Diabetic nephropathy is associated with increased urine excretion of proteases plasmin, prostasin and urokinase and activation of amiloride-sensitive current in collecting duct cells

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ABSTRACT

Background. Diabetic nephropathy (DN) is associated with hypertension, expanded extracellular volume and impaired renal Na⁺ excretion. It was hypothesized that aberrant glomerular filtration of serine proteases in DN causes proteolytic activation of the epithelial sodium channel (ENaC) in the kidney by excision of an inhibitory peptide tract from the γ subunit.

Methods. In a cross-sectional design, urine, plasma and clinical data were collected from type 1 diabetic patients with DN (n = 19) and matched normoalbuminuric type 1 diabetics (controls, n = 20). Urine was examined for proteases by western immunoblotting, patch clamp and ELISA. Urine exosomes were isolated to elucidate potential cleavage of γENaC by a monoclonal antibody directed against the ‘inhibitory’ peptide tract.

Results. Compared with control, DN patients displayed significantly higher blood pressure and urinary excretion of plasmin(ogen), prostasin and urokinase that correlated directly with urine albumin. Western blotting confirmed plasmin, prostasin and urokinase in urine from the DN group predominantly. Urine from DN evoked a significantly larger amiloride-sensitive inward current in single collecting duct cells compared with controls. Immunoblotting of urine exosomes showed aquaporin 2 in all patient samples. Exosomes displayed a virtual absence of intact γENaC while moieties compatible with cleavage by furin only, were shown in both groups. Proteolytic cleavage by the extracellular serine proteases plasmin or prostasin was observed in DN samples predominantly.

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Conclusion. DN is associated with increased urinary excretion of plasmin, prostasin and urokinase and proteolytic activation of ENaC that might contribute to impaired renal Na⁺ excretion and hypertension.

Keywords: albuminuria, exosome, hypertension, proteinuria, sodium

INTRODUCTION

Diabetic nephropathy (DN) is a common complication that affects 10–30% of diabetic patients [1] and is characterized by albuminuria, hypertension and kidney tissue changes that predict an adverse outcome [2]. The mechanisms that underlie the hypertension accompanying long-term diabetes are not known. Previous studies showed that the epithelial sodium channel (ENaC) is activated proteolytically by urine from patients with albuminuria through aberrantly filtrated plasminogen, activated to plasmin in urine [3]. Plasmin turns ‘near silent’ ENaC channels active by proteolysis [4]. At plasmin concentrations below 4 μg/mL, proteolytic cleavage by plasmin is mediated by glycosylphosphatidylinositol (GPI)-anchored prostasin and, at higher concentrations, by a direct action of plasmin [5]. Constitutive activation of renal ENaC is sufficient to precipitate hypertension as evidenced by Liddle’s syndrome [6]. Prostasin is GPI-anchored to principal cells, but the enzyme also circulates in plasma [7]. It is not known whether circulating prostasin is aberrantly filtered with plasmin in diabetes with albuminuria. Plasmin is present in urine from diabetic rats [8] and patients with type 2 diabetes [9], but it is not known whether higher levels are excreted in overt DN. A likely activator of plasminogen in urine, urokinase-type plasminogen activator (uPA), has been detected in increased amounts in rats with DN [10]. The human counterpart has not been examined. It is thought that subsequent to intracellular proteolytic cleavage of γENaC by furin in the synthesis pathway, extracellular cleavage by plasmin/prostasin results in the putative release of an ‘inhibitory’ 43-amino acid peptide tract from γENaC and channel activation [3, 4]. Proteolytic cleavage of γENaC has been shown in oocyte systems [8] and in human kidney tissue [11]. Whether γENaC cleavage status is changed in human kidney in proteinuria accompanying diabetes is not defined. It has become possible to investigate this question non-invasively by the surrogate marker of urinary exosomes which are 40–100 nm particles released from the apical membrane of renal epithelial cells and excreted in urine [12, 13]. It was hypothesized that DN is associated with augmented urine excretion of urokinase, plasminogen/plasmin and prostasin which is reflected by increased abundance of proteolytically activated γENaC in exosomes. The hypothesis was addressed by investigating type 1 diabetic patients with and without DN (controls) in a cross-sectional design with respect to (i) urinary excretion of uPA, plasminogen/plasmin and prostasin, (ii) urinary protease activity, (iii) ability to evoke ENaC current in cortical collecting duct cells upon addition of urine and (iv) proteolytic cleavage of γENaC in urinary exosomes as detected by a monoclonal antibody directed against the ‘inhibitory’ peptide tract within γENaC [5, 11].

MATERIALS AND METHODS

Study participants

This cross-sectional, observational study included 19 type 1 diabetic patients with DN (nephropathy group) and 20 type 1 diabetic patients without nephropathy (control group) enrolled from Department of Endocrinology, Odense University Hospital, Denmark. DN was defined as urinary total protein excretion of ≥300 mg/24 h. The control group had urinary total protein excretion of <30 mg/24 h. The inclusion criterion was type 1 diabetes with or without DN. Exclusion criteria were pregnancy or lactation, clinically relevant organic or systemic disease including malignancy, medication with amiloride or glucocorticoids and lack of understanding of study or capability to cooperate.

Ethical considerations

The study was approved by The Ethical Committee of The Region of Southern Denmark (Project-ID: S-20120061). All patients gave written informed consent to participate in the study.

Blood pressure recordings

Twenty-four hour ambulatory blood pressure monitoring was obtained using blood pressure monitors from A&D Medical (TM-2430 ambulatory blood pressure monitors, A&D Medical, CA, USA). Devices have been approved and validated for clinical use [14].

Materials

Plasma, spot urine and 24-h urine samples were frozen and stored at −80°C. Samples were thawed on ice, and aliquots were prepared and stored at −80°C until experiments were performed.

Plasma and urine analyses

Aliquots of the urine samples were centrifuged at 10 000 r.p.m. (Mikro 200, Hettich Lab Technology, Germany) for 1 min and the supernatant was used. Biochemical analyses were performed on an Abbott Architect c8000 or c16000 device following the manufacturer’s instructions. HaBAc levels were determined by high-performance liquid chromatography (HPLC, Tosoh G8, Alere). Urinary creatinine concentrations were determined by spectrophotometry using Microlab 300 (Vital Scientific BV, AC Dieren, NL). Total plasminogen [abbreviated: plasmin(ogen)], prostasin and urokinase (uPA) were measured using ELISA kits: plasminogen (Cat# IHPLGKT-TOT; Innovative Research, Novi, MI, USA); prostasin (Cat# CSB-EL018825HU; Cusabio Biotech Co., China) and urokinase (ab108917; Abcam, CA, USA). Prior to ELISA for urinary prostasin, urine was centrifuged at 4°C for at least 14 min at 4000g while filtrated and concentrated 5-fold using centrifugal filters (molecular weight cut-off 10 kDa, Amicon Ultra-4 10 K Centrifugal Filter Devices, Merck Millipore Ltd., A&D Medical, CA, USA). Devices have been approved and validated for clinical use [14].
Urinary exosomes

Immediately upon voiding, 3.4 mL of 100 mmol/L NaN₃ and a mixture of protease inhibitors [100 µL 1 mmol/L leupeptin and 500 µL 100 mmol/L phenylmethanesulphonyl-fluoride (PMSF, Sigma)] were added to 100 mL spot urine before storage at −80°C. Urine samples were thawed at 4°C, vortexed thoroughly and centrifuged at 3000 g for 30 min (Sorvall RC 26 Plus). The supernatant was further processed by ultracentrifugation at 45 000 r.p.m. (220 000g) at 4°C for 100 min (Beckman Ultracentrifuge L-70). The pellet was resuspended in 100 µL homogenization buffer added Roche Complete Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics GmbH, Germany).

Western immunoblotting

Urine or the aprotinin purification eluate was subjected to western immunoblotting as described previously [17]. The membrane was incubated with primary antibodies: plasminogen (ab 6189-100; Abcam, MA, USA, 1:5000); prostasin (cat. no. 15527-1-AP; Proteintech Group, IL, USA, 1:1000); urokinase (ab8473; Lot. no. GR106505-1, Abcam, 1:2000); γENaC (mabinhis) [5, 11], 1:2000; aquaporin 2 (AQP2; c17 sc-9882, Lot. no. K1711, Santa Cruz Biotechnology, TX, USA, 1:2000) for 1 h at room temperature or at 4°C overnight. HRP-conjugated secondary antibodies were applied (DakoCytomation, Denmark, 1:2000). For exosome protein immunoblots, loaded amount was normalized to urinary creatinine.

Urinary protease activity

Urinary protease activity was evaluated using 10% Zymogram (gelatin) gels as described previously [17] with the only exception that urine was concentrated five times as described above.

RESULTS

Patient characteristics

The mean age, diabetes duration and gender distribution did not differ between diabetes with nephropathy and diabetic control group (Table 1). Retinopathy status was similar between groups. The nephropathy group had significantly higher 24-h urinary total protein excretion and higher systolic, but not diastolic, ambulatory blood pressure despite a higher number of antihypertensive drugs, whereas creatinine clearance was reduced compared with the control group (Table 1). Twenty-four hour urinary sodium excretion and urine Na⁺/K⁺ ratio were not different between the two groups (Table 1).

Patients with DN display increased urine serine protease excretion

In the nephropathy group, urinary plasmin(ogen) excretion normalized to creatinine was significantly higher compared with the control group (Figure 1A). Urinary plasmin(ogen) concentration correlated significantly with urinary albumin concentration (P < 0.0001, r² = 0.55, Figure 1C; 4 of the 18 control samples displayed values within the detection range of the assay and these samples were included). There was a significant correlation between urinary plasmin(ogen)/creatinine-ratio and 24-h ambulatory mean arterial blood pressure (P = 0.02, r² = 0.19, not shown). The urinary prostasin/creatinine-ratio was significantly higher in nephropathy than the control group (Figure 1B). Urinary prostasin concentration correlated significantly with urinary albumin concentration (P < 0.0015, r² = 0.37, Figure 1D; 5 of the 19 control samples were within detection range of the assay and were included). Plasma plasmin(ogen) did not differ between groups (Figure 1E). Plasma prostasin was significantly higher in nephropathy than the control group (Figure 1F). There were no correlations between 24-h urinary Na⁺ excretion and urinary plasmin or prostasin levels or blood pressure (data not shown). Western immunoblotting of creatinine-normalized

Single cell (CCD M1) patch-clamp experiments

Whole-cell patch-clamp experiments were conducted on the M-1 cortical collecting duct cell line (ATCC, Boras, Sweden) as previously described [17] with the only exception that neither alpha₂-antiplasmin nor heat denaturation was applied in this study.

Statistical evaluation

Data were tested for normal distribution and log-transformed if not normally distributed. Log-normally distributed data are presented in semi-logarithmic diagrams with geometric means. Normally distributed data are presented as means ± SEM. Nephropathy and control groups were compared by unpaired Student’s t-test. Correlation was evaluated using Pearson correlation (linear dependence). The means of three groups were compared by one-way ANOVA followed by the Bonferroni multiple comparison post hoc test. P < 0.05 was considered statistically significant. GraphPad Prism 6.04 for Windows was used.
urine samples from 12 of the 19 nephropathy patients (examples in Figure 2A) displayed bands migrating with molecular weights corresponding to intact plasminogen (83–88 kDa), active intact plasmin (∼80 kDa) and plasmin subunits (57, 26 kDa). In five samples, only active plasmin was present. Pure human plasmin subjected to the same procedure migrated at an apparent molecular weight of 75 kDa. Urine samples from the diabetic control group (n = 18) showed no detectable plasmin (ogen) (examples in Figure 2A). Nephropathy patients consistently displayed bands at ∼40 kDa representing prostasin in urine samples (Figure 2B). Four of the seven urine samples from diabetic controls showed 40 kDa bands (examples in Figure 2B). Western immunoblotting showed bands at 50 kDa corresponding to urokinase (uPA) in urine samples from 18 of the 19 nephropathy patients, but from none of the control patients (n = 20) (examples in Figure 2C). The uPA/creatinine concentration ratio in spot urine was significantly higher in nephropathy than the control group by ELISA (Figure 2D, P = 0.006). Urine uPA concentration correlated significantly with albumin (Figure 2E, P = 0.016, r² = 0.24; 5 of the 19 control samples were within the detection range and included). Gelatin zymography demonstrated urinary protease activity associated primarily with nephropathy patients. Resolution at molecular sizes was poor with the long digestion period applied in the assay, but products migrating similar to pure human plasmin at 75 kDa was present in 11 of the 19 nephropathy patients versus 4 of the 19 control samples (examples in Figure 3A–C).

**Effect of urine from patients with DN on whole-cell inward current**

Cortical collecting duct cells (M1) were used as biosensors to investigate the effect of urine on whole-cell inward current. The inward current evoked by exposure to urine from the nephropathy group was three times higher than that of the control group (Figure 4, n = 5 in both groups, P < 0.0001). Moreover, treatment with amiloride (2 µmol/L) before addition of nephropathy urine abolished the inward current produced by urine (n = 5, P < 0.0001).

Prostasin and proteolytically cleaved γENaC in urinary exosomes

The collecting duct-specific water transporter, AQP2, was detected in urine exosome fractions with the expected molecular size at 25 and 37 kDa corresponding to native and glycosylated forms in seven of the eight randomly selected patients from the nephropathy group and 10 of the 10 patients from the diabetic control group (Figure 5A and B). There was no significant difference between groups in the abundance of exosome-associated AQP2 by densitometry in creatinine-normalized urine samples (not shown). Immunoblotting for prostasin performed on the same urine exosome fractions demonstrated a 40-kDa prostasin band in 5 of the 8 patients from the nephropathy group and 9 of the 10 patients from the control group, respectively, with no significant difference (examples in Figure 5C). Western immunoblotting of human kidney cortex tissue homogenate pool used as a positive control showed bands at 100, 75 and 37 kDa with a monoclonal mouse antibody directed against an epitope within the inhibitory peptide of human γENaC [5, 11]. This corresponds to intact full-length human γENaC, furin-only cleaved γENaC and γENaC cleaved proteolytically by an extracellular serine protease only (Figure 5D and E). In exosomes, three nephropathy patients and no controls showed intact γENaC at 100 kDa (Figure 5D). In the nephropathy group, six of the eight samples displayed prominent bands at ∼75, 50–60 and 37 kDa, respectively, whereas only four of the seven samples from the diabetic control group showed bands at 75 and 60 kDa (Figure 5E). Most consistent difference was a band at 37 kDa corresponding to ENaC, furin-only cleaved ENaC and ENaC at 100 kDa.

Table 1. Baseline patient characteristics of type 1 diabetic patients with (nephropathy) or without DN (control)

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Nephropathy</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Females</td>
<td>7/19</td>
<td>7/20</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.8 ± 3.0</td>
<td>52.7 ± 2.6</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>32.8 ± 3.0</td>
<td>33.9 ± 2.8</td>
</tr>
<tr>
<td>HbA1c % (mmol/mol)</td>
<td>8.4 ± 0.5 (68 ± 5.4)</td>
<td>8.0 ± 0.3 (64 ± 0.1)</td>
</tr>
<tr>
<td>24-h urinary total protein excretion (g/24 h)</td>
<td>1.8 ± 0.5***</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Urinary albumin/creatinine-ratio (mg/g)</td>
<td>960.4 ± 258.0***</td>
<td>5.7 ± 2.2</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min)</td>
<td>56.9 ± 7.0***</td>
<td>101.3 ± 6.3</td>
</tr>
<tr>
<td>24-h urinary sodium excretion (mmol/24 h)</td>
<td>155.8 ± 18.1</td>
<td>171.4 ± 16.7</td>
</tr>
<tr>
<td>24-h diastolic blood pressure (mmHg)</td>
<td>0.41 ± 0.06</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>Antihypertensive drugs**</td>
<td>3.0 ± 0.4**</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Hypertensive patients</td>
<td>19/19</td>
<td>13/19</td>
</tr>
<tr>
<td>Degree of diabetic retinopathy*</td>
<td>3.4 (95% CI: 2.9–3.9)</td>
<td>3.0 (95% CI: 2.5–3.5)</td>
</tr>
<tr>
<td>Diabetic neuropathy</td>
<td>3/19</td>
<td>2/20</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.9 ± 1.0</td>
<td>26.7 ± 0.9</td>
</tr>
</tbody>
</table>

*Number of antihypertensive drugs including diuretics, but excluding amiloride and spironolactone.

**Hypertension was defined as 24-h systolic and/or diastolic blood pressure >130/80 mmHg, respectively, or use of ≥1 antihypertensive drugs.

Average degree of diabetic retinopathy. If the degree of diabetic retinopathy differed on the two eyes, the highest degree was used.

*P < 0.05, **P < 0.01, ***P < 0.001.
to distal cleavage in nephropathy patient exosomes, which was not observed in controls (Figure 5D and E). One sample from the nephropathy group and three control samples, respectively, were positive for AQP2 and prostasin, but negative for γENaC.

**DISCUSSION**

The present cross-sectional study demonstrates, in two groups of type 1 diabetic patients with similar diabetes duration (~30 years), that nephropathy is associated with (i) insufficient blood pressure control despite an average use of three antihypertensive agents; (ii) higher urine excretion of active serine proteases plasmin, urokinase and prostasin that correlated directly with urine albumin; (iii) augmented proteolytic activity in urine; (iv) ability of urine to evoke amiloride-sensitive inward current in renal cortical collecting duct cells and (v) more ‘distally’ cleaved γENaC in urinary exosomes. Observations are compatible with aberrant glomerular filtration of proteases and proteolytic activation of γENaC. The present data are observational and cross-sectional and predict an in vivo contribution of ENaC to impaired sodium excretion and insufficient blood pressure control in DN. The effect of the ENaC blocker amiloride has been tested in type 2 diabetes patients with resistant hypertension. A significant blood pressure reduction (9.8/3.4 mmHg) was found in a placebo-controlled study by Saha et al. [18] in African Americans. The combination of spironolactone and amiloride was superior compared with treatment with either drug alone [18]. Multiple indirect observations are in accordance with impaired sodium excretion, potentially ENaC-mediated, as an independent contributor to extracellular volume expansion and increased blood pressure in diabetes: (i) plasma renin activity, angiotensin II and aldosterone are either normal [19] or suppressed [20], (ii) atrial natriuretic peptide is increased [20], (iii) extracellular volume is expanded [19]; (iv) exchangeable body sodium is increased 10–15% [21]; (v) blood pressure is more salt-sensitive in microalbuminuric diabetic patients compared with diabetics with normoalbuminuria [22, 23] and (vi) volume expansion produced by water immersion [24–26] or saline...
infusion [27] in type 1 diabetic patients results in impaired renal sodium excretion compared with non-diabetic controls. Whether these observations are due to augmented ENaC activity has not been tested directly. This study further supports the notion that plasminogen is lost across an injured glomerular barrier and activated subsequently in urine to plasmin in diabetes with nephropathy, similar to patients with type 2 diabetes and microalbuminuria [9], preeclampsia [17], adult [3] and childhood nephrotic syndrome [28] and a rat diabetes model [8]. The nephropathy patients displayed hypertension and received multiple drugs (Table 1). The study was not designed to evaluate whether the urine proteases differed in normotensive versus hypertensive diabetic patients. However, there was a correlation between urinary plasminogen/creatinine-ratio and blood pressure not observed with prostasin or with uPA that is compatible with a causal relation. This study demonstrates increased urine excretion of soluble prostasin and uPA with plasminogen. This serine protease cocktail present in pre-urine in soluble form with high local concentration at the collecting duct apical membrane provides activating

**FIGURE 2:** (A) Western immunoblotting of crude spot urine samples demonstrating intact plasminogen (83–88 kDa), active plasmin (∼80 kDa) and plasmin subunits (57, 26 kDa) in urine from type 1 diabetic patients with DN. The matched control group showed no detectable plasminogen. HP is pure human plasmin control. (B) Western immunoblotting of crude spot urine samples that display a single band migrating at ∼40 kDa compatible with prostasin in four samples from nephropathy and in one of the four control patients. Human placenta tissue (PL, 3 µg protein) was used as positive control. (C) Urine urokinase-type plasminogen activator (uPA) was detected in crude spot urine samples by western immunoblotting as a band migrating at ∼50 kDa in samples from the nephropathy patients, but not the control diabetic patient group. ELISA confirmed higher uPA content in spot urine samples from nephropathy than control patients (D); numbers are geometric means. uPA concentration correlated significantly with urine albumin concentration (E).

**FIGURE 3:** (A–C) Gelatin zymography demonstrated urinary protease activity in nephropathy urine samples in 11 of 19 versus 4 of 19 samples in control group, respectively. Pure human plasmin (HP) was used as positive control. The figure shows 10 representative nephropathy and 9 control samples of which 4 nephropathy (lanes numbered 1, 3, 4 and 9) and 3 controls (lanes numbered 12, 13 and 17) were considered positive, respectively. MW, molecular weight marker.
potential at the γENaC ectodomain. Accordingly, urine from DN patients activated amiloride-sensitive current in a bioassay approach similar to previous observations with urine from other albuminuric diseases [3, 9, 17, 28]. Recent observations suggest that circulating prostasin may originate predominantly from the liver [7], but the physiological significance of the increased plasma prostasin measured in the present patients is not clear. Urokinase also circulates in plasma and this study confirms observations of increased urine uPA in diabetic rats [8] and implies a significant contribution of aberrantly filtered urokinase to pre-urine in albuminuria.

Proteolytic processing of γENaC was shown in human nephrectomy tissue [11] and cleavage of γENaC by prostasin/kallikrein was observed only in proteinuric patient kidneys consistent with our hypothesis [11]. In this study, γENaC proteolysis was studied by immunoblotting with urinary exosomes, a surrogate marker for kidney tissue. Collecting duct-derived exosomes were released at a similar rate as documented by the presence of AQP2 in roughly similar abundance in the two groups. Of note, prostasin was detected also at fairly similar levels in the exosome fraction from nephropathy and non-nephropathy patients. This would be in

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**FIGURE 4:** (A and B) In single murine cortical collecting duct cells (M1 cell line), urine from type 1 diabetic patients with DN induced significantly larger whole-cell inward current compared with the matched diabetic control group (n = 5 in both groups). (A) A representative original current trace from a single cell before (grey line) and after (black line) exposure to urine from a subject with DN, respectively. (B) Pre-treatment with amiloride (2 µmol/L) abolished the inward current produced by nephropathy urine (n = 5). pA, picoampere; Nephropathy + A, nephropathy urine with amiloride in the bath solution.

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**FIGURE 5:** (A and B) Western immunoblotting of protein contained in urine ultracentrifugation pellets with exosomes showed a positive signal for aquaporin 2 in its native (25 kDa) and glycosylated forms (37 kDa) in both diabetic control patients and in nephropathy patients and thus displayed a significant contribution derived from principal cells in collecting ducts. (C) Western immunoblotting of exosome pellet revealed a band at ~40 kDa compatible with prostasin in exosomes from both the nephropathy and control group. Human placenta tissue (PL, 3 µg) was used as positive control. (D and E) Western immunoblotting of exosome pellet for γENaC with a monoclonal mouse antibody directed against the inhibitory domain of human γENaC. Human kidney cortex tissue pool (HC, 40 µg) served as positive control and revealed bands at 100, 75 and 37 kDa corresponding to non-cleaved human γENaC, furin-only cleaved γENaC and γENaC cleaved proteolytically by an extracellular serine protease, e.g. plasmin or prostasin. In exosome fraction from type 1 diabetic patients with DN, all products were represented, but the most consistent findings were the absence of intact ENaC at ~100 kDa and prominent bands at ~37 kDa. In exosome fraction from four of seven diabetic non-nephropathy patient urines, the blotting revealed bands at 75 and ~60 kDa, but with no products at ~37 kDa and three lanes did not show any product at all.
accompany with a similar shedding rate and membrane abundance for the constitutive, GPI-anchored principal cell associated prostasin in the two groups [29]. In hypertensive patients, urinary exosome-associated prostasin was not significantly different from hyperaldosteronism patients, which suggests a rather constant membrane association and shedding [30]. In contrast, in crude urine, soluble prostasin excretion was significantly higher in samples from the nephropathy group. Because prostasin was detectable in plasma, and crude urine prostasin correlated highly significantly with urinary albumin, this could reflect aberrant filtration of soluble prostasin similar to plasminogen. The exosome association of prostasin and its activation by plasmin would predict more proteolytic activity and ENaC cleavage in nephropathy patients.

Tasin and its activation by plasmin would predict more similar to plasminogen. The exosome association of pros-

The dominence of the 37 kDa moiety in nephropathy compatible with increased distally acting protease exposure [11]. It should be noted that mab_inhib detects an epitope in the inhibitory tract between the furin and prostasin cleavage sites, and cleavage by both proteases would therefore lead to release of this peptide into the urine. Thus, the apparent absence of γENaC epitope in AQP2- and prostasin-positive samples would imply full release of the epitope within the ‘inhibitory peptide’ of γENaC. This study shows that ENaC analysis in urine exosomes is feasible, but larger patient materials should be sampled to allow safe conclusions regarding difference in exosome ENaC abundance and dual ENaC cleavage between patient categories and degrees of proteinuria and its correlation to Na⁺ excretion and blood pressure. There was no significant difference between 24-h urine Na⁺ excretion and Na⁺/K⁺ ratio in the two groups. Salt intake was estimated based on a single 24-h urine sample, which does not provide information on chronic salt intake or ‘salt sensitivity’. Conclusions regarding functional interplay between urinary plasmexcretion, ENaC activity and salt intake in DN should be based on interventional studies. In perspective, a ‘clamped’ hyperactive ENaC could explain impaired sodium excretion and salt sensitivity of the blood pressure in DN. Plasmin excretion in urine may assist defining which subgroups of diabetics will benefit from reductions in dietary salt intake. Because amiloride inhibits both ENaC and uPA [31], this diuretic seems to be an attractive addition to reach blood pressure treatment targets in DN.

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CONFLICT OF INTEREST STATEMENT

None declared.

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