Generation of Urinary Albumin Fragments Does Not Require Proximal Tubular Uptake

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ABSTRACT

Urinary albumin excretion is an important diagnostic and prognostic marker of renal function. Both animal and human urine contain large amounts of albumin fragments, but whether these fragments originate from renal tubular degradation of filtered albumin is unknown. Here, we used mice with kidneys lacking megalin and cubilin, the coreceptors that mediate proximal tubular endocytosis of albumin, to determine whether proximal tubular degradation of albumin forms the detectable urinary albumin fragments. After intravenous administration of ^{125}I-labeled mouse albumin to knockout and control mice, we examined kidney uptake of albumin and urinary excretion of both intact albumin and its fragments using size exclusion chromatography. In control mice, all labeled albumin eluted as albumin fragments in the urine. In megalin/cubilin-deficient mice, we observed decreased uptake and degradation of albumin and increased urinary excretion of intact albumin; we did not, however, detect a decrease in the excretion of albumin fragments. These results show that the generation of urinary albumin fragments occurs independently of renal tubular uptake and degradation of albumin, suggesting that the pathophysiological implications of changes in urinary albumin fragments require reevaluation.

J Am Soc Nephrol 23: 591–596, 2012. doi: 10.1681/ASN.2011101034

Albuminuria is one of the single most sensitive and commonly used tests of renal dysfunction. It is established as a marker and risk factor not only in kidney disease but also in diabetes mellitus and cardiovascular disease.[1] Using high-performance liquid chromatography, it has been shown recently that animal and human urine contain both immunoreactive albumin and additional immunounreactive albumin fragments, which are not detected with conventional assays.[2–4] It was estimated that >99% of the total albumin was excreted in fragmented, low-molecular mass forms (<10 kD). It has further been hypothesized that the excretion of the albumin fragments is the result of proximal tubule lysosomal activity[5,6] and that reduced excretion of albumin fragments signifies dysfunction of the tubular uptake and degradation pathway.[7,8] These observations may have important implications for the diagnosis and follow-up of diabetes as well as in renal and cardiovascular diseases, because they suggest that albumin assays that include quantitation of immunounreactive albumin fragments in urine are more sensitive in the detection of albuminuria and that a change in the excretion of urinary albumin fragments is a potentially important marker of tubular dysfunction.[9] Indeed, altered excretion of urinary albumin fragments has been detected in diabetic nephropathy,[9,10] glomerular disease,[11] and IgA nephropathy.[12] However, to understand the potential and significance of the excreted albumin fragments as a marker of disease, it is crucial to establish the site of formation and the potential functional changes in the kidney responsible for a change in urinary excretion of albumin fragments.

The two multiligand endocytic receptors megalin and cubilin are responsible for the endocytic recovery of filtered albumin by the proximal tubule.[13] Essentially, no albumin uptake can be detected in proximal tubules after the disruption of either one of these two receptors,[14,15] and both cubilin- and megalin-deficient mice, therefore, excrete intact albumin in the urine, whereas the highest excretion of albumin is found in combined megalin/cubilin-deficient mice.[16] After endocytosis, it is generally recognized that albumin, like other filtered proteins, undergoes lysosomal degradation into amino acids.[17,18] If albumin fragments are the result of endocytosis and lysosomal degradation, the excretion of albumin fragments in the urine, thus, should be dependent on functional megalin and cubilin. Such direct relationship between the excretion of protein fragments in the urine and receptor function, however, has never been established.

Received October 28, 2011. Accepted December 7, 2011.

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

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Here, we have used conditional megalin/cubilin-deficient mice with an efficient inactivation of cubilin and megalin expression in the kidney[16] to investigate if the endocytic uptake and degradation of albumin by proximal tubular cells is involved in the formation of urinary albumin fragments. Cubilin- and megalin/cubilin-deficient mice excrete increased amounts of immunoreactive albumin in the urine (Figure 1A), consistent with a loss of uptake of filtered albumin by proximal tubular cells. To trace intact and degraded forms of albumin, we injected the mice intravenously with ^{125}I-labeled mouse albumin. In line with the role of megalin and cubilin as the major albumin receptors in the kidney proximal tubule, we found a marked reduction in the total radioactivity of the kidneys of cubilin- and megalin/cubilin-deficient mice compared with control mice (Figure 1B) and only subtle changes in the urinary excretion of total radiolabel (Figure 1C). The collected urine samples from control and megalin/cubilin-deficient mice were also analyzed by size exclusion chromatography, which separates albumin into intact, high molecular weight (HMW) and fragmented, low molecular weight (LMW) forms. Consistent with previous reports,[6,19] we found that the label in the urine of control mice eluted as LMW fragments with no detectable intact, HMW-labeled albumin (Figure 2A). Notably, no change in the excretion of albumin LMW fragments could be observed in the urine of cubilin- or megalin/cubilin-deficient mice (Figure 2, B and C). In the urine of megalin/cubilin-deficient mice, small amounts ($3.06\% \pm 0.7\%$, n=5) of the excreted radiolabel were intact HMW albumin (Figure 2C), whereas no HMW albumin was observed in the urine of cubilin-deficient mice, suggesting that the amount of labeled intact albumin excreted was below the detection level using our radiolabeled tracer. This finding is in agreement with the observation of minor changes in the total urinary excretion of radiolabel by the mice (Figure 1C), because only a minor amount of the activity represents intact albumin. Importantly, only very small amounts of albumin LMW fragments (<1%) could be detected in the ^{125}I-labeled albumin tracer before injection into the mice (Figure 2D). Consequently, a minor fraction (<10%) of the LMW fragments found in the urine could potentially originate from the injected tracer. The above findings, therefore, show that, although ^{125}I-albumin uptake by the proximal tubular cells is dependent on megalin/cubilin-mediated endocytosis, formation of the urinary degradation products of albumin occurs independently of megalin/cubilin-mediated uptake in the kidney.

To exclude that albumin degradation occurs in the kidney independently of megalin/cubilin-mediated endocytosis of albumin, we injected the mice with an albumin conjugate (dye-quenched [DQ] albumin). DQ-albumin only fluoresces when it is degraded intracellularly and therefore, can be used to visualize albumin degradation in proximal tubular cells *in vivo*.[20] Accordingly, intracellular DQ-albumin labeling of proximal tubular cells was prominent 30 minutes after intravenous injection in control mice (Figure 3A). However, virtually no fluorescence could be observed in the kidney tissue sections of megalin/cubilin-deficient mice, correlating with the lack of megalin and cubilin expression (Figure 3B). This finding conclusively shows that megalin/cubilin-mediated endocytosis is crucial for the intracellular degradation of albumin by proximal tubular cells and verifies that no other pathway is involved in the intracellular generation of albumin fragments by the proximal tubule in the absence of megalin and cubilin.

Because our data suggest that the origin of the urinary albumin fragments is extrarenal, we also aimed to identify the source of degraded albumin. No formation of LMW albumin fragments could be observed on *in vitro* incubation of the ^{125}I-albumin probe with fresh blood or urine (data not shown); however, small amounts of labeled albumin LMW fragments appeared in the plasma of control mice collected at 90 minutes after injection, and they were still observed after 360 minutes (Figure 4). The LMW albumin observed during this time period must be formed after injection of labeled albumin, because no LMW albumin was observed at 30 minutes. This finding suggests that formation of albumin fragments can occur independently of renal degradation and raises the possibility

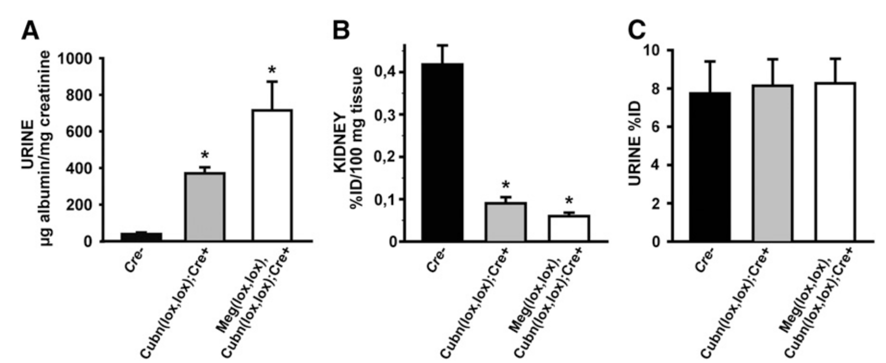

Figure 1. Increased urinary excretion and reduced kidney uptake of albumin by megalin/cubilin-deficient mice. (A) Urinary immunoreactive albumin excretion (detected by ELISA) relative to creatinine content (microgram albumin per milligram creatinine) by control (Cre^-), cubilin-deficient ($Cubn^{lox/lox}$;Cre^+), or megalin/cubilin-deficient ($Meg^{lox/lox}$,$Cubn^{lox/lox}$;Cre^+) mice. (B) Kidney tissue uptake of ^{125}I-labeled albumin (percent injected dose (%ID) per 100 mg kidney tissue) 6 hours after intravenous injection in control (Cre^-), cubilin-deficient ($Cubn^{lox/lox}$;Cre^+), or megalin/cubilin-deficient ($Meg^{lox/lox}$,$Cubn^{lox/lox}$;Cre^+) mice. (C) Urinary excretion of radiolabel (percent injected dose) collected for 6 hours after intravenous injection in control (Cre^-), cubilin-deficient ($Cubn^{lox/lox}$;Cre^+), or megalin/cubilin-deficient ($Meg^{lox/lox}$,$Cubn^{lox/lox}$;Cre^+) mice. The graphs show mean values from all mice in each group (n=5), and error bars indicate SDs. *Significantly different from control mice ($P<0.01$).

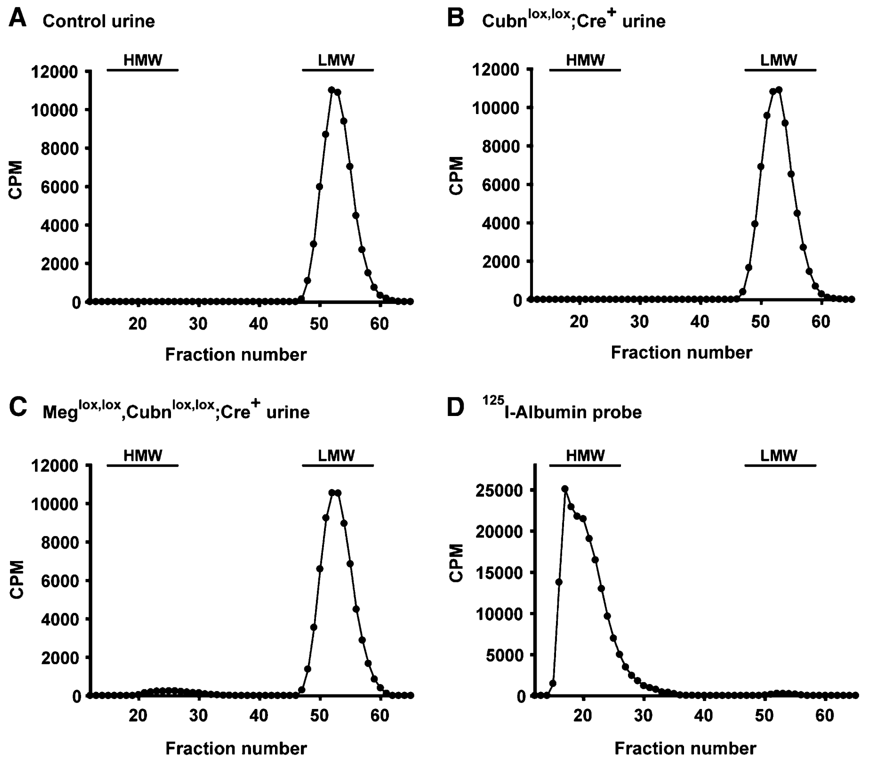

Figure 2. Excretion of albumin fragments in the urine of control and megalin/cubilin-deficient mice. Elution profile of size exclusion chromatography on Sephadex G-100 of ^{125}I-labeled albumin in urine samples from (A) control (Cre^{-}), (B) cubilin-deficient, or (C) megalin/cubilin-deficient mice. Urine was collected for 6 hours after the intravenous administration of ^{125}I-labeled albumin. Albumin was separated into high molecular weight (HMW) or low molecular weight (LMW) forms. (D) Only trace amounts (<1%) of albumin fragments were found in the elution profile of the ^{125}I-labeled albumin probe before injection. The shown elution profiles are representative of at least five independent experiments of each group of mice.

that the labeled LMW albumin fragments found in the urine could result from glomerular filtration. Assuming that the fragments are freely filtered, these fragments must be continuously generated to maintain plasma levels. Based on our data during the period of 30 minutes to 6 hours after injection, we found a time-weighted average of 1.97 cpm/μl LMW albumin in plasma (Figure 4D). With an averaged GFR in mice of ~288 μl/min,[21] the free filtration of even this very low plasma activity would result in the excretion of >150,000 cpm LMW albumin after 6 hours. Thus, this finding could readily account for the ~77,000 cpm observed in the urine collected from control mice (Figure 1C). Our data are, therefore, consistent with a small fraction of about 1% of albumin in plasma found in a LMW form, which is excreted in the urine. This finding is in agreement with previous studies that identified small amounts (<2%) of LMW albumin fragments in normal human plasma.[22] Up to 5% of albumin was found in a fragmented form in plasma samples from nephrotic syndrome patients.[11] Other reports have, by mass spectrometry analysis, further identified albumin fragments in normal plasma[23,24] as well as plasma of patients with focal segmental glomerulosclerosis,[25] uremia,[26] and diabetes.[10] This finding stands in contrast to studies based on tritium-labeled albumin, where the majority of these studies found no LMW albumin in plasma,[19,27] although small amounts of tritium-labeled fragments in plasma have been reported.[9] In this study, we have used albumin with a higher specific activity than the mentioned studies, which likely explains why we were able to detect the low amounts of labeled LMW albumin found in plasma.

It is the notion that the early detection of proximal tubular cell dysfunction by urinary measurements of LMW proteins may offer a sensitive means of monitoring kidney function and disease progression that has led to the interest in LMW albumin fragments as potential markers.[9] The megalin/cubilin-deficient mice excrete several LMW protein markers, such as α_1-microglobulin, β_2-microglobulin, and retinol binding protein,[16] associated with proximal tubule dysfunction.[28] However, because no change in the urinary excretion of LMW albumin fragments was observed in these mice, our findings challenge the concept that the formation of urinary albumin fragments reflects proximal tubule function and lysosomal degradation, which has been proposed based on studies with tritium-labeled albumin in diabetic patients and animal models.[7–9,29] A study in diabetic patients found no correlation between the urinary excretion of albumin fragments and β_2-microglobulin, also supporting the concept that changes in the excretion of urinary albumin fragments is not associated with proximal tubule dysfunction.[10] Based on the finding of albumin fragments in plasma, it is, therefore, more likely that changes in urinary excretion of albumin fragments reflect alterations in extrarenal albumin metabolism and/or glomerular filtration.

In conclusion, the presented data suggest that urinary albumin fragments do not originate from intracellular degradation in the kidney proximal tubule but that these fragments instead may be filtered from plasma into the urine. The pathophysiological significance of changes in urinary albumin fragments should, therefore, be cautiously interpreted. Furthermore, we have shown that the megalin/cubilin–receptor complex is essential not only for the tubular uptake but also for the proximal tubule cell degradation of filtered albumin.

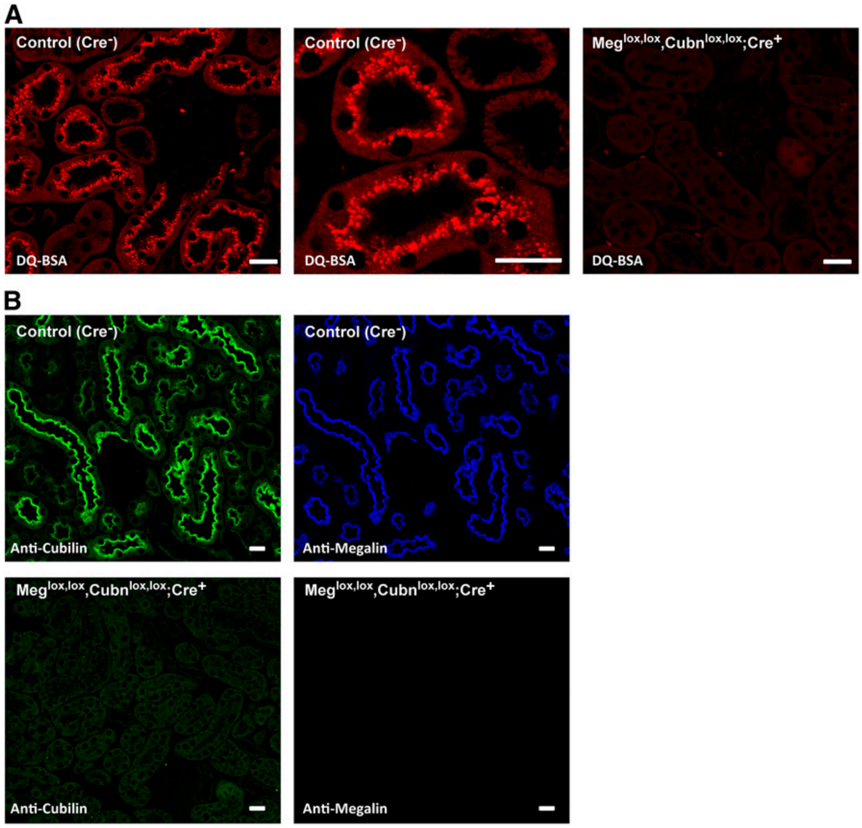

Figure 3. Albumin endocytosis and degradation *in vivo*. (A) Confocal microscopic analysis of perfusion-fixed kidney slices of DQ-albumin–injected control (Cre^-) or megalin/cubilin-deficient ($Meg^{lox/lox}$,$Cubn^{lox/lox}$;Cre^+) mice. The degradation-dependent DQ-albumin fluorescence (red) could only be detected in control mice, where it was localized in intracellular vesicles (enlarged view). (B) Immunostaining for cubilin (green) and megalin (blue) was detected in kidney slices of control (Cre^-) mice but not in megalin/cubilin-deficient ($Meg^{lox/lox}$,$Cubn^{lox/lox}$;Cre^+) mice. Scale bars, 20 μm. The shown images are representative of a similar analysis of three mice in each group.

CONCISE METHODS

Breeding of Conditional Knockout Mice

Conditional megalin/cubilin ($Meg^{lox/lox}$, $Cubn^{lox/lox}$;Cre^+) and cubilin-deficient mice ($Cubn^{lox/lox}$;Cre^+) with the Cre-recombinase gene driven by the Wnt4 promoter were produced as described previously.[16] Genotyping was made on tail DNAs by PCR as described previously.[15] Cre-negative littermates were used as controls. All mice were on a mixed C57BL/6-129/Svj background and 8–12 weeks of age at the time of use. Mouse breeding and handling were carried out in a certified animal facility according to provisions by the Danish Animal Experiments Inspectorate.

Urinary Albumin/Creatinine Measurements

Urine samples were collected at 24 hours in metabolic cages in the presence of a mix of proteinase inhibitors (Complete; Roche, Hvidovre, Denmark). Urine creatinine concentrations were determined using the Creatinine Companion kit (Exocell, Philadelphia, PA), and albumin concentrations were determined using a mouse albumin ELISA quantification kit (Bethyl Labs, Montgomery, TX).

Labeling of Albumin with Radioactive Iodine

Mouse serum albumin (Sigma, St. Louis, MO) was labeled with radioactive ^{125}I (^{125}I; GE Healthcare, UK) using the chloramine-T method.[30] The labeled preparation was applied to a Sephadex G-25 (PD-10) column (Amersham Biosciences, UK) to separate it from free label. In the collected fractions, the label was >98% protein bound as determined by TCA precipitation, and the specific activity of the radiolabeled albumin preparation was 3.2×10^4 cpm/ng.

Injection of ^{125}I-Albumin in Mice

Cubilin-deficient, megalin/cubilin-deficient, or control mice were anesthetized with isofluoran for a few minutes for the intravenous injection of 1×10^6 cpm ^{125}I-mouse albumin in 0.2 ml saline. The mice were placed in metabolic cages for 6 hours for urine collection in the presence of proteinase inhibitors as described previously.[15] At the end of the 6-hour urine collection, the mice were anesthetized, and the remaining urine in the bladder, the kidneys, and a blood sample were collected into a syringe containing heparin. Blood and urine samples were centrifuged for 10 minutes at 1600 × *g*. Specimens of plasma (20 μl), urine (50 μl), and the whole right kidney were counted on a γ-counter (Packard Biosciences, Berkshire, UK). Urine and plasma samples were further analyzed for intact (HMW) and degraded (LMW) ^{125}I-albumin by size exclusion chromatography. For time-course analysis of plasma samples, control mice were injected with ^{125}I-mouse albumin as described above, and blood was collected after 30, 90, 180, and 360 minutes.

Size Exclusion Chromatography

Size exclusion chromatography was performed on a Sephadex G-100 column (1.6 cm inner diameter×34 cm length; GE Healthcare) run with PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) at 20 ml/h at 4°C. Plasma (0.25 ml) or urine (0.5–1 ml) were loaded onto the column, and 100 fractions of 1.5 ml each were collected and analyzed for radioactivity on a γ-counter (Packard Biosciences). Before fractionation, the urine and plasma samples were stored at −20°C for up to 2 weeks. Similar results were obtained with fresh or stored urine and plasma samples (data not shown). For *in vitro* incubation, a 100,000-cpm ^{125}I-albumin probe was incubated with 0.5 ml fresh blood or urine for 16 hours at 37°C and analyzed by size exclusion chromatography as described above.

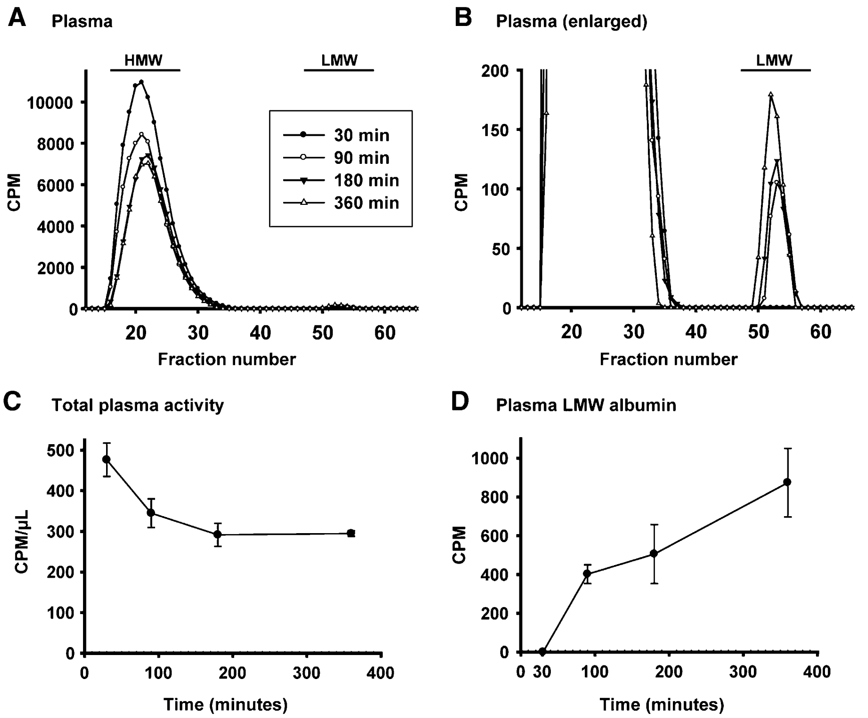

Figure 4. Intact and fragmented albumin in the plasma of control mice. (A) Elution profile of the size exclusion chromatography on Sephadex G-100 of ^{125}I-labeled albumin in plasma samples (250 μl) from control (Cre$^-$) mice at 30, 90, 180, and 360 minutes after the intravenous administration of 1×10^6 cpm ^{125}I-labeled albumin. Plasma albumin mainly eluted in a high molecular weight (HMW) form, although small amounts of low molecular weight (LMW) forms could be detected. (B) Enlarged view of the graph in A showing the LMW forms of ^{125}I-labeled albumin in plasma. The shown elution profiles in A and B are mean values (n=3). (C) Plasma concentration curves of the total label in plasma. (D) LMW albumin label in the plasma samples (250 μl) at the different time points from the above described experiment. This label corresponds to a time-weighted average of 1.97 cpm/μl LMW albumin in plasma in the period of 30 minutes to 6 hours after injection. Points in C and D represent means ± SDs (n=3).

Injection and Visualization of DQ-BSA

Anesthetized mice were injected intravenously with 10 μg/g body wt DQ-BSA (Invitrogen, Carlsbad, CA) dissolved in saline. After 30 minutes, the kidneys were fixed by retrograde perfusion through the abdominal aorta with 2% parformaldehyde and processed as reported.[15] Images of kidney tissue sections were analyzed directly or after immunostaining using a confocal laser-scanning microscope (LSM 510-META; Carl Zeiss, Göttingen, Germany) and processed using Zeiss Zen software (2009, Light Edition).

Immunohistochemistry

For immunohistochemistry, perfusion-fixed kidney sections were processed as reported using sheep antimegalin and rabbit anticubilin.[15]

Statistical Analyses

All data were expressed as mean ± SD. Data were compared between groups using unpaired *t* test. $P<0.01$ was considered statistically significant.

ACKNOWLEDGMENTS

We thank Hanne Sidelmann, Inger Blenker Kristoffersen, and Pia Kamuk Nielsen for excellent technical assistance.

The work was supported in part by the Lundbeck Foundation, the Danish Medical Research Council, Novo Nordisk Foundation, the European Commission EUNEFRON (FP7, GA#201590), the Danish Kidney Association, and the Aase and Ejnar Danielsens Foundation.

DISCLOSURES

None.

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See related editorial, "Is the Albumin Retrieval Hypothesis a Paradigm Shift for Nephrology?" on pages 569–571.