Urinary extracellular vesicles and tubular transport

Crissy F. Rudolphi, Charles J. Blijdorp, Hester van Willigenburg, Mahdi Salih and Ewout J. Hoorn

Department of Internal Medicine, Division of Nephrology and Transplantation, Erasmus Medical Center, Rotterdam, The Netherlands

Correspondence to: Ewout J. Hoorn; E-mail: e.j.hoorn@erasmusmc.nl

ABSTRACT

Tubular transport is a key function of the kidney to maintain electrolyte and acid-base homeostasis. Urinary extracellular vesicles (uEVs) harbor water, electrolyte, and acid-base transporters expressed at the apical plasma membrane of tubular epithelial cells. Within the uEV proteome, the correlations between kidney and uEV protein abundances are strongest for tubular transporters. Therefore, uEVs offer a noninvasive approach to probing tubular transport in health and disease. Here, we review how kidney tubular physiology is reflected in uEVs and, conversely, how uEVs may modify tubular transport. Clinically, uEV tubular transporter profiling has been applied to rare diseases, such as inherited tubulopathies, but also to more common conditions, such as hypertension and kidney disease. Although uEVs hold the promise to advance the diagnosis of kidney disease to the molecular level, several biological and technical complexities must still be addressed. The future will tell whether uEV analysis will mainly be a powerful tool to study tubular physiology in humans or whether it will move forward to become a diagnostic bedside test.

Keywords: aldosterone, aquaporin, biomarker, chronic kidney disease, exosomes, hypertension

INTRODUCTION

Urinary extracellular vesicles (uEVs) provide a noninvasive approach to studying cell biology in the kidney tubule and urinary tract. These nanosized membrane particles can be classified into two types (Fig. 1). The first type of uEV includes exosomes (≤200 nm) released by the fusion of multivesicular bodies with the cell membrane. The second type is released by outward budding of the cell membrane, resulting in microvesicles (100–1000 nm) or—in the case of dying cells—apoptotic bodies. Because exosomes and microvesicles largely overlap in terms of size, density, and protein markers, it is difficult to separate them. In practice, therefore, uEVs are either studied as one heterogeneous group or alternatively classified as small (≤200 nm) or large uEVs (>200 nm) [1]. Most uEV research focuses on proteins and nucleic acids included within the vesicles. The study of either component requires isolation (separation) of uEVs, often performed by ultracentrifugation or ultrafiltration, sometimes supplemented with further purification by size exclusion chromatography or density gradient centrifugation (Fig. 1). Other techniques include hydrostatic filtration dialysis, precipitation, immunocapture, and acoustic trapping. These techniques are not inclusive, and they are time-consuming and introduce variability among samples. Specifically in urine, uromodulin and albumin can interfere both with separation and with subsequent characterization of uEVs using proteomics and RNA sequencing [1, 2]. Recently, new techniques have been developed that circumvent these limitations by characterizing uEVs directly in whole urine. Examples of such techniques are nanoparticle tracking analysis, time-resolved fluorescence immunoassay (TRFIA), and EVQuan (Fig. 1) [2, 3]. Of note, analysis of uEVs in spot urines requires normalization to correct for concentration differences. Currently, the most appropriate method to normalize uEV count is by correction for urine creatinine (similar to the albumin-to-creatinine ratio) [2]. Other relevant considerations for uEV quantification are uEV excretion by the urinary tract (beyond the kidney) and degradation of EVs with extensive processing.

These uEVs have been shown to be a rich source of potential biomarkers for many kidney-, bladder-, and prostate-related diseases [1]. In addition, uEV cargo has been analyzed to assess tubular transport, which will be the focus of this review. Indeed, in the first proteomic analysis of uEVs already several tubular transport proteins have been identified, including sodium-potassium cotransporter 2 (NKCC2), sodium chloride cotransporter (NCC), epithelial sodium channel (ENaC), and aquaporin 2 (AQP2) [4]. Subsequent studies showed that γ-ENaC is present only in its cleaved form in uEVs, while α-ENaC is present both in its full-length and cleaved forms [5, 6]. All three isoforms of NCC are detected in uEVs [7]. It is assumed that the abundance of these transporters in uEVs mirrors the abundance in the kidney, but a direct comparison between selected transport proteins in uEVs and kidney tissue obtained from human cancer nephrectomies failed to identify...
a correlation between kidney–uEV protein abundances [8]. In a more controlled setting, however, a recent large-scale proteomics study in rats did identify significant and strong correlations between kidney and uEV protein abundances, especially for tubular transporters [9]. This finding suggests that the uEV cargo does reflect tissue pathways and tubular transporter activity but that several technical and biological aspects of uEV excretion must be considered to identify such signals. In the case of the human nephrectomy study, uEVs were isolated before surgery, and it is therefore conceivable that transporter abundances changed during surgery [10]. In addition, we recently showed that nephron mass determines uEV excretion rate, implying that a measure of nephron mass should be included when comparing tubular transporters among patients [11]. To decrease and ultimately overcome the issue of variability among uEV studies, guidelines for reporting on isolation, characterization, and normalization have been developed by EV-TRACK (etvtrack.org) and the Urinary Task Force of the International Society for Extracellular Vesicles [1, 12]. Recent reviews have covered uEV biomarkers in other kidney diseases and kidney transplantation as well as the therapeutic use of EVs [13, 14]. Here, we review which tubular transporters have been identified and characterized in uEVs, how EVs may regulate tubular transporters, and whether uEV cargo may reveal disease mechanisms and serve as biomarkers for kidney disease and hypertension.

**Tubular transporters in uEVs**

In addition to the apical transporters in uEVs reported by Pisitkun et al. [4], other apical transporters detectable in uEVs are sodium hydrogen exchanger 3 (NHE3) [15],...
FIGURE 2: Overview of the major apical tubular transporters identified in uEVs, including disease associations. uEVs are secreted by the tubular epithelial cell of all nephron segments and will therefore carry segment-specific transporters (depicted by the corresponding colors). The analysis of these tubular transport has been pursued in various disorders, including tubulopathies, hypertension, and kidney injury. The figure was created with BioRender.com.
bicarbonate and ammonium chloride reduced uEV-AQP1 [28]. This finding suggests a direct effect of acetazolamide on cells expressing AQP1.

Regulation of tubular transport by EVs

Protected by their lipid bilayer, EVs can carry proteins, microRNAs (miRNA), lipids, and other signaling molecules that neighboring and distant cells can take up. It is technically challenging to study the release of EVs by tubular epithelial cells and their subsequent effect on more distal target cells in vivo. Instead, several in vitro studies have provided important indications for EV-mediated cell–cell communication between tubular epithelial cells. The concept of a role for EVs in cell–cell communication assumes that specific molecular cargo will be loaded during altered physiology. Indeed, a proximal tubular cell line secreted more apical and basal EVs when challenged by inflammation, and both EV populations had distinct molecular profiles and functional properties [29].

Several studies have shown that miRNAs play a key role in EV-mediated cell–cell communication. One study identified 276 mature miRNAs in human uEVs and found that the predicted targets for these miRNAs were membrane transporters and their regulators [30]. Indeed, treating proximal tubular cells with human uEVs decreased messenger RNA levels of SLC38A2 and the encoded protein SNAT2, an amino acid transporter. Treating collecting duct cells with the same uEVs reduced the renal outer medullary potassium channel and its regulatory kinases, SGK1 and WNK1. The role of EVs in cell–cell signaling was demonstrated more directly in a study using a collecting duct cell line [31]. When EVs were isolated from desmopressin-treated cells and administered to its regulatory kinases, SGK1 and WNK1. The role of EVs in cell–cell communication was demonstrated more directly in a study using a collecting duct cell line [31]. When EVs were isolated from desmopressin-treated cells and administered to untreated cells, AQP2 and water transport increased in the recipient cells. To investigate the possibility of proximal-to-distal communication, Jella et al. [32] hypothesized that EVs from proximal tubule cells can regulate ENaC activity by delivery of the glycolytic enzyme GAPDH, which in turn can modify intracellular calcium signaling. Single-channel patch clamp recordings showed that the proximal EVs reduced ENaC open probability in Xenopus cells and isolated split-open tubules. This effect was blunted after applying EVs that were transfected with a GAPDH inhibitor. Another study hypothesized that EVs play a role in modifying ENaC activity through purinergic signaling [33]. To address this behavior, EVs were isolated from a proximal tubular cell line, collecting duct cell line, aldosterone-treated cells, and from urine of patients with primary aldosteronism. The proximal EVs downregulated the ectonucleoside CD39 in the collecting duct cells, likely by increasing specific miRNAs. In turn, this increased extracellular adenosine triphosphate (ATP) and downregulated α-ENaC. The effects of applying EVs from the three other sources to the collecting duct cells were opposite. These EVs decreased extracellular ATP and upregulated α-ENaC, possibly by increasing 14-3-3 proteins and ATPase activity. Together, these studies show that different types of EV cargo can alter tubular transporter status in recipient cells and possibly also tubular transport.

Disease associations

The identification of tubular transporters in uEVs raises the possibility of studying them as biomarkers for kidney disease and hypertension (Fig. 2). This hypothesis has been investigated in inherited tubulopathies, hypertension, primary aldosteronism, preeclampsia, and kidney injury.

Inherited tubulopathies

The analysis of uEVs in patients with inherited tubulopathies (here focused on renal tubular disorders of salt and water transport) serves as proof of principle to analyze whether the affected kidney transporters show the same anticipated pattern in uEVs. Indeed, in patients with Bartter syndrome type 1 and Gitelman syndrome, NKCC2 and NCC were either low or absent in uEVs, depending on whether the alteration affected stability, processing, or trafficking of the cotransporter [3, 34–36]. In patients with Gitelman syndrome, uEV analysis also reflects the changes that occur in unaffected nephron segments to compensate for salt wasting. Although NCC was absent or reduced, NHE3, β-ENaC, and pendrin were increased both in uEVs and in kidney biopsies of patients with Gitelman syndrome [37]. Conversely, uEV abundances of NCC and pNCC were increased in patients with familial hyperkalemic hypertension, a mirror image of Gitelman syndrome caused by genetic variants in NCC regulating kinases or ligases, including WNK1, WNK4, KLHL3, or cullin-3 [3, 38]. In a case study of a child with a novel CUL3 variant, uEV analysis showed reduced cullin-3 and increased NCC [39]. Compared with healthy participants, patients with inherited or acquired distal renal tubular acidosis showed reduced levels of the B1 subunit of the V-ATPase in uEVs and failed to increase its abundance after an acid load [17]. In patients with inherited nephrogenic diabetes insipidus, residual uEV-AQP2 abundance correlated well with the severity of the phenotype [40, 41]. Thus, in inherited tubulopathies, the predicted alterations in kidney transporter abundance are reflected in uEVs. To move these insights to clinical application, an uEV precipitation method was developed that allows protein analysis in frozen, small-volume (1–2 mL) urine samples. The diagnostic utility of this approach was illustrated by showing reduced NCC abundance in patients with genetically confirmed Gitelman syndrome but also in patients who had only a single SLC12A3 variant [42]. The TRFIA technique also allows uEV-transporter analysis with small-volume urine samples in patients with inherited tubulopathies and does so by combining an uEV-capture antibody and a secondary antibody against the transporter of interest [3].

Hypertension

Given the close link between kidney salt transport and blood pressure regulation, uEV analysis may provide valuable insights into salt sensitivity, diuretic responsiveness, or the presence of aldosterone excess (Table 1). In 41 patients with untreated mild hypertension, salt sensitivity was assessed after switching from a low- to a high-salt diet and defined as 10% or more increase in mean ambulatory blood pressure. Twenty-four–hour urinary sodium was effectively reduced (44 mmol/day) and increased (162 mmol/day) after the diets,
and the renin-angiotensin system was effectively suppressed on the high-salt diet. NKCC2 and NCC abundance in uEVs, however, did not correlate with salt resistance or salt sensitivity [43].

In another study that also included patients with untreated hypertension, uEV-NCC increased after treatment with hydrochlorothiazide but not valsartan, suggesting an angiotensin II–dependent mechanism [44] (Table 1). The increase in uEV-NCC was greater in patients whose blood pressure responded to hydrochlorothiazide and whose plasma potassium levels decreased, suggesting that the antihypertensive effect is related to the magnitude of compensatory NCC upregulation. In kidney transplant recipients, the calcineurin inhibitors (CNIs) cyclosporine and tacrolimus have been shown to increase NCC activation by phosphorylation and thereby contribute to CNI-induced hypertension. This effect is also reflected by a higher total NCC and pNCC abundance in uEVs [45, 46] (Table 1). In fact, higher pretreatment total NCC and pNCC abundance in uEVs predicted a better blood pressure response to thiazide diuretics in kidney transplant recipients with hypertension [46]. Together, these findings illustrate that pNCC is predominantly regulated by plasma potassium, which also explains the inverse relationships between dietary potassium intake with pNCC and blood pressure (Table 1) [23]. Although thiazide diuretics are generally well tolerated, some patients develop hyponatremia. Patients with thiazide-induced hyponatremia have lower NCC and higher AQP2 in uEVs than patients who use thiazides but do not develop hyponatremia [47]. The antidiuretic effect of thiazide diuretics appears to be mediated by prostaglandin E2 rather than vasopressin. Patients with thiazide-induced hyponatremia had a higher abundance of SLCO2A1 in uEVs, a prostaglandin transporter that is expressed in the collecting duct. It is incompletely understood how prostaglandins and SLCO2A1 increase AQP2 and thereby mediate the antidiuretic effect of thiazide diuretics. After recovery from thiazide-induced hyponatremia, AQP2 and NCC remained higher in uEVs than in thiazide and nonthiazide users without hyponatremia, suggesting that this uEV profile may predict who is at risk for thiazide-induced hyponatremia.

**Primary aldosteronism**

Primary aldosteronism is a common cause of secondary hypertension that is often accompanied by hypokalemia. An uEV analysis in primary aldosteronism is of interest because it may shed light on the complex interactions among aldosterone; plasma potassium; and the regulation of NCC, ENaC, pendrin, and prostasin. In patients with primary aldosteronism, uEV-NCC and uEV-pNCC were higher than in patients with essential hypertension (Table 1) [20], and uEV-pendrin and uEV-prostasin were reduced after treatment of primary aldosteronism [21, 22]. NCC, pNCC, and WNK4 increased in uEVs isolated from patients who received the mineralocorticoid fludrocortisone as part of the diagnostic workup for suspected primary aldosteronism [48]. Of interest, the plasma potassium concentration strongly and negatively correlated with NCC, pNCC, and WNK4 abundance in uEVs [48]. Similarly, uEV-pendrin correlated negatively with plasma potassium and positively with plasma aldosterone [49]. During co-administration of fludrocortisone and potassium chloride, uEV-pendrin decreased, suggesting that the suppressive effects of potassium outweigh stimulation by mineralocorticoids [49]. Patients with glucocorticoid excess and hypertension resulting from Cushing syndrome had higher pNKCC2, pNCC, and NCC abundances in uEVs than healthy volunteers [50]. The higher uEV sodium co-transporter abundances were specifically observed in patients who also had a suppressed renin-angiotensin system, suggesting that glucocorticoids increased sodium reabsorption through sodium co-transporters, causing hypertension with suppressed renin and aldosterone. Similar to patients with primary aldosteronism, plasma potassium correlated negatively with uEV-NCC and uEV-pNCC [50]. These studies illustrate that even in disorders with actual or apparent mineralocorticoid excess, the ‘potassium switch’ is still operative and results in lower levels of NCC and pendrin in uEVs.

**Preeclampsia**

Although the pathophysiology of preeclampsia is incompletely understood, placental disturbances are considered to cause podocyte injury with subsequent proteinuria and salt-sensitive hypertension. In 49 women with preeclampsia, nephrin levels in podocyte-derived uEVs were significantly lower compared with healthy pregnant women and correlated with the degree of proteinuria [51]. In addition, plasma levels of hemopexin were decreased in preeclampsia and correlated negatively with the ratio of podocinα1-to-nephrinα ENaCs, suggesting that podocyte injury may be the result of free fetal hemoglobin in maternal plasma. Proteinuria may directly contribute to hypertension because aberrantly filtered plasma proteases can activate ENaC through proteolytic removal of an inhibitory peptide tract in γ-ENaC or cleavage by prostasin. Women with preeclampsia had higher levels of pNKCC2, α-ENaC, and cleaved γ-ENaC in uEVs compared with healthy pregnant and nonpregnant controls [52]. In another study, however, no differences in uEV-ENaC were found between...
hypertensive and healthy pregnant women, likely because plasma aldosterone was elevated in both groups compared with nonpregnant controls [6].

Kidney injury

Before uEVs were extensively characterized, NHE3 was analyzed in ultracentrifuged urine samples isolated from patients with different forms of acute kidney injury (AKI) [15]. In patients with acute tubular necrosis compared with patients with pre-renal or intrinsic causes of AKI, uEV-NHE3 was selectively increased and outperformed fractional sodium excretion and urinary retinol-binding protein. A relative increase in uEV-NHE3 was also observed after donor nephrectomy, which was attributed to compensatory hypertrophy in the proximal tubule [11]. After 5/6 nephrectomy in rats (as a model for chronic kidney disease [CKD]), a new population of large vesicles (1000–20 000 nm, called 'large renal tubular EVs') was found both in the proximal tubules and in urine [53]. Proteomic analysis identified several proximal tubular transporters, including AQP1, SGLT1, SGLT2, V-ATPase, megalin, cubulin, Na+/K+ ATPase, SLC34A1, and SLC3A1; NHE3, however, was not identified. The authors speculated that the formation of these large renal tubular EVs may explain the loss of proximal tubular functions that can occur in CKD.

Other studies that analyzed tubular transporters in uEVs in the context of kidney injury mainly focused on aquaporins. When rats were treated with gentamicin, uEV-AQP2 decreased before the development of a urinary concentrating defect and a rise in plasma creatinine [54]. After cisplatin treatment in rats, uEV-AQP2 also decreased before a rise in plasma creatinine, while uEV-AQP1 first increased, and then decreased [55]. After bilateral and unilateral ischemia reperfusion injury in rats, AQP1 and AQP2 abundances decreased both in kidney and in uEVs, which coincided with polyuria [56]. In humans, uEV-AQP2 and, to a lesser extent, uEV-AQP1 decreased the first day after kidney transplantation, again coinciding with hypotonic polyuria and returning to control levels by day 6 [57]. In summary, in these studies, a lower uEV-AQP2 preceded or coincided with the urinary concentrating defect and sometimes also predicted more generalized nephrotoxicity. In addition, uEV-AQP1 and uEV-AQP2 were reduced in patients with CKD stage G4 and G5 compared with earlier stages of CKD and healthy participants, but no analysis of urinary concentrating ability was performed [58]. In contrast, in a rat model of nephrotic syndrome, uEV-AQP2 was decreased in the setting of reduced urine output; the authors explained this by a vasopressin escape phenomenon [59]. Although none of these studies quantified uEVs to analyze the uEV excretion rate, the uEV markers TSG101 and ALIX were analyzed in several of the studies as a proxy for uEV number [54, 56–59].

In contrast to the reduced uEV-AQP1 and uEV-AQP2 levels, uEV-TSG101 and uEV-ALIX often increased during kidney injury. A possible explanation is the occurrence of hypoxia, which increases EV excretion through hypoxia-inducible factor-1α [56]. Although the uEV excretion rate is related to nephron mass [11], it is yet unclear how uEV excretion rate is affected by different forms of acute or chronic kidney injury.

Perspectives

This review illustrates the opportunities that uEVs have provided to noninvasively study protein abundance and modifications of kidney tubular transporters in animals and humans. It is important to emphasize that uEV protein abundance is not always the same as transporter activity in the kidney. A good example is the increase in uEV-NCC during NCC inhibition with thiazide diuretics [44, 46]. Another unanswered question is whether an increase in transporter abundance in uEVs means that more transporters per uEV are excreted or simply more uEVs [10]. The uEV release rates may also differ per nephron segment and may be influenced by cell surface area, metabolism, and local osmolality. This behavior reiterates the importance of quantifying uEVs to analyze uEV excretion rate. The recent discovery that nephron mass determines the uEV excretion rate is also relevant in this regard and should be followed up by studies that investigate uEV excretion rate during the course of kidney disease [11].

An increasing number of techniques are available for uEV isolation, quantification, and characterization (Fig. 1). With this expanding arsenal, however, it has become clear that each technique leads to a different uEV yield and EV subtype isolation and may even create artefacts [2]. A critical view, therefore, could be that uEVs have failed to deliver because of these biological and technical complexities, leading to an unfavorable signal-to-noise ratio. Yet, tubular transporter profiling in uEVs has largely recapitulated physiologic regulation in the kidney despite the fact that this regulation is acute and governed by multiple factors. To fully address the untapped potential of uEVs, a better understanding of the factors that influence uEV biogenesis and excretion rate is required. Furthermore, uEV isolation and characterization techniques should be developed that are high throughput, standardized, and reproducible [1]. Whether the analysis of uEVs will find its way to clinical application remains to be seen, although our personal bias is that uEVs hold the promise to advance the analysis of kidney disease to the molecular level [60].

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

CONFLICT OF INTEREST STATEMENT

None declared.

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