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THE ROLE OF ALTERED CALCIUM AND mTOR SIGNALING IN THE PATHOGENESIS OF CYSTINOSIS

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Background

Several lines of evidence suggest that cystinosin, next to its cystine-transporting function, has a role in cellular signaling pathways. In this project we focus on two closely interfering pathways, namely on Ca^{2+} signaling and on mTOR (mammalian target of rapamycin) signaling.

The cytosolic [Ca^{2+}] is involved in the regulation of mTOR via Ca^{2+}/calmodulin-dependent protein kinase and AMP-activated protein kinase. Thus, abnormal Ca^{2+} signaling can be an upstream event in the pathogenesis of cystinosis, analogous to the other lysosomal storage disorders, and might alter mTOR activity.

On the other hand, in yeast, the cystinosin analog ERS1 interacts with the components of the EGO (exit from growth arrest) complex, located on the vacuole and controlling TOR signaling, a major signaling pathway that integrates metabolic processes with nutrient availability and growth-factor signaling. Inhibiting TOR signaling induces autophagy. Indeed, signs of increased autophagy have been reported in cystinosis. Our preliminary results indicate that mTOR is down-regulated in cystinosis cells and that further inhibiting mTOR by rapamycin augments cystine accumulation.

In addition we have detected an abnormal distribution of endo-lysosomes in cells lacking or overexpressing cystinosin, which would suggest a role of cystinosin in controlling lysosome distribution and movement. These processes are under control of the GTPase Rab7, which in turn can regulate the mTOR pathway through its role in regulating the morphology and function of late endosomes/lysosomes.

Based on this background information and our preliminary data, we aim to investigate the role of Ca^{2+} and mTOR signaling in the pathogenesis of cystinosis.

Specific aims

A. We will specifically study the mechanism of altered Ca^{2+} signaling in cystinosis cells. To this end we will study expression of Ca^{2+}-regulating proteins and Ca^{2+} fluxes in the endoplasmic reticulum (ER) and endo-lysosomal compartment.
B. We will focus on the mechanism of altered mTOR signaling/autophagy in human cystinosis cells.
C. Based on the obtained results we will test appropriate chemical compounds interfering with Ca^{2+} and mTOR signaling for improving cystinosis phenotype.

Results

1. Study of ATP-induced Ca^{2+} release in control and cystinosis PTEC cells

Previous experiments performed in our group indicated altered Ca^{2+} signaling in cystinosis cells proximal tubular epithelial cells (PTEC). Extracellular ATP stimulates Ca^{2+} release from the ER in a concentration-dependent manner by increasing 1,4,5-triphosphate (IP_3) production with subsequent activation of IP_3R. Ca^{2+} release was measured as the rise in free cytosolic [Ca^{2+}] induced by extracellular ATP in a medium containing 1.5 mM Ca^{2+}. Cystinosis cells demonstrated a significantly lower Ca^{2+} release from the ER at all tested ATP concentrations. Moreover, in cell lines carrying a homozygous deletion of the *CTNS* gene, the decrease in cytosolic [Ca^{2+}] after the initial peak was faster than in cells carrying a heterozygous deletion.

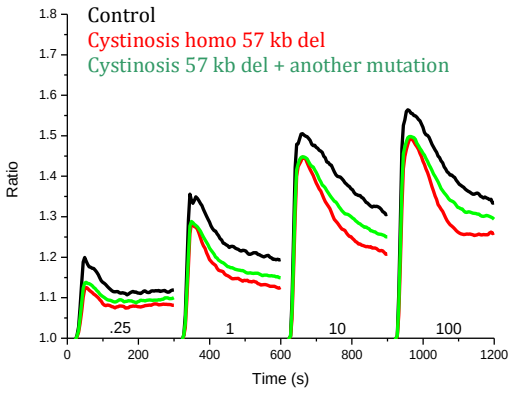


Fig. 1. Traces of [Ca^{2+}] as a function of time after addition of 0.25, 1, 10 and 100 µM ATP in a 1.5 mM Ca^{2+}-containing medium in control (n=4), cystinosis patients with a homozygous 57 kb deletion (n=4), and cystinosis patients with a heterozygous 57 kb deletion plus another mutation (n=6). Each line represents the mean of at least 10 measurements per cell line. Differences between control versus cystinosis at all conditions: $p < 0.001$.

To further study agonist-induced Ca^{2+} release in PTECs, we selected a number of cell lines, obtained from urine sediments and characterized in our laboratory (see Table 1).

Table 1. Cell lines used for agonist-induced Ca^{2+}-release experiments.

Cell line	Phenotype	Genotype
PT10.5	Control	Normal
PT14.4	Control	Normal
PT33.5	Control	Normal
PT34.8	Control	Normal
PT2.1	Cystinosis	Homozygous 57 kb deletion
PT46.2	Cystinosis	Homozygous 57 kb deletion
PT53.3	Cystinosis	57 kb deletion + c.18-21 del
PT13.5	Cystinosis	57 kb deletion + loss of function point mutation
PT13.5 3F-CTNS	Rescue	Introduced 3Flag-CTNS
PT13.5 3F-CTNS LKG	Rescue	Introduced 3Flag-CTNS LKG

To analyze whether differences in ATP-induced Ca^{2+} release demonstrated in Figure 1 were due to Ca^{2+} entry from the extracellular compartment or were due to its release from the ER, we loaded cells with Fura-2 AM, a Ca^{2+}-sensitive dye and then treated them with EGTA-containing Ca^{2+}-free Krebs buffer to remove the extracellular Ca^{2+}. After that, Ca^{2+} release was induced by adding 4 different concentrations of ATP in the same buffer. Finally, 1 M of extracellular Ca^{2+} was added to visualize the maximal intracellular [Ca^{2+}] reachable in the cells. Control and cystinosis cells were grown in 96-well plates, and each measurement was repeated in total 6 times during one experiment and the results from 3 independent experiments were averaged to obtain the final values.

Strikingly, in the experimental conditions described above, no difference in the amplitude of the response to the maximal ATP concentration (100 µM) was observed between the control and cystinosis cell lines. Such difference, demonstrated previously in PTEC cells could be explained by a different design of the experiment, in which no Ca^{2+} was present in the extracellular medium. At the same time, the EC_{50} of ATP was significantly lower for cystinosis cells indicating a more pronounced response of these cells to extracellular ATP. One tested cystinosis cell line with 57 kb deletion

combined with another mutation (c.18-21 deletion) in *CTNS* gene showed a slightly lower EC_{50} than the control cell lines (Figure 2).

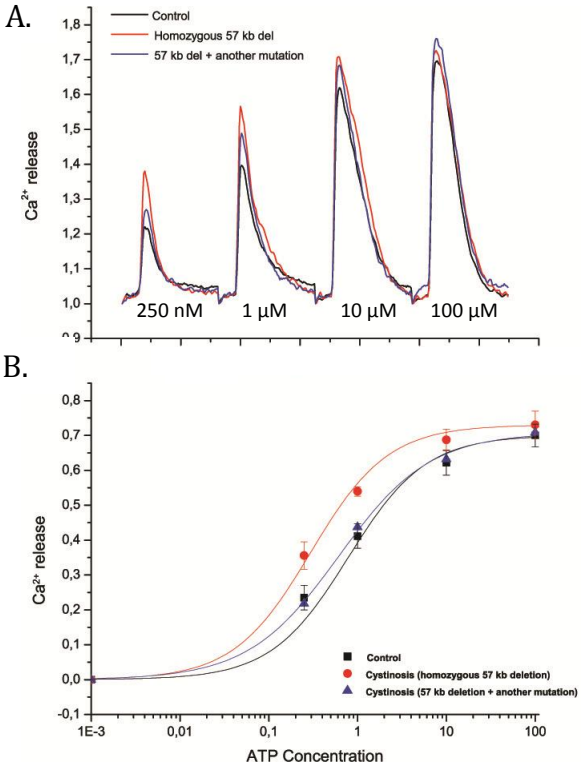


Fig. 2. A. Traces of [Ca^{2+}] after addition of 0.25, 1, 10 and 100 µM ATP after binding of extracellular Ca^{2+} with EGTA in control (n=3), cystinosis patients with a homozygous 57 kb deletion (n=2), and cystinosis patients with a heterozygous 57 kb deletion plus another mutation (n=1). Each line represents the mean of at least 3 independent measurements per cell line. Differences between control versus cystinosis at all conditions: $p < 0.05$.
B. Average EC_{50} values of 3 control, 2 cystinosis with homozygous 57 kb deletion, and 1 cystinosis with 57 kb deletion + another mutation cell lines demonstrated a prominent difference in sensitivity of these cells to ATP ($p<0.05$).

Average EC_{50} values of 4 control and 2 cystinosis (with homozygous 57 kb deletion) cell lines demonstrated a prominent difference in sensitivity of these cells to ATP (Figure 2B, 3A).

We next investigated the effect of CTNS and its isoform CTNS LKG, in which a part of cytosolic c-terminus is substituted by a longer sequence containing LKG motif, introduced into cystinosis cells using a lentiviral construct for stable expression. Such rescue cell lines were available in our group for one cystinosis line bearing a 57kb deletion on one allele and a loss-of-function point mutation on the other (PT13.3). In accordance with previous observation, rescued cells demonstrated increased EC_{50} values in comparison to the original cystinosis line, with the CTNS LKG isoform having a lesser effect than the major CTNS isoform (Figure 3b).

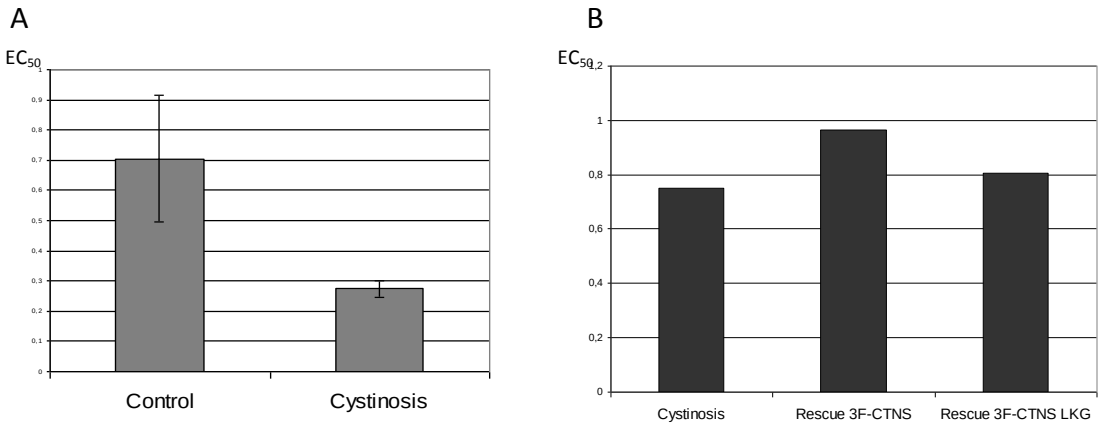


Fig. 3. A. Mean EC_{50} of ATP of control and cystinosis cell lines ($p<0.05$)
B. Mean EC_{50} of ATP in cystinosis cell lines rescues with a lentiviral construct containing mock, 3F-CTNS and 3F-CTNS LKG isoform.

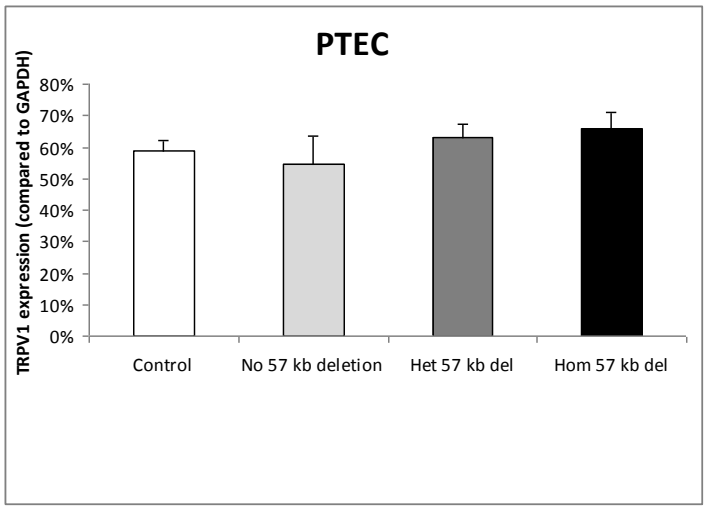
These observations clearly indicate an altered Ca^{2+} signaling in cystinosis cells in response to ATP. However, the mechanism of this effect remains to be investigated. In the future experiments we will evaluate agonist-induced Ca^{2+} release from the ER by means of Ca^{2+} imaging in single cells. This will allow more accurate measurement of cellular response to various agonists.

We will also study the effect in rescued cell lines deriving from cells with homozygous 57 kb deletion of *CTNS* gene and in cells with *CTNS* downregulated by specific shRNA to exclude the possibility of cell-specific effects and lessen the initial variability of the parameters. To reveal possible alterations in Ca^{2+} content of the ER and late endosomes/lysosomes, we will perform ratiometric measurements using compartment-specific fluorescent Ca^{2+} sensitive probes, as well as disrupting agents and ionophores. To study the mechanism of altered Ca^{2+} release in response to ATP in cystinosis versus control cells, we will measure the effect of IP_3, which is downstream from ATP signaling. We will assess Ca^{2+} release from the ER in response to IP_3 by loading the stores with radioactive $^{45}Ca^{2+}$ with subsequent quantification of

its release that will allow us an accurate quantitative measurement of the effect. Also, the input of cystine accumulation will be evaluated by pre-treating the cystinosis cells with the cystine-lowering drug cysteamine.

2. Study of TRPV1 functionality in PTEC cells

One of the most prevalent mutations causing cystinosis in the European population is the homozygous 57 kb deletion, which also affects the first two non-coding exons of the TRPV1 gene. Therefore, alterations in Ca^{2+} signaling in these patients could, in principle, be linked to a possible loss of function of the TRPV1 cation channel, permeable for Ca^{2+} ions after activation in response to certain stimuli. A previously performed by our group qPCR analysis of *TRPV1* gene expression in cystinosis PTECs and fibroblasts demonstrated the presence of *TRPV1* mRNA, indicating that its transcription was unaffected by the 57 kb deletion (Figure 4). To assess the functionality of TRPV1 in renal cells, we performed a series of free cytosolic [Ca^{2+}] imaging experiments in collaboration with Dr. Joris Vriens. Ca^{2+} entry was measured in response to capsaicin (a specific TRPV1 agonist) and 50 µM OAG (a TRPC6 agonist) and 100 µM ATP as a positive control. The experiments were performed on control and cystinosis PTEC cells and control and cystinosis podocytes with a homozygous 57 kb deletion. No response to capsaicin was seen both in control and in cystinosis PTEC cells, indicating that TRPV1 was not functional in this cell type. Similarly, no response to TRPV1 agonists was observed in control and cystinosis podocytes that were tested both by means of single-cell Ca^{2+} imaging and Patch Clamp technique. Importantly, cells responded with an increase of cytosolic [Ca^{2+}] to ATP. Furthermore, both cystinosis and control podocytes increased their [Ca^{2+}] in response to OAG stimulating TRPC6 selectively expressed on the podocytes, but not on the PTEC.



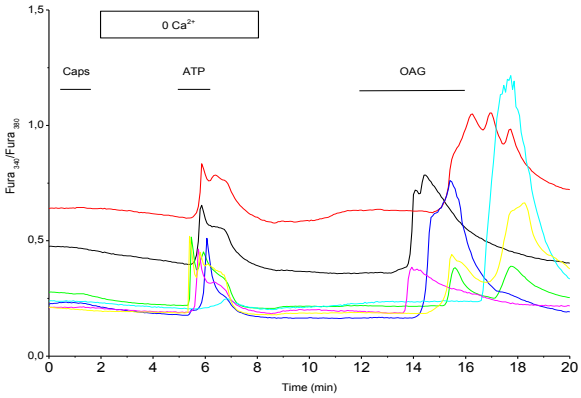


Fig. 4. TRPV1 does not interfere with Ca^{2+} signaling in PTEC and podocytes.
Above: qPCR analysis demonstrated unaltered transcription of the TRPV1 gene in PTEC cells with homozygous 57 kb deletion.
Below: cytosolic [Ca^{2+}] was not influenced by TRPV1 agonist capsaicin in podocytes, but increased in response to ATP and OAG (TRPC6 specific agonist).

Thus, differences in function of TRPV1 were not responsible for differential response of cystinosis and control cells to ATP.

3. Study of mTOR signaling and autophagy in control and cystinosis PTEC cells

Autophagy rate was evaluated by quantitative measurement of LC3-II protein, a well-known marker of autophagosomes. In accordance with literature data, cystinosis cell lines had a significantly higher level of LC3-II accumulation after treatment with bafilomycin A1 (Figure 5).

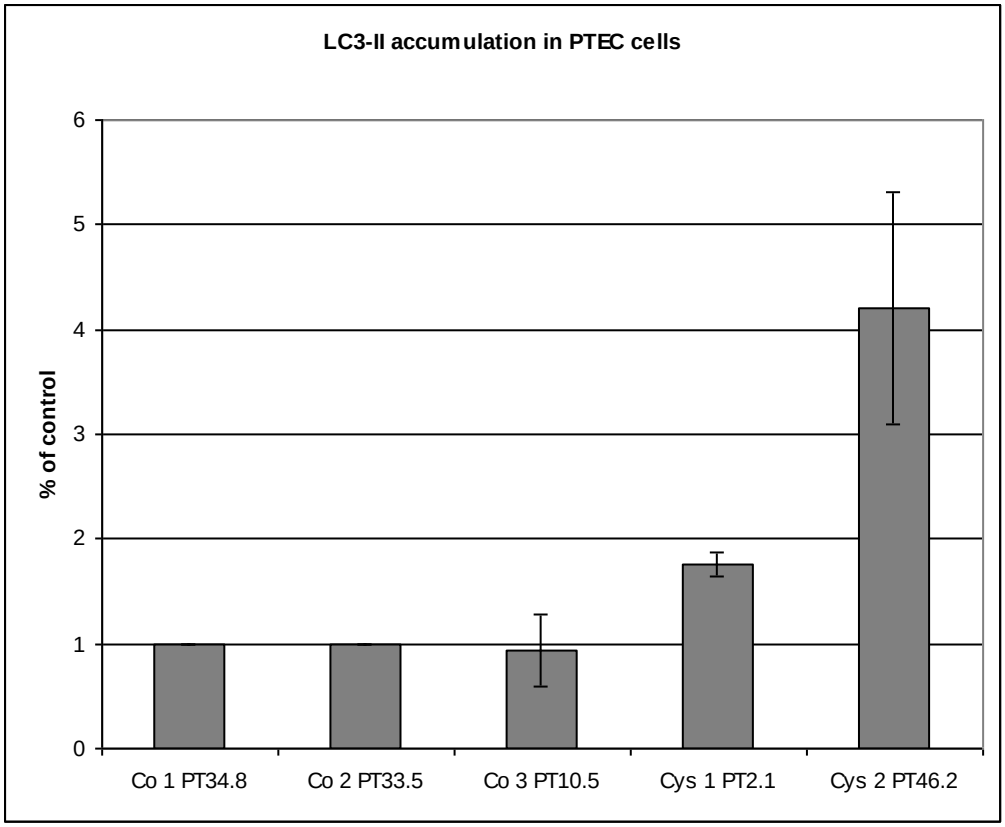


Fig. 5. LC3-II accumulation is increased in cystinosis cells with 57 kb homozygous deletion.

The thorough screening of control (n=4) and cystinosis cell lines (n=6) demonstrated large variability in mTOR activity measured as a ratio of pS6/S6. The variability reflected the high sensitivity of the cells to slight changes in current nutritional status, confluency of the cell monolayer or percentage of dividing cells. To tackle the problem of variability, we are going to apply the following approaches: 1) studying mTOR activity in stably rescued cell lines by introducing 3Flag- and GFP-tagged CTNS and CTNS LKG genes using lentiviral vectors; 2) using cell lines with a down-regulation of the *CTNS* gene with specific shRNAs. We will next study the effects of nutrient starvation and subsequent stimulation with nutrients in control and cystinosis PTECs.