FXYD2 and Na,K-ATPase Expression in Isolated Human Proximal Tubular Cells: Disturbed Upregulation on Renal Hypomagnesemia?

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Abstract Autosomal dominant renal hypomagnesemia (OMIM 154020), associated with hypocalciuria, has been linked to a 121G to A mutation in the FXYD2 gene. To gain insight into the molecular mechanisms linking this mutation to the clinical phenotype, we studied isolated proximal tubular cells from urine of a patient and a healthy subject. Cells were immortalized and used to assess the effects of hypertonicity-induced overexpression of FXYD2 on amount, activity and apparent affinities for Na\(^+\), K\(^+\) and ATP of Na,K-ATPase. Both cell lines expressed mRNA for FXYD2a and FXYD2b, and patient cells contained both the wild-type and mutated codons. FXYD2 protein expression was lower in patient cells and could be increased in both cell lines upon culturing in hyperosmotic medium but to a lesser extent in patient cells. Similarly, hyperosmotic culturing increased Na,K-ATPase protein expression and ATP hydrolyzing activity but, again, to a lesser extent in patient cells. Apparent affinities of Na,K-ATPase for Na\(^+\), K\(^+\) and ATP did not differ between patient and control cells or after hyperosmotic induction. We conclude that human proximal tubular cells respond to a hyperosmotic challenge with an increase in FXYD2 and Na,K-ATPase protein expression, though to a smaller absolute extent in patient cells.

Keywords Proximal tubular cells · Hypomagnesemia · FXYD2 · Na,K-ATPase

Introduction

Autosomal dominant renal hypomagnesemia (OMIM 154020), associated with hypocalciuria, is a disorder which has been linked to a 121G to A mutation in the FXYD2 gene (Meij et al. 2000). This mutation leads to a G41R substitution in the transmembrane domain of the FXYD2 protein (Na,K-ATPase \(\gamma\)-subunit). FXYD2 belongs to a family of seven mammalian members, all of which share the FXYD motif in the N-terminal domain. All family members have been shown to be able to associate with Na,K-ATPase and to alter its intrinsic properties (Crambert et al. 2002, 2005; Lubarski et al. 2005; Delprat et al. 2007; Beguin et al. 1997, 2001, 2002; Li et al. 2005; Arystarkhova et al. 1999; Therien et al. 1999). This together with their tissue-specific expression pattern has led to the hypothesis that they function as cell- and tissue-specific regulators of Na,K-ATPase.

Association of FXYD2 with Na,K-ATPase alters the apparent affinity for K\(^+\) in a membrane potential–dependent manner (Arystarkhova et al. 1999; Beguin et al. 1997).
and reduces Na\(^+\) affinity (Arystarkhova et al. 1999; Beguin et al. 2001). The apparent affinity for ATP is also altered (Therien et al. 1999). The G41R substitution in the transmembrane domain of FXYD2, which is linked to hypomagnesemia, has been demonstrated to disrupt the association between Na,K-ATPase and FXYD2 (Meij et al. 2003; Pu et al. 2002). The molecular mechanism by which this lack of association leads to hypomagnesemia is not yet resolved. Thus far, studies of these molecular mechanisms have been hampered by the absence of a suitable human renal cell model. This limitation can, in principle, be overcome by the possibility of culturing renal cells derived from urine (Laube et al. 2005; Racusen et al. 1995).

In the present study, we cultured and characterized proximal tubular epithelial cells (PTECs) immortalized with human papillomavirus E6/E7 genes (HPV16 E6/E7) from the urine of a hypomagnesemia patient and compared their properties with those of cells derived from the urine of a healthy control. To this end, we determined the expression levels of FXYD2 and the Na,K-ATPase \(\alpha\)-subunit, the maximum ATPase activity and the apparent Na\(^+\), K\(^+\) and ATP affinities of Na,K-ATPase under normo- and hyperosmotic culturing conditions. We show that the Na,K-ATPase \(\alpha\)-subunit and FXYD2 can be upregulated by exposing the cells to hyperosmolality, yet the absolute expression levels remain lower in patient cells compared to control cells. The increase in Na,K-ATPase expression is proportionate to an increase in maximal Na,K-ATPase activity, whereas it is not accompanied by changes in the apparent affinities for Na\(^+\), K\(^+\) or ATP of the enzyme.

**Materials and Methods**

**PTE Cell Line Generation**

Urine was collected from a patient (35 years old, male) in whom the G41R mutation was confirmed previously (Meij et al. 2000) and used to obtain proximal tubular cells. Urine of a healthy subject (12 years old, male) was used for obtaining control proximal tubular cells. Proximal tubular cells were isolated as described previously (Wilmer et al. 2005). Urine was centrifuged (223×g, 5 min, room temperature) within 5 h after collection. After washing in phosphate-buffered saline (PBS) and a second centrifugation step, urine sediment was resuspended in 3 ml PTEC culture medium (DMEM–Ham’s F12; Cambrex Biosciences, East Rutherford, NJ) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin (GIBCO, Grand Island, NY), 100 U/ml streptomycin (GIBCO), ITS (Sigma, St. Louis, MO; 5 \(\mu\)g/ml insulin, 5 \(\mu\)g/ml transferrin and 5 ng/ml selenium), 36 ng/ml hydrocortisone (Sigma), 10 ng/ml epidermal growth factor (EGF, Sigma) and 40 pg/ml triiodothyronine (Detrisac et al. 1984). The suspension was transferred to a 25-cm\(^2\) tissue culture flask and placed at 37°C in a 5% CO\(_2\) incubator. The medium was refreshed every 2–3 days. To maintain proliferation, cells at passage 4 or less were transfected with plasmid DNA, containing the HPV16 E6/E7 genes, using the amphotropic packaging cell line PA 317 (Ryan et al. 1994). The proximal tubular origin of cultured cells was confirmed by visual morphology and by flow-cytometric analysis using CD13 (aminopeptidase N) antibodies, specific for PTECs.

**Cell Culture**

Cells were cultured at 37°C in a humidified 5% CO\(_2\)/95% air atmosphere in PTEC culture medium (osmolality 286–356 mosmol/kg H\(_2\)O), supplemented with 50 mg/ml gentamycin (GIBCO). For hyperosmotic induction of FXYD2, cells were grown to 100% confluence, after which they were grown for another 2 days in medium supplemented as indicated in “Results.” Care was taken not to cause changes in the pH of the culture medium. Patient and control cells were analyzed under the same conditions, to exclude experimental artifacts.

**RNA Isolation and cDNA Synthesis**

Cells were harvested by trypsinization, washed in PBS (500×g, 1 min, 4°C) and resuspended in 500 μl TRIzol (Invitrogen, Carlsbad, CA). After addition of 50 μl chloroform and 0.2 mg/ml glycogen, the suspension was vortexed for 10 s, incubated for 10 min at 4°C, vortexed again and centrifuged (12,000×g, 10 min, 4°C). Clear supernatant (200 μl) was transferred to a small tube and 200 μl of propanol-2 was added. The suspension was vortexed for 10 s, incubated for 10 min at 4°C, vortexed again and centrifuged (16,000×g, 30 min, 4°C). The RNA pellet was washed in 70% EtOH, air-dried and taken up in 40 μl of DEPC water. cDNA was synthesized using 300 ng of isolated RNA as template and random primers.

**FXYD2 cDNA Detection**

FXYD2 cDNA was amplified for detection using specific primers for human FXYD2a (atgactggtttgtcatgacctg), human FXYD2b (atgacaggtggacagctg) and the redundant reverse primer for human FXYD2 (ttggtctacatcattgt). PCR products were analyzed by agarose gel electrophoresis and sequencing.

**Crude Membrane Preparation**

Cells were harvested by trypsinization, washed in PBS (500×g, 1 min, 4°C) and resuspended in 500 μl H\(_2\)O
containing protease inhibitor (1 tablet/10 ml, per the manufacturer’s protocol; Roche, Indianapolis, IN). After repeated freezing and thawing to lyse the cells, DNA was broken down by incubation with 100 U/ml Turbo DNase (10 min, 4°C; Ambion, Austin, TX), after which the membranes were isolated by centrifugation (16,000×g, 30 min, 4°C). The pellet was resuspended in 50 μl PBS and stored at −20°C.

Membrane Preparation

Cells were harvested by incubation in PBS/10 mM EDTA at 37°C for 1 h, after which they were scraped from the culture flasks and collected. After centrifugation (2,000×g, 5 min), the cell pellet was resuspended in 5 ml PBS/10 mM EDTA (pH 7.0) containing protease inhibitor (per the manufacturer’s recommendations). Cells were lysed by repeated freezing and thawing and homogenized by pulling through a needle. The homogenate was centrifuged (200×g, 5 min), after which the membranes were isolated from the supernatant by centrifugation (100,000×g, 60 min). The pellet was resuspended in 500 μl H2O, lyophilized, taken up in 400 μl buffer (250 mM sucrose, 50 mM Tris–HCl [pH 7.0] and 2 mM EDTA) and stored at −20°C.

Protein Determination

Protein concentrations were quantified with the modified Lowry method according to Peterson (1983) using bovine serum albumin as a standard.

Western Blotting

Membrane fractions were solubilized in sample buffer and analyzed on SDS–PAGE gels containing 15% acrylamide according to Laemmli (1970). For immunoblotting, the separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). FXYD2 subunits were detected with an antibody against the C-terminal end (Or et al. 1996), kindly provided by Dr. S. Karlish (Rehovot, Israel), whereas the α-subunit of Na,K-ATPase was detected with antibody C356-M09 (Koenderink et al. 2003). Primary antibodies were detected using an antirabbit secondary antibody, labeled with horseradish peroxidase (Dako, Copenhagen, Denmark).

ATPase Activity Assay

ATPase activity was determined using a radiochemical method. For this purpose, 0.1 mg of membranes was added to 100 μl of medium containing (in mM) 0.8 MgCl2, 0.1 EGTA, 0.2 EDTA, 1 TrisN3 and 50 Tris–HCl (pH 7.0), as well as various concentrations of activating cations and Mg-[γ-32P]-ATP as indicated in “Results.” Ionic strength was kept constant with choline chloride. After incubation for 30 min at 37°C, the reaction was stopped by adding 500 μl 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid, and after 10 min at 0°C, the mixture was centrifuged for 10 s at 10,000×g. To 0.15 ml of clear supernatant, containing the liberated inorganic phosphate (32Pi), 3 ml OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added, and the mixture was analyzed by liquid scintillation analysis. In general, blanks were prepared by incubating in the absence of membranes. Na,K-ATPase activity is presented as the difference in activity between total ATPase (cations as indicated) and Mg2+-ATPase (no Na+ and K+ present, with 10 mM ouabain present).

Analysis of Data

Differences were tested for significance by means of Student’s t-test. K0.5 values were determined by analyzing the plots using the nonlinear curve-fitting program (Hill equation function) of Origin 6.1 (OriginLab, Northhampton, MA).

Results

PTE Cell Line Generation

After culturing of urine sediments, colonies with cobblestone morphology appeared in urine samples of both healthy control and hypomagnesemia subjects. Cultured cells were immortalized by transfection with HPV E6/E7 genes. Although PETC markers were positive (see “Materials and Methods”), we cannot with absolute certainty exclude mixed cell populations.

cDNA Characterization

RNA was isolated from patient and control cell lines, both of which were induced by an increase in osmolality of 200 mosmol/kg H2O using sucrose. cDNA was synthesized and subsequently analyzed by performing PCRs with primers designed to pick up human FXYD2a and FXYD2b. Analysis of the PCR products on agarose gel indicated the presence of both FXYD2a and FXYD2b cDNA, which was confirmed by sequencing (data not shown). Sequencing furthermore revealed that the control cell line had only a guanine at position 121, whereas the patient cell line had both a wild-type guanine signal and a mutant adenine signal in both FXYD2a (Fig. 1) and FXYD2b (data not shown). The latter indicates that patient cells are heterozygous and that both alleles are transcribed.
Hyperosmotic Challenges

The crude membrane isolations of the cell lines were analyzed on SDS–PAGE, to visualize the presence of FXYD2 and the catalytic subunit (α) of Na,K-ATPase. After several passages at normal osmolality, the amount of FXYD2 in the membrane fractions diminished (data not shown), in line with literature data (Capasso et al. 2001; Wetzel et al. 2004) showing that various cell lines originating from the kidney do not express FXYD2 unless they are exposed to hyperosmotic medium. Indeed, exposure of our cell lines to hyperosmotic medium (raised by 200 mosmol/kg H₂O with NaCl) raised the amount of FXYD2 observed on Western blot, with little or no effect on the amount of expressed α-subunit (Fig. 2, lanes 1 and 2). Exposure of the cells to an additional 200 mosmol/kg H₂O choline chloride (Fig. 2, lane 4) gave similar results as 200 mosmol/kg H₂O NaCl. Exposure to an additional 200 mosmol/kg H₂O sucrose (Fig. 2, lane 5) showed an even larger increase in FXYD2 expression. In contrast, exposure to neither an additional 200 mosmol/kg H₂O sodium acetate (Fig. 2, lane 3) nor 200 mosmol/kg H₂O urea (Fig. 2, lane 6) increased FXYD2 expression. In addition, expression of FXYD2 seemed to be lower in patient cells under all conditions compared to control cells.

Na,K-ATPase Expression and Activity

The presence of the α-subunit and FXYD2 in samples used for ATPase activity measurements was visualized on Western blot (Fig. 3). When cultured in hypertonic medium (additional 200 mosmol/kg H₂O sucrose), the relative intensity of α-subunit and FXYD2 staining increased 1.7 ± 0.2-fold and 5 ± 1.0-fold for control cells and 1.3 ± 0.5-fold and 6.1 ± 2.3-fold for patient cells, respectively. The expression level of FXYD2 in patient cells was only 35 ± 10% of that in control cells (normosmotic conditions). Also, the expression levels of the Na,K-ATPase α-subunit seemed to be higher in the control cell line.

In the presence of ATP, Na⁺ and K⁺, the total Na,K-ATPase activity measured in isolated membranes increased for both patient and control cells (Fig. 4a), though the increase was less in the patient cells. Control fractions showed a maximum activity of 2.86 ± 0.11 μmol Pi mg⁻¹ protein h⁻¹ from noninduced cultures and 4.89 ± 0.07 μmol Pi mg⁻¹ protein h⁻¹ from induced cultures. The membrane
fractions from patient material showed a maximum activity of 2.37 ± 0.24 l mol Pi mg⁻¹ protein h⁻¹ from noninduced cultures and 3.02 ± 0.18 l mol Pi mg⁻¹ protein h⁻¹ from induced cultures. Mg²⁺-ATPase activity, measured in the absence of Na⁺ and K⁺ and in the presence of ouabain, was virtually the same for control and patient material and did not alter upon induction. When corrected for the relative intensity of isolated α-subunit on Western blot (Fig. 3), the samples showed no significant differences in activity (Fig. 4b). This finding indicates that the increase in ATPase activity can be fully explained by the sucrose-induced increase in Na,K-ATPase α-subunit expression.

In membrane samples from noninduced control cultures, the apparent affinities for ATP (Fig. 5), Na⁺ (Fig. 6) and K⁺ (Fig. 7) were 0.54 ± 0.05, 9.1 ± 0.8 and 1.38 ± 0.26 mM, respectively (Table 1). Essentially the same values were found after induction with sucrose (0.51 ± 0.04, 8.7 ± 0.6 and 1.24 ± 0.1 mM, respectively). Corresponding values obtained with samples from noninduced (0.68 ± 0.07, 8.8 ± 0.7 and 1.79 ± 0.18 mM, respectively) and induced (0.59 ± 0.05, 8.3 ± 0.9 and 1.61 ± 0.11 mM, respectively) patient cultures did not significantly differ from control cultures.

Discussion

FXYD2 (Na,K-ATPase γ-subunit) is a kidney-specific regulator of Na,K-ATPase. Dominant renal hypomagnesemia associated with hypocalciuria has been linked to a 121G → A mutation in the FXYD2 gene, resulting in a G41R substitution in the protein itself. Since the relation between the mutation and hypomagnesemia is not understood yet, we isolated proximal tubular cells from the urine of a healthy control subject and a patient carrying the above mutation and converted these into immortalized cell lines. mRNA of both FXYD2α and FXYD2β was shown to be present and the 121G to A mutation, associated with hypomagnesemia, was detected in the patient cell line. At the protein level we show that both cell lines contained FXYD2 and the catalytic α-subunit of Na,K-ATPase. We furthermore show that both proteins can be upregulated by culturing the cells under hypertonic conditions.

The induction of FXYD2 in our cell lines is in agreement with the literature (Capasso et al. 2001; Wetzel et al. 2004), as is the decline in FXYD2 protein over prolonged time periods when normal medium is used. The absence of any induction by 200 mM urea, which diffuses freely through the cells, indicates that these cell lines express FXYD2 particularly when cultured in an environment with...
extracellular hyperosmolality. This indicates the existence of a regulatory system in which FXYD2 is upregulated to modulate the function of Na,K-ATPase so that it better suits the cellular environment. Moreover, upregulation of FXYD2 is Na\(^+\)-independent and Cl\(^-\)-dependent. This result confirms the data of Capasso et al. (2003), who showed that in IMCD3 cells chloride stimulates expression of FXYD2 and activates JNK in response to hypertonicity.

On the mRNA level we show the presence of FXYD2a and FXYD2b, as well as their mutated forms, in patient cells. Unfortunately, the antibody used in this study cannot distinguish between these two splice variants, nor can it distinguish between wild-type and G41R mutant FYXD2.

Earlier work using recombinant protein obtained from cRNA-injected *Xenopus laevis* oocytes revealed a distinct difference in molecular mass between wild-type and mutant protein on SDS–PAGE (Meij et al. 2003). However, in our isolated cells we could not detect this difference in gel migration. A similar lack of difference in gel migration was found in HeLa cells transfected with either wild-type FXYD2b or G41R-mutated FXYD2b (Pu et al. 2002). We therefore cannot say with certainty that both wild-type and mutant FYXD2 are present in protein form. If the mutated FYXD2 is quickly degraded after upregulation, only the wild-type FYXD2 will be observed. In previous studies it was shown that the routing of FXYD2-

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**Fig. 5** Effects of ATP on Na,K-ATPase activity. ATPase activity was measured in membrane preparations as described in “Materials and Methods” in the presence of 0.1 mM EGTA, 0.2 mM EDTA, 0.8 mM MgCl\(_2\), 1.0 mM Tris–N\(_3\), 100 mM NaCl and 10 mM KCl, at pH 7.0. Ionic strength was kept constant with choline chloride. Samples from noninduced cultures are represented by open symbols. Samples from cultures induced with an additional 200 mosmol/kg \(\text{H}_2\text{O}\) of sucrose are represented by closed symbols. Shown are means ± se of three enzyme preparations.

**Fig. 6** Effects of NaCl on Na,K-ATPase activity. ATPase activity was measured in membrane preparations as described in “Materials and Methods” in the presence of 0.1 mM EGTA, 0.2 mM EDTA, 0.8 mM MgCl\(_2\), 1.0 mM Tris–N\(_3\), 10 mM KCl and 2.0 mM MgATP at pH 7.0. Ionic strength was kept constant with choline chloride. Samples from noninduced cultures are represented by open symbols. Samples from cultures induced with an extra 200 mosmol/kg \(\text{H}_2\text{O}\) of sucrose are represented by closed symbols. Shown are means ± se of three enzyme preparations.
G41R is impaired and that it can prevent the correct routing of wild-type FXYD2 (Meij et al. 2000; Cairo et al. 2008). Immunohistochemical scanning of patient cells might indicate if FXYD2 is indeed retarded. However, due to high intracellular background signals with the current antibody, we were unable to draw any conclusion about the localization of FXYD2 (data not shown).

Na,K-ATPase activity measurements on isolated membranes showed no significant changes in cation or ATP affinities between the two cell lines, irrespective of induction. The wild-type cell line showed a significant increase in total ATPase activity, but this can be explained by the increase in the total amount of Na,K-ATPase \( \alpha \)-subunit. When ATPase activity was corrected for the amount of \( \alpha \)-subunit detected on Western blot, all samples displayed the same activity, regardless of origin or induction state, indicating that an increase in Na,K-ATPase activity is due to upregulation of Na,K-ATPase. Whereas these findings are in agreement with an earlier study in which the \( \alpha \)-subunit and subsequent activity were increased in a primary culture of human proximal tubule cells exposed to hypertonicity (Yordy and Bowen 1993), they are at odds with studies performed by Wetzel et al. (2004). The latter authors show a reduction in the maximal activity of Na,K-ATPase after hyperosmotic induction of NRK-52E cells in the presence of 10% FCS. We revealed stimulation of Na,K-ATPase activity (also in the presence of 10% FCS), which correlated with the amount of expressed \( \alpha \)-subunit. Whether this discrepancy is due to differences in cell type or induction of FXYD2 splice variants is unclear.

The major difference we found between the patient and control cell lines was the lower amount of FXYD2 expressed per milligram of protein in the isolated membranes obtained from the patient cell line under the various conditions compared to the control cell line under similar conditions (Fig. 3). The mutated FXYD2 is retarded in the Golgi network (Meij et al. 2003) and probably broken down more rapidly than the wild-type FXYD2. In heterozygous cells, oligomers between wild-type and mutant FXYD2 may be unable to leave the Golgi network, leading to increased breakdown (Cairo et al. 2008). Recently, it was shown that FXYD2 and the Na,K-ATPase \( \alpha \)-subunit do not traffic together to the plasma membrane and, thus, assemble at the plasma membrane to form a complex (Piñol-Roma et al. 2008). We therefore can conclude that the reduced Na,K-ATPase response to hypertonicity in patient cells is not due to interaction with the mutated FXYD2. It is likely that the absolute decrease of hyperosmolarity induced upregulation of FXYD2 and that the Na,K-ATPase \( \alpha \)-subunit in isolated patient cells also happens in vivo. Reduced overexpression of the FXYD2 subunit in response to hypertonic stress can affect cell survival or might lead to changes in intracellular ATP levels (Capasso et al. 2006). Eventually, this decrease of

### Table 1

| ATP affinity \((\text{mm})\) | Na\(^+\) affinity \((\text{mm})\) | K\(^+\) affinity \((\text{mm})\) |
|--------------------------|---------------------|-----------------|
| Control \(--\)           | 0.54 ± 0.05         | 9.1 ± 0.8       | 1.38 ± 0.26     |
| Control \++              | 0.51 ± 0.04         | 8.7 ± 0.6       | 1.24 ± 0.10     |
| Patient \(--\)           | 0.68 ± 0.07         | 8.8 ± 0.7       | 1.79 ± 0.18     |
| Patient \++              | 0.59 ± 0.05         | 8.3 ± 0.9       | 1.61 ± 0.11     |

Values are derived from Figs. 5, 6, and 7.
upregulation will have adverse effects on magnesium homeostasis by some hitherto unknown mechanism.

Although we discuss only two single cell lines here, we analyzed the first cell line to be created from cells isolated from the urine of a patient with the G41R mutation linked to dominant renal hypomagnesemia associated with hypocalciuria. We show that FXYD2 can be induced in these cells and that in hypertonically challenged patient cells the amount of Na,K-ATPase α-subunit and FXYD2 is reduced. As such, these cell lines represent a valuable tool for research into the disorder. Isolating and producing cell lines from other patients is the next step for future experiments.

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