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Proximal Tubular Expression Patterns of Megalin and Cubilin in Proteinuric Nephropathies

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Introduction: Receptor-mediated endocytosis is responsible for protein reabsorption in the proximal tubules. For albumin this process involves at least 2 interacting receptors, megalin and cubilin. Albumin is not usually present in the urine, indicating a highly efficient tubular reuptake under physiological conditions. However, early appearance of albuminuria may mean that the tubular system is overwhelmed by large quantities of albumin or that the function is impaired.

Methods: To better understand the physiological role of megalin and cubilin in human renal disease, renal biopsies from 15 patients with a range of albuminuria and 3 healthy living donors were analyzed for proximal tubular expression of megalin and cubilin using immunohistochemistry (IHC) and semi-quantitative immune-electron microscopy. Their expression in proteinuric zebrafish was also studied.

Results: Megalin and cubilin were expressed in brush border and cytoplasmic vesicles. Patients with microalbuminuric IgA nephropathy and thin membrane disease had significantly higher megalin in proximal tubules, whereas those with macro- or nephrotic-range albuminuria had unchanged levels. Cubilin expression was significantly higher in all patients. In a proteinuric zebrafish nphs2 knockdown model, we found a dose-dependent increase in the expression of tubular megalin and cubilin in response to tubular protein uptake.

Discussion: Megalin and cubilin show different expression patterns in different human diseases, which indicates that the 2 tubular proteins differently cooperate in cleaning up plasma proteins in kidney tubules.

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Megalin[1] (also known as Lrp2, low-density lipoprotein-related protein 2) and cubilin[2] are 2 large transmembrane proteins expressed on the surface of proximal tubular epithelial cells, where they are central to the endocytic reabsorption of many plasma proteins filtered across the glomerular capillary wall.[3–6] This mechanism is evolutionarily conserved and exists also in zebrafish.[7]

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Under a normal physiological condition, some albumin molecules pass through the glomerular filtration barrier[8,9] but are reabsorbed by the proximal tubular epithelium.[10] Consequently, albumin is not present in the urine of healthy individuals, and the presence of minute amounts of protein is considered as a sensitive marker of a dependent risk factor for future progression of renal disease.[11] The mechanism behind these observations is unclear, but increasing the load of protein taken up and recycled by the proximal tubular cells potentially contributes to the development of inflammation and fibrosis.[12]

In proteinuric diseases such as IgA nephropathy (IgAN) and minimal change nephrotic syndrome, large amounts of plasma proteins, including albumin, enter

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the tubules.[13] However, the role of megalin and cubilin[14] is unclear. Despite multiple studies reported in cells,[15–17] mice,[18–21] rats,[22,23] and dogs,[24,25] very little is known about the role of megalin and cubilin in humans.[26,27] Available data rely on the phenotypes of rare genetic diseases resulting in dysfunctional megalin (Dent's disease,[28–30] Lowe's syndrome,[28,29] and Donnai-Barrow syndrome[5,31–34]) or cubilin (Imerslund–Gräsbeck syndrome[35–38] and Fanconi-bichel syndrome[39]). However, it remains unclear how normal megalin and cubilin respond to increased protein load in common human kidney diseases.

We hypothesized that the expression of proximal tubular megalin and cubilin may correlate to the degree of albuminuria. In this study, we used human kidney biopsies from albuminuric nephropathies to study the subcellular localization and the role of megalin and cubilin in patients with albuminuria of different degree. As controls, we used healthy kidney donor biopsies. Furthermore, a proteinuric zebrafish model was used to confirm our findings.

MATERIALS AND METHODS

Ethical Statement

Approval was obtained from the Ethical Committee in Stockholm, Sweden, before the initiation of the study.

Patients

This study included a cohort of 15 patients (mean age 30 years, 10 patients are male) with kidney diseases selected from incident patients who underwent diagnostic renal biopsy at the Karolinska University Hospital between 2000 and 2013. We chose IgAN with different degrees of albuminuria and compared the results with a disease resulting in nephrotic-grade albuminuria and one nonalbuminuric (thin membrane disease, TMD). We also classified the patients according to the albuminuric levels. Group 1: albuminuria <300 mg/24 hours group: TMD (nonalbuminuric, n = 3) and microalbuminuric IgAN (>30 mg/24 hours and <300 mg/24 hours, n = 3);[40,41] group 2: albuminuria >300 mg/24 hours and <3500 mg/24 hours group: macro-albuminuric IgAN (n = 3);[41] group 3: albuminuria >3500 mg/24 hours group: nephrotic-albuminuric IgAN (n = 3) and minimal change nephrotic syndrome (n = 3). Biopsies from 3 healthy living kidney donors were used as controls. These biopsies were taken immediately after removal of the kidney from the donor and before flushing with preservative solution. All patients' characteristics are shown in Table 1.

Kidney Biopsies

For light microscopy, the specimens were fixed in 4% phosphate buffered formalin, and then dehydrated and embedded in paraffin according to standard procedures. Sections of 1.5 μm were cut on a microtome and stained with hematoxylin and eosin, periodic-acid Schiff, Ladewig's trichrome, and periodic acid silver. Materials for diagnostic transmission electron microscopy were fixed in a mix of 2.5% glutaraldehyde and 0.5% paraform aldehyde and embedded in an epoxy resin. The blocks were cut into approximately 60-nm-thick sections and evaluated under a transmission electron microscope.

The histopathology of the included patients' biopsies was re-examined by an experienced nephropathologist to confirm the respective diagnoses. Tubular atrophy and interstitial fibrosis were semi-quantified according to Banff criteria.[32] Thus, tubular atrophy was graded between 0 and 3: 0 = none, 1 = affecting 0 to 25% of the cortical area, 2 = affecting 26% to 50%, and 3 = affecting >50%. Interstitial fibrosis was graded 0–3: 0 = affecting 0 to 5% of the cortical area, 1 = affecting 6% to 25%, 2 = affecting 26% to 50%, and 3 = affecting >50%.

Biochemical Methods

Biochemical analyses were performed on serum albumin, serum creatinine, and albuminuria using routine methods at the Department of Clinical Chemistry, Karolinska University Hospital at Huddinge.

Generation of Proteinuric Zebrafish

The proteinuric zebrafish model was generated by morpholino-mediated knocking down of nphs2 as described earlier.[42] The morpholino antisense oligo (MO) targeting nphs2 (5′-TAGACTTACCT TCTCCAGGTCCCTC) and a standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA) were obtained from GENE TOOLS, LLC, Philomath, OR. Two doses (50 μM and 100 μM) of nphs2 MO were injected into 1- or 2-cell embryos, respectively. As a control, 100 μM of control MO was used for injection. In general, 2 nl of MO solution with the concentrations above was microinjected into the yolk of an embryo. In the knockdown experiments, we used embryos from a wild-type AB zebrafish line, which is maintained and raised in the zebrafish core facility, Karolinska Institute, as described.[43] In the larval stage, nphs2 knockdown in zebrafish is unlikely to lead to human-like massive proteinuria due to a relatively low level of plasma proteins. It has been shown that albumin appears to be absent in zebrafish plasma due to the lack of the gene encoding albumin in its genome, but instead a plasma vitamin D binding protein, which may have a similar function to albumin in zebrafish,[44] exists and shows 40% homology (amino acid similarity by the Blast comparison) of mammalian albumin. An antibody

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