CALL FOR PAPERS | Renal Solute Cotransporters and Exchangers

Differential cystine and dibasic amino acid handling after loss of function of the amino acid transporter b\(^{0,+}\)AT (Slc7a9) in mice

Andrea Di Giacopo,1* Isabel Rubio-Aliaga,1* Alessandra Cantone,2 Ferruh Artunc,3 Rexhep Rexhepaj,4 Isabelle Frey-Wagner,4 Mariona Font-Llitjós,5,7 Nicole Gehring,1 Gerti Stange,1 Isabel Jaenecke,5 Nilufar Mohebbi,1 Ellen I. Closs,2 Manuel Palacin,6 Virginia Nunes,7,8/2 Hannelore Daniel,3 Florian Lang,3 Giovambattista Capasso,2 and Carsten A. Wagner1

1Institute of Physiology-Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich, Zürich, Switzerland; 2Department of Internal Medicine, Chair of Nephrology, Second University of Naples, Naples, Italy; 3Department of Physiology, University of Tübingen, Tübingen, Germany; 4Molecular Nutrition Unit, Technical University of Munich, Freising, Germany; 5Department of Pharmacology, University Medical Center of the Johannes Gutenberg University, Mainz, Germany; 4IRB Barcelona, Department of Biochemistry and Molecular Biology, University of Barcelona and U731 CIBERER, Barcelona, Spain; 7Medical and Molecular Genetics Center, Institut d’Investigació Biomèdica de Bellvitge (IDIBELL), L’Hospitalet de Llobregat, Spain; 8Department of Physiological Sciences II, University of Barcelona, Spain; and 9U730 CIBERER, Barcelona, Spain

Submitted 19 April 2013; accepted in final form 4 October 2013


First published October 9, 2013; doi:10.1152/ajprenal.00221.2013.—

Cystinuria is an autosomal recessive disease caused by mutations in the cystine transporter Slc7a9. Cystinuria is characterized by cystine stones. Cysinuria causes 1–2% of kidney stone formation in adults and 6–8% of kidney stone formation in children. Mutations in Slc7a9 cause different types of cystinuria (6).

AMINO ACIDS ARE ESSENTIAL for the organism as building blocks of proteins and as precursors of neurotransmitters, nitric oxide, glucose, or hormones, among other functions. The interplay between different peptide and amino acid transporters is essential to ensure the delivery of these diverse structures, i.e., cationic, anionic, aromatic, and neutral amino acids to the different cells. The physiological importance of a particular transporter is most clearly revealed when dysfunction leads to disease in humans (for a review, see Ref. 6). Aminoacidurias are among the inherited diseases caused by mutations in amino acid transporters and are characterized by excessive loss of single amino acids or groups of amino acids in the urine (6, 7).

Cystinuria is an autosomal recessive disease characterized by the hyperexcretion of cystine and the dibasic amino acids arginine, lysine, and ornithine into urine and the formation of cystine stones. Cysinuria causes 1–2% of kidney stone formation in adults and 6–8% of kidney stone formation in children. Mutations in Slc3a1 (rBAT) and Slc7a9 (b\(^{0,+}\)AT) cause different types of cystinuria (6).

rBAT is the so-called heavy subunit mostly responsible for the translocation and stabilization of the heteromeric amino acid transporter at the apical membrane of epithelial cells in the renal proximal tubule and small intestine. b\(^{0,+}\)AT is the light and catalytic subunit responsible for the transport of amino acids (5, 30, 31, 45, 46). rBAT belongs to the Slc3 family together with the other heavy subunit of heteromeric transporter systems, 4F2hc (Slc3a2) (21). In vivo, b\(^{0,+}\)AT is the only light subunit known to bind to rBAT. It belongs to the Slc7 family like all other light subunits, LAT1 (Slc7a5), LAT2 (Slc7a8), y\(^{+}\)LAT1 (Slc7a7), y\(^{+}\)LAT2 (Slc7a8), Asc-1 (Slc7a10), and xCT (Slc7a11), that bind to 4F2hc to form various heteromeric transporter systems (see review, 21).

rBAT and b\(^{0,+}\)AT are linked by a disulfide bridge and form system b\(^{0,+}\)AT that mediates the exchange of extracellular dibasic amino acids; GFR; peptide transporter; SLC7A9; cystine

* A. Di Giacopo and I. Rubio-Aliaga contributed equally to this work.

Address for reprints and other correspondence: C. A. Wagner, Institute of Physiology—Zürich Center for Integrative Human Physiology (ZIHP), University of Zurich, Winterthurerstrasse 190, CH-8075 Zurich, Switzerland (e-mail: wagnerca@access.uzh.ch).

http://www.ajprenal.org

1931-857X/13 Copyright © 2013 the American Physiological Society

F1645
amino acids and cystine for intracellular neutral amino acids. This active transport has three main driving forces: the electric potential across the membrane, high intracellular concentrations of neutral amino acids, and the intracellular reduction of cystine to cysteine in the cytoplasm. System b\(^{0,+}\) is expressed in the small intestine and in the S1 and S2 segments of the proximal tubule in the kidney, where >90% of cystine reabsorption occurs (10, 15, 34). It is generally accepted that luminal system b\(^{0,+}\) together with basolateral system y\(^{+}\)LAT1 (y\(^{+}\)LAT1/4F2hc and y\(^{+}\)LAT2/4F2hc) are responsible for the transport of cationic amino acids from the intestinal or tubular lumen into the blood despite the presence of hCAT-1 in the basolateral membrane (11, 23). In nonepithelial cells, other Slc7 members, the cationic amino acid transporters CATs (SLC7A1–3) and mainly CAT-1 are the main responsible transporters for the delivery of cationic amino acids into cells. Disruption of Slc7a1 in mice leads to death 1 day after birth (32), as the delivery of arginine and ornithine into cells is crucial to maintain protein synthesis and enzymatic reactions.

Lack of apical transport of cationic amino acids in epithelial cells leads to cystinuria (6), and mutations in the basolateral transporter y\(^{+}\)LAT1, the transporter responsible for basolateral export of cationic amino acids, leads to another inborn amino-aciduria, lysinuric protein intolerance (43). Disruption of rBAT (Slc3a1) in the mouse by N-ethyl-N-nitrosourea mutagenesis leads to type I cystinuria, i.e., hyperexcretion of dibasic amino acids and cystine as well as urolithiasis (33). Targeted disruption of b\(^{0,+}\)AT in mice causes non-type I cystinuria (18). This mouse model shows massive urinary excretion of cystine, arginine, ornithine, and lysine, but only 82% of the homozygous mice developed crystalluria and 42% cystine lithiasis, suggesting that modifiers could affect the clinical outcome also observed in patients. Moreover, the incidence of other renalopathies like tubular dilatation, tubular necrosis, glomerular fibrosis, and chronic interstitial nephritis is higher in knockout mice compared with their wild-type littermates (19). The development of these mouse models has helped in the understanding of these aminoacidurias, but further characterization is needed to understand the renal and intestinal consequences of losing Slc7a9 function.

Despite the important role of rBAT/b\(^{0,+}\)AT in the intestinal absorption of cystine, arginine, lysine, and ornithine, no drastic alterations of plasma amino acid levels have been observed either in the affected patients or in these mice. Therefore, it has been suggested that the peptide transporter PEPT1 could compensate for the intestinal uptake of these amino acids by translocating di- and tripeptides containing cationic amino acids and cystine (12, 18). Nevertheless, the consequences of losing system b\(^{0,+}\) function at the molecular level of other amino acid transporters have not been studied yet. Additionally, the role of b\(^{0,+}\)AT in the kidney is not completely understood, as polymorphisms in the human SLC7A9 gene have been associated in two genome-wide association studies (GWAs) with estimated glomerular filtration rate levels and susceptibility to chronic kidney disease (8, 25). Thus we further investigated the Slc7a9 knockout model to examine the expression of different amino acid transporters and peptide transporters in the intestine and kidney and also performed amino acid transport studies. Our data demonstrate that the lack of b\(^{0,+}\) AT function in the small intestine and the kidney reduced the reabsorption of system b\(^{0,+}\) substrates whereas only minor effects on gene expression of other amino acids and peptide transporters were observed. Additionally, reduced fractional excretion of system b\(^{0,+}\) substrates indicates that transporters located in distal segments of the kidney and/or metabolic pathways may partially compensate for Slc7a9 loss of function.

MATERIALS AND METHODS

Mice. Slc7a9\(^{-/-}\) mice were generated as described previously (18). Animals were maintained at 22 ± 2°C and a 12:12-h light-dark cycle with access to tap water and a standard rodent diet ad libitum (Provimi KLBA, Kaiseraugst, Switzerland). Mice were bred on a C57BL/6 genetic background. For these experiments, Slc7a9 heterozygous animals were mated. All procedures applied throughout this study were conducted according to the Swiss animal welfare laws and guidelines for animal care and approved by the Zurich Veterinary Office (Kantonales Veterinäramt) under the reference number 03/2011. For mRNA and protein analysis, male mice (10–14 wk old) were anesthetized with ketamine-xylazine, and the kidney and the small and large intestines were removed rapidly. After freezing in liquid nitrogen, the tissues were stored at −80°C until further analysis.

mRNA analysis by semiquantitative real-time RT-PCR. Isolation of total RNA from the mouse kidney, duodenum, jejunum, ileum, and colon was performed using an RNasy Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturer’s instructions. Frozen tissues were homogenized in a mixture containing β-mercaptoethanol (Sigma, Buchs, Switzerland) and RLT-Buffer (Qiagen) with a resulting concentration of 1%. After centrifugation, DNase and RDD Buffer (Qiagen Rnase-Free DNase Set, Qiagen) were added to 200-μl aliquots of each homogenized sample. The RNA was eluted, and, after quality control and quantification with an ND-1000 Spectrophotometer (NanoDrop Technologies), each RNA sample was diluted to a final concentration of 100 ng/μl and stored at −80°C.

cDNA was synthesized from 300 ng total RNA of each extract using a TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The primers for the Slc1a4, Slc6a14, Slc6a19, Slc7a1, Slc7a2, Slc7a3, Slc7a9, Slc7a11, Slc15a1, and Slc15a2 genes were designed using Primer3 (Whitehead Institute for Biomedical Research, Steve Rozen, and Helen Skaletsky); the primers for Slc3a1, Slc3a2, Slc7a7, Slc7a8, Slc7a9, and hypoxanthine guanine phosphoribosyl transferase (HPRT) genes were designed using Primer Express software from Applied Biosystems. The sequences of the primers and probes are listed in Table S1 (all supporting information for this article is available on the journal website). Probes were labeled with the reporter dye FAM at the 5′-end and the quencher dye TAMRA at the 3′-end (Microsynth, Balgach, Switzerland). The preparation of 20-μl PCR volume was performed by blending 3 μl cDNA as a template, 25 μM of the forward and reverse primers, 5 μM of the labeled probes, 10 μl of the TaqMan Universal PCR Master Mix, and RNase-free water up to the final volume. Thermal cycles were set for denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/elongation at 60°C for 1 min with auto ramp time. All reactions were run in triplicate. The threshold was set to 0.06 to analyze the data; this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. The gene expression of the Slc members investigated was calculated in relation to hypoxanthine guanine phosphoribosyl transferase (HPRT). Relative expression ratios were calculated as 2^[(CtHPRT-Cttest gene)], where Ct represents the cycle number at the threshold 0.06.

Western blotting. PEPT1-2 (Slc15a1, -2) protein expression analyses were performed using renal brush-border membrane vesicles (BBMVs). BBMVs were prepared from male mice aged 10–14 wk by an EGTA/Mg\(^{2+}\) precipitation method, as described previously (2).
BBMV protein (5–10 μg) was solubilized in 3% Laemmli buffer (100 mM Tris, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT). SDS-PAGE was performed on a 10% polyacrylamide gel. Proteins were transferred electrophoretically from gels to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) using a wet transfer system (Mini Trans-Blot Cell, Bio-Rad Laboratories, Hercules, CA). After blocking with 5% skim milk powder in Tris-buffered saline containing 0.1% Tween 20 for 60 min, the blots were incubated with the respective primary antibodies [rabbit polyclonal anti-rat PEPT1 IgG, 1:5,000 (29); rabbit polyclonal anti-mouse PEPT2 IgG, 1:5,000 (29); and monoclonal anti-mouse β-ACTIN (1:5,000, Sigma, Buchs, Switzerland)] overnight at 4°C. After washing and subsequent blocking, blots were incubated with the corresponding secondary antibody (goat anti-rabbit antibody, 1:5,000, linked to alkaline phosphatase, Promega, Madison, WI) and mouse antibody linked to alkaline phosphatase (1:5,000, Promega, Madison, WI) for 1 h at room temperature. After washing and 5-min incubation in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5), antibody detection was performed with CDP-Star (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s instructions. Measurements were performed with the Las-4000 Imaging Analysis System (Fujifilm Medical Systems), and the images were analyzed with Advanced Image Data Analyzer software (Raytest) to calculate the ratio/intensity of the protein of interest/intensity of actin.

Clearance experiments. The glomerular filtration rate (GFR) was measured by inulin clearance. A thiotubatbarbital sodium (Inactin, Sigma-Aldrich) intraperitoneal injection (120 mg/kg body wt) was given as an anesthetic. The animals were then surgically prepared as follows. After a tracheotomy, the left carotid artery and left jugular vein were cannulated with polyethylene (PE) tubing. The arterial catheter was then connected to a pressure transducer to monitor blood pressure and to take blood samples, while the venous catheter was connected to a syringe pump for saline infusion. After replacement of surgical fluid loss with isotonic saline, the mice were given a priming dose of 10 μCi of [methoxy-1H]-inulin, followed by a maintenance infusion in isotonic saline containing 10 μCi/ml at a rate of 0.4 ml/h. The bladder was cannulated with a PE-50 tube for urine collection. After a 60-min balance period, 30-min urine samples were collected. Blood samples were taken after each clearance period. A total of four urine and blood collections were made. GFR was calculated by standard methods.

Amino acid analysis. Urine and plasma samples for the different experiments were collected individually and immediately frozen. Thawed urine samples were diluted with a Li-citrate sample dilution buffer, pH 2.20 (1 vol urine:3 vol buffer or 1 vol urine:9 vol buffer in case of high amino acid concentration). Plasma samples were also diluted with the Li-citrate sample dilution buffer, pH 2.20 (1 vol plasma:4 vol buffer). Samples were filtered through centrifugal filter devices with 3-kDa molecular weight cut-off (Nanosep, Pall, East Hills, NY) before analysis, and the injection volume was 75 μl. Amino acids were separated by ion-exchange chromatography with a Biochrom BC30 amino acid analyzer (Biochrom, Cambridge, UK) employing a Li-citrate buffer system and ninhydrine post-column detection. Creatinine concentrations in urine were determined with the Creatinine Liquicolor Jaffé reagent (Rolf Greiner BioChemica, Flacht, Germany) according to the manufacturer’s instructions.

Ussing chamber technique. Ussing chamber experiments were performed as previously described (37). For analysis of electrogentic intestinal amino acid transport, proximal (10–15 cm post-pylorus) jejunal segments were mounted into a custom-made mini-Ussing chamber with an opening of 0.00769 cm². Under control conditions, the serosal and the luminal perfusate for jejunal experiments contained (in mM) 115 NaCl, 2 KCl, 1 MgCl₂, 1.25 CaCl₂, 0.43 KH₂PO₄, 1.6 K₂HPO₄, 5 Na pyruvic acid, 25 NaHCO₃, and 20 mannitol (pH 7.4, NaOH). In some experiments, sodium was replaced by choline in equimolar ratios. Where indicated, the respective amino acid at the indicated concentration was added to the luminal perfusate at the expense of mannitol. In all Ussing chamber experiments, the transepithelial potential difference (Vt) was determined continuously and transepithelial resistance (Rt) was estimated from the voltage deflections (ΔVt) elicited by imposing test currents of 1 μA (37). The resulting Rt was calculated according to Ohm’s law. The resistance of the empty chamber was subtracted from Rt. By means of Rt and Vt, the resulting current was calculated in the presence of luminal mannitol or glucose according to Ohm’s law. The resulting amino acid-induced current was the difference of the calculated current in the presence and absence of amino acids.

Uptake studies in renal and intestinal BBMVs. Renal and jejunal BBMVs were prepared from male mice aged 10–14 wk by an EGTA/Mg²⁺ precipitation method, as described previously (2). Final pellets were suspended in buffer comprising 20 mM HEPES and 300 mM mannitol and adjusted to pH 7.4 with Tris. Single data points correspond to uptake experiments under the described conditions performed in BBMVs isolated from one mouse. Each experiment was performed at least in duplicate.

To assess the transport sensitive to membrane potential, BBMVs were preloaded with 10 mM potassium gluconate and amino acid uptake was measured in the presence of 1% (vol/vol) valinomycin.

Uptake was measured at 37°C after 1- and 120-min (equilibrium) incubation with either 0.1 mM L-[35S]-cystine (PerkinElmer, Schwarzenbach, Switzerland), 1 mM L-[1H]-arginine (PerkinElmer), 1 mM L-[3H]-lysine (PerkinElmer), or 1 mM L-14Cglutamine (PerkinElmer) in the presence of 100 mM NaCl or 100 mM KCl. L-Cystine uptake was measured in the presence of 1 mM diamiode to guarantee its stability. Additionally, to assess the influence of neutral amino acids on the uptake, BBMVs were preloaded with either 10 mM L-phenylalanine or L-glutamine. Data were analyzed by an ANOVA model using genotype, presence of Na⁺ gradient, and preincubation with valinomycin or neutral amino acids as factors and considering their interaction and the effect of the interindividual variation between animals.

Statistical analysis. Statistical analysis was performed using R 2.15.2 (42). Three-way ANOVA followed by multiple comparison analysis, the Mann-Whitney U-test, or Student’s t-test were used as indicated to test statistical significance. Multiple comparison analysis was performed using the library nlims and the contrast library (26, 36). Statistical significance was considered at P values <0.05. Data are represented as means ± SE. Graphical representation of the data was made with the lattice (39) and the sciplot (28) library and Adobe Photoshop CS6 Extended (Adobe Systems).

RESULTS

Amino acids in plasma and urine in mice after loss of system b₀⁺ function. Slc7a9⁻/⁻ animals were generated as described previously (18) and backcrossed to a C57BL6/J genetic background. These mice showed a similar urinary amino acid profile to the one previously observed in a mixed genetic background 129Ola-C57BL/6 (18), i.e., a drastic hyperexcretion of cystine, lysine, arginine, and ornithine and a slight hyperexcretion of glutamine/glutamate compared with their wild-type counterparts (Fig. 1A). In contrast, few alterations could be observed in the plasma amino acid profile; glutamine/glutamate, histidine, and serine were slightly increased in plasma compared with wild-type littermates (Fig. 1B). This difference was not observed in the previous analysis (18) and could be due to the different genetic backgrounds or environmental conditions.

Intestinal phenotyping of Slc7a9⁻/⁻ mice. Although few alterations were observed in plasma amino acids levels, we next tested whether electrogenic transport of dibasic amino acids was changed in the small intestine after knocking down
Slc7a9. The transepithelial electrogenic transport of l-lysine, l-arginine, and dl-ornithine was assessed using the Ussing chamber technique in jejunal sections, a site of high expression of system b0,+ (15). The induced currents after addition of l-lysine in the presence of Na⁺ were almost abolished in Slc7a9⁻/⁻ mice compared with their wild-type counterparts (Fig. 2A). However, l-arginine-induced currents were reduced only by half in Slc7a9⁻/⁻ mice (Fig. 2B), and the ornithine-induced currents were similar between genotypes (Fig. 2C). The electrogenic transport measurement suggests that arginine and ornithine are transported via other routes apart from system b0,+ in the intestine when the light subunit b0,+AT is not expressed. In the absence of a Na⁺ gradient, no significant differences between genotypes were observed for l-lysine-induced currents. Na⁺-independent l-arginine-induced currents tended to be lower (by 60%) and Na⁺-independent dl-ornithine-induced currents tended to be higher (by 60%) in Slc7a9⁻/⁻ mice than in their wild-type counterparts, a difference, however, not reaching statistical significance.

Since Ussing chamber experiments do not allow distinguishing between transport processes occurring at the apical and basolateral membrane, we performed uptake studies in jejunal BBMVs to analyze only apical transport activities. The uptake of l-lysine was diminished by 75% in BBMVs from Slc7a9⁻/⁻ mice compared with wild-type mice (in the presence of a Na⁺ gradient and preincubation with valinomycin to impose a potential difference and glutamine to allow the exchanger activity of system b0,+ (Fig. 2D). Both the presence of a Na⁺ gradient and preincubation with glutamine led to a stimulation of l-lysine uptake in the wild-type but not in knockout mice. In contrast, no significant difference was observed in BBMV l-arginine uptake between genotypes (in the presence of a Na⁺ gradient and preincubation with glutamine) (Fig. 2E).

Next, we investigated the expression of the heavy subunits rBAT (Slc3a1) of heteromeric system b0,+. As shown in Fig. 3A, no differences in rBAT mRNA expression between genotypes were observed in any intestinal section. Similar results were obtained in investigating the second heavy chain 4F2hc (Slc3a2) (Fig. 3B). 4F2hc forms complexes with the light subunits y⁺LAT1 (SLC7A7) and LAT2 (SLC7A8) to build system y⁺L and system L, respectively. All three subunits are highly expressed in the small intestine (15). LAT2 showed no differences in expression between the two genotypes (Fig. 3G). A slight reduction in the expression of y⁺LAT1 in the small intestine was observed, but this decrease was statistically significant only in the ileum (Fig. 3F). This slight decrease may suggest a reduced transport of cationic amino acids to plasma to compensate for the reduced uptake at the apical membrane of enterocytes. ATB⁰⁺ (SLC6A14) is responsible for the reabsorption of cationic and neutral amino acids and β-alanine in the colon (5). Here, we found that it is also expressed in the ileum but not detectable in the duodenum (Fig. 3H). However, neither in the ileum nor in the colon could differences between the genotypes be observed. System y⁺ is important for the absorption of cationic amino acids in nonepithelial cells. Therefore, here we investigated whether differences in the intestinal expression of CAT-1 (Slc7a1), CAT-2 (Slc7a2), and CAT-3 (Slc7a3) can be observed. Expression of CAT-1 in the intestine is similar between genotypes (Fig. 3C). In the duodenum, CAT-2B expression was significantly reduced in the knockout animals compared with wild-type mice (Fig. 3D). Although to our knowledge it has not been previously observed in the human intestine (44), we could detect low-expression CAT-3 in the three intestinal segments. A similar CAT-3 expression pattern was found in all intestinal segments in both genotypes (Fig. 3E).
System b^0,+ is an exchanger which transports cationic amino acids and cystine into the enterocyte in exchange for cytoplasmic neutral amino acids. Therefore, we next looked for the expression of the major neutral amino acid transporter in the apical membrane of enterocytes, B^0AT1 (Slc6a19). We could observe a trend toward higher mRNA expression in the ileum of Slc7a9^{-/-} mice compared with wild-type animals (44% higher) (Fig. 3I). However, at the protein level no differences between genotypes could be observed (data not shown). Differences in B^0AT1 mRNA expression levels between genotypes were not observed in the jejunum or duodenum either. Last, we investigated the gene expression of the peptide transporter Pept1 (Slc15a1), which has been suggested to compensate for the loss of function of amino acid transporters in diseases like Hartnup or cystinuria (12). However, no differences between genotypes could be found in any intestinal section (Fig. 3J).

**Uptake of cystine and cationic amino acids in renal BBMV.** Next, we looked at the epithelial cells in the kidney. Using renal BBMV, we observed that the uptake of L-cystine is diminished by 70% in BBMV from Slc7a9^{-/-} mice compared with wild-type mice (in the presence of a Na^+ gradient and preincubation with valinomycin) to impose a potential difference (Fig. 4A). Addition of 1 mM diamide to the uptake buffer ensured the measurement of only L-cystine uptake. The presence of a Na^+ gradient slightly stimulated L-cystine uptake in wild-type but not in knockout mice. In the knockout animals, the measurements were comparable in all conditions, indicating that L-cystine uptake measured under these different conditions is solely due to the presence of SLC7A9.

Similar results were observed for arginine (Fig. 4B), another substrate of system b^0,+. L-Arginine uptake was diminished by half compared with wild-type animals (in the presence of a Na^+ gradient and preincubation with valinomycin) to impose a potential difference (Fig. 4B). Addition of 1 mM diamide to the uptake buffer ensured the measurement of only L-arginine uptake. The presence of a Na^+ gradient slightly stimulated L-arginine uptake in wild-type but not in knockout mice. In the knockout animals, the measurements were comparable in all conditions, indicating that L-arginine uptake measured under these different conditions is solely due to the presence of SLC7A9.

Furthermore, a slight stimulation of L-arginine transport was observed in jejunal brush border membrane vesicles (BBMVs). A: current induced by addition of 20 mM L-lysine to the lumen perfusate was almost abolished in the Slc7a9^{-/-} mice jejunal sections compared with their wild-type counterparts (in the presence of Na^+). No difference between genotypes was observed in the absence of Na^+. In wild-type animals, the current induced by the presence of L-lysine was significantly increased by adding Na^+ to the perfusate. **p < 0.001, differences between genotypes. ##p < 0.001, differences between the absence and presence of Na^+ using the Mann-Whitney test. B: current induced by addition of 20 mM L-arginine to the lumen perfusate was significantly reduced by half in the Slc7a9^{-/-} mice jejunal sections compared with their wild-type counterparts (in the presence of Na^+). No significant differences were observed between genotypes in the absence of Na^+. **p < 0.05 for differences between the genotypes using the Mann-Whitney test. C: addition of 20 mM DL-ornithine to the lumen perfusate induced similar currents in Slc7a9^{-/-} and wild-type jejunal sections in the presence and absence of Na^+. No significant differences were observed between genotypes in the absence of Na^+. *p < 0.05 for differences between the genotypes using the Mann-Whitney test. D: uptake of 1 mM L-[3H]lysine after 1-min incubation time in the presence and the absence of a Na^+ gradient and with and without preloading with glutamine (10 mM). Uptake was measured in the presence of 1% (vol/vol) valinomycin. ANOVA and multiple comparison analysis results are depicted as **p < 0.01, difference between genotypes; §p < 0.05, difference between preloading conditions; ##p < 0.01, difference between the presence and absence of Na^+ gradient. E: uptake of 1 mM L-[3H]arginine after 1-min incubation time in the presence and the absence of a Na^+ gradient and with and without preloading with glutamine (10 mM). Uptake was measured in the presence of 1% (vol/vol) valinomycin. Values are means ± SE; n = 4–8 animals/group. Values for wild-type animals are depicted in gray and white for Slc7a9^{-/-} mice.

**Fig. 2.** Characterization of the loss of function of the mouse light subunit b^0, AT (Slc7a9) in the intestine using the Ussing chamber technique and uptake studies in jejunal brush border membrane vesicles (BBMVs). A: current induced by addition of 20 mM L-lysine to the lumen perfusate was almost abolished in the Slc7a9^{-/-} mice jejunal sections compared with their wild-type counterparts (in the presence of Na^+). No difference between genotypes was observed in the absence of Na^+. In wild-type animals, the current induced by the presence of L-lysine was significantly increased by adding Na^+ to the perfusate. **p < 0.001, differences between genotypes. ##p < 0.001, differences between the absence and presence of Na^+ using the Mann-Whitney test. B: current induced by addition of 20 mM L-arginine to the lumen perfusate was significantly reduced by half in the Slc7a9^{-/-} mice jejunal sections compared with their wild-type counterparts (in the presence of Na^+). No significant differences were observed between genotypes in the absence of Na^+. **p < 0.05 for differences between the genotypes using the Mann-Whitney test. C: addition of 20 mM DL-ornithine to the lumen perfusate induced similar currents in Slc7a9^{-/-} and wild-type jejunal sections in the presence and absence of Na^+. No significant differences were observed between genotypes in the absence of Na^+. *p < 0.05 for differences between the genotypes using the Mann-Whitney test. D: uptake of 1 mM L-[3H]lysine after 1-min incubation time in the presence and the absence of a Na^+ gradient and with and without preloading with glutamine (10 mM). Uptake was measured in the presence of 1% (vol/vol) valinomycin. ANOVA and multiple comparison analysis results are depicted as **p < 0.01, difference between genotypes; §p < 0.05, difference between preloading conditions; ##p < 0.01, difference between the presence and absence of Na^+ gradient. E: uptake of 1 mM L-[3H]arginine after 1-min incubation time in the presence and the absence of a Na^+ gradient and with and without preloading with glutamine (10 mM). Uptake was measured in the presence of 1% (vol/vol) valinomycin. Values are means ± SE; n = 4–8 animals/group. Values for wild-type animals are depicted in gray and white for Slc7a9^{-/-} mice.
observed in the wild-type animals in the presence of a Na⁺ gradient. Moreover, as system B⁰,⁰⁺ expressed in heterologous cell systems functions as an obligatory exchanger for cystine and cationic amino acids with neutral amino acids (9, 35), we investigated here the effect of preloading BBMVs with high concentrations of neutral amino acids in the intravesicular space on arginine uptake. Both preincubation of BBMVs with 10 mM L-phenylalanine and 10 mM L-glutamine led to stimulation of L-arginine uptake in wild-type mice in the presence and absence of a Na⁺ gradient. In the knockout animals, the measurements were comparable in all conditions, indicating that L-arginine uptake measured under these different conditions is solely due to the presence of SLC7A9. Thus the exchanger activity by neutral amino acid observed in L-arginine uptake is completely abolished in the renal epithelial cells in the absence of SLC7A9 protein.

In contrast, uptake of L-lysine was diminished by 70% in BBMVs from Slc7a9⁻/⁻ mice only after preincubation of the BBMVs with 10 mM L-glutamine compared with wild-type mice (in the presence of valinomycin to impose a potential difference) (Fig. 4C). This difference in transport activity was observed in the presence and absence of a Na⁺ gradient. In wild-type animals, preincubation of BBMVs with 10 mM L-glutamine led to stimulation of L-arginine uptake in the presence and absence of a Na⁺ gradient. Moreover, stimulation of L-lysine transport was observed in the wild-type animals in the presence of a Na⁺ gradient.

As previously mentioned (Fig. 1A and Ref. 18), glutamine excretion in urine is increased in Slc7a9⁻/⁻ mice. Therefore, we looked for glutamine uptake in the BBMVs. No difference was observed for L-glutamine uptake between genotypes. The presence of a Na⁺ gradient stimulated L-glutamine uptake to a similar extent in both genotypes (Fig. 4D).

Renal function in Slc7a9⁻/⁻ mice. To further assess renal amino acid transport in Slc7a9⁻/⁻ mice, we performed clearance experiments. The GFR was calculated based on the measured inulin clearance. As shown in Fig. 5A, insert, Slc7a9⁻/⁻ mice showed a reduced GFR of ~30% compared with wild-type animals. No differences could be observed between the genotypes for ammonium in plasma and urine as well as plasma urea levels (Fig. 5, B and C). Next, the fractional excretion of amino acids was estimated using the plasma and urine amino acid concentrations (Fig. 1, A and B), and the GFR values were calculated by using inulin. For most amino acids, no differences between genotypes could be detected, and the fractional excretion values ranged between 0.5 and 2.5%. As expected, cystine, arginine, ornithine, and lysine showed different fractional excretion values between genotypes (Fig. 5A). Arginine was almost completely excreted in Slc7a9⁺/⁺ mice compared with wild-type animals. However, lysine showed ~35% fractional excretion, and the least affected amino acids were ornithine and cystine, showing 16 and 11% excretion, respectively. Those amino acids were almost completely reabsorbed in wild-type animals. These data suggest that the loss of function of system B⁰,⁰⁺ creates differences in the amino acid handling, suggesting compensation by different transport systems or metabolic pathways.

Expression analysis of renal peptide and amino acid transporters in Slc7a9⁻/⁻ mice. As shown in the intestine, no statistically significant difference in the gene expression of the heavy subunits rBAT (Slc3a1) and 4F2hc (Slc3a2) was ob-

Fig. 3. Gene expression analysis by real-time PCR in intestinal sections after the loss of function of the mouse light subunit b⁰,⁰⁺ AT (Slc7a9). A: the heavy subunit rBAT (Slc3a1) of system b⁰,⁰⁺ showed similar gene expression in the intestine of wild-type and Slc7a9⁻/⁻ mice. B and C: the heavy subunit 4F2hc (Slc3a2) and the cationic amino acid transporter CAT-1 (Slc7a1) showed similar gene expression in the intestine of wild-type and Slc7a9⁻/⁻ mice. D: gene expression of CAT-2B (Slc7a2) was significantly reduced in the duodenum of Slc7a9⁻/⁻ mice compared with their wild-type counterparts. No statistically significant differences were observed in jejunum and ileum. E: cationic amino acid transporter CAT-3 (Slc7a3) showed similar gene expression in the intestine of wild-type and Slc7a9⁻/⁻ mice. F: gene expression of the light subunit from system y¹L y⁺ LAT1 (Slc7a7) was significantly reduced in the ileum of Slc7a9⁻/⁻ mice compared with their wild-type counterparts. No statistically significant differences were observed in duodenum and jejunum. G: the light subunit from system L, LAT2 (Slc7a8), showed similar gene expression in the intestine of wild-type and Slc7a9⁻/⁻ mice. H and I: no differences were observed in the gene expression of ATB⁰,⁰⁺ (Slc6a14) and B⁰AT1 (Slc6a19) in the intestine of Slc7a9⁻/⁻ compared with wild-type mice. J: peptide transporter Pept1 (Slc15a1) showed similar gene expression in the wild-type and Slc7a9⁻/⁻ mice. Values are means ± SE; n = 5 animals/group. Values for wild-type animals are depicted in gray and white for Slc7a9⁻/⁻ mice. DD, duodenum; JJ, jejunum; IL, ileum; CO, colon. Differences between genotypes: *p < 0.05 and ***p < 0.001 using the Mann-Whitney test.
served between genotypes (Fig. 6A). Moreover, the catalytic subunits γLAT1 (Slc7a7), γLAT2 (Slc7a6), LAT2 (Slc7a8), and xCT (Slc7a11) also showed no statistical significant differences between genotypes in their gene expression (Fig. 6, A–C). We further could detect the ATB0,+ (Slc6a14) transcript in the kidney with similar expression levels in wild-type and Slc7a9−/− mice (Fig. 6B). The cationic amino acid transporters (CATs) 1–3 (Slc7a1–3) also showed similar expression levels in both genotypes as the neutral amino acid transporter B0AT1 (Slc6a19). In the kidney, we further investigated the expression of Asct1 (Slc1a4), which mediates the transport of cysteine and neutral amino acids. No differences were observed between the genotypes (Fig. 6C). Finally, we investigated the expression of the peptide transporters Pept1 (Slc15a1) and Pept2 (Slc15a2). Pept1 is the high-capacity/low-affinity peptide transporter mainly expressed in the small intestine, but it has also been reported to be expressed to a lesser extent in the S1 segment of the renal proximal tubule (13, 41). In the Slc7a9−/− mice, the levels of Pept1 mRNA are significantly decreased compared with their wild-type counterparts (Fig. 6B). However, Pept1 protein level analysis revealed no differences between genotypes in the apical membrane of kidney epithelial cells (Fig. 6E). The major peptide transporter in the kidney is the low-capacity/high-affinity peptide transporter PEPT2, which is located in the S2 and S3 segments of the proximal tubule (13, 41). The Pept2 transcripts showed significantly higher levels in the Slc7a9−/− mice than in their wild-type counterparts (Fig. 6C), but no differences could be observed for Pept2 between genotypes at the protein level (Fig. 6D).

**DISCUSSION**

b0,+AT is the catalytic amino acid transporter subunit for cystine, lysine, ornithine, and arginine located in the apical membrane of renal and intestinal epithelial cells. This transport is electrogenic and accepted as Na+ independent. The hallmark after loss of function is hyperexcretion of these amino acids in the urine and kidney stone formation. Additionally, SLC7A9 variants have been associated with estimated GFR levels and susceptibility to chronic kidney disease in GWAS studies (8, 25). Here, we found that loss of function of Slc7a9 in mice leads to reduced GFR calculated with inulin. This reduction could be due to the increased incidence of glomerular fibrosis and glomerulonephritis observed previously in knockout compared with wild-type animals (19). Despite changes in the GFR, only the fractional excretion of cystine, ornithine, arginine, and lysine were altered, but for cystine, ornithine, and lysine to a lesser extent than expected.

The intestinal consequences of oral arginine and lysine loading help in the clinical differentiation between the different types of cystinuria (17). Thus it is not surprising that in non-type I cystinuria (due to mutations in SLC7A9 and the model investigated here), electrogenic lysine transport in the intestine was completely abolished in the absence of the catalytic subunit of system b0,+ whereas arginine and ornithine showed a residual or even unchanged activity. Similarly, the uptake of lysine in jejunal BBMVs was markedly diminished in the absence of the catalytic subunit of system b0,+ whereas arginine showed unchanged activity. The high fractional excretion of arginine in Slc7a9−/− mice and the renal brush-border membrane uptake results confirm that system b0,+ is the major transporter responsible for the reabsorption of arginine in the kidney. In the small intestine, residual currents elicited by the application of arginine and the jejunal BBMV observations may indicate additional transport pathways that need to be identified at the molecular level. Furthermore, loss of Slc7a9 resulted in a 80% reduction of lysine-induced currents and 75% of lysine uptake, indicating that system b0,+ is the major if not the only electrogenic transport pathway for this amino acid in the intestine. In contrast, reduction in fractional renal lysine excretion was lower than expected since it is assumed that b0,+ is the only luminal transport system for the reabsorption of cationic amino acids across the brush-border membrane. Moreover, uptake...
studies in renal brush-border membrane showed that system b0,+ plays a major role in the renal reabsorption of lysine but probably other systems also contribute to renal reabsorption. Similarly, the fractional excretion of ornithine was unexpectedly low. Thus other transporters in the proximal tubule or in later nephron segments may play a role in the reabsorption of these amino acids. At least for arginine and lysine, active transport in the loop of Henle has been detected by microperfusion (14). Here, we investigated all the known cationic amino acid transporters at the molecular level, the y+L, the y+H11001, and the ATB0,+ systems. However, our data could not explain the differences observed in the handling of arginine and lysine after disruption of Slc7a9 in mice. Additionally, certain metabolic pathways could contribute to this compensation. The renal fractional excretion of cystine was also lower than expected. Cystine contributes to glutathione (GSH) metabolism in renal cells. GSH is a major antioxidant and the most abundant low-molecular-weight peptide in cells (16). GSH has also been suggested to be a cysteine reservoir for the organism. The liver is the net synthesizer for GSH, but kidneys also play a major role in GSH homeostasis by removing GSH from plasma and providing the three amino acids required for its synthesis. Currently, the proposed mode of removing GSH from blood by renal cells is by extracellular catabolism of GSH through the action of γ-glutamyl transpeptidase (γGT) and dipeptidases that release the free amino acids and mainly the dipeptide Cys-Gly. As none of the GSH transporters known today is able to overcome the large outwardly GSH electrochemical gradient (1), uptake of Cys-Gly and of the free amino acids cysteine/cystine, glutamine, and glycine is of major importance for glutathione synthesis. Cysteine and glycine are the rate-limiting amino acids. Cysteine availability in the cell depends on cysteine transport and cystine reduction. The amino acid transport system ASC also

by 10.220.32.246 on August 15, 2017 http://ajprenal.physiology.org/ Downloaded from
transports cysteine (4, 5). Even though we did not find any alteration in the gene expression of the cysteine transporters Asct1 (Slc1a4) and the cystine transporter X_c− (4F2hc/Slc7a11), we cannot exclude an increased rate of cysteine absorption due to higher reduction of cysteine to cystine under these conditions. Therefore, most probably b0,+AT plays an important role in delivering cysteine by transporting cystine into the cells. In the absence of b0,+AT function no cystine uptake was measured in the renal apical membrane vesicles, and therefore the cells may have increased Pept2 gene expression to compensate the reduced cystine uptake by increasing Cys-Gly uptake. Indeed, PEPT2 contributes to the reabsorption of amino acids from GSH breakdown as it is critical for Cys-Gly reabsorption (22). Here, we found increased Pept2 gene expression levels, but no increases in PEPT2 protein levels in the apical membrane.

In humans, mutations in SLC6A19 lead to Hartnup disease (24, 40). Cystinurias patients show no or only mild malnutrition symptoms, presumably because the intestinal peptide transporter PEPT1 compensates for the reduced amino acid uptake by absorbing these amino acids as di- and tripeptides. However, in Slc6a19-deficient mice uptake of carnosine (a PEPT1 substrate) in intestinal sections was not upregulated (3). Here, we did not observe any increased gene expression level of Slc15a1 in any intestinal section. These results are not surprising and do not deny the possible role of PEPT1 in compensating for the loss of function of Slc6a19 or Slc7a9. Changes in PEPT1 expression and function are only measurable by changing hormone concentrations, adding pharmacological compounds with similar structure, alterations in the H+ gradient across epithelial cells, in pathological situations that compromise the brush-border membrane, or when administering a high protein load (for a review, see Ref. 38). In other situations, as PEPT1 is a high-capacity transporter with a wide substrate specificity, the importance of PEPT1 is not measurable by functional or expression analysis. This was confirmed after disruption of Slc15a1 in mice. These mice showed altered amino acid plasma levels but the drastic difference in amino acid absorption was only revealed after administration of a high-protein load (29).

Ornithine-induced currents in the intestine are not appreciably affected by disruption of b0,+AT function. The fractional excretion also indicated that other transporters are involved in ornithine absorption and reabsorption or its metabolic functions compensate for the loss of b0,+AT function. Ornithine is rapidly metabolized to arginine in the intestine or transported to the blood by the CAT cationic amino acid transporters (11). As shown here, CAT-3 is also detectable in the intestine apart from CAT-1 and CAT-2B. The Slc7a9 knockout animals showed reduced expression of CAT-2B in the duodenum, which could account for a decreased release to the blood.

Classically, system b0,+ has been defined as an exchange system that transports cationic and neutral amino acids independently from sodium (9). Our results from the BBMV uptake studies demonstrate that the reported exchange of dibasic amino acids with neutral amino acids is completely abolished in the absence of SLCT7A9. Surprisingly, in the BBMV uptake studies we observed that the presence of an inwardly directed sodium gradient slightly modulates transport of cystine and arginine. Similarly, a marked difference was observed in the l-lysine-induced transepithelial current in the presence and absence of Na+. Also, the intestinal apical l-lysine uptake was stimulated in the presence of a Na+ gradient but only when the BBMVs were previously loaded with a neutral amino acid. Probably, this Na+ dependency could be due to Na+ modulation of system b0,+ as observed in renal BBMVs. This sodium-dependent transport activity is abolished in Slc7a9−/− mice, suggesting that it requires b0,+AT subunits. At least in the case of cystine, these results are consistent with old BBMV studies (27) and micropuncture studies (20) in proximal tubules demonstrating a sodium-dependent component of cystine reabsorption. Also, expression of the rBAT subunit in Xenopus laevis oocytes induces cystine- and cationic amino acid-elicited currents that are partially sodium dependent (Wagner CA, Bröer S, Lang F, unpublished observations), suggesting that the transport activity of system b0,+ in renal and intestinal epithelial cells may be modulated by Na+.

b0,+AT is an exchanger of cationic amino acids for neutral amino acids. After disruption of Slc7a9, we observed increased urinary excretion of glutamine, probably due to the increased plasma concentrations and enhanced glomerular filtration. Although we could not distinguish in our amino acid analysis between glutamine and glutamate levels, we believe that these changes are due to glutamine and not glutamate changes as glutamine is the most abundant amino acid in plasma (in mice ~400 μM glutamine vs. 40 μM glutamate) (29) and could account for the increased concentration from 0.53 ± 0.02 mM in wild-type to 0.80 ± 0.10 mM in Slc7a9−/− mice. The main renal and intestinal amino acid transporter for neutral amino acids is B0AT1. Disruption of Slc6a19 in mice abolished uptake of glutamine in the intestine and drastically reduced renal uptake of glutamine (3). Here, we did not observe increased expression of B0AT1 in the intestine or in kidney, therefore the increased plasma levels observed after Slc7a9 disruption could be due to increased glutamine reabsorption by other transporters. Asct2 is not responsible for this compensation as it could not be detected recently in the kidney applying several different methods (3). Moreover, we could not measure any difference in glutamine uptake in the renal apical membranes between Slc7a9 knockout and wild-type animals. 4F2hc/LAT2, ATB0,+ and 4F2hc/0,+LAT2 seem not to contribute to the increased plasma levels at least from the gene expression analysis data. A decrease in ileal y LAT1 gene expression could account for less removal of glutamine from the blood, contributing partly to its increased plasma levels. However, as the high concentrations of glutamine in plasma are mostly maintained by de novo synthesis rather than (re)absorption, the absence of Slc7a9 provokes most probably some changes in glutamine interorgan metabolism that may lead to higher glutamine in plasma, such as higher release from muscle and adipose tissue or decreased uptake from blood in the kidney (mediated by system N).

In conclusion, minor changes in transporter gene expression and changes in the fractional excretion of amino acids point to an interaction of SLC7A9 with other transporter and metabolic pathways. This role is not surprising as its substrates, cystine, arginine, lysine, ornithine, and glutamine, have major and different roles in the organism. Lower fractional excretion of cystine and lysine than expected in Slc7a9−/− mice and previous findings on Slc15a2 knockout suggest an important role of both transporters in ensuring cysteine delivery to the kidney cells for glutathione metabolism, which might provide a link to
loss of function of Slc7a9. Further investigations are required to verify these hypotheses.

ACKNOWLEDGMENTS

We thank Johanna Welzhofer (Molecular Nutrition Unit, Technical University of Munich, Munich, Germany) for technical support in amino acid analysis; François Verrey (Institute of Physiology, University of Zurich, Zurich, Switzerland) for providing us with the anti-B0AT1 antibody; and Britta Spanier (Molecular Nutrition Unit, Technical University of Munich, Munich, Germany) for providing the anti-PEPT1 and anti-PEPT2 antibody.

Present address of I. Frey-Wagner: Div. of Gastroenterology and Hepatology, Univ. Hospital Zürich, Univ. of Zürich, Zürich, Switzerland.

Present address of N. Mohebbi: Div. of Nephrology, Univ. Hospital Zürich, Zürich, Switzerland.

GRANTS

This work was supported by an FP-6 EU project (EUGINDAT) grant to M. Palacin. V. Nunes, F. Lang, H. Daniel, and C. A. Wagner, by MINECCO (SAF2009-12606-C02-01 and 02) to M. Palacin and V. Nunes, 09SGR1490 from the Catalonian government to V. Nunes, and SGR2009-1355 from the Catalonian government to M. Palacin.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


