Progress report of Cystinosis Research Network grant

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.

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).

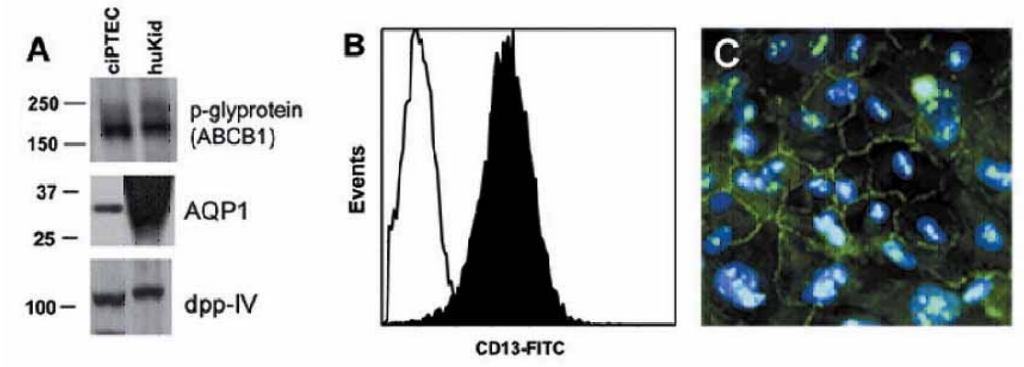

Figure 1.Characterization of ciPTEC.
(A) Expression of p-glycoprotein, AQP1 and dppIV demonstrated by Western Blot. (B) Expression of aminopeptidase N demonstrated using CD13-FITC and analyzed using flow cytometry. (C) Epithelial tight junction protein ZO-1 demonstrated by immuno-fluorescence microscopy.

Additionally, 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.

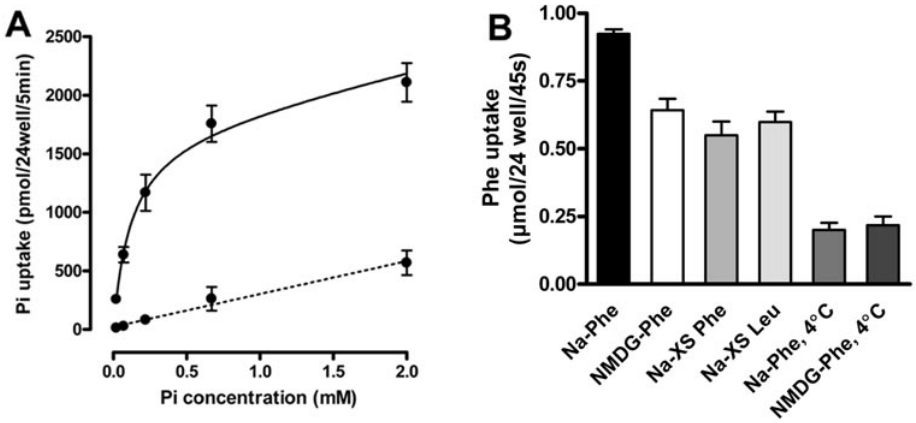

Figure 2. Sodium dependent transport in ciPTEC.
(A) Concentration dependent phosphate transport in ciPTEC measured using $^{32}PO_4$ in presence (black line) or absence (dashed line) of sodium. (B) Uptake of phenylalanine was inhibited in absence of Na (with NMDG), or by excess unlabelled Phe, Leu or at 4°C.

Intracellular ATP content was measured in 6 cystinotic and 7 control ciPTEC clones by using ATP Bioluminescence Assay KIT (Roche®) (see figure 3A). In both control and cystinotic ciPTEC, ATP levels were decreased after 10 days culturing at 37C. Cell viability was stable for at least 21 days, measured using a resazurin assay (not shown). Mean intracellular ATP levels were significantly more decreased in cystinotic ciPTEC compared to control ciPTEC after 10 days culture at 37°C (49% versus 75% of initial value, p<0,05), pointing to disturbances in ATP metabolism in cystinotic cells (figure 3B and 3C).

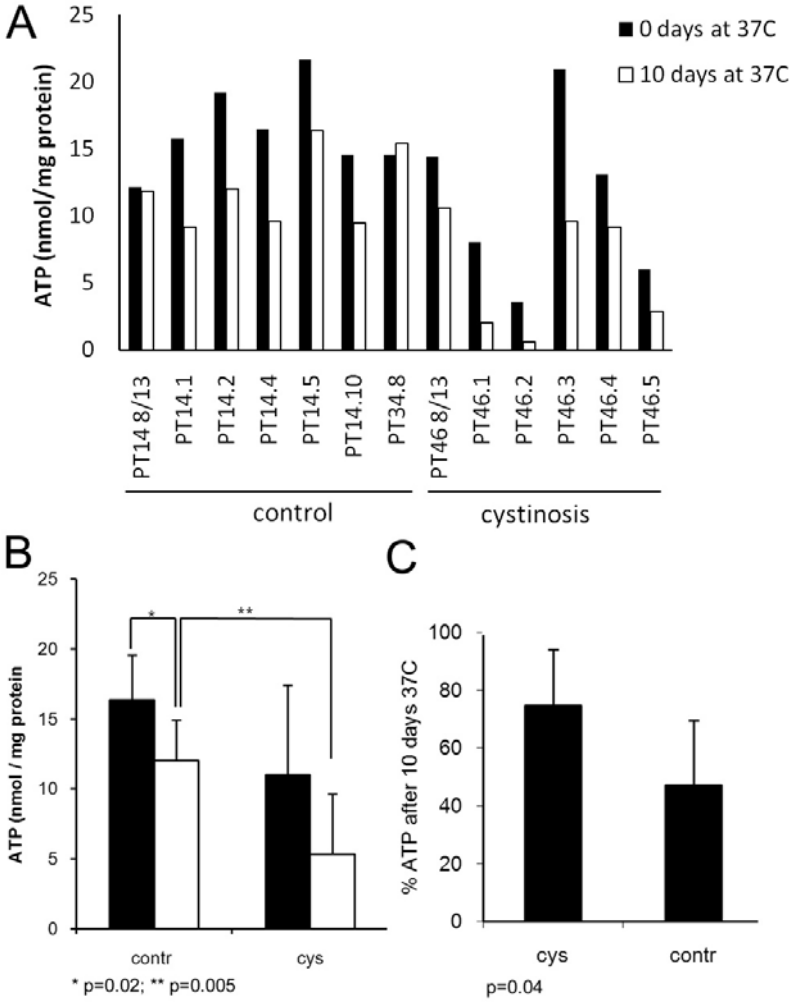

Figure 3. Intracellular ATP content in control and cystinotic ciPTEC clones. *(A) ATP levels in different ciPTEC clones immediately after transferring cells from 33 to 37°C (white bars) and 10 days after culturing at 37°C (black bars); note: 14.1 is donor n°14, clone n°1; (B) Mean ATP levels in cystinotic and control clones. (C) % intracellular ATP after 10 days culture at 37°C compared to 33C.*

Activity of Na,K-ATPase was determined by ^{86}Rubidium ($^{86}Rb^{+}$), 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. Despite significant ATP decrease in all cystinotic cell lines, activity of Na,K-ATPase was decreased only in two cystinotic clones compared to the controls (see figure 4).

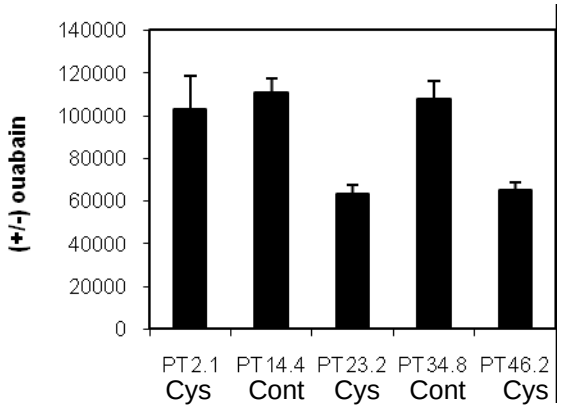

Figure 4. Na,K-ATPase activity in ciPTEC
Uptake of $^{86}Rb^{+}$ in cystinotic and control ciPTEC is presented as specific Na,K-ATPase activity, by subtracting $^{86}Rb^{+}$ uptake in presence of ouabain.

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. Lower concentrations do not decrease cystine levels completely.

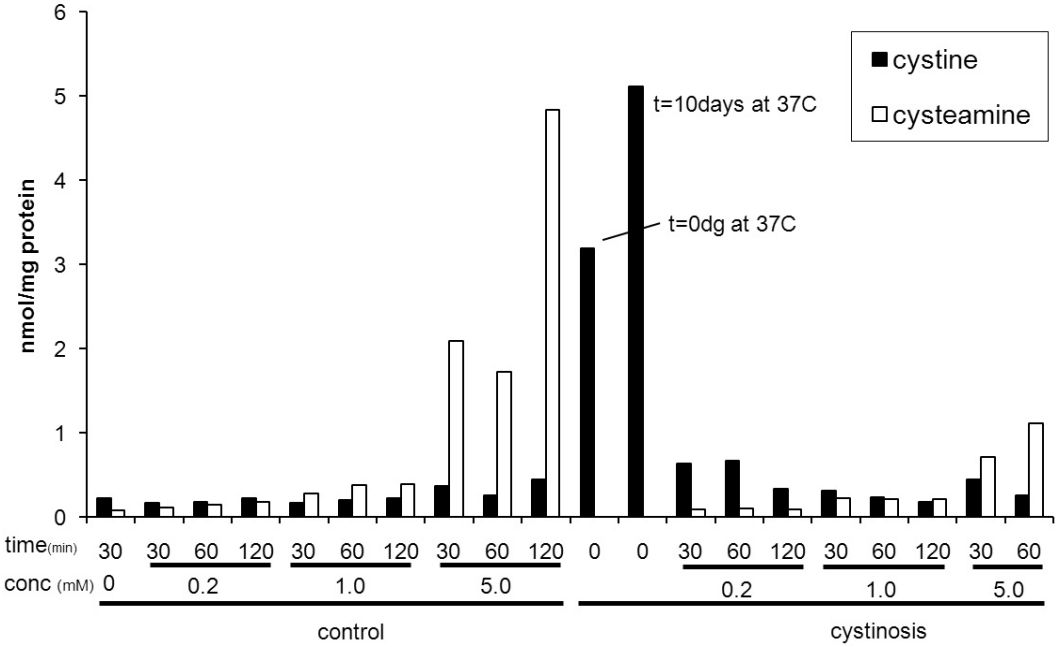

Figure 5. Cystine and cysteamine concentrations in cystinotic and control cells after incubation with cysteamine. *Concentrations of cystine and cysteamine in control and cystinotic cells after incubation for 30, 60 or 120 minutes with 0.2, 1.0 or 5.0 mM cysteamine. Both cystine and cysteamine are measured by HPLC.*

To study the mitochondrial function in ciPTEC, we will inhibit glycolysis by deoxyglucose (DOG). Preliminary experiments were performed to determine the optimal conditions to inhibit glycolysis maximal, while viability is intact. Cells were incubated with DOG while lactate production was measured in supernatant and viability was measured using resazurin assay (Figure 7)

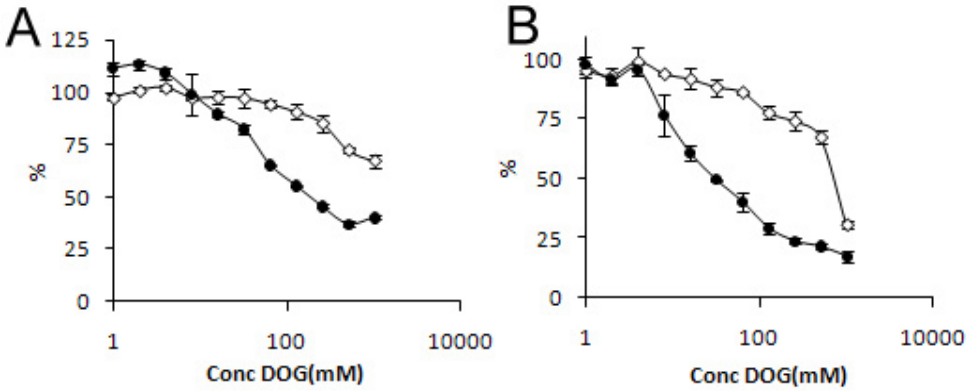

Figure 6. Glycolysis inhibition and viability in ciPTEC*. In both control (A) and cystinosis (B) ciPTEC inhibition of glycolysis was studied by adding DOG to the culture medium for 4 hours. Decrease of lactate production (closed circles) was inhibited at lower concentrations of DOG then the viability was decreased (open circles).*

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).

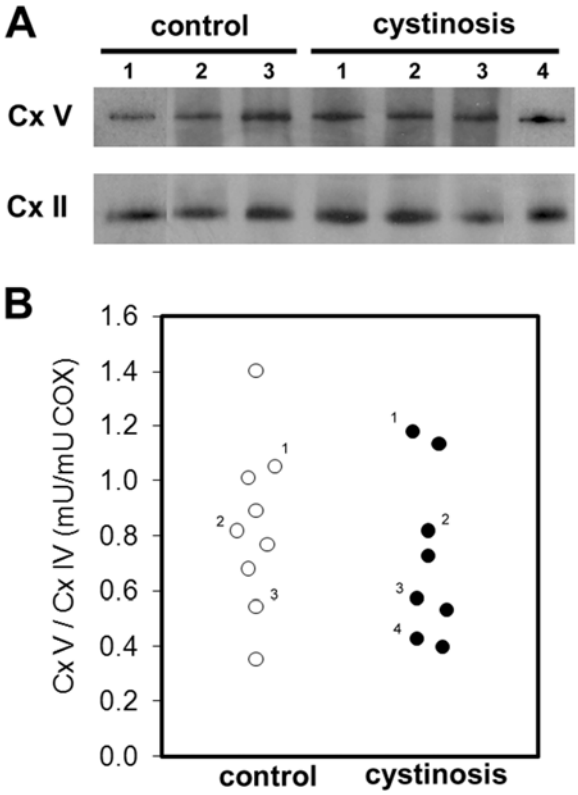

Figure 7. Complex V expression and activity in fibroblasts.
(A) Expression of complex V and loading control complex II was determined in enriched mitochondrial fractions of control (n=3) and cystinotic (n=4) fibroblasts using blue native gel electrophoresis. (B) Complex V activity was measured in isolated 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.

At present we are continuing our research to elucidate the ATP metabolism in cystinotic ciPTEC further. 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.

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 31P magnetic resonance spectroscopy (MRS) examination, which was performed in a 3T MR spectrometer using a dedicated home-built 31P 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. 31P 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 (γATP/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.

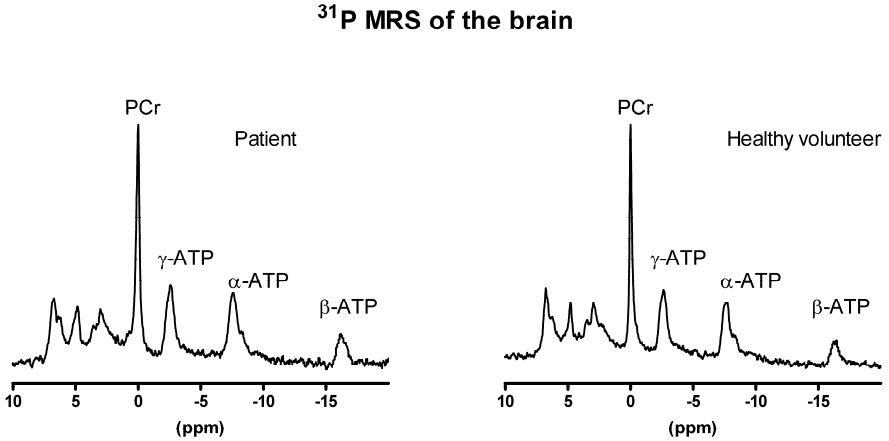

Figure 7. Brain ATP content in one patient with cystinosis and healthy adult control.

Measurements of ATP in the liver of cystinotic patients were not possible until now because the specific “liver” coil for 31P 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 31P MRS. We plan to be able to start with patient’s measurements in the second part of 2008.