Methods

Animals. The animal experiments employed young, female Munich Wistar Froemter (MWF) rats from a breeding colony at the University of Regensburg (110 to 160 g). The animals were fed a standard diet and kept on a 12:12-h light-dark cycle.

Animal preparation. For multiphoton microscopy experiments, rats were anesthetized with an intraperitoneal injection of 113 mg/kg thiobutabarbital (Inactin, Sigma-Aldrich Chemie GmbH, Germany). Body temperature was maintained at 37.5°C by placing the animals on an operating table with a servo-controlled heating plate. The right carotid artery or the femoral artery was catheterized with hand-drawn polyethylene tubing for continuous measurement of arterial blood pressure and heart rate. A catheter connected to an infusion pump was inserted into the right jugular or femoral vein for an intravenous infusion of either saline or angiotensin II-solution and injection of drugs and the fluorescent dye. For the imaging session, the left kidney was exposed by a small flank incision

Multiphoton microscopy. Experiments were performed using a ZeissLSM 710 NLO confocal fluorescence microscope (Carl Zeiss Jena GmbH, Jena, Germany), which was equipped with an applicable warming plate to maintain the animal's body temperature at 37°C. Excitation was achieved using a Chameleon Ultra-II MP laser (Coherent Deutschland GmbH, Dieburg, Germany) at 860 nm with a laser power of 7% of 3200 mW. Sixteen-bit 1024x1024 (visualization of endocytotic vesicles) or 100x500 (glomerular flow measurements) pixel images were obtained by applying a 40x long distance (LD) C-Apochromat 40/1.1 water objective. Emission was collected using external detectors (nondescanned detectors) with filterset 1 (green channel): beamsplitter 500-550, longpass (LP) 555 and filterset 2 (red channel): beamsplitter P 565-610 including mirror). Detector settings were kept constant for all measurements: For the green and red channel, master gain was 600/500, digital gain was 10/10 and offset was 0/0.

Visualization and quantification of albumin endocytosis by podocytes. Podocytes were visualized by negative imaging (8) during a continuous infusion of Lucifer Yellow (5 mg/ml, 2 µl/min). To label the vasculature, a 5 mg/ml solution of Alexa Fluor 594 BSA conjugate (Invitrogen GmbH, Darmstadt, Germany) dissolved in PBS was first purified by dialysis for 3 days (Spectra/Por Float-A-Lyzer, 50 kDa, Spectrum Europe B.V. Breda, Netherlands) and then concentrated using Nanosep® Centrifugal (VWR International GmbH, Darmstadt, Germany) and injected i.v. (1.3 µl/g). Proximale tubules were visualized by collecting autofluorescence. To investigate the effects of Ang II on podocyte albumin endocytosis, increasing doses (10, 30, 60 and 80 ng/kg/min) of Ang II (Phoenix pharmaceuticals, Inc.) were each infused for 30 min into seven animals. Z-stacks of 5-7 glomeruli per animal and per dose of Ang II were acquired. Subsequent, to analyze the fate of the endocytic vesicles within a single podocyte, z-stacks over time were obtained for 30 min during continuous infusion of 80 ng/kg/min Ang II.

For *in vivo* colocalization studies of albumin and acid cell compartments, vasculature was labeled using FITC-albumin conjugate (40 mg/ml, 1.5 µl/g BW, Sigma-Aldrich) and acid cell compartments were stained with 30 µl/animal of 1:5 diluted lysotracker-solution (Invitrogen).

To investigate the role of the AT1 receptor, losartan (9.9 µg/kg BW) was injected i.v. before starting the Ang II infusion, in five experiments. Furthermore five animals were pretreated with gentamicin, a competitive inhibitor of megalin, for 5 days (100 mg/kg/day, i.p.), before the Ang II dose response experiments.

Image analysis. A total of 10 representative layers of the z-stack were analyzed. Amira 5.4.5 (Visualization Sciences Group, Mérignac Cedex) was used to reconstruct and calculate the volume of the albumin-containing vesicles.

In order to investigate the fate of the vesicles, z-stacks over time were analyzed using the ZEN2010 software. The vesicle fluorescence intensity in the green (Lucifer Yellow) and

red channel (Alexa Fluor 594 albumin) was determined within 10 minutes, using regions of interest (ROI, 20 pixels), placed in a depth of 15 µm underneath the kidney capsule. For the determination of the subpodocyte space/plasma-intensity-ratio, 20 pixel ROIs were set in the subpodocyte space and in the capillary loops in a depth of 15 µm underneath the kidney capsule and Alexa Fluor 594 albumin intensities were measured.

Glomerular capillary flow. The dose-dependent effects of Ang II (0, 10, 30, 60 and 80 ng/kg/min) on glomerular capillary flow were determined in five animals, using MPM (25). Glomerular vasculature was stained using Alexa Fluor 594 albumin, which labels the plasma, whereas the red blood cells appear as dark unstained objects inside the capillaries. The red blood cells velocity (V) was visualized and measured by performing a time (t) series of 500 fast longitudinal line scans, applying a pixel dwell time of 1.97 µs. The total distance (X) was 20 µm within the central axis of the capillary, displaying the movement of the RBC as dark bands within the Xt-scan. The velocity [µm/ms] of the RBC is inversely proportional to the slope of the evolved bands, which was calculated as $\Delta X/\Delta t$. To determine the diameter (d) of the measured capillaries, a z-stack of each glomerulus was obtained. The diameter was then indicated as the mean of 3 single vertical measurements along the line scan area in the capillary. Glomerular capillary flow [nl/min] was calculated using the following formula:

$$\text{Flow} = V*((d/2)^2*\pi).$$

In vitro analysis of fluorescent dyes. To investigate the pH sensitivity of the fluorescent probes, 4 µl of Lucifer Yellow (2.5 mg/ml) and Alexa Fluor 594 albumin (5 mg/ml) stock solutions were each diluted in 100 µl of PBS of pH 7.2; 6.0; 5.0; .4.5 and 4.0, respectively. The solutions were incubated at 37°C for 10 minutes, before fluorescent intensities were measured using a Nano Drop 3300 Fluorospectrometer (Thermo Fisher Scientific Inc., Waltham).

Antibodies. Immunostainings were performed for light microscopy and for electron microscopy. The following primary antibodies were used: rabbit anti-podocin IgG (1:200,

Sigma-Aldrich), guinea pig anti-megalin IgG (1:100, generous gift of Franziska Theilig), goat anti-albumin IgG (1:500, Abcam) and rabbit anti-caveolin-1 IgG (1:100, Abcam). As secondary antibodies for immunostainings, either Alexa Fluor 488 or Rhodamin(TRITC)-conjugated donkey anti-rabbit IgG (Jackson Immuno Research), Alexa Fluor 488 or Rhodamin(TRITC)-conjugated donkey anti-goat IgG (Jackson Immuno Research) and Cy2 conjugated goat anti-guinea pig IgG (Abcam) was used. For electron microscopy Alexa Fluor 488 and 1.4 nm gold conjugated rabbit F (ab) anti- goat (Abcam) was used.

Fixation. Animals were perfused with 4% paraformaldehyde solution in phosphate buffered saline (pH 7.2) at a constant perfusion pressure of 130 mm Hg. Subsequently, the fixed kidneys were either processed for electron microscopy or paraffin sections. Human, paraffin-embedded kidney tissue was a generous gift of Prof. Reichhold ().

Immunostaining for light microscopy. Fixed kidneys were first dehydrated by ascending alcohol solutions (70, 80, 90, and 100% methanol), followed by 100% isopropanol for 2x30 minutes each and embedded in paraffin. Areas from the middle of the kidney were sliced (2-5 μm) and blocked with either 10% horse serum/1% BSA/PBS or 5% milkpowder/0.04% triton/PBS for 20 minutes at room temperature. Sections were then incubated with the first antibody overnight (4°C). Following three washing steps (PBS), the secondary antibodies (diluted 1:400) were applied for 90 minutes and the sections were then mounted with glycergel (DakoCytomation), before imaging on an Axiovert observer microscope (Zeiss).

Immunostaining for electron microscopy. Perfusion-fixed kidney slices of Ang II-infused rats were further fixed in 4% paraformaldehyde and 0.1 M sodium-cocadylat buffer for one day. After washing with 0.1 M sodium-cocadylat buffer, the kidney slices were dehydrated in ascending ethanol dilutions (70, 90 and 100%) for 2x20 minutes each at 4°C and embedded in LR white resin (Agar scientific). Ultrathin sections (100 nm) were collected on single slot pioloform-coated nickel grids (Agar scientific) and incubated on drops of a

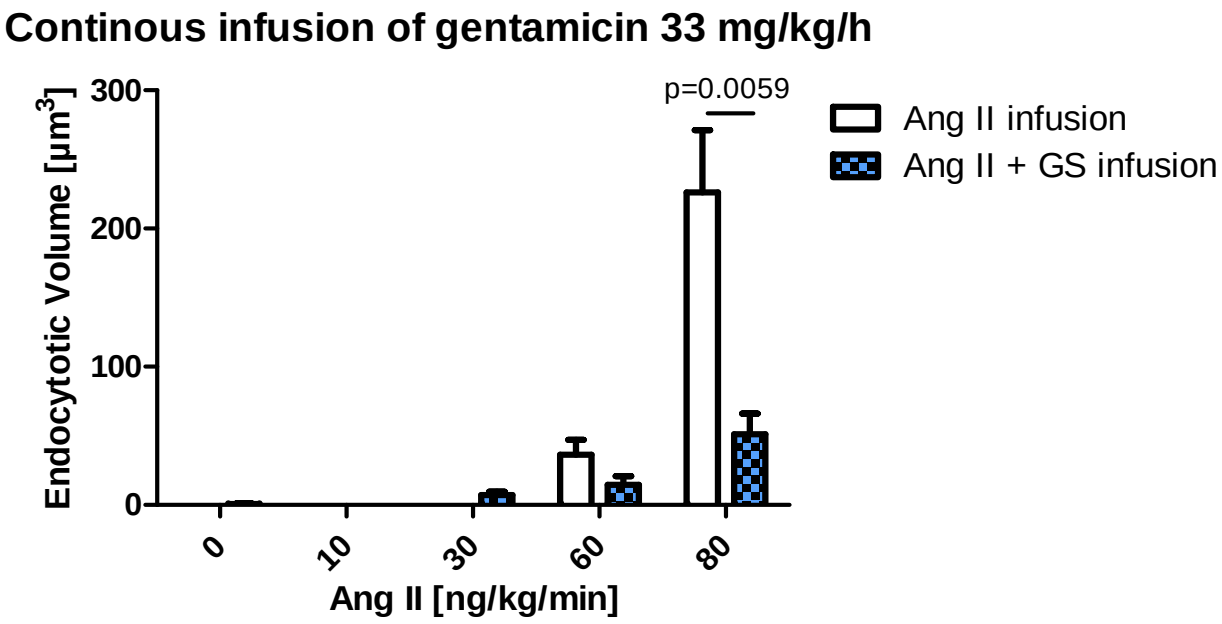
blocking solution (5% milkpowder/PBS) for 15 minutes, washed (0.5% milkpowder/PBS) and then incubated with the first antibody (1:50) for 60 minutes. After washing (0.5 milkpowder/PBS) the sections were incubated on drops with the second antibody (1:50) for another hour and fixed with 2% glutaraldehyd/PBS for 5 minutes. Next, the gold staining was intensified during silver incubation (0.73% silverlactat/5.6%hydrochinon/25.5%citrat/23.5%sodium-citrat/33%gummi arabicum/H2O) for 17 minutes before washing (0.5 milkpowder/PBS). Finally, the grids were counterstained for electron microscopy with 1% aqueous uranyl acetate/H2O and visualized using a JEOL JEM-2100F Feldemissions-Transmissionselektronenmikroskop (Jeol).

Urinary exosomes isolation. Albumin-containing vesicles were isolated from the urine of Ang II-infused rats using total exosomes isolation reagent (Invitrogen). The rats were injected with Alexa BSA before Ang II-infusion, which was used to visualize the albumin-content of urinary exosomes by MPM.

Statistics. Data were further analyzed by ANOVA with Bonferroni post hoc test using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA). All data are given as means ± SEM. $P<.05$ was considered significant.

Study Approval. All of the animal experiments were conducted according to the National Institutes of Health's *Guidelines for the Use of Laboratory Animals and approved by local authorities.*

Supplemental figure 1. Ang II-induced albumin endocytosis is reduced in the presence of the competitive megalin inhibitor gentamicin (short-term administration of gentamicin). Data show the result of a continuous administration of gentamicin (33 mg/kg/h) starting 60 min before the infusion of Ang II (80 ng/kg/min). The volume of the albumin-containing vesicles in podocytes was quantified by 3D reconstruction. Endocytotic activity of podocytes is largely blunted in the presence of the competitive megalin inhibitor gentamicin.



Supplemental figure 2. Immuno-labeling of podocin is restricted to the glomerulus when used in single stainings. In contrast to what was observed when the podocin antibody was used in double stainings (fig. 5, showing some cross-reactivity with the proximal tubule), no tubular staining was present in single stainings.