Intravital Imaging Reveals Angiotensin II–Induced Transcytosis of Albumin by Podocytes

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ABSTRACT

Albuminuria is a hallmark of kidney disease of various etiologies and usually caused by deterioration of glomerular filtration barrier integrity. We recently showed that angiotensin II (Ang II) acutely increases albumin filtration in the healthy kidney. Here, we used intravital microscopy to assess the effects of Ang II on podocyte function in rats. Acute infusion of 30, 60, or 80 ng/kg per minute Ang II enhanced the endocytosis of albumin by activation of the type 1 Ang II receptor and resulted in an average (±SEM) of 3.7±2.2, 72.3±18.6 ($P<0.001$), and 239.4±34.6 μm^3 ($P<0.001$) albumin-containing vesicles per glomerulus, respectively, compared with none at baseline or 10 ng/kg per minute Ang II. Immunostaining of Ang II–infused kidneys confirmed the presence of albumin-containing vesicles, which colocalized with megalin, in podocin-positive cells. Furthermore, podocyte endocytosis of albumin was markedly reduced in the presence of gentamicin, a competitive inhibitor of megalin-dependent endocytosis. Ang II infusion increased the concentration of albumin in the subpodocyte space, a potential source for endocytic protein uptake, and gentamicin further increased this concentration. Some endocytic vesicles were acidified and colocalized with LysoTracker. Most vesicles migrated from the capillary to the apical aspect of the podocyte and were eventually released into the urinary space. This transcytosis accounted for approximately 10% of total albumin filtration. In summary, the transcellular transport of proteins across the podocyte constitutes a new pathway of glomerular protein filtration. Ang II enhances the endocytosis and transcytosis of plasma albumin by podocytes, which may eventually impair podocyte function.

J Am Soc Nephrol 27: 731–744, 2016. doi: 10.1681/ASN.2014111125

Albuminuria is a hallmark of kidney disease of various etiologies and usually caused by a deterioration of the integrity of the glomerular filtration barrier. Albuminuria is a symptom of glomerular disease but also, a cause; thus, it is involved in the development of renal interstitial fibrosis and eventually, a decline in kidney function.[1] The glomerular filtration barrier consists of the fenestrated endothelium, the basement membrane, and the podocytes. Lesions of the podocytes, such as podocyte effacement, detachment, or microvillus transformation, inevitably lead to the development of albuminuria.[2]

Patients with albuminuria, in most cases, benefit from a blockade of the renin angiotensin system (RAS), and RAS inhibition has antiproteinuric effects, even in individuals without hypertension.[3] In a recent study, angiotensin II (Ang II) increased the glomerular filtration of an FITC-labeled Ficoll with a molecular mass between 70 and 400 kD.[4] In a more direct study, using intravital microscopy, these data were largely confirmed for the plasma protein albumin.[5] Thus, the sieving coefficient of albumin increased markedly after infusion of Ang II in healthy nonhypertensive rats, and this effect was mediated by the angiotensin II type 1 (AT1)

Received November 24, 2014. Accepted May 11, 2015.

Published online ahead of print. Publication date available at www.jasn.org.

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receptor and partly counteracted by the AT2 receptor; furthermore, the changes in albumin filtration in response to Ang II were largely independent of changes in mean arterial BP.[5]

In this study, we addressed the consequences of the Ang II–induced albumin leakiness of the glomerulus for the function and structure of the filtration barrier.[6] We hypothesized that an increased flow of albumin across the filtration barrier may force the podocytes to clear the basement membrane and/or the subpodocyte space (SP) of accumulating albumin. In line with our hypothesis, cultured immortalized human podocytes were shown to perform caveolae-dependent endocytosis of albumin,[7] and albumin-containing vesicles could be observed by electron microscopy in mouse, rat, and human tissue.[8]

Using intravital multiphoton microscopy (MPM) and electron microscopy, we found that Ang II infusion promotes the endocytosis of albumin by podocytes. The podocyte albumin endocytosis during Ang II infusion was mediated by the AT1 receptor, megalin dependent, and facilitated by an increase in the albumin concentration of the SP. Moreover, the podocytes released albumin-containing vesicles into the urinary space. Furthermore, analysis of the urine showed the excretion of albumin-containing vesicles of podocyte origin. Our data suggest a novel mechanism of transcellular plasma protein transport across the filtration barrier and emphasize the crucial role of this pathway during proteinuria.

RESULTS

Ang II Induces the Endocytosis of Plasma Albumin by Podocytes

We recently used MPM to show that acute Ang II infusion increases the sieving coefficient for albumin in the normal glomerulus. To assess the consequences of the increased flow of albumin across the filtration barrier for podocyte function, we infused increasing concentrations of Ang II in rats. Mean arterial pressure (MAP) was 98.9±5 mmHg at baseline and rose to 102.4±4, 109.8±4, 120.1±5, and 132.7±3 mmHg for 10, 30, 60, and 80 ng/kg per minute Ang II, respectively (n=7 each). The Ang II–mediated increase in MAP was accompanied by a slight decrease in glomerular capillary flow, which declined from 10.3±1.6 nl/min at baseline to 8.1±1.4, 8.0±1.2, 7.9±1.1, and 7.0±1.2 nl/min during infusion of 10, 30, 60, and 80 ng/kg per minute Ang II, respectively (n=29 each; P=0.002, P=0.02, P=0.02, and P<0.001, respectively, versus baseline). Plasma Ang II concentrations increased from 92±22 pg/ml at baseline to 582±108 pg/ml during the infusion of 80 ng/kg per minute Ang II (n=4).

During baseline conditions, there was no visible albumin endocytosis by podocytes. By contrast, Ang II infusions of 10, 30, 60, and 80 ng/kg per minute increased the total volume of three–dimensional reconstructed albumin–containing vesicles to 0, 3.7±2.2, 72.3±18.6 (P<0.001), and 239.4±34.6 μm^3 (P<0.001), respectively (n=40 for each concentration) (Figure 1C). Podocyte endocytosis of albumin was abolished when the animals were injected with the AT1 receptor antagonist losartan (9.9 μg/kg; n=25) before starting the infusion of Ang II (Figure

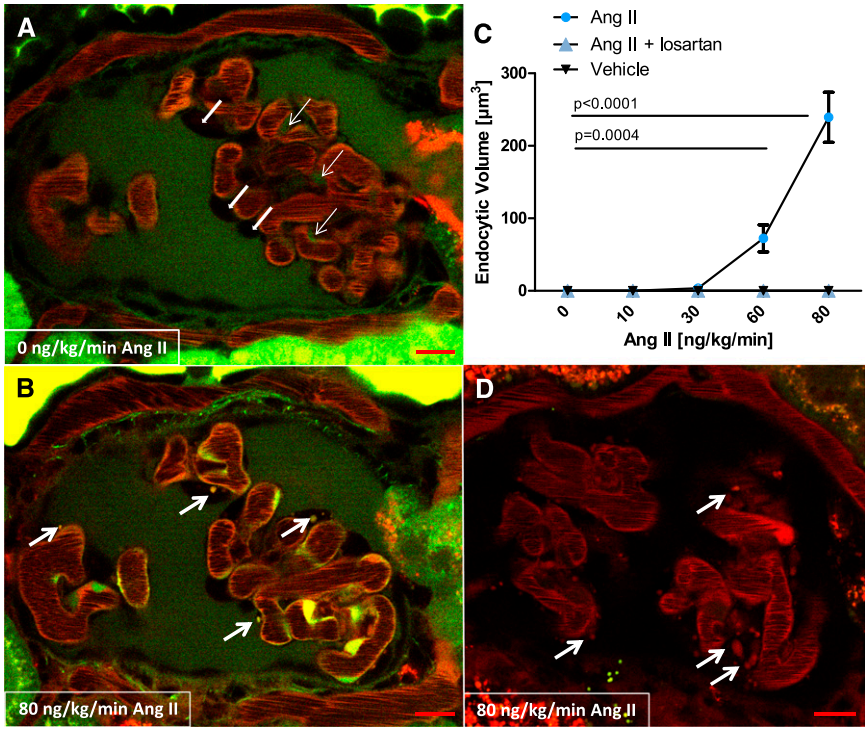


Figure 1. Ang II–induced albumin endocytosis by podocytes is mediated by the AT1 receptor. (A) MPM image of a rat glomerulus during baseline conditions. The vasculature was stained with Alexa bovine serum albumin (BSA) (red). To visualize podocytes, Lucifer Yellow (LY), a freely filtered small molecular weight dye, was continuously infused. Podocytes are excluded from the staining and appear as dark objects at the outer margin of the glomerular capillaries (bold arrows). Mesangial cells take up LY and accumulate a green/yellow color (thin arrows). Scale bar, 10 μm. (B) The same glomerulus during continuous infusion of 80 ng/kg per minute Ang II. Orange vesicles within the podocytes (arrows) indicate that Alexa BSA and LY are colocalized in the endocytic vesicles. Scale bar, 10 μm. (C) Podocyte albumin endocytosis as a function of the Ang II dosage and in the presence of the AT1 receptor antagonist losartan. The volume of the albumin-containing vesicles in podocytes was quantified by three-dimensional reconstruction. (D) MPM image of a rat glomerulus during infusion of 80 ng/kg per minute Ang II without coinfusion of LY. Bowman's space appears black, and the cell bodies of the podocytes are invisible. Albumin endocytosis is detectable as red vesicles (arrows) at the outer margin of the glomerular capillaries. Scale bar, 10 μm.

1C). In some experiments, podocytes were visualized using MPM by negative imaging during constant infusion of Lucifer Yellow (LY) (Figure 1A).[9] Images obtained during these experiments revealed a colocalization of LY and Alexa BSA in the Ang II–induced endocytic vesicles, suggesting that podocytes perform fluid-phase uptake. The formation of the endocytic vesicles was restricted to the capillary aspect of the podocytes (Figure 1B) and absent during control experiments, when 0.9% saline was infused into the animals (Figure 1C) (n=15). Immunohistochemistry of kidney sections of Ang II–infused rats showed that the albumin-containing vesicles are found in podocin-positive cells in the glomerulus (Figure 2C). Electron microscopy images of kidney sections of the Ang II–infused animals further confirmed that the endocytic vesicles contained albumin. As shown in Figure 3, immunogold staining of albumin was localized in vesicular structures within the podocytes as well as in the proximal tubule.

Podocyte Albumin Endocytosis Is Megalin Dependent

Albumin endocytosis by cultured human podocytes is caveolae dependent.[7] Our immunostainings confirmed the expression of caveolin-1 in the podocytes (Figure 4A) of the Munich Wistar Froemter (MWF) rat and revealed that glomerular albumin colocalized with caveolin-1 (Figure 4B). Because megalin colocalizes with caveolae in various cell types[10] and because the proximal tubular uptake of filtered albumin is mediated by the megalin/cubilin complex,[11–13] we hypothesized that megalin may be involved in the uptake of albumin by podocytes. We used immunohistochemistry to assess the expression of megalin in podocytes of the MWF rat. As can be seen in Figure 4C, megalin was expressed in rat podocytes and colocalized with the slit–membrane protein podocin. Similar results were obtained with human kidney tissue (Figure 4D). To assess the function of megalin in the podocytes, we used gentamicin as a competitive inhibitor of megalin–dependent albumin endocytosis.[14] When rats were pretreated with gentamicin (100 mg/kg body wt intraperitoneally daily) for 5 days, the dose–dependent albumin endocytosis induced by Ang II infusion was markedly reduced compared with vehicle-pretreated animals. The total volume of endocytic vesicles after infusion of 60 and 80 ng/kg per minute Ang II averaged 1.5±1.5 and 22.5±6.3 μm^3 (n=27 each) for the gentamicin groups and 72.3±18.6 and 239.4±34.6 μm^3 for the vehicle-pretreated groups, respectively (P=0.003 and $P<0.001$, respectively) (Figure 5).

Considering possible unspecific toxic effects of the chronic gentamicin treatment, we used a second approach and acutely infused gentamicin at a rate of 33 mg/kg per hour. The gentamicin infusion was started 1 hour before the infusion of Ang II and maintained throughout the experiment. Again, podocyte albumin uptake was attenuated by gentamicin treatment. In response to acute gentamicin infusion, the endocytotic volumes during infusion of 60 and 80 ng/kg per minute Ang II were reduced to 14.7±6.3 (P=0.24) and 51.10±15.0 μm^3 ($P<0.01$), respectively, compared with 72.3±18.6 and 239.4±34.6 μm^3, respectively, for the vehicle-pretreated groups (Supplemental Figure 1).

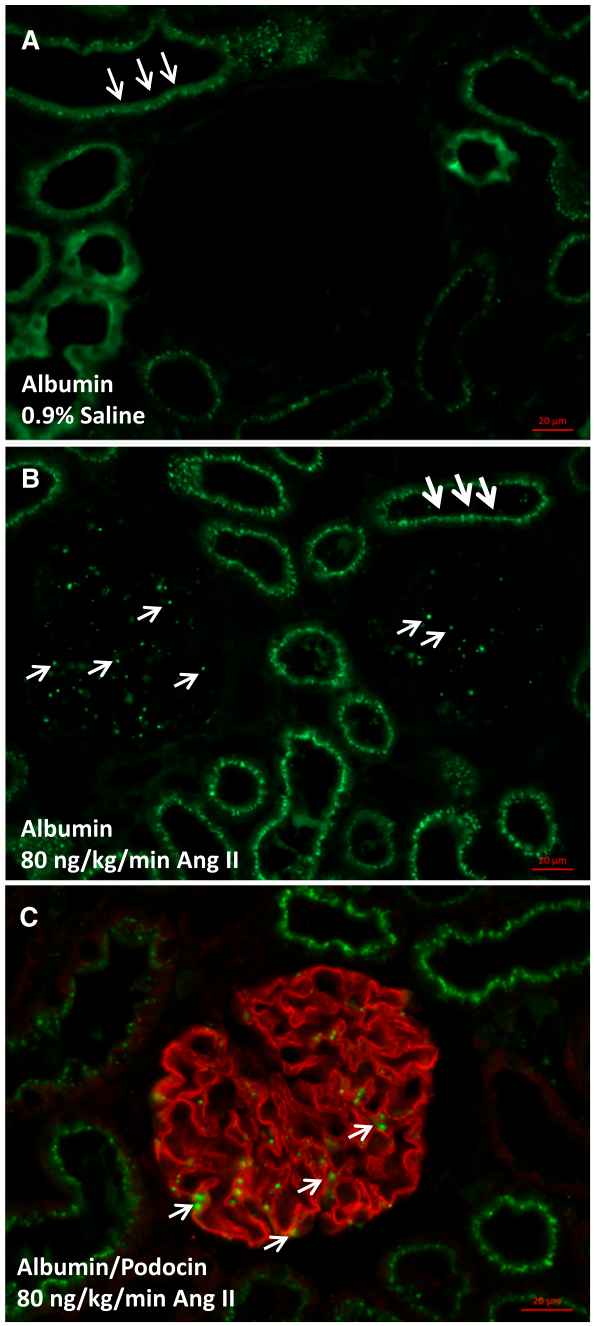


Figure 2. Endocytic vesicles in podocytes contain plasma albumin. (A) Immunostaining for albumin in a control rat kidney. A bright green albumin staining is visible in the proximal tubules (arrows), where filtered albumin is retained by endocytosis. No albumin is visible within the glomerulus. (B) Immunostaining for albumin in the kidney of an Ang II–infused rat. Intensive staining is found in the proximal tubules (bold arrows) and vesicles within the glomerulus (arrows). (C) Albumin-containing vesicles (green) are found in podocin-positive cells (red).

Finally, immunohistochemistry on renal sections of Ang II–infused rats revealed the colocalization of glomerular albumin

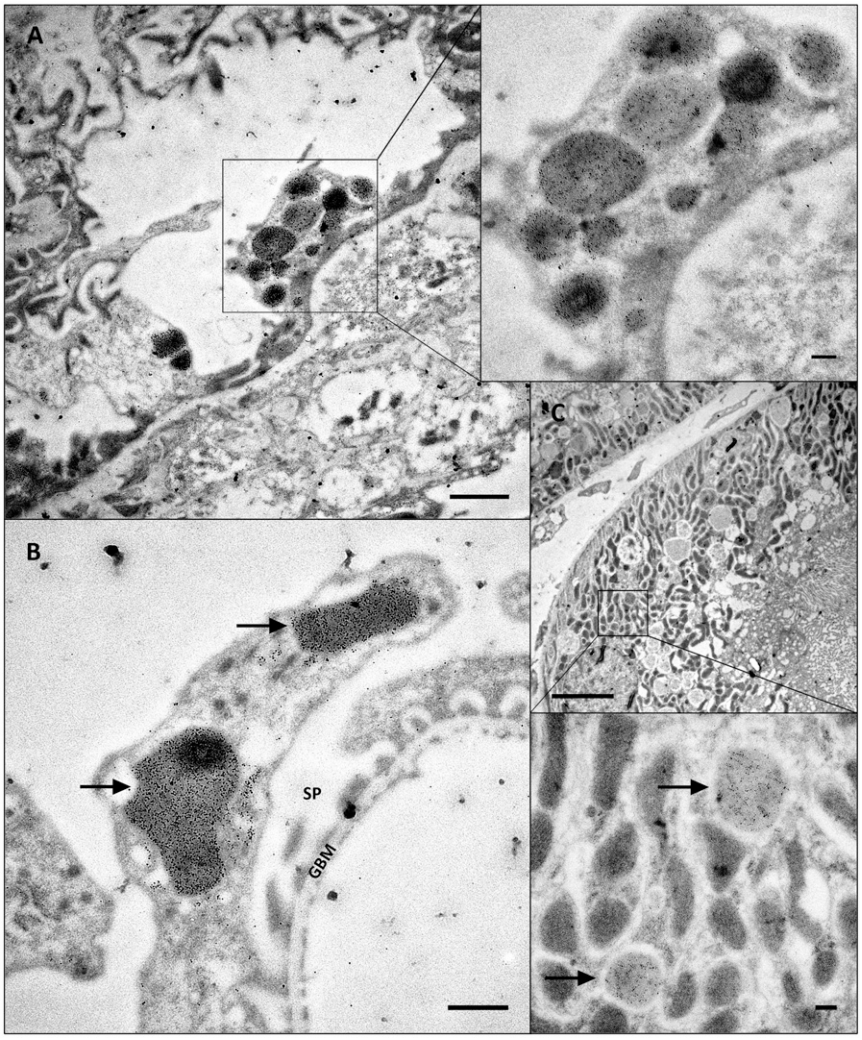


Figure 3. Endocytosed plasma albumin is localized in vesicular structures in podocytes. Electron microscopy of ultrathin LR White–embedded kidney sections of an Ang II–infused rat (immunogold labeling of albumin). (A) Podocyte filled with multiple albumin-containing vesicles. Scale bar, 1 μm; 200 nm in Inset. (B) Podocyte with two large albumin–containing vesicles (arrows). GBM, glomerular basement membrane; SP, subpodocyte space. Scale bar, 500 nm. (C) Albumin is located in endocytic vesicles (arrows) within the proximal tubules. Scale bar, 4 μm; 200 nm in Inset.

and megalin (Figure 5D). These data suggest that the Ang II–induced albumin endocytosis by podocytes is largely if not completely megalin dependent.

Ang II Increases the Albumin Concentration in the SP

Because the formation of endocytic vesicles in response to Ang II apparently occurred at the capillary aspect of the podocytes, we next determined if Ang II leads to changes in the albumin concentration in the SP, which is considered a compartment of the filtration barrier with restricted fluid movement (Figure 6A).[9,15] The fluorescence intensity in the SP relative to that in the blood plasma did not change during infusion of 0.9% saline (NS; n=7) (Figure 7B). By contrast, the albumin concentration in the SP increased by 19.4% after infusion of 80 ng/kg per minute Ang II compared with baseline (P=0.002; n=20) (Figure 6B). When the rats were pretreated with gentamicin, the albumin concentration in the SP increased even further during Ang II infusion compared with Ang II–infused and vehicle-pretreated rats (+34.8±7%; P=0.02; n=10) (Figure 6B). These data suggest that Ang II causes an accumulation of albumin below the podocyte cell body and that the endocytic activity of the podocyte may contribute to the clearance of the filter.[16–18]

Endocytosed Albumin Is Partly Located in Acidified Vesicles

The albumin-containing vesicles had two fates. One subset was acidified over time as judged by the reduction of red channel fluorescence intensity, which corresponded to the Alexa BSA–derived fluorescence. Thus, within 10 minutes of vesicle formation, the fluorescence intensity in the red channel gradually decreased by 41±5%, whereas the green channel, which indicated coendocytosed LY, remained unchanged (n=7 each) (Figure 7, A–C). We further investigated the fluorescent dyes as a function of pH *in vitro*. When incubated at pH values of 7.2, 6.0, 5.0, 4.5, and 4.0 for 10 minutes, we found a linear decline in the fluorescence intensity of Alexa BSA with decreasing pH of the solution (r^2=0.90; P<0.001). In contrast, the fluorescence intensity of LY was independent of the pH (r^2=0.007; NS) (Figure 7C). A decrease in the fluorescence intensity of Alexa BSA of approximately 40% corresponded to a pH of 4.5 compared with a pH of 7.2, which is similar to the content of lysosomal vesicles.[19] Furthermore, the *in vivo* application of LysoTracker, a marker of acidified compartments, revealed that the albumin-containing vesicles colocalized with LysoTracker (Figure 7E).

Albumin-Containing Vesicles Are Released into the Urinary Space

The second subset of vesicles grew quickly in size, migrated from the capillary to the urinary aspect of the podocytes, and was eventually released into the urinary space (Figure 8). As a consequence, floating albumin–containing vesicles could be observed on detachment from podocytes (Figure 8E). The transpodocyte passage was a highly dynamic process and occurred within 3.5±0.6 minutes (n=10) of the vesicle appearance. The fluorescence intensities within this subset of vesicles did not change significantly over time (100% at baseline versus

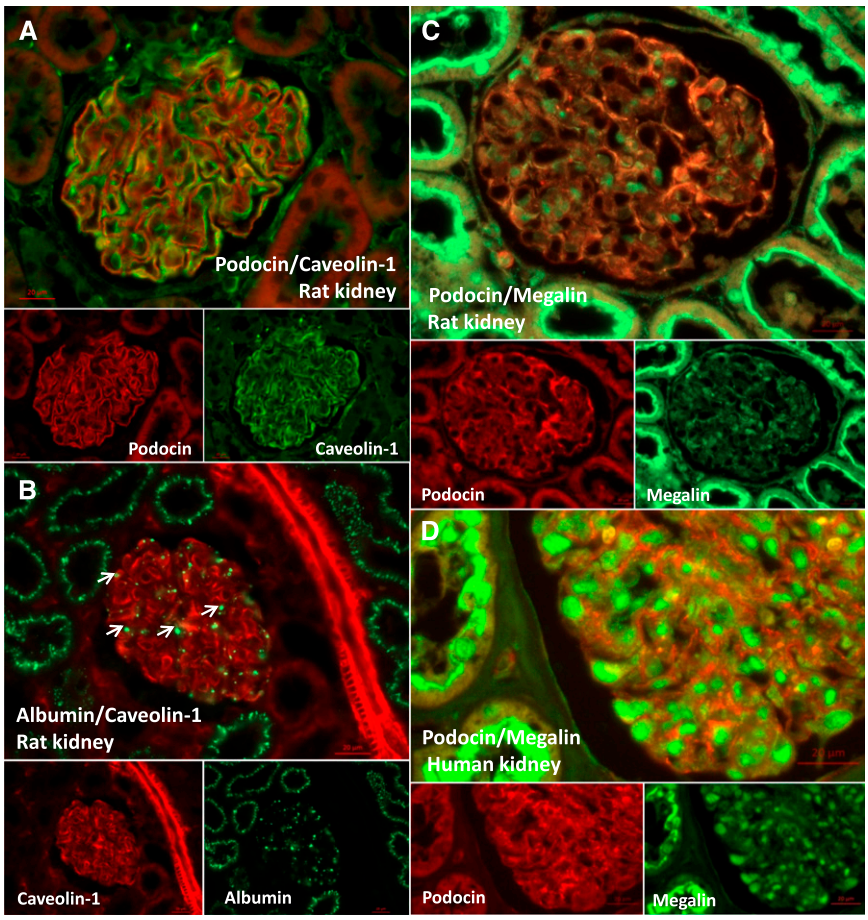


Figure 4. Podocytes express caveolin-1 and megalin. (A) Immunostaining confirms the colocalization of podocin (red) and caveolin-1 (green). (B) Glomerular albumin vesicles of an Ang II–infused rat kidney colocalize with caveolin-1. Immunostaining for megalin (green) and podocin (red) in glomeruli of (C) rat and (D) human kidneys.

93.5%±7.5% before release into the urinary space; n=10; NS), and it was 7.5-fold higher compared with the intensities in the podocyte cytoplasm. This finding could be because of the rapid and dynamic transcellular transport of their albumin content into the urinary space. Furthermore, the considerable increase in size observed in some vesicles was caused by the docking and fusion of smaller vesicles with larger ones (Figure 8B). Electron microscopy of Ang II–infused rats further revealed single albumin–containing vesicles bending into the urinary space (Figure 9A), and subsequently, numerous detached albumin–containing vesicles were found in the Bowman's space (Figure 9B). On the basis of the average surface area of the released vesicle of 190 μm^2, the release of albumin-containing vesicles by podocytes into Bowman's space will lead to a considerable membrane loss. Nevertheless, no apparent podocyte cell shrinkage was observed, suggesting an efficient membrane resynthesis.

Podocyte Albumin Release in Response to Acute Ang II Infusion Is Not Associated with Cell Death

Ang II exposure and albumin endocytosis have been reported to induce apoptosis in podocytes.[20,21] Therefore, we next investigated if albumin release by podocytes in our experimental setup of acute Ang II–mediated albumin endocytosis may be related to apoptosis/necrosis. To label functionally compromised cell nuclei, we injected propidium iodide before Ang II infusion. Although there was abundant podocyte albumin endocytosis during infusion of 80 ng/kg per minute Ang II, no propidium iodide–positive cells were detected within the glomerulus (Figure 10A). In contrast to what was observed during Ang II infusion, propidium iodide accumulated in the nuclei of podocytes and other glomerular cells on laser–induced tissue lesions (Figure 10C).

Quantitative Analysis of Plasma Albumin Endocytosis by Podocytes

Endocytosed plasma albumin was subject to either acidification/degradation or release into the urinary space. We next determined the relative contribution of albumin release versus vesicular acidification on the basis of the following calculation. The mean vesicular Alexa Fluor 594 albumin fluorescence intensity during infusion of 80 ng/kg per minute Ang II was 15,603±945 relative Units (rU) (n=33). The volume of vesicles before release averaged $0.00024 \pm 6.7 \times 10^{-5}$ nl. A subset of 47.4% of the investigated podocytes performed albumin release, and the average number of release events per release-positive podocyte within 20 minutes was 1.78±0.24. The number of podocytes per rat glomerulus is approximately 380.[22] Consequently, the total released volume per glomerulus was $0.474 \times 380 \times 1.78 \times 0.00024 = 0.076$ nl. Accordingly, the total amount (volume × fluorescence intensity) of transcellular albumin filtration per glomerulus within 20 minutes was 0.076 nl×15,603 rU=1190 nl×rU. The average volume of acidified vesicles was $2.7 \times 10^{-5} \pm 7.1 \times 10^{-6}$ nl. Within a timeframe of 20 minutes, 68.4% of the podocytes in the field showed an average of 1.77±0.31 albumin-containing vesicles per cell. Consequently, the amount of albumin, which was subject to vesicular acidification, was $0.68 \times 380 \times 1.77 \times 2.7 \times 10^{-5}$ nl×15,603=194 nl×rU. In summary, 86% [1190/(1190+194)] of the endocytosed albumin was released into the urinary space on transcellular movement, whereas 14% was acidified and presumably degraded.

Relative Contribution of Transcellular Versus Paracellular Albumin Filtration

Because our study suggested that Ang II induced transcellular albumin filtration, we aimed to quantify the relative contribution of transcellular and paracellular albumin filtration

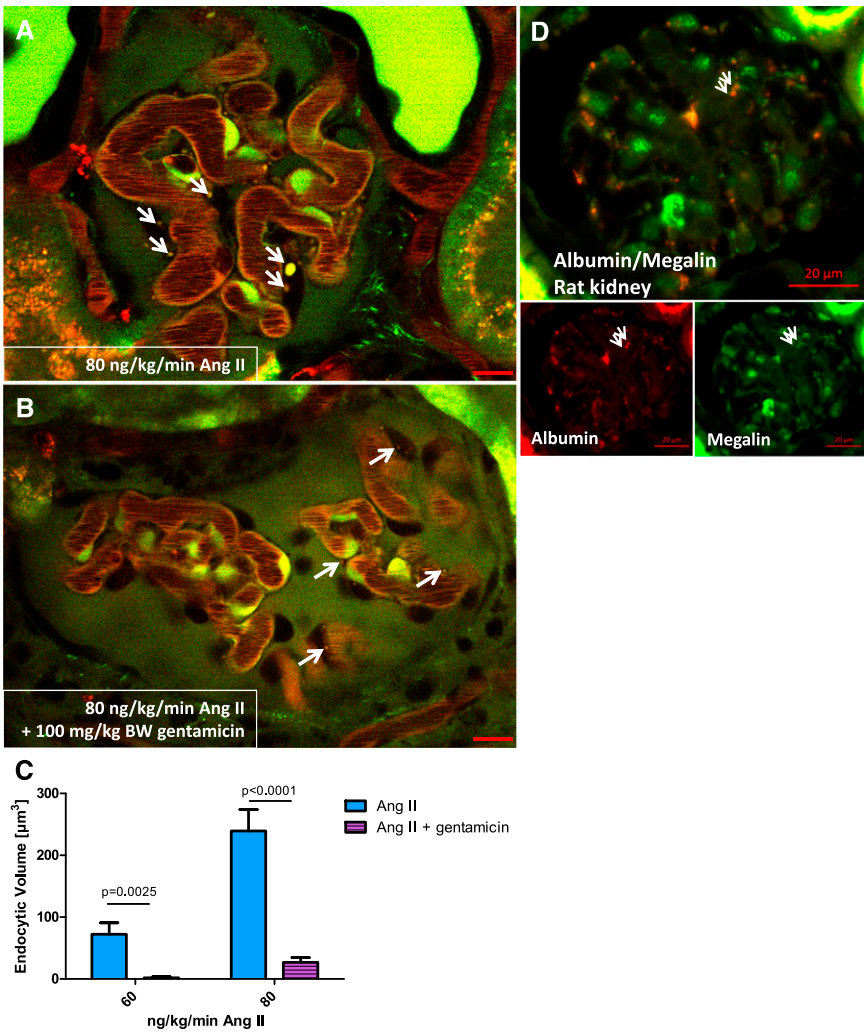


Figure 5. Ang II–induced albumin endocytosis is reduced in the presence of the competitive megalin inhibitor gentamicin. (A) MPM image of a rat glomerulus during infusion of 80 ng/kg per minute Ang II. The vasculature was stained by Alexa BSA. The podocytes show large albumin–containing vesicles (arrows). Scale bar, 10 μm. (B) MPM image of a glomerulus of a gentamicin-treated rat (100 mg/kg per day intraperitoneally) during infusion of 80 ng/kg per minute Ang II. Only small albumin–containing vesicles are visible within the podocytes (arrows). BW, body weight. Scale bar, 10 μm. (C) Quantification of the Ang II–induced albumin endocytosis by podocytes with and without gentamicin treatment (100 mg/kg per day intraperitoneally). (D) Colocalization of megalin-expressing cells (green) and albumin-containing vesicles (red).

over a period of 20 minutes. During infusion of 80 ng/kg per minute Ang II, the mean fluorescence intensity of Alexa Fluor 594 albumin in Bowman's space was 51.1±11.1 rU (n=27). Assuming a single nephron glomerular filtration rate of 11 nl/min during Ang II infusion,[5] the total amount of albumin filtered along the paracellular pathway per glomerulus was 51.1 rU×11 nl/min×20 min=11,251 nl×rU. With the amount of transcellular filtered albumin (1190 nl×rU), the transcellular/paracellular albumin filtration ratio was 1190/11,251=0.106. Thus, during Ang II infusion, approximately 10% of the total albumin filtration involved transcellular movement.

Urinary Vesicles Contain Plasma Albumin and Proteins from the Apical Membrane of Podocytes

Considering the release of endocytosed albumin, we next isolated albumin-containing vesicles from the urine of Ang II–infused rats. The urinary albumin–containing vesicles were investigated using MPM and revealed the existence of small vesicular structures containing the albumin–conjugated fluorescent dye (Figure 11A). In addition to FITC-labeled albumin, the urinary vesicles contained programmed cell death 6 interacting protein, an established exosomes marker protein,[23] and the podocyte apical membrane protein podocalyxin, whereas podocyte foot process protein podocin was absent, which was determined by Western blotting (Figure 11, B–D).

DISCUSSION

We recently used MPM to show that acute Ang II infusion increases the sieving coefficient for albumin in the normal glomerulus and that this effect of Ang II is largely pressure independent.[5] Here, we assessed the consequences of the increased flow of albumin across the filtration barrier for podocyte function. We found that Ang II infusion acutely enhanced the endocytosis of albumin by podocytes in a dose-dependent manner. We could further confirm the existence of Ang II–mediated albumin endocytosis by podocytes using immunohistochemistry and electron microscopy.

Glomerular vacuolization is a known phenomenon during proteinuria and has been reported in humans with FSGS.[24] Data obtained from human, rat, and mouse kidneys suggested that plasma proteins, such as IgG, ferritin, and albumin, may be localized in podocyte vacuoles.[8,17,25–30] Commensurate with these findings, podocytes were suggested to clear the glomerular filtration barrier from proteins.[17] Our results are in agreement with these studies and further reveal that an Ang II–mediated increase in albumin flux across the glomerular membrane enhances the endocytosis of albumin by podocytes in the normal glomerulus. More importantly, our results, for the first time, show that this process is megalin dependent and visualized that the endocytosed albumin may be released into the urinary space by podocytes.

The multiligand receptor megalin plays an essential role in the reabsorption of filtered albumin by the proximal tubule.

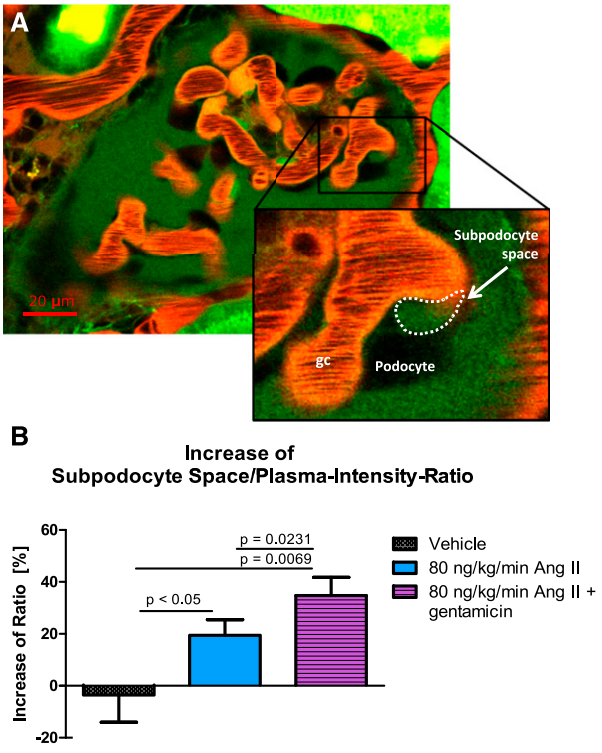


Figure 6. Ang II increases the concentration of plasma albumin in the SP. (A) MPM was used to visualize the SP during continuous infusion of LY. The glomerular vasculature (gc) was stained red (Alexa BSA). Scale bar, 10 μm. (B) Changes in the albumin concentrations in the SP during Ang II infusion were measured as the SP/plasma fluorescent intensity ratio of Alexa BSA.

The expression of megalin and cubilin has been localized in rat and human podocytes,[31–33] and subepithelial immune deposits were observed in rats infused with antimegalin IgG.[16] Our immunohistochemical data are consistent with these studies and confirm the expression of megalin in human and rat podocytes. Furthermore, our data showed that albumin is located in vesicle-like structures, which colocalized with megalin within the podocytes. Using MPM, we showed that the Ang II–mediated endocytosis of albumin by podocytes was significantly reduced by about 90% when the animals were pretreated with gentamicin, a competitive megalin inhibitor. These results suggest that the *in vivo* endocytosis of albumin by podocytes occurs in a megalin-dependent manner.

The Ang II–mediated formation of albumin-containing vesicles occurred on the luminal side of the podocytes and was accompanied by an increase of the albumin concentration in the SP. The Ang II doses used in this study caused a slight decrease in glomerular capillary flow. A reduction in flow favors the diffusion of macromolecules, such as proteins, over a biologic membrane,[34,35] and this biophysic effect may contribute to the rise in the albumin concentration in the SP. We conclude that the increased albumin flux over the glomerular filtration barrier may trigger the podocytes to endocytose the increased amount of albumin and consequently, clear the filter. Accordingly, the transcellular transport of albumin along the podocyte cell body may be significantly involved in the reported Ang II–mediated increase in the albumin sieving coefficient and subsequently, the rise in urinary albumin excretion.[5] Contraction of the podocyte foot processes in response to Ang II may further modulate the paracellular flux of albumin and consequently, alter its concentration in the SP.[36] Congruent with this assumption, we found that the albumin concentrations in the SP were increased when the animals were pretreated with gentamicin, suggesting again that the clearance function of podocytes is megalin dependent. Increased concentrations of albumin in the SP during Ang II infusion may also, in part, be related to Ang II–induced changes in the barrier function of the endothelium and its glycocalyx.[37] Thus, alterations in the composition and thickness of the glomerular endothelial glycocalyx may increase the leakiness of the filtration barrier for plasma proteins without changes in podocyte function.[38,39] Such changes in the barrier function of the endothelium may, for example, be caused by an increased local generation of reactive oxygen species and changes in local vascular endothelial growth factor (VEGF) concentration. Podocytes generate reactive oxygen species in response to Ang II, and Ang II has been shown to induce VEGF synthesis in podocytes through the p38 MAP kinase pathway.[40,41] Furthermore, podocyte–specific VEGF–deficient mice are proteinuric without apparent podocyte damage.[42]

In accordance with a clearance function of podocytes, the blockade of the fetal Fc fragment receptor, which is relevant for intracellular albumin vesicle sorting, caused a reduction in proteinuria in nephrotic rats.[25] Furthermore, fetal Fc fragment receptor-deficient mice accumulated IgG in the glomerular basement membrane, further supporting the assumption that podocytes have a clearance function for the glomerular filtration barrier.[16,17]

We found that endocytosed albumin is partly localized in acid cell compartments, which colocalized with the lysosomal marker LysoTracker *in vivo*. We, therefore, conclude that podocytes may degrade albumin in lysosome–like acidified vesicles. In keeping with this interpretation, cultured human urine–derived, podocyte–like epithelial cells were shown to degrade albumin in lysosomes.[43] In addition to lysosomal degradation, we showed that albumin is subject to a transcellular transport through the podocyte into the urinary space. Transcellular transport of proteins by podocytes has been suggested as a novel pathway of protein filtration during states of disease[25] and a potential mechanism of protein clearance from the SP.[17] Nevertheless, the phenomenon itself has never been visualized *in vivo*. Our results showed that, in addition to the classic paracellular pathway, albumin is transported into the urinary space along the transcellular pathway and that this pathway accounts for approximately 10% of the total glomerular albumin filtration. Our MPM and electron microscopy data further suggest that the actual mechanism of transcellular filtration includes the release of albumin-containing vesicles.

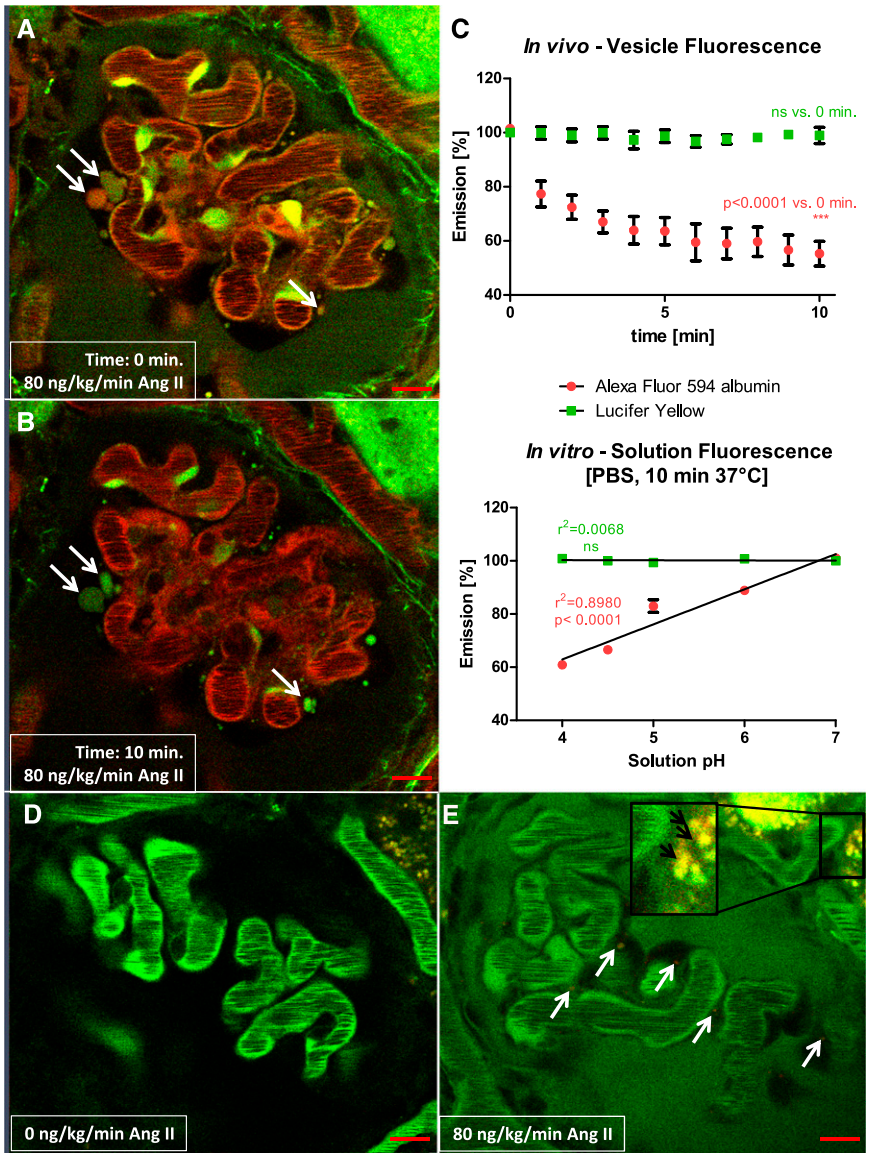


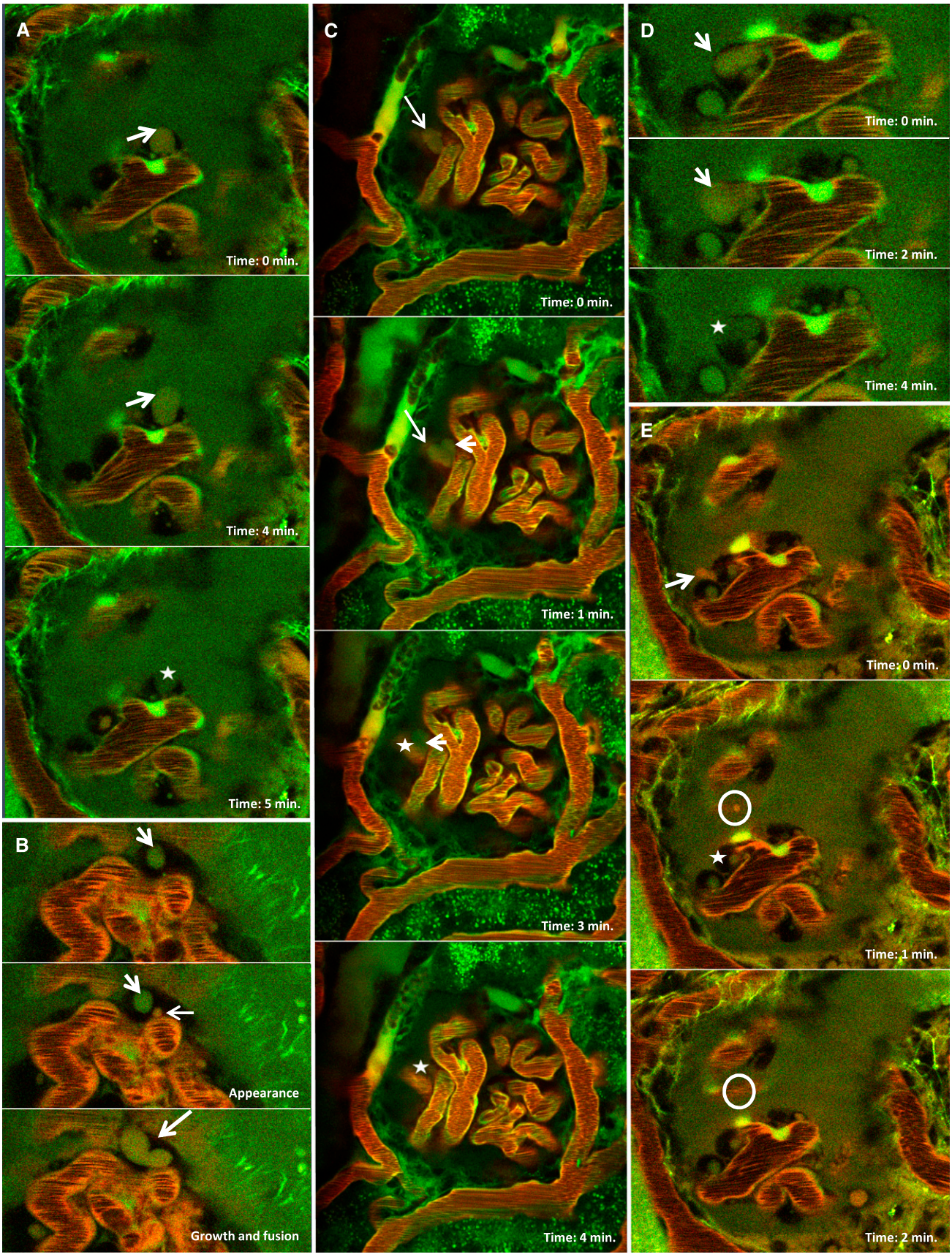
Figure 7. A subset of endocytic vesicles in podocytes is acidified. (A) MPM image of a glomerulus during infusion of 80 ng/kg per minute Ang II. Large orange vesicles are visible in the podocytes (arrows), indicating the colocalization of Alexa BSA and LY. Scale bar, 10 μm. (B) Ten minutes later (during continuous infusion of 80 ng/kg per minute Ang II), the color of the same endocytic vesicles appeared bright green, and the intensity of the red fluorescence of Alexa BSA declined. Scale bar, 10 μm. (C, upper panel) *In vivo* quantification of the vesicle fluorescent intensities of LY and Alexa BSA in podocytes over a period of 10 minutes. (C, lower panel) *In vitro* quantification of the fluorescent intensities of LY and Alexa BSA as a function of the pH. Glomerulus during (D) baseline and (E) infusion of 80 ng/kg per minute Ang II. Glomerular vasculature was stained using FITC-conjugated albumin (green). (E) Endocytic vesicles colocalize with LysoTracker (red), a marker of acidified compartments. Albumin and LysoTracker also colocalize in the proximal tubules (black arrows in Inset). Scale bar, 10 μm.

Thus, albumin is released in membrane-enclosed vesicles into the urinary space by podocytes. These vesicles could be observed when floating in Bowman's space after being released by podocytes. Accordingly, we isolated albumin-containing vesicles from the urine of Ang II–infused rats. These urinary vesicles contained podocalyxin, suggesting they were derived from the apical membrane of podocytes.[44] However, podocin, which is predominantly expressed in the podocyte foot processes, was absent from urinary vesicles. This finding is congruent with the observations made by *in vivo* imaging and the use of electron microscopy, suggesting that the release of albumin-containing vesicles was limited to the apical aspect of podocyte cell bodies.

On the basis of results of the propidium iodide experiments, necrosis or late-stage apoptosis most likely is not the cause for the release of large albumin–containing vesicles; however, early apoptotic blebbing needs to be considered as a source of albumin–containing vesicle formation.[45] This assumption is favored, by the in part large size of the albumin-containing vesicles. However, the concentration of albumin in the released vesicles was substantially higher compared with the cytoplasmic concentration. To our knowledge, endocytotic vesicles are usually not involved in the development of apoptotic blebs, but blebs are formed directly at the plasma membrane.[46,47] Furthermore, we observed no membrane protrusions into Bowman's space and no formation of membrane blebs containing low–cytoplasmic albumin concentrations. Such blebbing events would be readily detectable because of the contrast between the black podocyte cytoplasm and the yellow fluorescence in Bowman's space during LY infusion. In view of these observations, membrane blebbing presumably is not the primary pathway of albumin release by podocytes.

Although the exact identity of the albumin-containing vesicles remains to be determined, the endocytosis of albumin by podocytes followed by the release of albumin in membrane-containing vesicles leads to a net membrane loss. This process presumably is energy inefficient and may initiate a long-term deterioration of podocyte function when large amounts of plasma proteins are cleared from the filtration barrier.[48]

Membrane shedding has been postulated as a sign of podocyte injury.[44] Similar to what was found in proximal tubular cells,[1] *in vitro* studies in human urine–derived podocytes revealed that an albumin overload triggers a proinflammatory response.[21] Furthermore, when rats were infused with a solution containing


A
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Appearance
Growth and fusion
C
Time: 0 min.
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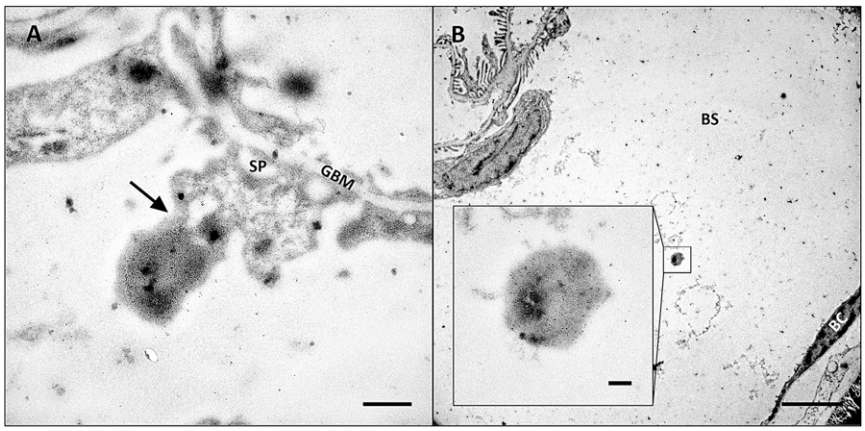

Figure 9. Electron microscopy confirms the release of albumin-containing vesicles into the urinary space (immunogold labeling of albumin). Electron microscopy images of an Ang II–infused rat kidney. (A) A podocyte vesicle containing albumin is bending into the urinary space before release (arrow). GBM, glomerular basement membrane; SP, subpodocyte space. Scale bar, 500 nm. (B) Subsequent to its release, an albumin-containing vesicle is found in the Bowman's space. BC, Bowman's capsule; BS, Bowman's space. Scale bar, 4 μm; 200 nm in Inset.

high concentrations of albumin, the animals developed proteinuria and showed signs of albumin deposition in the basement membrane and foot process effacement, suggesting that a prolonged overload of the endocytic machinery causes podocyte injury.[48] It seems, therefore, likely that an increase in albumin uptake by podocytes induces a proinflammatory response, suggesting that the podocyte release of endocytosed albumin is an early sign of pending podocyte injury. However, on the basis of negative *in vivo* propidium iodide staining, we consider it unlikely that vesicular albumin release by podocytes in the acute settings of our study was related to cellular injury. Nevertheless, the phenomenon of albumin transcytosis, which in our study, was observed in a healthy kidney, may, in the long run, initiate podocyte injury.

To our knowledge, this report provides the first *in vivo* evidence for the release of albumin-containing vesicles by podocytes. Although the paracellular pathway through the slit diaphragm is considered to be the classic pathway into the urinary space for proteins that have crossed the endothelium and the basement membrane, the transcellular passage across podocytes seems to constitute a new pathway across the filtration barrier. These *in vivo* observations are congruent with the findings in immortalized human podocytes *in vitro*. Thus, cultured human podocytes performed caveolae–dependent albumin endocytosis and alternatively, lysosomal degradation or transcytosis.[7]

In summary, Ang II increases the leakiness of the glomerular filtration barrier for albumin and initiates the endocytosis of albumin by podocytes in an AT1- and megalin-dependent manner. The increased movement of albumin across the filtration barrier is partly mediated by the transport of albumin along the transcellular pathway and likely facilitated by high albumin concentrations in the SP. Podocytes release albumin-containing vesicles into the urinary space on endocytosis. The endocytic activity of podocytes induced by Ang II may participate in the long-term deterioration of podocyte function.[48] From these data, we suggest that a reduction in the podocyte endocytic activity may contribute to the renoprotective effects of RAS inhibitors independently of their hemodynamic effects.

CONCISE METHODS

More detailed information can be found in Supplemental Material.

Animals

The animal experiments used young female MWF rats (110–160 g).

Multiphoton Microscopy

Imaging procedures used a Zeiss LSM710 NLO Confocal Fluorescence Microscope (Carl Zeiss) with a Chameleon Ultra-II MP Laser (Coherent) at 860 nm. Imaging setup, disposition of fluorescent probes, and animal preparation were performed as previously described[5]; 1024×1024-pixel images were acquired.

Visualization and Quantification of Albumin Endocytosis by Podocytes

Podocytes were visualized by negative imaging[9] during a continuous infusion of LY (5 mg/ml and 2 μl/min). To investigate the effects of Ang II on podocyte albumin endocytosis, 1.3 μl/g body wt purified Alexa Fluor 594 albumin solution (Invitrogen)[5] was injected, and increasing doses (10, 30, 60, and 80 ng/kg per minute) Ang II (Phoenix Pharmaceuticals) were each infused for 30 minutes. *Z* stacks of five to seven glomeruli per animal and per dose of Ang II were acquired. To analyze the fate of the endocytic vesicles within a single

Figure 8. Podocytes release albumin-containing vesicles into the urinary space. The vasculature was stained by Alexa BSA, and podocytes were visualized by MPM during continuous LY infusion. (A, C, and D) A subset of vesicles increased in size before being released into the urinary space (arrows). The release of albumin-containing vesicles (star) occurred within a few minutes after the vesicle appearance. (B) In close proximity to a vesicle (thin arrow), another small vesicle appears at the luminal side of the same podocyte (bold arrow). Shortly afterwards, the two vesicles are fusing into a larger vesicle (arrow). (E) A vesicle is bending into the urinary space (arrow). After being released, a floating vesicle is appearing in Bowman's space.

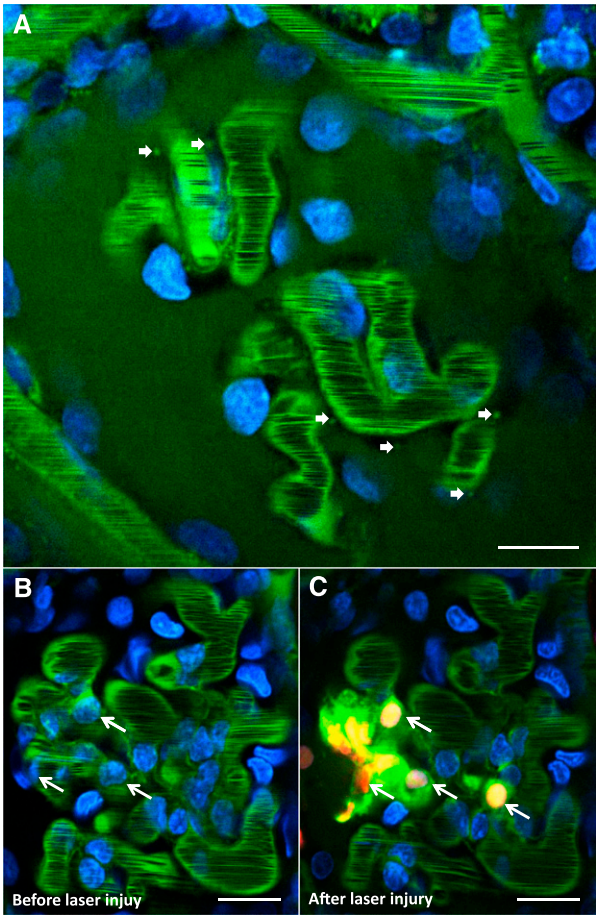

Figure 10. Podocyte albumin release during Ang II infusion is not related to cellular injury. (A) *In vivo* propidium iodide (red) staining after infusion of 80 ng/kg per minute Ang II. Cell nuclei were stained blue by Hoechst 33342. Arrows indicate Alexa BSA–containing vesicles within podocytes. No propidium iodide staining is visible. As a positive control, glomeruli were exposed to higher laser power (900 nm, 70% laser power, maximal zoom, and 2 seconds of exposure). Scale bar, 20 μm. Glomerulus (B) before and (C) 30 minutes after high–laser power exposure. The bright red and yellow nuclear staining (arrows in C) indicates injured cells with uptake of the DNA-intercalating dye.

podocyte, *Z* stacks over time were obtained for 30 minutes during continuous infusion of 80 ng/kg per minute Ang II.

To investigate the role of the AT1 receptor, losartan (9.9 μg/kg body wt) was injected intravenously before starting the Ang II infusion in five experiments. Five animals were pretreated with gentamicin, a competitive inhibitor of megalin, for 5 days (100 mg/kg per day intraperitoneally) before the Ang II dose response experiments. In a second experimental setup, gentamicin was continuously infused intravenously (33 mg/kg per hour) during the experiment starting 1 hour before the Ang II infusion.

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 body wt; Sigma-Aldrich), and acid cell compartments were stained with 30 μl per animal 1:5 diluted LysoTracker solution (Invitrogen).

To address possible apoptotic effects of Ang II on the podocytes, propidium iodide (0.66 mg/kg body wt; Invitrogen) was injected 30 minutes before the Ang II infusion. As a positive control, a laser-induced lesion (900 nm, 70% laser power, maximal zoom, 2 seconds of laser exposure) was set within the glomerulus to visualize the propidium iodide staining of nonvital cell nuclei.

Quantification of the Contribution of Paracellular Versus Transcellular Albumin Filtration

First, mean fluorescence intensities of Alexa Fluor 594 albumin within the Bowman's space, the glomerular capillary lumen, and endocytotic vesicles were empirically determined during four experiments of continuous infusion of 80 ng/kg per minute Ang II. Second, the mean vesicular volume before release into Bowman's space and the volume of acidified vesicles were measured by three-dimensional reconstruction using AMIRA 5.4.5. The percentage of endocytotic active podocytes on the basis of the total number of identifiable podocytes was calculated. From this subset of podocytes, we extrapolated to the total number of podocytes/glomerulus on the basis of data in the literature.[22] Third, within a *Z* stack over 20 minutes, we determined the average shedding events and the number of acidified vesicle. To estimate the single-nephron GFR during Ang II infusion, we referred to our own published data.[5]

Glomerular Capillary Flow

The dose-dependent effects of Ang II on glomerular capillary flow velocity were determined using MPM.[49] A *Z* stack of each glomerulus was acquired to determine the diameter of the capillaries and calculate the glomerular capillary flow.

Image Analyses

In total, 10 representative layers of the *Z* stack were analyzed. Amira 5.4.5 (Visualization Sciences Group) was used to reconstruct and calculate the volume of the albumin-containing vesicles. *Z* stacks over time were analyzed using the ZEN2010 software.

In Vitro Analysis of Fluorescent Dyes

To investigate the pH sensitivity of the fluorescent probes, stock solutions of LY and Alexa BSA were each diluted in PBS of pH 7.2, 6.0, 5.0, 4.5, and 4.0. The solutions were incubated at 37°C for 10 minutes before fluorescent intensities were measured using a Nano Drop 3300 Fluorospectrometer (Thermo Fisher Scientific).

Antibodies

Immunostainings were performed for light microscopy and electron microscopy. The following primary antibodies were used: rabbit antipodocin IgG (1:200; Sigma-Aldrich), guinea pig antimegalin IgG[50] (1:100), goat antialbumin IgG (1:500 or 1:50; Abcam, Inc.), and rabbit anticaveolin-1 IgG (1:100; Abcam, Inc.). As secondary antibodies for immunostainings, either Alexa Fluor 488 or Rhodamin (TRITC) –conjugated donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories), and Cy2–conjugated goat anti–guinea pig IgG (Abcam, Inc.) were used. For electron microscopy, Alexa Fluor 488 and 1.4-nm gold–conjugated rabbit F (ab) anti–goat (1:50; Abcam, Inc.) were used.

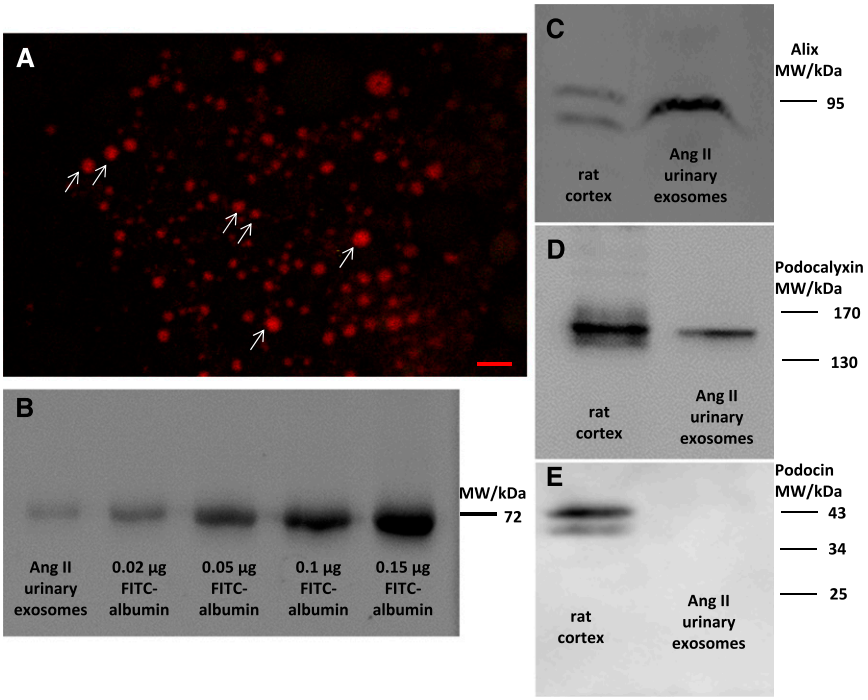

Figure 11. Urinary vesicles contain fluorescent-labeled albumin and podocyte markers. Vesicles were isolated from the urine of rats, which were injected with either (A, arrows) Alexa BSA or (B) FITC–albumin before the Ang II infusion. (A) MPM visualizes intact Alexa albumin–containing vesicles recovered from the urine. Scale bar, 10 μm. (B) Urinary vesicles (lane 1) and increasing concentrations of an FITC–albumin standard on a 10% SDS-PAGE gel visualized by ultraviolet imaging. (C–E) Western blot analysis was performed to determine the content of vesicles. (C) Programmed cell death 6 interacting protein was identified in the urinary albumin, which is involved in the biogenesis of exosomes. (D and E) The presence of podocalyxin and the lack of podocin suggest that albumin release by podocytes occurs at the apical membrane of the cell body rather than in the slit diaphragm region. MW, molecular mass.

Fixation

Animals were perfused with 4% paraformaldehyde solution in PBS (pH 7.2) at a constant perfusion pressure of 130 mmHg. Subsequently, the fixed kidneys were processed for either electron microscopy or paraffin sections. Human paraffin–embedded kidney tissue was a gift from Stephan Reinhold.

Immunostaining

For light microscopy, perfusion-fixed kidneys were embedded in paraffin, sliced (2–5 μm), and stained. The staining was then visualized with an Axiovert Observer Microscope (Carl Zeiss). For electron microscopy, kidneys were embedded in LR White Resin; 100-nm-thick sections were sliced and collected on single–slot pioloform nickel grids before staining and visualized using a JEOL JEM-2100F Feldemissions-Transmissionselektronenmikroskop (Jeol).

Plasma Ang II Levels

Plasma was obtained by tail bleeding before and immediately after the Ang II dose-response experiments. To stabilize the peptide, aprotinin (10 μl per 100 μl blood; Sigma-Aldrich) was added to the blood sample before centrifugation for 20 minutes at 4°C. Ang II levels in the plasma were then measured by an Angiotensin II ELISA (Phoenix Pharmaceuticals) according to the manufacturer's instructions.

Urinary Vesicles Isolation

Albumin-containing vesicles were isolated from the urine of Ang II–infused rats using total exosomes isolation reagent (Invitrogen) or by ultracentrifugation. To visualize the albumin content of urinary albumin–containing vesicles, some of the rats were injected with either Alexa BSA or FITC–albumin before Ang II infusion. The fluorescent albumin in the vesicles was then visualized by either MPM or a Fusion Fx7 Spectra Imager (Vilba) after plotting on a 10% SDS-PAGE gel.

Western Blotting

For the identification of marker proteins of the urinary vesicles, electrophoresis of the samples was performed on a 10% or 7% SDS-PAGE gel and then transferred to a nitrocellulose membrane (Thermo Fisher Scientific). The following primary antibodies were used: rabbit antipodocin IgG (1:50; Sigma-Aldrich), rabbit antipodocalyxin (Santa Cruz Biotechnology), and goat anti–programmed cell death 6 interacting protein IgG (1:50; Santa Cruz Biotechnology). Labeled secondary antibodies were visualized using Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific) according to the manufactures' instructions.

Statistical Analyses

Data were further analyzed by ANOVA with Bonferroni *post hoc* test using Graph Pad Prism 5 (GraphPad Software). All data are given as means±SEM. $P<0.05$ 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 were approved by local authorities.

ACKNOWLEDGMENTS

This study was supported by Deutsche Forschungsgemeinschaft Grant SFB699/B7.

DISCLOSURES

None.

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This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2014111125/-/DCSupplemental.