Kidney

On the Origin of Urinary Renin
A Translational Approach


See Editorial Commentary, pp 831–833

Abstract—Urinary angiotensinogen excretion parallels albumin excretion, which is not the case for renin, while renin’s precursor, prorenin, is undetectable in urine. We hypothesized that renin and prorenin, given their smaller size, are filtered through the glomerulus in larger amounts than albumin and angiotensinogen, and that differences in excretion rate are because of a difference in reabsorption in the proximal tubule. To address this, we determined the glomerular sieving coefficient of renin and prorenin and measured urinary renin/prorenin 1) after inducing prorenin in Cyp1a1-Ren2 rats and 2) in patients with Dent disease or Lowe syndrome, disorders characterized by defective proximal tubular reabsorption. Glomerular sieving coefficients followed molecular size (renin>prorenin>albumin). The induction of prorenin in rats resulted in a >300-fold increase in plasma prorenin and doubling of blood pressure but did not lead to the appearance of prorenin in urine. It did cause parallel rises in urinary renin and albumin, which losartan but not hydralazine prevented. Defective proximal tubular reabsorption increased urinary renin and albumin 20- to 40-fold, and allowed prorenin detection in urine, at ≥50% of its levels in plasma. Taken together, these data indicate that circulating renin and prorenin are filtered into urine in larger amounts than albumin. All 3 proteins are subsequently reabsorbed in the proximal tubule. For prorenin, such reabsorption is ≥100%. Minimal variation in tubular reabsorption (in the order of a few %) is sufficient to explain why urinary renin and albumin excretion do not correlate. Urinary renin does not reflect prorenin that is converted to renin in tubular fluid. (Hypertension. 2016;67:927-933. DOI: 10.1161/HYPERTENSIONAHA.115.07012.) • Online Data Supplement

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Renal clearance of solutes occurs through a combination of glomerular filtration, tubular secretion, and tubular reabsorption. The molecular weight (MW) cut-off for glomerular filtration is thought to be 30 to 50 kDa.1 Urinary albumin (MW, 67 kDa) is widely accepted as a measure of damage to the glomerular filtration barrier (GFB). Recently, urinary levels of renin–angiotensin system (RAS) components have been suggested to reflect the activity of the intrarenal RAS, independently of GFB damage. This particularly concerns angiotensinogen and renin.2–4 Indeed, angiotensinogen synthesis has been demonstrated in the proximal tubule,5 suggesting that renal angiotensin (Ang) II production might occur independently of circulating (hepatic) angiotensinogen. Yet, the urinary excretion pattern of angiotensinogen (MW, 55–65 kDa) is identical to that of albumin in a wide variety of patients.4,6–10 Moreover, 2 landmark studies, making use of a kidney-specific angiotensinogen knockout mouse, revealed that, both under normal and pathological conditions, renal Ang II production depends entirely on plasma-derived (ie, hepatic) angiotensinogen.11,12 These studies suggested a new concept, namely that it is filtered, plasma-derived angiotensinogen that accumulates in the proximal tubule, rather than locally synthesized angiotensinogen, and that this filtered angiotensinogen contributes most to renal Ang II production.12 Indeed, filtered angiotensinogen largely, if not completely, is intact angiotensinogen (and not cleaved, des-Ang I-angiotensinogen), truly allowing a contribution to renal angiotensin generation.8 Pohl et al13 have suggested that tubular angiotensinogen uptake occurs via endocytosis, in a megalin-dependent manner.

Renal tubular renin expression is believed to occur more distally, in the collecting duct, in addition to its classical expression...
in the juxtaglomerular apparatus. Importantly, urinary renin excretion does not correlate with albumin excretion, except when the GFB is damaged and circulating renin levels are greatly elevated (eg, during preeclampsia). Given its MW (48 kDa), circulating renin is likely to pass through the glomerulus. To what degree this also applies to prorenin (MW, 54 kDa), the inactive precursor of renin, remains to be determined. Prorenin could be detected in urine of preeclamptic women but not in urine of hypertensive patients or diabetic subjects, despite the fact that the latter have greatly elevated circulating prorenin levels. One possibility is that prorenin, once filtered, is converted to renin in tubular fluid. If so, this would explain why there is usually no prorenin in urine, and why urinary renin levels do not correlate with urinary albumin levels. Here, it should be kept in mind that renin, like angiotensinogen, is also endocytosed by the proximal tubule in a megalin-dependent manner.

In this study, we set out to delineate the origin of urinary renin. To do so, we used 3 approaches, based on the hypothesis that renin and prorenin, given their smaller size, are filtered in larger amounts than albumin, and that all 3 proteins are subsequently reabsorbed in the proximal tubule. First, the glomerular sieving coefficients (GSCs) for renin and prorenin versus albumin were determined, both under normal conditions and after doxorubicin-induced GFB damage. Second, urinary renin levels after the induction of prorenin production in the liver were studied, using Cyp1a1-Ren2 rats, ie, transgenic rats, which express the mouse Cyp1a1-Ren2 gene exclusively in the liver in an inducible manner. Third, blood and urine of I3C animals treated with losartan or hydralazine, were assessed by one-way ANOVA with post hoc Dunnet correction. Data with a nonnormal distribution were logarithmically transformed before ANOVA. Differences between renin and total renin levels were assessed by Wilcoxon signed-ranks tests. Correlations between urine/plasma concentration ratios (%100%) of parameters were assessed by Spearman correlation coefficient. \( P<0.05 \) was considered statistically significant. All statistical analyses were performed using SPSS (IBM SPSS Statistics 20, Chicago, IL).

**Results**

**Glomerular Filtration of Renin and Prorenin in Normal and Albuminuric Mice**

The average GSC of mouse albumin in healthy C57/Bl6 mice was 0.00417±0.00043. The GSC of recombinant human prorenin was significantly greater than that of albumin (Figure 1). The GSC of recombinant human renin was significantly greater than that of both albumin and prorenin. In doxorubicin-treated mice, the GSC of each protein was greater than that in normal mice. Both prorenin and renin showed significantly greater GSCs than albumin, whereas the difference in GSC between prorenin and renin was diminished in doxorubicin-treated mice (Figure 1).

**Urinary Renin and Prorenin and Renal Megalin Expression After Hepatic Prorenin Induction in Cyp1a1-Ren2 Rats**

As reported previously, inducing hepatic prorenin synthesis with I3C in Cyp1a1-Ren2 rats increased mean arterial blood pressure from 111±4 to 197±6 mmHg (\( P<0.01 \); n=9–11). Hydralazine partly (150±4 mmHg; n=4) and losartan fully (121±7 mmHg; n=7) prevented this rise. I3C exposure increased urinary volume from 10±1 to 57±13 mL/d (\( P<0.05 \)), and the urinary creatinine concentration decreased accordingly from 5.9±0.3 to 0.6±0.2 mmol/L (\( P<0.05 \)). Losartan and hydralazine normalized urinary volume to 1±1 and 9±1 mL/d, despite the fact that only losartan normalized mean arterial blood pressure. Unfortunately, we did not monitor drinking behavior, thus not allowing us to conclude whether the volume normalization during hydralazine was because of a reduction in water intake. The net creatinine excretion per day was identical in all groups (data not shown).

Compared with control (n=4), I3C exposure (n=5) increased plasma renin 20-fold, and plasma total renin 200-fold, confirming that its effect predominantly concerned prorenin (Figure 2A). On top of I3C, losartan (n=4) and hydralazine (n=4)
additionally increased plasma renin and total renin ≈2- and ≈20-fold although these changes were significant for hydralazine only (\(P<0.05\) versus I3C). The I3C-induced rise in plasma renin and prorenin was accompanied by a ≈200-fold rise in urinary renin levels (\(P<0.05\); Figure 2B) and a ≈1000-fold rise in urinary renin excretion (\(P<0.05\); Figure 3A). Hydralazine partially prevented this rise (Figure 3A), without affecting the urinary renin levels, whereas losartan fully prevented it. Under all conditions, urinary total renin levels were not significantly different from urinary renin levels, suggesting that urine did not contain prorenin (Figure 2B).

As expected based on the rise in renin levels, I3C lowered plasma angiotensinogen levels, particularly in combination with losartan and hydralazine (Figure 4A). In fact, after hydralazine, plasma angiotensinogen was close to 0. The urinary levels of angiotensinogen rose ≈10-fold after I3C although this did not reach significance versus control (Figure 4B), and urinary angiotensinogen excretion increased ≈100-fold (Figure 3B). I3C in combination with losartan or hydralazine reduced urinary angiotensinogen excretion to that of control animals.

I3C with or without antihypertensive treatment did not affect plasma albumin levels (Figure 5A). Urinary albumin levels rose ≈100-fold after I3C (\(P<0.05\)), and urinary albumin excretion rose ≈500-fold (\(P<0.05\); Figure 3C). Losartan fully and hydralazine partially prevented this rise (Figures 3C and 5B).

The urine/plasma concentration ratio of albumin correlated with that of angiotensinogen (\(r=0.63; P<0.05\)) but not with that of renin (\(r=0.18; P=0.46\); Figure 6).

I3C treatment tended to reduce renal megalin expression (Figure 7) although this decrease became significant only (\(P<0.05\)) in the presence of hydralazine. Losartan prevented this phenomenon.

**Figure 2.** Plasma (A) and urinary (B) renin (white bars) and total renin (black bars) levels in transgenic Cyp1a1-Ren2 rats before (control, CO) and after treatment with indol-3-carbinol (I3C), with or without concomitant exposure to losartan (LOS) or hydralazine (HYD). *\(P<0.05\) versus CO; †\(P<0.05\) versus I3C; and ‡\(P<0.05\) versus I3C-LOS. Data are mean±SEM of \(n=4–5\).

**Figure 3.** Urinary excretion (per 24 hours) of renin (A), angiotensinogen (B), or albumin (C) before (control, CO) and after treatment with indol-3-carbinol (I3C), with or without concomitant exposure to losartan (LOS) or hydralazine (HYD). *\(P<0.05\) versus CO; †\(P<0.05\) versus I3C; and ‡\(P<0.05\) versus I3C-LOS. Data are mean±SEM of \(n=4–5\).

**Plasma and Urinary RAS Components in Patients with Dent Disease or Lowe Syndrome**

Plasma samples were obtained from 2 men with Dent disease (because of a CLC5 mutation) and 2 men with Lowe syndrome (because of a OCRL mutation, age 24–47 years), and in 3 of these men, 1 or more urine samples could additionally be obtained (7 urine samples in total). All patients were normotensive, but 1 patient used an angiotensin-converting enzyme inhibitor for proteinuria.

Urinary total renin levels were higher than urinary renin levels in all 7 urine samples, and thus, the urine of these patients did contain prorenin. Figure 8 shows that urinary renin levels were comparable with plasma renin levels (range, 68–275%), whereas urinary prorenin levels were ≈2-fold lower than plasma prorenin levels. Angiotensinogen and albumin levels in urine were 1% to 2% of the concomitantly measured plasma levels. Urinary aldosterone levels (6966 pg/mL) were higher than plasma aldosterone levels (485 pg/mL). Plasma creatinine levels averaged 284±72 μmol/L.
This study shows that the GSCs of renin, prorenin, and albumin correlate with their molecular size, albumin displaying the lowest GSC and renin the highest. The GSCs of all 3 proteins increased after inducing GFB damage with doxorubicin, and under this condition, the GSC of prorenin was no longer different from that of renin. This is suggestive of a decrease in glomerular size selectivity after exposure to doxorubicin. Severe prorenin-induced hypertension also resulted in rises in urinary renin and albumin. Because this was not accompanied by changes in nephrin expression or Wilms tumor staining (both suggestive for GFB damage),

Indeed, blood pressure lowering prevented or diminished these rises, complete normalization occurring only when blood pressure had been normalized completely (with losartan). Remarkably, even a >200-fold elevation of circulating prorenin, with or without hypertension, did not result in detectable prorenin levels in urine. Only when megalin-dependent tubular prorenin reabsorption was absent, did urinary prorenin become detectable. These data support the concept that urinary renin is plasma derived and does not represent activated prorenin of plasma or renal origin. Circulating prorenin apparently does filter into the proximal tubule but is fully reabsorbed in a megalin-dependent manner.

Multiphoton imaging previously revealed that the GSC of angiotensinogen amounted to 25% to 50% of that of albumin despite the fact that its MW is comparable with that of albumin. Because there are no reasons why the GSC of albumin would be up to 4-fold higher than that of angiotensinogen, the authors suggested that this difference is because of the presence of low-MW fragments in their albumin preparation. Such fragments would have a GSC of 100%, and a fragmented fraction of labeled albumin of only 0.19% would have been enough to explain the difference. Thus, in reality, the GSCs of albumin and angiotensinogen are most likely comparable. Our earlier data in humans fully support this view: in hypertensive subjects, the albumin and angiotensinogen levels were $\approx 0.05\%$ of their concomitant plasma levels. This study now reveals that the urinary albumin and angiotensinogen levels in patients with disabled tubular reabsorption are up to 1% to 2% of their plasma levels, ie, 20- to 40-fold higher than normal. It is important to note that the plasma angiotensinogen levels in these patients were identical to those in hypertensive patients. These data, therefore, demonstrate that normally >95% of filtered angiotensinogen and albumin is reabsorbed. Given the strong correlation between the urinary/plasma concentration ratios of albumin and angiotensinogen in a wide variety of patients, as well as in healthy pregnant women (who display greatly elevated plasma angiotensinogen levels) and women with preeclampsia, it is highly likely that the process underlying tubular reabsorption (megalin-dependent endocytosis) is identical for both proteins. Our data in Cyp1a1-Ren2 rats stress the importance of normalizing the urinary angiotensinogen levels versus plasma angiotensinogen levels. Obviously, this is also true for urinary renin. Indeed, after I3C prorenin induction, particularly in combination with losartan or hydralazine, circulating renin levels became so high that circulating angiotensin started to get depleted. This resembles the situation in heart failure patients treated with RAS blockers, or the application of excessive RAS blocker doses. An additional reason for the high renin levels during hydralazine may be that this drug, through an interaction with CYP1A2, induces the generation of autoantibodies, thereby potentially worsening renal pathology. The virtual absence of circulating angiotensinogen during hydralazine explains why the urinary angiotensinogen levels in the hydralazine-treated rats were normal, while simultaneously urinary renin was excessively elevated.

Urinary renin levels in humans amount to $\approx 0.05$ to 5
This study now reports urinary renin levels in patients with Dent disease or Lowe syndrome that are up to 68% to 275% of plasma renin. This is 20- to 40-fold higher than normal, identical to the rise observed for albumin and angiotensinogen in these patients. Blocking tubular reabsorption with lysine yielded a 100-fold rise in urinary renin, without affecting plasma renin. Combined with the larger GSC for renin versus albumin, these data indicate that circulating renin is readily filtered into urine and subsequently reabsorbed for 90% to 98%. Small variations in reabsorption (in the order of a few %) may easily double or triple urinary renin. Such variations, if not exactly paralleled by the variation in albumin reabsorption, could explain why urinary renin excretion does not always correlate with urinary albumin excretion.

An alternative explanation for the different patterns of renin excretion versus albumin excretion is the release of renin into urine from the collecting duct. Yet, synthesis at this site largely concerns prorenin. Thus, such release, if occurring, would require the conversion of prorenin to renin. The same would apply to the possibility that urinary renin is in fact circulating prorenin that has been converted to renin within the tubular fluid. This study allowed us to address these 2 scenarios. First, when elevating circulating prorenin several 100-fold in Cyp1a1-Ren2 rats (which is far above the rises observed in human physiology, which are <10-fold, and rather maximally 3- to 5-fold), urinary renin excretion rose ≈1000-fold. A comparable (=500-fold) rise was observed for albumin, whereas prorenin remained undetectable in urine. Had conversion of filtered, circulating prorenin contributed to urinary renin, the rise in urinary renin should have been many orders above that of albumin, given the much greater rise in circulating prorenin. Second, and most important, urine of the patients with Dent disease or Lowe syndrome did contain prorenin, albeit in amounts that, when corrected for its plasma concentration, were ≈2- to 3-fold lower than those of renin. This difference fits perfectly with the reduced GSC of prorenin versus renin under normal circumstances. Clearly, therefore, prorenin is filtered in the glomerulus, but its tubular reabsorption is virtually 100%, leaving no detectable prorenin in urine under normal circumstances. Early studies in mice, which observed prorenin in urine only when tubular reabsorption was blocked with lysine, complement this view. In summary, these findings argue against prorenin–renin conversion in tubular fluid and also suggest that urinary renin is not activated prorenin from the collecting duct, at least under the conditions of this study.

The circulating levels of renin and prorenin in patients with disabled proximal tubular reabsorption were 2- to 3-fold above normal, suggestive for RAS activation in these patients. This might relate to the polyuria in patients with Dent disease or Lowe syndrome. RAS activation also resulted in elevated plasma aldosterone. The urinary aldosterone levels in these patients were >1 order of magnitude above the plasma aldosterone levels. This is not different from previous studies and reflects the well-known fact that aldosterone, as a small molecule, is readily filtered in the kidney, and subsequently concentrated in urine, without being reabsorbed. As a result, aldosterone concentrations in urine correlate with those in plasma, in full agreement with their common (adrenal) origin.

Finally, the partial normalization of the elevated urinary excretion of renin and albumin by hydralazine in the I3C-treated Cyp1a1-Ren2 rats could be because of the fact that it did not fully normalize blood pressure. However, because Ang II suppresses the protein expression of megalin via its type 1 receptor, an alternative explanation is that RAS blockers lower urinary protein not only because they lower blood pressure, but also because they increase reabsorption of proteins via megalin. Conversely, intrarenal RAS activation will lower megalin expression, thereby increasing urinary renin levels. The elevated urinary renin levels in patients with diabetes mellitus, in whom such intrarenal RAS activation is well-known to occur, might be the consequence of this phenomenon. Our data on megalin expression are in complete agreement with this concept: expression decreased after RAS stimulation, particularly in the presence of hydralazine and losartan prevented this.

**Perspectives**

Urinary renin originates in the circulation. Because of their smaller size, both plasma renin and prorenin are filtered into urine.
in larger amounts than plasma albumin. All 3 proteins are subsequently reabsorbed by >90% in the proximal tubule. Remarkably, for prorenin, such reabsorption is virtually 100%, and there is no evidence for its conversion to urine in tubular fluid. Minor differences in tubular reabsorption (in the order of a few %) are sufficient to explain why urinary renin and albumin excretion, unlike urinary angiotensinogen and albumin excretion, are unrelated. Such differences may relate to the absolute molecular size and charge. An attractive concept would be that the presence of the prosegment, ie, the 43 amino acids that represent the difference between renin and prorenin, plays a decisive role. Such a role has also been demonstrated with regard to prorenin binding to the (pro)renin receptor. Glycosylation is less likely to contribute to this phenomenon because Ren2 prorenin is nonglycosylated (unlike the recombinant human renin and prorenin preparations applied in the present study) and yet it behaved in exactly the same manner as prorenin in the human and mouse studies: it remained undetectable in urine despite excessive rises and high blood pressure-induced reninuria and albuminuria.

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**Disclosures**
None.

**References**


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ON THE ORIGIN OF URINARY RENIN. A TRANSLATIONAL APPROACH.

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SUPPLEMENTAL INFORMATION

MATERIALS and METHODS

Glomerular filtration of renin and prorenin in normal and albuminuric mice
Experiments with mice were approved by the Institutional Animal Care and Use Committee of the University of Southern California (USC). Animals had free access to food and water throughout the experimental period. Male and female 3- to 5-week-old C57/Bl6 mice from established animal colonies at the University of Southern California were used for experiments (originally purchased from the Jackson laboratory, Bar Harbor, ME). One group of animals served as control group, and one group of animals received an intravenous injection with doxorubicin (25 mg/kg) to induce glomerular filtration barrier damage. At day 4-5 after doxorubicin administration, animals were injected with proteins conjugated with Atto565 maleimide (Sigma-Aldrich, St. Louis, MO) as described before. The dosages of each protein were 6.7 mg/kg for albumin (Sigma-Aldrich), 6.7 mg/kg for recombinant human prorenin, and 0.3 mg/kg for recombinant human renin (the latter two were a kind gift of dr. W. Fischli, Actelion, Allschwil, Switzerland). GSC was measured by multiphoton microscopy as described before.

Urinary renin and prorenin after hepatic prorenin induction in Cyp1a1-Ren2 rats
The rat experiments were performed in accordance with guidelines issued in the ‘Guide for the Care and Use of Laboratory Animals’ (2010), and approved by the institutional Animal Care and Use Committee of Maastricht University. Animals were housed with free access to food and water throughout the experimental period and were housed under controlled conditions of temperature (21°C). Regular 12-hour diurnal cycles were maintained. Transgenic young Cyp1a1-Ren2 rats were obtained from an internal breeding stock, originally derived from animals supplied by the Centre of Cardiovascular Science, University of Edinburgh, UK. In transgenic Cyp1a1-Ren2 rats, the mouse Ren2 gene is placed under control of a cytochrome P450 promotor, Cyp1a1, on the Y-chromosome. The transgene is expressed primarily in the liver. Transcription of mouse prorenin can be induced by adding the natural xenobiotic indole-3-carbinol (I3C) to the diet. After withdrawal from I3C, the production of mouse prorenin stops. Rats were fed a diet containing 0.3% I3C (Sigma-Aldrich) from 4-8 weeks of age. Since this also induces severe hypertension, the rats were treated with either placebo, the angiotensin II type 1 receptor antagonist losartan, or hydralazine (n=4-11/group). Losartan (MSD, Oss, the Netherlands) was dissolved in PBS (GIBCO, Life Technologies, Carlsbad, CA) and administered at a dose of 20 mg/kg per day, via subcutaneously implanted osmotic minipumps (MODEL 2004 Alzet, Durect Corporation, Cupertino, CA). Osmotic minipumps were implanted under isoflurane anesthesia (1-4% Forane, Abbott House, Berkshire, UK) and buprenorphine analgesia (0.03 mg/kg s.c.). Hydralazine (Sigma-Aldrich) was administered via drinking water at a dose of 100 mg/kg per day. Rats were weighed, and drinking water, with the appropriate dilution of hydralazine, was prepared every other day. In addition, an osmotic minipump containing hydralazine at a dose of 3 mg/kg per day was implanted, for maximum effect and equal procedures between groups. For comparison, a subset of age-matched transgenic Cyp1a1-Ren 2 rats received normal rat chow (Sniff, Soest, Germany) and was treated with placebo. After 4 weeks of treatment rats were placed in metabolic cages to collect 24-hour urine. Additionally, mean arterial blood pressure (MAP) was measured in conscious state, as described before. Afterwards, rats were sacrificed by exsanguination via the abdominal aorta to collect blood. Blood was collected in tubes containing EDTA and aprotinin, centrifuged, and stored at -20°C. Kidneys were cut into 8 equal pieces and frozen in liquid nitrogen.
Plasma and urinary RAS components in patients with Dent’s disease or Lowe syndrome

The experimental protocol of this study was approved by the local research ethics committee. Spot urine samples (n=7) were obtained from 3 patients with Dent’s disease or Lowe syndrome visiting the outpatient clinic, frozen and stored at -80°C. Blood (n=5) was obtained from 4 patients during routine blood sampling, collected in tubes containing EDTA, centrifuged at 3,000 g for 10 minutes, and stored at -80°C. In case >1 urine or blood sample per patient was collected, the urine or plasma levels measured in these patients were averaged, to obtain one urine and one plasma concentration per person.

Biochemical measurements

Renin concentration was measured by enzyme-kinetic assay, by quantifying angiotensin (Ang) I generation in the presence of excess sheep (human samples) or porcine (rat samples) angiotensinogen. Total renin was measured after incubation with trypsin (which converts prorenin into renin) coupled to Sepharose, and the prorenin concentration was subsequently calculated by subtracting renin from total renin. In the human samples, ng Ang I/ml.hr activities were converted to pg/mL concentration as described before. Angiotensinogen was measured as the maximum quantity of Ang I generated during incubation, at pH 7.4 and 37°C, with recombinant human renin (human samples) or rat kidney renin (rat samples) in the presence of a mixture of ACE, angiotensinase, and serine protease inhibitors. Rat albumin was measured by ELISA (AssayPro, St. Charles, MO).

Aldosterone was measured by solid-phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). Human albumin and creatinine were measured at the clinical chemical laboratory of the Erasmus MC using standard protocols. Megalin expression was assessed by immunoblotting as reported previously. Kidneys were cut into 8 equal pieces, and one piece was homogenized on ice in isolation buffer to perform immunoblotting as reported previously. The antibody against megalin was a kind gift of dr. Erik I. Christensen, Aarhus University, Aarhus, Denmark (1:20.000). β-actin (1:50.000; Abcam) was used for normalization of protein levels. Protein was visualized using horseradish peroxidase-conjugated secondary antibodies (1:3000; Bio-Rad, Veenendaal, The Netherlands). Signals were detected by chemiluminescence (Pierce, Rockford, IL) and quantified using ImageQuant LAS 4000 (GE Healthcare, Diegem, Belgium).

REFERENCES

