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Original Paper

Altered Renal Expression of Relevant Clinical Drug Transporters in Different Models of Acute Uremia in Rats. Role of Urea Levels

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Key Words
Uremic toxins • Oat1 • Oat3 • Organic anion transporters • Acute kidney injury

Abstract
Background/Aims: Organic anion transporter 1 (Oat1) and 3 (Oat3) are organic anion transporters that play critical roles in the body disposition of numerous clinically important drugs. We investigated the effects of acute uremia on the renal expression of Oat1 and Oat3 in three *in vivo* experimental models of acute kidney injury (AKI): induced by ischemia, by ureteral obstruction and by the administration of $HgCl_2$. We also evaluated the influence of urea in the expression of these transporters in proximal tubular cells suspensions. ***Methods:*** Membranes were isolated from kidneys of each experimental group and from cell suspensions incubated with different urea concentrations. Oat1 and Oat3 expressions were performed by immunoblotting. ***Results:*** A good correlation between uremia and the renal protein expression of Oat1 and Oat3 was observed *in vivo*. Moreover, the incubation of isolated proximal tubular cells with different concentrations of urea decreases protein expression of Oat1 and Oat3 in plasma membranes in a dose-dependent manner. ***Conclusion:*** The more severe the renal failure, the more important is the decrease in protein expression of the transporters in renal membranes where they are functional. The *in vitro* study demonstrates that urea accounts, at least in part, for the decreased expression of Oat1 and Oat3 in proximal tubule plasma membranes.

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Introduction

The organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs, including anti-HIV therapeutics, antitumor drugs, antibiotics, anti-hypertensives and anti-inflammatories [1, 2]. Therefore, understanding the regulation of these transporters has profound clinical significance.

Several OAT members have been cloned. These OATs are expressed in distinct tissues and cell membranes. In the kidney, organic anion transporter 1 (Oat1) and 3 (Oat3) are organic anions/α-ketoglutarate exchangers. They are involved in the energetically linked basolateral entry of organic anions into the proximal tubule cells of the kidneys. Their substrate selectivity is markedly broad. Oat1 is detected exclusively in the proximal tubules and Oat3 is localized in the proximal tubule, cortical and medullary thick ascending limb of Henle´s loop, connecting tubules, and cortical and medullary collecting ducts [1-3].

Oat1 and Oat3 are involved in the uptake process of organic anions, the rate limiting process for renal secretion. We have described modifications in the renal transport of organic anions in the presence of AKI induced by ischemia [4], by ureteral obstruction [5] and by $HgCl_2$ [6, 7]. The changes in pharmacokinetics due to altered renal elimination of drugs may have a direct and adverse effect on the therapeutic safety and efficacy of many important drugs.

Uremia is caused by small, dialyzable uremic toxins for which urea serves as a surrogate marker. In chronic renal failure, uremic toxins accumulate in the serum because of impaired renal clearance [8]. It has been described that chronic uremia is able to modify the expression and activity of different membrane transporters [9-13] being several of them implicated in the renal handling of uremic toxins [14, 15]. In AKI, acute uremia is observed; but to our knowledge there are no reports analyzing the direct effects of acute uremia on the expression and activity of different membrane transporters. The aim of the present work was to evaluate the possible influence of acute uremia and the role of urea levels in the renal expression of Oat1 and Oat3 by means of *in vivo* and *in vitro* studies. To achieve this aim we analyzed:

- the renal expression of Oat1 and Oat3 in relation to urea plasma levels in three *in vivo* experimental models of AKI.
- the direct influence of urea in the expression of Oat1 and Oat3 in proximal tubular cells suspension.

Materials and Methods

Experimental animals

Ethical approval. Animals were cared for in accordance with the principles and guidelines for the care and use of laboratory animals, recommended by the National Academy of Sciences and published by the National Institute of Health (NIH publication 7th edition revised 1996) and recommended by regulations of the local ethics committee. All experimental procedures were approved by the Faculty of Biochemical and Pharmaceutical Sciences Institutional Animal Care and Use Committee.

Animals. Male Wistar rats aged from 110-130 days old were used throughout the study. All animals were allowed free access to a standard laboratory chow and tap water, and housed in a constant temperature and humidity environment with regular light cycles (12 h) during the experiment.

Three *in vivo* experimental models of acute uremia were employed:

Ischemic acute kidney injury. Animals were anesthetized with sodium thiopental (70 mg/kg body weight, i.p.). As previously described [4], both kidneys were exposed through flank incisions and mobilized by being dissected free from the perirenal fat. Both renal pedicles were occluded with a smooth-surface vascular clip for 60 min (IR_{AKI} group, n = 4). All the studies were performed after 60 min of reperfusion. Control rats were subjected to sham operations identical to the ones used for IR_{AKI} group without occlusion of both renal pedicles.

Nephrotoxic acute kidney injury. Rats were treated with a single injection (s.c.) of $HgCl_2$ at a dose of 5 mg/kg body wt. (w/v in 1 mL saline/kg) (NT_{AKI} group, n = 4) [6]. Control rats received the vehicle alone (1 mL saline/kg). The studies were performed 18 h after the injection.

Post-renal acute kidney injury. For surgical procedures the animals were anaesthetized with sulfuric ether and ureters' ligation and release were performed as previously described [5]. The abdominal cavity was opened, and 2–0 silk ligature was placed at both proximal ureters (t=24 h) (PR_{AKI} group, n = 4). The ureteral obstruction was released after 24 h and the animals were kept alive for 24 h thereafter. After this time the studies were performed. Corresponding control groups were treated the same, except that no ureteral obstruction was performed.

As all the tested parameters for control groups were similar, we decided to consider them as one group (Sham group, n = 6) to facilitate analysis of the results.

On the day of the experiment the animals were anesthetized with sodium thiopental (70 mg/kg body wt., i.p.).

Urea determinations

On the day of the experiment, blood was withdrawn by cardiac puncture of each group of animals. Plasma samples were used to measure urea levels. Plasma urea levels were determined employing a commercial kit (Wiener Laboratory; Rosario, Argentina).

Preparation of kidney plasma membranes

Crude plasma membranes were isolated from kidneys of each experimental group as previously described in our laboratory [16]. Homogenates samples were separated for transporters abundance analyses and total protein levels. The homogenate was then centrifuged (1200 g x 15 min, 4ºC). The supernatant was aspirated and spun for 15 min at 22000 g. The supernatant was discarded and the fluffy beige upper layer of the resulting pellet, representing the crude plasma membranes, was resuspended in the remaining supernatant. Aliquots of the membranes were stored immediately at -80 ºC until use. Each preparation represents tissue from one animal. Four preparations were obtained for each experimental group. Protein quantification of samples was performed using the method of Sedmak and Grossberg [17].

Preparation of cortical tubule cell suspensions

Cortical tubule cell suspensions were prepared as described by Bertorello [18]. Briefly, after the kidneys were removed, from a different set of control animals (n = 4), the cortex was isolated, and the tissue minced on ice to a paste-like consistency. The tissue was incubated with 0.7 mg/mL collagenase in 10 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mM N-2-hydroxyethylpiperazine-NV-2-ethanesulfonic acid (HEPES) and 24 mM $NaHCO_3$ (pH 7.4). The incubation was carried out in a shaking water bath for 30 min at 37°C in an atmosphere of 95% O_2, 5% CO_2. Incubation was terminated by placing the tissue on ice and pouring through graded sieves (180, 75, 53 and 38 μm pore size) to obtain a cell suspension. The cells were washed three times by centrifugation at 400 rpm for 3 min in order to separate the remaining blood cells and traces of collagenase and were then kept on ice. Cells were resuspended to yield a protein concentration of approximately 1–2 mg/mL and were immediately used after preparation. The vast majority (approximately 80%) of the kidney cortex consists of proximal tubule cells, so the suspension obtained contains mostly proximal tubular cells [18]. Viability of the cells was tested under light microscopy after the addition of Trypan blue. Approximately 95% of the freshly isolated cells excluded the dye. Viability of the cells was maintained during a 2-h period independently of the absence or the presence of different urea concentrations. Moreover, there were no changes in the cells aspect under light microscopy before and after urea treatment. Protein quantification of samples was performed using the method of Sedmak and Grossberg [17].

Cortical tubular cell suspensions were employed to evaluate the direct effect of urea levels on Oat1 and Oat3 expression. We used this preparation because this is an excellent, physiological experimental model. Moreover, the available cell lines from renal cortical tubules do not express Oat1 and Oat3 and primary culture of renal proximal tubular cells lose the expression of these transporters, at least to our knowledge.

Incubation of cell suspensions

Urea was dissolved in the incubation media (Krebs-Henseleit buffer) prior to the addition of cell suspensions. All incubations were performed at 37°C with constant agitation and exposition to 95% O_2–5% CO_2. Cells were exposed to the following Urea concentrations in the incubation media (in g/L): 0 (U-0 group), 0.75 (U-0.75 group), 1.5 (U-1.5 group), and 3 (U-3 group). These concentrations parallel those obtained in plasma from animals of the three AKI experimental models studied. After a 2-h incubation period, cells were centrifuged, rinsed for three times with Krebs-Henseleit buffer and further used.

Plasma membranes from isolated proximal tubular cells

Cells were homogenized in buffer (300 mM mannitol, 12 mM HEPES/Tris and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) as described by Aslamkhan et al. [19]. The homogenate was centrifuged at 250 g for 15 min. The supernatant was then centrifuged at 20500 g for 20 min to collect crude plasma membranes.

Electrophoresis and immunoblotting

Immunoblotting and subsequent densitometry for Oat1, Oat3 and β-actin were performed in renal crude membranes (20 μg), cell lysates (6 μg) and in crude plasma membranes (3 μg) from proximal tubular cells as previously described [4-6, 16], using a commercial rabbit polyclonal antibody against rat Oat1 (Alpha Diagnostic International, San Antonio, TX, USA) or a non-commercial rabbit polyclonal antibody against rat Oat3 [3] or a commercial mouse monoclonal antibody against human β-actin (Alpha Diagnostic International, San Antonio, TX, USA). Immunoblotting and subsequent densitometry for alkaline phosphatase was performed in renal crude membranes (20 μg) as previously described [6] using a commercial rabbit polyclonal antibody against alkaline phosphatase (Sigma, Saint Louis, MO, USA). Blots were processed for detection using a commercial kit (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, USA). Membranes were stained with Ponceau Red to confirm equal protein loading and transfer between lanes as previously described [4-6, 16]. The abundances of Oat1 and Oat3 were normalized to β-actin and considered as percentage of the mean control value for each gel.

Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, Mo., USA) and were analytical grade pure.

Statistics

Statistical analysis was performed using an unpaired t test. When variances were not homogeneous a Welch's correction was used. $p<0.05$ was considered significant. Values are expressed as means ± S.E.M. For these analyses, GraphPad (San Diego, CA) software was used.

Results

Figure 1.A. shows a significant decrease in Oat1 protein expression in renal membranes from all AKI models as compared with Sham group. Figure 1.B. shows a significant decrease in Oat3 protein expression in renal membranes from all AKI models as compared with Sham group.

Oat1 and Oat3 protein expression in membranes of the three *in vivo* experimental models of acute uremia were plotted as a function of the respective urea plasma levels observed in each model. A good correlation was observed between urea plasma levels and Oat1 protein expression in membranes ($r= -0,99$; $p<0.05$) and, urea plasma levels and Oat3 protein expression in membranes ($r= -0,98$; $p<0.05$) (Fig. 2).

Values of alkaline phosphatase abundance in renal membranes from all AKI models were evaluated. Alkaline phosphatase abundance was not modified in NT_{AKI} group as previously described by Di Giusto et al. [6], significantly increased ($p < 0.05$) in IR_{AKI} group (207 ± 7%), and significantly decreased ($p < 0.05$) in PR_{AKI} group (75 ± 2%) as compared with Sham group (100 ± 4%). The heterogeneous changes in the abundance of Oat1, Oat3 and alkaline phosphatase observed in renal membranes from all AKI models underline the selectivity of the response.

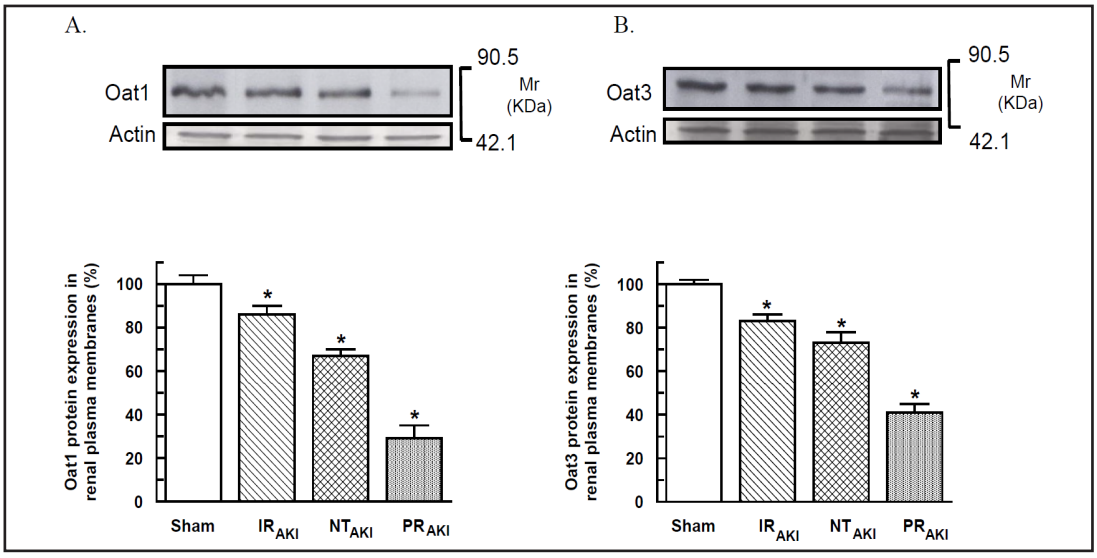

Fig. 1. Oat1 and Oat3 protein expression in renal membranes. Total plasma membranes from kidneys of Sham, IR_{AKI}, NT_{AKI} and PR_{AKI} were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (8.5 %) and blotted onto nitrocellulose membranes. Oat1 was identified using a commercial polyclonal antibody and Oat3 was identified using a non-commercial polyclonal antibody as described in Materials and Methods Section. Densitometric quantification of Oat1 (A.) and Oat3 (B.). Sham levels were set at 100%. Each column represents mean ± S.E.M. (*) $p<0.05$. Kaleidoscope Prestained Standards of molecular mass corresponding to bovine serum albumin (90.5 kDa) and to carbonic anhydrase (42.1 kDa) are indicated in the right of the Figure.

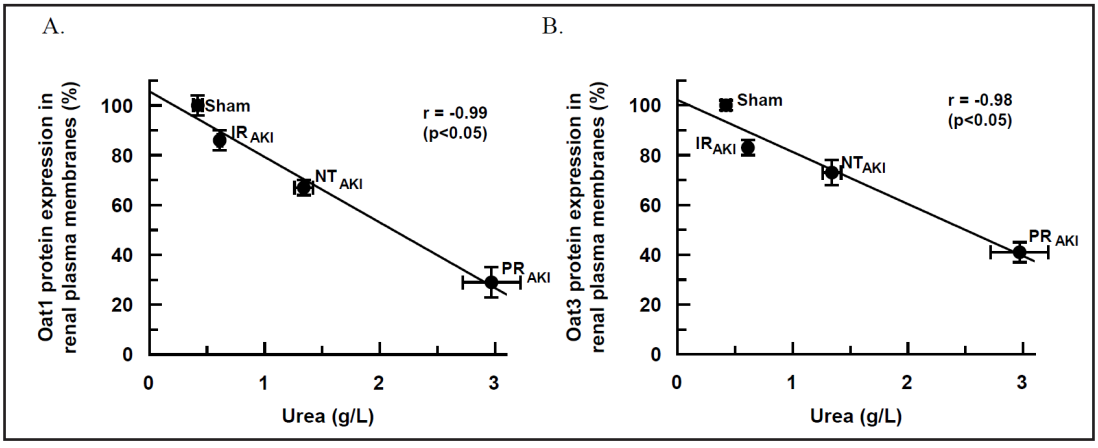

Fig. 2. A. Correlation between Oat1 protein expression in membranes and urea plasma levels in Sham, IR_{AKI}, NT_{AKI} and PR_{AKI} rats. B. Correlation between Oat3 protein expression in membranes and urea plasma levels in Sham, IR_{AKI}, NT_{AKI} and PR_{AKI} rats. Values are expressed as means ± S.E.M. $p<0.05$.

Figure 3 shows Oat1 and Oat3 protein expressions in plasma membranes from proximal tubular cells suspension incubated with different concentrations of urea. A significant decrease in Oat1 (Fig. 3.A.) and in Oat3 (Fig. 3.B.) abundance in membranes of cells incubated with 1.5 g/L (U-1.5 group) and 3 g/L (U-3 group) of urea was observed.

In order to evaluate if the down-regulation observed in plasma membranes expression of Oat1 and Oat3 is also observed in the total cellular expression of these transporters, Oat1 and Oat3 abundances were determined in whole cell lysates. There was no difference between groups in Oat1 (Fig. 4.A.) and in Oat3 abundances in whole cell lysates (Fig. 4.B).

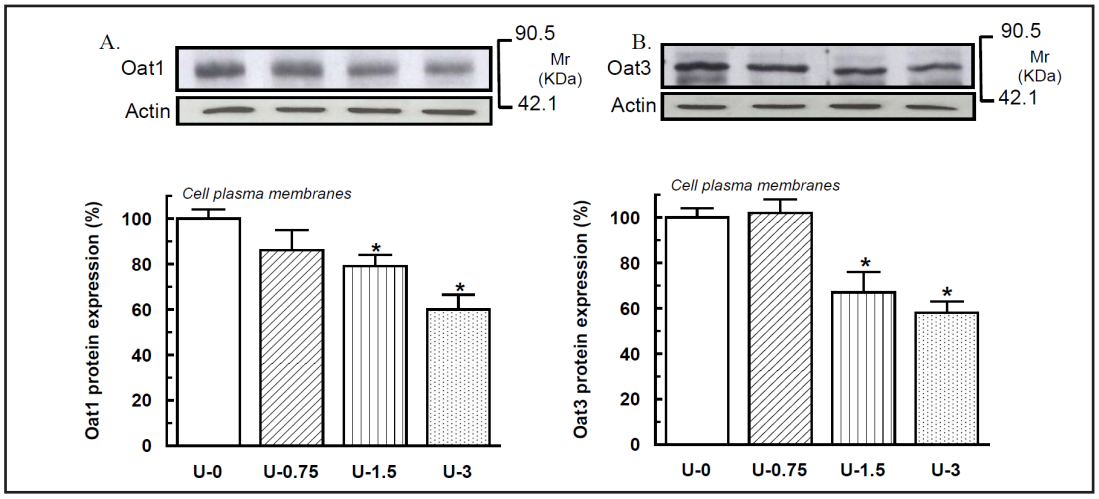

Fig. 3. Oat1 and Oat3 protein expression in plasma membranes from a cell suspension after the exposure to different Urea concentrations in the incubation media (in g/L): 0 (U-0 group), 0.75 (U-0.75 group), 1.5 (U-1.5 group), and 3 (U-3 group). Plasma membranes from proximal tubular cells were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (8.5 %) and blotted onto nitrocellulose membranes. Oat1 was identified using a commercial polyclonal antibody and Oat3 was identified using a non-commercial polyclonal antibody as described in Materials and Methods Section. Densitometric quantification of Oat1 (A.) and Oat3 (B). Sham levels were set at 100%. Each column represents mean ± S.E.M. from experiments carried out in triplicate on four different preparations for each experimental group. (*) $p<0.05$. Kaleidoscope Prestained Standards of molecular mass corresponding to bovine serum albumin (90.5 kDa) and to carbonic anhydrase (42.1 kDa) are indicated in the right of the Figure.

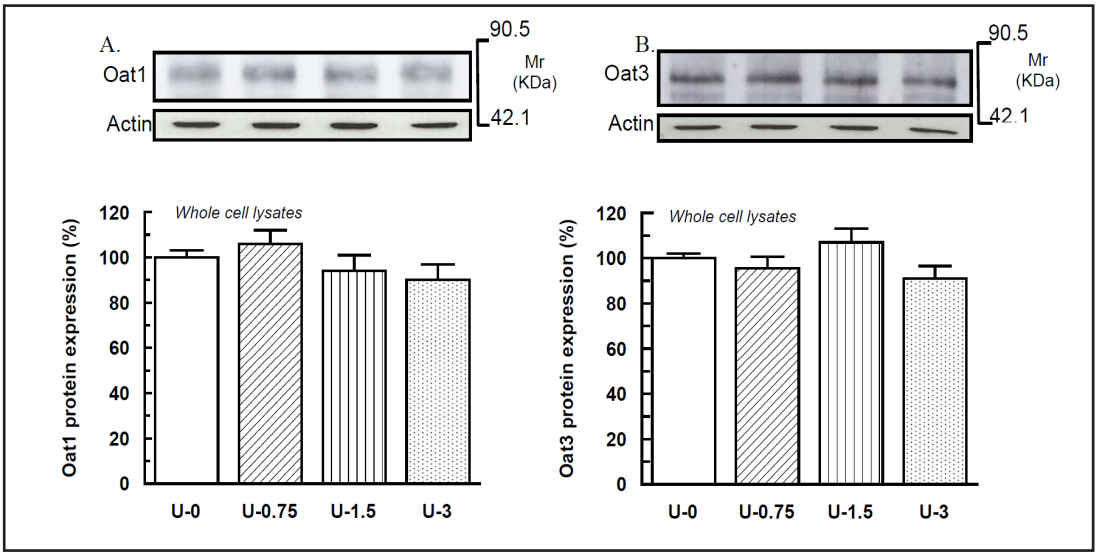

Fig. 4. Oat1 and Oat3 protein expression in whole cell lysates from a cell suspension, after the exposure to different Urea concentrations in the incubation media (in g/L): 0 (U-0 group), 0.75 (U-0.75 group), 1.5 (U-1.5 group), and 3 (U-3 group). Whole cell lysates from proximal tubule were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (8.5 %) and blotted onto nitrocellulose membranes. Oat1 was identified using a commercial polyclonal antibody and Oat3 was identified using a non-commercial polyclonal antibody as described in Materials and Methods Section. Densitometric quantification of Oat1 (A.) and Oat3 (B). Sham levels were set at 100%. Each column represents mean ± S.E.M. from experiments carried out in triplicate on four different preparations for each experimental group. Kaleidoscope Prestained Standards of molecular mass corresponding to bovine serum albumin (90.5 kDa) and to carbonic anhydrase (42.1 kDa) are indicated in the right of the Figure.

Discussion

The Oat transporter family is one of the major routes for body drug clearance/detoxification [1, 2]. Oat1 and Oat3 are both involved in the organic anion transport into the proximal tubular cell at the basolateral membrane [1-3]. Since transporters play important roles in drug disposition and elimination, it is important to pay special attention on their change in renal failure. Moreover, Oat1 and Oat3 have been implicated to some degree in the renal handling of uremic toxins [14, 15, 20].

It has been demonstrated that chronic uremia affects the expression and function of intestinal, hepatic and renal drug transporters via uremic mediators, thus altering their pharmacokinetics. In fact, interactions between uremic toxins and liver transporters, and interactions with transport proteins at the blood–brain barrier have been described [11-13]. Moreover, with the development of chronic renal failure and the subsequent accumulation of these products, renal secretion of organic anions is altered [9, 12]. Functional alterations in renal excretion of organic ions are of clinical importance, especially when drugs with a narrow therapeutic range are used. Serious renal diseases have an impact over renal disposition of a wide kind of organic ions by means of a decrease in glomerular filtration and tubular transport functions. We have reported altered elimination of organic anions in different experimental models of acute uremia in rats [4-6]. Consequently, an altered secretion adds to a poor glomerular filtration in AKI. Thus, the drug levels in blood are altered, with a greater likelihood of drug interactions, with an altered efficacy and increased probability of appearance of adverse effects. This is especially important in polymedicated patients with AKI.

Uremic toxins that accumulate in chronic renal failure may modulate transporter function, directly, by inhibiting the transport function and indirectly, by regulating the transporter expression level [11, 12, 21, 22]. To our knowledge there are no reports regarding the direct effects of acute uremia on the expression of different membrane transporters. Additionally, proximal tubular cells are also exposed to elevated concentrations of urea as a consequence of the altered renal function. In this way, urea has been usually conceived as a marker of uremic toxin removal, rather than being a toxin itself. However, it has been described that urea may modulate different protein abundances in renal cells [23-25].

At this point it was of interest to evaluate if it exits a direct influence of urea plasma levels, which are present at high and different levels in the three *in vivo* experimental models of acute uremia, mentioned above, on the renal expression of Oat1 and Oat3.

We found an important decrease of Oat1 and Oat3 protein expression in renal membranes of the three *in vivo* experimental models of acute uremia. Both transporter proteins, Oat1 and Oat3, are considered important in the excretion of many endogenous or xenobiotic solutes. Therefore, an alteration in their transport function could affect renal clearance and lead to accumulation of toxic compounds. It is important to highlight that the protein expression in renal plasma membranes is the parameter related with the function of these transporters. In fact, in these AKI *in vivo* experimental models, where there is evident renal damage, a down-regulation of Oat1 and Oat3 protein expression in renal plasma membranes was observed, and it was related to a decline in organic anions elimination as previously described [4-6].

We then studied a possible correlation between urea plasma levels and renal expression of Oat1, and urea plasma levels and renal expression of Oat3. We found a good correlation between uremia and Oat1 and Oat3 levels in renal plasma membranes. Since urea is a marker of altered uremic toxin removal, these data suggest that nitrogenous waste products might be involved in the regulation of both carriers expression in the three *in vivo* experimental models of acute uremia. In fact, urea might be involved in the regulation on the expression in membranes of both transporter proteins.

In order to evaluate the role of urea in the regulation of both Oat1 and Oat3 expression, we used a proximal tubule cell suspension incubated with solutions containing different

urea concentrations. We found decreased expression of Oat1 in membranes from proximal tubular cells while no difference was observed in whole lysates. This decrease was detected at urea concentrations of 1.5 and 3 g/L in the incubation media.

A similar pattern was observed for Oat3. We found decreased expression of Oat3 in membranes from proximal tubular cells while no difference was observed in whole lysates. This decrease was also detected at urea concentrations of 1.5 and 3 g/L in the incubation media.

These results obtained in cells parallel those obtained from *in vivo* studies. In the three AKI experimental models the expression of both transporters, Oat1 and Oat3, was decreased in renal membranes. In all these models uremic mediators, including high levels of urea, are present and might be involved in the observed alterations. In AKI, a mixture of uremic toxins is present and the combination of retention solutes can have cumulative, or even synergistic, effects on anion transport in the proximal tubule. On the other hand, from the *in vitro* study, we observed decreased expression of Oat1 and Oat3 in membranes from cells exposed at different urea concentrations demonstrating a direct role of urea in these alterations.

The decreased expressions of Oat1 and Oat3 in cell membranes without changes in whole lysates suggest an internalization of membrane transporters or an inhibition of the recruitment of preformed transporters into the membrane. Connected to this, it has been described that short-term activation of protein kinase C inhibits Oat1 and Oat3 transport activity by reducing its surface expression through enhancing the rate of Oat1 and Oat3 internalization from cell surface to intracellular compartments without affecting the total expression of the transporters [26-28]. Further more specific studies will be performed to assess if these proteins are endocytosed, to evaluate their destination compartments and to define if they are recycled to the membranes when urea levels are normalized.

The role of urea levels in the expression of other membrane transporters (located in the basolateral or in the apical side) is an interesting topic for further studies. Future tasks of importance will also be to evaluate the effects of urea levels on non-xenobiotic transporters in order to define if urea regulation is related or not to the kind of substrate transported by these carriers.

In summary, we have tried to determine whether urea levels could be responsible for the decrease in protein expression of renal transporters, Oat1 and Oat3, in the presence of acute uremia. In fact, we have demonstrated a good correlation between uremia and the protein expression of Oat1 and Oat3 in renal membranes. From this study, two main novel findings could be deduced. The more severe the renal failure (expressed as increased urea plasma levels), the more important is the decrease in protein expression of the transporters in renal membranes where they are functional. Moreover, we have demonstrated that the incubation of isolated proximal tubular cells with different concentrations of urea decreases protein expression of Oat1 and Oat3 in a dose-dependent manner. This *in vitro* study demonstrates that urea accounts, at least in part, for the down-regulation of Oat1 and Oat3 in plasma membranes observed in the three different AKI experimental models.

Because active transport processes such as those performed by Oat1 and Oat3 are particularly efficient at removing highly protein-bound solutes, and this is the same category of uremic toxins that pose problems with conventional dialysis methods, it is conceivable that, if an up-regulation of Oat1 and Oat3 expression were achieved by therapeutic manipulation, as described for other transporters [29, 30], in a dialysis patient, a window of increased uremic toxin clearance may ensued. In this connection, the additional up-regulation of apical transporters by therapeutic manipulation should contribute to the secretion into the tubular lumen of uremic toxins. Such strategies, currently studied in our laboratory, although intriguing, should be approached with caution.

Understanding the mechanisms of reduced drug clearance in the presence of uremia would be important to guide dose adjustment in patients who suffer renal injury and in polymedicated patients. Polymedication in the elderly is a common and serious problem that needs to be reviewed and evaluated continuously. Knowledge of pharmacokinetics including drug transport mechanisms could allow safer use of therapeutic agents.

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Disclosure Statement

None of the authors has competing interests with respect to any issue of the respective study.

Abbreviations

AKI (acute kidney injury); DMEM (Dulbecco's modified Eagle's medium); HEPES (N-2-hydroxyethylpiperazine-NV-2-ethanesulfonic acid); OAT (organic anion transporter family); Oat1 (Organic anion transporter 1); Oat3 (Organic anion transporter 3); PMSF (phenylmethylsulfonyl fluoride).

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