Isolation and perfusion of rat inner medullary vasa recta

Kristen K. Evans, C. Michele Navata, and Thomas L. Pannabecker
Department of Physiology, University of Arizona Health Sciences Center, Tucson, Arizona

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Evans KK, Nawata CM, Pannabecker TL. Isolation and perfusion of rat inner medullary vasa recta. Am J Physiol Renal Physiol 309: F300–F304, 2015. First published June 10, 2015; doi:10.1152/ajprenal.00214.2015.—Outer medullary isolated descending vasa recta have proven to be experimentally tractable, and consequently much has been learned about outer medullary vasa recta endothelial transport, pericyte contractile mechanisms, and tubulovascular interactions. In contrast, inner medullary vasa recta have never been isolated from any species, and therefore isolated vasa recta function has never been subjected to in vitro quantitative evaluation. As we teased out inner medullary thin limbs of Henle’s loops from the Munich-Wistar rat, we found that vasa recta could be isolated using similar protocols. We isolated ~30 inner medullary vasa recta from 23 adult male Munich-Wistar rats and prepared them for brightfield or electron microscopy, gene expression analysis by RT-PCR, or isolated tubule microperfusion. Morphological characteristics include branching and nonbranching segments exhibiting a thin endothelium, axial surface filaments radiating outward giving vessels a hairy appearance, and attached interstitial cells. Electron microscopy shows multiple cells, tight junctions, and either continuous or fenestrated endothelia. Isolated vasa recta express genes encoding the urea transporter UT-B and/or the fenestral protein PV-1, genes expressed in descending or ascending vasa recta, respectively. The transepithelial NaCl permeability (383.3 ± 60.0 × 10⁻⁵ cm/s, mean ± SE, n = 4) was determined in isolated perfused vasa recta. Future quantitative analyses of isolated inner medullary vasa recta should provide structural and functional details important for more fully understanding fluid and solute flows through the inner medulla and their associated regulatory pathways.

Keywords: renal medulla; renal blood flow; renal hemodynamics

SIGNIFICANT INSIGHTS INTO renal vascular physiology and pathophysiology can be achieved by more fully understanding the basic physiology of the descending and ascending vasa recta (5, 16, 18). Regulation of medullary blood flow rates and blood distribution patterns involves a host of endocrine and paracrine systems as well as contractile cells associated with descending vasa recta (16, 22). Endothelins (ET-1, ET-2, and ET-3) are potent vasoconstrictors of outer medullary descending vasa recta (25), and the ET₉ receptor subtype plays an important role in regulation of medullary blood flow (9). Common to descending vasa recta in both outer and inner medulla are the water channel aquaporin-1 (AQP1) and the facilitative urea transporter UT-B, and common to ascending vasa recta and fenestrated capillaries is PV-1, a protein associated with the fenestral diaphragm (8, 13, 26, 29). While isolation of rat or human outer medullary vasa recta and tissue slice preparations have made it possible to study the roles of regulatory pathways and membrane proteins in endothelial transport, pericyte contraction, and endothelial interactions (7, 16, 24), individual inner medullary vasa recta have never been isolated from tissue parenchyma of any species for in vitro functional studies.

The peak osmolality of the rat outer medulla is about twice that of systemic plasma (~600 mosmol/kgH₂O) whereas the peak osmolality of the inner medulla reaches ~10-fold that of plasma. This corticomedullary osmolality gradient is paralleled by a steep PO₂ gradient, which is related in part to different degrees of active transport along the corticomedullary and lateral axes (6). The outer medullary architecture is significantly different from that of the inner medulla as are the inner medullary fluid and solute compartment-to-compartment flows and tubulovascular interactions (8, 11, 20, 21, 28–30). On this basis alone, it might be predicted that functional characteristics of outer and inner medullary vasa recta are significantly different from each other.

We discovered that, while teasing out thin limbs of Henle’s loops for isolated tubule perfusion (12), vasa recta could also be isolated in a similar fashion. With subillumination on a stereomicroscope stage, vasa recta take on an appearance that is distinct from thin limbs, allowing us to identify and collect individual blood vessels and prepare them for brightfield and electron microscopy, RT-PCR, and isolated tubule microperfusion. Using these techniques, we establish the feasibility of more fully characterizing transport pathways, regulatory mechanisms, and structural proteins of isolated inner medullary vasa recta.

METHODS

Animals. Male Munich-Wistar rats (average age ~120 days; average wt ~400 g) were reared in the University Animal Care facility at the University of Arizona, Tucson, AZ, and provided with chow (Teklad 7001) and water ad libitum. Animals were euthanized with CO₂. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and were approved by the University of Arizona Institutional Animal Care and Use Committee.

Vasa recta dissection and identification of segments on the basis of structural features. Vasa recta were teased from the isolated inner medulla in a solution consisting of 280 mM sucrose/10 mM HEPES, adjusted to pH 7.4 with Tris-base and gassed with 100% O₂ before dissection. Dissection was carried out without the aid of enzymatic agents at 4°C under a stereomicroscope using reflected light below the dissection dish. Upper and lower vasa recta segments were teased from the upper and lower 50% of the inner medulla, respectively. Vasa recta were teased by first pinning down the medullary parenchyma with a dissecting needle (held in one hand) then peeling off successive layers of tissue with a pair of forceps (held in the other hand). Single vasa recta were then isolated from other tubular structures using fine needles.

Vasa recta are distinguished from thin limbs of Henle’s loops primarily by their hairlike surface projections and by their cell type (Fig. 1). Descending thin limbs have nuclei that protrude into the lumen, whereas ascending thin limbs have round flat nuclei (12). These nuclei were often not clearly recognizable in isolated vasa recta. These differences are shown most clearly when the tubule segments
are viewed under a compound microscope with brightfield (Fig. 1) or differential interference contrast optics, but they can also be seen under a stereomicroscope.

Electron microscopy. Isolated vasa recta or thin limbs were drawn into a glass Pasteur pipette and deposited in the cavity of a depression slide and fixed with 2.5% glutaraldehyde and 2% paraformaldehyde for at least 2 h at 4°C. An equal volume of 3% agar in 0.2 M PIPES was then added to the cavity and mixed with the fixative. After hardening, the agar with vessel or tubule was trimmed, postfixed with 1% osmium tetroxide, incubated in 2% aqueous uranyl acetate for 20 min, and embedded in Spurrs. Thin sections were stained with 3% lead citrate and imaged using a Tecnai Spirit.

Vasa recta gene expression. Single vasa recta segments (length 500–1,000 μm) were characterized by detection of mRNA coding for UT-B, PV-1, ETB, and the water channels AQP1 and AQP2. Each segment was transferred with 5–10 μl of buffer into a 0.5-ml microcentrifuge tube and lysed with 10 mM L-arginine/1% Triton X-100, pH 2.5. Lysate cDNA was synthesized using Maxima H Minus Reverse Transcriptase (Thermo Scientific, Waltham, MA) and oligo (dT)17 primer and amplified for 40 PCR cycles with Phire II polymerase (Thermo Scientific) on an Eppendorf Mastercycler (Eppendorf, Hauppauge, NY). UT-B (accession no. NM019346) was amplified with 5′-gcttgctgtcacttcc-3′ (sense), 5′-gagctgcaagactgggctt-3′ (anti-sense), 5′-gctttgtcgctgtaccttcc-3′ (sense), 5′-gacttggccttggagctgcaagactg-3′ (anti-sense), ETB (accession no. XM006252431) was amplified with 5′-ctgtggtggtcactagtgtgtc-3′ (sense), 5′-tgtgctgctgtgctgtgctgtg-3′ (anti-sense), AQP1 (accession no. NM012778) was amplified with 5′-caggacttagtgggctt-3′ (sense), 5′-caggacttagtgggctt-3′ (anti-sense), and AQP2 (accession no. NM120909) was amplified with 5′-caggcagagactgtgct-3′ (sense), 5′-caggcagagactgtgct-3′ (anti-sense). Correct PCR products were verified by size on gels stained with ethidium bromide. Specificity of each primer set was validated by sequencing whole tissue products.

Perfusion of isolated tubule segments. Tubule segments were perfused in vitro in a temperature-controlled chamber (37°C) (2, 12). The upstream portion of the tubule was drawn into a holding pipette, which contained a perfusion pipette and an exchange pipette. The downstream end of the tubule was drawn into a holding pipette that had a tip of appropriate diameter to form a complete seal between perfusate and bath solution. The perfusion rate was ~15–30 nl/min, and the bath was superfused at ~1 ml/min. Tubule dimensions were determined by ocular micrometer.

Transepithelial NaCl permeability measurements. NaCl permeability (PNaCl) was determined from the unidirectional 22Na flux resulting from a 125 mM lumen-to-bath NaCl concentration gradient. The perfusion solution consisted of (in mmol/l) 125 NaCl, 25 N-methyl-D-glucamine, 2.5 K2HPO4, 2 CaCl2, 1.2 MgSO4, 5.5 glucose, and 5 m. The bath solution was identical except 236 mM mannitol replaced 125 mM NaCl, producing solutions of nearly equal osmolalities (~290 mosmol/kgH2O) and preventing osmotic volume flux. Unidirectional lumen to bath Na flux (JNa; pmol·min⁻¹·mm tubule length⁻¹) was calculated using the following equation: JNa = C0V0 − C1V1, where C0 and C1 are the Na concentrations in the perfusate and collectate, respectively, and V0 and V1 are the perfusion rate per unit tubule length and the collection rate per unit tubule length. As no volume marker was included, volume flux was not measured. V0 and V1 were assumed equal.

PNaCl (cm/s) was calculated for lumen-to-bath fluxes from the following equation: PNaCl = JNa/(μDBC), where D is the tubule diameter and 6C = 6C1 − 6C2/ln (6C1/6C2), where 6C1 is the Na concentration gradient at the perfusion end of the tubule and 6C2 is the Na concentration gradient at the collection end of the tubule (3, 12). Net volume flow is zero in the absence of a transepithelial osmolality gradient and tracer backflux was negligible as short tubule segments were used, and tracer concentration in the trans compartment was <5% of that in the cis compartment. We did not measure the transendothelial electrical potential difference and so cannot account for any possible effect of potential on Na flux. The transepithelial electrical potential of Sprague-Dawley isolated perfused outer medullary vasa recta was ~0 mV (17).

Statistical analysis. Data combined from three or more samples are reported as means ± SE; n is the number of replicates.

RESULTS

Inner medullary vasa recta dissection. Thirty vasa recta were isolated from 23 adult male Munich-Wistar rats and were prepared for light level or electron microscopy, quantitative PCR to characterize expression of the urea transporter UT-B, the water channel AQP1, and/or the fenestral protein PV-1, and isolated tubule microperfusion to determine transepithelial NaCl permeabilities. Dissection techniques are comparable to those used for isolating inner and outer medullary thin limbs of Henle’s loops (4, 12) and outer medullary vasa recta (17). No morphological characteristics were recorded with light level microscopy that distinguished descending vasa recta from ascending vasa recta; however, the presence of pericytes in descending vasa recta of the rat outer intermediate medulla and outer medulla of rat has been documented (22), and these serve as a key indicator for dissection and isolation of mouse and rat
outer medullary descending vasa recta (15). Red blood cells were rarely seen in inner medullary blood vessels. Rat inner medullary descending and ascending vasa recta both have extensive nonbranching portions as well as one or more branching points that connect to a capillary plexus as shown previously with three-dimensional reconstruction (8, 20, 29). The diameters of isolated inner medullary vasa recta were variable, and while some were significantly <20 μm, the average diameter of perfused vasa recta was ~24 μm.

**Morphological characteristics of isolated vasa recta.** Isolated vasa recta observed with brightfield microscopy included branching segments (not shown) and nonbranching segments (Fig. 1) exhibiting a thin endothelium. The primary characteristics observed during dissection that distinguished vasa recta from thin limbs were the filaments that project out from the vessel surface giving them a hairy or bushy appearance that contrasted with the relatively smooth surface of thin limbs (Fig. 1). Interstitial cells were sparsely attached to most isolated vasa recta along their lengths. Electron microscopy of isolated vasa recta in transverse sections variably showed multiple cells and either continuous or fenestrated endothelia. The vas rectum in Fig. 2A shows few fenestrations (arrows in the Inset), suggesting that it may be a descending vasa rectum transitioning into an ascending vas rectum. A type II descending thin limb that was identified during dissection on the basis of structural criteria (12) shows numerous tight junctions and microvilli (Fig. 2B), characteristics common in type II descending thin limbs of several species (19).

**Vasa recta gene expression.** Transcripts for UT-B and PV-1 are expressed in single isolated vasa recta as determined with RT-PCR (Table 1) and gel analysis of PCR products (not shown). Future studies of gene expression in length-normalized segments can be conducted with quantitative PCR and deep sequencing of RNA species as has been done for isolated nephron segments (10, 12). As it stands, we show that RT-PCR can be useful for identification of UT-B- and PV-1-expressing segments, or descending vasa recta and ascending vasa recta/capillaries, respectively. Inner medullary descending vasa recta coexpress UT-B and PV-1 protein for variable lengths immediately before joining the fenestrated capillary plexus (20). Segments coexpressing UT-B and PV-1 mRNA likely are descending vasa recta; segments expressing PV-1 and not UT-B mRNA likely are ascending vasa recta (Table 1). Some, but not all UT-B-positive segments express AQP1 mRNA (Table 1) as has been shown for protein expression (20). The endothelin receptor ETB was expressed in both UT-B-positive and UT-B-negative segments, whereas AQP2 was not detected (Table 1).

**P<sub>NaCl</sub> of isolated perfused vasa recta.** Four isolated vasa recta were dissected from the lower 50% of the inner medulla (lower ~2.5 mm) and were mounted on glass pipettes for microperfusion and determination of passive lumen-to-bath 22Na flux and P<sub>NaCl</sub> (Table 2). Both branching and nonbranching segments were perfused. The branching portions were either removed before transferring the unbranched portion to the perfusion chamber, or the branching portions were held within the collection pipette. Branching and nonbranching vasa recta exhibited a P<sub>NaCl</sub> of 383.3 ± 60.0 × 10<sup>−3</sup> cm/s (mean ± SE).

### Table 1. Gene expression in isolated nonbranching vasa recta

<table>
<thead>
<tr>
<th>Vessel ID</th>
<th>Vessel Location</th>
<th>Gene Transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Endothelin Receptor B</td>
</tr>
<tr>
<td>1</td>
<td>Upper IM</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>Lower IM</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Lower IM</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>Lower IM</td>
<td>+</td>
</tr>
</tbody>
</table>

Gene expression was determined with RT-PCR and analyzed by gel electrophoresis. IM, inner medulla.

### Table 2. P<sub>NaCl</sub> of isolated perfused rat inner medullary vasa recta

<table>
<thead>
<tr>
<th>Na Concentration</th>
<th>Collectate 22Na, μM</th>
<th>Perfusion 22Na, μM</th>
<th>Collectate total Na, mM</th>
<th>J&lt;sub&gt;NaCl&lt;/sub&gt;, pmol·min&lt;sup&gt;−1&lt;/sup&gt;·mm&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>P&lt;sub&gt;NaCl&lt;/sub&gt;, 10&lt;sup&gt;−3&lt;/sup&gt; cm/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segment</td>
<td>Vasa Recta Length, μm</td>
<td>Vasa Recta Diameter, μm</td>
<td>Collection Rate, nl·min&lt;sup&gt;−1&lt;/sup&gt;·mm&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>Vasa Recta</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>575</td>
<td>32</td>
<td>60.8</td>
<td>0.02</td>
<td>0.52</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>25</td>
<td>191.6</td>
<td>0.14</td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>175</td>
<td>20</td>
<td>117.9</td>
<td>0.10</td>
<td>0.40</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>17.5</td>
<td>216.4</td>
<td>0.31</td>
<td>0.47</td>
</tr>
</tbody>
</table>

| Mean  | 237.5 | 23.63 | 146.67 | 0.14 | 0.47 | 38.61 | 11,052.05 | 383.31 |
| SE    | 133.59| 3.69  | 40.93  | 0.07 | 0.03 | 18.60 | 2,410.52  | 60.04 |
| n     | 4     | 4     | 4      | 4    | 4    | 4     | 4         | 4     |

All segments were dissected from the lower 50% of the inner medulla. Branching portions of branching segments were either removed before perfusion, leaving a single unbranched segment, or were held along with the main branch within the collecting pipette. The total concentration of unlabeled Na in the flowing bath was 0 mM, and the total initial concentration of unlabeled Na in the perfusate was 125 mM. *Nonbranching segment. **Branching segment. See the text for definitions.
DISCUSSION

In this study, we show for the first time that it is feasible to isolate inner medullary vasa recta from tissue parenchyma in Munich-Wistar rats, making it possible to carry out physiological studies on a population of renal vasculature that has never been investigated using in vitro techniques. We found that with light level microscopy, the morphological characteristics of inner medullary vasa recta are well defined and segments can clearly be distinguished from thin limbs of Henle’s loops. Isolated vasa recta can also be analyzed with electron microscopy. The mRNA from isolated vasa recta is sufficiently abundant and stable to permit analysis of gene expression in single segments by RT-PCR. Finally, we showed that isolated segments can be cannulated and microperfused in vitro for study of membrane transport properties.

The $P_{\text{NaCl}}$ that we measured in vasa recta in vitro from the lower 50% of the inner medulla was higher than values measured in vasa recta from the terminal ~2 mm of the inner medulla of young female Munich-Wistar rats using in vivo microperfusion (17). In our study, it is possible that NaCl efflux from the lumen exceeds mannitol influx driven by the mannitol bath to lumen gradient, thereby creating an osmolar gradient (see METHODS). Depending on the water permeability of the endothelium and magnitude of reabsorptive fluid flux, $P_{\text{NaCl}}$ could be overestimated. In the in vivo study mentioned above, $P_{\text{Na}} (\times 10^{-5} \text{ cm/s})$ of descending vasa recta was 75 and that of ascending vasa recta was 115; values for $P_{\text{urea}}$ were nearly identical to $P_{\text{Na}}$ (17). The differences in $P_{\text{Na}}$ in the two studies could reflect gender and/or age effects as well as functionally different populations of vasa recta. A significant accumulation of isotope in the perivascular interstitium with in vivo measurements could lead to underestimation of $P_{\text{Na}}$; however, boundary effects were considered unlikely as $P_{\text{Na}}$ and $P_{\text{urea}}$ were significantly different from each other (17). It has been suggested on the basis of functional studies that Na and urea transendothelial fluxes are primarily paracellular in the papilla (17). Structural studies support this idea as UT-B protein, the only known urea transporter in this segment, is only weakly expressed in vasa recta in the terminal 2 mm of the Munich-Wistar rat, and most vasa recta are fenestrated (8, 29). In the outer inner medulla and outer medulla, while the transendothelial Na flux is largely paracellular, the urea flux is primarily carrier mediated, passing by way of the urea transporter UT-B (14, 17, 23).

Although we have established the feasibility of obtaining and perfusing isolated vasa recta segments from the inner medulla, a number of potential complications may be encountered in future studies. J) It may be problematic to distinguish inner medullary descending and ascending vasa recta from each other based solely on morphological characteristics, making it difficult to subsequently identify genes that are selectively expressed within each of these segments. However, in future studies pericytes may prove to be a key marker for distinguishing descending vasa recta as they do for outer medullary descending vasa recta (15, 22). 2) A significant range in vasa recta diameter was observed, although not quantified, with the smallest being at most 50% the diameter of the largest. Thus a number of isolated vasa recta had a diameter too small to cannulate and perfuse with our current perfusion apparatus, yet it was still possible to collect them for gene analysis. A more rigorous characterization of vasa recta morphology using light level microscopy will be beneficial for more accurately categorizing different populations of vasa recta. 3) Collected samples of isolated vasa recta are unlikely to be completely pure populations of vascular endothelia and may include pericytes, interstitial cells, and cells that are derived from other tubular segments, such as collecting ducts. Fenestrated capillaries are anchored along the entire collecting duct length by endothelial projections (20). These projections interface with the plasma membrane of collecting duct cells as well as other structures (1, 20, 27). Tissue damage as a result of teasing and collecting duct cell carry-over during transfer of segments into PCR reaction tubes may be inevitable. To monitor collecting duct principal cell contamination, we measured AQP2 expression in RT-PCR analyses. Specific protocols will need to be further developed to identify, minimize, and account for other potential contaminants. Future studies should provide insights into expression of genes and proteins that are associated with membrane transport, cell growth and metabolism, and hormone regulation of cell processes.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.K.E., C.M.N., and T.L.P. conceived and designed the research; K.K.E., C.M.N., and T.L.P. performed experiments; K.K.E., C.M.N., and T.L.P. analyzed the data; K.K.E., C.M.N., and T.L.P. interpreted the results of experiments; K.K.E., C.M.N., and T.L.P. drafted the manuscript; K.K.E., C.M.N., and T.L.P. edited and revised the manuscript; K.K.E., C.M.N., and T.L.P. approved the final version of the manuscript; T.L.P. prepared figures.

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