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RENAL EXPRESSION AND URINARY EXCRETION OF Na+/DICARBOXYLATE COTRANSPORTER 1 (NaDC1) IN OBSTRUCTIVE NEPHROPATHY. A candidate biomarker for this pathology.

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"RENAL EXPRESSION AND URINARY EXCRETION OF Na^+/DICARBOXYLATE COTRANSPORTER 1 (NaDC1) IN OBSTRUCTIVE NEPHROPATHY. A candidate biomarker for this pathology"

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Abstract

Obstructive nephropathy is characterized by alterations in renal function that depends on the degree and type of obstruction. To increase the knowledge about the physiopathological mechanisms involved in the renal damage associated with bilateral ureteral obstruction (BUO), we studied the renal expression and function (as urinary citrate excretion) of sodium-dependent dicarboxylate cotransporter (NaDC1) in rats. In addition, we evaluated the urinary excretion of NaDC1 as a candidate biomarker of this pathology.

Male Wistar rats underwent bilateral ureteral obstruction for 1 (BUO1), 2 (BUO2), 5 (BUO5) and 24 (BUO24) hours or sham operation. After 24 hours of ureteral releasing, traditional parameters of renal function and citrate levels were determined and NaDC1 levels were evaluated in total renal homogenates, apical plasma membranes and urine by electrophoresis and Western blotting.

Traditional parameters of renal function were only modified in BUO5 and BUO24. The renal expression of NaDC1 was decreased in BUO5 and BUO24, with a concomitant increase in urinary excretion of citrate. Moreover, the urinary excretion of NaDC1 increased after short times of ureteral obstruction (BUO1 and BUO2) and was positively correlated with the time elapsed after obstruction.

The results obtained from the renal expression of NaDC1 could explain an adaptive mechanism to prevent the formation of kidney stones by increasing the levels of citrate, a calcium chelator. The urinary excretion of NaDC1 could be postulated as an early biomarker of obstructive nephropathy that also gives information about the duration of the obstruction.

Keywords: bilateral ureteral obstruction; biomarker; citrate; NaDC1; obstructive nephropathy.

Introduction

Obstructive nephropathy is a serious and common pathology often reversible, which can occur anywhere in the urinary tract as a result of anatomical injuries, functional disorders or kidney stones. This pathology has marked effects on glomerular hemodynamic, tubular function and renal parenchyma structure [25]. After ureteral obstruction, modifications in energy and substrate metabolism in renal cells have been reported, such as a decrease in oxidative and an increase in anaerobic respiration [10]. Krebs cycle intermediates are responsible for approximately a 15 % of renal oxidative metabolism [20]. The availability of Krebs cycle intermediates has been ascribed crucial for the survival of proximal tubule cells during renal ischemia and reperfusion damage [29]. The levels of various Krebs cycle intermediates are regulated in the kidney by the sodium-dependent dicarboxylate cotransporter (NaDC1, *SLC13/A2*) [16,18].

NaDC1 is expressed in the apical membrane of proximal tubule cells. The primary function of this transporter is to reabsorb the filtered Krebs cycle intermediates such as succinate, α-ketoglutarate and citrate, which are the main energy source of the cell. Besides, the flow of dicarboxylates generated by NaDC1 is critical for the efficient function of other transporters, such as the organic anion transporter Oat5, which reabsorbs or secretes exogenous and endogenous compounds by the counter-transport of 4 and 5 carbon dicarboxylates [16]. Physiologically, NaDC1 is considered an important modulator of urinary citrate concentrations. Citrate is involved in the regulation of renal oxidative metabolism and, moreover, is a calcium chelator that inhibits the formation of kidney stones [9].

It is well-known that altered renal function, independently of the etiology, implicates complex modifications in the expression of several proteins. It has been reported that ureteral obstruction is associated with a marked decrease in the expression of several sodium transporters along the nephron, such as type 3 Na^+/H^+ exchanger, type 2 Na-Pi cotransporter, Na-K-ATPase, type 1 bumetanide sensitive Na-K-2Cl cotransporter and the thiazide-sensitive cotransporter. The downregulation of these transporters contributes to the increased sodium urinary excretion, to the urinary concentrating defect and to the postobstructive polyuria observed in this pathology [11,12].

Our group has experience in working with experimental models of obstructive nephropathy [5,26-28]. In addition, we performed the first detection of NaDC1 in urine samples [7], and in subsequent studies we demonstrated the occurrence of changes in urinary excretion and renal expression of NaDC1, under pathological conditions such as ischemia/reperfusion and extrahepatic cholestasis [4,7].

Based on the previously mentioned and considering that there is no knowledge about the alterations of NaDC1 under conditions of obstructive renal disease, the aim of this work was to study the renal expression and function of NaDC1 in rats with bilateral ureteral obstruction (BUO) of different evolution. This work contributes to increase the knowledge about the physiopathological mechanisms involved in the renal damage associated with obstructive nephropathy. Moreover, the urinary excretion of NaDC1 (NaDC1u) was also evaluated and postulated as a biomarker of the ureteral obstruction evolution time.

Methods

Materials

Reagents were acquired from Sigma-Aldrich (St. Louis, Mo., USA) and were analytical grade pure. The antibodies against Oat1 were purchased from Alpha Diagnostic International (San Antonio, TX, USA). Noncommercial polyclonal antibodies against NaDC1 were gently provided by Prof. Anzai, Department of Pharmacology, Graduate School of Medicine, Chiba University, Japan.

Experimental animals

Male Wistar rats (110-130 days old and 350-380 g body weight) were used throughout the study. The animals had free access to food and water and were kept under standard conditions of humidity and temperature, with regular light cycles (12 h) during the experiments. All animal experiments were carried out in agreement with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985). The experimental protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario (Resol. 484/2015). The surgical procedure consisted of an obstructive nephropathy model based on complete obstruction of both ureters (bilateral ureteral obstruction, BUO), as previously described [5,26-28]. The animals were weighed and anaesthetized with sodium thiopental (70 mg kg^{-1} b.w., i.p). Through incisions on both sides of the abdominal cavity, the ureters were exposed and ligated with vascular clamps. Four groups were studied:

BUO1: rats with 1 hour of ureteral obstruction (n=5)

BUO2: rats with 2 hours of ureteral obstruction (n=7)

BUO5: rats with 5 hours of ureteral obstruction (n=6)

BUO24: rats with 24 hours of ureteral obstruction (n=6)

Obstruction was considered successful when the ureteral diameter was > 2 mm and there was evident hydronephrosis. At t=0, the ureteral ligation was released and the rats were placed in metabolic cages during 24 h (t=24) in order to collect urine. Corresponding control groups (Sham groups), were treated similarly for each experimental group, except that no ureteral obstruction was performed. As no differences were observed between them in all parameters studied, we used a unified Sham group (n=11) in a similar way to that previously reported [6, 28]. The urinary volume was determined by gravimetry and expressed as µL per minute per 100 g b.w. (Vu; μL min^{-1} $100g^{-1}$). Urine samples were centrifuged at 3000 g for 10 min to remove cellular debris and the supernatant was used for all the urinary assays.

At t=24, animals were weighed and anesthetized. Blood samples were obtained by cardiac puncture. The blood was centrifuged at 3000 g for 10 min to achieve plasma separation. The kidneys were removed, decapsulated, weighted and stored at -80°C until use. Euthanasia was performed by overdose of anesthesia and subsequent thoracotomy. Figure 1 summarizes the experimental scheme.

Biochemical determinations

Blood samples were used to measure urea, creatinine (Cr_p), osmolytes and citrate plasma levels. Urine samples were used to determine creatinine (Cr_u), total proteins, glucose, alkaline phosphatase activity (AP_U), osmolytes and citrate levels.

The urea, creatinine, glucose and total proteins concentrations, as well as AP_U, were determined with optimized spectrophotometric methods employing commercial kits (Wiener Laboratory, Rosario, Argentina). The citrate levels were determined employing a citric acid enzymatic kit (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany) as previously described [4,22]. Osmolalities (Osm) was measured in a freezing point osmometer (Osmomat 030, Gonotec, Germany).

Fractional excretion of water (FE% H_2O), fractional excretion of osmolytes (FE% Osm) and fractional excretion of citrate (FE% Citrate) were calculated by conventional formulae.

In addition, the urines were used to evaluate the NaDC1 abundance by electrophoresis and Western blotting.

Preparation of total homogenates and apical membranes from kidneys

The kidneys were removed, decapsulated and minced using homogenate buffer in 30 g/100 mL proportion (ice-cold 50 mM mannitol, 5 mM EGTA, 2 mM Tris HCl buffer (pH 7.10), 1 mM phenylmethylsulfonyl fluoride (PMSF)). The preparations were homogenized for 5 min at top speed in a *Glas-Col* homogenizer, and aliquots of homogenate samples were separated, for analyses of transporter abundances and total protein levels, and were stored at -80°C until use.

Apical membranes were separated by Mg/EGTA precipitation as previously described [6,8,19]. $MgCl_2$ (12 mM, final concentration) was added to the homogenate and the mixture

was stirred for 15 min in an ice bath. The homogenate was then centrifuged (3,000 g, 15 min, 4°C). The supernatant was delicately separated and centrifuged again (28,000 g, 40 min, 4°C). The obtained pellet was mixed with experimental buffer (50 mM mannitol, 10 mM Hepes-Tris (pH 7.50), 1 mM PMSF) and centrifuged once more (800 g, 15 min, 4°C). The supernatant was finally centrifuged for 45 min at 28,000 g, and the pellet material, representing apical membranes, was resuspended in experimental buffer. Membranes were stored immediately at −80°C until use. The purity of apical membranes was ascertained through analyses of marker proteins, AP for apical membranes and the Organic Anion Transporter 1 (Oat1) for basolateral membranes. AP activities were determined in apical membranes and in homogenates using a commercial Kit (Wiener Lab, Argentina) and Oat1 abundances in apical membranes and in homogenates were assayed by electrophoresis and Western blotting as described below. The activity of AP was enriched in apical membranes approximately seven to eight times relative to that in their respective homogenates in all experimental groups studied. The enrichment of Oat1 was approximately 1.5 to 1.9 in Sham group as well as in all BUO experimental groups.

Electrophoresis and Immunoblotting

Homogenates (20 μg of protein), apical membranes (16 μg of protein), and urine (10 μL) samples from Sham and BUO rats were heated at 100°C for 3 minutes in the presence of 1% 2-mercaptoethanol and 2% SDS. Samples were applied to 8.5% polyacrylamide gel, separated by SDS-PAGE, and then electroblotted to nitrocellulose membranes. To confirm the correct transfer and verify equal protein loading, staining with Ponceau Red was used as previously reported [8,17,19]. In this connection, Romero-Calvo et al. [17] clearly demonstrated that routine quantification of Ponceau staining is validated as an alternative to

actin blotting. The nitrocellulose membranes were then incubated with 5% nonfat dry milk in PBST buffer (PBS with 0.1% Tween 20) for one hour at room temperature. After being rinsed with PBST, the membranes were incubated with non-commercial rabbit polyclonal antibodies against rat NaDC1 overnight at 4°C or with commercial rabbit polyclonal antibodies against rat Oat1. Specificity of NaDC1 antibodies has been described elsewhere [18].

Membranes were incubated for one hour at room temperature and under stirring, with a peroxidase-coupled goat antirabbit IgG (Amersham, Buckinghamshire, UK). After consecutive rinses with PBST and PBS, NaDC1 or Oat1 bands were detected by chemiluminescence using commercial kits (ECL enhanced chemiluminescence system, Pierce, Thermo Fisher Scientific, USA) and were quantified by densitometry with commercial software.

Histopathological and Immunohistochemistry Studies

A different set of experimental animals were used for histopathological studies. Kidneys were briefly perfused with saline, followed by perfusion with periodate–lysine–paraformaldehyde solution (0.01 M $NaIO_4$, 0.075 M lysine, 0.0375 M phosphate buffer, with 2% paraformaldehyde, pH 6.20) through a cannula introduced in the abdominal aorta. The kidney slices were immersed in periodate–lysine–paraformaldehyde solution at 4°C overnight. After that, they were treated with different solvents and embedded in paraffin. Then, 4 μm thick paraffin sections were cut. After deparaffining, some sections were processed for routine staining with hematoxylin-eosin and others were treated for NaDC1 immunohistochemistry. The sections were incubated with non-commercial antibodies against NaDC1 overnight at 4°C. Subsequently, the counterstain was performed with

hematoxylin and finally the sections were mounted, for further examination by conventional microscopy.

Statistical analysis

For densitometry of immunoblots, the GelPro Analyzer software (Gel-Pro Analyzer^{TM}; Media Cybernetics, Maryland, USA) was employed. Samples from obstructed kidneys were run on each gel with corresponding Sham kidneys samples and normalized to Pounceau red. Abundances of NaDC1 and Oat1 in the samples from the experimental animals were calculated as percentages of the mean Sham value for that gel. The abundance of NaDC1 in urine was normalized to urinary creatinine values as previously described [6,7,19].

Statistical analysis between the different experimental groups was performed by an analysis of variance (one-way ANOVA). When F value was significant ($P < 0.05$), the group means were analyzed using Newman Keuls test for significant differences.

To evaluate the correlation between NaDC1u and obstruction time, the correlation coefficient (r^2) was calculated. A non-linear regression adjusted to the Michaelis-Menten equation was estimated.

The results were expressed as mean ± standard error (SEM). For these analyses commercial GraphPad software was used.

Results

To characterize the experimental model, kidney weight and traditional parameters of renal function were measured. The kidney weight and the urine volume showed significant increases in BUO5 and BUO24 groups (Table 1). To evaluate possible tubular injury, total urine proteins, urine glucose, fractional excretion of water, fractional excretion of osmolytes and urine AP activities were determined. Proteins, glucose and the AP in urine were related to urinary creatinine concentrations in order to correct for variations in urine production as previously reported for urinary transporters and enzymes (5-8,19). Determinations of urinary biomarkers alone are not sufficient because normal physiological modifications in water excretion can dilute or concentrate urinary proteins and metabolites. Creatinine is eliminated in urine at relative constant velocity allowing it to be used to normalized urinary excretion of a particular compound. Total urine proteins, urine glucose, fractional excretion of water, and fractional excretion of osmolytes showed a marked and significant increase in BUO24 rats (Table 1). On the contrary, no modifications by the different time periods of ureteral obstruction as compared with sham group of rats were observed in urine AP activities as it is shown in Table 2. Urea (Fig. 2a) and creatinine (Fig. 2b) serum levels were significantly increased in BUO24 animals. Histological studies from kidneys of Sham and BUO rats (Figure 3) revealed that renal architecture was preserved in Sham, BUO1 and BUO2 groups. A marked interstitial fibrosis, characteristic of this pathology, was observed in BUO5 and BUO24 animals. Kidneys of BUO24 group showed tubular dilatation, as previously described in this experimental model [5,24,26-28]. The variations observed in both histological samples and renal function parameters indicate that

obstructive renal damage may be detected with these parameters in BUO5 and BUO24 groups.

The renal expression levels of NaDC1 were analyzed by immunoblotting techniques. The studies were performed in total homogenates and apical membranes from Sham and BUO renal tissue samples. NaDC1 levels in homogenates and in apical membranes, showed no modifications after short times of obstruction. On the contrary, BUO5 and BUO24 groups presented decreases in NaDC1 levels (Figure 4). Immunohistochemistry studies corroborated the results obtained by western blotting. NaDC1 showed an intense labeling in the apical membrane of proximal tubule cells from Sham, BUO1 and BUO2 kidneys. This labeling decreased in BUO5 and BUO24 groups (Figure 5).

Oat1 abundance, a transporter protein located in basolateral membranes of proximal tubule cells, was assayed in whole renal homogenates by immunoblotting techniques. The expression of this protein was not significantly modified after different times of ureteral obstruction as compared with Sham rats (Sham = 100 ± 3, BUO1 = 102 ± 10, BUO2 = 97 ± 3, BUO5 = 104 ± 18, BUO24 = 75 ± 6).

AP activities, an enzyme expressed in apical membranes of proximal tubule cells, were also evaluated in renal homogenates and in apical membranes by a spectrophotometric technique. As it is shown in Table 2, no statistically significant differences between experimental groups were detected for AP activity in homogenates. On the other hand, AP activity in membranes was lower in BUO24 group as compared with Sham one.

The heterogeneous changes in the activity of AP and in the abundance of Oat1 and NaDC1 detected in the renal tissue of BUO rats underline the selectivity of response.

To evaluate the function of NaDC1, fractional excretion of citrate was determined in all experimental groups. BUO5 and BUO24 rats showed a significant increase in fractional excretion of citrate compared with the other groups as it is shown in Figure 6.

NaDC1u was determined using the Western blotting technique. Densitometric quantification of NaDC1 from urine was expressed as arbitrary units relative to urinary creatinine concentration in order to correct for variations in urine production as previously described above for urine proteins, glucosuria and urine activity of AP. The results obtained are shown in Figure 7a. NaDC1u showed a significant increase in all BUO groups. Figure 7b shows the non-linear regression of NaDC1u against obstruction time in Sham and BUO rats. A good correlation between these two variables (r^2= 0.9126, $p \leq 0.05$) was obtained. As stated above, AP urinary excretion was not modified in all experimental groups and glucosuria was only increase in BUO24. Therefore, since a homogeneous pattern of alterations in these different parameters of tubular injury is not observed, the selectivity of the response for the urinary excretion of NaDC1 stands out.

Discussion

Obstructive nephropathy is an important clinical condition, of acute and frequently reversible development. It is a frequent cause of renal failure that affects both the elderly and infant population, with a wide spectrum of possible etiologies, from congenital malformations to renal calculi. Ureteral obstructions cause renal damage of varying degrees depending on the duration and severity of the disease. Furthermore, after releasing off the obstruction, consequences of the urine flow disruption might remain [10]. In our

experimental model, different rat groups were subjected to a specific time period of BUO, and the studies were performed 24 hours after the release of obstruction. The BUO experimental model used in this work has been employed in several opportunities in our laboratory and also by other authors, being validated as an appropriate experimental model of obstructive nephropathy [5,11,12,24,26-28]. The traditional parameters used to measure renal function were altered in response to renal damage after BUO. Urea and creatinine plasma levels were markedly increased in BUO24. Total urine proteins, glucosuria, fractional excretion of water and fractional excretion of osmolytes markedly increased in BUO24. The kidney weight and the urine volume significantly increased in BUO5 and BUO24 groups. In parallel, the renal architecture presented alterations which began to be observed in BUO5 group. Therefore, rats with BUO of 5 and 24 hours duration developed signs and symptoms of obstructive renal disease, similar to that described [5,11,12,24,26-28].

The pathophysiology of ureteral obstruction is multifarious and it involves complex alterations in the expression of multiple proteins [11,12,21]. Li et al. [11,12] have demonstrated a downregulation in the major sodium transporters along the nephron in experimental models of obstructive nephropathy. NaDC1 is also a sodium transporter located in the apical membrane of renal proximal tubule cells (S1, S2 and S3 segments). This carrier has a relevant function in the regulation of Krebs cycle intermediates in the kidney, which are essential for tissue repair after renal damage [16,29]. NaDC1 has substrate specificity for 4- and 5-carbon dicarboxylates (succinate, α-ketoglutarate) as well as tricarboxylates (citrate). The transport is electrogenic, coupled to the movement of three sodium per divalent dicarboxylate and highly dependent on pH [3]. In the kidney, the

functions of NaDC1 reported to have clinical significance are the regulation of urinary citrate concentrations and the generation of a gradient of dicarboxylates that allows the correct function of some renal transporters, such as the organic anion transporter Oat5 [1,16]. We have reported an upregulation of renal NaDC1 in correlation with a decrease in citrate urinary excretion in the presence of extrahepatic cholestasis [4]. On the contrary, we observed a decrease in the expression of NaDC1 in kidneys from rats with ischemic renal damage [7]. In order to increase the knowledge of physiopathological mechanisms involved in the renal injury observed in obstructive nephropathy, we evaluated the expression of NaDC1 in homogenates and in apical membranes from kidneys after different time periods of both ureters complete obstruction. When total homogenates and apical membranes were analyzed, we observed no differences in the renal expression of NaDC1 between BUO1 and BUO2 groups. On the contrary, NaDC1 showed significant decreases in both total homogenates and apical membranes from BUO5 and BUO24 animals. These results could indicate that, when the damage is more severe, the synthesis and/or degradation process of the protein may be altered. Notwithstanding, the similar decrease observed in NaDC1 protein expression both in homogenates and in apical membranes from BUO5 and BUO24 groups may also be due to the dump of this protein in the tubular lumen. This possibility is one of the reasons that encouraged us to evaluate the NaDC1 levels in urine.

To study the function of NaDC1, we evaluated the urinary excretion of one of its main substrates, citrate. The fractional excretion of this metabolite was increased in BUO5 and in BUO24. Considering that one of the main functions of NaDC1 is to reabsorb the di/tricarboxylates, the increase in urinary excretion of citrate could be a consequence of the decreased NaDC1 levels in the apical membrane of proximal tubular cell observed in the same experimental groups.

In recent years, research on biomarker panels for acute kidney injury has rapidly grown. Many of the proteins currently considered as biomarkers of obstructive nephropathy give us scarce information about the degree of damage generated in the obstructed kidney and about the elapsed time after the onset of the obstruction [2,13,23]. Urinary detection of NaDC1 was first described in a previous study of our group [7]. Thus, we also evaluated NaDC1 levels in urine from rats with obstructive nephropathy and determined if there was any grade of correlation between urinary levels of this carrier and the degree of renal damage. We observed that NaDC1u increases in all experimental groups. The fact that NaDC1u increases in BUO1 and BUO2 when traditional markers of renal function were still not modified allows us to postulate this parameter as an early biomarker of renal damage in this pathology. Moreover, it was observed that as the ureteral obstruction period increased, NaDC1u was higher. This behavior would seem to indicate the presence of a positive correlation between these two variables. Indeed, the regression analysis confirmed the positive correlation between "NaDC1u" and "obstruction time", with a high degree of significance (r^2= 0.9126; $p \leq 0.05$). In addition, when the obstruction was prolonged (BUO24), the abundance of NaDC1 in urine reached a plateau, which could be interpreted as a limit in the amount of protein that can be dumped in the urine. These results indicate that the time period of obstruction determines the amount of NaDC1 that appears in the urine. Therefore, by knowing the urinary abundance of this transporter, we could predict how long the renal obstruction lasted. It is important to remark that obstructive nephropathy is an evolving pathology in which the kidney deterioration goes ahead after obstruction release. In this sense, it has been shown that the time of obstruction release is a relevant factor in the prognosis of long-term impairment of renal function. Taking into account that the duration of the obstruction is one of the determining factors in kidney worsening,

NaDC1u could be an excellent biomarker of the time elapsed after the beginning of the obstructive insult.

The scientific approach to pathologies of high complexity should be oriented, firstly, to elucidate the cellular and molecular pathways that regulate these alterations; and secondly, to develop tools to determine the onset and magnitude of renal damage. In this connection, in this work we first reported a decrease in renal expression of NaDC1 accompanied by an increase in urinary excretion of citrate. Citrate is one of the most abundant metabolites in urine that prevents the calcium crystals precipitation, their agglomeration and growth [14,15]. As previously mentioned, obstructions of the urinary tract may be congenital or acquired. Critically ill patients, transplanted or those who undergo surgical procedures in organs adjacent to the kidney could develop some physical condition that temporarily blocks the urine flow, like kidney stones. Therefore, the increase in urinary excretion of citrate could respond to a compensatory mechanism of the organism to try to prevent any situation of urinary tract obstruction. Second, we demonstrated that NaDC1u increases before any other parameter indicating that there is a renal alteration due to a previous obstruction and we also observed the positive correlation between the NaDC1u and the time period of obstruction. This is relevant because knowing the urinary concentration of NaDC1 would allow an early detection of the obstructive nephropathy and could also provide information about the time elapsed since the pathology began in order to reverse more rapidly the renal disease. Nevertheless, it is well known that once renal damage has reached certain level, renal function will irreversibly decline independently of the presence or absence of the primary events that have initiated the process. Further studies will help us to determine if NaDC1u could also allow the identification of a threshold above which renal impairment is irreversible despite the obstruction release of the urinary ducts.

In summary, we demonstrate in the present work that:

-the renal expression of NaDC1 is decreased after bilateral ureteral obstruction, with a concomitant increase in urinary excretion of citrate. This could be an adaptive mechanism aim to prevent the formation of renal stone,

-the urinary excretion of NaDC1 increases after short times of ureteral obstruction and it is positively correlated with the time elapsed after obstruction. Thus, this parameter could be postulated as an early biomarker of obstructive nephropathy that also gives information about the duration of the obstruction.

Conflict of interest

All the authors have no conflicts of interest to declare.

Author Contribution

R.V.C. performed most of the experiments, statistical analyses and was involved in writing the manuscript. M.J.S. and E.C.N assisted with some experiments. A.B. co-directed the study and was involved in manuscript writing. A.M.T. designed and directed the study and was involved in the manuscript writing. All authors reviewed the manuscript.

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Table 1: Renal weight (Rw), urine volume (Vu), urinary total proteins, urinary glucose, fractional excretion of water (FE% H_2O) and fractional excretion of osmolytes (FE% Osm) in Sham and BUO rats.

	Sham (n=11)	**BUO1** (n=5)	**BUO2** (n=7)	**BUO5** (n=6)	**BUO24** (n=6)
Rw (g)	2.30 ± 0.03	2.39 ± 0.07 [e]	2.44 ± 0.06 [e]	2.62 ± 0.04 [a,e]	3.30 ± 0.11 [a,b,c,d]
Vu (μL min^{-1} 100g^{-1})	2.25 ± 0.24	3.23 ± 0.40 [e]	3.20 ± 0.29 [e]	4.59 ± 0.33 [a,e]	6.80 ± 1.08 [a,b,c,d]
Total proteins (g/g Cr)	0.83 ± 0.07	1.44 ± 0.13 [e]	2.01 ± 0.21 [e]	1.92 ± 0.19 [e]	5.08 ± 0.89 [a,b,c,d]
Glucose (mg/g Cr)	58 ± 21	47 ± 21 [e]	84 ± 15 [e]	62 ± 24 [e]	196 ± 49 [a,b,c,d]
FE% H_2O	0.48 ± 0.04	0.72 ± 0.06 [e]	0.79 ± 0.06 [e]	1.15 ± 0.08 [e]	11.70 ± 2.88 [a,b,c,d]
FE% Osm	1.99 ± 0.07	2.16 ± 0.14 [e]	3.08 ± 0.31 [e]	2.87 ± 0.16 [e]	15.27 ± 2.94 [a,b,c,d]

Results are expressed as means ± SEM. (Sham= sham-operated rats; BUO1= 1 hour of ureteral obstruction; BUO2= 2 hours of ureteral obstruction; BUO5= 5 hours of ureteral obstruction; BUO24= 24 hours of ureteral obstruction). Fractional excretion of water: FE% H_2O= (V_u/Cl_{Cr}) x 100. Fractional excretion of osmolytes: FE% Osm= (Osm_u x V_u/Cl_{Cr} x Osm_p) x 100. Cl_{Cr}: creatinine clearance; Osm_u: urinary osmolytes; Osm_p: plasma osmolytes. [a] $p<0.05$ vs. Sham; [b] $p<0.05$ vs. BUO1; [c] $p<0.05$ vs. BUO2; [d] $p<0.05$ vs. BUO5; [e] $p<0.05$ vs. BUO24.

Table 2: Alkaline Phosphatase activities in renal homogenates (AP_H), in apical membranes (AP_{AM}) and in urine (AP_U) from Sham and BUO rats.

	Sham (n=11)	**BUO1** (n=5)	**BUO2** (n=7)	**BUO5** (n=6)	**BUO24** (n=6)
AP_H (mUI/mg Prot.)	366 ± 118	358 ± 13	361 ± 38	347 ± 32	263 ± 12
AP_{AM} (mUI/mg Prot.)	2812 ± 245	2625 ± 252	2605 ± 98	2533 ± 209	1737 ± 140 [a]
AP_U (mUI/ mg Cr)	123 ± 17	135 ± 5	155 ± 18	128 ± 18	126 ± 26

Results are expressed as means ± SEM. (Sham= sham-operated rats; BUO1= 1 hour of ureteral obstruction; BUO2= 2 hours of ureteral obstruction; BUO5= 5 hours of ureteral obstruction; BUO24= 24 hours of ureteral obstruction). [a] $p<0.05$ vs. Sham; [b] $p<0.05$ vs. BUO1; [c] $p<0.05$ vs. BUO2; [d] $p<0.05$ vs. BUO5; [e] $p<0.05$ vs. BUO24.

Legends to Figures

Figure 1: Experimental design. Animals were randomly divided into four groups and anesthetized with sodium thiopental (70 mg kg^{-1} b.w., i.p). The surgical procedure consisted of obstructive nephropathy model based on complete obstruction of both ureters (bilateral ureteral obstruction, BUO). (a) BUO1: rats with 1 hour of ureteral obstruction (n=5), (b) BUO2: rats with 2 hours of ureteral obstruction (n=7), (c) BUO5: rats with 5 hours of ureteral obstruction (n=6) and (d) BUO24: rats with 24 hours of ureteral obstruction (n=6). For each experimental group, Sham animals were treated similarly, except that no ureteral obstruction was performed. At t=0, the ureteral obstruction was released and the rats were kept alive for 24 hours thereafter (t=24) in metabolic cages. The day of the experiment, animals were anaesthetized again and all samples were collected.

Figure 2: Urea (a) and creatinine (Cr) (b) plasma levels in Sham (n=11) and bilateral ureteral obstruction (BUO) rats, with 1 (BUO1, n=5), 2 (BUO2, n=7), 5 (BUO5, n=6) and 24 (BUO24, n=6) hours of complete ureteral obstruction. Results are expressed as means ± SEM. [a] $p<0.05$ vs. Sham; [b] $p<0.05$ vs. BUO1; [c] $p<0.05$ vs. BUO2; [d] $p<0.05$ vs. BUO5; [e] $p<0.05$ vs. BUO24.

Figure 3: Histological sections of kidneys from Sham (a), BUO1 (b), BUO2 (c), BUO5 (d) and BUO24 (e) rats. Hematoxylin-eosin staining. It is possible to observe the presence of interstitial fibrosis (arrows) in BUO5 and BUO24 groups. Cell desquamation and tubular dilatation (arrow heads) can be observed in BUO24 group. No histological alterations were observed in BUO1 and BUO2 kidneys. These pictures are representatives of typical samples obtained from four animals from each experimental group. Bars: 40 μm.

Figure 4: NaDC1 protein expression in renal tissue from Sham (n=11) and BUO rats (BUO1, n=5; BUO2, n=7; BUO5, n=6; BUO24, n=6). Total homogenates (20 μg proteins) (a) and apical membranes samples (16 μg proteins) (b) were separated by SDS-PAGE (8.5%) and blotted onto nitrocellulose membranes. NaDC1 were identified using

noncommercial polyclonal antibodies as described in Materials and Methods. Densitometric quantification of NaDC1 immunoblotting from homogenates (c) and apical membranes (d). Representaive Pounceau stained nitrocellulose membrane for homogenates (e) and for apical membranes (f). Results are expressed as means ± SEM. [a] $p<0.05$ vs. Sham; [b] $p<0.05$ vs. BUO1; [c] $p<0.05$ vs. BUO2; [d] $p<0.05$ vs. BUO5; [e] $p<0.05$ vs. BUO24. Kaleidoscope prestained standards of molecular mass (Mr) corresponding to bovine serum albumin (80,4 kDa) and to carbonic anhydrase (38,9 kDa) are indicated in *the right of the figure*.

Figure 5: Immunohistochemistry for NaDC1 in renal tissue from Sham (a), BUO1 (b), BUO2 (c), BUO5 (d) and BUO24 (e) rats. Serial sections from each rat kidney were stained using a non-commercial antibody against NaDC1. NaDC1 labeling was observed in the apical membranes in proximal tubule cells (arrows). In BUO5 and BUO24 rats, it can be seen that NaDC1 is reduced, compared with the staining observed in the proximal tubules of the other groups. These pictures are representatives of typical samples obtained from four animals from each experimental group. Bars: 40 μm.

Figure 6: Fractional excretion of citrate (FE% Cit) in Sham (n=11) and BUO rats (BUO1, n=5; BUO2, n=7; BUO5, n=6; BUO24, n=6). Fractional excretion of citrate: FE% Citrate= $(Cit_u \times V_u/Cl_{Cr} \times Cit_p) \times 100$. V_u: urinary minute volume; Cit_u: urinary citrate; Cit_p: plasma citrate; Cl_{Cr}: creatinine clearance. [a] $p<0.05$ vs. Sham; [b] $p<0.05$ vs. BUO1; [c] $p<0.05$ vs. BUO2; [d] $p<0.05$ vs. BUO5; [e] $p<0.05$ vs. BUO24.

Figure 7: (a) NaDC1u excretion from Sham and BUO rats (BUO1, n=5; BUO2, n=7; BUO5, n=6; BUO24, n=6). Urine samples (10 μL) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (8.5%) and blotted onto nitrocellulose membranes. NaDC1 were identified using noncommercial polyclonal antibodies as described in Materials and Methods. Densitometric quantification of NaDC1 (a) immunoblotting from urine was expressed as arbitrary units relative to urinary creatinine concentration in order to correct for variations in urine production. Results are expressed as means ± SEM. [a] $p<0.05$ vs. Sham; [b] $p<0.05$ vs. BUO1; [c] $p<0.05$ vs. BUO2; [d] $p<0.05$ vs.

BUO5; [e] $p<0.05$ vs. BUO24. **(b)** Correlation between NaDC1u and obstruction time. The correlation coefficient (r^2) was 0.9126 ($p \leq 0.05$, n=5), applying non-lineal regression.