Biochemical and Biophysical Research Communications 407 (2011) 355–359

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reduced phosphate transport in the renal proximal tubule cells in cystinosis is due to decreased expression of transporters rather than an energy defect

Mary L. Taub [a,*], James E. Springate [b], Facundo Cutuli [a]

[a] Department of Biochemistry, School of Medicine and Biomedical Sciences, University at Buffalo, 3435 Main Street, Buffalo, NY 14214, United States
[b] Department of Pediatrics, School of Medicine and Biomedical Sciences, University at Buffalo, 3435 Main Street, Buffalo, NY 14214, United States

ARTICLE INFO

Article history:
Received 1 March 2011
Available online 8 March 2011

Keywords:
Cystinosis
Renal proximal tubule
Phosphate transport
Primary culture
siRNA

ABSTRACT

Nephropathic cystinosis is an autosomal recessive disorder caused by mutations in the *CTNS* gene [1], which encodes for a transporter (cystinosin) responsible for cystine efflux from lysosomes. In cystinotic renal proximal tubules (RPTs), the defect in cystinosin function results in reduced reabsorption of solutes by apical Na^+/solute cotransport systems, including the Na^+/phosphate (Pi) cotransport system [2]. However the underlying molecular mechanisms are unknown, given the lack of an appropriate cellular model. To obtain such a model system, we have knocked down cystinosin with siRNA in primary RPT cell cultures. An 80% reduction in cystinosin strongly inhibited Na^+ dependent Pi uptake (70%). Although this finding could be explained by a direct effect on transporters as well as by altered energetics (the ATP level dropped by 52%), our results demonstrate a lack of involvement of Na, K-ATPase, and a reduction in the number of NaPi2a transporters.

1. Introduction

Nephropathic cystinosis is an autosomal recessive disorder caused by mutations in the *CTNS* gene encoding for cystinosin, a transporter responsible for the efflux of cystine from lysosomes [3]. During the first year of life, patients with infantile cystinosis develop the Fanconi syndrome, characterized by severe fluid and electrolyte imbalances [2]. The Fanconi syndrome can be attributed to defective reabsorption of a number of solutes by the renal proximal tubule (RPT), including phosphate (Pi), bicarbonate, amino acids and glucose. Consequences include rickets, acidosis, and end-state renal disease.

Despite an extensive number of studies, it is still unclear why the transport of solutes by a number of apical Na^+/solute cotransport systems is reduced, and the Fanconi syndrome emerges in cystinosis. In order to study this problem previously, rabbit RPT cells were incubated with cystine dimethyl ester (CDME), which causes cystine to accumulate in lysosomes [4]. The result was a generalized decrease in the activity of apical secondary active transport system [4]. The decreased transport was attributed to a primary defect, altering the activity of the Na^+/Pi cotransport system, and a decline in the level intracellular Pi affecting ATP synthesis. However, recently the conclusions obtained in the studies with CDME have been questioned, because CDME was found to directly inhibit mitochondrial ATP production [5]. Thus, a new in vitro approach is needed.

In this report the effect of a cystinosin knock-down on a well-characterized primary rabbit RPT cell culture system is examined. The results of these studies indicate that the activity of the Na^+/Pi cotransport system decreases after knocking down cystinosin. We evaluate whether this decrease in the activity of the Na^+/Pi cotransport system can be explained by a decrease in the ATP level affecting the Na^+ gradient, and/or by an alteration directly affecting the Na^+/Pi cotransporter itself.

2. Materials and methods

2.1. Materials

Hormones, human transferrin, the ATP assay kit, X-ray film, and other chemicals were from Sigma Aldrich Chemical Corp (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DME), Ham's F12 Medium (F12), soybean trypsin inhibitor, lipofectamine, primers, double stranded stealth siRNA and sodium green tetraacetate were obtained from Invitrogen Corp. (Carlsbad, CA). The sequence of the sense strand of 509 cystinosin siRNA was GAC AAU ACG UCU UGC UGC CCA GUU A, and the sequence of the equivalent scrambled siRNA was GAC GCA UUU CUG UCG ACC CGA AUU A. Class IV collagenase was from Worthington (Freehold, NJ). Mouse monoclonal antibodies against the cystinosin (sc-21712) and β-actin (sc-69879) were from Santa Cruz Biotech (Santa Cruz, Calif.), and

* Corresponding author. Address: Department of Biochemistry, 140 Farber Hall, University at Buffalo, 3435 Main Street, Buffalo, NY 14214, United States. Fax: +1 716 829 2132.
E-mail address: biochtau@buffalo.edu (M.L. Taub).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.03.022

Rabbit anti-human NPT2a (Cat. #NPT27A) was from Alpha Diagnostics (San Antonio, Tx). Goat anti-mouse Horse Radish Peroxidase (HRP) conjugate, Goat anti-rabbit HRP, Immun-Star HRP Substrate, nitrocellulose, acrylamide, and other electrophoresis reagents were from Bio-Rad Corp. (Hercules, Calif.). Sulfo-NHS-ss-biotin was from Pierce/ThermoScientific (Rockford, Ill), and Streptavidin Sepharose beads from Amersham/GE Healthcare (Piscataway, NJ). The Prism 5 Program was obtained from GraphPad Software, Inc. (San Diego, CA). $^{86}Rb^{+}$ (>1 μCi/μg), ^{32}P-Pi (8500 Ci/mM), ^{14}C-methyl-α-D-glucopyranoside (α-MG) (331 mCi/mmol), ^{14}C-3-o-methyl-D-glucopyranoside (3-o-MG) (56.4 mCi/mmol) were from Perkin Elmer Corp. (Billerica, Ma).

2.2. Primary RPT cultures

The basal medium for primary RPT cells consists of a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F12 medium (F12) (pH 7.4) containing 15 mM HEPES, 20 mM Na^{+} bicarbonate, penicillin (92 units/ml) and kanamycin (0.01%) (DME/F12) [6]. Immediately prior to use, the medium was further supplemented with bovine insulin (5 μg/ml), human transferrin (5 μg/ml), and 50 nM hydrocortisone (Medium RK1) [6]. Water used for medium and growth factor preparations was purified using a Milli-Q deionization system. Iron oxide in 0.9% NaCl was autoclaved and diluted with PBS prior to use [7].

To initiate primary rabbit RPT cell cultures [6,8], a kidney from a male, New Zealand White rabbit (2–2.5 kg) was perfused via the renal artery, first with phosphate buffered saline (PBS), and subsequently with sterile 0.5% (w/v) iron oxide. The renal cortex was sliced, homogenized with a sterile Dounce homogenizer (loose pestle), and the homogenate (consisting of nephron segments) was sequentially passed through a 253 μ and an 83 μ mesh. The RPTs and glomeruli on the 83 μ mesh were transferred into a 50 ml conical tube containing DME/F12. The glomeruli (with bound iron oxide) were removed with a sterile stir bar, giving a tubule preparation that stained positively for γ-glutamyltranspeptidase.

RPTs were incubated in DME/F12 containing 0.05 mg/ml collagenase class IV and 0.5 μg/ml soybean trypsin inhibitor for 2 min at 23° C, washed twice by centrifugation, re-suspended in DME/F12, and plated in 35 mm dishes containing Medium RK-1. The medium was changed the day after plating and every 2 days thereafter.

2.3. Treatment of primary cultures with cystinosin siRNA

The primary RPT cells were transfected with 200 nM of cystinosin siRNA or scrambled control siRNA using lipofectamine (10 μl/35 mm dish) after 4 days in culture. The transfection was repeated the following day (day 5 in culture) and 2 days later (day 7 in culture). Cultures were used for experiments on day 8.

2.4. RealTime PCR

RNA was purified from the cultures using an RNA-4PCR kit (Ambion, Austin, TX). After removing genomic DNA using TURBO DNAse I (Ambion), cDNA was synthesized from the RNA using reverse transcriptase. Transcripts were amplified in an iCycler using iQ Sybr Green Supermix (Biorad, Hercules, Ca.) containing 5 μM forward and reverse primers complementary to appropriate cDNA templates. Ct values were calculated (with the Bio-Rad IQ5 program), and relative mRNA levels quantitated as described by Pfaffl [9] using GAPDH mRNA as an internal control.

The primers were designed by using Primer-BLAST (NCBI website), and synthesized (Invitrogen). Included amongst the cDNAs used for primer design are rabbit cystinosin (Ensembl Assession Number ENSOCUT00000008001), CGCGAGCTTGATGTAGGAG, ATTGCTTCCTGCTGCTCT; and rabbit GAPDH (Assession Number L23961, RABGLY3PHO), GCCCTCAATGACCACTTTGT, TCATGACAAGGTAGGGCTCC.

2.5. Preparation of cell lysates for electrophoresis

Primary RPTs were washed with ice-cold PBS at 4 °C, and solubilized in RIPA lysis buffer (20 mM Tris, pH 7.5, 1% Triton X-100, 120 mM NaCl, 1 mM EDTA) supplemented with1 mM phenylmethylsulfonyl fluoride (PMFS), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM Na^{+} ortho-vanadate. The cell lysates were removed from the culture dishes with a rubber policeman, and transferred into microfuge tubes.

2.6. Cell surface biotinylation studies

Cell surface biotinylation studies were conducted by a modification of the method of Gottardi and Caplan [10,11]. To summarize, primary RPTs were grown on Transwell inserts (24 mm, 0.4 μm polycarbonate membranes, Corning #3412) in Medium RK1 until achieving confluence (10–12 days). Prior to the biotinylation, the medium was changed, and 2 h later the monolayers were placed at 4 °C. After washing twice with PBS containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (PBS–Ca–Mg), the monolayers were incubated for 25 min. with sulfo-NHS-ss-biotin (0.5 mg/ml) in10 mM triethanolamine, pH 9.0, 2 mM $CaCl_2$ and 150 mM NaCl. This step was repeated. The monolayers were washed twice with ice-cold PBS–Ca–Mg containing 100 mM glycine (incubating 20 min during the second wash), followed by two washes with PBS–Ca–Mg. Finally, the monolayers were solubilized in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, containing 1 mM PMFS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM Na^{+} orthovanadate (1 h; 4 °C). The lysate was removed with a cell scraper, and centrifuged (14, 000g; 10 min; 4 °C).

After rotating the lysates (100–250 μg of protein [12]) overnight with Streptavidin Sepharose beads, they were washed 3 times with lysis buffer, twice with a high salt wash buffer (lysis buffer containing 0.1% Triton X-100 and 500 mM NaCl), and once with 10 mM Tris, pH 7.5. Biotinylated proteins were eluted by heating 5 min at 100 °C.

2.7. Western analysis

Samples equalized with regards to protein [12] were separated in 7.5% SDS/polyacrylamide gels in parallel with molecular weight markers, and transferred to nitrocellulose in a Bio-Rad Trans-Blot Apparatus. The blots were blocked 1 h in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (TTBS), incubated 2 h with primary antibody in TTBS, washed (6×; TTBS), and incubated 45 min with an HRP conjugated-secondary antibody. After washing (7×; TTBS), blots were incubated with Immun-Star HRP Luminol/Enhancer and bands visualized with Bio Max MS2 Film. Blots were reprobed with an anti β-actin antibody to control for equal loading. The X-ray films were scanned with a Bio-Rad Scanning Densitometer, and the relative band intensity quantitated (Quantity One; Biorad).

2.8. Uptake studies

Monolayers in 35 mm dishes were washed twice at 23 °C with uptake buffer (containing either 150 mM NaCl, 1.2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 10 mM MES/Tris, pH 7.5, or the same buffer containing KCl in lieu of NaCl). Subsequently, monolayers were incubated 30 min in uptake buffer containing [^{32}P] – Phosphate (Pi) (1.5 μCi/ml) and 1 mM unlabeled Pi at 23 °C [13], followed by three washes with uptake buffer, and solubilization in RIPA lysis buffer.