, where mesangial intercellular spaces are much wider (Fig. 3A). These observations further support the existence of fluid filtration from the JG AA and the bulk fluid flow in the JGA.

As a noninvasive alternative to existing methods, multiphoton in vivo imaging can be used for the quantitative visualization of basic renal functions (10, 14, 39). In a recent innovative method (10) LY, a widely used extracellular fluid marker (6), was a superior tool to measure glomerular filtration and tubular flow rate due to its low molecular weight, excellent water solubility, and intense fluorescence. Compared with the demanding conventional micropuncture methods (38), this technique monitors the fluorescence of a single iv bolus of LY in real-time and noninvasively, so tubular flow, blood flow, and macula densa function are intact. In the present study, LY was useful to visualize fluid filtration from the AA lumen to the JGA interstitium with high temporal resolution (Fig. 3, and video supplement). The LY iv bolus appeared in the lumen of AA within 5 s, and LY was detected around the AA and in the extraglomerular mesangium before the remainder of the circulating LY filtered into the Bowman’s capsule, early proximal tubule, or appeared in peritubular capillaries (Fig. 3, A-D). LY fluorescence was detected in both the extraglomerular and
intraglomerular mesangium (Fig. 3A). This is consistent with the anatomical continuity between these two areas, and also with the fenestrated and filtering intraglomerular capillaries (25). The movement of LY in the renal interstitium was most likely due to both fluid filtration and diffusion. However, the fact that LY appeared in the JGA interstitium before it was filtered in the glomerulus is the unequivocal evidence of bulk fluid flow rather than the movement of LY in the interstitium. Based on LY fluorescence, fluid filtration from the AA was specific for the quinacrine-labeled, renin-positive, and fenestrated JG portion of AA (Fig. 3, A–F). In contrast, low LY intensity was detected in the interstitium around the proximal, upstream segment of AA (Fig. 3, B and E). Interestingly, the maximal LY fluorescence in the JGA interstitium was 15–20% of that in the AA plasma (Fig. 3D). This is a very significant amount and supports the existence of an intense fluid flow through the MD plaque from the base of the cells toward the tubular lumen which was evident in real-time images (video supplement). The morphological basis for the filtration of fluid from the Bowman’s capsule back into the JGA interstitium is most likely the presence of Bowman’s space and podocyte foot processes in the JGA facing the extraglomerular mesangial interstitium (25). Fluid flow through the MD base to the tubular lumen is an unexpected finding which conflicts the generally accepted salt reabsorptive model of MD function (3). However, the fluid flow from the JGA AA lumen and the Bowman’s space into the JGA interstitium appear to be important determinants of the JGA fluid balance and fluid composition. The concentration of MD-derived autacoids is certainly modified by the significant and dynamic JGA fluid flow that was directly visualized in the present study. This supports the earlier hypothesis of Rosivall et al. (28, 30) on the so-called short-loop feedback mechanism. According to this hypothesis, the TGF and renin release signals (humoral mediators) generated by the classic, long-loop MD feedback are rapidly modulated by the JGA interstitial fluid flow originating from the vascular components of the JGA. However, the present findings are clearly in disagreement with the existing paradigm on the stable JGA environment which is thought to be an ideal milieu for humoral signal transmission (2, 32). In contrast, the relatively high volume and rapid physiological changes of JGA fluid flow can almost certainly wash MD-derived autacoids (ATP, adenosine, PGE2, NO, etc.) quickly out of the JGA. Maneuvers that should alter JGA fluid flow, such as acute volume loading, changes in osmolality, and elevations in interstitial pressure indeed modulate TGF sensitivity (5, 16, 17).

In summary, the present in vivo studies visualized for the first time the filtration of fluid from the fenestrated, renin-positive JG AA segment into the JGA interstitium. Fluid flow in the JGA was significant in volume and rapidly responsive to hemodynamic changes in the JGA. Fluid flow was also observed in the MD area and it appeared to be an important factor in MD cell function. We propose that the functional significance of the JGA fluid flow is twofold: it may help filter the released renin into the renal interstitium (endocrine function) and it may also alter TGF and renin signals in the JGA.
(hemodynamic function). These studies also suggest that several aspects of the current model of JGA environment and JGA function need to be reevaluated.

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REFERENCES