Microalbuminuria in Type 1 Diabetes Is Associated With Enhanced Excretion of the Endocytic Multiligand Receptors Megalin and Cubilin

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OBJECTIVE — Proteinuria is the hallmark of diabetic nephropathy; yet, glomerular histology does not fully explain mechanisms contributing to proteinuria. Our objective was to identify proteins in the urine of individuals with type 1 diabetes and microalbuminuria that might implicate a mechanistic pathway operative in proteinuria.

RESEARCH DESIGN AND METHODS — Using a GeLC/MS platform proteomics approach, we compared the urine proteome from 12 healthy nondiabetic individuals, 12 subjects with type 1 diabetes yet normal urinary albumin excretion rates, and 12 subjects with type 1 diabetes and microalbuminuria (type 1 diabetes + microalbuminuria).

RESULTS — The abundance of megalin and cubilin, two multiligand receptors expressed in kidney proximal tubule cells and involved with the reuptake of filtered albumin and megalin/cubilin ligands, was significantly increased in type 1 diabetes + microalbuminuria urine, compared with both nonalbuminuric groups.

CONCLUSIONS — Aberrant shedding of megalin and cubilin could contribute to albuminuria in diabetes and to deficiency states of important vitamins and hormones.

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Excess urinary albumin excretion (UAE) (30–299 mg/day), termed microalbuminuria, portends incipient diabetic nephropathy. Mechanisms contributing to proteinuria in diabetic nephropathy are incompletely understood but likely involve pathology within the glomerulus, including endothelial cell injury, glomerular basement membrane thickening, loss of slit diaphragm veracity, and podocytopenia (1). Additionally, in the proximal tubule (PT), decreased protein reabsorption likely occurs (1,2); data from diabetic animals suggest that altered PT handling and diminished albumin retrieval contribute to albuminuria (3,4). Because ~70% of the urinary proteome originates from kidney or genitourinary tissues (5,6), we used the GeLC/MS platform proteomics approach to compare the urine proteome from *1*) nondiabetic individuals, *2*) subjects with type 1 diabetes yet normal UAE, and *3*) subjects with type 1 diabetes and microalbuminuria (type 1 diabetes + microalbuminuria), so as to identify proteins that might implicate a mechanistic pathway operative in proteinuria.

RESEARCH DESIGN AND METHODS

— This study was conducted as a subanalysis of a study described elsewhere (7); 12 of 143 originally described subjects had type 1 diabetes + microalbuminuria (UAE ≥30 mg albumin/g creatinine, herein designated as group 3). Two additional subgroups (matched to group 3 for age and sex) were identified and designated group 1: healthy, nondiabetic, nonalbuminuric subjects (n = 12); and group 2: subjects with type 1 diabetes and normal UAE (<30 mg albumin/g creatinine; n = 12). Group characteristics are available in Table A1 in an online-only appendix at http://care.diabetesjournals.org/cgi/content/full/dc09-0112/DC1.

An SDS-PAGE to liquid chromatography–tandem mass spectrometry (GeLC/MS) platform was used to compare 24-h urine samples from subjects in groups 1–3. liquid chromatography–tandem mass spectrometry allows for identification of proteins in urine; relative protein abundance was quantified by spectral counting (5,8). For each subject, a urine aliquot corresponding to 1,000 μg urine creatinine was calculated. Within each group, these 12 aliquots were pooled into four pools (two female + two male pools of three subjects each); each pool was independently analyzed. ANOVA was used to ascertain differences between groups. Linear relationships between data were analyzed using the Pearson product moment correlation.

RESULTS

— Groups 1–3 were comparable for sex, age, BMI, blood pressure, and glomerular filtration rate (Table A1 in the online appendix). Groups 2 and 3 were comparable for disease duration and displayed higher A1C and continuous glucose monitoring system glucose and lower C-peptide values compared with controls. Per study design, group 3 alone exhibited increased UAE.

Over 150 protein signatures were identified by GeLC/MS analysis to be either *1*) only identified in group 3 urine or *2*) present in groups 1 and 3 urine but exhibiting at least two times greater frequency, by spectral counting, in group 3

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Table 1—*Comparisons and correlations of megalin, cubilin, and their ligands in urine*

	Comparisons*					Correlations	
Protein/ligand	1: Control	2: Type 1 diabetes	3: Type 1 diabetes + microalbuminuria	1 vs. 2	1 vs. 3	2 vs. 3	Megalin vs. ligand	Cubilin vs. ligand
Megalin	5.63 ± 1.60	3.13 ± 3.94	20.13 ± 9.19	NS	<0.05	<0.001	—	$R = 0.88$; $P < 0.0001$
Cubilin	2.63 ± 3.78	1.50 ± 2.98	20.13 ± 15.8	NS	<0.01	<0.001	$R = 0.88$; $P < 0.0001$	—
Angiotensinogen	ND	ND	0.25 ± 0.71	—	—	—	—	—
TBG	ND	ND	0.63 ± 0.92	—	—	—	—	—
IGF2R	ND	ND	1.13 ± 1.55	—	—	—	—	—
APOA1	ND	ND	2.0 ± 1.77	—	—	—	—	—
Transthyretin	ND	ND	3.38 ± 1.77	—	—	—	—	—
VDBP	0.5 ± 0.76	1.13 ± 1.46	3.63 ± 1.69	NS	<0.01	<0.05	$R = 0.433$; $P = 0.04$	$R = 0.399$; $P = 0.05$
Clusterin	ND	1.13 ± 1.55	4.88 ± 3.04	—	—	<0.05	—	—
RBP4	2.25 ± 2.05	2.25 ± 2.77	8.88 ± 5.25	NS	<0.05	<0.01	$R = 0.444$; $P = 0.03$	NS
EGF	8.75 ± 6.39	7.38 ± 1.77	22.0 ± 10.04	NS	<0.05	<0.05	$R = 0.663$; $P < 0.001$	$R = 0.682$; $P < 0.001$
Serotransferrin	8.5 ± 8.72	13.88 ± 13.62	90.38 ± 45.14	NS	<0.001	<0.01	$R = 0.791$; $P < 0.001$	$R = 0.574$; $P < 0.01$
Albumin	213.4 ± 83.7	244.8 ± 168.7	515.5 ± 140.9	NS	<0.01	<0.05	$R = 0.748$; $P < 0.001$	$R = 0.614$; $P = 0.001$

*Data are averaged mean spectral counts ± SD. Statistical significance was defined as $P < 0.05$. APOA1, apolipoprotein A-1; EGF, epidermal growth factor; IGF2R, IGF-2 receptor; ND, none detected; NS, not significant; TBG, thyroxin binding globulin; VDBP, vitamin D binding protein; RBP4, retinol binding protein 4. Detailed proteomic methodology is provided in the online appendix.

compared with group 1 ($P < 0.05$). The most abundant proteins in all groups were albumin, uromodulin, and α-1-microglobulin/bikunin precursor, respectively. Two PT endocytic receptors, megalin (i.e., LRP2) and cubilin, active in the reuptake of filtered small-molecular-weight proteins (9), were among the 20 most abundant proteins in group 3 urine (see Table A2 in the online appendix) and were significantly more abundant in urine from group 3, compared with groups 1 or 2 (Table 1). Neither receptor was identified in the 20 most abundant proteins in groups 1 or 2. Megalin and cubilin form a complex on the cell surface of PT cells (9). Using data from all groups, megalin and cubilin mean spectral counts were highly correlated, consistent with evidence that they form a cellular complex and are co-excreted in urine (Table 1).

Megalin is a membrane-bound protein, and the extracellular domain can be shed from PT cells (10). Analysis of the peptide spectra generated by GeLC/MS revealed that >99% of the peptide fingerprinting covered regions of the extracellular domain. Moreover, the most COOH-terminal peptide detected in all groups extended to amino acid 4304 within the extracellular domain, suggesting that urinary megalin represents predominantly the extracellular component.

Megalin and cubilin participate in the reuptake and endocytosis by PT cells of a number of proteins including albumin. Several megalin and/or cubilin ligands were elevated in or only detected in urine from group 3 (Table 1). Analyzing data from all groups, the urinary abundance of albumin correlated with the urinary abundance of both megalin and cubilin (Table 1). Several other megalin/cubilin ligands also correlated with megalin/cubilin abundance (Table 1).

CONCLUSIONS — Megalin is an ~600-kDa single-spanning transmembrane glycoprotein belonging to the LDL receptor family (9). This endocytic receptor is primarily expressed in polarized epithelial cells including PT cells of the kidney and is a multiligand receptor with many ligands including albumin, vitamin-binding proteins, carrier proteins, lipoproteins, hormones, and drugs (9). Cubilin is an ~460-kDa peripheral membrane glycoprotein with no transmembrane component (9). Also expressed in renal PT cells, cubilin binds several ligands in common with megalin including albumin, vitamin D binding protein,

transferring, and lipoproteins (9). Together, these receptors are responsible for the tubular clearance of most filtered proteins. In fact, over ~95% of filtered albumin is reabsorbed in the PT primarily via the megalin-cubilin complex (2). Because megalin and cubilin are involved in reuptake of albumin and smaller proteins from the glomerular filtrate, diminished functioning of these receptors in diabetes could contribute to proteinuria. Animal and clinical investigations support this hypothesis by demonstrating selective proteinuria in conditions of megalin or cubilin loss of function (11–13).

Studies have shown that LRP family members, including megalin, are shed from cell surfaces by matrix metalloproteinases (10). Matrix metalloproteinase activity is elevated in type 1 diabetes urine (7), and matrix metalloproteinase expression is altered in the diabetic kidney (14). Thus, enhanced matrix metalloproteinase activity in the parenchyma and/or tubular lumen of the diabetic kidney might result in shedding of the megalin/cubilin complex from PT cell surfaces. Secondary to megalin and cubilin loss, isolated deficiencies of certain small-molecular-weight proteins and their ligands (i.e., vitamins) would be predicted.

Certain study limitations exist. This study was a sub-analysis of a preexisting dataset and was not intended to predict disease progression, but, rather, to identify proteins that might infer a mechanistic pathway operative in diabetic nephropathy. Confirmation that "megalin-uria" or "cubilin-uria" correlates with or predicts microalbuminuria will require measurement of megalin and cubilin in individual specimens from a larger prospective cohort. At present, however, there are no immunometric assays for megalin or cubilin to allow for quantitative assessment.

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