Two-photon excitation fluorescence imaging of the living juxtaglomerular apparatus

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1Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, and 3Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki 444-8585, Japan; and 2Nephrology Research and Training Center, Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

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Peti-Peterdi, János, Shigeru Morishima, P. Darwin Bell, and Yasunobu Okada. Two-photon excitation fluorescence imaging of the living juxtaglomerular apparatus. Am J Physiol Renal Physiol 283: F197–F201, 2002. First published January 29, 2002; 10.1152/ajprenal.00356.2001.—Recently, multiphoton excitation fluorescence microscopy has been developed that offers important advantages over confocal imaging, particularly for in vivo visualization of thick tissue samples. We used this state-of-the-art technique to capture high-quality images and study the function of otherwise inaccessible cell types and complex cell structures of the juxtaglomerular apparatus (JGA) in living preparations of the kidney. This structure has multiple cell types that exhibit a complex array of functions, which regulate the process of filtration formation and renal hemodynamics. We report, for the first time, on high-resolution three-dimensional morphology and Z-sectioning through isolated, perfused kidney glomeruli, tubules, and JGA. Time-series images show how alterations in tubular fluid composition cause striking changes in single-cell volume of the unique macula densa tubular epithelium in situ and how they also affect glomerular filtration through alterations in associated structures within the JGA. In addition, calcium imaging of the glomerulus and JGA demonstrates the utility of this system in capturing the complexity of events and effects that are exerted by the specific hypertensive autacoid angiotensin II. This imaging approach to the study of isolated, perfused live tissue with multiphoton microscopy may be applied to other biological systems in which multiple cell types form a functionally integrated syncytium.

Table 1. Composition of luminal perfusion solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>Low-NaCl</th>
<th>Hypotonic</th>
<th>Ringer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>25</td>
<td>25</td>
<td>135</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>NMDG</td>
<td>110</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td>Cyclamic acid</td>
<td>110</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Osmolality, mosmol/kgH₂O</td>
<td>300</td>
<td>210</td>
<td>300</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
</tr>
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NMDG, N-methyl-D-glucose.

THE PROCESS OF GLOMERULAR filtration and its control by local hemodynamics and the hormonal milieu involve the complex interaction of a number of different cell types. Regulation of glomerular filtration occurs at the juxtaglomerular apparatus (JGA), a complex structure that consists of a number of different cell types, including vascular smooth muscle cells, secretory granular epitheloid cells, vascular endothelium, mesangial cells, and macula densa (MD) tubular epithelial cells (2). MD cells, in the distal nephron, function as specific sensor cells that detect changes in tubular fluid osmolality and/or salt concentration via specific transport mechanisms (3) and send signals through mesangial cells in the JGA. These signals effect afferent arteriolar smooth muscle cells and renin granular cells to control preglomerular vascular resistance [tubuloglomerular feedback (TGF)] and renin release, respectively (1–3, 13). It has been difficult to study these cellular interactions within the JGA in living preparations, given the constraints of existing technologies.

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Recently, multiphoton excitation laser scanning fluorescence microscopy has been developed (8, 14) that is particularly applicable to deep optical sectioning of living tissue samples. This technique offers a tremendous increase in image resolution vs. conventional confocal microscopy. Importantly, it can optically section through an entire glomerulus (glomerular diameter =100 μm), thus providing the ability to directly study structures and cellular components that lie deep within the glomerulus. This new technology was used in the present work to visualize the living perfused JGA.

MATERIALS AND METHODS

Isolated, perfused afferent arteriole-cortical thick ascending limb preparations were used as previously described (10, 11). Preparations were visualized using a two-photon laser scanning fluorescence microscope (MRC1024MP, Bio-Rad) composed of a mode-locked titanium-sapphire laser (Tsunami, Spectra Physics), and a photo-diode pump laser (Millenium, Spectra Physics). Individual preparations were transferred to a thermoregulated Lucite chamber mounted on a Zeiss AxioLab inverted microscope and visualized using a ×40 water-immersion lens. The fluorescent membrane-staining dye 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH; 5 μM, Dojin) or the Ca2+-sensitive fluorophore indo 1-acetoxymethyl ester (indo 1-AM; 5 μM, Molecular Probes, Eugene, OR) was added to the tubular and/or arteriolar perfusion solutions (Table 1) for a loading period of 5 min to visualize cellular structures or monitor intracellular Ca2+ concentration ([Ca2+]i). After the loading period, TMA-DPH or indo 1-AM was removed. TMA-DPH fluorescence was captured using a band-width emission filter at 430 ± 15 nm in response to a two-photon excitation wavelength of 755 nm. Indo 1 fluorescence was measured at emission wavelengths of 453 and 405 nm in response to two-photon excitation wavelength of 720 nm. A 405 ± 17-nm band-pass filter, placed before detection by a photomultiplier tube (PMT; channel 2), was used to select indo 1 emissions that increase with increasing [Ca2+]i, whereas a 453 ± 2-nm band-pass filter, placed before a second PMT (channel 1) was used to select isosbestic emissions of indo 1 that are independent of [Ca2+]i. After ~15-min incubation with control perfusion solutions, fluorescence intensities for both wavelengths stabilized at a constant level. Ratiometric (405 nm/453 nm) images were collected and analyzed using a time series function of the LaserSharp1.2 software (Bio-Rad) with the most frequently used application of cycle time 10-s, for 30 cycles.

RESULTS

Glomerular and JGA morphology. We examined three-dimensional (3D) morphology of the perfused, living glomerulus with attached tubular segments using optical Z-sectioning. Figure 1 represents midlevel sections (a complete Z-series in 2-μm steps is available in a supplementary file), using a cell membrane
marker (TMA-DPH) or an intracellular fluorophore (indo 1). Both morphology and cytosolic parameters, e.g., \([\text{Ca}^{2+}]_i\), of individual cells that comprise the glomerulus can be studied with high resolution. Afferent and efferent arterioles, glomerular capillary loops, the proximal tubule and cortical thick ascending limb, and MD cells can be identified and visualized in striking detail. In addition, it was also possible to visualize the process of filtration across the capillary wall into the Bowman’s capsule. In terms of the MD cells, we show for the first time (Fig. 2), 3D imaging of a perfused MD plaque, consisting of 24 individual cells.

**Real-time imaging of the JGA during TGF.** We visualized MD cells as well as the entire JGA area during activation of the TGF mechanism. Images are shown in Figs. 3 and 4 [videos (time-series images taken every 10 s) of the same preparation are available in supplementary files]. Increasing tubular perfusate osmolality and NaCl concentration ([NaCl]), which mimics normal physiological TGF activation, produced a remarkably fast and reversible MD cell swelling. MD cell swelling is most likely due to a high level of NaCl transport into these cells. It should also be noted that a reversible blebbing or expansion of the MD apical membrane occurred during TGF activation (Fig. 3b). Thus two-photon imaging has revealed what may be a novel rapid process of membrane insertion + retrieval that occurs in MD cells during TGF. Figures 4 and 5 demonstrate morphological changes in the JGA during TGF activation. Parallel with MD cell swelling, swelling/contraction of cells in the final part of the afferent arteriole was observed that caused an almost complete collapse of the arteriolar lumen and a shrinkage of capillary loops and the entire glomerulus. This is the first evidence for a “sphinixceter-like” response of the terminal intraglomerular afferent arteriole. These intraglomerular morphological changes are absent when the efferent arteriole is perfused, suggesting the lack of efferent arteriolar vasoconstriction during TGF.

**Effects of ANG II.** In another effort to apply this new technology to the JGA, we measured morphological and dimensional changes in arterioles and glomerular capillary loops in response to the vasoactive peptide ANG II in the presence or absence of the type 1 ANG II receptor (AT1) blockade with candesartan. We also tested the effects of another vasoconstrictor hormone, norepinephrine (Fig. 6). ANG II (10^{-8} M), added to the arteriolar perfusate, significantly constricted afferent...
(AA) and efferent arterioles (EA). These vascular effects were prevented by coadministration of $10^{-6}$ M candesartan. ANG II also caused a significant reduction in the glomerular capillary loop diameter (Fig. 6). Vascular reactivity was well preserved in these studies, because addition of $10^{-6}$ M norepinephrine at the end of each experiment significantly constricted both AA and EA.

Figure 7 demonstrates changes in glomerular morphology and $[Ca^{2+}]_i$ in response to ANG II. ANG II ($10^{-8}$ M) added to the perfusate significantly increased $[Ca^{2+}]_i$ in AA, EA, mesangial cells, and podocytes. In the case of intraglomerular mesangial cells, there was a $35.2 \pm 11.7\%$ increase in the indo 1 fluorescence ratio $(n = 5)$ with addition of ANG II that had been antagonized by $10^{-6}$ M candesartan.

**DISCUSSION**

Despite the key role that the JGA plays in the regulation of renin release, TGF, and control of glomerular filtration and blood flow (1, 2, 13), it has been very difficult to examine single cells of the JGA even with conventional videomicroscopy (4–7). However, we now report that with two-photon microscopy, one can visualize single cells of the living JGA and glomerulus in real time and in striking detail.

With simultaneous increases in tubular osmolality and [NaCl], MD cells produced a significant cell swelling. This was primarily due to an increase in cell height (Fig. 3), consistent with an earlier work (4). However, in contrast to the studies of Gonzalez et al. (4) we found much larger increases in MD cell volume. Remarkably, these cells are capable of almost doubling their initial cell volume within 30 s despite a concomitant increase in luminal osmolality. This cell swelling is primarily due to NaCl entry into the cell, because the same change in the tubular perfusate osmolality at constant [NaCl] did not produce any significant change in cell volume.

We also examined TGF activation on intraglomerular elements. For the first time, we observed a sphincter-like constriction of the afferent arteriole in the terminal intraglomerular segment of this vessel. During TGF, the vascular diameter of this sphincter decreased to $0$. Compared with this strong vasoconstrictor response, we observed only minor changes in vascular diameter in more proximal extraglomerular segments of the afferent arteriole (Fig. 5). These studies indicate that the principal effector site for TGF occurs in the terminal-intraglomerular afferent arteriole. These studies also suggest that measurements of proximal afferent arteriolar diameter, as used by others (5, 12), may not accurately reflect TGF responses. One can further speculate that the minor vasoconstriction seen in the proximal afferent arteriole may be, at least in part, a myogenic response that is a consequence of the terminal-TGF-mediated sphincter activity. Also, the TGF-mediated reduction in intraglomerular afferent arteriolar diameter is more consistent with the magnitude of in vivo TGF responses obtained with micropuncture (9). Further studies are needed to identify the cell type in the afferent arteriole that constitutes this sphincter-like structure.

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**Figure 5.** Comparison of changes in afferent arteriolar diameter in intra- and extraglomerular segments. Tubular NaCl concentration was increased from 25 (osmolality 210 mosmol/kgH$_2$O, NaCl substituted by NMDG; a) to 135 mM (osmolality 300 mosmol/kgH$_2$O; b). Tissue was stained with TMA-DPH. Bar = 10 μm.

**Figure 6.** Effects of ANG II alone or with AT$_1$ receptor blockade candesarten (Cand) and norepinephrine (NE) on AA, EA, and glomerular capillary loop (GCL) internal diameter (ID). *P < 0.05, nonsignificant compared with control (ctrl) in each group (n = 5 each).
In other experiments, we tested the effects of ANG II on glomerular and JGA morphology, because ANG II is a well-known modulator of renal hemodynamics and kidney function (9). In addition to the expected arteriolar vasoactive effects, to our knowledge this is the first study that demonstrates an increase in mesangial cell Ca$^{2+}$ in the intact JGA. Thus these studies suggest that ANG II, perhaps through activation of mesangial cells and podocytes, may modify the glomerular capillary filtration surface area and that these effects are inhibited by AT$_1$ receptor blockade with candesartan. This approach has allowed us to identify a novel intraglomerular sphincter-like effector site for TGF. It has offered a powerful new tool for investigating the structural and cellular components that regulate the process of glomerular filtrate formation and renal hemodynamics.

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Supplementary digital video files are available at http://ajprenal.physiology.org/cgi/content/full/283/1/F197/DC1.

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