

Study of ATP metabolism in human cystinotic proximal tubular cells and in humans with cystinosis in vivo

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Key objective 1. Study of intracellular ATP status, energy generating capacity and consumption in temperature sensitive immortalized PTEC cell lines (ciPTEC)

1.1. Measurement of ATP content in ciPTEC.

To study ATP metabolism in cystinosis, we have developed conditionally immortalized proximal tubular cell lines (ciPTEC). By collecting urine sediments of healthy controls and patients with cystinosis, cell lines were obtained by transfecting exfoliated cells with temperature sensitive SV40T and human telomerase TERT genes as described in the grant proposal. To determine proximal tubular origin in more detail, ciPTEC were further characterized (see figure 1). Expression of specific proximal tubular markers p-glycoprotein, aquaporin-1 (AQP-1), dipeptidylpeptidase IV (ddp-IV), aminopeptidase N and epithelial tight junction protein ZO-1 confirmed their proximal tubular origin of selected clones (Wilmer et al, submitted).

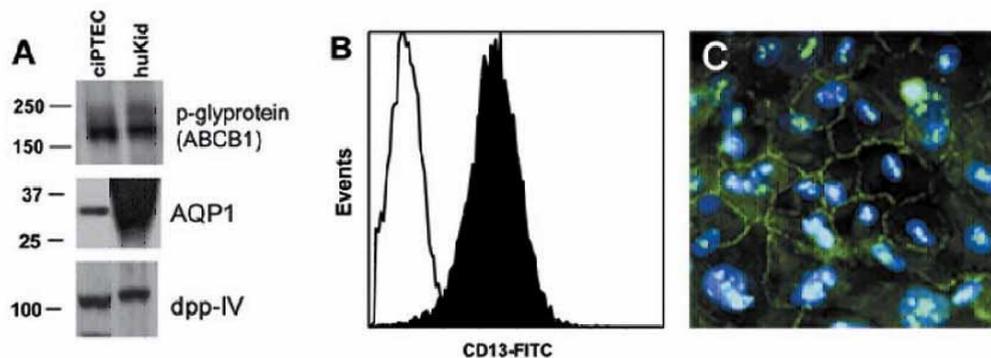


Figure 1. Characterization of ciPTEC.

(A) Expression of p-glycoprotein, AQP1 and dppIV demonstrated by Western Blot. (B) Expression of aminopeptidase N demonstrated using CD13-FITC and analyzed using flow cytometry. (C) Epithelial tight junction protein ZO-1 demonstrated by immuno-fluorescence microscopy.

Functionally sodium-dependent transporters were demonstrated in these cell lines (see figure 2). Taken together, these characteristics indicate ciPTEC are from proximal tubular origin and maintain their transport function.

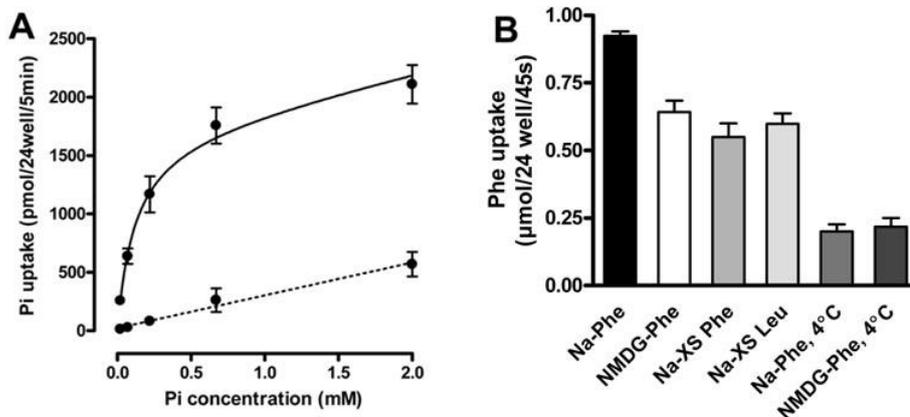


Figure 2. Sodium dependent transport in ciPTEC.

(A) Concentration dependent phosphate transport in ciPTEC measured using $^{32}\text{PO}_4$ in presence (black line) or absence (dashed line) of sodium. (B) Uptake of phenylalanine was inhibited in absence of Na (with NMDG), or by excess unlabelled Phe, Leu or at 4°C.

Intracellular ATP content was measured in three independent experiments by using ATP Bioluminescence Assay KIT (Roche®). Our preliminary results in 2 cystinotic and 2 control cell lines show decreased intracellular ATP content in cystinotic cells (22 and 24 nmol/pg protein in cystinosis versus 45 and 43 nmol/mg protein in controls). These experiments will be extended to a larger amount of cell lines which will allow us performing of statistical analysis.

Mitochondrial and cytosolic ATP production capacity in control and cystinotic ciPTEC was determined using transfection with mitochondrial (mluc) or cytosolic targeted luciferase (cluc). After transfection with mluc or cluc, cells were perfused with luciferin while monitoring light emission, which is ATP dependent. After reaching a stable signal, cells were perfused using digitonin (DIG), to calibrate for cell transfection efficiency. To test the functionality of this test, sodium iodo-acetate (SIA) is added in one set of experiments, which inhibits glycolysis. This method allowed us to show that mitochondrial ATP production capacity was not altered between control and cystinotic ciPTEC (Figure 8). Only the addition of SIA resulted in a significant decrease of ATP production.

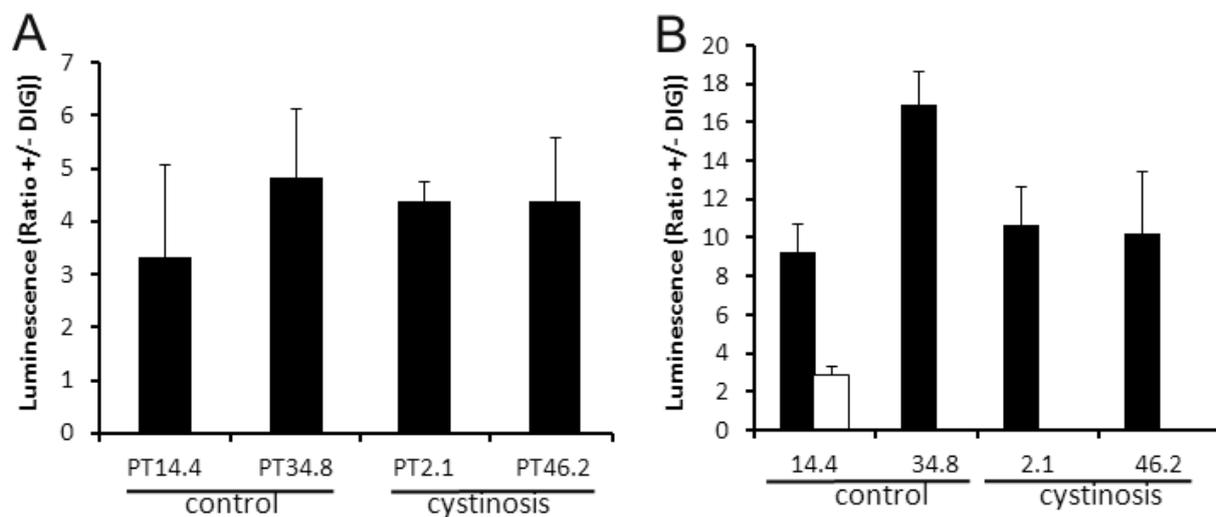
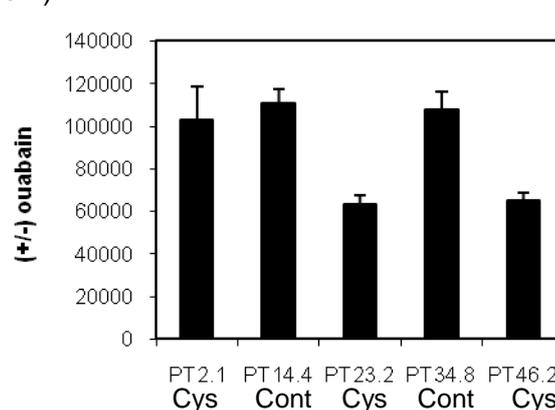


Figure 8. Mitochondrial (A) and cytosolic (B) ATP production in control and cystinotic ciPTEC. Data are expressed as ratio luminescence before and after DIG permeabilization (black bars). White bar indicates cytosolic ATP production in control 14.4 in presence of SIA (0.05mM).

Activity of Na,K-ATPase was determined by $^{86}\text{Rb}^+$, a congener of K^+ , uptake in intact cystinotic and control cell lines as previously described [Munzer et al. 1994]. Specific Na,K-ATPase activity is determined by measuring uptake in presence and absence of inhibitor ouabain. The activity of Na,K-ATPase was decreased in two of three cystinotic clones compared to the controls (see figure 4).

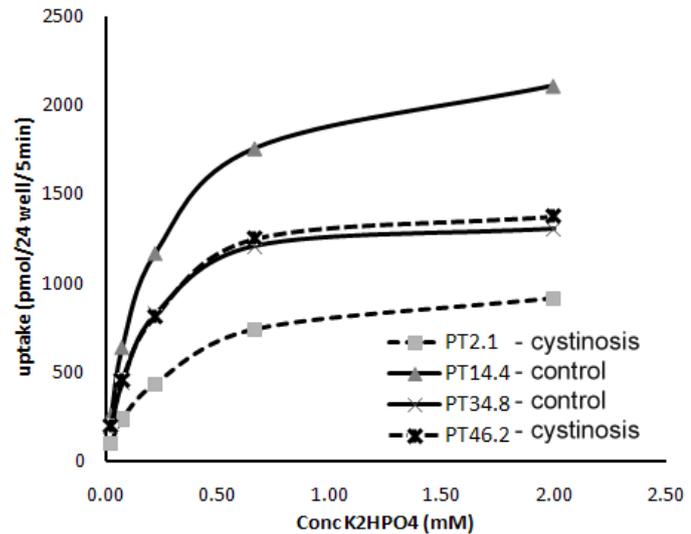
Figure 4. Na,K-ATPase activity in ciPTEC

Uptake of $^{86}\text{Rb}^+$ in cystinotic and control ciPTEC is presented as specific Na,K-ATPase activity, by subtracting $^{86}\text{Rb}^+$ uptake in presence of ouabain.



To determine whether ATP decrease in cystinotic cells could be caused by decreased phosphate reabsorption, we assessed a $^{32}\text{PO}_4$ uptake experiment in 2 control en 2 cystinotic ciPTEC. The results indicate that all cell lines reabsorb phosphate in a concentration dependent manner. The results demonstrate an overlap between control and cystinotic ciPTEC (figure 11), requiring further studies.

Figure 11. Phosphate uptake in control and cystinotic ciPTEC. ciPTEC cultured for 10 days at 37C are incubated with radioactive labeled K_2HPO_4 at various concentrations.



At present, we are increasing the amount of cell lines, because of divergent results in the current population of 2 control ciPTEC and 2 cystinotic ciPTEC. Conditionally immortalized cell lines of additional patients and controls are currently further characterized. Table 1 shows an overview of the study population at this moment.

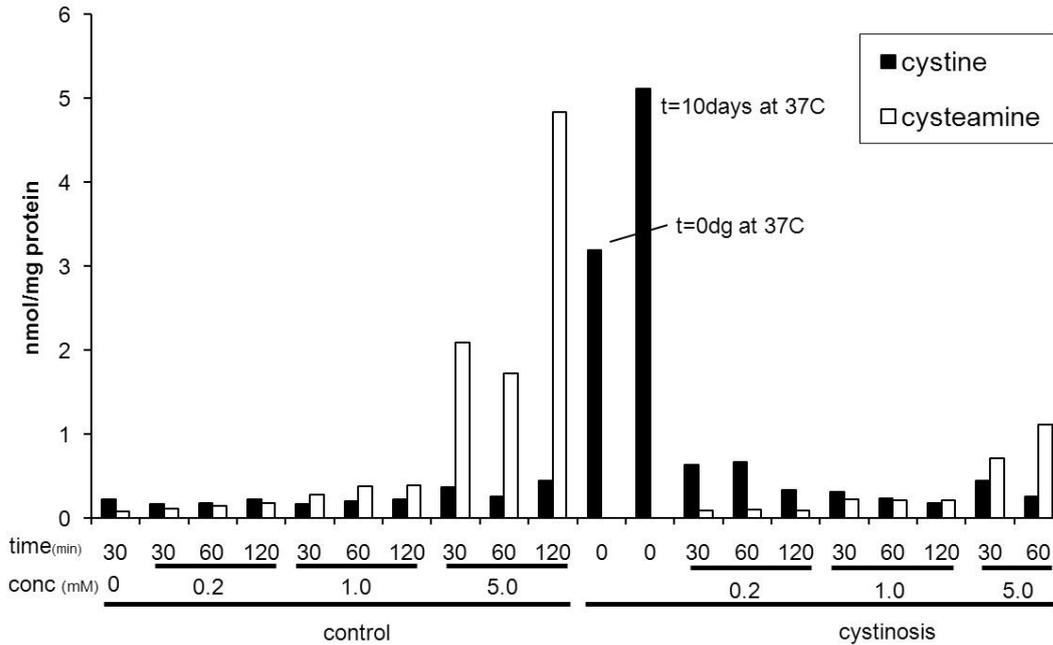
	code	immortalized	subcloned	characterized
healthy controls	PT10	yes	yes	no
	PT14	yes	yes	yes
	PT33	yes	yes	no
	PT34	yes	yes	yes
cystinosis (hom57kb)	PT2	yes	yes	yes
	PT13	yes	no	no
	PT41	yes	yes	no
	PT46	yes	yes	yes
	PT55	yes	no	no
cystinosis (het57kb)	PT24	yes	yes	no
	PT53	yes	yes	no
	PT48	yes	yes	no
	PT54	yes	yes	no
cystinosis (other mutation)	PT4	yes	yes	no
cystinosis (mutation not detected)	PT25	yes	yes	no
	PT47	yes	yes	no

Table 1. Study population. Cystinotic patients are divided according to their mutation in CTNS gene. Indicated is whether obtained cells lines are immortalized, subcloned and/or characterized at this stage.

1.2. Influence of cysteamine on intracellular ATP concentrations in cystinotic cells.

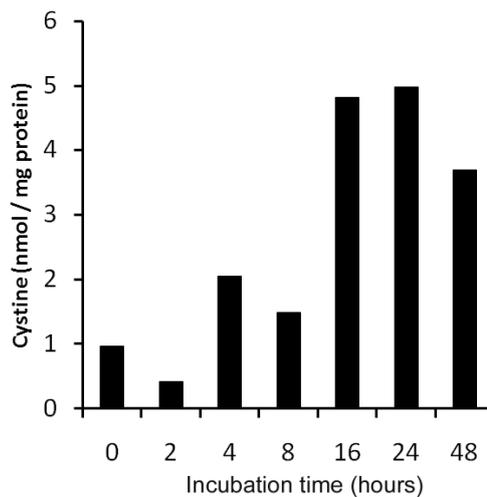
To investigate whether intracellular cystine depletion by cysteamine in cystinotic cells can restore intracellular ATP content, we determined the optimal cysteamine concentrations and incubation time for maximal cystine depletion. The results of these experiments are shown in figure 5, indicating that incubation of cystinotic cells with 1mM cysteamine for 60min results in cystine levels comparable to control cystine levels. Additionally, we have investigated the cystine depletion at 1mM cysteamine with increasing incubation times. The results indicated that cystine is depleted for up to 8 hours after addition of cysteamine (figure 9).

Figure 5. Cystine and cysteamine concentrations in cystinotic and control cells after



incubation with cysteamine. Concentrations of cystine and cysteamine in control and cystinotic cells after incubation for 30, 60 or 120 minutes with 0.2, 1.0 or 5.0 mM cysteamine. Both cystine and cysteamine are measured by HPLC.

Figure 9. Cystine depletion by cysteamine. Cystine levels are determined at different time points after addition of 1mM cysteamine in tissue culture medium at time point 0.



Intracellular ATP levels are measured in control and cystinotic ciPTEC after incubation with or without cysteamine (1mM) for up to 5 days. Because cystine levels increased after 8 hours, cysteamine and culture medium is refreshed every 6 hours to prevent cystine accumulation. In this assay, both cystinotic cell lines have decreased ATP levels, which are not restored by cysteamine treatment (figure 10). To be conclusive, this assay will be repeated with additional cell lines derived from other patients and controls.

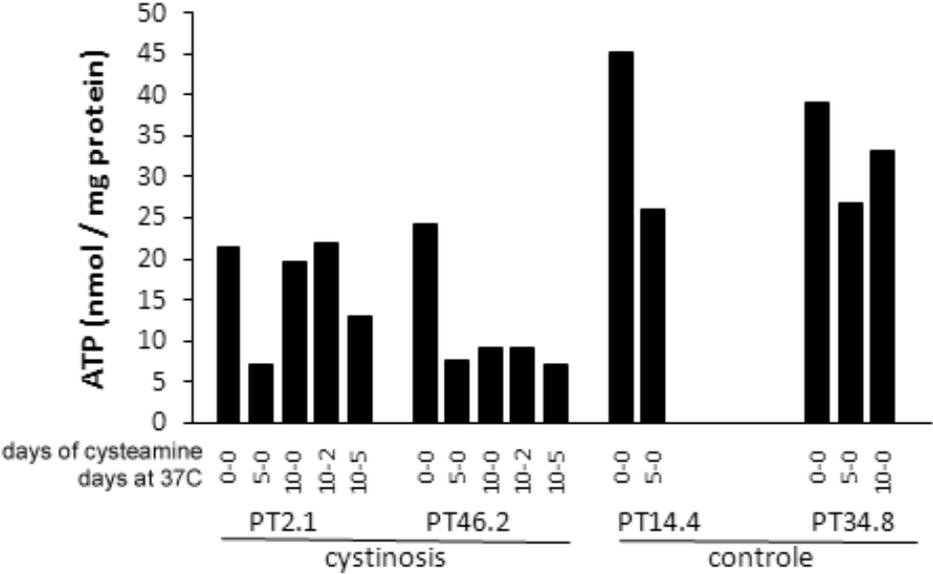


Figure 10 ATP levels after cysteamine treatment. ATP levels are analyzed in cell homogenates using Roche luminescence ATP kit. Cells are cultured at 37C for up to 10 days. At day 5 or day 8, cells are treated with cysteamine (1mM), with refreshment of culture medium with cysteamine every 6 hours to prevent the accumulation of cystine.

1.3. To finalize our study in cystinotic fibroblasts (Levtchenko et al. 2006), we measured the expression and activity of mitochondrial complex V activity. This measurement was not available in our laboratory at the time of this study. As shown in figure 7, both expression and activity of complex V was not different between cystinotic and control cells (Wilmer et al. 2008).

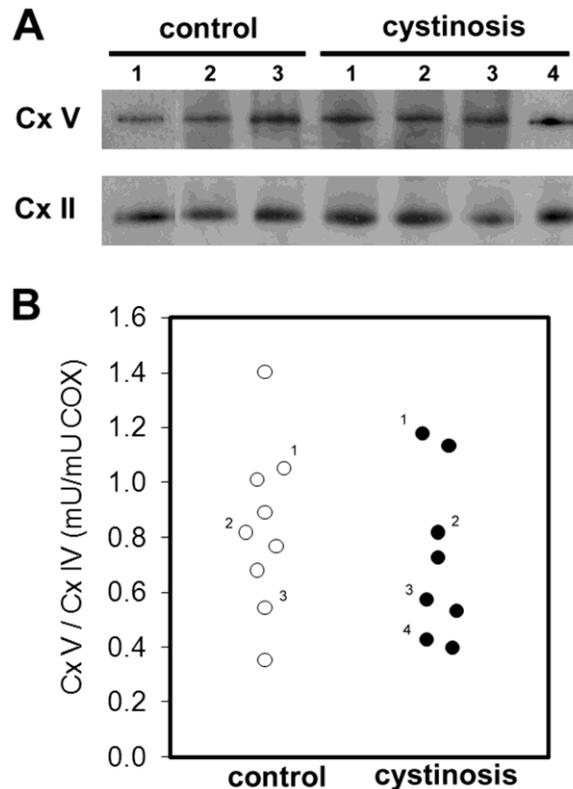


Figure 7. Complex V expression and activity in fibroblasts.

(A) Expression of complex V and loading control complex II was determined in enriched mitochondrial fractions of control (n=3) and cystinotic (n=4) fibroblasts using blue native gel electrophoresis. (B) Complex V activity was measured in isolated mitochondria of control (n=9) and cystinotic (n=8) fibroblasts using an enzymatic assay and normalized for complex IV activity. Control and patient's samples loaded in figure A correspond to numbers indicated in figure B.

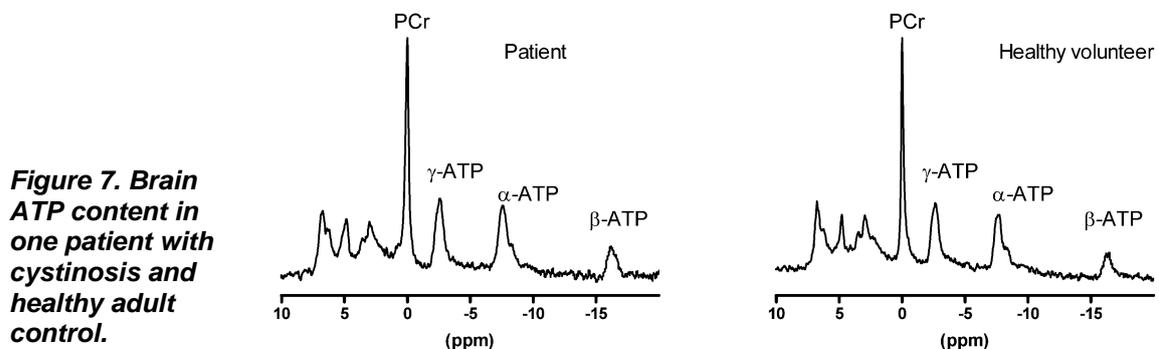
Future plans

At present we are continuing our research for further elucidating whether ATP metabolism is disturbed in cystinotic ciPTEC. For this purpose the amount of cell lines has been extended. We will measure ATP levels after incubation with cysteamine and glycolysis inhibitor DOG. Furthermore, mitochondrial ATP production will be measured. These findings will elucidate the status of mitochondrial and glycolytic metabolism in cystinotic proximal tubular cells and will conclude whether alterations in ATP metabolism are responsible for the phenotype observed in cystinosis. The role of disturbed phosphate transport and the influence of cysteamine treatment will be studied in 2009. This work will contribute to the PhD thesis of M. Wilmer, which will be finalized in 2009.

Key Objective 2. Measurement of intracellular ATP status in liver tissue of cystinotic patients by phosphorus nuclear magnetic resonance spectroscopy (MRS)

The method for *in vivo* measurement of ATP in brain was established at our department of Radiology. In order to assess the *in vivo* ATP content in the brain a cystinosis patient underwent a ^{31}P magnetic resonance spectroscopy (MRS) examination, which was performed in a 3T MR spectrometer using a dedicated home-built ^{31}P RF coil. Spectra were recorded using a pulse-acquire sequence with an adiabatic pulse for excitation, 16 scans and a repetition time of 5 seconds. Apart from localization by the RF coil no additional volume selection was applied. ^{31}P MR spectra of the brain of both a healthy volunteer as well as the patient are depicted in figure 7. Quantitative analysis of the signal intensities by time-domain fitting in the jMRUI software package shows a slightly lower ratio of gamma-ATP and phosphocreatine ($\gamma\text{ATP/PCr}$) for the patient than for the healthy volunteer, 0.75 versus 0.90 respectively. Despite of this result, it cannot yet be concluded that the ATP content in brain of cystinosis patients is lowered as more subjects have to be measured.

^{31}P MRS of the brain



Measurements of ATP in the liver of cystinotic patients were not possible until now because the specific “liver” coil for ^{31}P MRS was not available and the coil for brain measurements appeared not to be suitable. However, we prefer liver ATP content for comparing ATP homeostasis between cystinotic and control individuals because cystine accumulation is more pronounced in the cystinotic liver than in the brain. Furthermore, brain measurements are less comfortable for the patients who have to be closed in a small chamber isolating their head. Technically, ATP measurements in the liver are also preferable, because no buffering of ATP with phosphocreatine (PCr) occurs in the liver. This metabolic difference with brain (and muscle) might make differences between cystinotic and control subjects more pronounced.

During 2008 the department of radiology was developing and validating a “liver” coil for ^{31}P MRS. The permission of the Ethical Board is requested. Because of the technical difficulties with ^{31}P MRS apparatus, we postponed patient’s measurements to 2009.