Abstract

Background. Organic anion transporters (OATs) are located on either the basolateral or the apical membrane of the proximal tubule cell and mediate the absorption and secretion of various drugs and endogenous metabolites. It has been shown that cellular damage in acute kidney injury (AKI) involves three forms of injury: sublethal damage resulting in loss of cell polarity, cell death through apoptosis and necrosis. We hypothesize that cellular mistargeting of OAT proteins in AKI will change the profile of OAT proteins in urine.

Methods. Thirty AKI patients were included in the study. AKI was defined by clinical course, daily urine output, response to fluid repletion, urinary sediment, fractional excretion of sodium (FeNa) and urine osmolality. Urinary OAT1, OAT3 and OAT4 protein abundance was measured from semiquantitative immunoblots of urine membrane fraction samples (exosome) collected from patients with AKI and from control subjects.

Results. Although all patients studied reached a similar severity of renal failure measured by serum creatinine, some of them recovered from AKI with supportive care only, while others required renal replacement therapy (RRT). OAT1 and OAT3, which are normally localized in the basolateral membrane of the proximal tubule cell, were detected at low levels in urine from control subjects and were increased significantly in all patients with AKI. OAT4 protein, which is normally localized in the luminal membrane of proximal tubule cells, was present in abundance in urine of control subjects. Interestingly, in patients with AKI who eventually recovered, urinary OAT4 was found to be significantly lower than in controls, while in patients who needed RRT, it was higher than in controls.

Conclusions. We have shown that OATs are mistargeted in AKI. The urinary OAT protein profile can help us to learn about the pathophysiology of the disease and might be a marker of AKI severity. AKI patients with early reversible proximal tubular damage will have high urine OAT1 and OAT3 and low OAT4, while patients with severe AKI will have high urine OAT1, OAT3 and OAT4.

Keywords: acute kidney injury; exosome; organic anion transporter 1, 3, 4; proximal tubule; renal replacement therapy

Introduction

Cellular damage in acute kidney injury (AKI) involves three forms of injury perceived as a continuum: sublethal damage resulting in loss of cell polarity, cell death through apoptosis and necrosis/necroptosis (reviewed in [1–4]). Proximal tubules are particularly susceptible to the ischemic damage in AKI [5–9]. Ischemia disrupts the actin cytoskeleton that anchors the Na/K-ATPase pump to the basolateral membrane, allowing some of the pumps to redistribute onto the luminal membrane [10, 11] and leading to interference with normal ion transport. Recovery of function is associated with the return of Na/K-ATPase pumps to the basolateral membrane [10]. Beta-1 integrins are normally polarized to the basal cell membrane where they maintain attachment of cells to the extracellular matrix. Ischemic injury results in the redistribution of integrins to the apical membrane with consequent shedding of tubule cells [12]. The loss of epithelial polarity also leads to the loss of another basolateral membrane surface protein, the complement inhibitor, Crry, which permits activation of the alternative complement pathway resulting in the recruitment of macrophages and neutrophils [13, 14].

The human organic anion transporters (OATs) OAT1, OAT3 and OAT4 have been previously characterized [15–19]. OATs are located on either the basolateral or the apical side of the proximal tubule [20, 21] and mediate the absorption and secretion of various endogenous metabolites and drugs including non-steroidal anti-inflammatory drugs, loop and thiazide diuretics, β-lactam and sulfonamide antibiotics and antivirals [22–24]. OAT1 was suggested to be the p-aminohippurate (PAH)/decarboxylate exchanger [15–17, 25]. Both hOAT1 and hOAT3 proteins were detected in the basolateral membrane of the proximal tubules [20]. OAT4 was localized to the apical membrane of the proximal tubules cell [21]. It has been previously shown that apical plasma membrane proteins are excreted into urine through the process of exosome formation [26–31]. In this study, we measured OAT protein abundance in urinary exosome fractions. We hypothesize that cellular mistargeting of OAT proteins in AKI will change the profile of OAT proteins in urine.
Materials and methods

Study population

Thirty consecutive patients admitted to the internal medicine, surgery departments or intensive care unit and presented to nephrology consultative service due to AKI were enrolled in the study. The inclusion criteria were (i) AKI, defined as a 2-fold increase in serum creatinine in patients with normal basal renal function or 50% increase in serum creatinine from baseline concentration in patients with chronic renal insufficiency. (ii) Renal dysfunction did not improve after correction of possible renal causes. (iii) Granular and epithelial cell casts were found in urinary sediment. Exclusion criteria were (i) pre-existing advanced chronic renal failure—baseline serum creatinine >2 mg/dL, (ii) urinary output <100 mL/24 h, (iii) pre-renal azotemia, defined as renal failure that rapidly improved after volume repletion or improvement in cardiac output, (iv) obstructive uropathy identified by kidney sonography and (v) acute glomerulonephritis or acute tubulointerstitial nephritis suspected by clinical features and urinalysis. A flowchart of patient enrollment is presented in Figure 1. Renal replacement therapy (RRT) consisted of intermittent hemodialysis. Indications for RRT were refractory fluid overload, uncontrollable hyperkalemia, severe metabolic acidosis or development of symptoms associated with uremia (for example, altered mental status, bleeding diathesis or gastrointestinal symptoms).

Two control groups were used in the study: six age-matched hospitalized patients with normal kidney function and three healthy volunteers. Pooled urine of healthy volunteers was used in all runs of western blots, serving as an internal control. The study was approved by the Sheba Medical Center IRB-Helsinki Committee.

Data collection

For each AKI patient, the following data were collected: age and sex, baseline serum creatinine, 24-h urinary output, response to fluid challenge, peak serum creatinine, serum urea, serum urea-to-creatinine ratio, serum sodium, urine creatinine, urine sodium, fractional excretion of sodium (FeNa), urine osmolality, urine sediment, renal outcome (recovery/dialysis/survival) and patient outcome. Peak serum creatinine was defined as the highest serum creatinine level before RRT was started or the highest creatinine level after which kidney function improved spontaneously.

Membrane fraction preparation

Isolation of membrane fraction of urine (exosome) was performed as previously described [32, 33]. Normal volunteers and patients without urinary catheter urinated into a sterile plastic bottle. Urine was then transferred into 50-mL plastic tube. From patients with a urinary catheter, fresh urine (last 1–2 h collection) was taken into 50-mL plastic tube. One tablet of probenecid inhibitor mixture (Roche Diagnostics, Indianapolis, IN) and 3.4 mM NaN3 were added to 50 mL urine. Samples were immediately frozen and stored at −80°C until use. After defrosting and extensive vortexing, urine samples were centrifuged at 17 000 g for 15 min to remove whole cells and other debris, and the pellet was discarded. The supernatant was centrifuged at 200 000 g for 60 min using a Beckman L8-I ultracentrifuge with rotor 70Ti to obtain a membrane fraction. Pellets were resuspended in isolation solution containing 250 mmol/L of sucrose and 10 mmol/L of triethanolamine. Each sample was mixed with Laemmli buffer and heated to 95°C for 5 min.

Semi-quantitative immunoblotting of OAT proteins in urine

Twelve microliter membrane fraction samples from controls or patients with AKI were loaded onto gel. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Separated proteins were transferred electrophoretically from the gel onto polyvinylidene difluoride membrane. After a 1-h 5% milk blocking, membranes were probed overnight at 4°C with rabbit anti-OAT1 (Alpha Diagnostic International, San Antonio, TX), mouse anti-OAT3 (Abcam, Cambridge, UK), rabbit anti-OAT4 (Alpha Diagnostic International) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Membranes were washed with 0.1% Tween-20 in Tris-buffered saline and incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Jackson ImmunoResearch, Suffolk, UK) for 1 h and developed using an enhanced chemiluminescence method. To test for specificity of labeling, competition experiments were performed in which anti-OAT1 and anti-OAT4 antibodies were pre-incubated with the specific peptides (Alpha Diagnostic International) containing the target sequence for the antibody. Quantitative data were obtained by scanning the bands and measuring the band density using Photoshop Image Software. The amount of urinary OAT was normalized to GAPDH protein level.

Statistical analysis

The patients were separated into three groups: patients without AKI (6 patients), Group A—patients who had renal function recovery without RRT (17 patients) and Group B—patients who needed RRT (13 patients). The main analysis was performed using normalized values of urinary OATs. The distributions of all continuous variables were tested for normality using the Shapiro–Wilk test in subgroups separately. After that, all variables were log transformed and the distributions of the new variables were again tested for normality. The distributions of all log-transformed variables except age were much closer to normal than the distributions of the original variables. The distributions of the continuous variables were presented as median and interquartile range. All regressions and tests for the continuous variables were done using the log-transformed variables. We used unpaired t-test with Satterthwaite’s correction for unequal variance for comparisons between the groups. The relationships of OAT1, OAT3 and OAT4 with potential predictors were assessed using multiple linear regression of the log-transformed normalized OAT measure as the dependent variable and age, gender, shock, diabetes, sepsis and serum creatinine as covariates. The potential ability of the three OATs as independent indicators of RRT need was assessed using multiple logistic regression analysis with logarithm of normalized OATs as the covariates and RRT as the outcome. The distributions are graphically presented as box and whisker plots and scatterplots. All P-values are two tailed and P < 0.05 was reported as significant. All calculations were done using STATTA 10 SE.

Results

Group data

Mean age of the control group patients (patients without AKI) (n = 6) was 78.2 years and AKI patients (n = 30) 66.5 years (P = 0.12). Mean serum creatinine was 0.8 mg/dL in control group patients and 6.4 mg/dL in AKI group at the peak of AKI. According to AKI course, patients were separated into two groups: Group A—patients who recovered without RRT and Group B—patients who needed RRT. AKI patient characteristics are listed in Table 1. There were no statistically significant differences between the two groups for age, sex, admission type, in-hospital mortality rate and relevant laboratory variables concerning renal function: daily urinary output, serum creatinine,
FeNa, urine osmolality and urinalysis measured at the peak of AKI. From 17 patients who had renal function recovery without RRT, 4 patients died during hospitalization. Three of them died from sepsis and one from heart failure. Seven of 13 patients who required RRT died within 2 weeks after the beginning of dialysis—6 from sepsis and 1 from cardiogenic shock. One patient did not improve and continued RRT after discharge from the hospital. Kidney function of five additional patients improved sufficiently to stop dialysis during hospitalization.

Validation and specificity of semiquantitative immunoblot of OAT protein in urine membrane fractions

OAT band density was found to be linearly related to amounts of separated and transferred OAT protein. As shown in Figure 2, OAT1 (Figure 2A) and OAT4 (Figure 2B) proteins were detected with increasing band intensity for gel loading ranging from 5- to 20-μL membrane fraction samples. To test for specificity of the antibody, competition experiments were performed in which the antibody was pre-incubated with the antigen peptide containing the target sequence for the antibody. The band disappeared in the presence of the specific peptide of anti-OAT1 (Figure 2A) and anti-OAT4 antibodies (Figure 2B). These results confirmed that the 62-kDa molecular weight band represents OAT1 protein and the 75-kDa molecular weight band represents OAT4 protein. Anti-OAT3 antibody showed a single band of ~55 kDa, as described by the supplier (Abcam), therefore competition experiments were not performed for this antibody.

Urinary OATs protein abundance in controls, AKI patients who improved without RRT (Group A) and AKI patients who needed RRT (Group B)

The amount of OAT protein in the urine was studied by means of immunoblots of membrane fractions extracted from urine samples of controls and patients with AKI at the peak of serum creatinine. Typical blots are shown in Figure 2C. OAT1 and OAT3 proteins, which are basolateral, were detected at low levels in control samples and increased significantly in both groups of AKI patients, while the apical OAT4 was present in abundance in urine of controls and in Group B but was low in Group A patients.

Urinary OAT protein levels normalized to urinary GAPDH in controls, AKI patients who improved without RRT (Group A) and AKI patients who needed RRT (Group B)

Figure 3 presents plots of urinary OAT protein values before normalization (Figure 3A) and after normalization to GAPDH (Figure 3B) and to urine creatinine (Figure 3C). OAT1 increased 6-fold in all AKI patients compared to pooled control. OAT3 increased 8-fold in Group A and 4-fold in Group B AKI patients. OAT4 decreased 0.4-fold in Group A and increased 2.3-fold in Group B AKI patients compared to pooled control (Figure 3B).

Statistical analysis was performed using normalized values of urinary OAT ratios to pooled control after log transformation of the variables was done. The t-tests for logOAT1 were as follows: AKI versus non-AKI patients: P = 0.0022; Group A versus Group B patients: P = 0.8135. logOAT3: AKI versus non-AKI patients: P = 0.0004; Group A versus Group B patients: P = 0.8077. logOAT4: AKI versus non-AKI patients: P = 0.0062; Group A versus Group B patients: P = 0.0209.

We did not find any significant dependence of normalized OATs on gender, age, shock, diabetes, sepsis and serum creatinine (data not shown).

The ability of OATs normalized to GAPDH to separate the three groups is illustrated by Figure 4. It can be seen that OAT1 and OAT3 normalized to GAPDH separate patients with and without AKI. Logistic regression analysis leads to
full separation of the patients with and without AKI, while OAT3 (Figure 4B) makes almost perfect separation. Using combinations of OAT3 and OAT4 (or OAT1 and OAT4) as indicators of RRT necessity, we obtained sensitivity of 76.92% (69.23%) and specificity 94.12% (82.35%) for couples OAT3 and OAT4 (OAT1 and OAT4), respectively.

Urinary OAT protein levels normalized to urinary creatinine in controls, AKI patients who improved without RRT (Group A) and AKI patients who needed RRT (Group B)

The pattern of OAT protein in urine normalized to urinary creatinine concentration at the peak of AKI was similar to the pattern found with normalization to GAPDH (Figure 3C).

Statistical analysis was performed using normalized values of urinary OAT ratios to pulled control after log transformation of the variables was done.

The t-test for logOAT1 was as follows: AKI versus non-AKI patients: $P = 0.0257$; Group A versus Group B patients: $P = 0.0906$. logOAT3: AKI versus non-AKI patients: $P = 0.0000$; Group A versus Group B patients: $P = 0.7994$. logOAT4: AKI versus non-AKI patients: $P = 0.1635$; Group A versus Group B patients: $P = 0.0012$.

We also performed the analysis of OATs normalized to urine creatinine as indicators of the subgroups of AKI. The results were similar to those for OATs normalized to GAPDH (data not shown). For separation between RRT and non-RRT patients, we obtained a sensitivity 61.54% (76.92%) and specificity 94.12% (94.12%) for couples OAT1 and OAT4 (OAT3 and OAT4), respectively.

Urinary OAT profile of representative patients who recovered from AKI

Patient 1. A 51-year-old man in remission of acute myeloid leukemia was hospitalized due to sepsis. Serum creatinine in admission was 5.0 mg/dL, while his basic creatinine was 0.9 mg/dL. His urine output was 400 mL/day and he did not improve with the infusion of normal saline 2 L/day. His urine sediment showed granular brown casts, FeNa was 3.2% and urine osmolality was 332 mosmol/kg. Several days after admission, the patient’s renal function started to improve. Consecutive urine samples were measured for OATs at the peak of AKI and before discharge from the hospital, when serum creatinine dropped to 1.7 mg/dL. Figure 5A presents the profiles of urinary OATs at peak of AKI and after improvement.

Patient 2. An 81-year-old woman with a history of chronic lymphocytic leukemia was admitted to the hospital due to urosepsis. Serum creatinine, which was 1.0 mg/dL at baseline, increased gradually to 5.7 mg/dL due to septic shock and she was presented to the nephrology consultation service. Her urine output was 1300 mL/day, but she did not improve with infusion of 2 L/24 h normal saline. Her urine sediment showed granular brown casts, FeNa was 15.1% and urine osmolality was 280 mosmol/kg.
Consecutive urine samples for OATs measurement were taken at the peak of AKI and 2 weeks later, when serum creatinine decreased to 1.1 mg/dL. Urinary OATs profile at peak of AKI and after improvement is shown in Figure 5B.

Patient 3. A 20-year-old previously healthy man was hospitalized because of vomiting and oliguria after participation in a long distance running as a preparation for army service. Serum creatinine at admission was 8.5 mg/dL and serum creatine phosphokinase was 1620 U/L. He received 3 L of fluids intravenously during 6 h, however, his urine output remained ~100 mL/day. His urine sediment showed granular brown casts, FeNa was 0.5% and urine osmolality was 315 mosmol/kg. Several days after the admission, the patient’s renal function improved and he was discharged from the hospital. Consecutive urine samples for OAT measurements were taken at the peak of AKI and 2 months later, when the patient visited our nephrology outpatient clinic for follow-up. His serum creatinine was 1.1 mg/dL. Urinary OATs profile at peak of AKI and after remission is presented in Figure 5C.

In all the three patients, urinary OAT1 and OAT3 at the peak of AKI were found to be higher than control values. After improvement, urinary OAT1 and OAT3 decreased. Urinary OAT4 was lower compared to control value at the peak of AKI and rose after recovery.

Discussion

OATs are located on either the basolateral or the apical side of the proximal tubule and mediate the absorption and secretion of various drugs and endogenous metabolites. OAT1 and OAT3 are located at the basolateral membrane of proximal tubules [20], while OAT4 was detected on the apical side of the proximal tubules [21]. Previous studies showed that OAT transporters might change their distribution in ischemic kidney injury. Immunohistochemical analyses of hOAT1 in cadaveric renal allografts 1 h after reperfusion have shown altered distribution of hOAT1 to cytoplasm or reduced expression in proximal tubule cells. In rat models of ischemia/reperfusion-induced AKI decreased expression of rOAT1 and rOAT3 messenger RNA (mRNA) and protein, together with decreased Na/K-ATPase expression were found [34]. This resulted in accumulation of indoxyl sulfate, a uremic toxin transported by the OATs. Furthermore, the decrease in OAT1 and OAT3 expression correlated with reduced clearance of PAH in an animal model of ischemia–reperfusion injury [35, 36] and in patients 3–7 days after transplant [37]. These changes appeared reversible, as reperfusion resulted in eventual restoration of PAH clearance and OAT1 and OAT3 expression [35].
It was previously shown that apical plasma membrane proteins are excreted through the process of exosome formation [26–31]. Therefore, in this study, the measurement of OAT proteins abundance in urinary exosome fractions was performed. Our findings suggest that OATs change their normal distribution in AKI patients. OAT1 and OAT3, which are not present in the urine of controls, were detected in urine of AKI patients. OAT4, which is normally present in urine of control, was found to be low in AKI patients who recovered without RRT and high in patients who needed RRT. These findings suggest that OAT proteins change their localization because of general mistargeting and perhaps lack of energy in the tubular cells in AKI. Based on sequential OAT protein abundance measurement in patients with AKI, we can speculate that OATs return to their normal location when AKI resolves.

It has been found that during ischemic injury, the proximal tubule Na/K-ATPase, which is normally located at the basolateral membrane of proximal tubule cells, is partly redistributed to the apical plasma membrane in this segment [38]. Ischemic insult in rats is associated with a marked decrease in abundance of several sodium transporters along the nephron, such as Na+/H+ exchanger isoform 3 (NHE3), NaPi-II, Na/K-ATPase and NBC [39]. Although a decrease in mRNA expression was demonstrated [40–42], the reduced content of protein in acute renal failure (ARF) may also be partly non-genomic. It could be directly related to cellular injury and dysfunction occurring after severe ischemia, including disruption of the cytoskeleton, the membrane structure and function and the signaling pathways, or perhaps to loss of transporters due to sloughing of the brush border, as was suggested for NaPi-II [42]. Few works have measured urine levels of the transporters in AKI. NHE3, which is localized in the apical membrane of renal proximal tubular cells and thick ascending limb cells, was found to be increased in urine of AKI patients with acute tubular necrosis compared to controls and patients with pre-renal azotemia [29], indicating a possible increase in NHE3 release during tubular cell apoptosis/necrosis.

Our study adds important information regarding tubular response to ischemic injury. We show that patients, with similar AKI severity defined by serum creatinine and by other clinical and laboratory parameters, had a different clinical course: some of them needed RRT, while others improved spontaneously. Based on our data, we suggest that urine OAT profile at the peak of AKI might help to distinguish between the two groups. Patients who improved spontaneously had high urine OAT1 and OAT3 and low OAT4 compared to the control subjects. This finding could be explained by the translocation of basolateral OAT1 and OAT3 to apical membrane and their loss in urine, while OAT4 turns inward and hence decrease in the urine. The loss of cell polarity and mistargeting of the transporters might happen as a result of adenosine triphosphate depletion during early renal ischemia. Patients who needed RRT had high urine OAT1, OAT3 and OAT4. This finding may indicate increased exosomal OATs release due to tubular cell apoptosis, as was suggested for urinary exosomal fetuin-A elevation in AKI [30]. Using combinations of urinary OAT3 and OAT4 (or OAT1 and OAT4) as indicators of the necessity of RRT, we obtained a good sensitivity of 76.92% (69.23%) and specificity 94.12% (82.35%).

Recently, several protein biomarkers have been evaluated as non-invasive indicators of kidney injury. The examples include kidney injury molecule-1 (KIM-1), which was found to be a sensitive urinary biomarker for AKI [43], serum cystatin C [44], urinary interleukin-18 (IL-18) [45] and urinary neutrophil gelatinase-associated lipocerin (NGAL) [46]. It was shown that serum cystatin C, urine IL-18 and urine KIM-1 are indicators of established AKI, while serum cystatin C, urine NGAL, IL-18, glutathione-S-transferase-π and γ-glutathione-S-transferase are sensitive for early diagnosis of AKI [47]. Our results can complete this list with new potential candidates for estimation of AKI severity and outcome.

One of the pitfalls of the study is the normalization of specific urinary protein level. Normalizing a specific urinary protein concentration to urine creatinine takes into account differences in urinary flow rate. However, under non-steady-state conditions as AKI, the urine creatinine excretion rate changes over time and therefore results in an underestimation or overestimation of the protein excretion rate. The most accurate method suggested is to
quantify specific proteins by the collection of timed urine specimens to estimate the actual excretion rate [48]. However, the authors of this work themselves admit that this method has obvious practical limitations. Alternatively, the excretion of specific proteins, for example exosomal proteins, can be normalized by the excretion of exosomal markers such as Alix or TSG101 that are found in all exosomes [33]. We normalized urinary OAT abundance values to the housekeeping protein GAPDH, which was also found in the exosome [32], and to urine creatinine. Using normalization to urinary GAPDH and to urinary creatinine, we received a similar pattern of OAT protein distribution in AKI patients.

Although a decrease in Oat1/3 mRNA expression was demonstrated [49], based on our findings in humans, we speculate that reduced OAT1 and OAT3 protein expression in kidney tissue in ischemia–reperfusion injury that was found in rats [34–36] might be at least partly explained by loss of the transporters into urine.

PAH is an ideal tool for determining renal plasma flow in intact humans [50]. It is transferred from renal circulation to urine predominantly by active secretion by OAT1 that is located on the basolateral membrane [51]. Study performed on human renal allograft showed that there is no reduction in renal plasma flow during post-ischemic ARF [52]. The authors suggested that redistribution of Na/K-ATPase due to a loss of cell polarity could limit PAH transport [52]. In agreement with this hypothesis, our data show that mistargeting of OAT1, the PAH transporter, in AKI might also impair the active proximal secretion of PAH by the OAT.

![Fig. 5. Comparison of urinary OATs levels in three typical AKI patients at the peak of AKI and after improvement.](image-url)

*Urinary OAT protein profiles in AKI*
The loop diuretic furosemide, which is commonly used in AKI cases, gains access to its tubular site of action mainly by proximal tubular secretion. Both OAT1 and OAT3 contribute to normal renal secretion of furosemide [53]. Studies have shown that AKI patients with diuretic response to furosemide have less severe acute renal failure [54, 55]. Although we did not find differences in abundance of urinary OAT1 and OAT3 between two groups of AKI patients, we can speculate that patients who respond to furosemide have less prominent OAT proteins mistargeting compared to patients who do not respond to furosemide.

In summary, based on our finding, we suggest that OATs are mislocalized in AKI. In patients with AKI, urinary OAT protein profiles might also help to differentiate reversible proximal tubular damage from severe tubular necrosis or apoptosis. It could be interesting to investigate the effects of OAT mistargeting on the handling of various drugs in patients with AKI.

Conflict of interest statement. None declared.

References


Received for publication: 13.12.10; Accepted in revised form: 14.8.11